Cancer Metastasis – Biology and Treatment 20

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Cal Roskelley *Editors*

Genomic Instability and Cancer Metastasis

Mechanisms, Emerging Themes, and Novel Therapeutic Strategies

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Preface

In 1975, the mortality rate for all cancers in the United States was 199 deaths per 100,000 people in the population [1]. The most recent statistics available (2006–2010) indicate that the current mortality rate is 176.4 deaths per 100,000 people [2], which represents a modest 11 % reduction in the past 35 years. It has been estimated that approximately 90 % of cancer deaths arise from the metastatic spread of primary tumors [3]. Thus, any future improvement in the overall cancer mortality rate will depend upon a more thorough understanding of the properties that enable the metastatic process.

Cancers are diverse and complex tissues. Conceptually, it is proposed that cancers possess shared hallmarks [4], but it may be more correct to consider cancer as an evolving, heterogeneous, and dynamic entity [5] that is responsive to a plethora of selective events, both intrinsic to the tumor (e.g., hypoxia) and extrinsic (e.g., combination chemotherapy). Within the framework of an evolving tissue, it is clear that the extent of heterogeneity may provide selective advantages. Moreover, scientists have long known that cancer cells display significant intra-tumoral heterogeneity at the genetic level [6], which falls under the umbrella term of genomic instability. Therefore, it is reasonable to propose the interplay between tumor heterogeneity, which may be enabled by genomic instability, and an enhanced ability to withstand the selection pressures applied throughout the metastatic process.

This book connects cancer metastasis with genomic instability in a comprehensive manner through four sections. Section 1 outlines the fundamental mechanisms that occur at tissue, cellular and molecular levels and regulate the processes of cancer metastasis, genomic stability, and DNA damage response, respectively. Section 2 discusses the model systems that will enable our better understanding of the metastatic process and genomic instability through experimentation performed *in silico*, *in vitro*, and *in vivo*. Section 3 reviews emerging themes and frameworks for the understanding of the contributions of non-tumor cells to the metastatic process (ex., tumor microenvironment, mechanotransduction, and immunomodulation). Finally, Section 4 discusses new therapeutic approaches designed to overcome the unique challenges presented by the heterogeneous and metastatic tumor.

Section 1 takes a reductionist approach to describe the mechanisms responsible for the maintenance of tissue and cellular integrity starting with an examination of

the tumor tissue, followed by a discussion of processes that prevent instability at the genome level, and ending with a discussion of molecular pathways that act at the base-pair level to maintain integrity. Drs. Rodenhiser and Chambers (London Regional Cancer Clinic, Canada) introduce the metastatic tumor as an evolving tissue that presents many challenges, including diversity and dynamic heterogeneity, which must be interrogated in the clinic if novel treatments are to be successful. Dr. Connell and colleagues (University of British Columbia, Canada) outline the pathways in the normal cell, or the malignant tumor cell, that are responsible for the prevention of genomic instability and, when compromised, the promotion of heterogeneity. Dr. El-Khamisy and colleagues (University of Sheffield, UK and Helmy Institute, Egypt) review the diverse molecular pathways that fall under the umbrella term of the DNA damage response, and discuss the intimate relationship between loss of function in each of these pathways and cancer predisposition.

More complete knowledge of cancer metastasis, and the complex interplay between regulators that are found in the cancer cell, the tumor tissue and within the organism inflicted with cancer, will be born from studies that encompass *in silico*, *in vitro*, and in vivo model systems, each of which are reviewed in Section 2. Dr. Costes and colleagues (Lawrence Berkeley National Laboratories, USA) utilize 3D automated foci detection and computational modeling to understand and track the properties and kinetics for repair of DNA double strand breaks. Chapter 4 outlines how integration of mathematical models with irradiation data, a highly quantitative and reproducible manner to induce DNA damage, enables the synthesis of new knowledge in the fields of cancer initiation, detection and progression. In Chapter 5, Dr. Bennewith and colleagues (BC Cancer Agency and Dalhousie University, Canada) discuss a variety of animal models and the strengths and considerations when using these models to address specific research questions, which span from high-throughput analysis of novel compounds to dissection of the relative contributions of individual gene products during defined stages of cancer metastasis, from local invasion to distal entrenchment and expansion.

In 2000, Hanahan and Weinberg outlined six hallmarks of cancer [7]; only one of the six original hallmarks of cancer (i.e., they stimulate the growth of blood vessels to supply nutrients to the tumor) identified a property that was extrinsic to the tumor cell. It is increasing clear, however, that non-tumor cells, in the cancer tissue and the patient afflicted with cancer, are critical to cancer progression and metastasis, including, but not limited to, the role of tumor-stroma interactions and the tumor microenvironment, mechanical cues provided from the environment to the tumor, and immunomodulation. Section 3 reviews these emerging themes in the field of tumor microenvironment and cancer metastasis. In Chapter 6, Dr. Calvin Roskelley (University of British Columbia, Canada) provides an overview of microenvironmental control of cancer metastasis while recent advances in the fields of mechanotransduction in the tumor and immunosurveillance are detailed by Dr. Nelson and colleagues (Princeton University, USA) and Dr. Gregor Reid (University of British Columbia, Canada) in Chapters 7 and 8, respectively.

Prevention and treatment of metastatic tumor spread may represent the most significant challenge of medical oncology. In Chapter 8, Dr. Reid reviews the cross-talk

between immune cells and cancer cells; the controlled regulation of these dynamic processes through chemical or cell-based therapies may allow for improved immunosurveillance of metastatic cells. While tumor heterogeneity enabled through genome instability likely provides the tumor advantages against conventional chemotherapeutic and irradiation treatments, it is hoped that molecular-targeted therapies can turn the table by targeting pathways that are non-essential in normal tissue but, due to the loss of parallel pathways, are essential to tumor cells. In Chapter 9, Dr. McManus and colleagues (University of Manitoba, Canada) utilize colorectal cancer as the framework within which to introduce the concept of synthetic lethality and review the recent therapeutic advances gained through the targeting of deficient DNA repair pathways. Finally, a significant hurdle to the success of any systemic therapy, including those that may eventually be used to combat metastatic disease, is the efficient and specific delivery of the therapeutic agent to the target cell or tumor. In Chapter 10, Drs. Hauser-Kawaguchi and Luyt (University of Western Ontario, Canada) review the emerging field of nanomedicine and the utility of nanoparticles for improved cancer imaging and drug delivery.

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Contents

Chapter 1
Cancer Metastasis: Tracking and Attacking a Moving Target

David I. Rodenhiser and Ann F. Chambers

Abstract The effective treatment of metastatic cancer is complicated by both the diverse set of dysregulated molecular pathways contributing to cancer progression and the challenge of aiming clinical therapies at a seemingly unpredictable moving target. From an evolutionary perspective, metastasis can be considered as a process during which novel cell populations are generated that can exploit the unique tissue environments they encounter at a secondary tissue site and in response to treatment. In this review, we explore metastasis as a consequence of evolution on the scale of tumor cells within the individual patient. The survivability of any individual cancer cell, and as a consequence, the success of any broad-based or targeted therapy to treat that patient, may best be understood in terms of selective advantage and phenotypic changes resulting from genomic drift among cells from the original tumor. These drivers of evolution can generate successful metastatic cells that either survive as dormant cells, or thrive as secondary tumors during the time course of the disease. The metastatic target is thus dynamic, requiring a dynamic approach to treatment. Here we will discuss the growing information about heterogeneity and evolution of metastatic cell populations, and how this information impacts on treatment strategies that will be needed to combat metastatic disease.

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Introduction

While the past 30 years of the genomics revolution have dramatically advanced our knowledge of the molecular mechanisms of cancer, successful treatment of metastatic disease has been limited. This is despite the development of landmark synthetic approaches such as the Hallmarks of Cancer [1, 2] that have provided the conceptual framework within which to integrate the complex characteristics of cancer causation and the potential targets for novel treatment strategies. All cancers can be linked together mechanistically as involving interconnected pathways that can be independently (and simultaneously) activated (or disrupted). Paradoxically, the common, acquired molecular variants that are the genesis of these altered hallmarks also define the uniqueness of cancer phenotypes among tumor types as well as among the individuals within whom these cancers develop and thrive.

Notwithstanding these advances in knowledge, and the specific successes made in understanding, diagnosing and treating many primary cancers, two critical roadblocks exist for curing these diseases, in general, and metastatic cancer, in particular. The first involves the cellular and genomic complexity and plasticity of heterogeneous cancer cell populations that exist as dynamic and unstable entities that evolve over time. Second, the timing, rate and sites of these evolutionary processes (within the originating tissue or at a distant secondary site) are dependent on the genomic makeup of that individual cell and the selective pressures that are placed on it. This level of complexity also changes temporally, as cancer cells acquire the ability to metastasize, with new selection pressures yielding novel, more unstable subclones of metastatic cells that can be selected for by that new environment. Unfortunately, the inaccessibility of these dispersed metastatic cells, as either camouflaged micrometastases or dormant cells, makes detection and treatment of micrometastatic disease nearly impossible. This lag period between dispersal and detection of rogue cancer cells permits the development of heterogeneity to go ahead unnoticed, with the genesis of novel clones that differ from the parent tumor and are better suited to survival in the face of new microenvironmental stresses. This paradigm of clonal evolution in the metastatic context offers both challenges and opportunities to effectively detect, treat, control and/or cure metastatic disease.

Introduction to Metastasis—The Clinical Problem

Cancer that is detected early, before it has spread, is more readily treated with success, when compared to cancer that has already metastasized at the time of diagnosis. Metastatic cancer is generally not considered to be curable, although long-term

survival with metastatic disease is increasingly feasible. However, even cancer that is detected "early" can subsequently recur. Once it has metastasized, it is then more difficult to treat with success. Thus, for example, many patients with early breast cancer are treated first with local therapies (surgery, radiation), in some cases followed by often-aggressive adjuvant therapy designed to treat presumed, but clinically undetected, micrometastatic disease, to attempt to prevent metastatic recurrence. The decision to use adjuvant therapy is generally population-based, with groups of patients that are believed to be at higher risk for subsequent recurrence being more likely to be offered (and benefit from) adjuvant therapy. Recent and ongoing efforts at molecular profiling of groups of patients is leading to gene signatures that are both associated with poor vs. good outcome (suggesting the degree of potential benefit from aggressive initial therapy), as well as signatures that are predictive for responses to specific therapies (suggesting which patients should vs. should not be treated with specific therapies) (reviews, [3–6]). In spite of this improved ability to assess the potential for recurrence, as well as likelihood of response to given therapies, these therapy decisions are imprecise, such that many individual patients who likely did not need this treatment may be over-treated, while other patients, with "favorable" tumors, are not treated with adjuvant therapy but may subsequently relapse. Thus, our understanding of the risk of metastatic recurrence in individual patients is inexact and requires information specific to the genotype of the patient's primary tumor as well as the generation of genetic roadmaps laying out the likely paths of clonal development that lead to the genesis of novel metastatic cells in their relevant environmental contexts.

The logistical steps undertaken in cancer metastasis have been well established from extensive data gleaned from experimental studies (reviews, [7, 8]). These steps include the acquisition of a locally invasive phenotype, entry into blood or lymphatic vessels in the primary tumor, escape into these circulatory systems, systemic delivery to new organs, extravasation from these vasculature channels, and re-initiation of growth of the tumor in these new sites. However, many of the steps in metastasis likely have occurred prior to our current ability to diagnose cancer [9], suggesting that the latter steps of the process (primarily growth in ectopic, secondary organs) may be the more appropriate targets for therapeutic intervention [10, 11].

Coupled with the challenges of deciding on initial therapy for localized disease, it is becoming increasingly recognized that some cancers can recur years or decades after apparently successful primary therapy, due to the process of tumor dormancy (reviews, [7, 12–14]). Much ongoing work is aimed at clarifying mechanisms that regulate entry into tumor dormancy, as well as cessation of dormancy and re-initiation of growth of dormant cancer. However, considerable clinical challenges still remain in determining which cancers are likely to enter into clinical dormancy, and what factors (inherent to the tumor cells, the host or specific organ microenvironments, or modifiable lifestyle factors), can lead to release of growth constraints on dormant cancer (reviews, [14–17]). The relationship between cancer dormancy and the evolutionary nature of cancer progression discussed here remains poorly understood. Dormant cells maintain a degree

of normalcy in that they possess a benign phenotype programmed by a molecular signature that lacks the final genetic aberrations that tip those cells towards becoming a novel metastatic cancer. Furthermore, those molecular changes that have occurred reflect the constraints of the new environment in which they populate with subsequent changes necessary to permit release from dormancy at that secondary site. However, the fact that cancer can be maintained in a functionally dormant state, often for many years, suggests that understanding the inherent regulatory mechanisms within these dormant cells could offer new therapeutic strategies to maintain dormant cancer in a chronic, indolent state, in spite of the evolutionary forces that are inherent to cancer.

We have learned a great deal over the past decades about molecular aspects of cancer biology. This information has been translated into new medicines targeted to the molecular defects that have been identified and these agents have shown significant clinical benefit, with some having reducing toxicity compared to earlier therapies (reviews, [8, 18]). Much of this success has come primarily for patients with cancer that is localized at the time of diagnosis. Essential to these successes have been technologies that inform early diagnosis. Often, these patients may be treated aggressively, with the hope of totally eradicating the tumor, first by local therapy followed by adjuvant treatment to eliminate presumed but undetected micrometastatic disease [19]. This approach has led to apparent survival benefits for many patients with localized disease. Biologically, it is recognized that some patients may be at risk for very late recurrences, and may benefit from long-term therapy. This has been shown particularly in hormone responsive breast cancer, where patients have been shown to benefit from long-term endocrine therapy, which can have low enough toxicities to warrant long-term use, although the toxicities associated with treating many patients to benefit a sub-population are still of concern (reviews, [13, 20, 21]).

In contrast to cancer that is localized to the primary site, metastatic cancer is often treated quite differently, with less aggressive, sequential use of therapies predicted to have some effect, at least transiently, against a given patient's tumor [22]. This strategy is based on the current recognition that cure of metastatic cancer may not be possible, while prolongation of survival may be achievable. Indeed, some progress has been made in extending life for patients with metastatic disease. For lung, breast, prostate and colorectal cancers, for example, progress has been made in decreasing cancer-specific mortality overall [23], although improved survival for patients with metastatic disease has been only "modest" [24].

In spite of these limited gains, metastatic disease is still regarded as ultimately incurable. Such current paradigms of treatment of metastatic disease are not adequate in successfully tackling this disease. In spite of the significant progress made in understanding molecular and genetic factors that play a role in many different cancer types, we still have major unmet medical needs in treating metastatic cancer, preventing metastatic recurrence in successfully treated local disease and delaying metastatic recurrence. These challenges will require new ways of thinking about the nature of metastatic disease and new therapeutic strategies to counter it.

Clonal Heterogeneity and Evolution as a Hallmark of Cancer and Metastasis

Until recently, the working paradigm of cancer development has been based on the idea that cancer can progress linearly, from benign, early cancers, through increasingly aggressive, locally invasive disease, culminating in frank metastatic disease. This progression was initially noted at the histological level, which still forms the basis of the initial assessment of a tumor and its appropriate therapy. Histological progression is increasingly associated with defined molecular changes that functionally underpin clinical progression, following the paradigm set forth for colorectal cancer by Vogelstein and colleagues in the 1990's [25]. These molecular changes are also used clinically to target appropriate therapies to individual patients, when such targeted therapies are available. The potential drivers of this progression, as outlined in the Hallmarks of Cancer [1, 2], are complex with respect to the cellular pathways upon which they impact, but ultimately these genomic changes result in the generation of diversity and selection for increasingly aggressive and treatment-resistant phenotypes.

One significant conceptual advance towards a better understanding of metastasis is to consider the inherent nature of metastatic disease as a persistent, evolutionary process, possessing the consequences and challenges of successfully treatment of disease that is, by its very nature, temporally and spatially dynamic. Such a model for clonal evolution of a cancer cell population is modeled in the context of Darwinian natural selection, whereby the cancer cells represent somatic cell species that adapt to changing environment. This perspective of an evolutionary process underpinning cancer development was first put forth nearly 40 years ago. In his groundbreaking paper from 1976, Peter Nowell proposed that tumor progression results from acquired genetic variability within an original clone of cancer cells, allowing the sequential selection of more aggressive sublines [26]. He proposed that carcinogen-induced changes in a normal progenitor cell produce daughter cells with growth advantages initiating the process of clonal expansion. In this context, clonal expansion within tumors is not necessarily linear, but rather is branched (Fig. 1.1). Multiple competing subclones of tumor cells are generated that differ cytogenetically, genomically and ultimately differ at the transcriptome level. Genetic instability in these daughter cells leads to the further generation of clonal variants: some of which die, while others establish themselves as subpopulations that are suited to their particular microenvironment. The process repeats itself, as new clones are generated from the surviving progenitors and are selected for through interactions with the host microenvironment. Inherent within this selected, viable cell population are cells with silent genetic variants, and permissive epigenetic changes, which could be selected for at a later time, when the cells are exposed to a newer, more advantageous environment. Recently, Nik-Zainal and colleagues have reconstructed the genomic evolution of 21 breast cancer genomes and have shown that subclonal diversification is prominent, with most mutations being found in just a fraction of tumor cells [27]. Every tumor has a dominant subclonal lineage, representing more

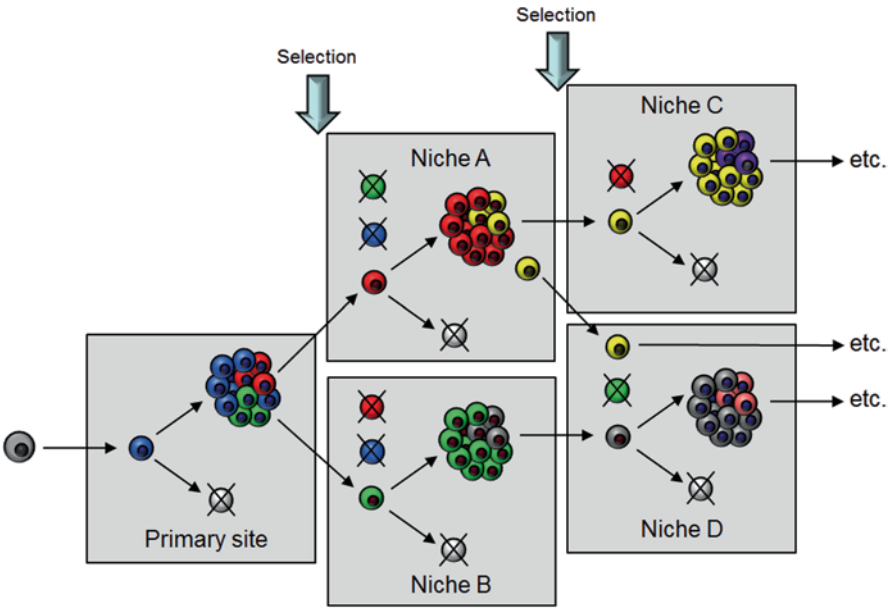

Fig. 1.1 Model of clonal evolution. Cancer cell clones arise and are selected through interactions between the cell's genotype/ phenotype and the environmental pressures impacting on those cells. These selection pressures may be diverse, changing the microenvironment around the cells, altering gene expression, involving agents that damage DNA (generating genomic instability) and may include the chemotherapies that eliminate some cells while letting other clonal populations to expand. Cell colors infer to populations of cells with distinct genomic characteristics. '*X*' indicates dead cells. *Grey* boxes (*Niches*) indicate individual environmental conditions within which clones can survive, including as metastatic cells. Some clones (in *yellow*) may be able to fit and evolve in multiple niches. (Figure based on Nowell [26])

than half of the tumor cells, although minimal expansion of these subclones occurs until many hundreds to thousands of mutations have accumulated. This invokes a model of long-lived, but sparse, lineages of cells passively accumulating mutations until poised for tumor dominance. Expansion of the dominant subclone to a detectable tumor mass may therefore represent the final rate-limiting step in breast cancer's development, triggering diagnosis.

Key features of tumor progression, which were noted early on, are heterogeneity within, and between tumors, and the evolution of this heterogeneity over time. Initially, it was considered that a population of cancer cells could include stable phenotypes that would endow some cells with increased ability to metastasize relative to other cells [28]. However, work in the 1980's introduced the idea of 'dynamic heterogeneity', i.e. the idea that variations within a cancer population might themselves be unstable [29–33]. By applying Luria-Delbruck fluctuation analysis techniques to expanding populations of cancer cell lines, these studies instead suggested that a key feature of highly aggressive cancer cell populations was a high degree of phenotypic 'plasticity', when compared to less aggressive populations. This plas-

ticity would endow cancer cells with the adaptability that would favor survival and proliferation in new microenvironments, such as found in ectopic, metastatic sites. Highly 'successful', aggressive cancer cell populations thus may have an increased capacity to generate transient variants that are able to survive and form metastatic colonies. This 'plasticity' is a population-based phenomenon, giving rise perhaps stochastically to individual cells, at any moment in time (and position in the body), that are able to thrive as metastasis-initiating cells. Recently, Meacham and Morrison have discussed the idea of tumor cell plasticity in the context of the cancer stem cell concept, supporting the idea that plasticity of cancer cell populations could support progression to more aggressive states [34].

One might ask why cancer occurs at all, given the cellular surveillance and repair pathways tasked with maintaining genomic and cellular integrity. In their review on clonal evolution in cancer, Greaves and Maley have posited that cells in tissue ecosystem habitats have evolved over millennia to ensure multicellular function of the collective cells comprising the individual while restraining clonal expansion of renegade cells [35]. Thus, a fine balance is struck such that mechanisms of self-renewal, proliferation and growth are regulated within limits that permit their survival, particularly in response to environmental change or damage, without 'excess' leeway in these responses that could tip survival in favor of genetically damaged, rogue cells that can proliferate uncontrollably and put the multicellular organism at risk. As a consequence, extensive and interconnected cellular pathways regulate the cell cycle, proliferation, DNA repair, senescence and differentiation of cells, in an effort to maintain the functionality of the multicellular cell collective, while weeding out any rogue cells that begin to deviate from their appropriate programming. Clinically benign cancer cells that do come into existence have gained and maintained (epi) genetic changes providing a selective growth advantage for those individual cells so that they may eventually expand into multiple clones that co-populate the primary tumor site. It is the exposure to cues from a permissive microenvironment acting on these variant cells within that population that eventually leads to the expansion of a cancer cell clone that is 'successful' in that context of that novel environment.

The challenges of treating metastatic disease are due in good measure to this heterogeneity described above. Recent evidence has suggested that such intratumoral heterogeneity occurs both in the primary tumor and within the metastases themselves, hindering personalized-medicine strategies that depend on results from single tumor-biopsy samples. The literature provides evidence of clonal heterogeneity, genomic diversity and in some cases, the emergence of treatment-resistant subclones within primary tumors and metastases. For example, tumor specimens analyzed from patients with high-grade serous ovarian cancer (HGSC), exhibited widespread intratumoral variation in mutation, copy number and gene expression profiles, with key driver alterations in genes present in only a subset of samples [36]. Furthermore, reconstruction of evolutionary histories indicated that diversity may arise at early stages of tumorigenesis. Similarly, primary triple-negative breast cancers (TNBCs) have been shown to exhibit a wide and continuous spectrum of genomic evolution. While certain somatic mutations (TP53, PIK3CA and PTEN)

seem to be clonally dominant compared to other genes, in some tumors their clonal frequencies are incompatible with founder status [37]. This same group showed that single nucleotide mutational heterogeneity occurred in ER (a)-positive metastatic lobular breast cancer, with multiple mutations identified in metastases that were not identified in the DNA from the primary tumor of the same patient, which arose 9 years earlier [38]. In addition, Yachida and colleagues have shown that clonal heterogeneity contributes to pancreatic cancer progression, in that distant metastases arose from clones that were genetically distinct from the parental clones, yet these metastatic subclones were also present within the primary carcinoma [39]. This suggests that from the mixture of unique but related subclones within a tumor, some clones can preferentially expand and seed future metastatic populations.

In their study on primary and metastatic renal cell carcinomas, Gerlinger and colleagues integrated data from several genomic platforms to show branched evolutionary tumor growth across multiple regions with primary tumors and associated with their metastases [40]. Mutational intratumor heterogeneity was seen for multiple tumor-suppressor genes, with several genes (SETD2, PTEN, and KDM5C) undergoing multiple distinct and spatially separated inactivating mutations within a single tumor. These genomic changes suggested that convergent phenotypic evolution had occurred. Furthermore, a phylogenetic tree could be generated that revealed that metastatic tumors were derived from one of two subclone branches within one sector of the primary tumor, while the other branch diversified into other primary tumor regions. The observation that mutations shared with metastatic sites were detected at higher frequencies than were mutations shared with other primary-tumor regions, has implications for diagnosis and treatment, in that single tumor-biopsy samples likely underestimate the tumor genomics landscape.

Translating Heterogeneity into the Clinic: Tracking and Attacking a Moving Target

Significant gains in knowledge have been made regarding the molecular causes of cancer and in identifying potential targets to attack that are clinically relevant to finding a cure [1, 2]. Yet, as previously described, any overall successes have been muted by the fundamental nature of cancer as a highly mutable set of diseases that are complex intra-tumorally, within a patient (and among patients) and temporally, as tumors evolve and as metastases develop. Furthermore, patients can differentially respond to treatments (based on their cancer genotypes), and likely will experience reoccurrence as new cancer cell subclones repopulate tissue niches abandoned by clones that have succumbed to therapies, or find new previously un-hospitable niches. How then, to outsmart an adversary that constantly camouflages itself and changes when detected?

One strategy is to gather as much information as possible about the tumor prior to treatment. To date, most treatments have failed with respect to long term survival because in many cases the diagnostic criteria have been simplistic (unimodal; i.e.

ER status, HER2 status, recorded as ‘positive’ or ‘negative’, etc.) thereby failing to accurately characterize a tumor, its inherent heterogeneity and/or its subtypes and somatic driver mutations. Confounding the situation, treatment decisions in the metastatic setting are often based on features that were noted in the primary tumor, although there is a growing recognition of the importance of biopsy and characterizing of metastases, when feasible (e.g., [41, 42]). To this end, attempts to define cancer subgroups and their molecular drivers have recently been published, which in many cases involve generating integrated views of the genome and transcriptome from representative numbers of patients [43–46]. For example, Yachida and colleagues showed in pancreatic cancer that the number of intragenic mutations in KRAS, CDKN2A and TP53 and immunolabeled for CDKN2A, TP53 and SMAD4 protein products in each carcinoma were correlated to clinicopathologic feature [39]. Carcinomas with only one to two driver alterations were enriched for those patients with the longest survival [39]. As well, Curtis and colleagues undertook an integrated analysis of copy number and gene expression in 2000 primary breast tumors and provided a novel molecular stratification of the breast cancer population [47]. These ten integrative subgroups overlapped somewhat with existing PAM50 subgroups and also had distinct clinical outcomes, particular in the context of long term survival. However, their ten subgroups still maintained certain degrees of heterogeneity within their tumor classification.

The keys to these studies have been the use and integration of multiple, high resolution platforms that include but are not limited to exome sequencing, genomic deep sequencing, copy number analysis and gene expression analyses [43]. Furthermore, there is the necessity to undertake multi-regional analysis to identify spatially distinct tumor regions [40]. Correlating spatially and temporally separated tumor specimens within and across patient samples can map tumor diversity within patients, define causative driver mutations, expose potential evolutionary trajectories prior to treatment and better inform personalized therapies in those patients throughout the treatment course [36]. As well, the use and integration of newer technologies to map epigenetic changes at the microRNA and DNA methylation levels will further inform therapeutic choices [48, 49].

Ultimately, two scenarios for success are possible. First, the full weight of these technologies may identify essential driver mutations that may be critical to the survival of the cancer and, once therapeutically targeted, could lead to clinical success with the obliteration of that disease (possessing that unique genomic signature) in that individual. More likely, however, is that such a driver mutation may not be essential to cells in all cancer clones, leaving the possibility of relapse after aggressive treatment due to expansion of a new cancer from the remaining clonal populations in the primary tumor or from until-now dormant metastases (Fig. 1.2). Hence, when necessary, the concept of ‘cure’ must include developing strategies to turn cancer into a chronic disease. This will necessitate several steps. First, early diagnosis is necessary, since by definition this suggests that clonal heterogeneity is less extensive and the emergence of metastatic cells has been minimized. In addition, early diagnosis should be augmented by manipulating the patient’s environment by minimizing environmental exposures (i.e. UV, ionizing radiation, carcinogen exposure)

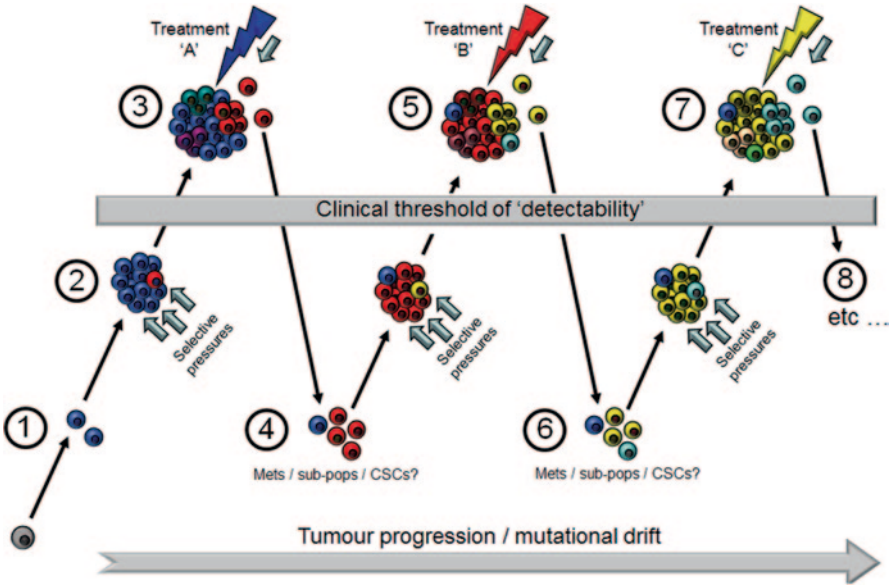

Fig. 1.2 Clinical relevance of clonal heterogeneity and tumor progression. Here the branching evolution model of cancer cells is overlaid with a clinical context. This model implies that at different times during the course of their disease, patients have different cancers at the genomic level that must be targeted appropriately. Cancer cell clones expand from a progenitor (*Steps 1, 2*), eventually becoming detectable clinically (*Step 3*), and are assessed and treated on the basis of characteristics related to the major clone in the tumor at that point in time. Tumor response to treatment may suggest treatment success, however surviving cells (*Step 4*) may eventually expand until cancer relapse is confirmed. The new, genomically unique tumors require different treatment strategies (*Step 5*; *different colored lightning bolt*). This process may repeat itself (*Steps 6, 7, 8*) as new cancer clones arise

that may contribute to genomic instability and further drive clonal evolution in pre-existing dormant or residual cancer cells, post-treatment. Second, continual surveillance is essential. Promising new methods involving the isolation of circulating tumor cells can provide a real-time liquid biopsy that allows assessment of genetic drift, molecular characterization and identification of actionable genomic targets, within the total population of cancer cells present in the body [50]. In addition, the presence of cell-free tumor-specific DNA in the peripheral blood from patients with metastatic disease can also be used to identify alterations in tumor burden and to monitor therapy response [51].

To conclude, our research efforts to date have led to great strides being made in acquiring mechanistic knowledge related to cancer biology, the synthesis of critical conceptual paradigms such as the cancer hallmarks, and the application of new genomic technologies that can be applied to the growing field of personalized medicine. The role of clonal heterogeneity in cancer offers a conceptual framework within which to consider cancer as an ever-evolving entity requiring integrative approaches that map cancer evolution both spatially and temporally. In some cases,

these integrative approaches may identity novel pathways and hallmarks for which off-the-shelf agents do not exist, opening new treatment paradigms for investigation and drug development. Ironically, the present challenges we experience in understanding clonal heterogeneity as a mechanism underpinning metastatic progression may ultimately offer the best opportunities for long term successes in treating cancer patients. Translation of this knowledge into the clinic continues, but as is the case in learning any new language, we are still developing our vocabulary.

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Chapter 2
The Generation, Detection, and Prevention of Genomic Instability During Cancer Progression and Metastasis

Helen Chen, Christopher Maxwell and Marisa Connell

Abstract Genome stability is tightly regulated through the cell cycle. Aberrations in genome structure and sequence are a hallmark of malignancy and these changes can allow abnormal cells to escape the regulatory mechanisms that would otherwise direct these cells into apoptosis or senescence. When genome instability occurs, it can happen as large or small structural changes in the genome, changes in gene expression, or even changes at the epigenetic level. There are many environmental factors that can induce DNA damage and strain the machinery that is responsible for maintaining genome stability. In some cases, such as UV light or chemical carcinogens, it is possible to avoid these factors and thus reduce the risk of cancer. But, in other instances, hereditary mutations impair the function of genes and their products, which normally protect the stability of the genome. While genomic instability offers selective advantages to the tumor, the tumor-specific loss of these pathways may provide therapeutic opportunities, which could be personalized through knowledge of the specific types of genomic instability that characterize an individual's tumor.

Keywords Genomic instability · Epigenome stability · DNA damage

Abbreviations

BER	Base excision repair
BFB	Break fusion break
CDK	Cyclin dependent kinase
CGH	Comparative genomic hybridization
CIN	Chromosome instability
CpG	C-phosphate-G
CRC	Colorectal cancer

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DDR	DNA damage response
DNA	Deoxynucleic acid
DSB	Double strand break
EMT	Epithelial to mesenchymal transition
HDR	Homology directed repair
LOH	Loss of heterozygosity
MET	Mesenchymal to epithelial transition
MIN or MSI,	Microsatellite instability
MMR	Mismatch repair
mtDNA	Mitochondrial DNA
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NIN	Nucleotide instability
PCR	Polymerase chain reaction
SAC	Spindle assembly checkpoint
UV	Ultraviolet

Introduction

Cancer is a genetic disease. Tumor cells contain multiple mutations, ranging from single nucleotide changes to large-scale structural and numerical alterations of chromosomes. Collectively, these mutations are referred to as genome instability, which may be predisposed through inherited, germ-line mutations, as in the case of *p53, BRCA1* and *BRCA2*, or acquired as somatic mutations throughout an individual's lifetime. Among the currently known cancer causing genes, 82 are associated with germ-line mutations, 474 are associated with somatic mutations and 513 are associated with chromosomal alterations [1]. These mutations promote cell growth, inhibit cell death and are propagated through accelerated cell division. Moreover, specific genetic alterations among cancer cells can give rise to sub-populations of cells with growth advantages, as in the case of a metastatic cancer cell or a cancer stem cell. These aggressive cells often arise during later stages of tumorigenesis, and their genome may significantly differ from the initial tumor population. However, regardless of how or when these genetic mutations are acquired, alteration of critical genes can change a once-normal cell into a cancerous cell that divides uncontrollably, and additional genomic changes may allow them to gain further growth advantages and eventually spread throughout the body. Thus, the molecular process behind tumorigenesis can be viewed as the accumulation and evolution of genomic alterations.

During the life cycle of a normal cell, genome maintenance is tightly regulated to prevent neoplastic transformation or tumorigenesis. There are various caretaker processes throughout the cell cycle that strategically minimize genome instability, including high-fidelity DNA replication, accurate chromosome segregation and cell

cycle checkpoints. In addition, other mechanisms, such as DNA damage response (DDR) pathways, telomere stability and epigenome maintenance, prevent non-mutational genetic changes. Defects in these gene products and processes will compromise their ability to monitor genomic alterations and execute the appropriate damage responses, which include repair, induction of senescence or apoptosis. In most cases, alteration of a particular gene product is not sufficient for transformation but may fast-track a subset of pre-cancer cells to acquire additional genomic changes that allow them to gain further growth advantages. Accumulation rates and the types of genomic alterations may vary in distinct subsets of cancer cells, contributing to the heterogeneity observed in cancer.

The notion of genomic instability as a hallmark of malignancy has intrigued cancer biologists for over a century, from Theodore Boveri's hypothesis that highlighted chromosomal aberrations as the cause of cancer [2, 3], to the discovery of the "Philadelphia chromosome" that leads to the activation of the *Abl* gene [4] and then the identification of the first familial breast cancer susceptibility gene, *BRCA1* [5, 6]. High-resolution arrays, such as comparative genomic hybridization (CGH) and whole-genome sequencing, have identified recurrent alterations as well as genomic heterogeneity within clinically similar cancers. For example, genomic and epigenomic profiling allows for more precise classification of breast cancer subtypes, as well as the prediction of subtype-specific therapeutic targets [7].

However, despite our technological advances, we are still baffled by questions posited following Boveri's observation of genetic imbalances in sea-urchin eggs, such as: (1) how and when does genome instability occur?; (2) how many mutations does it take to cause cancer?; (3) is genome instability the driver for tumorigenesis, or simply a passenger of disease progression?; (4) what role does genome instability play in cancer evolution and metastasis?; and (5) how do we take advantage of this shared trait of all cancer cells to uncover new paradigms for prevention, diagnosis and responsive therapy?

Genomic Instability in Cancer

Genomic instability is a characteristic of all cancers and encompasses a variety of genetic alterations ranging from single nucleotide differences to large-scale changes at the chromosomal levels [8]. Genomic instability can be divided into three categories based on the degree and type of genetic alteration.

a. Nucleotide Instability (NIN)

Nucleotide instability includes base substitutions, deletions and insertions of one or a few nucleotides. These alterations result when errors occur during DNA replication or when the repair machinery malfunctions, such as nucleotide excision repair (NER) and base excision repair (BER) [8]. These alterations can cause dramatic

changes to gene structure and expression. For example, missense mutation in the K-*ras* gene occur in over 80 % of primary exocrine pancreatic tumors and their corresponding metastases [9]. NIN may also arise in mitochondrial DNA (mtDNA), and instability of mtDNA occurs in a variety of human cancers, including colorectal (CRC), gastric and lung [10].

b. Microsatellite Instability (MIN or MSI)

Microsatellites are short, two to six base-pair simple, or tandem, sequence repeats of DNA located throughout the genome. MIN occurs when the DNA mismatch repair (MMR) system is impaired, which results in the expansion, contraction, deletion and random insertion of microsatellites [11]. The MMR system identifies and binds to the mismatch, and excises the erroneous nucleotide and repairs the mismatch. MIN has been documented in a variety of cancers, including gastric, ovarian, lung, endometrial, and CRC [12–17]. To date, five MIN markers have been recommended by the National Cancer Institute for disease screening in patients susceptible to Lynch syndrome. MIN occurs in approximately 15 % of all CRC, which include both hereditary and sporadic forms of CRC, and are associated with a better prognosis than non-MSI tumors [18].

c. Chromosomal Instability (CIN)

CIN is the most prevalent form of genomic instability, observed in over 90 % of all malignancies, and is detected throughout the entire neoplastic transformation process, from premalignant lesions to metastatic lesions [19]. For example, chromosome 10 is often lost in glioblastomas, resulting in the inactivation of the tumor-suppressor gene, *PTEN* [20]. CIN refers to alterations of segments of chromosomes, or whole chromosomes, in terms of their structure or number, including amplifications, deletions, translocations, insertions, inversions, loss of heterozygosity (LOH) and homozygous deletions [8]. Change in chromosome numbers is a condition known as aneuploidy, while chromosome translocation involves the fusion of different chromosomes, or of two distant segments on the same chromosome, resulting in a chimeric chromosome [8]. Finally, the ploidy of the entire genome may deviate from the standard 2N complement of chromosomes and give rise to polyploid cells. CIN in tumor cells alters the expression of thousands of genes, which may help to explain why CIN tumors have a poorer prognosis than either MIN or NIN tumors [21].

Telomere Maintenance in Cancer

Telomeres are unique G-rich repetitive sequences (TTAGGG) located at the ends of the eukaryotic chromosomes [22, 23]. Telomeres protect the ends of the chromosomes and preserve their integrity [24]. As telomeres will gradually shorten with

each round of cell division due to chromosome end-processing, telomere maintenance is necessary for continuous cell division and the addition of telomeric repeats must be catalyzed by telomerase reverse transcriptase (hTERT) [25]. In most somatic cells, insufficient telomerase activity will lead to telomere shortening and the induction of cellular senescence [26]. Thus, telomerase inhibition may be a promising target in cancer therapy [27–29].

Inhibition of telomerase activity in a variety of cancer cell lines resulted in accelerated telomere shortening, cell death and differentiation [27–29]. However, due to mutations in the tumor suppressor p53, cancer cells frequently bypass senescence, and continue to divide, which promotes genome instability due to chromosome fusions [24]. Telomere shortening will signal the DDR pathway [30, 31], which may promote CIN, tumor initiation and progression [32, 33]. As an example, telomerase knockout ($mTR^{-/-}$) mice significantly increase the incidence of spontaneous tumor formation (4-6 fold compared to the wild-type population) due to telomere shortening [34]. These $mTR^{-/-}$tumors have 3–18 fold more chromosome fusions and a two-fold increase in aneuploidy compared to $mTR^{+/+}$ tumors [34]. Conversely, telomerase activity also promotes tumorigenesis [35]. Thus, telomere-associated tumorigenic processes are stage specific; telomere shortening is critical to the accumulation of genetic mutations needed for cancer initiation while, in later stages, telomerase activity promotes cell proliferation necessary for cancer expansion and metastasis.

Epigenome Instability in Cancer

Epigenetics is defined as all heritable changes that may modify gene expression without affecting the primary DNA sequence, such as DNA methylation and chromatin remodeling. DNA methylation and histone modification are the most well understood epigenetic processes, and preservation of these epigenetic markers during cell division is vital for gene regulation. In cancer, epimutations may result in dysregulation of critical genes either independently, or in conjunction with deleterious genetic mutations. Moreover, these epimutations are inherited through clonal expansion, which can promote cancer initiation and progression [36, 37]. Although it is unclear whether these epigenetic alterations are causative or a consequence of tumorigenesis, it is certain that epigenome instability is a prominent feature in cancer.

a. DNA Methylation in Cancer

DNA methylation is a covalent modification where a methyl group is added to the carbon-5 position of cytosine nucleotides followed by a guanine (CpG) via a group of DNA methyltransferase (DNMT) enzymes [38]. DNA methylation can result in gene silencing, and occurs primarily at CpG islands within heterochromatin [38]. An individual cell's DNA methylation pattern is important for its ability to establish

tissue-specific gene expression or maintain pluripotency. Global DNA hypomethylation and site-specific hypermethylation are two key epimutations that occur in cancer [36]. DNA hypomethylation can lead to aberrant overexpression of oncogenes, such as *R-Ras* in gastric cancer, *S-100* in colon cancer [39] and *IGF2* in Wilms' tumor [40]. Hypomethylation of retrotransposons and specific repeat sequences can also result in genomic instability by promoting chromosome rearrangements [41, 42]. Global hypomethylation in many cancers, such as in the breast, brain and cervix, is positively correlated with increased grades of malignancy [43]. On the other hand, hypermethylation contributes to tumorigenesis by silencing the transcription of tumor suppressor genes, such as *Rb, BRCA1* and *p16* [36], which may act as a second hit as described by Knudson's hypothesis [44].

b. Histone Modification in Cancer

At the chromatin level, modifications of the four core histones, H2A, H2B, H3 and H4, regulate gene expression. These histone modifications include acetylation, deacetylation, methylation, phosphorylation and ubiquitination [45]. For example, methylation of histone H3 lysine 9 (H3K9), H3K27, H4K20, and H3 arginine 2 (R2) are indicative of heterochromatin formation and transcriptional repression, while acetylation of H3 and H4 promote euchromatin formation and gene transcription [45].

The global loss of H4K16ac and H4K20me3 has been recognized as a hallmark of almost all human cancers [46], while other changes in histone modification are used as prognostic markers [47–51]. These epigenetic marks are catalyzed by various histone-modifying enzymes, such as lysine methyltransferases, arginine methyltransferases, serine-threonine kinases, histone deacetylases and acetyltransferases [45]. Aberrant expression of these enzymes results in changes in histone modification, which can dysregulate subsequent DNA repair, gene transcription and growth-promotion. For example, histone deacetylase 1 (HDAC1) is reduced in gastric cancer, while reduced HDAC5 and HDAC10 associate with poor prognosis in lung cancer [52]. Changes in histone modification patterns can also lead to an overall change in the chromatin structure, which increases the risk of translocation of random transposons and chromosome breakage during mitosis.

c. Nucleosome Remodeling in Cancer

Nucleosomes are the basic unit for DNA packaging, and the architecture of these nucleosomes determines chromatin structure and the accessibility of regulatory DNA sequences to transcription factors [53]. Nucleosome remodeling includes the repositioning of the nucleosome, as well as changes in the content of histone proteins within the nucleosome [53]. In a cancer epigenome, the sliding of pre-existing nucleosomes and incorporation of new nucleosomes, determines chromatin

accessibility and gene expression [53]. For example, in hereditary nonpolyposis colon cancer, three *de novo* nucleosomes are present within the promoter CpG island of the *MLH1* gene, which is a homolog of the E. coli DNA mismatch repair gene [54]. Moreover, substitution of the canonical histone proteins with non-canonical histone proteins within the nucleosome can also influence nucleosome occupancy and gene expression [53, 54].

Crosstalk Between Genomic and Epigenomic Instability

Despite the differences between DNA mutations and epimutations in cancer, their effects on gene regulation are ultimately the same. Indeed, these two processes work symbiotically and synergistically with genetic mutations in epigenetic regulators resulting in epimutations, and vice versa. For example, hypermethylation of key tumor suppressor genes, such as *Rb, PTEN, BRCA1* and *MLH1,* is a common phenomenon observed in cancers [54, 55]. On the other hand, genetic mutations of key epigenetic modifiers, such as different DNMTs, histone modification proteins and chromatin remodeling proteins, can also impair epigenome stability [56]. Both genomic instability and epigenomic instability are phenomena observed in almost all cancers at every stage of cancer evolution [56, 57]. The crosstalk between these two phenomena adds complexity to cancer biology but also offers potential novel therapeutic targets [58, 59].

Mechanisms for Genome and Epigenome Stability

For a normal cell, the end goal of cell division is to accurately duplicate its genome and distribute its genetic material evenly between the two daughter cells. To maintain genome integrity during proliferation, four major mechanisms are in place: (1) high-fidelity DNA replication during S-phase; (2) accurate chromosome segregation during mitosis; (3) sporadic DDR throughout the cell cycle; and (4) quality control checkpoints regulating cell cycle progression (Fig. 2.1).

a. Error-Free DNA Replication

The duplication of the genome during S-phase is under tight regulation to ensure copy number and temporal (once per cell cycle) control, known as replication licensing [60, 61]. Replication licensing is highly conserved throughout evolution and is regulated by cyclin dependent kinases (CDKs). Replication is initiated with the assembly of a pre-replication complex assembled at the replication origin. Untimely initiation can cause re-replication and aneuploidy, and low replication-initiation density can lead to unfinished replication of the whole genome [62]. During

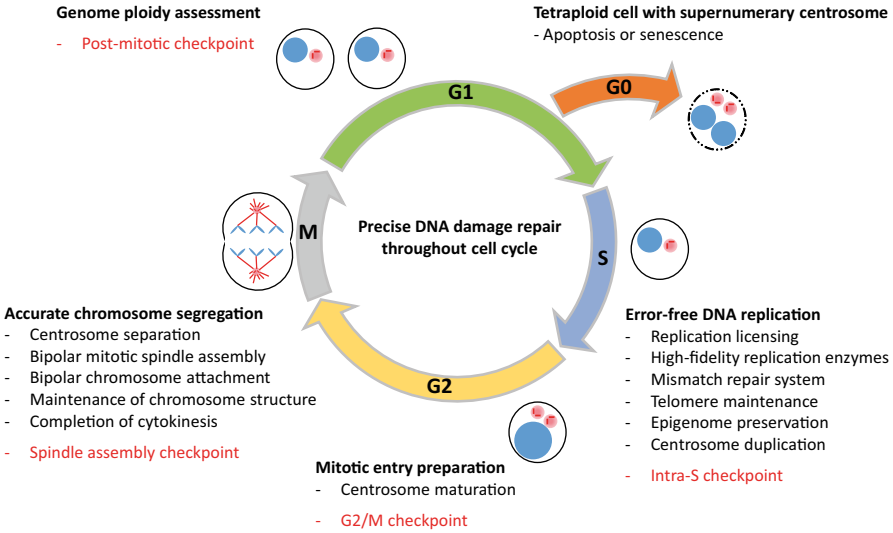

Fig. 2.1 Mechanisms to prevent genome instability throughout the cell cycle**.** The cell employs many mechanisms through the cell cycle to prevent genome instability. The important processes that occur during each phase of the cell cycle are highlighted in *black* while cell cycle checkpoints are highlighted in *red*

the extension phase of DNA replication, replication forks often pause and restart to ensure replication completion. Failure to restart these replication forks will result in double-strand breaks, single-strand DNA breaks and hyper-recombination, which all need to be resolved by the DDR pathway before mitotic entry to maintain genome stability [62]. Replication on the lagging strand occurs through the production of multiple short Okazaki fragments, these fragments need to be ligated to maintain the structure of the lagging strand [63]. Moreover, there is an RNA primer and a short DNA segment at the 5' end of each Okazaki fragment, which all needs to be removed prior to ligation of the Okazaki fragment [63]. Failure to remove these elements affects Okazaki fragment maturation, and can result in genomic alterations [63]. El-Khamisy and colleagues (Chapter 3) provide an in-depth discussion of the molecular control of DDR pathways.

b. Bipolar Spindle Assembly and Accurate Chromosome Segregation During Mitosis

After error-free DNA replication during S-phase, genome stability requires accurate chromosome segregation during mitosis. Chromosome segregation is a multi-step process, which requires faithful centrosome duplication, assembly of a bipolar mitotic spindle, proper attachment between the chromosomes and the mitotic spindle, and completion of cytokinesis. Dysregulation of any of these processes may result in aneuploidy.

The centrosome is the primary microtubule organizing center in the eukaryotic cell and is composed of a pair of centrioles surrounded by a cloud of proteins that promote microtubule nucleation and cilia formation [64, 65]. During mitosis, the centrosome directs mitotic spindle formation at the poles, which contributes to faithful chromosome segregation and cleavage furrow formation [65, 66]. Since the mitotic spindle is a bipolar structure, the centrosome must be duplicated only once during every cell cycle and in a semi-conservative manner. Thus, centrosome duplication occurs simultaneously with DNA replication during S-phase, and is tightly regulated.

Centrosome amplification, the presence of more than two centrosomes, is a common characteristic of almost all cancers, which frequently accompanies aberrant chromosome numbers [67]. The presence of supernumerary centrosomes during mitosis is problematic as it can cause multipolar mitoses, chromosome missegregation, cytokinesis failure and subsequent chromosome imbalances that promote tumorigenesis [67]. Due to dysregulation of various tumor suppressors and oncogenes, supernumerary centrosomes can arise from centrosome overduplication, *de novo* assembly, or previous mitotic failure [64, 67–70]. Centrosome duplication errors result from multiple daughter centrioles forming around a single mother centriole, or multiple rounds of centrosome duplication and paired centriole duplication [64, 67–70]. Extra centrosomes can also be made from *de novo* assembly, where an acentriolar centrosome is made without a pre-existing centriolar centrosome as the template [71–73]. Various mitotic catastrophes can also result in supernumerary centrosomes. For example, loss of mitotic spindle integrity due to inadequate crosslinking by microtubule-associated proteins can cause a centrosome to split with each individual centriole capable of functioning as a spindle pole, or the formation of an acentriolar spindle pole [74–79]; aborted cytokinesis, mitotic slippage and DNA damage are additional mechanisms for cancer cells to obtain extra centrosomes [80].

The presence of supernumerary centrosomes may not be good news even for cancer cells, as catastrophic aneuploidy can result in nonviable daughter cells [81]. However, cancer cells have developed mechanisms that overcome this fate by clustering multiple centrosomes [70, 81–84]. These mechanisms can dampen high level aneuploidy and extreme CIN to avoid programmed cell death [85, 86]. Given that centrosome clustering may be advantageous for the survival of cancer cells, this process may be an attractive and specific therapeutic target [83, 84, 87].

The mitotic spindle, a microtubule-based bipolar structure, is the cellular machinery responsible for the distribution of genetic material between the progeny cells. The mitotic spindle will capture the chromosomes at the kinetochores, align them along the cell's equator, and then pull them towards each spindle pole. Mitotic spindle integrity is a vital tumor suppressor pathway that requires microtubule crosslinking and motor protein movements to establish spindle length, position and orientation [88–92]. Bipolar chromosome attachment generates tension across the sister kinetochores, and this signals for chromatid separation and mitotic progression. Erroneous attachments engage the spindle assembly checkpoint (SAC), which prevents the onset of anaphase until the errors are corrected. However, if the SAC

fails to sense the misattached chromosome, the misattached/unattached chromatid will lag behind, which could mean the loss or gain of whole or part of a chromosome in the daughter cells [93, 94].

Cytokinesis partitions the cytoplasm of the mother cell between the two daughter cells concurrent with nuclear membrane formation. The process of cytokinesis includes the specification of the cleavage plane, ingression of the cleavage furrow, and abscission of the midbody, which are all heavily dependent on microtubule and actin networks. Cytokinesis failure may occur when mitotic spindle elongation and positioning are perturbed, thus disrupting delivery of activation signals to the cortex for cleavage plane formation [95]. Aborted cytokinesis will result in a cell with double the genetic material (tetraploid) and double the number of centrosomes. This tetraploid state is considered an intermediate for the aneuploid state frequently observed in cancer cells [96], and the fate of these tetraploid cells heavily depends on p53 [97–100].

Telomere-associated genome instability occurs when inappropriate DNA repair (i.e. non-homologous end joining (NHEJ) and homology directed repair (HDR)) takes place at dysfunctional telomeres. Inappropriate NHEJ produces dicentric chromosomes [30, 31], which are highly unstable with a tendency to break during mitosis [30, 31]. Repair of these new breaks can propagate new dicentric chromosome formation [30, 31]. This process is called the breakage-fusion-bridge (BFB) cycle, which can continue over multiple cell divisions and generate complex chromosomal rearrangements [101, 102]. This BFB cycle generally has three outcomes: (1) LOH due to breakage sites or asymmetric segregation of chromosomes after breakage; (2) gene amplification due to breakage sites; and (3) unbalanced translocations [30]. During HDR, inversions, deletions and translocations are generated when recombination occurs at a highly homologous stretch of telomeric DNA either on the same chromosome or between two different chromosomes [30]. Activation of the HDR pathway increases the formation of anaphase bridges, which must be resolved to prevent cytokinesis failure and aneuploid progenies [103]. El-Khamisy and colleagues (Chap. 3) provide an in-depth discussion of the molecular control of NHEJ and HDR pathways.

c. Cell Cycle Checkpoints

Cell cycle checkpoints are the cell's quality control mechanisms that coordinate the progression of the cell cycle and delay entry into the next stage in the presence of genome damage. Under circumstances where the detected genome damage is not fixed, these checkpoints will trigger senescence, mitotic catastrophe or apoptosis to eliminate high-risk cells. The G1/S (and post-mitotic) and G2/M checkpoints can recognize DNA lesions and abnormal chromosome structures, whereas the spindle assembly checkpoint is an intra-mitosis surveillance mechanism that monitors the interaction between chromosomes and the mitotic spindle.

The post-mitotic checkpoint monitors the state of the genome after the previous round of the cell cycle, and delays replication in the presence of damaged DNA.

This checkpoint is heavily dependent on p53, which is the most commonly mutated gene in cancer [104]. Cytokinesis failure during the previous round of cell division will result in tetraploid progeny with supernumerary centrosomes, a so-called "double-value cell" [3]. Tetraploid intermediates may play an important role in tumorigenesis [96, 105–109], and precede aneuploidy cells in a premalignant condition called Barrett's oesophagus [106]. Tetraploid cells isolated from $p53^{-/-}$ mouse mammary epithelial cells form tumours in nude mice [100].

p53 is a phosphoprotein found at low levels in normal cells. Upon DNA damage, p53 is stabilized and protein turnover is inhibited through phosphorylation by ATM (Ataxia telangiectasia mutated) and Chk1 (checkpoint kinase-1) [110–113]. The key transcriptional target of p53 is the cdk inhibitor, $p21^{Waf1}$ [111]. $p21^{Waf1}$ binds to the cyclin E/cdk2 complex to inactivate the kinase, which prevents the initiation of DNA synthesis and blocks G1-S progression [114]. Prolonged G1 arrest by p53 can result in cellular apoptosis or senescence [115, 116]. When p53 is compromised, tetraploid cells continue to proliferate leading to increasingly error prone divisions and aneuploid progeny [96].

The G2/M checkpoint senses DSBs and facilitates their repair by preventing mitotic entry. The checkpoint inhibits the mitosis-promoting complex, cyclin B1/cdc2 kinase, through inhibitory phosphorylation of cdc2 kinase [117–119]. If the G2/M arrest fails, the damaged chromosome will enter mitosis and may initiate a BFB cycle that can generate complex chromosomal rearrangements [101, 102]. Alternatively, the acentromeric fragment of the broken chromosome may be degraded, while the centromeric fragment may be repaired through the addition of a telomere region [120].

The spindle assembly checkpoint is a unique surveillance mechanism that does not sense DNA damage, but rather serves as a preventative measure against genome instability. During mitosis, sister chromatids are kept together by the cohesion complex, which is degraded at the metaphase-anaphase transition in an ubiquitin-dependent manner by the anaphase promoting complex/cyclosome. Correct bipolar attachment of chromosomes generates tension across the sister chromatids at the centromeres [121, 122]; the assembly checkpoint generates a wait signal in the absence of tension due to erroneous attachment, such as syntelic (both sister chromatids are attached to the same pole), monotelic (only one sister chromatid is attached) and/or absence of any attachments from either sister chromatid [123]. Upon assembly checkpoint activation, the mitotic checkpoint complex is loaded onto the kinetochores of the misattached chromosome, which inhibits the anaphase promoting complex/cyclosome and allows the cell to fix the erroneous attachment [121, 123].

The assembly checkpoint involves extensive kinase signaling pathways to sense misattachments, delay chromosome segregation and correct attachment errors. For example, Aurora kinase B detects chromosome misattachments [124, 125], but Aurora kinase A can override the checkpoint and enable cells to enter anaphase despite misattached chromosomes [126, 127]. In order for all 23 pairs of chromosomes to establish bipolar attachment during metaphase, a delicate balance is needed between stabilization of the correct kinetochore-microtubule attachment and turnover of misattachments by dynamic spindle microtubules. Polo-like kinase 1 decreases

kinetochore–microtubule dynamics to stabilize initial attachments during prometaphase, and removal of Polo-like kinase 1 from kinetochores during metaphase is necessary to maintain microtubules dynamics [128]. Finally, the checkpoint kinase BUBR1 inhibits the anaphase promoting complex/cyclosome through interactions with the mitotic checkpoint complex and the motor protein CENPE, which is required for microtubule attachment at the kinetochores [129, 130].

Mosaic variegated aneuploidy (MVA; OMIM: 257300) is a rare, hereditary condition due to biallelic mutations in BUBR1 and characterized by mosaic aneuploidy that leads to developmental defects and predisposition to cancer [131–136]. Both frameshift and missense mutations in the gene encoding BUBR1 have been identified in MVA families [135, 136], which result in truncated transcripts and reduced protein abundance [137]. Reduction in BUBR1 levels affect chromosome attachment and assembly checkpoint stringency, which leads to premature chromatid separation. As a result, individuals with MVA have a high incidence of childhood cancers, such as Wilms' tumour, rhabdomyosarcoma and leukemia. The severity of the disease phenotype in MVA patients reinforces the notion that the assembly checkpoint is crucial for the prevention of genomic abnormalities and cancer.

Prevention, Detection, and Prognosis of Genome Instability

Preventing DNA damage and genome instability can reduce the risk of developing cancer. Although there is no one way to prevent genomic damage, measures can be taken to reduce one's exposure to non-inherited sources and limit one's cancer risk.

a. Non-Inherited Sources

Many environmental factors, such as exposure to carcinogens, viruses, and diet, may lead to genome instability and eventually cancer. In the following section, we will discuss some of these environmental factors, how they lead to genome instability, and some methods that reduce exposure to these risk factors.

People are exposed to radiation daily, particularly in the form of solar radiation (ultraviolet (UV) light). UV-A light causes indirect DNA damage by producing free radicals and reactive oxygen species that go on to damage DNA, while UV-B light directly damages DNA by causing the formation of pyrimidine dimers [138]. DNA damage from UV light is a causative factor for melanoma and other skin cancers [139]. Fortunately, it is easy to take steps, like using a sunscreen that protects against both UV-A and UV-B light, which can cut the risk of melanoma in half [140].

Chemical carcinogens are found in the environment and can arise from many different sources. Some chemical carcinogens, like air pollution, cannot be completely avoided and, in these cases, it is important to limit exposure as much as possible. Other chemical carcinogens, like cigarette smoke, can be completely avoided. Cigarette smoke contains more than 20 carcinogenic chemicals [141] and smoking

causes at least 80 % of lung cancers and 20–30 % of pancreatic cancers [142, 143]. Benzene, a carcinogen in cigarette smoke, is linked to leukemia and other blood-related cancers [144] and is known to cause genome instability by inducing DNA strand breaks and other chromosome damage [145].

Human papillomavirus (HPV) can induce cancer of the cervix, vulva, vagina, penis, oropharynx, and anus. HPV can be passed on to children during birth and has been implicated in cases of sporadic retinoblastoma in children [146]. HPV causes cancer through expression of virus proteins or oncoproteins that promote oncogenesis. In HPV, the E6 and E7 oncoproteins cause aberrant proliferation [147–149], leading to centrosome duplication and eventual genome instability. Ectopic expression of E6 and E7 leads to structural and numerical chromosomal abnormalities, respectively [150–152]; similar abnormalities, such as enlarged nuclei, multinucleate cells, and tripolar mitotic spindles, are observed in clinical samples of cervical lesions. Vaccines that protect against several HPV strains, including HPV-16 and HPV-18 [148, 153], have been developed and their use may significantly reduce the prevalence of HPV-induced cancers.

Diet impacts DDR pathways as certain nutrients act as necessary cofactors. A dietary lack of folate, for example, can lead to the misincorporation of uracil into the genome rather than thymidine [154], which induces chromosome strand breaks and/or impairs excision repair. A reduced dietary intake, or low tissue/plasma levels, of Vitamin B6 associates with a higher risk of developing cancer while Vitamin B12 (folic acid) deficiency is linked to DNA damage, such as chromosome breaks, micronuclei formation, and DNA hypomethylation [155]. Additionally, low dietary intake of calcium, folate, nicotinic acid, vitamin E, retinol, and β-carotene and high intake of panthothenic acid, biotin, and riboflavin have been associated with increased genomic instability [156]. With respect to β-carotene, sufficient intake in one's diet correlated to a lower risk of MSI-H types of colon cancer [157]. Together, these data highlight the importance of a well-balanced diet in the maintenance of genome integrity.

b. Diagnosis of Genome Instability

The type and level of genomic instability may offer opportunities to personalize therapies, which will be discussed in greater detail in Chaps. 9 and 10. Methods to quantify genomic instability, including the structure of the chromosomes, genomic sequences, and/or gene expression, each have intrinsic advantages and disadvantages. In the following sections, and in Table 2.1, we outline some of the common methods used to identify genome instability. Chapter 4 will discuss in more detail some new methods for measuring and modeling DNA damage.

b.i. Diagnosing Large Scale Aberrations

Karyotyping images and arrays all chromosomes to measure aneuploidy, chromosome breaks, translocations, and inversions [158, 159]. Different types of staining

Table 2.1 Methods of determining genomic instability

Large aberrations				
	Karyotype	FISH	Array-CGH	Flow cytometry
Technique	Isolate entire chromosomes and determine ploidy with dyes	Label cells with DNA dye and fluorescence determines ploidy	Hybridize non-tumor and tumor samples. Identify balanced or unbalanced signal	Label cells with probes that bind chromosomes
Aberrations detected	Aneuploidy, inversions, chromosome breaks, translocations	Changes in ploidy	Changes in copy number	Changes in ploidy
Advantages	High sensitivity for mosaic cultures can detect balanced aberrations		Better resolution	Very accurate for ploidy
Disadvantages	Low resolution	Not automated cell sectioning and overlap	Detection of mutations is limited Low sensitivity in mosaic cultures	Limited to ploidy
Small aberrations				
	ISSR-PCR	AP-PCR	SNP Array	Genome sequencing
Technique	Amplify microsatellite regions. Compare PCR products between tumor and non-tumor samples	DNA amplified using random primers. Footprint is compared between tumor and non-tumor	DNA fragments labelled, hybridized to an array with known SNPs	Isolate genomic DNA and sequence
Aberrations detected	MIN, amplifications, deletions, translocations, insertions	Amplifications, deletions, insertions translocations	LOH, copy number changes	All mutations
Advantages		Uses small amounts of sample. No prior sequence knowledge required	Detects copy neutral events	Multiple mutations at one time
Disadvantages	Exact region difficult to identify	Reproducibility	Low sensitivity in mosaic cultures	Cost

Table 2.1 (continued)

	Expression analysis
Technique	mRNA is labelled and hybridized to an array. Fluorescence is compared between tumor and non-tumor samples
Aberrations detected	Mutations that cause changes in gene expression, including epigenetic changes
Advantages	Can easily identify involved genes. Certain profiles have been linked to particular prognoses

can be used to identify different characteristics of the chromatin. For example, Giemsa stain binds to heterochromatin and the banding patterns will indicate insertions, deletions, and other abnormalities. Spectral karyotyping, a type of fluorescence *in situ* hybridization, employs multiple fluorescent probes to uniquely identify chromosome pairs and determine changes in ploidy and rearrangements. Array-CGH also detects changes in copy number, such as insertions, deletions, and amplifications [160, 161] by hybridizing fluorescently-labeled DNA isolated from cancer cells to fluorescently-labeled DNA isolated from non-cancer cell. This method has better resolution than karyotyping or fluorescence *in situ* hybridization, but it cannot detect mutations, such as inversions and translocations that do not change the copy number.

b. ii. Diagnosing Small Scale Aberrations

There are several polymerase chain reaction (PCR)-based assays that identify genomic instability. Inter-simple sequence repeat PCR (ISSR-PCR) uses primers that have regions homologous to dinucleotide repeats to amplify microsatellite regions of DNA [162–166]. By comparing the gains and losses in amplified DNA bands between tumor and normal tissues, regions of genome instability can be identified. The advantage of this method is that it can detect smaller alterations however it may be difficult to find, or sequence, the altered DNA region. Arbitrarily primed PCR (AP-PCR) uses primers composed of random sequences and low stringency conditions to create genome footprints for normal and tumor tissues, which allows detection sequence changes, insertions, deletions, and amplifications [167]. This technique uses small amounts of DNA, and allows one to reamplify, clone, and/or sequence the resulting bands which aids in the identification of abnormal tumor sequences [168], however specificity and reproducibility can be challenges. Single nucleotide polymorphism (SNP) arrays can detect LOH and copy number changes [169, 170]. Although similar to array-CGH, SNP arrays have an advantage in that they can detect copy-neutral events. Due to their high resolution (i.e. over 1.8 million markers that span the entire genome), SNP arrays enabled discoveries such as a reduced level of genome instability in hereditary as compared to sporadic cases of retinoblastoma, which

contrasted with a popular model for hereditary retinoblastoma [171]. However, SNP arrays may not be sufficient to identify mutations in mosaic cultures. Whole genome sequencing will identify point mutations [172–174], but its high cost precludes it broad clinical application at this time.

Genome instability can also be determined by gene expression analyses. Several different profiles can identify cancer cells and some of these profiles are also predictive of prognosis. Habermann et al. [175] identified a 12-gene expression signature that distinguished between breast cancers with high or low genomic instability [175]. This signature can also predict prognosis in other cancer types [176]. A CIN measure, using either the top 25 or top 70 genes (CIN25 and CIN70 signatures respectively), correlated gene expression to the "total functional aneuploidy" in data sets from many different tumor types [177].

c. Genomic Instability and Cancer Prognosis

Genome instability is typically correlated with a poor prognosis [175–177]. Higher CIN is indicative of a poor prognosis in early invasive luminal HER2-negative and node negative breast cancer. Moreover, the 3q8pq20 subtype of oral cancer is prone to chromosome abnormalities and a higher rate of metastasis than non-3q8pq20 patients. However, genome instability is not always correlated with a poor prognosis. In sporadic colorectal cancer, patients with microsatellite instable cancers have better prognosis. Moreover, some cancers appear to have a threshold where genome instability no longer indicates a poor prognosis. In some ER-negative breast cancers, the CIN MCD4 cohort had better survival than patients that were classified in the intermediate and lower MCD cohorts [178]. This improved outcome was also seen in MCD4 patients that were treated with adjuvant chemotherapy. Thus, genomic instability may empower tumor heterogeneity but it may also negatively impact biological fitness.

Genetic Changes and Cancer Evolution

Carcinomas undergo a series of morphological changes to promote local invasion and distant migration. In preparation for local dissemination, tumor cells may undergo an adaption known as the "epithelial-mesenchymal transition" (EMT), which allows these epithelial cells to invade nearby blood and lymphatic vessels and migrate to distant sites [179-181]. Activation of the EMT program in tumor cells requires changes to the genome and/or epigenome, which promotes the turnover of adherens junctions, the expression of extracellular matrix degrading enzymes, and increased cell motility among other changes. Once these metastatic cells have reached their potential secondary colonization sites, they must pass through a reversal process called the "mesenchymal-epithelial transition" (MET). In theory, tumor

cells undergoing reversible EMT and MET programs suggests the new tumor colony should be identical to the cells in the primary tumor. However, multiple studies have identified genetic and epigenetic heterogeneity in metastatic cells, suggesting that not all tumor cells are capable of initiating metastasis or able to revert back to their previous epithelial stage during MET [182–184].

The process of neoplastic progression is an evolutionary process, similar to that observed during speciation, where heritable genetic variations can promote the survival of tumor cells through clonal expansion [185–187]. Mutations and epimutations that favor increased proliferation and resistance to apoptosis are highly favorable during clonal expansion of neoplastic cells. For example, amplification of Aurora kinase A can override the assembly checkpoint to prevent mitotic arrest and promote chromosome aberrations [126]. The tumor microenvironment changes as the disease progresses; dense population growth and over-consumption of local resources add selective pressures for dispersal and metastasis. In most solid tumors, the center of the tumor mass is often necrotic, hypoxic and densely populated [188, 189]. Metastatic cells, however, leave the primary tumor site and colonize secondary sites, which require certain fitness advantages. An aneuploid genome, and the heterogeneity it engenders, has been shown to promote metastatic properties [190]. On the other hand, tumor cells at the primary site may be selected for their ability to cope with the stressful local environment through metabolic reprogramming. For example, hypoxic cancer cells uses glucose as fuel and produce lactate as a waste product, which serves as fuel for their oxygenated neighboring cells [191–193]. This intratumor symbiosis enables tumor cell cooperation that promotes tumor growth. Lastly, cancer therapies apply artificial selection pressures over the tumor cell population. Thus, the mosaic genome and epigenome in cancer cells may seem chaotic, but, in its chaos, may in fact coordinate disease progression.

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Chapter 3
DNA Damage Response Pathways in Cancer Predisposition and Progression

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Abstract Cells are continually challenged by DNA assaults from endogenous and exogenous sources. Without appropriate repair, DNA damage can cause genome instability and the development of various diseases such as cancer, Immunodeficiency, neurological abnormalities, and premature aging. To maintain genome integrity, cells have evolved highly conserved defense mechanisms, collectively known as the DNA damage response (DDR), to identify the lesions, signal their presence, and activate the appropriate DNA repair pathway. In this chapter, we will discuss the recent advances in DDR, cancers with heritable defect in DNA repair, and secondary cancers developed following treatment with chemotherapeutic drugs that damage the DNA. We will focus on four major repair pathways with particular attention to the exploitation of recent knowledge to improve cancer therapy.

Keywords Double-strand breaks · Single-strand breaks · Reactive oxygen species · Synthetic lethality · Homologous recombination · Non-homologous end joining · Mismatch repair · DNA end processing · Fanconi Anemia

Abbreviations

5′ dRP	5′ deoxyribose phosphate
8-oxodG	7,8 dihydro-8-oxo-2′-deoxyguanosine
8-oxoGua	8-oxo-7,8-dihydroguanine
9-1-1	Rad9-Rad1-Hus1
AML	Acute myeloid leukemia
A-NHEJ	Alternative NHEJ
APE-1:	Apurinic endonuclease
aCGH	Array comparative genomic hybridization
B-CLL	B-cell chronic lymphocytic leukemia
BER	Base excision repair

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BIR	Break-induced replication
BMF	Bone marrow failure
C-NHEJ	Classical NHEJ
CRC	Colorectal cancer
DDR	DNA damage response
dHJ	Double Holliday junction
DSB	Double strand break
DSBR	DSB repair
dsDNA	Double-stranded DNA
EC	Endometrial cancer
EDM	Exonuclease domain mutations
FA	Fanconi anemia
FapyA	4,6 diamino-5-formamidopyrimidine
FapyG	2,6-diamino-4-hydroxy-5-formamidopyrimidine
FEN-1	Flap endonuclease-1
hESC	Human embryonic stem cell
HSC	Hematopoietic stem cell
HR	Homologous recombination
ICLs	Interstrand crosslinks
IDLs	Insertion-deletion loops
iPSC	Induced pluripotent stem cells
IR	Ionizing radiation
LS	Lynch syndrome
MGMT	Methylguanine methyltransferase
MIN or MSI	Microsatellite instability
MMR	Mismatch repair
MPG	N-methylpurine DNA glycosylase
NEIL1	Endonuclease VIII-like 1
NHEJ	Non-Homologous end joining
OGG1	8-oxoguanine glycosylase
PAR	Poly ADP-ribose
PARG	Poly(ADP-ribose) glycohydrolase
PARP	poly (ADP-ribose) polymerase
PNK	polynucleotide kinase
PUA	α, β-unsaturated aldehyde
ROS	Reactive oxygen species
RPA	Replication protein A
SCE	Sister chromatid exchange
SL	Synthetic lethality
SSA	Single-strand annealing
SSB	Single strand break
SSBR	SSB repair
SSDA	Synthesis-dependent strand annealing
ssDNA	Single-stranded DNA
t-AML	Therapy-related AML

TLS	Translesion synthesis
t-MDS	Therapy-related myelodysplastic syndrome
t-MDS/MPN	Therapy-related myelodysplastic/myeloproliferative neoplasms
t-MN	Therapy-related myeloid neoplasms
UAF1	USP1-associated protein
USP1	Ubiquitin-specific peptidase 1
UV	Ultraviolet
VUS	Variants of uncertain significance

Introduction

Damage to the DNA poses a threat to the survival of the organisms and the faithful transmission of genetic information to offspring. Genome integrity is continually challenged by both endogenous and exogenous factors that can lead to genomic instability. Endogenous DNA damage can arise from attacks by reactive oxygen species (ROS), which are normal cell metabolic byproducts. Additionally, misincorporation of dNTPs during replication and base deamination, depurination, and modification contribute to endogenous DNA breakage. Exogenous DNA damage, on the other hand, can arise from ionizing radiation (IR), ultraviolet (UV) light, and chemical exposure to genotoxic material like industrial chemicals [85, 35]. Additionally, conventional cancer chemotherapeutic agents work by inducing extensive DNA damage and, in turn, cell death. Because these agents do not target cancer cells exclusively, mutations may also be propagated to normal cells leading to the development of secondary cancers [31]. The most common forms of DNA damages are single base lesions and DNA single-strand breaks (SSBs), and the ones with the most deleterious effects are interstrand crosslinks (ICLs) and DNA double-strand breaks (DSBs) [131, 35]. To avoid such deleterious outcomes, cells have evolved defense mechanisms, collectively known as the DNA damage response (DDR) to identify lesions, signal their presence, and activate the appropriate DNA repair mechanisms that will work on fixing the damage with little to no loss. Distinct DNA lesions are repaired by different DNA repair pathways such as mismatch repair (MMR), SSB repair (SSBR), DSB repair (DSBR), and Fanconi anemia (FA) pathway. While each of these pathways process a particular set of lesions, there is also considerable overlap between the pathways [73, 138].

Cells defective in any of the DNA repair pathways generally demonstrate high sensitivity towards DNA damaging agents and this could be viewed as two sides of the same coin: one negative side, which is the predisposition to diseases like cancer, and the other positive side, which is portrayed in harnessing this knowledge for targeted therapy. In this chapter, we shall discuss the recent advances in selected DNA repair pathways, with particular attention to cancers with heritable defect in DNA repair and secondary cancers developed following treatment with chemotherapeutic drugs.

Mismatch Repair

The MMR pathway is a bidirectional excision-resynthesis system that corrects mismatches generated during DNA replication or homologous recombination (HR). MMR increase the fidelity of DNA replication by 1000-fold [112, 184]. Mismatches fall into two groups: base-base mispairs resulting from incorrect nucleotide insertion by DNA polymerases and insertion-deletion loops (IDLs) resulting from slippage of DNA polymerase at simple sequence repeats (microsatellite) [116, 126].

a.i.-Prokaryotic Mismatch Repair MMR is well conserved from bacteria to mammals. The primary bacterial proteins involved in MMR are designated MutS, MutL, and MutH. MutS recognizes mismatchs as homodimers. MutL (also called molecular matchmaker) facilitates the interaction between DNA-MutS-MutL and MutH [150, 194]. MutH cleaves GATC sequences selectively in the nascent strand, which remains transiently unmethylated because deoxyadenine methylase lags behind the replication fork [121]. The nicked strand is cleaved by one of four single-strand exonucleases (the 5′ → 3′ exonucleases ExoI and ExoX or the 3′ → 5′ exonucleases RecJ and ExoVII) [24]. The single-strand gap is bound by single strand-DNA binding protein (SSB). DNA polymerase III then completes the gap, and DNA ligase seals the nick [88].

a.ii.-Eukaryotic Mismatch Repair MMR in eukaryotes retains many of the key features of the *E. coli* MMR pathway. The MutS equivalents in humans (MSH) exist in two heterodimeric forms: MutSα (MSH2 and MSH6) that identifies base-base mismatches and small loops, and MutSβ (MSH2 and MSH3) that identifies larger loops, with some overlap in substrate specificity between the two MutS complexes [127]. Recently, MutSα has been shown to have strong bias for insertion loops repair, while MutSβ has an even stronger bias for deletion loops repair [195]. Human cells express more MSH6 than MSH3, leading to a MutSα: MutSβ ratio of 10:1 [51, 147]. Despite their redundant activities, both complexes are required for MMR, and defective or abnormal expression of MSH6 or MSH3 leads to spontaneous mutation (mutator phenotype) [51, 52, 79, 148].

Heterodimeric MutS complex (MSH2/6 or MSH2/3) recognizes the substrate, likely by recognizing increased flexibility at the site of the mismatch [95]. MutS complex then recruits MutL. MutL equivalents in humans exist in three heterodimeric forms: MutLα (MLH1–PMS2), MutLβ (MLH1–MLH3), and MutLγ (MLH1–PMS1). MutLα is the major MutL homolog that participates in MMR and has endonuclease activity [30]. MutL*α* binds several MMR proteins and modulates their activity in a mismatch-dependent manner [116, 126]. EXO1, the only eukaryotic exonuclease implicated in MMR to date, has an obligate 5′→3′ polarity, which seems inconsistent with the bidirectional MMR, but close analysis revealed that MutLα harbors cryptic endonuclease activity. PMS2 introduces a nick in the daughter strand 5′ or 3′ of the mismatch, and this nick serves as an entry point for the EXO1 that carries out the excision step [100, 101]. Consistently, MLH1–PMS2 is required for 3′ excision but not 5′ excision [41, 185, 255]. Replication protein A (RPA) protects the MMR excision intermediate from nuclease degradation, and the

excised DNA strand is resynthesized by Polδ [116, 126]. The basic human MMR system includes MutSα or MutSβ, MutLα, EXOI, PCNA, RFC (loads PCNA onto DNA), RPA, Polδ, and DNA ligase I [41, 255]. The 5′ to 3′ mismatch-directed strand excision requires only MutSα, EXOI, and RPA, whereas substrates with a 3' nick also require MutLα, PCNA, and RFC [56].

MMR in eukaryotic cells has to deal with the nucleosome to reach the mismatch. Previous reports have demonstrated that DNA mismatches within tightly associated nucleosomes, in contrast to naked DNA, are poor MMR substrates [125, 202], so there must be a signal that allows for a timely recruitment of MMR onto the nucleosome. Li et al. [124] have made a breakthrough by demonstrating that an epigenetic histone mark, H3K36me3, during G1- and early S-phase recruits the MutSα onto the chromatin before replication, independent of the presence of mismatch. Cells that lack STD2 (H3K36 trimethyltransferase) display microsatellite instability (MSI) and spontaneous mutation frequencies, characteristic of MMR-deficient cells.

a.iii.-Strand Discrimination Eukaryotic cells do not use methylation for strand discrimination, alternatively a nick in DNA can signal for strand-specific eukaryotic MMR *in vitro*. The first biochemical studies carried out with extracts of human or *Drosophila melanogaster* cells showed that covalently closed circular DNA substrates with a single mismatch were refractory to MMR, but a nick in either strand situated up to 1 kb away from the mismatch was necessary and sufficient to activate the MMR process [86, 223]. Discontinuous lagging strand synthesis of Okazaki fragments (aproximately 200 bp long in eukaryotes) introduces a high number of 5' DNA ends that discriminate the nascent lagging strand [25]. On the other hand, the leading strand is replicated in a continuous manner. This raises the question of how the MMR directs the nascent leading strand.

The answer to this question appears to lie in an interaction between MutLα and PCNA. RFC loads PCNA at 3′ primer termini (boundaries between double- and single-stranded DNA) with the same side facing the DNA terminus [154]. Mismatch made by DNA polymerase is detected by MutSα or MutSβ, which slides along the DNA, interacts with PCNA, and displaces the polymerase. The loading of MutLα generates a protein complex that travels towards the mismatch where MutLα can introduce nicks in the leading strand, which are used as loading sites for EXO1. Since only one strand of DNA has the correct orientation (5′ → 3′ or 3′ → 5′) for hydrolysis, the enzyme will cleave only a single strand [182, 183]. According to this model, on the leading strand, the MutLα/PCNA complex needs to travel from the 3′ terminus to the mismatch which could be hundreds of nucleotides away [202], so it was suggested that MMR is less efficient on the leading strand compared to the lagging strand, where strand discontinuities are available [169].

Recently, an additional mechanism was proposed for the nascent leading strand discrimination. During replication, more than one million ribonucleotides are introduced into the mouse genome [84, 193]. A similar situation occurs in *Saccharomyces cerevisiae*, where Polε (the leading strand polymerase) incorporates about four times more ribonucleotides than Polδ (the lagging strand polymerase) into the nascent DNA [140, 170, 171]. Recent reports have shown that RNase H2-dependent processing of the ribonucleotides incorporated by Polε acts as a signal

that can direct MMR to the nascent leading strand. Inhibition of RNase H2 has no effect on the lagging strand because of the high number of nicks introduced by Okazaki fragments. This mechanism has limited contribution to MMR fidelity because it requires that the mispair and the ribonucleotide are within 1 kb of each other [72, 139].

b. Mismatch Repair Deficiency, Microsatellite Instability, and Lynch Syndrome Microsatellites are short repetitive DNA sequences 1–6 [60]. Because of their repetitive sequence structure, microsatellites exhibit a particularly high mutation rate. During replication, DNA polymerases often fail to correctly duplicate the microsatellite repeats due to slippage, which results in IDLs [183]. This phenomenon is known as MSI and it is recognized as length changes in the microsatellites. The MSI status is determined using a panel of five microsatellites (BAT25, BAT26, D2S123, D5S346, and D17S250) [155].

In 1993, MSI was detected in about 10–15% of sporadic colorectal carcinomas as well as in >90% of Lynch syndrome (LS) patients, also referred to as hereditary nonpolyposis colorectal cancer (CRC) [183]. The finding that MMR deficiency in *Saccharomyces cerevisiae* induced MSI led to the suggestion that cancers with MSI might also have defects in MMR [97]. LS is a prevalent autosomal dominant hereditary cancer syndrome caused by heterozygous mutations in one of the MMR genes MSH2, MSH6, MLH1, or PMS2 [191]. The mutations in MMR genes that lead to truncation or deletion can be securely classified as pathogenic, but in a significant fraction of individuals suspected to develop LS, subtle alterations in MMR genes are identified such as missense mutations or mutations in splice sites. These types of mutation are called variants of uncertain significance (VUS) [191]. The pathogenesis of many of these VUS is not clear due to the absence of data on the consequences of these mutations on gene function. Many functional analysis assays have been developed *in vitro* and *in vivo* to identify the pathogenicity of VUS [50, 191]. Recently, using yeast as a model system, it has been demonstrated that more than half of the deleterious missense mutation in MSH2 result in lower levels of the protein due to ubiquitin-mediated proteasomal degradation (the primary ubiquitin ligase being san1). Increasing the expression of the unstable variants, deletion of san1, or the of proteasomal inhibitor restores MMR function [9].

MSI is known to occur due to defects in MMR genes, such as germline mutation in MSH2 or MLH1 in most LS cases and epigenetic silencing of MLH1 in most sporadic cases [19, 82, 118, 232]. Nevertheless, many colorectal and several other MSI-positive cancers do not have genetic or epigenetic defects in MMR genes. Recently, Li et al. [124] have shown that depletion of SETD2 impairs MutSα chromatin binding, leads to MSI, and increases the mutation rates. Intriguingly, they have found a renal cell carcinoma and a Burkitt's lymphoma cell line, both without defects in MMR genes but MSI positive, to be mutated in SETD2. This report provides an explanation for the discrepancy between the genotypes and phenotypes of such cancers. In addition, recent studies support the idea that defects in MMR pathway could be independent from defects in MMR genes. POLE and POLD1 are related B family polymerases, and they represent the main catalytic and proofreading subunits of the Polε and Polδ enzyme complexes [187, 168]. POLE and POLD1 contain a 3′–5′

exonuclease (proofreading) domain which recognizes and excises the mispair and in turn increases replication fidelity by approximately 100-fold. Recent reports have shown that POLE and POLD1 exonuclease domain mutations (EDMs) increase the susceptibility to CRC and, in the latter case, to endometrial cancer (EC). In addition, somatic POLE EDMs have been reported in sporadic CRCs and ECs [34, 177].

Microsatellites have been identified within the coding sequences of a number of genes [54, 55]. The DNA polymerase slippage within these coding sequences can induce frameshift mutations. In case of CRC genomes, cancer-associated genes frequently affected by MSI (e.g., TGFBR2, ACVR2A, and BAX) have been investigated [99, 146, 189]. Recently, Kim et al. [108] have provided a comprehensive analysis of the prevalence and functional consequence of MSI in CRC and EC. Using exome and whole genome sequencing, they have shown that recurrent MSI events in coding sequences have (1) elevated frameshift-to-inframe ratios, so they hypothesized that the genes inactivated by recurrent MSI may have tumor suppressor roles, and the high frame shift (nonneutral) could provide selection advantage on coding sequence (2) lower transcript levels than wild-type alleles, which may be due to RNA surveillance pathway that eliminates mRNA containing a premature stop codon, and (3) tumor type specificity.

Mismatch Repair, Monofunctional Alkylating Agent, and Therapy-Related Myeloid Neoplasms

Conventional chemotherapeutic agents used in clinics operate by inducing DNA damage in cancer cells. Unfortunately, normal cells are also targeted by these chemotherapeutic agents, which induce mutations and, in turn, the development of secondary cancers in normal cells. The most prevalent forms are therapy-related myeloid neoplasms (t-MN) account for about 10–20% of myeloid neoplasms and can be subdivided into therapy-related myelodysplastic syndrome (t-MDS), therapy-related acute myeloid leukemia (t-AML), and therapy-related myelodysplastic/myeloproliferative neoplasms (t-MDS/MPN) [218]. Based on the type of chemotherapeutic agents, two main subtypes of t-MN with different characteristic have been identified. The first subtype of t-MN is related to exposure to alkylating agents, and it is characterized by a long latency period of 3–10 years, a preceding myelodysplasia, and loss of all or parts of chromosomes 5 or 7 or both. The second subtype of t-MN is related to exposure to topoisomerase II poisons and is characterized by a short latency period of 1–3 years, often without a preceding myelodysplasia, and balanced chromosomal rearrangements involving MLL at 11q23 and t(15,17)(PML-RARA) [74, 178, 181].

Alkylating agents are divided into monofunctional (e.g., temozolomide, dacarbazine, and methylnitronitrosoguanidine (MNNG)) or bifunctional alkylating agents, such as nitrogen mustards (chlorambucil and cyclophosphamide), and chloroethylating agents (e.g., nimustine (ACNU), lomustine (CCNU), and carmustine (BCNU)) [113]. Base excision repair (BER) can repair the majority of the alkylated DNA adducts induced by monofunctional alkylators except for O6meG, which is

largely responsible for the cytotoxicity of this class of chemotherapeutic agents. Methylguanine methyltransferase (MGMT) can directly repair the O6meG by covalent attachment of the methyl group from the O6meG to a cysteine residue on MGMT, leading to irreversible inactivation of MGMT [71, 145, 162]. During replication, DNA polymerases frequently mispair O6meG with thymine, which, in turn, activates the MMR [213]. Interestingly, rather than repairing *O*6meG, MMR induces DNA damage signaling, cell cycle arrest, and apoptosis [83, 98, 158]. This means that the cytotoxicity of monofunctional alkylating agents requires a proficient MMR. Indeed, cells proficient in MMR and deficient in MGMT show high sensitivity to monofunctional alkylating agent, while cells deficient in MMR and MGMT are resistant to cell death and have increased mutation rates [18, 45, 204]. The mutator phenotype that characterizes MMR-deficient cells may accelerate t-MN development. Consistently, t-AML, which arises after exposure to alkylating agents, displays MSI [31].

The mechanism by which MMR mediates the cytotoxicity of monofunctional alkylating agents is not fully understood. Two models have been proposed, the "futile cycle" and "direct signaling" models. The "futile cycle" model suggests that since the MMR machinery can only target the newly synthesized DNA strand containing the mismatched thymine, the *O*6meG will never be removed and another thymine opposite to O6meG will be inserted in the following replication. The repeated excision and regeneration of O6meG:T mispairs will induce cytotoxic DNA DSBs. In this model, the ATR is indirectly activated after DNA damage [158, 250]. The "direct signaling" model suggests that MMR proteins binding to a O6meG:T mispair acts as scaffold for direct recruitment and activation of ATR DNA damage signaling pathway. This model has been supported by separation of function mutations in mice containing mutations in Msh2 and Msh6 ATPase domains, which are essential for MMR activation but not for MMR-dependent DNA damage-induced apoptosis. These mice showed that MMR activity can be inhibited without affecting MMR-induced DDR [130, 249, 252]. Interestingly, many studies have shown that RPA is not essential for MMR-dependent ATR activation [134, 175]. In contrast to "the direct signaling" model, a recent study showed that O6meG induced ATM and ATR activation, and inhibition of ATM and ATR sensitized the cells to monofunctional alkylating agent [58].

Single-Strand Break Repair

SSBs arise either through insult from the direct action of ROS or topoisomerases or indirectly as a result of an intermediate step in the BER pathway [29] where BER enzymes injure the sugar backbone in the process of removing the damaged base [28, 66]. Oxidations, deaminations, and alkylations occur at a very high rate of about 30,000 damages per cell per day.

a.i-Recognition The first step in SSBR is the detection of the break. The main protein involved in this step is poly (ADP-ribose) polymerase (PARP). PARP is rapidly

recruited to the DNA strand break and consequently activated [28]. PARylation, or the polymerization of ADP-ribose, of target proteins is thought to modulate their recruitment, stabilization, or activity at SSB sites [138]. However, the residence time of PARP at the site of break is very short because as soon as PARP is auto-ribosylated the charge repulsion allows for its dissociation [138]. PARP is then returned to its original conformation and is free to be recruited on a different break via the degradation of poly ADP-ribose (PAR) by poly(ADP-ribose) glycohydrolase (PARG) [230]. One significant protein in BER, which is thought to be recruited by PARP1, is the scaffold protein XRCC1 [59, 149]. XRCC1 and PARP1 together aid repair by forming complexes with other BER proteins to promote recruitment/retention of repair factors and chromatin modification [152].

a.ii.-Base Excision and End Processing BER works through the excision of the incorrect/damaged base through cleavage of the N-glycosidic bond by DNA glycosylases [152]. Different types of lesions are recognized by different gylcosylases. For example, 8-oxoguanine glycosylase (OGG1) works on 8-oxoGua and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) lesions, while Endonuclease VIII-like 1 (NEIL1) has higher selectivity towards FapyG and 4,6 diamino-5-formamidopyrimidine (FapyA) [152]. When removing the damaged base, BER enzymes incise oxidatively damaged DNA to create an SSB. This step is achieved using apurinic endonuclease-1 (APE-1) or bifunctional DNA glycosylases that are capable of both base removal and apurinic site incision. This step leaves behind not only an abasic site but also unconventional termini; these need to be processed and restored to the proper 3′-hydroxyl and 5′-phosphate before the repair via short-patch or long-patch BER proceeds. Next, DNA ends are processed, which is considered the most miscellaneous step of SSBR, where a number of enzymes are involved including polynucleotide kinase (PNK), APE-1, DNA polymerase, and flap endonuclease-1 (FEN-1) [28]. An abasic site formed as a result of a monofunctional glycosylase will be recognized and cleaved by APE-1, leaving behind 3′ OH and 5′ deoxyribose phosphate (5′dRP) termini [234]. Polβ possesses lyase activity, removing the 5′dRP at the nick [151]. However, if the glycosylase is bifunctional, its lyase activity cleaves the sugar-phosphate backbone leaving behind either an α, β-unsaturated aldehyde (PUA) or a phosphate group at the 3′ end. APE1 then removes the PUA creating a 3′ hydroxyl substrate for Polβ, while PNK, stimulated by XRCC1, removes the phosphate group at the 3′ end [234]. Both indirect and direct SSBs can be repaired by either of the BER pathways, short- or long-patch. The significant feature that distinguishes the two pathways from each other is the size of the repair patch—single nucleotide for short patch [49] and 2-12 nucleotides for long patch [68].

a.iii.-Gap Filling and Ligation Following restoration of conventional DNA termini, a single nucleotide or more is inserted by polymerases. *In vitro* studies suggest that the major polymerase involved in gap filling of short-patch repair is Polβ, while Polβ and Polδ/Polε are employed in long-patch repair [67, 186]. Whether Polβ requirement *in vivo* is due to its lyase or gap filing activity or both is not clear [215]. DNA ligases are now ready to seal the remaining nick. Long-patch ligation

is primarily mediated by Lig1, while short patch is mediated by the Lig3a-XRCC1 complex [28].

Single-Strand Break Repair and Cancer

When a single component of the SSBR is inhibited, repair is compromised. When this happens, mutagenic lesions may accumulate causing risk of cancer development. Cancer is one of the diseases that are closely related to oxidative stress caused by ROS. The result of the oxidation of the highly susceptible guanine base is 7,8 dihydro-8-oxo-2′-deoxyguanosine (8-oxodG). During DNA replication, this modified base favors pairing with adenine instead of cytosine; further DNA replication will then allow adenine to bond with thymine [206]. This leads to mutagenesis via the dreadful GC—TA transversion. Other modified bases as well as other mutations also occur; however, 8-OxoGua and GC—TA transversion are both better studied and occur more frequently. If not fixed, this transversion mutation may deactivate a tumor suppressor gene (e.g. p53) or activate an oncoprotein (e.g. RAS), leading to tumorigenesis [42]. If this SSB is not immediately recognized and properly repaired, it can and will develop into the more deleterious DSB when replication takes place. Sakumi et al. [201] showed that OGG1-knockout mice were predisposed to lung cancer. Another study showed the increased risk of uterine myomas transformation into malignant cells is related to the levels of 8-oxoGua [65].

The aim of studying the different pathways of repair is not only to recognize the factors that increase risk of disease development but also to target these pathways for therapy. The study by Trivedi et al. [227] showed that both elevated N-methylpurine DNA glycosylase (MPG) expression and siRNA-mediated loss of Polβ in human breast cancer cells increases the sensitivity towards temozolomide. An increased accumulation of 5′dRP caused by the overexpression of MPG and loss of the lyase Polβ (mentioned earlier) is suggested to be the cause of this sensitization [227]. Following the induction of alkylating damage, PARP hyperactivation, as a result of Polβ deficiency, will lead to BER failure due to the consequent NAD+ and ATP depletion [221]. Because the process of PARylation requires NAD+ as substrate, NAD+ biosynthesis inhibition could be used to sensitize cancer cells towards chemotherapy. Dual inhibition of BER and NAD+ biosynthesis was more effective in sensitizing temozolomide-resistant glioblastoma cells rather than targeting BER alone [75]. Further, PARG, which is responsible for PAR removal, could be a good target for sensitization; however, PARG inhibition is still not well characterized as a target for cancer therapy. Inhibitors of APE1 are divided into two classes: indirect inhibitors, like methoxyamine, which irreversibly bind to the AP site on the DNA and direct inhibitors, like lucanthone, which inhibits APE1 endonuclease activity [1, 44]. In preclinical studies, methoxyamine was found to potentiate the cytotoxic effects of temozolomide and pemetrexed [1, 44].

Emerging studies on the use of BER gene expression as sensitive predictors of cancer prognosis have become popular. Upregulation of APE1 is a feature of several malignancies with higher levels indicative of more aggressive tumors. The

expression level of APE1 could also be an indicator of therapeutic response [1]. Higher levels of APE1 may overcome damage caused by IR or chemotherapy [1]; therefore, combination therapy that targets BER candidates for a better therapeutic outcome may be useful. For example, depletion of APE1 along with the TDP1, a protein involved in the repair of stalled topoisomerase I, amplifies the sensitivity to MMS treatment [6]. Germline and tumour-specific polymorphisms in BER genes are related to the risk of cancer development [44], making these polymorphisms useful as predictive biomarkers in cancer. A number of meta-analyses and case-control studies have emerged, uncovering such polymorphisms associated with increased risk of cancer development. For example, polymorphism OGG1 S326C has been associated with an increased risk of CRC [159].

Double-Strand Break Repair

There are two distinct pathways that contribute to the repair of DSBs: HR and non-homologous end joining (NHEJ).

a. Homologous Recombination Repair of the DSBs where the DNA strand goes on a search for homologous sequences to synthesize the new DNA sequence is termed HR, which is believed to be an error-free process. There are four main models for HR including break-induced replication (BIR), synthesis-dependent strand annealing (SDSA), single-strand annealing (SSA), and double Holliday junction (dHJ). The latter is only seen in meiosis and will not be discussed here. When a nick is recognized, endonucleases resect or chew off the 5′ ends of both strands exposing the 3′ ends to create 3′ overhangs. This is followed by the binding and assembly of recombination proteins to form a presynaptic filament. The 3′ overhangs go on a search for similar or homologous sequences to copy and synthesize new DNA sequences. Once the homologous sequence is found, DNA synthesis is initiated by the invasion of the single strand into the double strand homologous DNA, usually a sister chromatid. After successful DNA synthesis is completed, separation, ligation, and removal of extra nucleotides bring this process to an end.

The MRN complex, which is composed of MRE11, RAD50, and NBS1, is employed early on in the process of HR. This complex works at the site of break to create the 3′ overhangs and to facilitate DDR signaling [93, 220, 253]. The single-strand DNA formed is then approached by RPA; together they form a recombinogenic intermediate. Both the MRN complex and RPA call on signaling proteins ATM and ATR, members of phosphatidylinositol 3-kinase-related kinases (PIKKs), which, through a series of phosphorylation cascades involving the cell-cycle checkpoint kinases CHK1 and CHK2, arrest the cell cycle to allow DNA repair to take place [119, 120]. Next, RAD51 displaces RPA on the single-stranded DNA (ssDNA) [103] and forms the nucleoprotein presynaptic filament in a process mediated by BRCA2 and PALB2 (also known as FANC). RAD51 is essential in the steps of homology searching and double-stranded DNA (dsDNA) invasion [216]; therefore, RAD51 foci are surrogate markers for HR.

a.i.-Break-Induced Replication When replication starts, single-strand lesions, which are very frequent as discussed earlier, may cause stalling of the replication fork. This, in turn, transforms the single-strand nick to the more serious DSB. The one-ended DSBR involved here is often called BIR—a part of the HR pathway. Previously, the BIR pathway was thought to be a semiconservative method where it copies short DNA stretches and would stop once the Holliday junction is resolved and replication would carry on normally. Contrary to the early model, once BIR is initiated, it continues to copy hundreds of kilobases—all the way to the telomere—through what is known as bubble migration [200]. This means that the very accurate S-phase replication process will be replaced by the erroneous BIR. It has been suggested that this error is due to the recruitment of low fidelity Translesion synthesis (TLS) DNA polymerases resulting in BIR-induced mutagenesis [47].

a.ii.-Synthesis-Dependent Strand Annealing Gene conversion by SDSA is a process where the newly synthesized sequence appears only in the repaired DNA, while no exchange in the flanking regions occurs, and thus, SDSA naturally leads to conservative repair [144]. This happens as the resolvases cut in a manner that produces noncrossover products giving rise to a different heteroduplex DNA configuration than that of the dHJ model associated with meiosis [144].

a.iii.-Single-Strand Annealing Another pathway that repairs two-ended DSBs is SSA, which takes place when the DSB occurs between two repeat sequences. The resection of the dsDNA exposes the two repeat sequences, and thus, homology is found within each other. Subsequently, annealing directly commences and the removal of the extra DNA sequences takes place leaving behind a DNA sequence that has lost a significant portion of its nucleotides (one of the repeat sequences and the nucleotides between the repeat sequences) [81], possibly giving rise to loss of heterozygosity.

Homologous Recombination and Cancer

Ongoing research aims to study inhibitory molecules that target different steps in the HR process; some of these inhibitory molecules made it to clinical and preclinical trials. For example, an inhibitor of ATR, NU6027, has been uncovered and found to reduce Rad51 foci and to boost cisplatin's cytotoxicity [180]. Similarly, two more ATR inhibitors have been studied; one is VE821, which was shown to promote sensitization to chemo- and radiotherapy in pancreatic cancer [64], and the second one is ETP46464. A PI3K inhibitor NVPBEZ235 has been identified as a potent ATM and ATR inhibitor [225] and is being tested in clinical trials as a chemo- and radiosensitizer [94]. Downstream of ATR, AZD7762, an inhibitor of CHK1, has been found to decrease the formation of Rad51 foci and decrease gene conversion. BRCA1 has been newly identified as a phosphorylation target of CDK1 and through this phosphorylation event, Rad51 foci are formed and gene conversion occurs. Therefore, inhibition of CDK1 using RO3306 or AG24322 is considered a

possible therapeutic target [32]. It is important to note that while the inhibition of kinases is considered a promising therapeutic target for cancer therapy, there are still several problems that need to be addressed. Because kinases do not act only on a single substrate, inhibition of these kinases will result in inhibition of several other pathways. Further, these inhibitors are nonselective, and so using them will definitely inhibit other kinases leading to off-target effects.

Cells that are defective in NBS1 show reduction in sister chromatid exchange (SCE). Moreover, knockdown of NBS1 sensitizes PARP-inhibited cells [153]. This is because PARP is an essential enzyme in SSBR as mentioned earlier, and its inhibition would elevate the level of SSBs and increase the likelihood of the formation of lethal DSBs. Mirin, an MRN complex inhibitor, works by blocking the exonuclease activity of MRE11 [53]. Inhibitors of RAD51, such as B02 [89] and RI-1 [21] have also been identified. Inhibitors of EGFR [127, 238] and tyrosine kinase BCR-Abl [33, 211] have been shown to inhibit the nuclear localization of Rad51 and BRCA1, which are key components of HR.

Cells do not normally rely on one pathway and parallel pathways allow for repair to proceed if one fails [94]. Interestingly, cancer cells tend to be deficient in one or more parallel repair pathways [80], a feature that is very crucial and can be exploited for therapy. Many therapies targeting cancer, either chemotherapy or radiotherapy, rely on creating DSBs to cause cell death, but also cause cytotoxicity in normal cells. Thus, understanding the extent of functional redundancy is crucial to optimize cancer therapy, which led to the birth of the concept of synthetic lethality (SL). SL is defined as the lethal synergistic effect that comes from blocking two different pathways that, if blocked separately, do not lead to death. SL in HR-mutant cells was first identified when PARP inhibitors were shown to sensitize BRCA-defective cells [20, 61]. The identification of defective HR could provide a foundation to stratify patients for PARP inhbitor treatment [94]. Subsequent studies showed the sensitizing effect of PARP inhibitors on cells defective in different HR genes. It was revealed that a deficiency in RAD51, RAD54, DSS1, RPA1, NBS1, signaling proteins ATR and ATM, CHK1, CHK2, FANCD2, FANCA, or FANCC sensitized the cell to PARP inhibition [153]. Many exceptions and challenges remain. For example, deficiency in RAD52 did not induce sensitivity to PARP inhibition, which was attributed to the fact that RAD52 is more involved in the SSA pathway rather than gene conversion, which is the main repair pathway involved in PARP inhibited DSBs [153]. SL will be covered in more detail in Chap. 9.

Since RAD51 is a key component of HR, it is currently being established as the mainstay marker for HR [163]. RAD51 foci are used in clinical trials to test the response of primary breast cancer cells to neoadjuvant chemotherapy. A study showed that patients that exhibited low RAD51 score, suggestive of defective HR, were likely to respond well to anthracycline-based chemotherapy [76]. Another study tested a BRCA1 and BRCA2 array comparative genomic hybridization (aCGH) classifier, the level of BRCA1 promoter methylation, BRCA1 mRNA expression, and EMSY amplification, collectively assessing BRCA dysfunction. The results showed that a BRCA2-like aCGH profile is a powerful predictor of chemotherapy response in ER-positive breast cancers [133]. However, BRCA1 abnormalities were

not predictive of better response in triple negative tumors [133]. Loss of heterozygosity can be used as a marker for defective HR genes through the generation of a DNA-based HR deficiency score and thus can be used to predict the sensitivity of tumors to PARP inhibitors [2].

Non-Homologous End Joining

During G0, G1, and early S phase of the cell cycle, NHEJ is regarded as the main mechanism for DNA repair and is responsible for nearly 85% of DSB repair processes during these phases [129]. HR was considered the only repair mechanism that is error-free and also the cell's first choice for repair. When HR is not possible, due to either lack of sister chromatid or defective HR genes, cells turn to the erroneous NHEJ pathway. However, recent studies suggest that it is NHEJ that is first called into action, and only when NHEJ is unsuccessful does HR take charge to proceed with an error-free repair [23]. It is now considered that NHEJ is, in fact, a complex process that is not, in the slightest, secondary to HR [80].

NHEJ utilizes microhomology (typically 1-4 nucleotides) on the two ends of the DSB to repair the break in a simple ligation process. This is known as classical NHEJ (C-NHEJ). However, sometimes the already exposed overhangs are not compatible with each other, and so, simple ligation is not an option. This is where alternative NHEJ (A-NHEJ) takes place. A-NHEJ makes use of what is known as end processing, resection and polymerization, to create compatible overhangs (typically 6-8 nucleotides) for ligation. A-NHEJ is therefore considered a mutagenic process as it leads to deletions and/or additions of DNA sequences. The C-NHEJ pathway consists of synapsis, then end-processing, and finally ligation [239]. Synapsis is important in NHEJ as it keeps the two ends in close proximity or in alignment with each other. Synapsis is thought to rely on the ring shape of the Ku heterodimer, which consists of Ku70 and Ku86. Moreover, DNA-PKcs –in addition to the binding ability of the nucleosome itself-is suggested to play a similar role in synapsis [129]. Next, Ku's binding to the broken DNA end facilitates the binding of DNA-PKcs. This binding activates the DNA-PKcs's kinase function, which is rather important for the phosphorylation of Artemis, a nuclease used in end resection, and DNA-PKcs' autophosphorylation allows for its dissociation from the DNA. Autophosphorylation defects of the DNA-PKcs do not allow the subsequent steps of NHEJ to take place [240]. This may then redirect the cell towards HR instead of NHEJ. As with resection, gap filling using Polμ creates compatible ends for ligation. Finally, XRCC4 mediates the interaction of the DNA ends with LigIV.

In the absence of the Ku heterodimer, A-NHEJ takes over the repair process. The characteristic feature for A-NHEJ is its repair through microhomology. However, this microhomology between the DNA ends is created at the expense of DNA sequences that are deleted in the process. The exonucleases responsible for this are thought to be CtIP and Mre11 [23]. Once end resection to uncover microhomology is complete, LIGIII or, to a lesser extent, LIGI seals the two ends.

Non-Homologous End Joining and Cancer

Translocations, whether caused by C-NHEJ or A-NHEJ, are considered a hallmark of cancer. Most translocations do not actually lead to fusion genes of a neomorphic nature that drive the cell into a hyperproliferative state, and so most of the translocations do not contribute to tumorigenesis. However, increased frequency of translocations in a tumor is considered an indicator of bad prognosis [23]. Because of the yet poorly understood A-NHEJ mechanism, it is unclear how much microhomology is utilized by the classic and alternative mechanisms to allow us to differentiate between them. Not only this, inconsistent results have been published suggesting that C-NHEJ is, in fact, responsible for most translocations that occur. In other words, science is still lacking solid evidence as to which pathway is responsible for these translocations, partially due to the poor understanding of the mechanisms in question.

As with HR, NHEJ repair pathway can be exploited for cancer therapy. Inhibitors that target different proteins known to be involved in NHEJ are under investigation. This inhibition is thought to redirect the cell towards more faithful pathways, such as HR. Inhibitors of DNA-PKcs are studied as part of combination therapy with chemotherapeutic agents [233]. NU7026 and NU7441, inhibitors of DNA-PKcs, enhanced the sensitivity of poor prognosis B-cell chronic lymphocytic leukemia (B-CLL) to topoisomerase II poisons [243]. In another study, NU7026 rendered pancreatic ductal adenocarcinoma cells not only sensitive to IR but also triggered apoptosis via the expression of cleaved caspase-3 [128]. Similarly, knockdown of NHEJ genes using shRNAs targeting Ku70 and Ku80 in 8988T cells boosted the cells' sensitivity to IR [128]. Known PI3Ks inhibitors LY294002 and Wortmannin also enhanced the cytotoxicity of DSB-forming agents; the former enhances the cytotoxicity to IR [199] and the latter to both etoposide and IR [16]. Further, in 2012, SCR7, an inhibitor of LIGIV, was discovered and shown to inhibit NHEJ by interfering with the ligase's DNA binding ability and so activating apoptotic pathways. In addition, the same study showed that SCR7 suppressed tumor progression *in vivo* [214]. As a form of SL, DNA-PK inhibitors could also be used in combination with chemotherapy in ATM-mutant tumours [138]. In fact, in the SL approach targeting HR (e.g. BRCA-2 deficient cells) through PARP inhibitors, resistance might occur. This resistance is attributed to deletions occurring in BRCA genes that could be mediated by A-NHEJ repair [57]. Interestingly, this resistance could be restored through NHEJ inhibition, a process called synthetic viability [17].

Moreover, the inhibition of DNA-PKcs in BRCA-deficient cells has been proven to decrease genome instability [179]. In a recent study, it was shown that knockdown of LIGIII, the ligase used for the error-prone A-NHEJ, sensitized KRAS-mutated leukemic cells to chemotherapy [77].

Selective inhibition of NHEJ factors in BRCA-deficient cells could be useful to treat cancer; however, total inhibition of NHEJ pathway will subject cells to massive DSB accumulation, which will ultimately lead to cancer progression. NHEJ could favor global genomic integrity at the price of some deletions that could go unnoticed due to their location at sequences with no known coding function [48].

Fanconi Anemia: A Cancer Predisposition Syndrome

Clinical Aspect of Fanconi Anemia

FA is a rare recessive disorder characterized by developmental abnormalities, bone marrow failure (BMF), and an increased cancer risk.

a. Developmental Abnormalities More than two thirds of FA patients have a broad spectrum of congenital abnormalities such as short stature, radial ray abnormalities, microcephaly, microphthalmia, and genital malformations [224]. The congenital malformations could be due to inappropriate increase in the p53-dependent apoptosis because of the inability of the FA-deficient cells to repair DNA damage during embryogenesis [135].

b. Bone Marrow Failure Anaemia as a consequence of BMF is usually the first life-threatening symptom with which individuals with FA present. During the first decade of life, most FA patients develop BMF, ranging from mild to severe [27, 117]. The fact that all blood lineages eventually become deficient strongly implies hematopoietic stem cell (HSC) dysfunction. Knockdown of FANCA and FANCD2 in human embryonic stem cells (hESC) results in reduced numbers of HSC and progenitor cells, suggesting an important role for the FA pathway during embryonic hematopoiesis [229]. Murine Fancc-/- mice show reduced numbers of fetal liver HSC and progenitor cell pool with decline in serial repopulating capacity [102].

The role of FA pathway in stem cell function was recently revealed by discovering that FA-deficient fibroblasts are refractory to reprogramming, (induced pluripotent stem cells (iPSC)). The reprogramming barrier of FA fibroblasts could be bypassed by either genetic complementation or reprogramming under hypoxic conditions [192, 164]. Recently, Yung et al. [254] have reported derivation of iPSC lines from a FANCC-deficient patient under normoxic conditions, but at much reduced efficiency. The FANCC-deficient patient-specific iPSC lines and FANCC-deficient hESC result in reduced number of clonogenic hematopoietic progenitors due to increased apoptosis in culture.

c. Cancer Predisposition FA patients bear a significant predisposition to develop cancer. Patients exhibit high risk of hematopoietic malignancies, including myelodysplastic syndrome and acute leukemia. Acute lymphoblastic leukemia is occasionally reported, but AML is the most common. Patients with FA have 800-fold higher risk of developing AML than the general population [197, 198]. FA patients have a common pattern of specific chromosomal abnormalities, frequent gains of the chromosomal regions (1q and 3q), and partial or complete loss of chromosome 7, which can be used as predictive markers [157]. FA patients also bear a significant predisposition to solid tumour formation. By the fourth decade of life, about one-third of FA patients will develop a solid tumor, but this may be an underestimate as many patients do not live long enough to develop a tumor [198]. Of the solid tumors diagnosed in FA patients, squamous cell carcinoma of the head and neck

and gynecological tract occur at a higher rate. These tumors are also associated with human papilloma virus, but the relative contribution of the virus to squamous cell carcinoma in FA patients is not clear [196].

FA Pathway

FA is caused by biallelic mutations in any of the 16 genes (FANCA, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, -N, -P, -O, or -Q) [15, 104]. FA proteins can be subdivided into three groups of proteins: core complex, ID complex, and downstream effectors proteins. FA core complex consists of eight proteins (FANCA, -B, -C, -E, -F, -G, -L, and -M) and five FA-associated proteins (FAAP20, FAAP24, FAAP100, MHF1, and MHF2). Approximately 90 % of FA patients have mutations in the eight FA core complex proteins [7, 36, 105, 122, 132, 247, 248]. The FA core complex is essential for monoubiquitination of the ID complex (FANCD2 and -I) by the E3 ubiquitin ligase FANCL. UBE2T functions as the E2 ligase, but it has not been associated with FA [46, 142]. Most FA core complex proteins, apart from FANCL and FANCM, do not have functional domains and they only act as a scaffold.

Monoubiquitinated ID complex recruits the downstream effector proteins which have affinity for the ubiquitin. Four of the downstream FA proteins (FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, and FANCO/RAD51C), also known as breast and ovarian cancer susceptibility genes, are necessary for HR to re-establish collapsed replication forks as a result of DSB formation during ICLs processing [87, 123, 231, 236, 244]. FANCP/SLX4 interacts with multiple nucleases, and FANCQ/ERCC4 is a structural endonuclease (discussed below). In addition, ubiquitin-specific peptidase 1 (USP1) and the USP1-associated protein (UAF1) are necessary for the completion of FA pathway by regulating the deubiquitination of ID complex [38, 107, 166, 174].

Replication-Dependent Interstrand Crosslink Repair

Despite the genetic and phenotypic heterogeneity of FA, the hallmark of all FA patient-derived cells is hypersensitivity to interstrand crosslinking agents such as mitomycin C, diepoxybutane, and cisplatin. Exposure of FA cells to interstrand crosslinking agents induces high levels of chromosomal aberrations, which are utilized as a diagnostic feature for FA [10, 11]. ICLs are highly toxic because they act as an absolute block to both DNA transcription and DNA replication, so they are widely used in anticancer therapies. ICLs can be repaired by replication dependent and by replication independent pathways [241]. FA pathway is specifically activated during S-phase, suggesting a role for FA pathway in the repair of ICLs during replication [5, 222]. In the following part, we will discuss the steps of ICL repair by FA pathway during S-phase.

a. Recognition Among FA proteins core complex, FANCM is of particular interest because it acts as a sensor of damage and mediator of checkpoint signaling [39, 40]. FANCM has an evolutionarily conserved helicase domain bearing ATP-dependent DNA translocase activity [69, 70, 156]. This ATPase activity is dispensable for core complex targeting and ID complex ubiquitination, but it is required for replication fork stability and efficient checkpoint response [14, 40, 91]. The ATP-independent DNA binding activity of FANCM is important for ID complex ubiquitination and for FA core complex recruitment to chromatin [106, 160, 245]. FANCM forms a complex with FAAP24 and MHF1/MHF2 with distinct DNA binding specificities; the former prefers ssDNA, whereas the latter prefers dsDNA [36, 210, 247].

When a replication fork encounters an ICL, polymerization is arrested. The FANCM–FAAP24–MHF1/2 complex recognizes the stalled replication fork and recruits the FA core complex, and the translocase activity of FANCM prevents the collapse of replication fork [36, 106, 210, 247]. Beside the FANCM–FAAP24–MHF1/2 complex, the MutS has a redundant role in activating the FA pathway and recognizing ICLs by enhancing the recruitment of the FA core complex [90, 237, 242].

b. Signaling Replication obstacles and stalled forks are signaled by ATR. ATR activation proceeds in two largely independent steps. First, a stalled DNA replication fork generates a stretch of ssDNA covered with RPA, which in turn recruits ATRIP-ATR. In parallel, the Rad9-Rad1-Hus1 (9-1-1) checkpoint clamp, loaded onto DNA by the RAD17-RFC clamp loader, recruits TOPBP1. Interaction of ATRIP-ATR with the TOPBP1 leads to S-phase checkpoint activation [26, 63]. Recently, FANCM was found to be required for the activation of ATR-mediated checkpoint signaling, and the translocase activity of FANCM is essential for this role [40, 91, 141, 203]. FANCM and FAAP24 interact with the checkpoint protein HCLK2, and the depletion of FANCM or FAAP24 results in a phenotype similar to cells lacking HCLK2, ATR, or CHK1, including a high rate of spontaneous DNA lesion, nuclear abnormalities and supernumerary centrosomes, and checkpoint defects in cells subjected to replication stress [40].

As we discussed above, the RPA covered ssDNA is critical for ATR activation, but ICL cannot accumulate a sufficient amount of a long stretch of ssDNA because the accumulation of the ssDNA requires the uncoupling of DNA polymerase and DNA helicase at the stalled replication fork, and both are blocked by the ICL, so how ICL induces ATR activation is puzzling. A recent study has answered this question by showing that FANCM-FAAP24 complex is required for the recruitment of RPA to ICL-stalled replication forks [91]. The activated ATR is essential for checkpoint signaling via CHK1 phosphorylation [26, 37, 63], and it is also required for the FA pathway activation through FANCI phosphorylation [96, 207]. Recently, two distinct ATR signaling subpathways following cellular exposure to interstrand crosslinking agents have been proposed. One is dependent on RAD17 and TOPBP1 in which the important substrate is CHK1. This pathway is also affected by the FANCM-FAAP24 complex. The second is dependent on FA core complex where

it functions in ATR activation by enhancing chromatin binding of ATRIP, and the important substrate of this pathway is FANCI phosphorylation which will then activate FANCD2 monoubiqutination [226].

The role of FANCM in cell signaling following exposure to interstrand crosslinking agents is not completely understood. The FANCM and FAAP24 have been implicated in checkpoint signaling in the above studies. However, Wang et al. [237] have recently found that FANCM and FAAP24 are not fully epistatic in repairing ICLs such that FAAP24 induces ATR-mediated checkpoint activation, while FANCM participates in recombination-independent ICL repair by promoting the recruitment of lesion incision activities, which requires FANCM translocase activity. In addition, the above reports placed FANCM upstream of ATR, but Singh et al. [209] have recently proposed that ATR precedes FANCM, because ATR phosphorylates FANCM (S1045) in response to genotoxic stress. This event is essential for the role of FANCM in FA pathway integrity, recruitment of FANCM to the DNA damaged site, prevention of premature mitotic entry, and the activation of CHK1 and G2/M checkpoints.

Coordination of Other DNA Repair Pathways During Interstrand Cross Link Repair

During ICL repair, FA pathway coordinates many repair pathways; excision repair, TLS, and HR. The FA pathway is essential for these processes as the nuclease and TLS steps depend on FANCD2 and its ubiquitination [111, 137, 190].

a. Nucleolytic Incision Following ICL recognition, it is thought that dual incisions are made 5′ and 3′ to the lesion. Many nucleases including FAN1, XPF, MUS81, SLX1, and SNM1A have been repoted to be implicated in ICL repair [78, 110, 165, 212, 217, 235].

a.i. FAN1 Many reports have identified FAN1 to be required for ICL repair [115, 136, 143, 205, 212]. FAN1 possesses intrinsic 5′-3′ exonuclease activity and endonuclease activity that cleaves nicked and branched structures [212]. FAN1 interacts specifically with the monoubiquitinated FANCD2 through its UBZ domain, and through this interaction it is recruited to chromatin [115, 136, 143, 205, 212].

Although the above reports support a role for FAN1 in the FA pathway, there is also strong evidence against such a role. Although FAN1-depleted cells are sensitive to interstrand crosslinking agents, ICL-dependent DSBs arise in these cells with normal frequency but persist longer than in wild type cells. This suggests that FAN1 may have nuclease functions following the ICL incision step [115, 143]. FAN1-null DT40 cells do not have elevated SCE frequencies, a feature of all other FA mutant DT40 cells. In addition, FA proteins and FAN1 appear to have nonepistatic functions because DT40 cells deficient in both FAN1 and FANCC or FAN1 and FANCJ,

have increased sensitivity to interstrand crosslinking agents compared to cells deficient in only FAN1. This suggests that FAN1 may participate in the processing of ICL independent from the classical FA pathway [251]. In support of an independent role for FAN1 in ICL repair from FA pathway, a study of four patients carrying a homozygous 15q13.3 microdeletion (which includes FAN1) showed that the patients had no characteristic symptoms of FA [228]. On the other hand, [256] identified mutations in FAN1 as a cause of karyomegalic interstitial nephritis, a disorder that serves as a model for renal fibrosis. Karyomegalic interstitial nephritis has none of the characteristic hallmarks of FA, but cells from patients with karyomegalic interstitial nephritis have significant sensitivity to interstrand crosslinking agents, which is complemented with wild-type FAN1.

a.ii. SLX4 SLX4/FANCP is a recently identified FA protein. Cells depleted of SLX4 are hypersensitive to interstrand crosslinking agents [43, 165]. Slx4-null mice have many key features of FA, including developmental defects, reduced fertility, and defect in the hematopoietic compartment [43]. SLX4 contains UBZ domain that is required for interaction with monoubiquitylated FANCD2 and for recruitment to DNA damage [246]. SLX4 functions as a scaffold for three structure-specific nucleases: XPF–ERCC1, MUS81–EME1, and SLX1. Genetic studies indicate that XPF-ERCC1 is the most relevant nuclease for ICL repair [12, 62, 109, 110, 165, 217]. The SLX4-dependent XPF-ERCC1 activity is critical for ICL repair but is dispensable for topoisomerase I poison-induced DNA damage repair. Conversely, SLX4-MUS81 is essential for topoisomerase I poison-induced DNA damage repair but is less critical for ICL repair. Mutation of SLX4 that inhibits interaction with SLX1 leads to partial resistance to interstrand crosslinking agents and topoisomerase I poison [110]. Recently, XPF was identified as FA protein (FANCQ). Whole exome analysis of unclassified FA individuals revealed biallelic germline mutations in XPF encoding gene (ERCC4) [15]. Interestingly, another patient with defect in XPF encoding gene was reported to have clinical features of three different DNA-repair disorders—Cockayne syndrome, xeroderma pigmentosum, and FA [104]. SNM1A may digest the unhooked oligonucleotides to create a better substrate for the TLS. SNM1A-depleted cells are sensitive to ICL agents [235].

Translesion Synthesis

Following incision of ICL, TLS is required to bypass the lesion. Since ICL lesion involves the two strands of the DNA, both strands cannot be used as the template. TLS uses low-fidelity polymerases to bypass the bulky damaged lesions to generate an intact template for HR-mediated repair. Monoubiquitylated ID complex is required for TLS steps during ICL repair [111]. PCNA acts as scaffold to which TLS polymerases bind [8]. Cells with defect in the TLS polymerases, Rev1 and Rev3, are hypersensitive to ICL agents [172, 173, 208].

Homologous Recombination

Following TLS, HR uses the produced intact template to repair the DSB generated by incision. Cells deficient in HR proteins are sensitive to interstrand crosslinking agents [13, 114, 161]. FA pathway is involved in HR activation, and cells lacking FA proteins are deficient in activating HR [167, 212]. In DT40 cells, FANCC and XRCC2 were shown to be epistatic in ICL repair [172]. However, FANCD2 and FANCI do not regulate chromatin loading of the key HR enzyme RAD51 [137].

In addition to promoting HR, many studies proposed that FA proteins actively suppress NHEJ. As we discussed above, the cell can repair DSB by HR or NHEJ. The choice depends on the cell cycle phase, where the HR works on S-phase and the NHEJ works on G1 phase. In addition, HR requires extensive resection to create a long 3′ overhang, but NHEJ requires little, if any, resection [92]. The FA pathway is thought to have a role in pathway choice by funneling the DSB created by ICL processing into HR. Inhibition of NHEJ components in FA-deficient cells suppresses hypersensitivity to interstrand crosslinking agents, diminishes chromosome breaks, and reverses defective HR [3, 176]. In contrast, deletion of 53BP1 or Ku80 increases the sensitivity of FANCD2-deficient cells to interstrand crosslinking agents than FANCD2 deficiency alone [22].

Another Replication-Dependent Pathway for Interstrand Crosslink Repair

Recently, a new mechanism for ICL repair during S-phase was revealed. HELQ, 3′–5′ DNA helicase with strand displacement activity, was found to participate in ICL repair independent from FA. HELQ helicase-deficient mice exhibit subfertility, germcell attrition, ICL sensitivity, and tumour predisposition. HELQ interacts directly with the RAD51 paralog complex BCDX2 and functions in parallel to the FA pathway to promote efficient HR at damaged replication forks. HELQ may also be involved in ATR-mediated CHK1 activation but not in ATR-mediated FANCD2 monoubiquitination. Thus, HELQ has a critical role in replication-coupled ICL repair, germ cell maintenance, and tumour suppression in mammals [4, 219].

Fanconi Anemia Pathway, Bifunctional Alkylating agent, and Therapy-Related Myeloid Neoplasms

ICLs induced by bifunctional alkylating agents result in the formation of DSBs which may lead to chromosomal rearrangements and in turn t-MN. Since t-MN arises in patients who have already developed one type of cancer, the inherited cancer susceptibility mutations may be involved in tumorigenesis [31].

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Chapter 4
Mathematical Modeling for DNA Repair, Carcinogenesis and Cancer Detection

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Abstract The constant damage of DNA in human cells is considered the main cause of aging and cancer. In this review, we discuss the most lethal form of DNA damage, the DNA double strand break (DSB), and how it relates to cancer. DSB sensor proteins in the nucleus detect DNA breaks within minutes following damage. These proteins are now routinely labeled by immunocytochemistry, and access to high throughput fluorescence microscopy and robotics open the door to rapid measurement of DSB levels in individuals. This method, often referred as the DSB foci assay, leads to images showing small bright spots at the site of each damage in the nucleus. We first discuss how energy consumption in the cell leads to detectable baseline levels of foci per cell measured in peripheral blood lymphocytes. Mathematical kinetics are then described to infer both genetic defects in DNA repair and environmental factors influencing these levels. We emphasize ionizing radiation, which is the principal environmental factor that increases DSB levels. Mathematical models associating a mutation probability for each DSB have been used to explain the dose dependence of cancer incidence observed after exposure to high doses of radiation. The main assumption in these models is that high mutation frequency can eventually lead to tumor suppressor gene deletion or oncogene amplification. We conclude by suggesting that the growing stream of genetic and phenotypic measurements related to DNA repair and DNA damage will lead to more accurate predictive tools for cancer risk and individualized cancer prevention.

Keywords Radiation Induced Foci · DNA double strand breaks · DNA repair kinetics · baseline DNA damage · cancer model · cancer risk · multi-stage clonal expansion model · cancer detection

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Abbreviations

ABM	Agent-based models
AT	Ataxia telangiectasia
DNA	Deoxyribonucleic acid
DSB	Double strand break
FA	Fanconi anemia
LNT	Linear-no-threshold hypothesis
IR	Ionizing radiation
PBL	Peripheral blood lymphocytes
RIF	Radiation-induced foci
ROS	Reactive oxygen species
SSB	Single strand breaks
TSCE	Two-stage clonal expansion
XP	Xeroderma pigmentosum

Introduction

Our DNA is vulnerable to injury by agents generated from within the cell as well as from external sources. Every day, each of the 10 trillion cells in the human body receives tens of thousands of DNA lesions that threaten the stability of our genome. These lesions include base modifications, DNA adducts, crosslinks, and single-stranded and double-stranded breaks. The efficiency of the repair of DNA damage is influenced by individual genetics, aging, and metabolism. Mammals have developed remarkably complex and in some cases redundant repair machinery to maintain genetic integrity for decades.

In this chapter, we will focus our attention on one specific class of DNA damage and how it impacts cancer risk. Namely, the DNA double-strand break (DSB) is unique in that both strands of the double helix are severed, thereby disrupting genome continuity. Because DSBs result in unconnected DNA termini, DSBs often lead to loss of genetic information in the forms of deletions, mutations, and/or translocations, all of which can be contributing factors to genome instability and the development of cancers [1–3].

Sources of DNA DSBs

Endogenous DNA damage can give rise to DSBs through a number of cellular processes. One source of DSBs is via reactive oxygen species (ROS), including superoxide, hydroxyl radicals, singlet oxygen, and hydrogen peroxide [4, 5]. ROS production is an inevitable byproduct of mitochondrial energy production and these molecules can diffuse from the mitochondria to the nuclei and cause DNA damage.

ROS can also be generated from exogenous sources like metal ions and phorbol esters and other reactive molecules [6]. Regardless of the source, ROS have the potential to induce DSBs by directly reacting with DNA to generate DSBs, but more often, unrepaired ROS-damage leads to DSBs through a variety of cellular processes. Different types of ROS-induced DNA damage can be transmuted into DSBs through aberrant processing by DNA repair enzymes, DNA replication machinery, and other transactions with damaged DNA [7]. For example, during base-excision repair of single chemically damaged base, several intermediates are formed that can lead to the formation of DSBs [8]. Likewise, two independent single-stranded breaks (SSBs) on opposite strands, but separated by less than 10 base pairs can result in a DSB. SSBs can also be converted to DSBs when encountered by the DNA replication machinery [9]. Additionally, repair of base mismatches in the DNA sequence generates SSBs as intermediates, which can result in DSB formation [10]. All of these mechanisms, and others, can give rise to DSBs in healthy normal cells and pose a significant challenge to genome integrity.

In addition to endogenous DNA damage and repair processes, many organisms initiate programmed recombinational events by purposely introducing DSBs in specific genomic contexts. An example in single-celled organisms is mating type switching in yeast, a programmed recombination initiated by DSBs introduced into the genome by the regulated activity of an endonuclease (reviewed in [11]). In mammals, DSBs are introduced to initiate V(D)J recombination and class switch recombination events in immune cells. These processes give rise to immunoglobulin and T-cell receptor immunodiversity and are essential for B and T cell maturation [12, 13]. Defects in these processes can lead to severe organismal consequences including immunodeficiency and cancer [14–16]. Adding to these specific endogenous DSBs, errors in other cellular processes, like DNA replication, incomplete dissociation before mitotic segregation and other errors in DNA transactions can cause sporadic DSBs in genomes.

Among exogenous agents causing DSBs, ionizing radiation (IR) is the most extensively studied and broadly applied in medical procedures (e.g. X-rays). IR causes DSBs by direct collision of charged particles, γ-rays or X-rays, with the DNA double helix. Secondarily, IR also interacts with water to cause the formation of a variety of ROS, which contribute significantly to radiation toxicity. For example, chromosomal aberrations resulting from radiation induced DSB can be reduced by 60% when OH radicals are being scavenged [17]. Importantly, many, if not most, cancer therapies are based on overwhelming the DSB repair capacity of cells, and toxicity is inherently biased towards rapidly dividing cells, such as the malignancy, immune cells, hair follicles, and cells lining the intestine. The cellular toxicity of DSBs has motivated the development of a number of radio-mimetic drugs that imitate radiation in that they cause large numbers of DSBs in living cells [18]. Radiation, and many chemotherapeutic drugs commonly used for cancer treatments, relies on the same mode of action, namely, causing overwhelming numbers of DSBs in cellular DNA. Ironically, cancer treatments based on inducing toxic levels of DSBs also cause highly clastogenic DNA lesions that can lead to cancer. The complexity of factors giving rise to DSBs, both from within cells and the

environment, together with a diverse set of biological pathways involved in DNA repair, demand consideration when designing mathematical models of this complex and medically relevant phenomena.

DNA Double Strand Breaks Baseline Levels

Before being able to interpret the impact of genotoxic treatment on DNA using DNA double strand break assays, one needs to establish baseline levels of DNA damage. This limits the kind of assay one can use. Classic DSB assays, such as pulse field gel electrophoresis [19] or the neutral comet assay [20], are direct measures of DNA fragments but they typically require high levels of DNA damage, have limited sensitivity for detecting low levels of DSBs, and can be difficult to replicate between independent laboratories. The seminal discovery that the histone variant H2AX was specifically modified only at sites of DSBs [21] gave rise to immunofluorescent techniques and a quantitative surrogate marker for radiation-induced DSBs in eukaryotic cells [22]. In contrast to the direct measurement of fragmented DNA (i.e. comet assays), quantifying phosphorylated H2AX (γH2AX) [21] or p53 binding protein 1 (53BP1) localized to DSB sites [23] is relatively simple. Importantly, quantification using this method is unambiguous and DSBs quantified as sub-nuclear spots, called "foci", have proven to be more reliable in detecting minor DNA repair defects in human cells [24].

Nuclear foci assays have been used extensively in laboratory settings, for quantifying DSBs in humans undergoing radiotherapy [25], and as a biomarker for aging and disease (reviewed in [26]). Remarkably, within seconds to minutes following IR, repair and checkpoint proteins are localized and/or modified at DSB sites, leading to the formation of radiation-induced foci (RIF). These rapid DNA damage responses typically reach a maximum at 0.5–1 h post-IR and diminish as DNA repair proceeds [21, 27–32]. The rapid appearance of γH2AX foci in cells after damage (seconds) has led to several mechanistic hypotheses to account for the speed and discrete localization of foci. A recent study suggests that chromatin structure instantly changes upon disruption of genome continuity, thereby immediately and locally activating kinases and dictating γH2AX foci size and location [33]. Regardless of the mechanism, the majority of studies quantifying DNA double strand breaks using the RIF assay have been based on manual scoring [34–39], which has led to statistical uncertainty [40], subjective bias, and lack of reproducibility. Recent high throughput approaches using true 3D automatic foci detection software [31] has led to reproducible RIF results that can be compared across different research laboratories [41]. In this latter work, we showed that spontaneous damage is occurring in a random manner leading to a number of DSB/cell following a Poisson distribution [41], and quantification matches human counts from individuals trained in foci recognition (illustrated in Fig. 4.1).

Assuming DSB are produced at a constant rate P and foci are resolved at a rate k, one can write the following mathematical law:

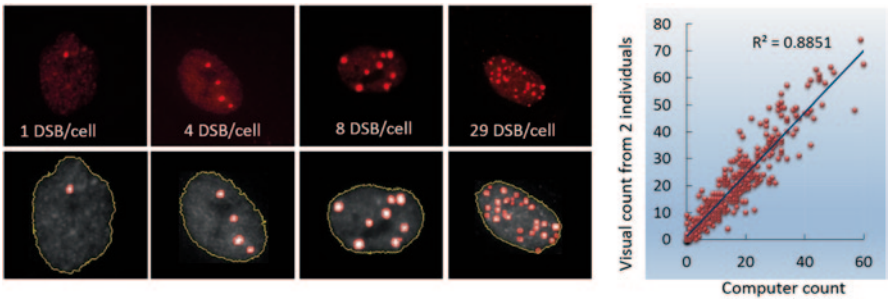

Fig. 4.1 Standardization and calibration of DSB detection using RIF assay. Images illustrate human breast cells exposed to IR and immunostained for 53BP1 as previously described [41]. Increasing doses of IR can be used to generate an expected linear number of DSB and can be quantified automatically using state of the art wavelet imaging tools. When comparing to gold standard (average human visual inspection done blindly by two individuals), the algorithm performs as well. The capacity of quantifying thousands of nuclei within minutes in a reproducible manner, has allowed standardization and high throughput quantification of DSB

$$\frac{dC}{dt} = P - kC \Rightarrow C(t) = \frac{P}{k}\left(1 - e^{-k.t}\right) \tag{4.1}$$

Where, C is the average number of DSB at time t in one nucleus and P is the constant rate of DSB being produced in the same cell under physiological conditions. Assuming the tissue of interest is at steady state for endogenous damage production, C should be constant. Therefore, we can resolve the spontaneous DSB rate at steady state:

$$\frac{dC}{dt} = 0 \Rightarrow C(t) = \frac{P}{k} = C_0 \Rightarrow P = kC_0 \tag{4.2}$$

To approximate the spontaneous DSB production, let us use the full range of reported values both for k, the repair rate, and $C_0$from the literature. It has been shown that repair kinetics can vary, depending on the type of lesion with half-lives as fast as ~5 min or as slow as 3 h [42, 43]. Studies on Italian children have reported spontaneous foci in peripheral blood lymphocytes (PBL) as low as 0.004 foci/cell [44]. In contrast, levels as high as 0.5–1 foci/cell in adult healthy donors PBL have been reported [45, 46]. This leads to a minimum spontaneous damage rate of 9.2E−4 DSB/h and a maximum of 5.2 DSB/h. In other words, during a 24 h time course, data suggest PBL may have to repair as many as 125 DSB or as few as 0.02. This is a large range of variation that should be reduced once the foci assay has been standardized across labs, by using validated imaging tools for spot quantification and by characterizing the same lymphocyte cell subtype to reduce cell specific bias [47]. Preliminary data on PBL, using our standardized approach, suggest 0.5 foci/cell (data not shown), suggesting the ambient level of DSB is ~10–50 DSB/day.

Age affects DSB levels, which have been shown to double or triple over a life time in healthy subjects [46, 48, 49]. These studies concluded that aging may cause accumulation of DSB and/or that repair capacity is reduced with age, with a known higher genomic instability for older groups. However, if one looks at healthy older individuals (age > 70), no significant increase compared to the youngest age group is observed [46], suggesting life style or genetics may be a determining factor for this aging process. It is also important to note in this study that telomere shortening was ruled out as a mechanism for increase DNA damage, contradicting previous studies [50, 51].

Evaluating DSB Repair Kinetics with the RIF Assay

Repair kinetics studies done with RIF use discrete time points in fixed specimens after the induction of damage with IR. However, foci formation at a DSB is not immediate and seems to occur asynchronously [41]. As such, there is a delay between DSB production and its detection via the foci assay. Such delay adds an important confounding factor when computing repair kinetics and we previously offered a mathematical correction for it [41]. We illustrated this issue by irradiating live breast cells expressing 53BP1 GFP and showing a cell with total of three RIF being produced over a 4-h time course (cumulative counts), but with a maximum of only two RIF at any time point (observable counts). In order to interpret RIF kinetics in an unbiased manner, we therefore had to introduce a mathematical formalism describing RIF formation as shown below:

$$\begin{cases} \dfrac{dC_0}{dt} = -k_1 C_0 \\ \dfrac{dC_1}{dt} = k_1 C_0 - k_2 C_1 \end{cases} \Rightarrow \begin{cases} C_0(t) = C_0(0)\cdot e^{-k_1 t} \\ \dfrac{dC_1}{dt} = k_1 C_0(0)\cdot e^{-k_1 t} - k_2 C_1 \end{cases}$$
$$\overset{C_1(0)=0}{\Rightarrow} \begin{cases} C_0(t) = \alpha D\cdot e^{-k_1 t} \\ C_1(t) = \dfrac{\alpha D k_1}{k_2 - k_1}\left(e^{-k_1 t} - e^{-k_2 t}\right) \end{cases} \tag{4.3}$$

where C_0 and C_1 are the average number of DSB and RIF per nucleus at time t, respectively and where RIF are formed at rate k_1 and they are resolved at rate k_2 and where α is the number of naked DSB/Gy before formation of RIF and D is the dose delivered to the cell. In this model, we assume that such chemical process, $DSB \xrightarrow{k_1} RIF \xrightarrow{k_2} RIF_{resolved}$, is irreversible. $C_1(t)$ in equation 4.3 can be used to fit the number of RIF at a given time (static measure). However, using time lapse imaging one can also measure the total number of RIF that have been produced since t = 0 (cumulated measure). This can be described mathematically as:

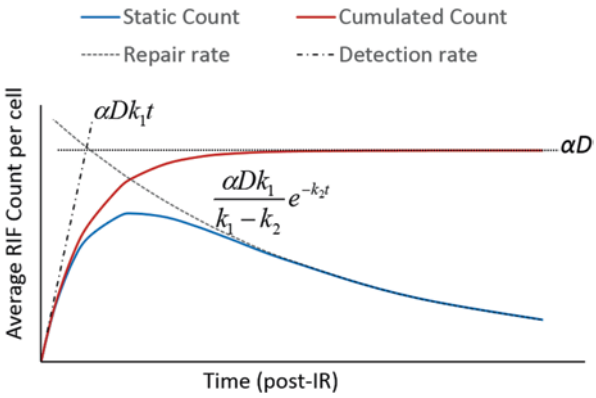

Fig. 4.2 RIF kinetics. Static counts are the average numbers of RIF per cell measured at a given time t. Cumulated counts are the total number of RIF observed since exposure to radiation at time 0

$$C_c(t) = \alpha D(1 - e^{-k_1 t}) \tag{4.4}$$

Equation 4.4 is derived simply by setting $k_2 = 0$ and using the same formalism as in equation 4.3.

Figure 4.2 illustrates the model.

Another model has also been proposed by Foray et al., where the model associates a repair probability unique to each DSB [52]:

$$C_1(t) = \frac{C_1(0)}{(1 + \beta \cdot t)^{(1+\alpha)}} \tag{4.5}$$

where α and β are the shape and location parameters for the Gamma probability distribution function. This approach yields more accurate repair rates and repair probabilities for the population of DSBs using Euler's Gamma function. However, this method does not predict the cumulated number of RIF throughout the whole kinetic, and can only fit the monotonically decreasing portion of the observable RIFs (see our previous review [39]).

From DNA Damage to Cancer

The link between DNA damage repair defects and cancer was first recognized with the study of the hereditary cancer-prone disease xeroderma pigmentosum (XP) when cells from XP individuals were found to be defective in repairing DNA SSBs induced by UV light [53]. Since that seminal discovery, a number of DNA repair genes and repair processes have been linked to many forms of cancer, aging, and disease (reviewed in part in [14, 54–56] and in Chap. 3). The γH2AX assay has been used to successfully identify repair defects in cells from individuals with genetic diseases like ataxia telangiectasia (AT), Nijmegen breakage syndrome [57],

XP [58], Fanconi anemia (FA) [59], and radiation sensitive severe combined immunodeficiency [24] among others. Immunostaining for γH2AX and flow cytometry detection was successfully used to identify individuals heterozygous for ataxia telangiectasia mutations [60] where flow cytometry screening and subsequent quantification of γH2AX foci with microscopy was utilized to assess DSB repair proficiency [61]. Investigation of γH2AX as a marker of inflammation-induced DNA damage [62], and as a potential marker of viral infection [63] that potentially suppresses DSB repair [64] are also reported. Of particular relevance was the discovery of breast cancer susceptibility proteins type 1 and 2 (BRCA1 and BRCA2) which are clearly linked to cancer and play essential roles in DNA DSB repair [65]. The sizeable body of work linking DNA repair defects to cancer risks effectively establish the simple paradigm that defective DNA repair leads to genomic instability, which in turn can lead to cancer.

In addition to this somewhat oversimplified paradigm, the effects of defective DNA repair can manifest as a number of pathologies. While a comprehensive review of all of the links between DNA repair and disease is beyond the scope of this chapter, here we point to a few intriguing associations. Neurological disorders are linked to DNA repair defective syndromes like AT and XP [66] and the DSB repair defective Ligase IV syndrome [67]. In addition, oxidative stress (including oxidative DNA damage) is associated with the neurodegenerative pathologies Alzheimer's and Parkinson's [68, 69]. Both cancer predisposition and early onset of aging pathologies are caused by defects in a highly conserved group of RecQ DNA helicases [70]. Defects in one such helicase, the WRN protein, cause a predisposition for a broad spectrum of cancers and an early onset of many aging pathologies. The WRN protein interacts with DSB repair proteins [71] and a number of other DNA repair pathways [72]. While the precise mechanistic links are still being discovered, it is clear that DNA repair defects impact genome stability and cancer predisposition in addition to a number of other important pathologies.

Epidemiological Study: The A-Bomb Survivors

The most documented argument favoring the correlation between DSB and cancer is the epidemiologic data from the Atomic Bomb survivors [73, 74]. These analyses were based on 17,448 first primary cancers (including non-melanoma skin cancer) diagnosed from 1958 through 1998 among 105,427 individuals. IR is known to induce DSB via the production of reactive oxygen species and direct DNA ionization. Therefore, increasing radiation doses increases in a linear manner the amount of DSBs. Similarly, cancer incidence from the A-bomb data show an increase with dose, but the linearity remains a very controversial topic [41, 73] and only significant doses of IR (>0.1 Gy), known to induce several DSB (at least 3), are currently required to establish a clear cancer risk. These epidemiological data most clearly demonstrate the impact of elevated DSBs on cancer risk and occurrence in a large human population.

Modeling Cancer Using DNA Damage

Modeling Radiation-Induced Carcinogenesis

When it comes to cancer risk from IR, the current risk model assumes the linear-no-threshold hypothesis (LNT), which implies that any amount of IR exposure is harmful. LNT is used to set dose limits for radiation occupational workers or the general public. The LNT is based mainly on data from the Japanese A-bomb survivors and secondarily on arguments involving the dose-response of surrogate endpoints. Gene mutations that are caused primarily by DSB are thought to be the initiating events of cancer (see Fig. 4.3). One poorly repaired DSB can lead to a point mutation while misrejoining of two DSBs leads to more complicated genome rearrangements. Physical laws suggest DSB frequencies are proportional to dose. Therefore, it is well accepted that point mutations are linear with dose since it requires only one DSB, whereas DSB misrejoinings are dependent to the dose squared [75]. In the dose-range of radiation cancer epidemiology, the quadratic term is almost always negligible, especially at low dose rates, as the first lesion is probably repaired before the second mutation occurs [76]. However, we have shown that DSB move into regularly spaced nuclear domains of 1.55 μm interval [41, 77]. Therefore, as the dose of IR increases, the probability of having two DSBs in the same repair center increases non-linearly, increasing further the risk of DSB misrejoining. Therefore, extrapolating risk linearly from high dose, as done with the LNT, could lead to overestimation of cancer risk at low doses.

Carcinogenesis is thought to occur in four interdependent stages as depicted in Fig. 4.3. The first stage is *Initiation* and is typically caused by chemical, physical, or biological agents, which irreversibly and heritably alter cellular genomes resulting in an enhanced growth potential. Mutation of tumor suppressor genes have been

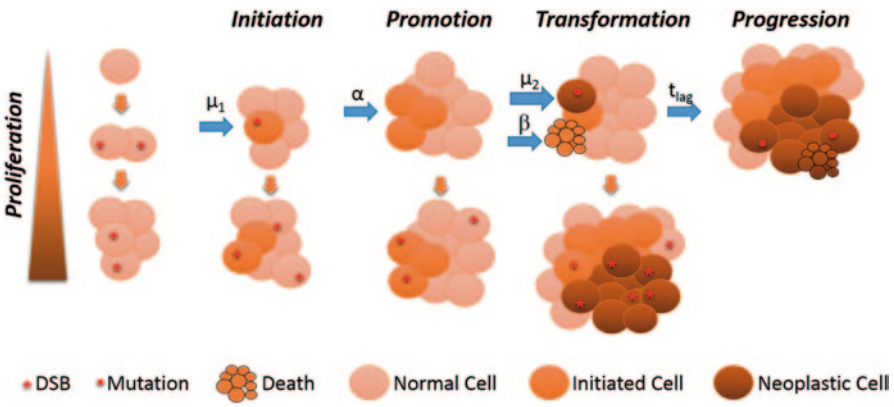

Fig. 4.3 The four stages of carcinogenesis and the role of DSB and mutation in this process. *Red circles* represent mutation affecting genes in the cancer process. (i.e. Tumor Suppressor Genes or Oncogenes)

thought to be the most likely initiating events, as originally introduced by Knudson [78]. This neoplastic potential is only realized, however, if the cell later undergoes *Promotion*, the second stage of carcinogenesis. *Promotion* is often thought to be the rate-limiting step in carcinogenesis since it has been shown that initiation alone is not sufficient to induce cancer [79]. Further genomic modifications due to genomic instability can lead to Transformation, which is often characterized by cell immortalization and oncogenic activation. Once a cell is transformed, additional proliferation leads to a full tumor. This last phase of carcinogenesis is defined as Progression.

In order to account for the observed power of age dependence in radiation-induced carcinomas, a multi-stage theory of carcinogenesis was first introduced [80, 81]. However, this model suggested 5 to 7 rate-limiting stages, which contradicted biological data. This discrepancy was resolved by the two-stage clonal expansion model (TSCE), which assumed a cell leads to a tumor by two separate mutations (Initiation and Transformation, with probability μ_1 and μ_2 respectively) and clonal expansion (Promotion and Progression) [82–84] (See Fig. 4.3). Promotion is characterized with a proliferation rate α, while Progression is modeled by a lag time (t_{lag}) required to reach a full tumor. DNA damage occurring spontaneously or via genotoxic stress can influence the rate of initiation, increasing the probability of developing cancer.

However, TSCE neglects the influence of intercellular and extracellular interactions in the tumor growth and predicts a final tumor that is unrealistic in that its cells are clonally identical. This is inconsistent with what is observed where tumors are heterogeneous and cell-cell and cell-extracellular matrix (ECM) interactions have been shown to influence tumor progression. In fact, a new paradigm for carcinogenesis has been gaining some credibility over the past two decades emphasizing a key role of cell-cell and cell-ECM interactions in the maintenance of tissue organization [85]. For example, during angiogenesis a tumor manages to communicate with its microenvironment to elicit the proliferation of endothelial cells to form blood vessels that will supply the tumor with oxygen. Another illustration of this paradigm is the existence of cancer susceptibility genes whose mutation affect genomic stability but associate with cancer only in certain tissues (e.g. BRCA1 for breast cancer, APC for colon cancer). This would suggest that the cellular and tissue context itself plays a role in causing the initiated cell to start proliferating.

Recent work on colon cancer introduced genomic instability in the TSCE model in order to better fit the data [86]. Fits were excellent but also suggested that radiation only played a small role in initiating genomic destabilization. The idea that non-mutational radiation effects play a critical role in destabilizing the genome is supported by the literature describing radiation-induced genomic instability as a non-targeted effect [87–89]. These types of multi-cellular interactions typically lack mathematical formalism due to the impossibility of reducing them to single entities such as cells. A new kind of formalism is required to adequately represent and model these multi-cellular interactions in a system.

Advances in computer science have engendered new approaches to model biological systems in ways that can formalize a system of interacting components. One such approach is agent-based models (ABM), which naturally describe complex adaptive systems as the results of interactive software objects in various contexts

[90]. Agents are non-deterministic codes originally developed for artificial intelligence. Each agent behaves individually in response to its situation on the basis of a set of contextual rules. In the case of representing cells within a tissue, agents may execute various behaviors appropriate for the system, such as proliferation, differentiation or death. A modeling framework that permits the integration of the different factors leading to homeostasis might include cooperation and competition, spatial organization, physical and molecular interactions between cells and their microenvironment, biological pathways, or genomic status. By modeling an irradiated tissue/organ/organism as a system of interacting cells that integrates these many factors, cancer can more realistically be analyzed as an emergent phenomenon of a perturbed system [91].

Our group has used ABM extensively to understand the emergent biological behaviors resulting from these complex multi-cellular interactions. For example, using ABM we showed that radiation could induce premature senescence of normal mammary epithelial cells, allowing for an accelerated outgrowth and selection of preneoplastic cells in culture [92]. We also used ABM to model the complex interplay between apoptosis, proliferation, and polarization needed to maintain a normal 3D structure of mammary epithelial cells [93]. Surprisingly, our simulations revealed a synergism between polarization and apoptosis in achieving growth arrest necessary to achieve a normal mammary acinar morphology. More recently, we used a multi-scale ABM to evaluate the effects of radiation on stem cell kinetics in the mammary gland during development [39]. The model made the prediction that irradiation during puberty, but not during adulthood, induces stem cell self-renewal and subsequent mammary stem cell enrichment, which in turn leads to estrogen receptor negative breast cancer. This prediction was validated functionally using *in vitro* and *in vivo* experiments, and may help explain why there is an elevated breast cancer risk after exposure to IR in young girls, but not adult women over the age of 40.

ABM hold great potential for modeling the increasing complexity of unraveling mechanism in carcinogenesis. Figure 4.3 depicts how one could integrate the impact of the microenvironment and inflammation in an ABM to predict tumor incidence *in silico*. One could assume that tumors arise via successive DNA mutation and that promotion are influenced by non-targeted effects (i.e. persistent effects on the microenvironment, modifying cellular behavior and baseline DNA damage level, modifying the frequency of both initiation and conversion (μ_1 and μ_2 terms respectively)). One can also generalize TSCE to more stages before a tumor arises, if necessary (i.e. Multi Stage Clonal Expansion).

Using Foci Assay as Biomarkers for Cancer Risk and Cancer Detection

Several studies have suggested that γH2AX quantification may be a powerful diagnostic tool for cancer risk and development and many other diseases [25, 26, 44, 94]. Elevated levels of γH2AX in premalignant lesions can induce cell cycle arrest and cellular senescence, thus serving a tumor-suppressive function [95–99]. These

findings suggested the use of the γH2AX assay may be an effective bioassay for early stage cancers [94]. Analysis of 30 human biopsies from colon, breast, ovary, liver and kidney cancers support the use of γH2AX in detecting early stage of tumor development [94] while other reports use γH2AX foci as a marker of cancer progression and treatment [100, 101]. Interestingly, sporadic breast cancer, gliomas, and lymphomas have been associated with genetic variations in the H2AX gene or changes in its promoter region [102–104].

Repair kinetics has also been explored as a way to predict radiation sensitivity and cancer risk. For example, primary lymphocytes from radiation sensitive mice with known defects in DNA repair (e.g. Balb/C and SCID mice) have RIF repair kinetics 1.5 to 6 times slower than resistant strains such as C57Bl/6 J (repair rates inferred from [105]). Similarly, ATM defects that result in persistent RIF [41, 106] are associated with radiation-induced carcinogenesis in mice [107], and increased toxicity from radiotherapy in ATM heterozygous patients [108, 109]. Additionally, DNA repair deficiencies are considered risk factors for both acute radiation toxicity and cancer, independently of the type of radiation [110–114]. Importantly, functional RIF kinetic assays may be sensitive enough to detect subtle genetic differences between individuals that would be hard to detect with more classic methodologies, such as single nucleotide polymorphism arrays. For example, recent studies on primary human fibroblasts, derived from 25 apparently healthy individuals and ten patients with DNA repair-defective syndromes have shown a wide variation in RIF levels and kinetics between individuals [115]. Our mathematical interpretation of RIF kinetics establishes novel metrics that can characterize such subtle differences. This approach may help identify genes that have not been previously implicated in DNA repair and individuals that are sensitive or cancer-prone in response to radiation exposure.

Some researchers have looked at DSB levels or DSB repair in lymphocytes as a potential indicator for cancer risk. The use of lymphocytes is ideal as they can be obtained in a relatively non-invasive manner. A recent study showed that baseline γH2AX foci levels in lymphocytes alone might be sufficient to detect individuals with breast cancer prior to treatment [116]. Elevated DNA DSB levels in lymphocytes of breast cancer patients have been observed previously, but using the comet assay [117, 118]. Others have additionally observed defective DNA DSB repair in lymphocytes in untreated patients with bladder [119], esophageal [120], and lung cancer [121]. These studies show great promise for the use of the γH2AX foci assay on lymphocytes for the detection and possible prevention of many cancers.

The development and broad application of the DSB foci assays brings major advantages to mathematical modeling of DNA damage and repair. First, this technology allows quantitative scoring of one specific type of DNA damage on a ‘per genome’ basis. Second, the assay is based on the cellular recognition of DSBs, not an estimate or average of broken molecules in a population of cells. These advantages permit accurate and precise measurements to be taken and compared between laboratories, samples, tissues, and individuals. This breakthrough technology will enhance the speed and accuracy of developing and testing mathematical models that

quantitatively describe DNA damage processes critical to cancer, aging, and other pathologies that are the focus of modern medicine.

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Chapter 5
Animal Models of Metastasis

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Abstract Tumour metastasis is a complex and dynamic process that is estimated to be associated with over 90% of cancer-related deaths. The various stages of the metastatic cascade are made up of interactions between metastatic tumour cells, the solid tumour microenvironment, host normal cells, and host tissue. While our understanding of tumour cell migration and invasion has been greatly improved using a variety of *in vitro* cell culture systems, the inherent complexity of the entire metastatic process is best studied using animal models. There are several *in vivo* models that are amenable to studying tumour metastasis, and this Chapter will focus on some of the more notable animal models that are commonly used in the metastasis field. We will first consider the zebrafish as a burgeoning model for tumour cell migration and invasion that is particularly well-suited for intravital imaging of labeled tumour cells to study mechanisms underlying metastatic tumour cell dissemination. We then discuss selected genetically engineered mouse models of metastatic cancer, which are powerful tools to elucidate the oncogenic potential of genes of interest. Murine tumour xenografts are also considered as the traditional "gold standard" models for metastasis research. We have included descriptions of strategies to artificially seed murine tissues with tumour cells to study the proliferation

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and survival of these cells in host tissues, and we provide an overview of quantitative methods to study the development of spontaneous metastases from solid tumour xenografts. We conclude with the use of patient-derived xenografts (PDXs), which are becoming increasingly common tools to validate experimental findings established in other model systems in an effort to enhance the clinical applicability of *in vivo* data.

Keywords Metastasis • Zebrafish • Genetically engineered mouse models (GEMMs) • Murine tumour xenografts • Patient-derived xenografts (PDX) • Biomarkers • Non-invasive imaging modalities • Personalized cancer therapeutics

Abbreviations

18FDG	18F labeled deoxyglucose
AR	Androgen response
CT	Computed tomography
EMT	Epithelial to mesenchymal transition
GEMM	Genetically engineered mouse model
MMTV	Mouse mammary tumour virus
LN	Lymph node
PB	Probasin
PDX	Patient-derived xenograft
PET	Positron emission tomography
PIN	Prostatic intraepithelial neoplasia
PyVmT	Polyoma virus middle T
SRC	Subrenal capsule
TAM	Tumour associated macrophage
TIC	Tumour initiating cell
TRAMP	Transgenic adenocarcinoma of the mouse prostate
WAP	Whey acidic protein
XT	Xenotransplantation

Introduction

Tumour metastasis is a complex, multi-step process [1] that is remarkably inefficient, with $<0.01\%$ of disseminated tumours cells estimated to eventually form macroscopic metastatic tumours. The process of metastasis can include local invasion of tumour cells into adjacent tissue, however the severe morbidity and mortality associated with metastatic disease is typically due to the development of metastatic tumour foci in tissues that are distant from the primary tumour. Tumour cells can metastasize through the lymphatic system to lymph nodes and/or through the bloodstream to a variety of distant tissues including the lungs, liver, brain, and bone marrow. A great deal of work has been devoted to understanding the genetic

and phenotypic characteristics of metastatic tumour cells [2], and tumour cells must have (or develop) the ability to migrate and invade in order to disseminate from a primary tumour. Metastasis begins with dissemination of tumour cells away from the primary tumour, which is a process that can involve tumour cell migration, invasion through extracellular matrix, and intravasation of tumour cells into the circulation. Most tumour cells that enter the bloodstream from a primary tumour mass die in the circulation before the cells can invade distant organs [3, 4]. Tumour cells that enter metastatic target organs can die or lie dormant for extended periods of time [5], with only a small proportion surviving and proliferating to form micrometastatic tumour foci. The continued growth of micrometastatic tumour foci into life-threatening macrometastatic tumours requires a switch to active angiogenesis [6–8] that is dependent upon active collaboration between the metastatic tumour cells and the surrounding host tissue. The role of host tissue in allowing (or promoting) metastatic tumour growth was first postulated by Stephen Paget in the late 1800s, with his 'seed-and-soil' hypothesis [9] suggesting that metastatic tumour cells (seeds) must enter suitable host tissues (soil) in order to grow into tumour metastases. Indeed, different tumour cell types preferentially form metastases in specific organs, and the site-specificity of metastatic growth seems to reflect the inherent (or acquired) properties of the disseminated tumour cells and the metastatic target organ itself. For example, expression of stromal-derived factor-1 (SDF1) by specific host tissues is thought to facilitate the invasion of tumour cells expressing CXCR4 [10], and the accumulation of bone marrow-derived cells in "pre-metastatic niches" have been shown to increase metastatic tumour growth in murine xenograft systems [11–13].

In order to study the complexity of the metastatic process and the role of the host in metastatic colonization, it is imperative to utilize animal models and there are several *in vivo* models that have been used to discover and study genes that are associated with metastasis. This Chapter will focus on some of the more notable animal models that are in common use in the metastasis field. The first section of this Chapter will focus on the zebrafish as a burgeoning model for tumour cell migration and invasion that is particularly well-suited for the intravital imaging of mechanisms underlying metastasis. This section is followed by a section describing selected genetically engineered mouse models of metastatic cancer and murine xenograft models, both of which have traditionally been considered the "gold standard" models for metastasis research. We will discuss methods to artificially seed tissues with tumour cells, and provide an overview of strategies to study spontaneous metastasis from tumour xenografts. The use of patient-derived xenografts that recapitulate clinical metastatic disease will also be considered.

Zebrafish as a Model for Metastasis

Zebrafish (*Danio rerio*) embryos develop rapidly and are transparent in the early stages of development, which has made them an attractive vertebrate model organism for developmental biologists modeling human diseases [14–16]. More recently,

scientists have used zebrafish as a model organism due to the ease at which their genomes can be manipulated, the inherent capacity to perform forward genetic screening and the convenience of conducting rapid loss of function studies using gene silencing morpholino antisense oligonucleotides. Over the last decade, cancer biologists have also begun to take advantage of these features to use zebrafish to model human malignancies, including as a model of invasion and metastasis by employing human tumour xenografts in the fish.

History of Human Tumour Xenotransplantation (XT) in Zebrafish

For xenotransplantation (XT) studies, cultured cells or primary tumours are fluorescently labeled and injected into zebrafish embryos. Since the developing larvae are transparent, the overall tumour burden and the location of the transplanted cells can be tracked using fluorescent microscopy. Fluorescent labeling is usually performed by either making stable cell lines that express fluorescent proteins or using a membrane stain (such as, CM-DiI or PKH2). Several anatomic sites within the embryo have been tested for injection location, including the duct of Cuvier and the fourth ventricle of the central nervous system, but the yolk sac has emerged as the preferential site of injection for xenotransplantation. Similarly, different zebrafish developmental stages have been assayed, with 24–48 h being the most common time point used for injections [17–19]. Importantly, zebrafish embryos are immunopermissive at early developmental stages, which facilitates xenograft work without requiring the induction of immunosuppression in the fish. While used less commonly, human cells can also be transplanted into the peritoneal cavity of adult zebrafish, however to avoid rejection by the immune system, the fish must undergo immune ablation with sub-lethal irradiation or dexamethasone treatment, prior to engraftment [20]. In the zebrafish model system, there are more limited possibilities for orthotopic injection of human cells to more closely mimic tumour interaction with an appropriate microenvironment, however glioblastoma cells have been injected into the hindbrain ventricle of the central nervous system [18]. Studies using primary human cell engraftment are limited, and include leukemic cells [21], as well as pancreatic, breast and prostate tumours [19, 22, 23]. A factor in the maintenance of zebrafish embryos bearing xenografts is the different temperature requirements of the embryos and the cancer cells. Human cancer cell lines are maintained at 37 °C, while zebrafish are maintained at 28 °C [24]. Empirical studies have found that incubation 34−35 °C enables the zebrafish to develop normally and the cancer cells continue to proliferate [17].

One of the main advantages of zebrafish XT is that the fish embryos are transparent and thus amenable to visualization of both tumours and host physiology using fluorescent microscopy of living embryos. As such, several transgenic lines of fish have been created which are particularly useful for direct observation of tumour-host microenvironment interactions. The *Tg(fli1-eGFP)* line expresses GFP

from the fli1 promoter for expression in the endothelial cells to highlight the fish vasculature; a powerful tool for studying tumour-associated angiogenic events [25] as well as tumour cell intravasation (or extravasation) into (or from) the vasculature [26, 27]. Other transgenic lines have been made to label immune cells, such as *Tg(mpx:GFP)* for neutrophils [28, 29], *Tg(mpeg1:eGFP)* for macrophages [30] and *Tg(cd41:eGFP)* for platelets and hematopoietic stem cells [31], which may be useful for investigating the contribution of the innate immune response to tumour invasion and metastasis.

A Tool for Mechanistic Metastasis Studies

Studies have been performed to evaluate the utility of zebrafish xenografts as models for metastasis. The first report of the successful xenotransplantation of human cancer cells in zebrafish occurred in 2005, where metastatic melanoma cells were injected into blastula stage embryos at 5h post fertilization. It was found that the melanoma cells retain a dedifferentiated state, migrate and divide in the developing fish [32]. In another early study, two pancreatic cancer cell lines derived from the same patient, one highly invasive and the other poorly invasive, were transplanted into zebrafish embryos. Two days after injection, the highly invasive line was found to have migrated throughout the embryo, whereas the poorly invasive line remained in one location [19]. Breast cancer cells were injected into zebrafish embryonic duct of Cuvier (directly into the circulation) and the pattern of invasion and metastasis mimicked what had been previously seen in mouse metastasis models [33]. Recently, a more extensive evaluation of this model system was performed using the transplantation of breast, prostate, colon and pancreatic cell lines of known invasive potential [22, 27]. These studies reveal that the zebrafish model reliably recapitulates the invasion data from *in vitro* studies and mouse metastasis models [22]. Taken together, these studies have demonstrated that the zebrafish xenograft model is faithful to the features of invasive, metastatic cancer.

Movement into and out of vasculature are essential steps in the metastatic cascade, but can be difficult to study in murine models, generally necessitating histological evaluation at discrete time points. Using transgenic fish that have fluorescent vasculature *Tg(fli1-eGFP)* and high resolution imaging techniques, the interaction between tumour cells and vasculature can be observed in real time. Furthermore, studies in zebrafish xenografts show direct links between the ability of tumour cells to induce host angiogenesis and their ability to metastasize. Breast cancer cells with low metastatic potential injected into zebrafish embryos promoted angiogenesis and became highly invasive under hypoxic conditions. This hypoxia-induced pathogenic angiogenesis was promoted by VEGF secreted by the tumour cells [34–36]. In an early study, breast cancer cells were injected into the peritoneal cavity of adult zebrafish (immunosuppressed with dexamethasone treatment) and had the ability to

invade in a RhoC dependent manner. Live imaging of the intravasation process showed that the tumour cells underwent cytoskeletal remodeling to form protrusive structures that invaded into newly formed vasculature [26]. High resolution microscopy has shown that tumour cells induce active remodeling of vessels at the sites of extravasation, rather than damage or induce vascular leakage, as has been previously thought. The remodeling is characterized by a clustering of endothelial cells around the tumour cells and a change in the architecture of endothelial cell-cell junctions. Furthermore, the expression of Twist, a transcription factor involved in epithelial to mesenchymal transition (EMT), promoted extravasation [34]. Similarly, E-cadherin (CDH1) knock-down in 4T1 murine mammary tumour cells was shown to increase dissemination throughout the zebrafish embryo, consistent with the promotion of EMT by loss of E-cadherin expression [27]. Live imaging has indicated that metastatic cells extravasate and colonize in areas high in neutrophils, and that the neutrophils actively remodel the collagen matrix to assist in the process. It was shown that the inhibition of VEGF signaling decreased tumour growth and angiogenesis, but paradoxically increased neutrophil migration and formation of micrometastases [29].

The zebrafish model system has also been used to dissect pathways relevant for metastasis, highlighting potential areas for therapeutic intervention. In particular, as compared to *in vitro* assays, the zebrafish provides host vasculature, innate immune cells and stroma that provide some of the host-tumour interactions that may play a role in tumour cell invasion and metastasis. Cell intrinsic factors that may promote metastasis and their interaction with the host tumour environment can also be explored. For example, several studies have demonstrated the importance of matrix metalloproteinases in invasion and metastasis. Galectin-3, which controls PAR-1 and MMP-1, was found to be increased in metastatic gastric cancer. Metastasis of gastric cancer cells in the zebrafish XT model system was inhibited by knockdown of galectin-3, Par-1 or MMP-1 [37]. Similarly, manipulation of another galectin, Gal-4, in pancreatic cancer cells causes changes in metastatic potential [38]. SMYD3 is overexpressed via epigenetic mechanisms in many cancers and is associated with increased MMP-9 expression. Knockdown of either SMYD3 or MMP-9 is capable of decreasing metastatic spread in zebrafish XT model [39]. Another factor, TGF-β, which has long been known to play a role in metastasis, has also been evaluated using zebrafish XT. Specifically, inhibition of TGF-β signaling, or downstream effectors, resulted in decreased metastatic potential in the zebrafish xenograft metastasis model [33].

The zebrafish model has also been leveraged to perform studies of cancer stem cells or tumour initiating cells (TICs) and their role in cancer progression and metastasis. For example, glioma cells or cancer stem cells derived from a glioma cell line were transplanted into zebrafish embryos and metastatic potential assessed. The stem cell population displayed higher metastatic potential, a process which was dependent on MMP-9 and dampened with an MMP-9 inhibitor [40]. TICs isolated from several prostate cell lines, as well as from primary prostate tumours, showed very high metastatic ability in the zebrafish xenograft model, even with very few transplanted cells [23].

Advantages and Disadvantages of Zebrafish XT as A Metastasis Model

Mouse models have been the gold standard for metastasis studies, although the zebrafish XT model system does offer some advantages. First, zebrafish XT does not require immune compromised animals. While human tumour xenograft studies have to be performed in mouse strains that lack a competent immune system, the zebrafish immune system does not completely mature until 28 days post-fertilization [41], negating the need for immunocompromised strains. Secondly, the direct visualization and live imaging of metastasis within zebrafish embryos can be readily performed due to their transparency. Third, their diminutive size and extraordinary fecundity (a single clutch can number in the hundreds of animals) can facilitate medium throughput screening of hundreds of embryos distributed into 96-well plates; a feat that is not possible with any other vertebrate animal model. The number of cells injected into zebrafish embryos is also low compared to murine xenografts, typically 50–100 cells [43–45], which may provide an advantage in cases where there are limited materials, such as with primary tissue transplants (e.g. from needle biopsy). Finally, automated or semi-automated whole animal imaging, combined with image analysis algorithms, enables quantification of metastatic spread and dissemination kinetics [27, 42].

Despite the advantages discussed above, zebrafish also present several possible disadvantages that should be considered. One of the chief concerns, as alluded to briefly above, is the possible lack of appropriate orthotopic sites for tumour-host interactions within the fish. Zebrafish lack lungs, prostate and mammary tissue and as such no orthotopic sites for tumours derived from these tissues exist in the fish, and thus conclusions drawn from xenograft studies using these tumours should be confirmed using other assays or models, such as mice. In addition, appropriate growth factors for the faithful proliferation of certain tumours may also be lacking (or in limited quantities) to sustain growth of tumour within fish. However, this challenge can be partly mitigated by supplementing growth factors directly in the embryo water of engrafted fish. Finally, zebrafish, despite sharing > 70 % genetic identify with humans, nonetheless lack several genes (e.g. the p14/ARF (CDKN2A) and PML tumour suppressors) and harbor at times multiple paralogs of human genes (e.g. there four Twist paralogs in zebrafish), which must be considered in interpreting results from zebrafish cancer models [46].

Future Studies

The zebrafish XT model system will continue to be used for mechanistic studies that will complement other model systems. However, to improve and expand the use of zebrafish XT some of the challenges regarding lack of appropriate orthotopic sites and genetic dissimilarities between fish and humans need to be addressed through new approaches. For example, co-injection of “helper-cells” or stroma expressing

appropriate tissue-specific factors may aid in engraftment of disparate tumour types for which the appropriate orthotopic tissue is lacking in the fish.

A second major area of future work using zebrafish XT will be the employment of human primary tumour xenografts. While the vast majority of experiments in zebrafish xenografts have used cultured cells, there is movement towards the injection of tumour cells, similar to patient-derived xenograft (PDX) models used in mice. The first such study used labeled primary pancreatic tumours that were transplanted into zebrafish embryos and found to be metastatic, whereas non-cancerous tissue showed no signs of invasion or metastasis [19]. In another study, a subpopulation of cells, the putative TIC population, was isolated from prostate tumours and transplanted into zebrafish [23]. In order to characterize invasive properties prior to xenografts, primary lung tumour cells were maintained in short term cell culture (5–10 days). The amount of invasion and micrometastases found in the zebrafish xenografts of the primary cultures mirrored the invasive potential found *in vitro* [22]. This approach has recently been pioneered for primary patient bone marrow-derived diagnostic leukemia samples, with the opportunity to personalize therapy by providing real time *in vivo* response data to specific targeted agents (Bentley et al., submitted).

Another burgeoning area employing the zebrafish XT model is chemosensitivity assays to define tumour-drug interactions and possibly even provide an avenue for personalization of therapy using PDX. Finally, the mechanistic insights gleaned from the metastasis studies using zebrafish XT will highlight which pathways can be targeted for anti-metastasis therapy. Therefore, future studies are also likely to leverage both the metastasis and chemosensitivity applications of the model system to evaluate potential novel anti-metastasis therapies.

Genetically Engineered Mouse Models of Metastatic Cancer

Genetically engineered mouse models (GEMMs) have been generated in an attempt to model spontaneous tumour formation and progression. As a tool for metastasis research, it is important the models mimic the progression from localized cancer to metastatic disease and that the metastatic lesions occur in the appropriate locations. While there are metastatic GEMMs for many cancers, including bladder, thyroid, lung, pancreas, and skin, this section will focus on genetic models of breast and prostate cancer. These models have been invaluable for study of the metastatic cascade and dissection of the factors which contribute to metastasis.

Breast Cancer

The most common sites for breast cancer metastasis are liver, brain, lung and bone. To induce mammary tumours, oncogenes such as Her2 (human epidermal growth factor receptor-2), MET (hepatocyte growth factor receptor), Wnt-1 (wingless-type

mouse mammary tumour virus integration site family member-1) can be overexpressed specifically in mammary epithelial cells. The mammary specific promoters used most often to drive these oncogenes to establish transgenic mice are the mouse mammary tumour virus (MMTV) or whey acidic protein (WAP). When Her2/neu was constitutively overexpressed in mammary epithelial cells under the control of the MMTV promoter, tumours arose with an average onset of 4 months and lung metastases were detected at high frequency [47]. Invasion and metastasis can be accelerated in the Her2/neu transgenic mice by the additional activation of Akt/PKB (phosphokinase B) [48]. Activating MET mutations cause mammary tumours and spontaneous metastasis to the lung, lymph nodes (LN), kidney and heart [49]. Metastasis in MMTV-Wnt-1 mice are rare at the time of initial tumour detection, however, after the primary tumour is removed, frequent lung and LN metastases are observed [50].

PyVmT (polyoma virus middle T) mice recapitulate breast tumour progression in humans from a pre-malignant state through to the development of metastatic mammary tumours [51]. PyVmT mice develop poorly differentiated mammary tumours with high penetrance of pulmonary metastases, and transplantation of spontaneously-derived PyVmT tumours into cleared mammary fat pads result in transplantable metastatic mammary tumours [52]. This model has been useful in studying various factors involved in breast tumour metastasis, including the importance of Akt/PKB signaling in tumour development and metastatic progression [53]. The role of host cells in promoting PyVmT metastasis has also been studied extensively, including the role of colony-stimulating factor-1 (CSF-1) induced tumour associated macrophages (TAMs) on primary tumour growth and metastasis [54, 55].

Mouse models with inducible, rather than constitutive, oncogene overexpression provide a means to study tumour progression and metastatic growth with tighter control over the induction of the oncogene. For example, transgenic mice that express the Her2 transgene under control of the tetracycline promoter create inducible overexpression of Her2/neu in the mammary gland upon treatment of the mice with doxycycline. Conditional activation of Her2/neu with doxycycline results in rapid onset of mammary tumours and pulmonary metastases. Both the primary and metastatic lesions continue to rely on Her2/neu activation as withdrawal of doxycycline results in regression [56]. Similarly, when Wnt-1 is conditionally overexpressed, tumours metastasize and subsequently regress upon removal of doxycycline [57]. However, recurrence of the regressed tumours differs in the two model systems, with tumour recurrence being much more frequent with the *Her2/neu* oncogene than with *Wnt-1* [58]. In the Wnt-1 inducible system, loss of a p53 allele decreases the number of tumours that regress while also increasing the frequency of recurrent tumours [57].

Mammary specific deletion of tumour suppressor genes has also been used to model breast cancer metastases. For example, p53-deficient mice generated using the WAP-Cre system resulted in estrogen receptor positive tumours that metastasized to the lungs and liver with a long latency [59]. Similarly, loss of BRCA1 (breast cancer-1, early onset) alone in the mammary gland produces tumours, albeit at low frequency and a long latency [60], and tumours that formed in this model demonstrated alterations in p53 expression. A combination of BRCA1 loss and loss

of one p53 allele produces tumours that metastasize to the liver and lungs with a shorter latency than with p53 deficiency alone [61]. Loss of both p53 and E-cadherin using the WAP-Cre system results in lobular tumours which metastasize to skin, lungs, liver, spleen, pancreas, GI tract and peritoneal cavity, with bone metastases also observed at low frequency [62].

Loss of PTEN (phosphatase and tensin homolog) and Her2 overexpression are genetic events that often co-occur in breast cancers, and GEMMs with Her2 overexpression results in tumours with the basal-like subtype that metastasize to the lungs [63]. In a similar model, control of Her2 expression and Cre recombinase were put under the control of the MMTV promoter in a bi-cistronic transcript to generate the PTEN-deficient "NIC" mice. The tumours in these mice were of the luminal subtype of breast cancer and there was an increase in metastatic lung lesions in these PTEN-deficient mice compared to the PTEN heterozygotes or the parental strain [64]. There was increased angiogenesis observed at the primary tumour site, which is postulated to contribute to the higher rates of metastasis [64].

Overall, there are several elegant mouse models that spontaneously, or after induction, form mammary tumours that can metastasize, and these models have provided important insights into the role of various oncogenes and proto-oncogenes in mammary tumour development. It should be noted that mice have ten mammary fat pads, all of which can develop mammary tumours in these models. Thus, experiments designed to study the effect of surgical primary tumour resection on the growth and development of residual metastatic disease is technically challenging in GEMMs, and murine xenograft models are better suited for these types of studies as will be discussed in sections 4.4–4.5 below.

Prostate Cancer

Prostate cancers metastasize primarily to bone and lymph nodes; however, there are no GEMMs that consistently and reproducibly metastasize to bone. Prostate specific expression of oncogenes is often achieved using the probasin (PB) promoter or the modified ARR_2PB promoter, which increases prostate gland specificity by adding two androgen response (AR) elements to the PB promoter. Valkenburg and Williams have authored an extensive review describing various mouse models of prostate cancer, including discussion of how closely each model recapitulates human prostate cancer [65].

PB driven expression of the SV40 T antigen is used to generate the TRAMP (transgenic adenocarcinoma of the mouse prostate) model. The TRAMP model follows progression of the human disease from prostatic intraepithelial neoplasia (PIN), to multifocal adenocarcinoma, followed by invasive metastases in lung and LN (rarely bone, kidney and adrenal glands) [66–68]. The Lady model is a modified version of the TRAMP model, and was created in 1998 using a longer PB promoter fragment linked to a deletion mutant of SV40 that only expresses the large T antigen (and not the small T antigen). Prostate tumours generated in Lady

mice are invasive and display features of neuroendocrine differentiation, but do not metastasize. Additional genetic alterations have been made to Lady and TRAMP models in an attempt to discover factors that contribute to altered metastatic potential in the two models. For example, overexpression of hepsin (cell surface serine protease often overexpressed in prostate cancer) in the Lady model has no impact on primary tumour formation, but promotes metastatic dissemination to lung, liver, and bone [69]. Similarly, expression of a dominant negative TGF-β receptor type II, thereby blocking TGF-β signaling, produces primary tumours of similar sizes, but with increased metastases compared to mice expressing T-antigen alone [70]. Prostate specific deletion of fibroblast growth factor receptor-1 (FGFR1) in the TRAMP background results in smaller tumours and less metastases, indicating its importance in prostate cancer progression [71].

Other transgenic models that mimic the progression of prostate cancer from PIN to metastasis include the PSP-KIMAP model, where the SV40 T antigen is knocked into PSP94 (prostate secretory protein 94), which is highly expressed in prostate cells. Metastases are observed in the lymph nodes, lung and liver [72]. Since PTEN is often lost in prostate cancer and in 40% of metastatic lesions [73], mice with prostate specific deletion of PTEN (using ARR_2PB-Cre) have been created. Prostate-specific deletion of PTEN results in PIN and eventually invasive prostate cancer with metastases in lymph nodes and lungs [74]. Similarly, SMAD4 is downregulated in high grade PIN and prostate cancer compared to benign prostatic hyperplasia [75], and deletion of SMAD4 in PTEN null mice increases metastatic potential [76]. Conditional inactivation of either p53 or retinoblastoma protein (Rb) individually in the prostate results in PIN and neoplasia, although combined loss of both tumour suppressors causes a more aggressive phenotype with neuroendocrine differentiation and distal metastases to the lymph nodes, liver and lung.

As with the mammary tumour GEMMs discussed above, the generation of prostate cancer GEMMs has greatly increased our understanding of oncogenes and tumour suppressor genes that are involved in prostate tumourigenesis and prostate cancer progression. Prostate cancer GEMMs that consistently metastasize to bone have not been reported and this is an important consideration since bone is the primary site of prostate cancer metastasis. The study of prostate tumour metastasis is therefore restricted to the use of prostate tumour xenografts as will be discussed in Sects. 4.4–4.5.

Future Directions

Current GEMMs offer a good framework for assessing the role of particular oncogenes, proto-oncogenes, and tumour suppressor genes in tumour development and progression, with several models also allowing study of the metastatic process. One important caveat to the use of GEMMs is that tumours result from ubiquitous expression of transgenes within the cells of a tissue, which does not mimic the state of human cancers where a minority of cancer cells arises in a milieu of untransformed cells.

There are also no currently available models for spontaneous brain cancer metastases, and the kinetics of GEMM tumour progression and metastasis is difficult to compare to patients in the clinic. Primary tumour volumes can limit the study of metastases, since mice can succumb to a large primary tumour burden prior to the detection of metastases in distant tissues and surgical resection of spontaneous primary tumours is often not possible. In these instances, tumour xenograft models allow researchers to more easily distinguish the role of a particular gene on growth of the primary tumour xenograft compared to the development and growth of tumour metastases.

Murine Xenograft Models

Murine xenografts have been used for decades to study primary tumour development, response to therapy, and the metastatic process. With the widespread use of tumour xenografts to study metastatic disease in mice, this section will focus on two important considerations for designing experiments to determine the effect of a particular gene on the metastatic process. We will first discuss different tumour cell implantation methods that have been employed to study the colonization of tissues with tumour cells, and will subsequently consider important methodologies for quantifying the development and growth of metastatic tumours in mice.

Implantation Sites and Artificial Models of Metastasis

Xenografted tumours were originally grown by implanting tumour cell suspensions in subcutaneous sites, although efforts to more closely model the natural tumour environment has led researchers to increasingly implant tumour xenografts in orthotopic sites. While orthotopic implantation of many tumour types can require surgical expertise (e.g., pancreatic tumours), there are several advantages inherent with implanting tumour xenografts in the tissue of origin. Murine skin is relatively poorly vascularized relative to most other organs, and there can be profound differences between subcutaneous and orthotopic tumour xenografts. The site of primary tumour implantation can have a profound effect on response of the tumour to chemotherapy [77], and transcription factors such as hypoxia-inducible factor-1 (HIF-1) can produce opposing effects on the growth of tumours implanted in subcutaneous or orthotopic sites [78]. Similarly, subcutaneously implanted tumours can be more poorly oxygenated than orthotopically implanted tumours of the same cell type [79], with orthotopic tumours thought to more accurately reflect the tumour microenvironment found in patients. Metastatic dissemination of tumour cells from a primary tumour can also be profoundly influenced by xenograft implantation site [80] with obvious implications for studying metastasis in pre-clinical models. In general, the use of murine tumour xenografts to study metastasis is appealing because the entire process of metastasis (from tumour cell dissemination to target organ colonization) is required to produce a measureable metastatic tumour.

Surgical resection of primary tumour xenografts can be performed in order to study the growth of spontaneous tumour metastases in the absence of the primary tumour, and to derive increasingly aggressive metastatic variants of tumour cell lines. Orthotopic breast tumours or melanomas are the most amenable to surgical resection, and removal of the primary tumour allows growth of disseminated tumour cells in metastatic tissues for periods of time that would otherwise be restricted by primary tumour volume. To generate clinically relevant metastatic variants of orthotopically implanted tumour cells, metastatic tumour foci are isolated from tissues after surgical resection of the primary tumour and re-implanted in the orthotopic site for repeated generations. This methodology has been used to generate derivatives of the original tumour cell line that spontaneously metastasize to the tissue of interest [81, 82]. Importantly, these spontaneously derived metastatic tumour cell variants have been used to assess fundamental differences between chemotherapeutic response of primary tumours and tumour metastases, and between pre-clinical tumour xenograft models and patients in the clinic [83].

In order to study the process of tumour cell colonization in greater detail, many groups rely on artificial distribution of tumour cells to metastatic target organs. Intravenous injection of tumour cells into the lateral tail vein has been used to directly seed lung tissue with tumour cells. Similarly, intrasplenic injections have been used to seed the liver with tumour cells, intraperitoneal injection of ovarian cancer cells is used to generate ascites and multiple metastatic nodules in the peritoneal cavity, and intracardiac injection of tumour cells are typically used to seed tissues throughout the mouse including the brain and bone. It is important to note that while each of these methods may produce tumours in target tissues (depending on the capacity of the injected tumour cells to colonize the tissue), none of these methods recapitulate (or require) early events of the metastatic process such as tumour cell migration, invasion, and intravasation. Seeding tissues with tumour cells has produced important contributions to our understanding of the genes involved in promoting metastatic growth. Systemic injection of tumour cells facilitates intravital imaging of tumour cell extravasation in tissues at defined timepoints after tumour cell delivery into the circulation. Also, tumour cell foci that grow in tissues after intravenous or intracardiac injection of tumour cells can be harvested and reinjected several times to select for tumour cells that preferentially colonize a particular tissue. Expression analyses of the resultant tumour cell lines have been used to identify many genes that mediate metastatic growth in tissues such as the lungs [84] or brain [85]. These models differ from the spontaneously metastasizing orthotopic tumour cell line derivatives discussed above, but have nevertheless provided important insights into molecular mediators of tissue colonization.

Regardless of the choice of murine xenograft model, the quality of data generated to describe the role of a particular gene in the process of metastasis is entirely dependent on the method selected to quantify metastatic growth. Common methods to measure experimental metastases range from enumeration of macroscopic metastases on the surface of a tissue to highly sensitive single cell analyses of disaggregated tissues that contain metastatic tumour cells.

Quantification of Metastatic Disease

The crudest methods to quantify metastases involve weighing tissues from tumour-bearing animals to determine the overall mass of the metastatic tumour burden, and counting macroscopic metastatic foci on the surface of the excised tissue. While these methods may be appropriate for quantifying large differences in metastatic tumour burden between experimental groups, these sorts of measurements are unable to determine how a particular gene is influencing the different steps of metastasis and would typically require continuation of experiments beyond humane endpoints for the animals unless the primary tumour is surgically resected. Non-invasive imaging modalities are far better alternatives for studying the development of macroscopic metastases, with the distinct advantage that metastatic tumour growth can be quantified in the same animal over time.

Positron emission tomography (PET) combined with computed tomography (CT) allows researchers to monitor metastatic tumour burden in the same mouse over time, and quantification of 18F labeled deoxyglucose (18FDG; an analogue of glucose) by PET is the most common method to detect metabolically active tumour metastases in animal models and the clinic. Detection of experimental radio-labeled antibodies against tumour cell specific markers is also facilitated by PET imaging, and the development of novel PET agents has the potential to provide improvements over 18FDG in terms of tumour specificity and the detection of tumour sub-types. Radio-labeled PET tracers emit positrons that interact with electrons to produce a pair of gamma photons traveling in opposite directions, and the detection of these gamma photons provides a means to localize PET signals. The resolution of PET imaging is on the order of a few millimeters, depending on the energy of the emitted positron (and hence the radioisotope used in the tracer) and the instrumentation used for imaging [86]. Overall, PET/CT imaging is an excellent modality to detect metastatic disease and is applicable to analyzing the metastatic growth of spontaneously derived tumours, which represents a distinct advantage over imaging modalities that are based on tumour cells that have been genetically modified to express fluorescent markers or bioluminescent enzymes (see below). However, the detection sensitivity of PET/CT is not amenable to quantifying microscopic metastatic disease, and PET/CT imaging requires access to the imaging equipment, appropriate expertise for image analyses, and a cyclotron to produce the radiolabeled PET tracers. The imaging and data analysis can also be labour intensive relative to non-invasive imaging modalities based on the detection and quantification of bioluminescent or fluorescent tumour cells.

Bioluminescent imaging is an alternative to PET/CT imaging that is applicable to model systems where the tumour cells can be genetically manipulated. Tumour cells are engineered to stably express a luciferase enzyme prior to introduction into mice, and intraperitoneal injection of the enzyme substrate (e.g., D-luciferin for firefly luciferase) causes the production of bioluminescent light from the tumour cells. Bioluminescence is detected and quantified by a high sensitivity camera coupled to an image analysis system, and the bioluminescent signal intensity can be monitored in the same mouse over time. Most systems allow imaging of five mice

simultaneously, and image analysis software to quantify photon emission from tumour cells is relatively straightforward to use. However, in the case of a mouse with metastatic disease, it is difficult to determine the source of bioluminescence-producing tumour cells *in situ*, and confirmation of the source of metastatic disease requires combination of bioluminescent imaging with CT scans or post-mortem comparative analyses. Large primary tumours tend to mask light emitted from smaller metastatic tumour foci, and emitted light can be quenched by tissues containing high levels of hemoglobin (e.g., liver). Regardless, bioluminescent imaging is increasingly being used to quantify metastatic disease in xenograft models of metastasis. While non-invasive imaging modalities allow researchers to monitor and quantify metastatic tumour growth in the same animal as a function of time, these methods necessarily require the development of macroscopic tumours dependent on the detection sensitivity of the imaging modality. Genes that affect earlier steps in the metastatic process or metastatic tumour cells that do not form large metastatic tumour foci are more readily studied using complimentary microscopic or single cell-based analyses.

Tissue sections stained with haematoxylin and eosin allow observation of metastatic tumour cell foci that can be enumerated and/or processed by image analysis software to determine overall metastatic tumour area in a given tissue section. Microscopic analyses of tissues provides important information about the relative size and number of metastatic tumour cell foci in a tissue, provided appropriate consideration is given to analyze multiple sections from increasing depth in the tissue in order to get an accurate (3-dimensional) indication of metastatic growth. Identification of smaller metastatic tumour cell foci can be challenging, and is often facilitated by staining serial sections for tumour cell specific markers (e.g., cytokeratins) or through the use of fluorescently-tagged tumour cells. Fluorescently-tagged tumour cells are also amenable to analyzing metastatic tumour cell content in disaggregated tissue by flow cytometry. However, it should be noted that some immunocompetent mouse strains (e.g., Balb/c mice) have been reported to mount an immune response against tumour cells expressing fluorescent proteins [87–89] and therefore the use of fluorescently-tagged tumour cells in a particular model system should be carefully considered [90]. The most accurate cell-based method to quantify metastatic tumour cells is to plate bulk cells derived from disaggregated tissues into *ex vivo* clonogenic assays. Clonogenic assays allow detection of single clonogenic tumour cells in an entire tissue provided the plating efficiency of the tumour cell lines *ex vivo* is taken into account. Transfection of tumour cells with drug-resistance markers prior to implantation in the mouse allows quantification of clonogenic metastatic tumour cells in disaggregated tissue without the staining background generated by fibroblasts and immune cells present in the disaggregated cell suspension.

Taken together, there are a variety of methods available to quantify tumour metastases in spontaneous and transplantable tumour xenografts. Traditional human cancer cell line-based xenograft models have provided insights into the pathogenesis and metastasis of cancer, resulting in the development of a handful of therapeutic agents for treatment of the disease. However, most of the data obtained on potential

cancer therapeutics with such *in vivo* models failed to translate into improvements in the management of cancer patients [91, 92]. Only about 5% of new potential anti-cancer drugs, that successfully passed preclinical *in vivo* tests, have significant efficacy in clinical trials and are approved for clinical usage by the U.S. Food & Drug Administration [93]. This discrepancy appears to stem from a lack of ability of established cancer cell lines to accurately predict the efficacy of anticancer agents in the clinic. This is thought to be due to increased cellular homogeneity after long-term culturing of the cell lines, contrasting with the heterogeneity of the parental cancers. Furthermore, cell line-based xenografts rarely possess the tissue architecture of the original cancer specimens from which the cell lines were derived and, consequently, do not properly represent the micro-environment as found in the original malignancies. There is therefore an urgent need for experimental cancer models that better represent the heterogeneity of human tumours and accurately predict clinical drug efficacies. In view of this, PDX models, generated by transplanting freshly resected patients' tumour tissue into immunodeficient murine hosts without an intermediate *in vitro* culture step, represent a major advancement.

Patient-Derived Xenografts

Implantation Sites

There are currently three common PDX graft sites used in immunodeficient mice to establish PDX models, namely the subcutaneous, orthotopic and subrenal capsule (SRC) sites. The subcutaneous graft site is the most commonly used site and has various advantages, including easy tissue implantation and monitoring of the developing tumour [94]. However, this site is known for a generally low engraftment rate, particularly in the case of some types of cancer [95]. This may be due to its well-known lack of vascularization and hence potentially inadequate nutrient supply that can lead to loss of cancer subpopulations. Also, the metastatic rates of subcutaneous xenografts are low, even for grafts derived from tumours that were highly metastatic in the patient. The orthotopic graft site provides a microenvironment similar to that of the original cancer and is theoretically the ideal graft site for testing spontaneous metastatic ability of cancer tissue. However, this site can have a limited xenograft carrying capacity depending on the tumour type, which can restrict the use of orthotopic implantation sites for establishment of models of some types of cancer. Successful engraftment at the orthotopic site was also found to be limited to highly advanced cancers, as found for the subcutaneous site [96]. PDX grafting in the SRC was first proposed for tissue implantation by Bogden [97]. A major advantage of the SRC graft site is the availability of an instant blood supply due to the high vascularization of the kidney [98, 99]. This provides high graft perfusion and the abundant supply of nutrients, hormones, growth factors and oxygen to the transplanted tissue is instrumental to the success of engraftment [100–104]. In addition, measuring the area of graft growing into the kidney parenchyma allows

quantitative examination of the local invasion ability of implanted tumours. As found with orthotopic grafting, the surgical procedure involved in SRC grafting is more difficult than subcutaneous implantation of tumour fragments, but the relatively high PDX take rate make SRC grafting a preferred site for PDX development.

Metastatic PDX Models of Various Types of Cancer

The study of mechanisms of cancer metastasis is hampered by limited access to clinical metastatic samples and the scarcity of *in vivo* models that show spontaneous, clinically relevant metastasis [105]. Since the first report of successful xenografting of a patient's tumour into nude mice in 1969, a number of PDX models derived from various types of primary and metastatic clinical samples have been established [94]. Some of the xenografts showed spontaneous, clinically relevant metastatic ability and provided unlimited sources of metastatic tissue for investigation of cancer metastasis mechanisms and identification of new therapeutic targets.

There is a long history of success with the establishment of colorectal PDX models [106, 107]. Recently, characterization of a large panel of colorectal PDX models showed that 13 out of 41 models (32%), established via orthotopic grafting, gave rise to metastases in mesenteric lymph nodes, liver or lung. Interestingly, the sites of metastatic dissemination of cancer cells in these models are similar to those commonly seen in the clinic [108]. PDX models showing similar spontaneous metastasis were also reported by other groups [109]. Orthotopic PDX models of pancreatic cancer have been also reported to retain stromal components and develop regional and distant metastases. Spontaneous metastases to liver and lung and development of malignant ascites have been observed in a number of studies [110–112].

Historically, the lowest success rates and metastatic abilities of PDX models have been observed in hormone-dependent mammary and prostatic carcinomas [113, 114]. However, recent efforts, involving orthotopic implantation of human breast cancer tissue with supplementation of estrogen, have resulted in improved engraftment efficiency and generated a number of improved PDX models [115, 116]. These models represent the diverse cancer subtypes observed in the clinic and, importantly, maintain essential features of the original tumours, especially their ability to metastasize to specific sites [115]. In the case of prostate cancer, a few PDX models derived from subcutaneous implantation of metastatic and/or castration-resistant cancer tissues have been described to show spontaneous metastasis to lymph node, liver and lung [113, 117, 118]. Recently, a new panel of PDX models developed via SRC grafting from needle biopsies, as well as primary and metastatic tissues, have shown a much higher engraftment rate [119–122]. Importantly, these models, including multiple models derived from biopsies of the same patient, showed various spontaneous metastatic abilities and provided valuable tools for studying intratumoural heterogeneity and underlying mechanisms of metastasis. Furthermore, recent studies of both breast and prostate cancers showed that the rate of engraftment could be used as a prognostic factor for patient survival time even for individuals with newly diagnosed disease who did not have detectable

metastases at the time of surgery. Thus, the PDX take rate in mice could potentially be used as a surrogate prognostic indicator of aggressiveness and risk of disease progression.

Advantages and Major Applications of PDX Models

There are a number of general advantages of PDX models compared to traditional cell line xenograft models. First, the PDX models retain histologic characteristics and certain intratumoural cellular heterogeneity seen in the parental tumours [115, 123–126]. Secondly, these PDX models, especially initially, maintain the human stromal component, and therefore are thought to more accurately represent the complex biochemical and physical interactions between the cancer cells and various components of their microenvironment as found in the original malignancies [94, 127]. Third, the PDX models preserve molecular characteristics of the original cancer, including chromosomal copy number variants [115, 128, 129], single-nucleotide polymorphisms [116, 130] and gene expression profiles [116, 126, 128, 131, 132]. Fourth, PDX models show responses to anti-cancer therapy that are more representative of patient tumour response in the clinic [133–135].

PDX models provide valuable tools for (i) addressing a variety of questions regarding the cause, progression, and therapy of cancer (e.g., for investigating the mechanisms of cancer metastasis and identifying new therapeutic targets), (ii) translational research (e.g., for efficacy and toxicity testing of potential and established anticancer therapeutic approaches and biomarker discovery) and (iii) personalized cancer therapy.

Application of PDX Models to the Study of Cancer Metastasis

PDX models retain most aspects of the human tumour microenvironment at an early passage, and they therefore provide opportunity to study the role of the solid tumour microenvironment in promoting metastatic growth that can complement work done in other tumour xenograft models. For example, the poorly oxygenated (hypoxic) microenvironment in solid tumours is known to be associated with treatment resistance, increased metastatic potential, and poor outcome. David Hedley and colleagues recently developed a series of orthotopically grown PDX models of pancreatic cancer and identified significant associations between hypoxia and aggressive growth or development of metastases in these models [112]. Furthermore, gene expression analysis showed increased expression of genes involved in cell survival and proliferation in the hypoxic models. This study indicated that hypoxia is a major adverse prognostic factor in pancreatic cancer patients and supports the development of hypoxia-targeting therapy.

Identification of metastasis-driving genes by a comparative gene expression profile analysis of primary and secondary clinical samples forms a major

challenge, since such samples are not homogeneous, but contain sub-populations of cancer cells that vary widely in metastatic abilities. To overcome this hurdle, a number of paired transplantable metastatic and non-metastatic prostate cancer PDX models have been successfully developed from individual patients' primary cancer tissues, such as the paired metastatic PCa1-met and non-metastatic PCa2 [120, 121], the LTL220M and LTL220N [122] and the LTL313 series PDX models [126]. Comparative gene expression analyses and bioinformatics and network analyses of these PDXs have led to identification of a number of cancer metastasis-associated genes [121, 136]. Similarly, differentially expressed miRNAs in a pair of metastatic and non-metastatic prostate cancer PDXs, LTL313B and LTL313H, have been identified, which likely include potential biomarkers for prostate cancer metastasis [137].

PDXs for Drug and Biomarker Discovery

In the era of targeted cancer therapy, it is important to evaluate drug efficacies using experimental models showing clinically relevant expression of molecular targets. Preservation of a patient's tumour genomic profile and tumour microenvironment in PDXs gives the opportunity to use primary patient tumour grafts as a model to improve the translation of preclinical therapeutic strategies in oncology. One preclinical setting in which PDXs can be extremely relevant is presented by evaluation of new potential drugs for cancer treatment. New drugs can be tested using a panel of PDXs which faithfully represent the heterogeneity of a specific cancer type, potentially leading to identification of the best treatment regimen for the particular cancer. To date, a large number of approved and pre-clinical anticancer drugs have been tested using PDX models, as recently reviewed [94, 138].

PDX models are also used for biomarker discovery. For example, taking advantage of a panel of metastatic colorectal PDX models, Bertotti et al. identified HER2 as a predictor of resistance to anti-epidermal growth factor receptor Cetuximab antibodies and revealed that the combined inhibition of HER2 and EGFR induced overt, long-lasting tumour regression [131]. The response to Cetuximab was also investigated using PDXs generated from various types of cancer, leading to identification of MET activation as a mechanism for drug resistance [139]. In addition, John J. Tentler et al. have identified activation of the Wnt pathway as a biomarker predicting resistance to a mitogen-activated protein kinase kinase MEK1/2 inhibitor, AZD6244, in Kras mutant colorectal cancer PDTX models, indicating a potential combination therapy strategy for the disease [140].

Personalized Cancer Therapy

Since each patient's cancer is unique, cancer therapy should ideally be tailored to individual patients. Choosing the most effective, least toxic regimen for a patient is

one of the major challenges faced by oncologists today [141]. In view of this, early generation PDX models of the patient's own malignancy may be useful for predictive drug efficacy testing for personalized cancer therapy. One attempt to use PDX models aiding in the selection of appropriate chemotherapeutic agents in personalized cancer therapy is the use of "mouse Avatars" [134, 135, 138, 142]. In a pilot study, Hidalgo et al. implanted various advanced solid tumours resected from 14 patients in immunodeficient mice, and treated them with 63 drugs in 232 treatment regimens. Overall, a significant correlation was observed between drug activity in the model and clinical outcome, both in terms of resistance and sensitivity. Importantly, using the treatments selected for each individual patient based on the results obtained with the corresponding PDX, 11 of 14 patients achieved a partial response. It should be noted that the treatments selected were not obvious and would not have been the first choice for a conventional second or third line treatment. This suggests that PDX models can be used to select effective personalized treatments. Recently, the same group integrated next-generation sequencing with the Avatar mouse model, identified tumour-specific mutation and copy-number alterations, and treated the PDX models with targeted drugs. It was reported that 6 out of 13 patients who received a personalized treatment achieved durable partial remissions, demonstrating that analysis of somatic genetic alterations combined with use of PDX models can be performed in the clinical setting and facilitate selection of specific treatments for personalized cancer therapy. A similar concordance between early generation engrafted tumours and original patient tumours has been established by different groups as well [128, 132, 143]. Although at present the sample size is too small to conclude if this approach is better than the standard-of-care approach for selecting a particular therapy, the robustness and accuracy of these systems in predicting anti-cancer drug efficacy in an individual patient will likely be validated in the near future.

Caveats and Future Prospects

At present, PDX models likely provide the most clinically relevant models of human cancer. However, like other models, PDXs have their inherent limitations and deficiencies. First, the most commonly documented limitation of all the xenograft models is the requirement to use immunodeficient mice which lack the human immune system. To circumvent this limitation, more sophisticated PDX models (humanized models) should be developed via cografting of tumour tissue along with bone marrow stem cells of the same patient. This combination may reconstitute components of the human immune system in mice and allow investigation of the role of the immune system in cancer metastasis and efficacy of immune-based therapies. Secondly, although PDX models are relatively stable for several generations, increasing histopathological and molecular differences between patient tumours and PDXs are foreseeable with continual passaging in the animals. It is therefore prudent to establish cryopreservation of PDXs at an early generation, ensuring preservation of the cellular and molecular characteristics of the original tumour and a

relatively robust supply of a particular patient's tumour. The maintenance of a successful PDX program requires ample financial resources and specialized expertise to reliably and reproducibly perform the PDX implants, which is a major factor hampering the widespread usage of PDX models compared to traditional cell line-based xenograft systems.

In summary, PDX models closely mimic the original cancers in terms of histopathology, tumour heterogeneity, chromosomal aberrations, gene expression profiles, tumour aggressiveness, and response to therapy. PDX models continue to be refined in order to increase take rate and to further enhance the already high clinical relevance of the system.

Concluding Remarks

While there are a variety of animal models that can be applied to the study of solid tumour metastasis, the choice of model system depends largely on the research question. Investigators seeking high-throughput analyses of genes or compounds that affect tumour cell migration may find that the zebrafish model is suitable for their purposes. Experiments designed to test the oncogenic potential of a particular gene may be suitable for combination with established genetically engineered mouse models, while dissecting the contribution of a particular gene to individual steps of the metastatic process is well-suited to the application of murine xenograft models. In order to maximize the clinical applicability of experimental findings, researchers are increasingly turning to patient-derived xenografts in order to validate experimental findings established in other model systems and in an effort to personalize cancer therapy.

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Chapter 6
Microenvironmental Control of Metastatic Progression

Calvin D. Roskelley

Abstract As solid tumors progress, the surrounding microenvironment is altered dramatically. This microenvironment contains stromal and immune cells, some resident and some newly recruited, that are often activated due to factors released by the tumor cells themselves. These activated cells then release soluble factors that feed forward on the tumour cells in a symbiotic manner. Activated cells also alter the deposition and processing of extracellular matrix molecules in the microenvironment which further affects both the genotype and the phenotype of tumor cells. More specifically, these microenvironmental alterations can have profound effects on the genome and epigenome of tumor cells as well as their signal transduction pathways, both biochemical and mechanical. All of these effects contribute to the invasion and progression of the metastatic tumor organ.

Keywords Microenvironment · Tumor progression · Stroma · Extracellular matrix

Abbreviations

CAFs	Cancer-associated fibroblasts
CIMP	CpG island hypermethylation phenotype
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
HIF1α	Hypoxia inducible factor 1alpha
MET	Mesenchymal to epithelial transition
MSI	Microsatellite instability
TAMs	Tumor-associated macrophages
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor

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Introduction

Given that the overwhelming majority of cancer victims succumb to the formation and expansion of metastatic lesions, an overarching goal of modern cancer research is to determine how changes in defined cohorts of oncogenes and tumor suppressors facilitate the emergence of the malignant phenotype within individual tumor cells and their progeny. As a result, identifying actionable targets that are the products of such dysregulated genes has become the cornerstone of rational cancer therapeutics. However, even when they are directed against bona fide targets, such therapies are often only partially effective and they are invariably susceptible to acquired resistance as the tumor evolves towards full blown malignancy. The recognition that many non-tumor cell autonomous events contribute to this progressive evolution, together with data generated by genome-wide profiling both at the transcriptomic and epigenomic levels, are starting to give us a systems-based picture of why this is the case. What is becoming increasingly clear from such studies is that the tumor microenvironment is a major multifactorial contributor to this progressive evolution [23].

In early *in situ* lesions that have been initiated by mutagenic insult, the microenvironment still closely resembles the normal tissue that the lesion arises in. In many cases, notably the breast [48], the prostate [56, 60], and the thyroid gland [25], this near 'normal' tissue microenvironment actually acts to suppress the further expansion of microcarcinomatous lesions. However, as the tumor progresses, the surrounding tissue microenvironment is replaced by an ever-changing milieu that is itself abnormal. Importantly, this abnormal microenvironment co-evolves with the tumor cells as part of the malignant tumor 'organ'. Rather than being suppressive, the microenvironment of the malignant tumor organ instead functions to promote the invasion and metastatic spread of the expanding lesion [5].

In addition to cellular differences, the microenvironment of the malignant tumor organ also differs in its extracellular components compared to the near normal microenvironment that surrounds early tumorigenic lesions. For example, factors released into the extracellular milieu by the tumor cells themselves can act to 'activate' nearby stromal cells in the surrounding microenvironment. The responding stromal cells can consist of those that already reside within the tissue affected or they can be recruited from other sites, most notably the bone marrow. The latter cells often have an ability to expand due to their progenitor characteristics which can further expand the activated stromal cell pool. This expansion and activation has been best documented in the case of cancer-associated fibroblasts (CAF's; [24]), although many other stromal cell types can also be 'activated' by factors in the tumor microenvironment.

CAF's are capable of modifying the acellular architecture of the tumor microenvironment by altering its insoluble extracellular matrix (ECM). This is achieved through changes in the production and deposition of matrix molecules as well as the alteration of the structure and interaction of those matrix molecules already present in the ECM. Examples include CAF-mediated changes in the deposition of

collagens, their extracellular processing by metalloproteases and their cross-linking by lysyl oxidases [11, 31]. CAF's also release soluble factors into the tumor microenvironment that act in a feed-forward way to further stimulate the growth of, or alter the phenotype of, tumor cells and to further recruit and activate more stromal cells. Tumor cell proliferation and survival can be facilitated by the CAF-mediated release of factors such as IGF-1, EGF, HGF and IL6, while a major tumor cell phenotype modifier that can be released by CAFs is transforming growth factor beta (TGF-β) (see epithelial-mesenchymal transformation section, below; [64]). Stromal cell recruitment and their feed forward activation can be initiated by the CAF-mediated release of cytokines such as CCL5 and SDF-1 [32, 35]. Importantly, the cytokines and growth factors that are released into the primary tumor microenvironment can act as short range paracrine factors as described above or they can also travel to distant sites and sow the microenvironmental soil to facilitate the expansion and local invasion of secondary metastatic lesions [76].

Acting in concert, soluble factors released by both the tumor cells and the activated stromal cells in the microenvironment can act over considerable distances to recruit immune cells to the lesion site. This immune infiltration, which has many of the hallmarks of a chronic inflammatory state, consists of varying numbers and ratios of lymphoid and myeloid cells, the precise nature of which depends on the tumor site involved and the malignant state of the lesion [59]. These infiltrates produce their own growth factors and cytokines that then further influence nearby and tumor and stromal cells in the manner described above. They also secrete proteolytic enzymes that can remodel the ECM [42]. Such remodeling can have profound effects on the tissue microenvironment as it can release and/or activate soluble factors that are normally sequestered within the insoluble portion of the ECM.

Soluble factors and ECM fragments released into the tumor microenvironment by proteolytic degradation can also act in a paracrine manner on nearby vascular endothelial cells and their surrounding pericyte stem-like population to initiate an angiogenic response. A critical factor that helps drive this angiogenic response is vascular endothelial growth factor (VEGF); [61], although VEGF-independent factors come to the fore as the malignant tumor organ evolves which has been confounding to targeted anti-angiogenic therapy development [63]. Regardless, the resulting formation of new blood vessels leads to an increase in blood supply that is critical to the malignant tumor organ's survival. Concurrently, the angiogenic process itself further alters the landscape of the tumor microenvironment because the resulting newly formed blood vessels are often tortuous, porous and 'leaky' which increases the hydrostatic pressure within the tissue. In addition, tumor cells are subjected to widely varying oxygen tensions depending how far they are from these new vessels [7, 19]. In areas of low oxygen, the resulting hypoxia induces metabolic alterations and a suite of gene expression changes in both the tumor and the stromal cells that lead to further microenvironmental changes in soluble factor production and ECM production and modulation that can affect a number of tumor characteristics including genome stability (see below). It is becoming increasingly clear that a wide variety of responses to metabolic alterations within the malignant tumor microenvironment are also mediated by the activated

stromal cells. For example, in response to the release of reactive oxygen species by tumor cell, nearby CAFs upregulate the expression of glycolytic enzymes which leads to their increased production and secretion of pyruvate and lactic acid that are then secreted and utilized by the surrounding tumor cells as a critical alternative energy source for their continued rapid proliferation [55].

While the cellular and acellular aspects of the microenvironment broadly influence tumor and stromal cell phenotypes, they also impinge upon specific processes that are critical for malignant progression. These include alterations to genomic stability, the epigenome, non-coding RNAs, immunomodulation, mesenchymal transformation and mechanotransduction. We provide specific examples below and expand on individual processes in the chapters that follow.

Hypoxia-Induced Genomic Instability

In normal tissues, the microenvironmental oxygen tension can be as high as 10%. In contrast, the oxygen tension within rapidly expanding locally advanced solid tumors is often less than 1% due in large part to their high metabolic rate [71]. The resulting hypoxia leads to the increased production of factors by both tumor and stromal cells that are transcriptional regulators, the prototype of which is hypoxia inducible factor 1alpha (HIF1α). These regulators both stimulate and suppress the expression of a wide variety of genes whose products modify the microenvironment to facilitate metastatic progression. These include the afore-mentioned angiogenic factor VEGF as well as osteopontin, an ECM protein that facilitates tumor cell invasion. The latter factor facilitates the movement of tumor cell cohorts even farther away from blood vessels which further exacerbates the hypoxic state locally within the lesion [43].

Hypoxia also acts to suppress the expression of a number of homologous recombination (HR) genes involved in repairing the DNA double strand breaks caused by ionizing radiation and radiomimetic drugs [44]. In some contexts this suppression is HIF1α-dependent [35] while in others it is not [4]. Interestingly, in some cases these two means of hypoxia-induced suppression can act on the same HR gene. This is the case, for example, with the suppression of BRCA1. Hypoxia also suppresses the expression of genes involved in DNA mismatch repair. This leads to an increase in spontaneous mutations that are associated with microsatellite instability (MSI) during experimental colorectal carcinoma progression [17, 62]. Furthermore, in human colorectal tumors HIF1α expression, which is used as an indication of hypoxia, and MSI are associated are associated with poor outcome/progression [21].

There is some evidence that hypoxia can also affect chromosome segregation during mitosis. Mechanistically, it appears that this occurs due to an alteration of the centrosome that leads to a defect in mitotic spindle formation [46]. Taken together, these and many other observations [43] indicate that hypoxia-mediated defects in DNA repair and chromosome segregation accelerate the genomic instability that

is already intrinsic to the growing tumor organ, thereby facilitating the continued evolution of malignant progression [9].

Epigenetic Dysregulation

The most widely studied epigenomic change that is correlated with tumorigenesis is the CpG island hypermethylation phenotype (CIMP). Essentially, this is a broad measure of suppressive promoter methylation that has been observed in bladder, breast, endometrial, gastric, colorectal, hepatocellular, lung, ovarian, pancreatic, renal cell and prostate carcinomas as well as leukemias, melanomas and gliomas. Drivers of this phenotype, including mutations in the isocitrade dehydrogenase-1 gene that result in the accumulation of the hypermethylating oncometabolite 2HG [70], are now being identified. However, while it is clear that the CIMP phenotype contributes to tumor formation, its role in tumor progression is less clear. For example, in colorectal cancer CIMP functionally contributes to the initial tumor formation but not tumor progression. Instead, a subsequent trend towards hypomethylation becomes more prominent as lesions progress from adenomas to invasive cancers [67]. In addition to contributing to a general increase in genomic instability, this hypomethylation has been shown to specifically trigger the production and release of soluble growth factors and modulators including insulin-like growth factor-2 (IGF2) and IGF2 binding protein-3 into the tumor microenvironment [29, 41]. These factors act in an autocrine fashion to increase tumor cell proliferation and invasion and they act in a paracrine fashion to activate stromal cells which, as was described above, have feed forward effects on tumour cells that contribute to malignant progression.

LINE-1 hypomethylation within the long interspersed nucleotide element-1 (LINE-1) often occurs in metastatic prostate [74] and metastatic endocrine pancreatic [10] carcinomas. While this epigenetic mark is often used as a general indicator of hypomethylation, it is also known to be functionally significant in tumor progression in a number of ways. Specifically, it can lead to the activation of adjacent genes as well as an increase in chromosomal instability [18, 65, 72] as well as genomic instability [1, 50].

Immunomodulation

Cytokines such as interleukin-4 and -13, produced by malignant and stromal cells within the tumor microenvironment in a manner that mimics the end stages of wound healing, cause an immune suppression that is tumor promoting. This is initiated, in large part, by the cytokine-mediated recruitment of monocytes to the lesion. These new recruits then differentiate into alternatively activated tumor-associated macrophages (TAMs) that skew towards an ‘M2’ phenotype that is immunosuppressive

[47, 59]. More specifically, alternatively activated TAMs do not exhibit the cytotoxicity of typical macrophages [54]. Instead, they release paracrine-acting factors such as the chemokine CCL22 [12] and they generate nitrogen species, particularly under hypoxic microenvironmental conditions [14], that suppress the infiltration and proliferation of T-lymphocytes into the tumor microenvironment. Immunosuppressive TAM's also secrete VEGF-A [40] which augments the hypoxia-induced increase in angiogenesis within the tumor microenvironment described above. Alternatively activated TAM's are an attractive anti-metastatic therapeutic target given their profound ability to facilitate tumor progression by contributing to an escape from immune surveillance while simultaneously promoting angiogenesis. Experimentally, TAMs can be targeted by blocking the cytokine CSF-1 [13, 45], which is required for the proliferation and differentiation all macrophage populations. Unfortunately, this approach is a very broad one and is likely to have long term side effects in patients. A more specific approach would be the reprogramming of TAM's into more conventional 'antigen-presenting' immune response-promoting macrophages that are known to have anti-tumor effect. Experimentally, this has been achieved using histidine-rich glycoprotein [57].

Interestingly, some cytotoxic drugs (eg. paclitaxel) can suppress the M2 TAM phenotype and skew it towards a pro-inflammatory M1 phenotype that is then antitumoral [34]. Thus, a goal of the field has been to identify mediators that drive this proinflammatory M1 TAM skewing in a predictable manner. One such mediator is the microRNA miR-511–3p [66]. Thus, non-coding RNA's are capable of modulating effectors in the microenvironment that play a critical role in tumor progression.

Immunomodulatory changes during tumor progression are discussed in more detail by Gregor Reid in Chap. 8 of this volume.

Mesenchymal Transformation

A major driver of tumor cell invasion is the epithelial-to-mesenchymal transformation (EMT; [69]). During the EMT process there is a breakdown in apical-basal polarity followed by the loss of adhesive junctions between stationary epithelial cells. The resulting individual cells acquire an anterior-posterior polarity and they become motile and mesenchymal [22]. Therefore, classical markers of this transformation are the loss of the epithelial cell-cell adhesion molecule E-Cadherin and the upregulation the mesenchymal intermediate filament protein vimentin. These changes, particularly at expanding tumor fronts, are often used as an indicator of invasive progression and the onset of the metastatic process [30].

During normal development, EMTs contribute to organogenesis and the formation of the body plan in a manner that is tightly regulated, both spatially and temporally [26]. For example, this occurs during gastrulation where a precisely controlled EMT leads to the production of invasive mesenchymal cells that move into the embryo and later re-aggregate to form the mesodermal condensations during

primary germ layer formation [39]. While microenvironmental organizing centers (eg. the primitive knot, Spemann's organizer) that regulate the position and timing of developmental EMT's have been identified, the precise nature of the instructive paracrine factors they release have still not been well characterized. In contrast, the core transcriptional machinery that acts to initiate the gene expression changes that initiate an EMT has been determined. This includes the Snail, Zeb and Twist transcription factors which act on the E-cadherin promoter to inhibit the gene's expression as well as stimulate the expression of secreted factors that further stimulate an EMT [52]. An example of the latter is platelet-derived growth factor (PDGF) which itself stimulates the localized production and activation of metalloproteases that degrade the ECM in the microenvironment to facilitate the migration and invasion of mesenchymal cells produced by the EMT [16].

During malignant tumor progression the precise spatial and temporal control of EMT is disrupted, most often because of an inappropriate accumulation and/or activation of EMT-inducing factors within the tumor microenvironment. One such factor is TGF-ß which, when it acts on epithelial-derived tumor cells (but not normal epithelial cells) stimulates the activity of the core EMT transcription factor complex [33]. TGF-ß is often produced and secreted by cancer-associated fibroblasts in the microenvironment [8]. Interestingly, TGF-ß that is produced by platelets and released into the microenvironment due to the leakiness of new vessels formed by angiogenesis can also contribute to transient tumor cell EMT at sites of thrombosis [36]. This has important implications for the movement of tumor cells from the stroma into the vasculature by a process known as intravasation. Additionally, it may also help explain why circulating tumor cells themselves can be mesenchymal [75]. The latter point is not trivial in terms of survival in the circulation as mesenchymally-transformed cells tend to be resistant to the suspension-mediated apoptosis that normally occurs when epithelial cells are detached from the ECM [27].

Other factors found in the tumor microenvironment that can act to stimulate the core EMT transcriptional machinery including the afore-mentioned PDGF produced by CAF's as well as WNT or WNT-like factors produced by recruited mesenchymal stem cells and interleukin-6 (IL-6) produced by TAMs [49]. Importantly, the removal of such factors can shift the tumor cell phenotype from the mesenchymal back to the epithelial in a process known as mesenchymal-epithelial transformation (MET). This often occurs during the later stages of metastatic progression where an MET is proposed to contribute to the colonization of distant sites after tumor cells have left the vasculature by extravasation. Such transient shifts between epithelial and mesenchymal phenotypes can also be regulated by the oxygen tension in the microenvironment given that hypoxia upregulates the core EMT transcriptional complex via the actions of HIF-1α [73]. Thus, the mesenchymal phenotype is often plastic, unless mutations within the E-cadherin gene and/or stable, epigenetically-driven changes in E-cadherin expression occur. As such, there are varying tumor microenvironment-driven metastable and stable states of mesenchymal transformation within tumor lesions that have important implications for therapeutic treatment strategies bent on reversing the process [68].

Mechanotransduction

Once they have acquired the ability to become invasive, either by varying degrees of mesenchymal transformation or by other means that can include either collective or single-celled amoeboid migration [20], tumor cells move through the tumor microenvironment by interacting with the ECM, the components of which are highly modified due to changes in component deposition, molecular cross-linking, and proteolytic processing within that microenvironment [58]. Ultimately, the molecular composition of the ECM greatly contributes to changes in motile phenotype of the invading tumor cells based on, for example, the soluble factors it sequesters and the specific nature of the cell surface integrins that it engages [28]. However, it is becoming increasingly clear that the mechanical properties of the ECM also play an important role in regulating the phenotype of the invading tumor cells. In this case, physical changes in the ECM can dramatically alter mechanical signals within tumor cells that influence proliferation, survival and the invasive phenotype itself [15]. Experimentally, this can be achieved by artificially crosslinking ECM components, particularly collagens, to increase the stiffness of the matrix which increases intracellular tension and integrin-mediated biochemical signaling within the tumor cell [38]. Thus, mechanical cues in the ECM are translated intracellularly by the cytoskeleton and signaling moieties that are modulated by tension applied through integrin-containing adhesion complexes. Such collagen cross-linking can be achieved by the actions lysyl-oxidase which is released into the tissue microenvironment by CAFs. In yet another example of a feed forward mechanism, lysyl oxidase-dependent collagen crosslinking will further activate CAF's themselves in an integrin signaling-dependent manner [3] and this effect can be so strong that it can facilitate tumor invasion and metastatic progression even when TGF-ß is removed [53].

Importantly, collagen crosslinking-dependent increases in radiologically observable mammographic 'density' is a major risk factor for breast carcinoma formation and progression [6]. The latter effect may be facilitated by the fact that ECM stiffness-mediated mechanotransduction augments the ability of soluble factors sequestered within the tumor microenvironment to efficiently induce an EMT [37].

Mechanotransduction events that contribute to tumor progression are discussed in more detail by Celeste Nelson's group in Chap. 7 of this volume.

Summary

It is now clear that the microenvironment that a tumor cell finds itself within can greatly affect its phenotype regardless its genotype. These microenvironmental effects are mediated by surrounding stromal cells, soluble factors, and the extracellular matrix all of which act together, with tumor cells, to form the tumor organ. Therefore, given the molecular and cellular complexity within the tumor organ, it is very difficult to predict the response of any one component of the organ to

a particular therapeutic treatment when that component is viewed in isolation. While this complexity can be extremely problematic when viewed from a reductionist point of view, particularly when it contributes to the failure of agents targeted against specific tumor cell-intrinsic oncogenes or tumor suppressors, it also provides myriad new therapeutic opportunities to halt the emergence of those microenvironment-dependent tumour progression phenotypes that contribute to the overwhelming majority of cancer deaths due to metastasis.

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Chapter 7
Mechanotransduction, Metastasis and Genomic Instability

Allison K. Simi, Alexandra S. Piotrowski and Celeste M. Nelson

Abstract Cells translate mechanical forces in the environment into biochemical signals in a process called mechanotransduction. In this way, mechanical forces direct cell behavior, including motility, proliferation, and differentiation, and become important in physiological processes such as development and wound healing. Abnormalities in mechanotransduction can lead to aberrant cell behavior and disease, including cancer. Changes in extracellular mechanical forces or defects in mechanosensors can result in misregulation of signaling pathways inside the cell, and ultimately lead to malignancy. Here, we discuss the ways in which physical attributes of the tumor microenvironment can promote metastasis and genomic instability, two hallmark features of cancer.

Keywords Mechanical stress · EMT · Stiffness

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
αSMA	α-smooth muscle actin
bFGF	Basic fibroblast growth factor
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular-signal-regulated kinase
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
GIN	Genomic instability

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IFP	Interstitial fluid pressure
ILK	Integrin-linked kinase
MET	Mechanoelectrical transduction
MLC	Myosin light chain
MMP	Matrix metalloproteinase
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
TAZ	transcriptional co-activator with PDZ-binding motif
TGF-β	Transforming growth factor β
VEGF	Vascular endothelial growth factor
YAP	Yes-associated protein

Introduction

Over a decade ago, Hanahan and Weinberg defined several features of cancer that they considered essential for the acquisition of a malignant phenotype, including replicative immortality, evasion of growth suppressors, evasion of apoptosis, stimulation of angiogenesis, stimulation of proliferation, and invasion and metastasis [1]. Since then, a flood of cancer research has led to modification and expansion of the proposed hallmarks; metastasis and genomic instability are two that persist [2]. Cancer is widely regarded as a disease of the cell, and cell behavior is directed by both biochemical and physical cues, which can work independently or synergistically [3]. Accordingly, the tumor microenvironment has been shown to affect tumor progression [4, 5]. This chapter focuses on the physical factors and mechanical forces that tumor cells encounter in the tumor microenvironment, which can in turn alter their behavior. Cells convert the physical signals they receive into biological responses via a process known as mechanotransduction [6].

Mechanotransduction involves both the external environment and internal signaling [7]. The transmission of external forces to intracellular signaling is centered on proteins that are activated by force, such as integrins [8, 9] and T-cell receptors [10]. Many cellular phenotypes, including morphology, motility, and proliferation, are governed by external mechanical forces [11–13]. Thus, mechanotransduction is central to a variety of physiologically normal processes, including embryonic development, differentiation, wound healing, and angiogenesis [14, 15]. Defects in mechanotransduction are known to be involved in several diseases, including cancer [16]. Understanding how defects in mechanotransduction affect tumor progression will add to our fundamental knowledge of cancer biology and may suggest new approaches for treatment.

How Mechanotransduction Regulates Normal Cell Behavior

Extracellular Factors Affecting Mechanotransduction in Normal Cells

Most cells are anchorage dependent: they need to adhere to a substratum to prevent apoptosis and promote cell cycle progression [17]. Thus, the mechanical microenvironment is important for cell survival. Cells sense their environment via conformational changes in mechanically responsive proteins, known as mechanosensors. Physical forces induce these conformational changes, which result in downstream signaling inside the cell [18, 14]. Forces can originate from a variety of features, including the rigidity of the extracellular matrix (ECM), static or dynamic fluid flow, and tissue growth [6]. These forces are further classified into specific types of loads that cells can detect. For example, forces incurred by blood flow include hydrodynamic pressure, shear stress, and cyclic strain, and all of these help regulate endothelial cell behaviors [19] such as cell reorientation [20].

Cells can also respond to mechanical loads by secreting biochemical factors, some of which result in subsequent ECM remodeling. Growth factors comprise one class of proteins that are important in this respect. Transforming growth factor β (TGF-β) is sequestered in the ECM, and is released when internal contractility of myofibroblasts is balanced externally by a stiff matrix, causing conformational changes in protein complexes embedded in the ECM. Free TGF-β starts a feed-forward loop, causing increased deposition of ECM proteins and additional (increased) expression of TGF-β [21]. Various other growth factors increase activity as a result of mechanical load, as evidenced by endothelial secretion of basic fibroblast growth factor (bFGF) in response to shear stress and hydrostatic pressure [22, 23]. Mechanical forces also regulate the expression of matrix remodeling proteins such matrix metalloproteinases (MMPs). This is seen in human monocytes/macrophages, which have been shown to increase expression of MMPs under cyclic strain, and thus contribute to ECM degradation [24].

Intracellular Factors Affecting Mechanotransduction in Normal Cells

There are several intracellular components involved in receiving mechanical signals and eliciting a response (Fig. 7.1). A feature that is particularly important to mechanical sensing is contractility; all cells have a network of cytoskeletal proteins (actin, microtubules, intermediate filaments) that aid in cell structure and mobility [17]. Cytoskeletal contractility creates a balance between intra- and extracellular forces acting on the cell, and thus is important for cells to be able to respond to forces in the surrounding microenvironment [25]. This balance exists so that

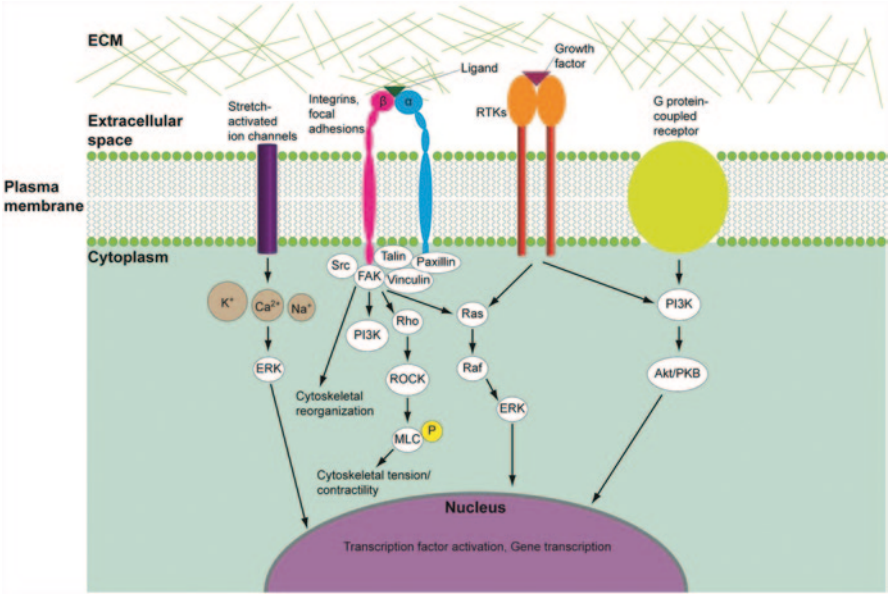

Fig. 7.1 Schematic of intracellular mechanotransduction pathways connecting the *ECM* to the *cytoplasm* and *nucleus.*

mechanical forces in the microenvironment and internal cellular tension can work together to regulate cell behavior, evident, for example, in changes in fibroblast proliferation when matrix stiffness and actomyosin contractility are decoupled [26]. Moreover, external mechanical stimuli help define the state of the cytoskeletal components through various pathways. For example, it has been shown that tensile forces regulate the expression of α-smooth muscle actin (αSMA), a gene important for cytoskeletal contractility, in osteoblasts [27], and that cytoskeletal tension in fibroblasts changes to match the stiffness of the substratum [28].

Communication between ECM and the cytoskeleton is mediated by mechanosensors, proteins or structures that can sense physical changes in the microenvironment and translate these into chemical signals inside the cell [15]. Mechanosensors are diverse and exist everywhere in the body, from ears to kidneys: mechanoelectrical transduction (MET) channels in cochlear hair cells respond to sound vibrations to induce the signaling necessary in auditory sensation [29], and primary cilia in renal epithelia respond to fluid flow to maintain homeostasis [30]. Yet the sensing mechanisms of many mechanosensors remain poorly understood.

The most well-studied mechanosensors are integrins, which contain extracellular, transmembrane, and cytoplasmic domains [31]. Integrins are composed of α- and β-subunits that form heterodimers [32]. Different types of integrins can bind to various ligands present in the ECM and induce signaling to regulate a variety of processes including attachment, migration, proliferation, and differentiation [33]. Through detection of external mechanical stresses, integrins promote changes in cytoskeletal structure and can activate signal transduction cascades [34–36]. Integrin

activity is also essential for the formation of focal adhesions, which act as centers of mechanotransduction [37]. Focal adhesions are protein complexes localized at the plasma membrane that link the ECM to the actin cytoskeleton. In addition to integrins, focal adhesions include hundreds of proteins, the most well-characterized of which are talin, paxillin, vinculin, focal adhesion kinase (FAK) and Src family kinases, which act as signaling molecules [38]. The formation of focal adhesions is regulated by both external forces and cytoskeletal contractility [39].

Other intracellular components involved in mechanotransduction include G proteins, receptor tyrosine kinases (RTKs), extracellular-signal-regulated kinases (ERKs), and stretch-activated ion channels [6].

G proteins are localized at focal adhesion sites and can undergo conformational changes induced by mechanical stress to promote cell growth. G proteins are activated in cardiac fibroblasts in response to stretch, as well as in endothelial cells and osteocytes in response to shear stress [40–42].

*RTK*s are transmembrane proteins that dimerize to become activated, and are involved in integrin-mediated mechanotransduction downstream of G proteins. Dimerization is triggered by binding of the receptor to extracellular ligands such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), leading to further signaling [43]. RTKs can also activate ERKs, which are important for gene expression and protein synthesis [44].

*ERK*s are kinases that play an important role in intracellular signaling, such as the activation of cytoplasmic and nuclear regulatory proteins. These kinases can be activated in response to mechanical stimuli. Shear stress and stretch have been shown to activate ERKs in aortic endothelial cells and pulmonary epithelial cells, respectively [45, 46].

Stretch-activated ion channels allow ions such as $Ca^{2+}$to move in and out of cells, which regulates several cellular processes. Cell stretching has been shown to increase intracellular levels of Ca^{2+} in several cell types [47, 48]. Intracellular Ca^{2+} levels are also important for the activation of other proteins in the mechanotransduction signaling cascade, such as ERKs [49].

Mechanotransduction and Metastasis

The invasion of primary tumors into their surrounding tissue and subsequent metastatic spread to other organs are among the largest obstacles to cancer treatment, and metastasis is the main cause of cancer-related deaths [50]. Metastasis relies on the ability of tumor cells to migrate from the primary tumor and form new lesions at distant locations [51]. Invasion and metastasis require physical interactions between malignant cells and the microenvironment, a process that inherently involves mechanosensing and mechanotransduction [16]. Both extracellular factors in the physical tumor microenvironment and intracellular factors within cancer cells contribute to mechanotransduction during invasion and metastasis. Identifying how mechanotransduction becomes abnormally regulated in cancer cells is necessary to understand the mechanisms that underlie invasion and metastasis.

Extracellular Factors Affecting Mechanotransduction in Tumors

The physical microenvironment within a solid tumor differs from that of normal tissue in several ways (Fig. 7.2): uncontrolled proliferation results in increased mechanical compression in a spatially restricted environment [52]; there is an increase in the production of ECM components (of which collagen is the most prevalent structural protein), which exhibit increased alignment, crosslinking, bundling, and stiffening [53, 54]; poorly formed blood vessels and the absence of functional lymphatics lead to increased interstitial fluid pressure (IFP) [155]. These changes in the extracellular environment can alter the behavior of tumor cells via mechanotransduction pathways, which are important for both invasion and metastasis. For example, mechanical compression can promote invasion and metastasis [55]. Compression has been shown to enhance cell-substratum adhesion in two-dimensional (2D) cell culture compression assays [52]. Moreover, compression can facilitate invasion by increasing the release and activation of ECM-degrading MMPs [56]. Mechanical loading can also alter cell shape and motility through compression-dependent changes in cytoskeletal dynamics [57].

The ECM is the framework for intercellular crosstalk, adhesion, and migration [58]. Solid tumors exhibit increased ECM stiffness and crosslinking, and changes in the structural components and mechanical properties of the ECM can promote an invasive phenotype in cancer cells [7, 16, 59]. For example, the mode by which tumor cells migrate is strongly dependent on the physical properties of the ECM [60].

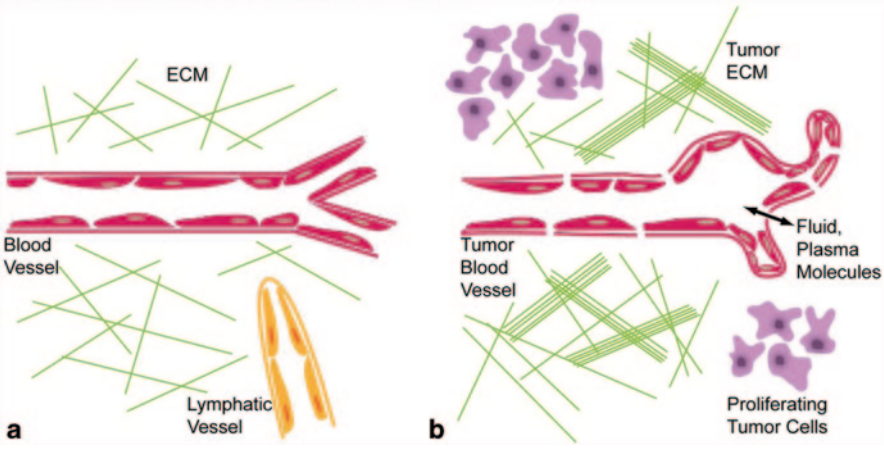

Fig. 7.2 Cartoon illustrating the physical changes in the tumor microenvironment compared to that of normal tissue. **a** Normal tissue microenvironment. The microenvironment in normal tissues contains linearized blood vessels that perfuse the tissue. *Lymphatic vessels* are present to drain excess fluids and maintain fluid homeostasis. *ECM* proteins make up the loose connective framework. **b** Tumor microenvironment. Poorly formed blood vessels *leak fluid* and *plasma macromolecules* into the interstitium. Many solid tumors lack a functioning lymphatic system. There are larger amounts of *ECM* proteins that are highly aligned, crosslinked, bundled, and stiffened. In addition, uncontrolled proliferation of cells in a confined space results in mechanical compression.

Changes in ECM composition and architecture also affect the distribution and activation of soluble factors (e.g., growth factors, cytokines, MMPs) that are themselves involved in cell behavioral changes and mechanotransduction [61]. ECM stiffness can promote the malignant behavior of tumor cells by increasing the expression and activity of adhesion receptors, thereby also activating mechanotransduction pathways [12]. For example, force has been shown to influence the development of focal adhesions since maturation of these complexes requires mechanical tension [62].

Increased ECM stiffness also directs cell behavior by increasing external resistance forces experienced by the cell [63]. Links to the ECM via integrins and focal adhesions can relay these stresses to the cytoskeleton, alter the balance of intracellular forces, and stimulate signal transduction cascades that influence cell behavior [7]. Moreover, increased ECM stiffness can disrupt epithelial polarity and induce migration and metastasis [64]. Cells have also been shown to migrate preferentially to regions of increased ECM stiffness via mechanotaxis/durotaxis [65, 66]. Finally, the crosslinking of ECM by lysyl oxidase, which can also stiffen the matrix and induce fibrosis, can promote tumorigenesis via enhanced integrin signaling [58].

ECM remodeling by tumor and stromal cells is important for both invasion and metastasis. For example, migrating tumor cells exhibit pericellular proteolytic degradation to make room for further migration [67]. Proteases such as MMPs are recruited to integrin assemblies and other adhesion receptors at the leading edge of a migrating cell to model and degrade the ECM [68]. Cancer cells have also been shown to realign their surrounding ECM perpendicular to the tumor boundary, altering its architecture for improved adhesion and migration, creating diverse routes for dissemination [69]. Migration is mediated by several types of proteolytic structures enriched with F-actin, β1-integrins, and MMPs, which are key players in mechanotransduction [70]. Single cell migration can also occur without proteolytic degradation under the mode of amoeboid migration [71]. The microscale architecture of the ECM, including the alignment of fibers and the location and size of pores, dictates the mechanisms of invasion and metastasis applied by cancer cells [72].

IFP and interstitial fluid flow have also been shown to affect the migratory and invasive behaviors of tumor cells [73, 156, 157]. In a three-dimensional (3D) culture model in which single tumor cells were suspended in ECM, fluid flow was shown to increase the percentage of migratory cells as well as their speed [73]. In a similar study, interstitial fluid flow was shown to result in the upstream migration of cancer cells as a result of asymmetry in matrix adhesion stresses needed to balance drag from fluid flow [74]. The stresses induced by flow created a gradient of integrin activation across the cells. Components of focal adhesions, including FAK, paxillin, and vinculin, localized at the upstream side of the migrating cells.

Intracellular Factors Affecting Mechanotransduction in Tumors

It is well known that changes in mechanotransduction promote invasion and metastasis [75]. The intracellular factors affecting mechanotransduction pathways in tumor cells may be altered in response to changes in the tumor microenvironment,

or to genetic mutations and changes in gene expression within the tumor cells. Intracellular mechanotransduction can, in turn, lead to changes in gene expression to promote invasion and metastasis.

Cytoskeletal reorganization is important for changes in cell shape and motility, and therefore migration and metastasis [16]. Cytoskeletal tension is primarily regulated by ERKs and the Rho family of small GTPases. One effector of Rho is Rho-associated kinase (ROCK), which regulates actin cytoskeletal contractility via myosin light chain (MLC) phosphorylation [64]. Rho activity has been shown to be elevated in some tumors, though decreases in its activity have also been reported [76, 77]. Cytoskeletal tension is also affected by the mechanical properties of the ECM, such as stiffness and crosslinking [7]. Increased matrix stiffness promotes the clustering of integrins and the formation of focal adhesions, in addition to increasing activation of FAK and ERK, and enhancing ROCK-mediated cytoskeletal contractility [64]. ROCK is also involved in the disruption of adherens junctions and moving the tail end of the cell behind the leading edge to assist in cell locomotion [78–80]. Moreover, cell migration involves the extension of membrane protrusions resulting from the cycling of actin polymerization and depolymerization, which are regulated by Rho GTPases via the cofilin pathway [81, 82].

ECM crosslinking has also been shown to result in the aggregation and clustering of integrins as well as enhanced signaling via phosphoinositide 3-kinase (PI3K) to induce invasion [58, 64]. Other components of focal adhesions have also been implicated in tumor progression, including Src, the activity of which has been shown to influence proliferation, invasion and metastasis [83, 84]. Src activation is required for ECM degradation during migration [85]. In 3D culture studies of breast tumor cells, Src activity increases the strength of cellular forces on the ECM as well as the duration and length of cell membrane protrusions [86].

Whereas some cells in the tumor become stiffer, metastatic cells are more deformable and exhibit reduced cytoskeletal stiffness [87]. Lower levels of integrin expression along with decreased adhesion to the ECM have been associated with oncogenic transformation [88, 60]. This increased deformability is correlated with enhanced metastatic potential. For example, enhanced deformability enables metastatic cells to move through tight spaces, such as between endothelial cells, during intravasation and extravasation [89].

In addition to regulating the cytoskeleton and associated proteins, mechanotransduction can lead to gene expression changes that promote invasion and metastasis. Cancer cells undergo a variety of genetic mutations and gene expression changes during tumor progression, which can affect their interactions with the microenvironment and subsequent mechanotransduction. Mechanotransduction itself is one source of changes in gene expression in cancer cells. A major way that mechanotransduction can affect gene expression is via the epithelial-mesenchymal transition (EMT). EMT, in which epithelial genes are downregulated and mesenchymal genes are upregulated, is thought to be an important mechanism in both invasion and metastasis [90, 91]. ECM stiffness has been shown to promote EMT, through which cancer cells acquire a migratory phenotype via a variety of pathways, some of which include key players in mechanotransduction, such as RTKs [92]. In one pathway,

EMT results from stiffness-mediated localization and signaling of Rac GTPases downstream of MMPs [93]. Mechanical stress and matrix rigidity can also induce EMT downstream of TGF-β [94, 95]. Furthermore, the activation of Rho GTPases is thought to contribute to EMT via the loss of adherens junctions between cells and the gain of mesenchymal characteristics [96].

Induction of EMT in tumor cells, which affects cytoskeletal organization and cell-cell and cell-matrix adhesions, can also alter how the cells sense exogenous forces, and therefore their responses to those forces [97, 98]. The downregulation of epithelial keratins results in reduced cytoskeletal stiffness and greater cell deformability, directly influencing the metastatic potential of tumor cells [99]. In addition to being more deformable than non-metastatic cells, metastatic cells also lose their anchorage dependence [100, 101]. Anoikis, or apoptosis induced by the loss of adhesion to the ECM, is suppressed in metastatic cells, allowing them to migrate and traverse through the bloodstream to distant organs [102, 103]. Anoikis is believed to be mediated by integrin signaling [104]. The activation of integrins and their associated proteins, including FAK and integrin-linked kinase (ILK), can suppress anoikis, indicating that mechanotransduction and apoptotic pathways are linked [105]. EMT can also suppress anoikis [106]. In particular, the downregulation of E-cadherin can protect cells against anoikis [107]. It is clear that several extracellular and intracellular components of mechanotransduction are altered in tumors, which promotes progression to invasive disease. Mechanotransduction, it seems, is another mechanism that can be hijacked to support malignant transformation.

Mechanotransduction and Genomic Instability

The term genomic instability (GIN) broadly describes the inability of a cell to pass on a copy of its DNA with fidelity. GIN can manifest itself in several ways, each the result of replicative stress caused by errors in DNA replication or the DNA damage response [108]. Microsatellite instability is the expansion or contraction of oligonucleotide repeats and results from mutations in mismatch repair genes [109, 110]; nucleotide excision-repair-related instability results from an impaired ability of the cell to remove and replace damaged nucleotides [111]; and chromosomal instability is a change in the structure or number of chromosomes, which typically occurs as a result of errors in DNA replication or mitosis [112, 113].

GIN is a defining feature of cancers, and is believed to be the driving force behind tumor progression. Various errors in DNA replication or repair processes lead to an abnormal genotype that continues to change with each generation of cells. As a result of GIN, tumors that originate from the same tissue and cell type can have wildly varying genetic profiles [114]. This intertumor heterogeneity, as well as subclonal heterogeneity within a single tumor, has been largely attributed to the Darwinian characteristics of cancer; that is, the evolution and adaptation of a cancer clone in response to external selective pressures [115]. Ultimately, this results in the acquisition of survival-enhancing features that allow a cancer to develop.

The local microenvironment is one source of pressure that results in GIN [116] and increased survival. Mouse embryonic stem cells exposed to radiation develop a high frequency of mutation *in vivo* but not in culture, suggesting that the microenvironment of the cells contributed to their development [117]. More specifically, both physical features of the tumor microenvironment as well as onslaughts by external agents have been shown to increase the frequency of mutation, thus increasing the chances that one of these mutations will affect maintenance of genomic integrity. Hypoxia is one hallmark characteristic of the tumor microenvironment known to play a role in promoting GIN. Hypoxia induces an elevated frequency of mutation in tumorigenic mammalian cell lines [118]. Similarly, exposure to heat and serum-starvation increases mutations in mouse mammary carcinoma cells [119]. Little is known about how GIN may arise from mechanical aspects of the microenvironment; the following describes a body of work that supports this idea.

Mechanical Forces Affect Mitosis and Cell Cycle Progression

One risk factor for the development of GIN is an increase in cellular proliferation, and hence the chance for DNA copy errors to arise. Recently, the mechanical properties of the microenvironment have been considered a major factor in its influence on cell behavior, specifically the regulation of cell cycle progression and mitosis and subsequent maintenance of the genome. Several studies have shown that modulating mechanical forces acting on cells can affect proliferation: mechanical stretch can reduce proliferation of podocytes [120], enhance differentiation and reduce proliferation of preadipocytes [121], and in endothelial cells, directed mechanical forces (specifically, shear and stretch) promote homeostasis but non-uniform forces can result in sustained pro-inflammatory and proliferative signaling [122]. These effects can be mediated by cell-cell contact, such as through VE-cadherin in endothelial cells [123].

The adhesion of a cell to its surroundings can alone induce changes in proliferation. Micropatterning techniques have been used to isolate the effects of cell spreading and cell-cell junctions from the effects of substratum adhesion on cell behavior. Such studies have revealed that E-cadherin is sufficient to induce epithelial cell proliferation via Rac1 signaling, and both proteins are required for cell-cell contact-dependent proliferation [124]. Similar findings hold for endothelial or smooth muscle cells via PI3K signaling [125]. Cytoskeletal structure and associated signaling have also proven to be important in cell-cell adhesion-mediated proliferation, based on studies regarding the role of VE-cadherin in vascular endothelial cells [126]. Additionally, simply varying the nature of the substratum also affects proliferative behavior. The basement membrane interacts differently with normal or cancerous epithelial cell lines, affecting growth and differentiation [127].

There is also evidence that mechanotransduction can influence various aspects of mitosis, and thus the segregation of the genome into daughter cells. Physical features of the microenvironment are one avenue of mechanical influence on

mitosis. For example, in HeLa cells (human cervical cancer cells), retraction fibers, which bind mitotic cells to the substratum, exert forces on the cell that dictate the orientation of the spindle during mitosis. This is mediated by regulation of the subcortical actin network [128]. Another study in HeLa cells similarly showed that the spatial distribution of ECM proteins helps determine the axis of division by regulating actin dynamics [129].

It would follow from these studies that mechanosensors and other intracellular mechanotransduction machinery are involved in the regulation of mitosis, and indeed this has been shown. Integrin-mediated adhesion is required for the cells to reorient the mitotic spindle parallel to the substratum [130]. Here again, cytoskeletal components are key communicators. G proteins and the motor protein dynein, both important in transmitting mechanical force, are also known to direct orientation of the spindle in development [131]. One can imagine that abnormal mechanical signaling, common to many diseases including cancer, could disrupt mitosis in a cell and thus generate genomically unstable progeny.

Mechanotransduction Regulates Biochemical Cues That Promote GIN

One way that mechanical stimuli ultimately promote changes in cell behavior is through intracellular signaling pathways that conclude with control of gene transcription. Genes regulated by mechanotransduction can affect a myriad of both normal and pathological processes in the body [14]. In the context of cancer, recent studies have suggested that important molecular targets of mechanotransduction include mitotic checkpoint genes and other cell-cycle regulators, which have long been associated with maintaining genomic stability [112, 132].

To discover mechanically-regulated genes associated with GIN, several studies have used polyacrylamide gels of varying stiffness to mimic the mechanical properties of the ECM, and thus determine the effects of substratum stiffness on cell behavior in culture [133]. Recent findings from these experiments show that the transcription factors YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif), which have implications in growth, proliferation, and differentiation, become activated in response to cytoskeletal tension and cell spreading induced by a stiff substratum [134]. In human mammary epithelial cells, expression of tumor suppressor phosphatase and tensin homolog (PTEN) is reduced in the presence of microRNA miR-18a, which is modulated by ECM stiffness [135]. PTEN is antagonistic to PI3K, a protein involved in many pathways important for cell growth and survival that promotes cancer when misregulated [136]. Polyacrylamide gels were also used to show that matrix rigidity induces integrin clustering in mammary epithelial cells, which induces the formation of focal adhesions and generates cytoskeletal tension. This in turn activates ERK and enhances EGF-dependent pathways that activate ERK, which is known for its involvement in cell cycle regulation [137, 64].

Other cell cycle-regulators are activated by adhesion to or disruption of the substratum. The protein p38 is best known for its role as a tumor suppressor, but also regulates mitotic entry and the spindle assembly checkpoint [138], and negatively regulates cell proliferation through a reactive oxygen species (ROS)-mediated response to stress [139]. When mammary epithelial cells lose adhesion to the substratum, p38 is activated and can induce apoptosis [140]. NM23-H1 is another protein associated with growth arrest, and this function was shown to be correlated with basement membrane assembly in human breast cancer cells [141].

Aside from the effects of mechanically-regulated gene transcription, cytokines and other signaling factors that contribute to cancer progression are often triggered by mechanical forces, and can induce GIN. For example, lung cancer cells show an increased production of ROS in response to shear stress [142]. ROS are well known to promote genetic mutations and cancer progression [143]. Furthermore, a xenograft of human skin overexpressing bFGF (in a cocktail with stem cell factor and endothelin-3) causes replication stress [144], the major source of GIN [108]. As previously described, bFGF is regulated by shear stress and hydrostatic pressure [23, 22].

Restructuring of the Stroma Results in GIN

In addition to signaling mediated by mechanosensors, cells can communicate with the microenvironment through various soluble factors that serve to restructure the surrounding stroma. In cancer, misregulation of these proteins has been linked to GIN. MMPs make up one class of proteins that remodel the ECM. Overexpression of MMPs can induce cell cycle progression, activate genotoxic pathways, and inhibit cytokinesis [145]. Furthermore, cells overexpressing MMPs often exhibit patterns of genomic irregularities [146]. The stroma is also heavily remodeled during the formation of new vasculature. Both cyclic and constant static stretch of endothelial cells increase the expression of vascular endothelial growth factor (VEGF) receptor and promote VEGF-induced proliferation, vasculogenesis, and angiogenesis [147]. VEGF has been shown to regulate the axis of division in endothelial cells, potentiating GIN [148]. Thus, through restructuring of the stroma, in addition to control of the cell cycle and associated proteins and cytokines by external forces, GIN is mediated by mechanotransduction in cancer cells.

Synopsis and Outlook

Aberrant mechanotransduction is a major contributor to tumor progression, metastasis, and GIN. Both mechanosensing and subsequent intracellular signaling alter properties of the cell that can lead to malignant transformation in cancer. Mechanotransduction is therefore important to study in order to understand the progression of this disease. Developing improved 2D and 3D cell culture models to mimic

the tumor microenvironment will enable us to determine the effects of abnormal mechanotransduction in cancer progression. Beyond experimental models, computational models can characterize the effects of mechanical stretch on cell behavior [121]. Others begin to account for intratumor heterogeneity when predicting therapeutic response [149]. However, current computational models cannot cope with mutational frequency of cancer cells, and thus there is a disconnect between investigations of the causes and consequences of this feature.

Although many of the proteins involved in mechanotransduction are known (e.g. integrins, cytoskeleton, myosins, kinases), the precise mechanisms by which a cell perceives the mechanical information of its environment remain unclear [150]. In addition, mechanical forces in the microenvironment are known to affect the cell cycle, and abnormal expression of cell-cycle regulators can result in GIN [132]; however, a clear mechanotransduction pathway linking these two events has not been elucidated. Similarly, current knowledge on the mechanosensing capabilities of stem cells is limited; verifying which forces, molecular pathways, and mechanosensing proteins are most important in directing construction of the stem cell niche and stem cell differentiation could lead to clinical applications (for example, targeting cancer stem cells) [151, 152].

Components of mechanotransduction pathways are starting to be considered as potential therapeutic targets. For example, it has been shown that the disruption of Rho or ERK signaling results in a reduction of cytoskeletal tension that leads to a decrease in tumor cell proliferation and the repression of malignant progression [16, 64]. Targeting Src activity could reduce proliferation, invasion, and metastasis [153]. Restoring anoikis response might curb metastasis [154], and the inhibition of collagen crosslinking and integrin signaling might reduce invasion. In addition, the mechanical properties of isolated metastatic cancer cells could be diagnostic indicators for prognosis. As we broaden our current understanding of mechanotransduction as it relates to both normal cell functions and disease, we will be able to integrate this knowledge into a synergistic treatment strategy for cancer.

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Chapter 8
Immunomodulation and Genomic Instability

Gregor Reid

Abstract The interaction between a developing tumor and the immune system is complex and dynamic, and comprises seemingly opposing activities. On one hand, the tumor-promoting effect of chronic inflammation has long been recognized and mechanisms contributing to this activity, including proliferative and anti-apoptotic signaling, tissue remodeling, and mutagenesis, are well described. In contrast, tumor-specific immune responses mediated by a variety of cell types and soluble factors have been shown to inhibit the progression of cancer. A full understanding of the interplay between these opposing forces will be required before clinical manipulation of the tumor immune environment can achieve consistent improvement in the outcomes for patients with cancer. The focus of this chapter is the influence of genomic instability on the pro- and anti-tumor immune activities that impact on cancer development at multiple stages of progression.

Keywords Inflammation · Immunoediting · Immunosurveillance · Microsatellite instability · Escape variant · DDR · NKG2D · Innate · Adaptive · Antigens

Abbreviations

AID	Activation-induced cytidine deaminase
BER	Base excision repair
CIN	Chromosomal instability
DAMP	Damage-associated molecular patterns
DC	Dendritic cells
DDR	DNA damage response
DNA	Deoxyribonucleic acid
Ig	Immunoglobulin
KIR	Killer-cell immunoglobulin-like receptors
M1	Type 1 macrophages
MDSC	Myeloid derived suppressor cells
MHC	Major histocompatibility complex
MIF	Migration inhibitory factor

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MMR	Mismatch repair
MSI	Microsatellite instability
MSI-H	High microsatellite instability
NK	Natural killer
TLR	Toll-like receptor
PAMP	Pathogen-associated molecular patterns
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
TAMS	Tumor-associated macrophages
Tfh	T follicular helper
T_{REGS}	Regulatory T cells

Introduction

Immune cells represent a significant component of the tumor microenvironment and as such can exert considerable influence over cancer progression. On one hand, it has been recognized since the observations of Rudolf Virchow in 1863 that cancer often arises at sites of prolonged inflammation, implicating immune mechanisms in the oncogenic process [1]. It is now estimated that approximately 25% of human cancers are associated with chronic inflammation and while mechanisms that may drive this process have been identified, the precise contribution of immune-mediated events to transformation and tumor progression remain incompletely understood [2].

In contrast to the tumorigenic effects of chronic inflammation proposed by Virchow, Paul Ehrlich postulated early in the twentieth century that the immune system could recognize and eliminate transformed cells and thus protect against the development of cancer [3]. Experimental support for immune recognition of cancer cells, obtained from the classical adoptive transfer studies of the 1950s, provided the foundation for Burnet and Thomas's seminal cancer immunosurveillance model, in which lymphocytes were responsible for reducing the incidence of cancer by eliminating continuously arising transformed cells [4]. Although this model has been extensively challenged for many years over the absence of empirical evidence, the basic premise has gained significant support from the results of experiments performed since the advent of molecularly defined immune-deficient mice [5, 6].

The clinical relevance of immune interactions with tumors, and insights into the mechanisms underlying the different progression scenarios, is provided by the strong correlation between immune cell infiltration and cancer prognosis. First reported for colorectal carcinoma [7–9], the influence of qualitative and quantitative differences in the composition of infiltrating immune cells on tumor progression is now recognized for several cancers [10–13]. In general, the presence of IFN-γ producing T cells at the invasive border and within the tumor stroma provides the greatest survival benefit, while a lack of infiltrating T cells correlates with poor prognosis. Indeed, in the case of colorectal cancer, the infiltration of T cells into the tumor is the most predictive for patient survival of all histologic and clinical

criteria. With ever more sophisticated evaluations of immune cell infiltration of tumors being reported, it is apparent that there is considerable variation between tumors and that the interaction is far from static, evolving significantly throughout tumor progression [14].

Although we now know much more about the cellular and molecular mechanisms of an immune response than Virchow and Ehrlich, a unified model of the interaction between the host immune system and the developing tumor remains elusive. A primary goal of current tumor immunology research is to resolve the apparent contradiction between the oncogenic and immunosurveillance activities of the immune system. The success of this endeavor will lead to more effective strategies to manipulate the immune microenvironment to achieve better outcomes for cancer patients. This chapter focuses on the mechanisms that balance pro-tumor chronic inflammation, which may enhance genomic instability, and anti-tumor immune activity associated with cancer prevention and an improved prognosis.

Inflammation and Cancer

Inflammation is a normal physiological response to infection, irritation or tissue damage that is associated with extensive cytokine and chemokine signaling, vascular changes, immune cell infiltration, and tissue remodeling. Granulocytes are the predominant cells infiltrating sites of acute inflammation, which generally resolve in a few days. Chronic inflammation, dominated by macrophage infiltration, results from conditions of incomplete pathogen or irritant clearance, or unchecked inflammatory signaling, and predisposes the host to a range of pathologies, including cancer [2, 15].

A variety of underlying causes of chronic inflammation have been linked to cancer, including infectious agents (e.g. Hepatitis viruses for liver cancer and helicobacter pylori for gastric cancer) [16, 17], irritants (e.g. asbestos for lung cancer) [18], autoimmunity (e.g. inflammatory bowel disease for colorectal cancer) [19], as well as unidentified ones (e.g. prostatitis for prostate cancer) [20]. In these cases, the chronic inflammatory response initiates and promotes the transition from premalignant state to full transformation and thus increases the incidence of cancer by establishing conditions beneficial to the expansion of mutated cells. However, inflammation is also associated with cancers that do not arise at sites of chronic inflammation. In these cases, oncogenic signaling itself, in the absence of external stimuli, may initiate the chronic inflammatory response that generates conditions that promote tumor progression (e.g. RET oncogene for thyroid cancer) [21].

Acute inflammation is driven by cells of the innate immune system, in concert with endothelial and epithelial cells and fibroblasts, which detect infection or tissue damage through germline-encoded pattern recognition receptors. These receptors, including the toll-like receptor (TLR) family, bind to conserved motifs present on infectious agents, known as pathogen-associated molecular patterns (PAMPs), or local cell products, the damage-associated molecular patterns (DAMPs) [22].

Signaling via TLR is mediated through MyD88 and culminates in the activation of the NF-κb transcription factor, a central regulator of inflammation that induces the production of cytokines and chemokines, adhesion molecules, matrix metalloproteases, and other inflammatory mediators.

The production of cytokines and chemokines and changes in vascular permeability lead to the rapid accumulation of leukocytes, primarily neutrophils but also macrophages, natural killer (NK) and dendritic cells (DC), at the injured site [23]. Activation of these infiltrating cells by cytokines and PAMP/DAMP recognition perpetuates the inflammatory response through the continued production of mediators such as reactive oxygen species (ROS). ROS play a central role in the inflammatory response, functioning both as signaling molecules and as a host-defense mechanism [24]. In addition, activation of DCs leads to stimulation of adaptive immune responses through the presentation of antigens from the injured site to T cells in the draining lymph nodes [25, 26]. This later event culminates in the accumulation of activated lymphocytes and the exertion of adaptive immune effector mechanisms at the injured site, contributing to the eradication of the underlying insult and resolution of inflammation.

An inability to successfully resolve the initial inflammatory response can lead to the development of chronic inflammation. Lasting in some cases for years, chronic inflammation sites are dominated by infiltration of macrophages and lymphocytes. Chronic inflammation is sustained by the persistent activation of NF-κB and STAT signaling pathways, leading to prolonged production of pro-inflammatory cytokines (e.g. TNF-α and IL-6) and reactive oxygen and nitrogen species (RONS) [27]. The resulting inflammatory milieu provides an environment supportive of cell survival and proliferation, angiogenesis and tissue remodeling. Such an environment is also conducive to the initiation, promotion, and progression of cancer [15, 28, 29].

The similarities between the immune environments generated by chronic inflammation in the presence or absence of cancer and tumors at sites previously devoid of inflammation suggest a mechanistic overlap in tumor promoting activities. The tumor-supportive immune environment is characterized the accumulation of M2-polarized tumor-associated macrophages (TAMs) [30], regulatory T cells (T_{REGS}) [31], and myeloid derived suppressor cells (MDSC) [32], and is rich in cytokines that promote cell survival and proliferation (e.g. IL-6 and IL-23) or impair anti-tumor immune activity (e.g. TGF-β and IL-10). This setting, initiated and maintained in large part by NF-κB- and STAT-driven transcription, results in the proliferation of cells with increasing levels of DNA damage and genome instability, leading to dysplasia that, in many cases, progresses to tumor development and metastasis.

Inflammation and Genomic Instability

While the chronic inflammatory environment provides proliferative and anti-apoptotic signaling and promotes the metastatic changes necessary for cancer progression, it is the ability to generate DNA changes and genomic instability that is the

driver of tumorigenesis. DNA lesions occur at low levels in most cells, but are more frequent at sites of chronic inflammation where the mutation rate is significantly higher than in normal tissues [33]. Elevated mutation rates are observed even in the absence of dysplasia, implicating this mutagenesis as a potential mechanism of tumor initiation [34]. In the absence of efficient repair, the accumulation of DNA alterations results in destabilization of the genome and generation of the mutator phenotype that is a hallmark of most cancers. Several inflammation-induced mechanisms contribute to the generation of genome instability.

(i) DNA Alteration Chronic inflammation is associated with the sustained production of RONS by activated phagocytes and epithelial cells [35, 36, 24]. The mutagenic activity of inflammatory phagocytic cells was originally defined using the classical Ames test [37, 38]. Revealingly, phagocytes derived from patients with defects in NADPH-oxidases did not demonstrate mutagenic activity, implicating ROS as primary mediators. Consistent with the similarities between inflammatory and tumor immune environments, TAMs were also mutagenic in the bacterial and mammalian assays [39, 40]. Subsequently, co-culture of an immortalized mouse fibroblast cell line with human neutrophils revealed the ability of the phagocytic cells to drive transformation of mammalian cells at a rate similar to that achieved by exogenous free radicals [41].

Although the most common DNA alterations are base changes, RONS can also generate gross changes in chromosomes via rearrangements, deletions, insertions and amplifications resulting from the cumulative effects of oxidation of DNA bases, proteins or lipids [36, 35]. Furthermore, the most common oxidation-induced base change, conversion of guanine to 8-hydroxyguanine, could change gene expression by altering regional methylation patterns [42]. Genetic alterations detected in the transformed cells generated by co-culture with TAMs included DNA strand breaks, sister chromatid exchange and mutations. These results were consistent with the long established correlation between the capacity of tumor promoting agents to induce cancer and their ability to induce inflammatory cell infiltration and production of RONS.

A frequently observed consequence of the activation of NF-κB signaling in epithelial cells by chronic inflammation is the ectopic expression of activation-induced cytidine deaminase (AID) [43]. A member of the apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like family, AID is involved in the generation of adaptive immune receptor diversity through hyper-mutation of the variable regions of immunoglobulin (Ig) genes [44]. In addition, AID also induces the site-specific double strand DNA breaks that are necessary for the Ig class switching that occurs during maturation of an immune response. While AID activity is normally restricted to the Ig gene locus, off-target mutation of non-Ig genes, including oncogenes and tumor suppressor genes, has been widely reported [45]. Evidence for the ability of ectopic AID expression to induce genomic instability is provided by the generation of lymphomas and carcinomas in a transgenic mouse model of ubiquitous AID expression [46]. Elevated expression of AID has been detected at the sites of most inflammation-associated carcinomas, including liver, gastric, colorectal and esophageal [43].

(ii) Inhibition of DNA Repair The low mutation rate in normal tissue is in part a reflection of the efficiency of DNA repair pathways, including mismatch repair (MMR) and base excision repair (BER). Chronic inflammation is associated with aberrations in several DNA repair pathways, which compounds the mutagenic activity at these sites and leads to genomic instability [36]. The reduced repair activity can be caused by RONS-mediated damage to repair enzymes or altered expression of repair genes as a result of inflammation-associated changes in methylation patterns and gene promoter activity. Inflammatory cytokines and RONS also induce expression of HIF-1α that in turn reduces expression of MMR proteins. Conversely, inflammation induces an increase in epithelial expression of two genes involved in BER, the primary pathway for repair of RONS-mediated DNA damage. Surprisingly, the increased activity of the AAG and APE1 enzymes correlated strongly with genomic instability [47]. Dysregulation of the MMR and BER pathways generates microsatellite instability (MSI) in regions of DNA rich in short repetitive sequences. Frameshift mutations in genes that contain such microsatellites, such as Bax and TGF-βRII, is thought to contribute to tumor development [48, 49].

(iii) Dysregulated Cell Cycle Checkpoints In addition to MSI resulting from inefficient DNA repair, chronic inflammation can also drive the generation of chromosomal instability (CIN) [15]. One of the primary mediators of CIN is the loss of the physiological mitotic checkpoints that normally inhibit the cell cycle during DNA repair or induce apoptosis if damage is excessive [50]. The p53 pathway is a frequent target of inflammation-induced dysregulation [51]. For example, the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) has been shown to inhibit the activity of the tumor suppressor proteins, p53 and retinoblastoma [52], a finding consistent with the clinical correlation between high MIF expression and poor prognosis [53–55]. In addition, RONS can inhibit p53 function and generate p53 mutations, and AID activity targets the p53 locus [56–59].

The ability of the chronic inflammation to activate both pathways that induce DNA abnormalities and those that nurture cells harbouring such lesions underlies tumor promotion by the immune microenvironment. The cumulative effect of these concurrent activities is the generation of a genome-unstable tumor cell population that provides the heterogeneity and plasticity that is the foundation for progression and metastasis of cancer [60]. Importantly, however, the generation of DNA lesions also serves as a signal to activate host immune surveillance mechanisms. The tumorigenicity of chronic inflammation is therefore countered by the induction of immune mechanisms that inhibit cancer progression (Fig. 8.1).

Immune Responses Against Cancer

Based on the correlation of tumor infiltration by various immune cells with clinical prognosis and the results of years of laboratory study, it is generally accepted that the most effective immune response for eliminating transformed cells and inhibiting

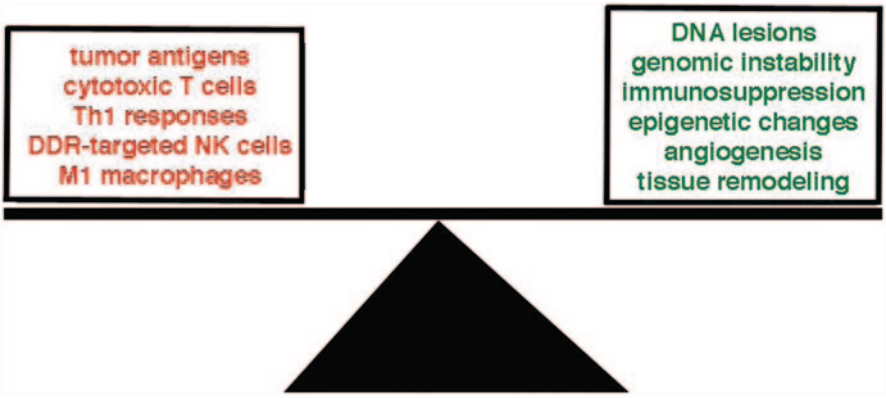

Fig. 8.1 Cancer in the balance. Chronic inflammation generates a microenvironment that includes both tumor promoting (*shown in green*) and tumor inhibiting (*shown in red*) immune activities. Tumor initiation, promotion and progression are influenced by the complex and dynamic interaction between these opposing factors. During immune-mediated equilibrium these activities are balanced, resulting in the survival, but not outgrowth of the established tumor. Effective targeting of these mechanisms, individually or in combination, may lead to improved patient outcomes by tipping the balance in favor of tumor elimination or sustained equilibrium

tumor growth is one dominated by the production of IFN-γ by $CD4^+$ and $CD8^+$ T cells, known as a Th1 response [61]. In addition to T cells, the tumor microenvironment during a Th1 response contains significant infiltration by natural killer (NK) cells, dendritic cells (DC), and type 1 macrophages (M1) and generates conditions that support the induction of tumor antigen-specific T cell cytotoxicity [62]. The role of specific lymphocyte subsets (e.g. T cells and NK cells), cytokines (e.g. IFN-γ and IL-12), and cytotoxic mechanisms (e.g. perforin, TRAIL) in protection against malignancy has been revealed by the increased tumor incidence in single gene-targeted immune-deficient mice.[63, 5] Confoundingly, components characteristic of a tumor-promoting immune environment, such as IL-6 and IL-23, are also often detected during Th1 anti-tumor responses, while many cells associated with productive anti-tumor immune responses have been reported to suppress such activity [64–66]. These apparent contradictions highlight the importance of the overall context of the immune response in determining the outcome, with variables such as tumor cell type, location, host immune status, and others, many of which are as yet unidentified, exerting significant influence.

Immune Recognition and Genomic Instability

The ability of the immune system to specifically recognize transformed cells is critical to mounting an effectively targeted anti-tumor response. In this regard, genomic instability contributes to such recognition through both the activation of

the DNA damage response (DDR) and the generation of mutated proteins. Detection of cells, via recognition of stress-related molecules by cells of the innate immune system [67], or through the presentation of tumor–associated antigens to adaptive immune cells [68], provides the means to discriminate altered tissue from normal tissue, enabling specific eradication of pre-malignant or malignant cells.

The role of NK cells as important innate immune effector cells in anti-tumor responses has been identified in many experimental systems and their presence in tumor infiltrates correlates with good prognosis [69]. Indeed, of all the immune cell subsets detected at cancer sites, NK cells are the least reported as being associated with tumor-promoting activities. Activation of NK cells is achieved when the balance of signaling through activating receptors, including NKG2D, NKp30 and NKp46, outweigh those received via inhibitory receptors, such as killer-cell immunoglobulin-like receptors (KIR) and CD94/NKG2A [70]. Following activation, NK cells exert a variety of effector mechanisms, including perforin/granzyme-mediated cell cytotoxicity and the production of cytokines that promote the generation of a Th1 response.

The occurrence of DNA lesions in cancer cells, resulting from chronic inflammation, oncogene activation, or other genotoxic stressors, can induce the DDR pathway [71]. Activation of the DDR cascade causes cell cycle arrest and initiates DNA repair pathways, enabling re-entry into the cell cycle, or senescence or apoptosis if the repair is unsuccessful. In addition, initiation of DDR can result in interferon production and the expression of ligands for the NK cell activating receptors, NKG2D and DNAM [72, 73]. Expressed at low levels on normal cells, these molecules are upregulated by cells exposed to DNA damaging agents and are frequently observed at higher levels on tumor cells. In addition to NK cells, NKG2D is often expressed on CD8 T cells, γ/δ T cells, and NKT cells; these are all cytotoxic cell types that have been associated with productive anti-tumor immune responses [74]. The increased expression of these ligands increases the sensitivity of cancer cells to killing by immune effector cells [75–77], and mice deficient in NKG2D or DNAM exhibit increased susceptibility to cancer [78, 79]. NKG2D binding to its ligand RAE-1 on the surface of tumor cells has also been implicated in NK cell-mediated clearance of senescent tumor cells [80]. Surprisingly, RAE-1 was expressed, independent of p53 status, on most tumor cells, but NK cells only eliminated those that had undergone p53-induced senescence. Critically, the senescent tumor cells secreted several chemokines, of which CCL2 was responsible for the NKG2D-independent migration of NK cells to the senescent tumors cells. In the absence of CCL2 production, there was limited NK infiltration into growing tumors. This finding highlights the importance of mechanisms of recruitment as well as targeting for immune-mediated elimination of cancer cells.

In contrast to the induction of innate immune responses, the altered expression of proteins on the surface of tumor cells is insufficient to generate a Th1 adaptive response. Recognition of cancer by T cells requires the presentation of peptide fragments of endogenous proteins in the context of major histocompatibility complex (MHC) class I or class II on the surface of tumor cells [81]. Furthermore, for the activation of naïve T cells, an additional costimulatory signal and appropriate

cytokine milieu must be provided at the time of the initial binding of the T cell receptor to the MHC-peptide complex [82]. As most tumor cells do not express costimulatory molecules or MHC Class II, cross-presentation of tumor antigens by dendritic cells is necessary for induction of an optimal T cell response. At the tumor site, cell death, often as a result of innate immune responses, initiates the recruitment of immune cells, including immature DCs that take up tumor proteins. Following antigen processing and migration to the draining lymph node, DCs present MHC Class I and II associated tumor antigens, along with costimulatory signals, to CD8 and CD4 T cells respectively [83]. Activated tumor-specific T cells then migrate to the tumor site and, in the case of a productive Th1 response, infiltrate the stroma and eliminate transformed cells based on their expression of appropriate MHC-peptide complexes.

The peptide fragments presented by MHC complexes dictate the tumor specificity of a T cell response. T cell responses directed at many tumor antigens have been detected, including responses against antigens expressed by both normal and transformed cells, and those that are unique to cancer cells [68]. The mutations produced by the chronic inflammatory environment could provide a rich source of potentially unique peptides that would be specific to the transformed cells. Indeed, MSI generated by impaired MMR leads to the generation of multiple frameshift mutations that represent neo-antigens to the host immune system [84–87]. Intriguingly, tumors with high MSI (MSI-H) demonstrate greater immunogenicity than microsatellite stable tumors, with greater infiltration by activated CD8 T cells, and this has been suggested as the mechanism responsible for the reduced incidence of metastasis and better prognosis of the MSI-H tumors [88–92]. Consistent with this hypothesis, T cells specific for frameshift–generated epitopes have been detected in colorectal cancer patients with microsatellite unstable malignant cells [87, 93].

Genomic Instability and Immune Editing

As described in the preceding sections, the interaction between the immune system and transformed cells can lead to a range of possible outcomes, of which Virchow's cancer promotion and Ehrlich's eradication represent the two extremes. The three-stage immune editing model of cancer progression was proposed in 2002 to reconcile the disparate influences of the immune system on cancer development [5]. The first stage, the elimination phase, resembles classical immunosurveillance, where newly arising transformed cells are specifically removed by the immune system. However, if this elimination is not complete, surviving transformed cells then enter the second stage, the equilibrium phase. During the equilibrium phase, there is a balance between tumor growth, often promoted by inflammatory mechanisms, and immune-mediated cytotoxicity that results in tumors that are present but not apparent. In the third stage of the model, the escape phase, loss of immune control over the nascent tumor leads to outgrowth and development of a clinically apparent

cancer. In this model, loss of equilibrium is the result of selection of tumor cell clones that have lost their sensitivity to, or block the generation of, protective immune mechanisms; the evolving tumor is thus edited by the immune system to be less immunogenic as it progresses to overt disease.

The majority of the experimental support for the immune editing hypothesis has come from tumor progression experiments in mice with targeted immune deficiencies [77, 94–96]. However, a considerable body of data consistent with this model has emerged from human cancer studies. The development of antibody and T cell responses against tumor antigens in patients has been widely reported, although these alone do not prove that immune control was exerted at any stage in cancer progression. However, many mechanisms that subvert anti-tumor immunity have been detected in emergent tumor cells, a scenario consistent with the immune-mediated selection of less immunogenic clones. These escape mechanisms include loss of MHC expression, upregulation of T cell inhibitory receptors, and release of soluble decoy ligands for NK cells [97–100]. Further evidence of an early equilibrium phase comes from patients with paraneoplastic syndromes in which immune responses directed at tumor antigens that are also expressed on neuronal cells trigger an autoimmune response [101, 102]. That the resultant neurologic dysfunction often presents significantly earlier than the underlying cancer suggests that the immune responses are being generated relatively early in tumorigenesis and may impede cancer progression. Finally, the previously described prognostic significance of tumor infiltrating immune cells implicates immune control as a significant modifier of cancer progression.

So where do the various immune modulatory effects of inflammation and genomic instability fit within the immune editing model of cancer progression? It is apparent that the onset of chronic inflammation precedes dysplasia in many instances, positioning this immune environment as an initiator of the tumor formation process. However, as mentioned earlier, some of the downstream effects of chronic inflammation may contribute to the induction of anti-tumor immune responses at this early stage. While it is obviously difficult to obtain clear evidence of successful cancer elimination, there are indications that anti-tumor immune activity is active during the very early stages of tumorigenesis. In the case of ulcerative colitis, an inflammatory condition that is highly associated with colon cancer, T cell responses specific for antigens expressed in dysplastic epithelium have been detected [103, 104]. This finding suggests that the immune activity against pre-malignant lesions may underlie the significantly lower incidence of colorectal colon cancer than ulcerative colitis [105, 104]. Importantly, this finding indicates that productive anti-tumor immune responses can be generated even after a chronic inflammatory environment has been established, an essential scenario if immune control of inflammation-driven cancer is to be clinically relevant. In keeping with early immune responses being induced by transformation events, T cells specific for frameshift mutations have been detected in cancer-free individuals with a genetic predisposition for colorectal cancer [106]. In the context of the immune editing model, the diagnosis of overt cancer is indicative that tumor escape has occurred. This is somewhat at odds with the prognostic significance of lymphocytic infiltration, which

suggests ongoing immune influence over cancer progression. While the precise nature of the association between immune infiltration and prognosis remains to be defined, it is an intriguing possibility that treatment of the cancer can re-establish a state of equilibrium, perhaps by restoring previously effective immune mechanisms or by the preferential elimination of escape variants [107].

Chronic inflammation provides several pathways for the escape of a tumor from immune control. Foremost amongst these is the generation of an environment that is inhibitory to productive anti-tumor immune responses. As described earlier, the presence of suppressive cell types, including MDSC and T_{REGs}, and cytokines, such as TGF-β and IL-10, may significantly undermine sustained T and NK cell-mediated cytotoxicity, allowing escape by simply removing the restraints. Such a scenario would not necessarily involve selection of resistant clones, and thus the tumor may still be amenable to immune control if the inhibitory environment could be altered [108]. Furthermore, the inflammatory environment provides additional features, such as vascular changes and extracellular protease activity, which could contribute to escape from local immune pressures through facilitation of metastasis.

The frequent loss or down-regulation of MHC class I expression on carcinoma cells suggests the outgrowth of immune-selected clones [97, 100]. MHC class I loss has been correlated with reduced T cell infiltration and poor prognosis [109]. While several mechanisms leading to MHC class I loss have been identified, frameshift mutations in β2-microglobulin, an essential component of class I complexes, are frequently observed in MSI-H colorectal cancer cells early in tumor progression [110–112]. While these early occurring mutations generate a heterogeneous pool of class I positive and negative tumor cells, subsequent progression and metastasis is associated with selection and uniform outgrowth of the class I-deficient tumors (Fig. 8.2).

Genomic instability has also been implicated in the reduction of lymphocyte infiltration in colon cancer patients, where chromosomal instability led to the loss of CXCL13 expression, which resulted in lower densities of B cells and T follicular helper (Tfh) cells at the invasive margins [14]. High expression of B cell and Tfh cell markers correlated with extended disease-free survival. The apparent contradiction between the increased immunogenicity observed for MSI-H tumors and the ability of genomic instability to generate escape variants highlights how little we currently understand the dynamic nature of the interaction between cancer cells and the immune system and emphasizes the need for longitudinal studies of individual tumor evolution to provide clearer insights.

Immune evasion strategies employed in one tumor environment may not always be advantageous in another, a scenario that may be particularly relevant to metastasis. The frequent loss of MHC class I by carcinoma cells may represent such an event. While loss of MHC expression reduces the efficacy of anti-tumor T cell-mediated cytotoxicity, a lack of class I increases the susceptibility of tumor cells to NK cell-mediated killing [113, 114]. As exposure to NK cells is likely to be increased during metastatic transit through blood or lymph, survival of the tumor cells during this migration may involve additional selective events. Interestingly, there is increasing evidence that platelets inhibit NK killing of tumor cells, both through the

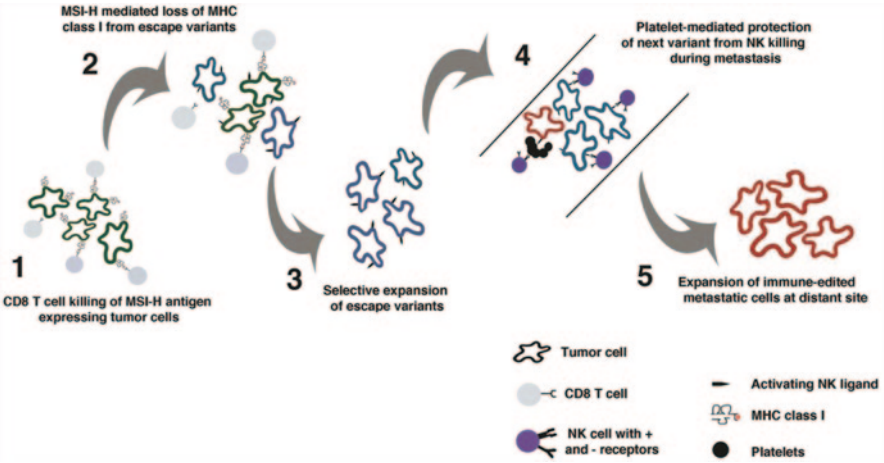

Fig. 8.2 MSI-H and immune editing. The immune-driven selection of tumor cells is a multi-step process, one that MSI-H can influence in multiple ways. MSI-H induced mutations can serve as tumor-specific antigens for T cell responses (*step 1*). In response to T cell-driven selective pressure, tumor cells that lose MHC class I expression, for example by MSI-H-mediated mutations, have a growth advantage (*step 2*) and become the dominant clones (*step 3*) During metastasis of these escape variants, those that acquire additional survival advantages to escape the new immune pressures encountered during transit (*step 4*) will form a tumor at distant sites (*step 5*)

release of soluble factors and by physical protection of the tumor cells; increased adhesion to platelets by metastatic tumor cells may represent a selected trait that enhances tumor escape variant survival during transit and overall metastatic success (Fig. 8.2) [115–117].

Conclusions

Cells of the immune system are a significant component of the tumor microenvironment, capable of either promoting or inhibiting the progression of cancer. While immune mechanisms that contribute to each of these outcomes have been identified, the dynamic interplay between the components of the immune environment during the progression of disease remains poorly understood. The complexity of these interactions is highlighted by the differing contributions made by the same components in different tumor settings or at different times in the tumorigenic process; this complexity will make the consistent manipulation of the immune environment for clinical benefit a challenging proposition.

Given the divergent effects of immune responses in the tumor environment, two broad strategies are being investigated to enhance patient outcomes; inhibition of tumor promoting immune activity and boosting of anti-tumor responses. Reduced cancer incidence associated with the use of anti-inflammatory agents provides

encouraging proof-of-principal evidence that the tumor-promoting inflammatory process can be clinically targeted [118–121]. While it is likely that achieving such outcomes against established cancer will be significantly harder, many approaches targeting the central pathways of chronic inflammation are currently under investigation for the ability to prevent cancer progression and metastasis [122–125].

The recent clinical successes with immune checkpoint blockade antibodies represent a significant milestone in the development of cancer immune therapies [126]. The improved patient outcomes achieved with monoclonal antibodies targeted to mediators of tumor-induced immune suppression demonstrates that evasion strategies can be overcome to re-activate protective anti-tumor immune responses. This success paves the way for the evaluation of strategies to address other escape pathways. Furthermore, the identification of commonly arising mutations in MSI-H cancers suggests that vaccine-based approaches to enhance T cell responses against these immunogenic tumors may be feasible [127]. Overall, our increasing understanding of the process of tumor progression has provided insights into how to target this process in patients. While we currently do not know enough to predict how well these approaches will work in each of the varied and complex tumor environments, there are encouraging signs that this work will result in improved outcomes for patients.

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Chapter 9
Synthetic Genetic Approaches in Colorectal Cancer: Exploiting and Targeting Genome Instability

Babu V. Sajesh, Amy L. Cisyk and Kirk J. McManus

Abstract Colorectal cancer is the third leading cause of cancer-related deaths throughout the world. Surgery is effective against early stage tumors but advanced stage tumors lack an effective targeting strategy. For nearly 50 years, 5-fluorouracil has been the standard of care for advanced disease, but the overall 5-year survival rate remains at only 6%. Accordingly, novel therapeutic strategies are urgently needed to decrease morbidity and mortality rates. Synthetic genetic approaches are well established in model organisms, and have recently garnered much attention in humans for their potential implications in cancer targeting. Synthetic lethality and synthetic dosage lethality are innovative strategies designed to specifically exploit and kill cancer cells based on the loss-of-function associated with tumor suppressors or the gain-of-function associated with oncogenes, respectively. By definition, these approaches are highly specific and restricted to tumor cells, and are expected to decrease side effects associated with current strategies. Both synthetic genetic approaches have been applied extensively in pre-clinical studies and numerous candidate drug targets have been identified, including some that have entered clinical trials. The focus of this chapter is to present the pathways that drive tumorigenesis in colorectal cancer, and describe how synthetic lethality and synthetic dosage lethality can exploit these origins for enhanced killing of tumor cells. Finally, we summarize the current status of the field and relate how these novel strategies can be custom-tailored to target advanced stage colorectal cancer as we enter the personalized medicine era.

Keywords Colorectal cancer · Metastatic disease · Chromosome instability · Genome instability · Therapeutic targeting · Treatment · Synthetic genetic approaches · Synthetic lethality · Synthetic dosage lethality · Personalized medicine

Abbreviations

5-FU	5-Flurouracil
APC	Adenomatous polyposis coli

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CIMP	CpG island methylator phenotype
CIN	Chromosome instability
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
KRAS	Kirsten rat sarcoma viral oncogene homolog
MMR	Mismatch repair
MSI	Microsatellite instability
nCIN	Numerical CIN
sCIN	Structural CIN

Introduction

The International Agency for Research on Cancer (World Health Organization) estimated that in 2012, there was 14.1 million newly diagnosed cancer cases in the world [1]. Amongst these cases, colorectal cancer (CRC) was the third most common cancer accounting for ~9.7% of all cases, or ~1.4 million new cases and ~700,000 deaths annually. CRC has significant burden for both males and females. In males, it ranks third behind lung and prostate cancer with ~746,000 new cases and 374,000 deaths annually while, in females, it ranks second behind breast cancer, with ~614,000 new cases and 320,000 deaths (Table 9.1). These statistics highlight the immediate need for novel therapeutic strategies and targets to better combat the disease.

CRC typically begins as a benign adenomatous polyp within the colon and/or rectum. Through the accrual of genetic insults, the polyps can develop into advanced stage adenomas with high-grade dysplasia that can progress into an invasive cancer, and may ultimately metastasize to lymph nodes and distant organs [2]. CRC staging is used as a metric to estimate the amount of penetration of the particular cancer. Staging is employed for diagnostic and prognostic purposes, and to determine the best treatment option. Five stages (stage 0, I, II, III and IV) are employed to describe

Table 9.1 Incidence and mortality rates of colorectal cancer

Location	Estimated incidence			Estimated mortality		
	Males	Females	Total	Males	Females	Total
World (2012) [1]	746,000	614,000	1,360,000	374,000	320,000	694,000
Australia[a] (2012/10) [147]	8760	7080	15,840	2205	1777	3982
Canada (2013) [148]	13,200	10,600	23,900	5000	4200	9200
United Kingdom[b] (2011) [149]	23,171	18,410	41,581	8520	7139	15,659
United States (2014) [150]	71,830	65,000	136,830	26,270	24,040	50,310

[a] Bowel Cancer; Estimated incidence and actual deaths reported for 2012 and 2010, respectively
[b] Bowel Cancer (Colon, Rectum and rectosigmoid junction); UK = England, Wales, Scotland and Northern Ireland

the extent of local invasion, the degree of lymph node involvement and whether the tumor has metastasized [3]. In stage 0 disease (i.e., carcinoma *in situ*), the cancer is confined to the innermost lining of the colon or rectum, whereas stage I tumors have grown into the inner wall of the colon (or rectum), but have not yet invaded beyond the wall. Stage II tumors have extended more deeply into or through the inner wall, and may involve proximal tissues, but do not show lymph node involvement. Stage III cancers exhibit lymph node involvement, while stage IV tumors are those that have begun to metastasize to distal sites including the liver and lungs [4].

Although the overall 5-year survival rate for newly diagnosed individuals in the United States is ~65%, survival rates differ substantially depending on the stage at initial diagnosis (Table 9.2) [5]. As expected, individuals with tumors diagnosed at early stages (I and II) fair better than those diagnosed at late stages (IV). For example, stage I and II disease is often cured through surgical intervention alone, while up to 74% of stage III disease is curable by surgery in combination with adjuvant chemotherapy [6, 7]. Although, recent advances in chemotherapy have improved survival and/or quality of life, stage IV disease is typically incurable [7] and has a 5-year overall survival rate of only 6% [5].

The clinical behavior of a CRC is multi-factorial as there are a myriad of environmental and host factors that contribute to the pathogenesis of the disease. A major challenge in the field has been to identify and characterize the molecular factors and pathways that initiate, promote and drive tumor formation. Indeed over the past 50 years, substantial progress has been made and many environmental, germ-line and somatic factors have been identified including the aberrant genes and biological pathways implicated in the pathogenesis of CRC. Consequently, one of the remaining challenges lies in the evolution and/or development of next generation therapeutics to better combat the disease.

Genome Instability and Its Role in Colorectal Cancer Development

For over a century, genome instability has been suspected to underlie tumorigenesis. In 1890, David von Hansemann, first reported abnormal mitotic figures in several epithelial cancers [8]. In 1914, Theodor Boveri postulated that tumors arise as a consequence of abnormal chromosome segregation [9]. He also theorized that tumor growth was based on incorrect chromosome combinations, and likely accounted

Table 9.2 Observed 5-year survival rates for colon and rectal cancer (Based on United States data)

Stage	Observed 5-year survival rate [5]	
	Colon cancer (%)	Rectal cancer (%)
I	74	74
II	37–67	32–65
III	28–73	33–74
IV	6	6

for the abnormal growth characteristics passed on to daughter cells. During the subsequent ~100 years, a wealth of fundamental and clinical research has substantiated the correlation between aberrant chromosome numbers in countless tumor types, yet very little information is available that describes the underlying aberrant biological processes and genes accounting for genome instability.

Genome instability is a hallmark of virtually all tumor types, including CRC. There is a growing body of evidence that suggests genome instability is a driver of the tumorigenic process and underlies the acquisition of tumor-associated genetic alterations [4]. Genome instability generally arises due to random mutations within critical genes, chromosome rearrangements [10–12] and epigenetic changes [13] that collectively provide a growth advantage to cells permitting them to proliferate, survive and spread [14]. However, the genetic and epigenetic aberrations that cause genome instability and drive tumor development may be the very targets that can be exploited through novel therapeutic strategies collectively referred to as synthetic genetic approaches (described below). What is clear, however, is that CRCs exhibiting genome instability can be classified into at least one of three general categories: 1) microsatellite instability (MSI), 2) chromosome instability (CIN), or 3) CpG island methylator phenotype (CIMP). Not only do these pathways contribute to the pathogenesis of tumors, but they also serve to differentiate the tumor cells from normal surrounding cells and tissues. Thus, if therapeutic strategies can be identified and developed that leverage and exploit these inherent differences, the therapeutic effect would be restricted to tumors and thus minimize side effects within the normal surrounding tissues. Accordingly, an awareness of these genome instability pathways, and the molecular players implicated therein, is necessary to develop synthetic genetic approaches.

a. Microsatellite Instability and DNA Mismatch Repair MSI arises due to defects DNA mismatch repair (MMR), which result in the accumulation of genomic mutations [15–17]. MSI is typically detected by measuring changes within microsatellite DNA [18], which are highly repetitive DNA sequences scattered throughout the genome. The highly repeated core sequence within a given microsatellite typically ranges from 1–6 base pairs in length, and is prone to DNA replication errors. Under normal conditions, single base pair mismatches and/or DNA slippage occurring within microsatellites are normally repaired by the MMR system, which, when compromised, leads to insertions and/or deletions that contribute to the tumorigenic process. While the number of repeats contained within a microsatellite varies from person to person, a given microsatellite within an individual remains constant. Consequently, extensive length variations for a given microsatellite in an individual is suggestive of an underlying MMR defect [18].

There are four predominant genes that encode functions within the MMR pathway, namely *MSH2*, *MLH1*, *MSH6* and *PMS2* (reviewed in [19]). These genes encode proteins that collectively function to identify and coordinate the repair of DNA mismatches, including those within microsatellites. However, if these functions become compromised, genome instability ensues. Because most of the genomic alterations stemming from MMR defects are typically small in size, and usually involve one to several bases, most MSI tumors maintain a karyotypically

stable or diploid to near-diploid state [20, 21]. Thus, MSI is also frequently referred to as a mutator pathway or phenotype [22].

MSI was first identified as an aberrant phenotype associated with CRC in 1993 [20, 23, 24]. Since then, numerous studies have determined that both inherited and somatic defects (i.e., somatic mutations and epigenetic silencing) within the MMR genes are contributing factors in the etiology of CRC (reviewed in [25]). For example, in Lynch syndrome, which accounts for 1–2% of all CRCs, individuals harbor a predisposition to develop CRC (and other tumor types) due to the inheritance of a defective MMR allele [26, 27]. DNA sequencing of Lynch Syndrome patients have shown that the majority of cases are associated with inherited defects in *MSH2* (~60%) or *MLH1* (~30%) with a minor proportion of cases attributed to defects in *MSH6* (5–10%) or *PMS2* (<5%) [28-30]. Interestingly, although MSI is associated with ~15% of sporadic CRCs, up to 60% of those cases are due to epigenetic silencing of *MLH1* rather than acquired mutations within the gene [31–34], indicating divergent molecular origins for inherited versus sporadic CRCs.

b. Chromosome Instability Is Highly Associated with Colorectal Cancer CIN is defined by an increase in the rate at which whole chromosomes, or large parts thereof, are gained or lost. CIN is an aberrant phenotype associated with up to 80% of all tumor types [35, 36], and is arguably best described in CRC, where it is associated with up to 85% of all sporadic and the vast majority of heritable forms of the disease [37–40]. Conceptually, CIN impacts tumor suppressors and oncogenes by decreasing or increasing gene copy numbers respectively, and/or by inducing structural rearrangements of chromosomes.

In general, tumors exhibiting CIN can be classified into two categories, numerical (nCIN) or structural (sCIN) (reviewed in [41]). Tumors with nCIN typically have increases in chromosome numbers, and most late stage CRCs typically exhibit between 60 and 90 chromosomes per cell [39]. Although the underlying defects leading to nCIN are not well understood, they often involve mutations in genes that regulate chromosome biology including, chromosome congression and segregation, DNA replication and cytokinesis [42]. On the other hand, tumors exhibiting sCIN frequently harbor abnormal chromosome numbers, but also display structural defects resulting from segmental duplications, deletions, or translocations. Like nCIN, the molecular basis of sCIN is poorly understood, but can be associated with aberrant telomere biology, *MYC* amplification or DNA repair defects [43, 44].

The terms CIN and aneuploidy are often used interchangeably, but they are in fact different terms. While aneuploidy refers to a "state" of abnormal DNA content (i.e., chromosome numbers), CIN describes the "rate" leading to aneuploidy. Although studies dating back to Boveri have demonstrated a correlation between aneuploidy and cancer [9], no empirical data clearly demonstrated an increase in the 'rate' at which aneuploidy occurred. In other words, the association of aneuploidy with certain cancers merely suggested an underlying CIN, but its actual nature and magnitude were never examined. In 1997, Vogelstein and colleagues [39] utilized a panel of diploid and aneuploid CRC cells to conclusively demonstrate that the aneuploidy observed in a large subset of CRCs reflected an underlying and persistent error in chromosome segregation that was accounted for by elevated rates in

chromosomal changes. This decisive work identified gains or losses in excess of 10^{-2} chromosomes per generation within the aneuploid CRC lines that did not occur within the diploid lines. These data suggest that cancer cells could be classified into MSI or CIN categories based solely on the observation of an aneuploid karyotype. This seminal work also addressed the long-standing debate over whether CIN contributed to tumorigenesis or was merely the result of the tumorigenic process (i.e., initiator vs. bystander). The observation that the MSI cell lines remained diploid and did not develop a CIN phenotype argued strongly against a bystander effect. Although the above information highlights the robust association between CIN and CRC, very little information is available that addresses its causal nature.

The classical CIN pathway involves the multi-step acquisition of specific genetic alterations and was first characterized by Vogelstein et al. [45]. It was initially proposed that an early mutation occurred within *APC* (adenomatous polyposis coli), a tumor suppressor that was believed to be the principle determinant of CIN [46, 47]. This belief was supported by previous DNA sequencing efforts that identified frequent *APC* mutations within CIN tumors [48]. However, subsequent sequencing efforts have identified CIN tumors without *APC* mutations, and non-CIN tumors with *APC* mutations [49]. These observations suggest that *APC* is unlikely to be the sole determinant of CIN, and accumulating evidence now suggests that there is a spectrum of genes, rather than a single gene, which when aberrantly expressed contribute to the CIN phenotype [12, 37, 50, 51]. Genes encoding proteins that function within pathways such as the spindle assembly checkpoint, sister chromatid cohesion, centrosome biology, DNA damage response, telomere regulation, and cell cycle checkpoints are strongly implicated in the pathogenesis of CIN [52]. Indeed, a large number of the genes that regulate these pathways are somatically mutated in CRCs exhibiting CIN (see [53]), and further supports their underlying involvement in CIN and the tumorigenic process.

In addition to its causal role in the pathogenesis of CRC, the presence of CIN (and aneuploidy) has significant clinical and treatment implications for the disease. Many features associated with CIN (e.g., loss of heterozygosity, the presence of an aneuploid karyotype, or gross chromosomal re-arrangements) are associated with metastatic potential in sporadic tumors [54]. The presence of CIN is also employed as a prognostic indicator, as it correlates with poor patient prognosis [55–58]. Finally, CIN has been shown to confer intrinsic multi-drug resistance in CRC cell lines [59] and many tumor types [60, 61].

c. CpG Island Methylator Phenotype (CIMP) and Its Role in Colorectal Cancer CIMP is an epigenetic phenomenon associated with gene regulation. Unlike MSI and CIN, CIMP does not inherently change the information encoded within the genome, but rather it imparts a level of regulation onto the genome. CIMP is characterized by the hypermethylation of CpG islands that typically reside within the promoter regions of genes, and results in transcriptional silencing (reviewed in [62]). CpG islands are DNA sequences, ranging from 500–2000 base pairs in size, that are rich in CG di-nucleotide repeats that localize within the 5' promoter region of genes [63]. In general, CpG hypermethylation recruits additional factors, such as histone deacetylases and chromatin remodeling proteins, that generally function to

compact the DNA, and render it resistant to transcriptional factors thus preventing gene expression [13].

CpG island methylation can occur naturally or associate with the progression and development of cancer. Type-A methylation refers to the natural age-related increase in global methylation that occurs as colonic epithelial cells age [64, 65], while type-C methylation is the cancer-associated form that is frequently observed in tumor samples. Extensive DNA methylation patterns are attributed to gene inactivation in numerous tumor types including CRC where hypermethylation is frequently observed in hundreds of genes, including DNA repair (e.g., *MLH1* in sporadic CRC) and tumor suppressor genes [64]. Although tumor hypermethylation patterns appear specific, it remains to be determined why certain loci become preferentially hypermethylated [64].

Overall, CIMP is associated with ~30% of sporadic CRCs and a modest increase in DNA methyltransferase activity correlates with CRC progression. However, the underlying mechanism(s) remain poorly understood [66] as silencing in cancer-related genes can occur independent of heterochromatic DNA compaction [67]. It should be noted that DNA methylation patterns are extremely stable. Therefore, they can be maintained through cellular division and transmitted to all daughter progeny [68]. Thus, it is likely that certain hypermethylation patterns, particularly those that confer a survival advantage, will be selected with time and will contribute to the development of CRC.

Current Therapeutic Strategies to Combat Colorectal Cancer

Overall, there are a number of current therapeutic strategies to combat CRC, but they can be generally classified into one of two categories: local or systemic. Local therapies include surgery and radiation therapy, and are designed to remove or destroy the cancer in or near the colon and rectum. Colonoscopies are the preferred method of surveillance for lesions, and permit the removal of polyps by surgical resection (e.g., snare polypectomy, cryosurgery, radiofrequency ablation therapy, etc.). Alternatively, laparoscopic or open surgeries can be performed to remove a portion of the bowel containing the tumor, which is frequently accompanied by the removal of proximal lymph nodes. These types of local therapies are most effective in early stages of the disease (0-II). Local therapies have limited applicability in advanced stages of the disease (Stages III or IV) especially those involving distal metastases (i.e., lymph node or tissues). Radiation therapy is often employed for palliative purposes, particularly in advanced rectal cancers or those with liver metastases [69–71].

More aggressive treatments are generally employed to treat advanced stage disease (stages III or IV), and are collectively referred to as systemic therapies. The overriding principle of systemic therapies is that a therapeutic agent is administered systemically so that it enters the circulatory system and can target cancer cells at the

primary site(s) within the colon and rectum, and also at metastatic sites. Systemic therapies include chemical (e.g., small molecule inhibitor, antimetabolite, DNA damaging agent, etc.) and biological (e.g., monoclonal antibody) agents (Table 9.3). Unfortunately, the therapeutic activity associated with these agents is not restricted to the tumor cells. Rather, many of the agents employed (e.g., 5-fluorouricil, irinotecan, oxaliplatin, etc. [see below]) interfere with DNA replication and are thus potentially toxic to all dividing cells. However, cancer cells are predominantly affected due to their rapid proliferation rates and heavy reliance on DNA replication. Due to a lack of target specificity, a number of side effects are often associated with these agents. These side effects can include alopecia (hair loss), anemia (low red cell counts leading to shortness of breath and fatigue), effects on the cells lining the digestive tract (nausea, vomiting, diarrhea, etc.), neutropenia (low white blood cell counts leading to increased risk of infection), organ damage or even the development of second cancers [72–75].

a. Current Systemic Approaches Although current chemical or biological treatment options in CRC are typically reserved for stage III and IV disease, some individuals with stage II disease receive treatments for prophylactic purposes. For over 50 years, 5-fluorouracil (5-FU) has been the mainstay of CRC treatments (reviewed in [76]). 5-FU is an anti-metabolite that was first synthesized in 1957 by Charles

Table 9.3 List of chemotherapeutic agents used to treat colorectal cancers

Chemotherapeutic agent	Therapeutic target[a]	Mechanism of action
Oxaliplatin	DNA	Prevents replication by forming inter-and intra-strand (DNA) crosslinks to prevent replication
Cetuximab	EGFR	Binds to EGFR and prevents signaling
Panitumumab	EGFR	Binds to EGFR and prevents signaling
5-Fluorouracil (5-FU)	TS	Pyrimidine analog and antimetabolite that binds and irreversibly inhibits TS to inhibit DNA replication
Capecitabine	TS	Pro-drug that is enzymatically converted to 5-FU; inhibits DNA replication
Tegafur	TS	Pro-drug that is enzymatically converted to 5-FU; inhibits DNA replication
Leucovorin[b]	TS (FU)	Stabilizes FU binding to TS
Bevacizumab	VEGF	Binds VEGF to inhibit angiogenesis
Ziv-Aflibercept	VEGF	Binds VEGF to inhibit angiogenesis
Irinotecan	TOP1	Inhibits DNA replication and transcription
Regorafenib	Multiple kinases[c]	Inhibits multiple kinases

[a] *VEGF* Vascular Endothelial Growth Factor, *TS* Thymidylate Synthetase, *EGFR* Epidermal Growth Factor Receptor, *TOP1* Topoisomerase 1

[b] Employed as an adjuvant therapy to FU

[c] Shown to inhibit the activity of RET, VEGFR1, VEGFR2, VEGFR3, KIT, PDGFR-α, PDGFR-β, FGFR1, FGFR2, TIE2, DDR2, TRKA, EPH2A, RAF-1, BRAF, $BRAF^{V600E}$, SAPK2, PTK5 and ABL at clinically relevant doses

Heidelberger [77]. The therapeutic activity of 5-FU stems from its ability to inhibit thymidylate synthetase, an enzyme required for the synthesis of thymidine, an essential nucleoside precursor required for DNA synthesis during replication or repair. More specifically, 5-FU prevents the formation of dTMP (deoxythymidine monophosphate), which prevents cell proliferation. Alternatively, capecitabine and tegafur, pro-drugs that are enzymatically converted into 5-FU, can be substituted. Although 5-FU primarily prevents DNA synthesis, it is also metabolized into fluorouridine triphosphate (FUTP) and can be readily incorporated within RNA, which leads to the disruption of normal RNA processing and function [76].

Currently, 5-FU is used extensively in the treatment of advanced stage CRC, but its efficacy as a single agent appears limited (~10–15%) [78]. To enhance treatment efficacy, many new combinatorial approaches involving 5-FU and additional compounds have been identified (Table 9.3). Additional chemical agents also include leucovorin (or folinic acid), which stabilizes 5-FU binding to TS [79], or irinotecan, which is a topoisomerase 1 inhibitor that functions by preventing DNA from becoming unwound during replication. Finally, oxaliplatin has been used in advanced disease, and is a platinum-based antineoplastic that forms DNA cross-links and inhibits DNA replication and transcription.

In addition to the systemic, chemical therapies described above, biological therapies (e.g., monoclonal antibodies) are frequently employed (Table 9.3). The prevailing concept is that the antibody binds to its cognate epitope, often a growth factor or cell surface receptor, to prevent ligand binding, protein dimerization or signaling from occurring. In CRC, biological therapies have begun to garner attention, and can generally be classified into one of three categories: 1) those that impact mitogenic signaling through the epidermal growth factor receptor (EGFR) (e.g., cetuximab and panitumumab), 2) those that bind vascular endothelial growth factor (VEGF) to prevent angiogenesis (e.g., bevacizumab and ziv-aflibercept), and 3) those that inhibit a large number of kinases and presumably affect numerous signaling cascades, including mitogenic, anti-apoptotic and pro-survival pathways (e.g., regorafenib). Many of these biological agents are now used in combination with 5-FU (or related) approaches and are beginning to show some efficacy [80–83].

Evolving Synthetic Genetic Approaches for Targeting Advanced Stage Colorectal Cancers

Many current chemical and biological therapeutic regimens involve the system-wide administration of an agent, whose requisite activity affects all dividing cells. These agents generally function by inhibiting various biological processes required for cell cycle progression and/or preventing oncogenic signaling pathways from functioning. As a result, all dividing cells, whether cancerous or not, are targeted, which is often associated with a diverse array of side effects. Accordingly, novel therapeutic strategies are required that minimize side effects through the more selective targeting of genetic and/or epigenetic factors that underlie tumor initiation, progression and development.

Over the past decade, there has been a fundamental shift away from the traditional pantropic approaches detailed above to more personalized approaches that target specific aberrant events driving tumor formation. Somatic mutations in genes that encode tumor suppressors, or oncogenes, whose disruption underlies genome instability (e.g., MSI, CIN and CIMP) are now widely recognized as significant genetic predispositions to tumorigenesis [10–12]. Typically, tumor suppressor genes (including DNA repair genes) are epigenetically silenced, deleted or accumulate non-synonymous mutations that adversely impact protein expression and/or function, while oncogenes are amplified or accumulate mutations that enhance the activity of the encoded protein (i.e., constitutive activity).

Synthetic genetic approaches can be subdivided into two categories: 1) synthetic lethal approaches, which exploit the loss-of-function associated with tumor suppressor genes (and DNA repair genes); and, 2) synthetic dosage lethal approaches, which exploit the gain-of-function associated with oncogenes. While these strategies are in their infancy, and many examples are in a pre-clinical development phase, a select few are already in various phases of clinical trials. Below are descriptions of the principles, concepts and examples of synthetic lethality and synthetic dosage lethality, particularly as they pertain to advanced stage CRC.

a. Synthetic Lethality—Introduction and Concept The term synthetic lethality was first coined by Dobzhansky in 1946, and describes the lethal genetic interaction observed when two independently viable homologous chromosomes were allowed to recombine in *Drosophila pseudoobscura* [84]. Synthetic lethality now simply defines the lethal combination of two independently viable gene mutations or deletions (Fig. 9.1). In essence, it describes a genetic interaction in which the outcome of a particular mutation or deletion is influenced by the presence of a pre-existing genetic predisposition or perturbation. Conceptually, synthetic lethal interactions occur via three basic mechanisms (Fig. 9.2): (1) partial ablation of two proteins contained within the same essential biological pathway (i.e., epistasis group) such that the pathway is non-functional; 2) ablation of two proteins contained within independent survival pathways required for viability; or, (3) ablation of two proteins contained within parallel pathways, which together impinge on an essential biological process.

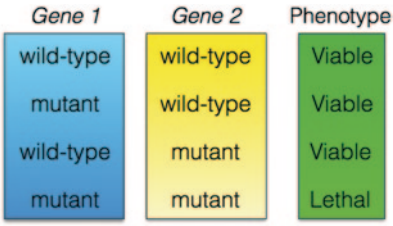

Fig. 9.1 The paradigm of synthetic lethality. Synthetic lethality defines a rare and lethal genetic interaction occurring between two genes. Mutually exclusive mutations occurring within either *Gene 1* or *Gene 2* are viable. A synthetic lethal interaction is defined if the simultaneous combination of *Gene 1* and *Gene 2* mutations results in death.

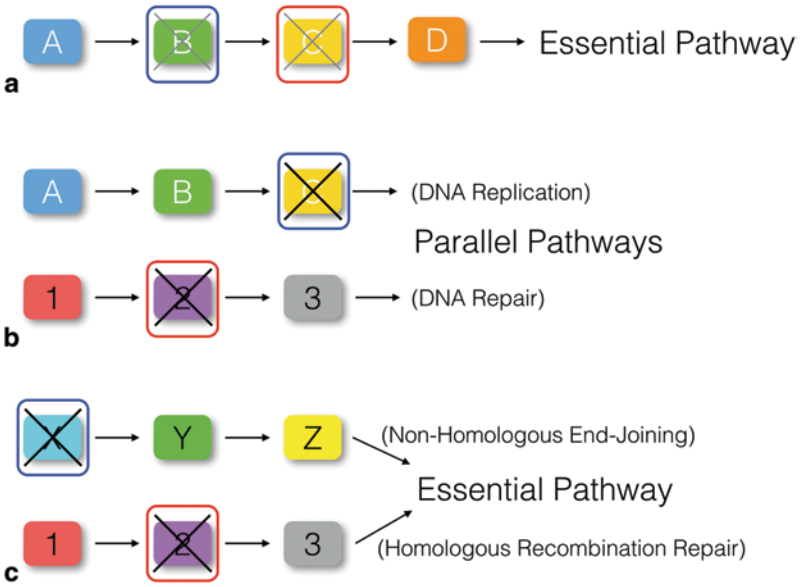

Fig. 9.2 Conceptual models of pathways underlying synthetic lethal interactions. Conceptual models depicting the mechanism(s) of synthetic lethal interactions: numbered/lettered rectangles represent gene products, somatically acquired (i.e., cancer) mutations are identified by red bounding boxes, while synthetic lethal interactors (i.e., drug targets) are identified with blue bounding boxes. **a** Partial ablation (*grey crosses*) of two independent functions encoded within a single essential pathway (i.e., epistasis group), such that the pathway is no longer functional. **b** Ablation (*black crosses*) of two functions encoded within two distinct parallel pathways. For example, a DNA replication defect would lead to DNA errors requiring repair, and inadequate repair will lead to death. **c** Ablation (*black crosses*) of two functions encoded within two separate pathways that together impinge on a single essential process. For example, DNA double strand breaks can only be repaired through two pathways, namely homologous recombination repair and non-homologous end joining—defects in both lead to cellular cytotoxicity.

Synthetic lethal studies are performed in a variety of genetically tractable model systems, including worms, flies and yeast (Fig. 9.3). Most recent approaches have employed deletion mutant arrays of budding yeast strains (collections of ~4700 non-essential gene deletion strains) to systematically interrogate all pair-wise gene combinations and produce comprehensive synthetic lethal interaction network data [85–89]. Over the past 15 years, these efforts have provided critical insights into biological function and pathway architecture, and have helped define molecular complex and epistasis group members [90–94]. However, the potential for these data to predict conserved interactions in human cancer has not yet been fully realized.

In 1997, Hartwell and colleagues [95] posited that cancer cells represent genetically sensitized cells that may be susceptible to drug therapies selectively targeting a second unlinked gene product (Fig. 9.4a). They suggested that synthetic lethal partners identified in model organisms could be used to identify conserved candidate interactions in a human cancer context (Fig. 9.4b). In support of this hypothesis, Dixon et al. [96] recently demonstrated a significant overlap between

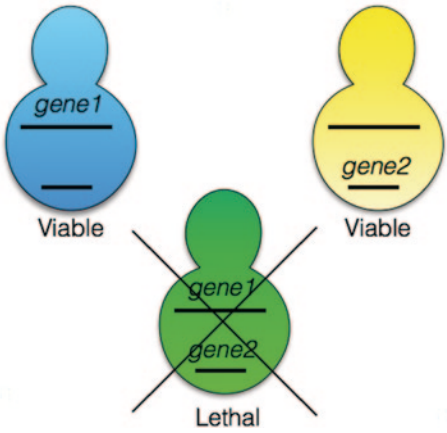

Fig. 9.3 Synthetic lethality in budding yeast. A schematic diagram depicting two independently viable yeast stains that are mutant for *gene1* and *gene2*. However, if these two genes are synthetic lethal interactors, the presence of both mutations within a single organism will produce a lethal phenotype. If slowed growth occurs, a synthetic sickness or synthetic growth defect is observed.

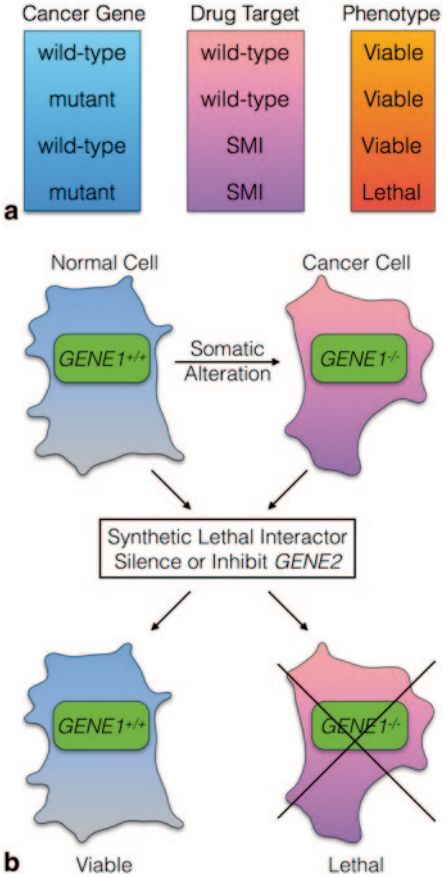

Fig. 9.4 Exploiting synthetic lethality for therapeutic benefit in a cancer context. **a** A conceptual model depicting the synthetic genetic therapeutic strategy in cancer. A specific cancer-associated mutation in a tumor suppressor gene by inhibiting the activity of a synthetic lethal interactor through the use of a small molecule inhibitor (*SMI*). **b** A normal cell is converted into a cancerous cell through the acquisition of mutations, some of which occur within defined tumor suppressor or DNA repair genes (*GENE1*). The hypomorphic function encoded by (epi-)genetic defects in *GENE1* can be therapeutically exploited by identifying and targeting a synthetic lethal interactor (*GENE2*).

synthetic lethal interaction data sets for two distantly related eukaryotes, while McManus et al. [97] provided the first empirical evidence of a conserved synthetic lethal interaction occurring in humans that was first identified in yeast (described below). Because many essential biological processes required to maintain genome integrity are evolutionarily conserved, synthetic lethal networks identified within model organisms may represent major untapped repositories.

a.i. Synthetic Lethality in Practice Perhaps the best studied and prototypic example of a human synthetic lethal interaction is that observed between *BRCA1/BRCA2* and *PARP1* (*poly ADP-ribose polymerase 1*). *BRCA1* and *BRCA2* are breast and ovarian cancer susceptibility genes that are also mutated in a large number of additional tumor types [98–102]. BRCA1 and BRCA2 function in the homologous recombination repair pathway, which is also referred to as the "error-free" DNA double-strand break repair pathway (reviewed in [103]). PARP1 functions in single-strand DNA break repair and, although it was traditionally believed to function in base excision repair [104, 105], it may participate in single-strand repair independent of base excision repair [106]. Nevertheless, PARP1 generally functions to detect DNA single-strand breaks and elicit a response through the ADP-ribosylation at the site of damage (reviewed in [107]).

In 2005, two independent research teams reasoned that PARP1 inhibition would lead to DNA single-strand breaks that would be converted into double-strand breaks during replication [108, 109]. They further posited that cellular cytotoxicity would ensue if the homologous recombination repair pathway was compromised by defects in *BRCA1* or *BRCA2*. As predicted, relative to controls, PARP1 silencing and inhibition enhanced killing within the *BRCA1*-and *BRCA2*-deficient cells, which was further substantiated in embryonic stem cells [109] and animal models [108], thus confirming PARP1 as a novel candidate drug target.

Since the initial characterization of a synthetic lethal interaction between *BRCA1*/*BRCA2* and *PARP1*, a number of small molecule inhibitor screens have been performed (see [3]). The efficacy of many PARP inhibitors (e.g., veliparib, CEP-9722, rucaparib, E7016, BMN-673, olaparib, etc.) are now being evaluated as either a mono- or combination therapy [110]. Olaparib (KuDOS Pharmaceuticals; KU-0059436 or AstraZeneca; AZD-2281) is perhaps the best studied amongst these inhibitors, and AstraZeneca recently initiated a Phase III clinical trial for ovarian cancer patients with *BRCA* mutations. Although many of the studies focus on hereditary breast and ovarian cancers, PARP inhibitors have clinical relevance in many tumor types. In CRC, for example, *BRCA1* and *BRCA2* are somatically altered in ~2.8 and ~6.6% of sporadic cases [99], respectively, which potentially represents ~3250 and ~7675 newly diagnosed Americans annually. Thus, the clinical potential of olaparib (and other PARP inhibitors) in managing cancers with *BRCA1* and *BRCA2* defects has broad-spectrum appeal.

As indicated above, McManus et al. [97] were the first to utilize synthetic lethal datasets from budding yeast to identify novel drug targets in human CRC. In 2009, they employed a cross-species approach to identify a conserved synthetic lethal interaction in a CRC context. Using reverse genetics, biochemistry and microscopy, they showed that diminished FEN1 (flap endonuclease 1) expression induced

cellular cytotoxicity specifically within *RAD54B*-deficient CRC cells, but not in isogenic controls, which identified FEN1 as a novel candidate drug target. *RAD54B* encodes an evolutionarily conserved helicase that functions in the homologous recombination repair pathway, and it is somatically altered in ~8.2% of CRCs (~9500 newly diagnosed Americans annually) [99]. FEN1 is an evolutionarily conserved flap endonuclease that functions in the removal of 5' overhangs during DNA repair, and in the processing of Okazaki fragments on the lagging strand during DNA replication. Subsequent work by van Pel et al. [111] expanded the synthetic lethal network of FEN1 to include additional genes that are somatically mutated in CRC, including *CDC4*, *MRE11A*, *SMC1A*, *SMC3* and *RNF20* [50]. The authors also performed a screen of 30,000 compounds and identified 13 novel FEN1 inhibitors with *in vitro* activity [111]. Of these, three were validated using cell-based assays and are now lead candidate compounds requiring additional pre-clinical testing and optimization prior to entering clinical trials.

More recently, the initial cross species candidate gene approaches have been expanded upon and additional synthetic lethal interactors (i.e., drug targets) have begun to be identified. For example, Sajesh et al. [112] employed hierarchical clustering on 692 yeast genes and identified the top 500 genetic interactions. Based on yeast datasets, several data-rich regions were identified including one that included all three evolutionarily conserved members of a superoxide radical (i.e., reactive oxygen species) detoxification pathway. *SOD1*, *CCS* and *PRDX2* encode proteins required to remove excess superoxide radicals through a two-step process. First, SOD1 dismutates superoxide radicals into hydrogen peroxide, an enzymatic reaction that requires the copper chaperone of SOD1 (CCS) to provide Cu^{2+} [113]. Second, PRDX2 reduces hydrogen peroxide into water and oxygen. The authors reasoned that SOD1 silencing or inhibition would cause the accumulation of excessive superoxide radicals that would produce DNA double-strand breaks. In cells with defective homologous recombination repair (e.g., *RAD54B*-deficient CRC cells), this damage would not be adequately repaired and death would result. In agreement with this hypothesis, the authors showed that *RAD54B*-deficient cells were hypersensitive to SOD1 silencing or inhibition, and thus confirmed SOD1 as a novel candidate therapeutic target.

Beyond the cross-species approaches detailed above, large-scale unbiased approaches have also been conducted to undercover novel candidate drug targets (i.e., synthetic lethal interactors) for many additional tumor suppressor genes. *TP53* is somatically altered in ~50% of CRC [114], and encodes a protein that normally functions to preserve genome integrity by inducing cell cycle arrests or apoptosis in the presence of DNA damage [115]. From a clinical perspective, the loss of P53 function is associated with poor prognosis and increased resistance to chemotherapeutic treatments [116–118].

Xie et al. [119] recently performed a genome-wide screen for synthetic lethal interactors of *TP53* in CRC cells. They identified 103 putative candidates of which two, *ATR* (Ataxia Telangiectasia and Rad3 related) and *ETV1* (ETS Translocation Variant 1), were confirmed as synthetic lethal interactors of *TP53*. While ATR is a serine/threonine kinase that functions in the DNA damage response [120], ETV1

is a transcription factor that regulates numerous biological processes including cell growth, proliferation and angiogenesis [121]. Using reverse genetics, cell-based and xenograft models, the authors showed that cellular proliferation was dramatically impeded within the *TP53*-deficient cells relative to controls [119]. Thus, both ATR and ETV1 were identified as novel candidate drug targets in *TP53*-deficient CRC cells.

Many of the synthetic lethal interactors detailed above warrant additional pre-clinical study and lead chemicals must be further optimized for *in vivo* efficacy and delivery using relevant animal models. Nevertheless, the identification of synthetic lethal interactors in CRC represents a critical first step towards migrating maximal numbers of candidate drugs into pre-clinical studies and clinical trials for advanced stage CRC.

b. Synthetic Dosage Lethality—Concept and Approach Oncogenic transformation provides a key growth advantage that drives tumor development. However, it may also represent an Achilles' heel that can be selectively targeted through synthetic dosage lethality. In 1996, Kroll and colleagues [122] developed a variation of the traditional yeast synthetic lethal screen in which they demonstrated that increased protein expression and/or activity caused lethality in a genetically sensitized, mutant yeast strain. The concept, termed synthetic dosage lethality, was based on previous observations in which lethality was observed following enhanced gene expression/function in specific mutant yeast strains. More specifically, *mcm3* (mini-chromosome maintenance 3) overexpression enhanced defects observed in *mcm2* mutants [123], while *orc6* (origin of replication complex 6) overexpression lowered the non-permissive temperature associated with cdc46–1 mutants [124]. Thus, synthetic dosage lethality, similar to synthetic lethality, may hold therapeutic potential in human cancers (Fig. 9.5).

Many human oncogenes (e.g., *KRAS*, *B-RAF*, *EGFR*, etc.) are either amplified or mutated in such a manner as to produce a constitutively active oncoprotein. This not only results in hypermorphic expression/function, but also serves to differentiate tumor cells from cells in normal surrounding tissues. Therefore, the genetic and epigenetic insults underlying the gain-of-functions associated with oncogenes may also be therapeutically exploited through the identification of a synthetic dosage lethal interactor. Although strategies employed to identify synthetic dosage lethal interactors are detailed elsewhere [125], what follows are brief examples of synthetic dosage lethal interactors identified for *KRAS,* an oncogene altered in CRC.

b.i. Synthetic Dosage Lethality in Practice KRAS (Kirsten Rat Sarcoma Viral Oncogene Homolog) is a proto-oncogene that encodes a membrane-associated guanosine triphosphate/diphosphate binding protein that is widely expressed in human cells. KRAS normally functions in intracellular signaling cascades, particularly through EGFR-signaling activation to regulate cell division, differentiation and apoptosis. Mutant *KRAS* is correlated with oncogenic transformation in CRC, and is observed in 35–45% of all cases (reviewed in [126, 127]). Approximately 95% of the *KRAS* mutations involve single bases within codon 12 or 13, which underlies single amino acid substitutions (e.g., G12D, G12V or G13D) [128]. These

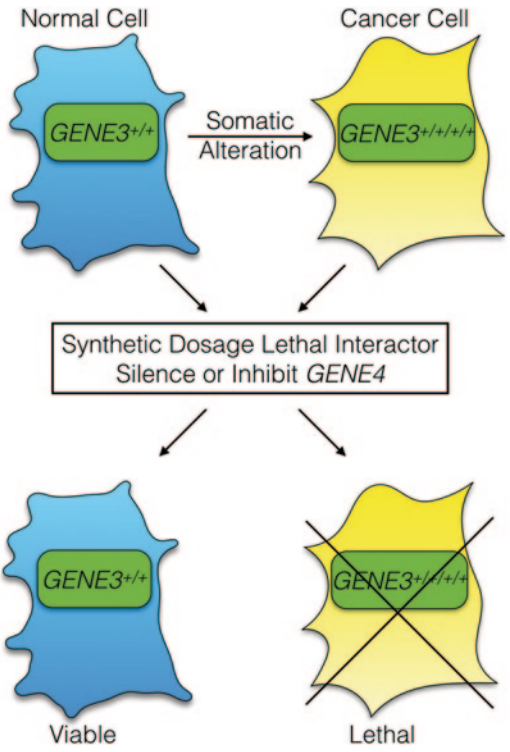

Fig. 9.5 Targeting oncogenes through synthetic dosage lethality. A normal cell is converted into a cancerous cell through the acquisition of mutations, some of which occur within a defined proto-oncogene (*GENE3*). Some oncogenes will arise through gene amplification or mutations (e.g., constitutively active) that confer hypermorphic function. The hypermorphic function encoded by (epi-)genetic defects in *GENE3* can be therapeutically exploited by identifying and targeting a synthetic dosage lethal interactor (*GENE4*).

substitutions render KRAS in a constitutively active GTP-bound state that produces a constant proliferation signal [129]. Because of its strong association with CRC, synthetic dosage lethal targets that can exploit oncogenic *KRAS* mutations are of extreme clinical interest.

In 2009, several large-scale screens identified novel candidate drug targets (i.e., synthetic dosage lethal interactors) that specifically exploited hypermorphic expression and/or function associated with oncogenic *KRAS* mutations [130–132]. Scholl et al. [132] performed high-throughput RNAi-based screens in a panel of human cancer cell lines harboring either wild type or mutant *KRAS*, and identified STK33 as an interactor. STK33 is a serine/threonine kinase that functions in cytoskeletal regulation through the phosphorylation of vimentin [133]. The authors showed that STK33 expression is essential for the survival and proliferation of various cell lines harboring *KRAS* mutations, but is dispensable in cell lines with wild-type *KRAS* [132]. Accordingly, they suggested that STK33 inhibition might be a strategy for therapeutic intervention in a broad spectrum of cancers. However, more recent studies have shown that STK33 expression is dispensable in certain cellular contexts with oncogenic *KRAS* mutations [134].

Luo et al. [131] performed a genome-wide RNAi-based screen, and uncovered multiple synthetic dosage lethal interactors of *KRAS*. Key amongst these genes were those that encoded mitotic-specific functions, including *CCNA2* (cyclin A2),

CDCA8 (borealin), *CASC5* (KNL-1), *KIF2C* (MCAK), components of the anaphase promoting complex (*ANAPC1*, *ANAPC4*, *CDC16* and *CDC27*), *SMC4* and *PLK1*. The large number of genes with established roles in mitosis led the authors to speculate that cells harboring *KRAS* mutations may experience heightened mitotic stress, and may exhibit hypersensitivity towards chemicals affecting mitosis. Indeed PLK1 silencing and inhibition (BI-2536) were both associated with increased sensitivity in *KRAS* mutant cell lines relative to isogenic controls, and was further validated in xenograft tumor models. These data suggest that PLK1, and perhaps many other mitotic proteins, may be candidate therapeutic targets. However, the clinical value of targeting PLK1 is currently unclear as PLK1 is an essential mitotic kinase under normal conditions [135, 136]. Thus, the use of PLK1 inhibitors in a treatment setting may be associated with adverse side effects. Nevertheless, a large number of additional candidates were identified that may hold therapeutic potential in *KRAS* mutant CRC contexts.

Sun et al. [137] employed a kinome-centered screen to identify candidates that could synergize with MEK inhibitors to selectively target cells harboring *KRAS* mutations. *KRAS* mutations reduce the sensitivity of cells to MEK inhibitors [138], and thus identifying conditions that re-sensitize tumors to MEK inhibition are highly desired. In this study, the authors showed that the mechanism accounting for the lack of MEK sensitivity was dependent upon the upregulation of ERBB3, a member of the EGFR family of tyrosine kinase receptors. Armed with the knowledge that ERBB3 heterodimerizes and forms active kinase complexes with other members of the ERBB family (e.g., ERBB1 [EGRF] or ERBB2), they showed that dual ERBB1/ERBB2 inhibitors (afatinib and dacomitinib) synergized with MEK inhibitors (selumetinib or trametinib) and resulted in decreased growth in cell-based CRC models. Subsequent *in vivo* xenograft models validated the combination therapy at least over the ~4-week study period. Thus, the authors conclude that *KRAS* mutated tumors may be resensitized to MEK inhibitors through the co-administration of ERBB1/ERBB2 inhibitors, although this remains to be tested clinically.

In addition to the above examples, a large number of studies have identified conditions (i.e., targets and chemicals) that can exploit oncogenic *KRAS* mutations [130, 134, 137, 139–142]. It is interesting to note that very few targets identified in any individual study are shared between studies. This may be due to the inherent differences in the experimental systems employed (i.e., RNAi-based libraries, experimental conditions, specific assays, etc.), the levels of sensitivity required to identify a positive candidate, or the genetic heterogeneity of the cellular contexts employed. Nevertheless, these studies have identified a myriad of synthetic dosage lethal interactors of *KRAS,* which will ensure that maximal targets are evaluated in the hopes of identifying as many new clinically-relevant targets and compounds as possible.

Conclusions—Evolution of Therapeutic Strategies at the Dawn of the Personalized Medicine Era

Novel therapeutic strategies and drug targets are needed to not only decrease morbidity and mortality rates associated with CRC, but to better target the tumor cells so as to minimize side effects within healthy cells and tissues. Synthetic genetic strategies potentially represent significant advancements over traditional pantropic approaches for targeting advanced stage CRCs. Synthetic lethality and synthetic dosage lethality are designed to specifically kill cancer cells based on the loss-of tumor suppressor function or the gain-of oncogene function. Both approaches have been applied extensively in cell-based screens and numerous candidate drug targets have been identified. In either case, the penultimate goal of the synthetic genetic screens is to identify candidates for which small molecule inhibitors will ultimately be developed (see [125]). Once a target is identified, an appropriate chemical screen can be devised to identify lead chemical compounds for subsequent validation, optimization and pre-clinical studies, prior to initiating clinical trials.

In theory, synthetic genetic approaches offer the ability to custom tailor the therapy to the individual and the tumor itself. With the decreasing costs and availability of next generation sequencing, it will become possible to generate sequence-specific data from tumor biopsies or circulating tumor cells that identify an aberrant molecular signature (i.e., aberrant tumor suppressor genes and/or aberrant oncogenes) that may be exploited through synthetic lethal and/or synthetic dosage lethal approaches. As we approach the dawn of the personalized medicine era, it will become possible to obtain critical information about the individual and their tumor(s) to better select a therapeutic strategy. It is conceivable that by identifying the specific defects in tumor suppressor genes and oncogenes, drugs can be identified and employed that exploit those very defects. This therapeutic concept will no longer treat advanced stage disease as a single entity with pantropic agents, but rather will treat each patient as an individual case.

Synthetic genetic approaches also represent significant advancements over traditional chemical and biological strategies as they potentially offer enhanced specificity. In this regard, the drug (i.e., synthetic genetic interactor) specifically exploits defects inherent to the tumor, and often seeks to target the molecular origins associated with genome instability (i.e., MSI, CIN or CIMP). Accordingly, these approaches not only serve to target and restrict the cytotoxic effects within tumor cells, but also decrease or eliminate side effects within healthy cells and tissues. Since metastatic disease often shares ‘ancestral’ aberrant genetic and epigenetic events with the primary tumor (particularly the loss of tumor suppressor functions), a major benefit of this approach is the potential to impact metastatic disease.

It may also become possible to identify appropriate combinatorial therapies that simultaneously exploit a number of the underlying genetic insults that may improve the current 6% 5-year overall survival rates for stage IV cancers [5]. For example, combining FEN1 and SOD1 inhibitors may produce additive or synergistic effects in tumors with *RAD54B*-defects. Alternatively, combinations targeting both syn-

thetic lethal (e.g., PARP1, FEN1, SOD1, etc.) and synthetic dosage lethal (e.g., ERBB2, MEK1, etc.) interactors could be developed that may even include traditional approaches, such as 5-FU (Table 9.3). Although highly speculative, these concepts must be formally evaluated in appropriate pre-clinical models before clinical trials can be initiated.

In summary, tumor heterogeneity and the development of drug resistance is a major concern in combating any tumor. With the advancement of DNA sequencing platforms and decreases in the associated costs, genetic information may direct patient care and treatment. For example, extensive DNA and chromatin immunoprecipitation sequencing (reviewed in [143]) efforts have already begun on CRC cell lines and for a select few patient samples [99], and is providing clinically-relevant insight into the genetic and epigenetic events associated with CRC [144–146]. However, the notion of a personalized medicine approach is in its infancy, as much of the pre-clinical studies are still ongoing and will have to make their way into clinical trials. Nevertheless, a select few clinical studies evaluating the efficiency of synthetic genetic approaches (e.g., PARP inhibitors) are currently underway. Thus, the clinical efficacy of these drugs and others that employ a synthetic genetic paradigm will remain to be determined.

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Chapter 10
Nanomedicine—Nanoparticles in Cancer Imaging and Therapy

Alexandra M. N. Hauser-Kawaguchi and Leonard G. Luyt

Abstract Nanomedicine refers to the application of nanotechnology in medicine, and endeavors to diagnose, treat, and/or monitor disease on a nanoscale. Cancer nanotechnology is a quickly evolving field of interdisciplinary research that involves the biomedical application of nanoparticles, which are nanoscale devices that are able to overcome biological barriers, specifically recognize a single type of cancer cell, and accumulate preferentially in tumors. Medical applications with nanoparticles are growing, as they have the potential to offer novel methods of non-invasive cancer detection, diagnosis, and treatment. Tumor targeting ligands, such as antibodies, peptides, or small molecules, can be attached to nanoparticles for targeting of tumor antigens and vasculatures with high affinity and specificity. In addition, diagnostic agents (i.e. optical, radiolabels, or magnetic) and chemotherapeutic drugs can be integrated into their design for more efficient imaging and treatment of the tumor with fewer side effects. Recent advances in nanomedicine raise exciting possibilities for future nanoparticle applications in personalized cancer therapy.

Keywords Nanomedicine · Nanoparticles · Nanoplatforms · Cancer · Cancer therapy · Targeted delivery · Drug delivery · Molecular imaging · Extravasation · Tumor targeting

Abbreviations

CEST	Chemical exchange saturation transfer
DOTA	1,4,7,10-tetraazacyclododecane-N, N'N'', N'''-tetraacetic acid
DOX	Doxorubicin
EPR	Enhanced permeability and retention
GRP	Gastrin releasing peptide

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HSA	Human serum albumin
ID/g	Injected dose per gram
MRI	Magnetic resonance imaging
MTX	Methotrexate
NIRF	Near infrared fluorescence
NPs	Nanoparticles
P-gp	P-glycoprotein
PEG	Polyethylene glycol
PET	Positron emission tomography
PLGA	D, L-lactide co-glycolide
PTX	Paclitaxel
RES	Reticuloendothelial system
RGD	Arginine-glycine-aspartic acid
SPECT	Single photon emission computed tomography
VAP	Vapreotide
VEGF/R	Vascular endothelial growth factor/receptor

Types of Nanoparticle Platforms

AuNP	Gold NP
CLIO	Cross-linked Iron Oxide NPs
CNT	Carbon nanotube
CPMV	Cowpea mosaic virus
IONPs	Iron oxide NPs
MnMEIO	Manganese-doped magnetism-engineered iron oxide
PAMAM dendrimer	Poly(amidoamine) dendrimer
QD	Quantum Dot
SPIO	Superparamagnetic iron oxide NPs
SWNT	Single-walled carbon nanotube
VNP	Viral Nanoparticle

Introduction

Nanomedicine refers to the application of nanotechnology in medicine, and endeavors to diagnose, treat, and/or monitor disease on a nanoscale. Specifically, cancer nanotechnology is a quickly evolving field of interdisciplinary research that involves elements of biology, chemistry, engineering, and medicine, with the aim of producing novel methods of noninvasive cancer detection, diagnosis, and treatment [1–3]. This is done with nanoparticles (NPs), which are nanoscale devices that are able to overcome biological barriers, specifically recognize a single type of cancer cell, and accumulate preferentially in tumors.

NPs are submicron-sized synthetic particles that range from one to hundreds of nanometers in diameter. They are considered to have great potential for medicinal

applications because they are stable, have large payloads, and have the capacity for multiple, simultaneous applications due to their size and high surface area:volume ratio [4]. In addition, their distinct size—bigger than many biological molecules, such as proteins, receptors, and antibodies, but 100 to 1000 times smaller than human cells—allows them to act uniquely when they are introduced *in vivo* for imaging and therapeutic purposes. The applications of nanoparticles in medicine are numerous: they can be administered by all routes; they can be developed and formulated rapidly; they increase the aqueous solubility of the attached drug; they protect the drug from degradation; they can release the drug at a controlled rate; they improve the bioavailability of the drug; they enable targeted delivery of the drug; and they decrease the adverse side effects of the drug. Indeed, these nanoscale particles are capable of unique interactions with biological molecules both at the surface and inside cells, which offers unlimited possibilities for future applications in cancer therapy.

Contemporary diagnostic classifications do not encompass the diverse heterogeneity of tumors and are incapable of identifying an appropriate method of treatment or projecting a patient's outcome. Current drug-related cancer treatments originated from traditional methods of drug-based disease treatments. However, most current anticancer drugs are nonselective, which results in their accumulation in both cancerous and normal cells. In addition, a tumor is often diagnosed at a later stage of its development, after it has metastasized to other parts of the body. Increasing concern over the toxicity of non-targeted chemotherapeutic drugs, which can cause severe tissue damage when localized in non-diseased tissue, has resulted in an interest in designing NPs capable of recognizing and targeting tumor tissue specifically. In doing so, NPs are now capable of delivering imaging and therapeutic agents to the target of interest—the tumor—and offer the potential for individualized cancer treatment.

This review will provide an overview of the most well studied nanoplatforms for imaging of tumors and the delivery of therapeutic drugs that are currently being employed. The most well studied nanoplatforms include quantum dots (QDs), liposomes, dendrimers, polymeric NPs, iron oxide nanoparticles (IONPs) and their derivatives, gold nanoparticles, and carbon nanotubes. This review will also explore the current challenges and exciting new approaches being utilized in the design of NPs for cancer imaging and therapy.

Nanoparticles with Medical Applications

Nanoparticles are constructed of a wide range of materials, from organic molecules such as liposomes, dendrimers or carbon nanotubes, through to inorganic structures including cadmium, iron oxide and gold. Table 10.1 describes the predominant nanoplatforms currently in use and provides examples of their applicability to nanomedicine.

Table 10.1 Nanoparticle characteristics and medicinal applications

NP platform	Composition and structure	Characteristics	Application	Examples	Ref
Quantum Dot	CdSe, CdTe	Intense intrinsic fluorescence and photostability	Imaging: optical fluorescence	1. PEG-conjugated QDs	[26]
		Toxicity concerns with material of construction		2. Antibody-conjugated QDs	
Liposome	Phospholipid	Amphiphilic, biocompatible	Imaging	1. Doxil (PEGylated liposomal DOX)	[26–30]
	Closed colloidal structure composed of lipid bilayer	Ease of modification	Drug delivery	2. Myocet (Non-PEGylated liposomal DOX)	
		Suppresses tumor growth and inhibits metastasis of lung cancer		3. DauoXome (Liposomal daunorubicin)	
Dendrimer	Various organic monomers	Highly branched for high ligand density	Imaging	1. PAMAM-MTX	[27, 31, 32]
	Hyperbranched synthetic polymer with regular pattern and repeated units	Ease of functionalization	Drug delivery	2. PAMAM-platinate	
		Biodistribution can be tuned			
		Structural and chemical homogeneity			
		Controlled degradation			
Polymeric NP	Linear polymer	Water-soluble	Drug delivery	1. Abraxane (Albumin Taxol)	[33–35]
		Biodegradable		2. Xyotax (PGA-Taxol)	
		Surface modification		3. PK1(HPMA-DOX)	
		Selective targeting of and accumulation in tumor			

Table 10.1 (continued)

NP platform	Composition and structure	Characteristics	Application	Examples	Ref
Iron oxide NP	Iron oxide	Magnetic surface	Imaging: contrast agent for MRI	1. TNT AntiEpCAM (polymer-coated IONP)	[36]
		Inherent MRI contrast agent		2. SPIO/DOX-NP	
		Biocompatible		3. SPIO/PTX-NP	
Gold NP	Gold	Easily conjugated surface	Imaging: contrast agent for MR	1. Aurimmune (CYT-6091)	[26]
		Spectroscopic advantages for sensitive tumor detection	Drug delivery	2. Verigene platform	
		Nontoxic	Radiation sensitizer for therapy		
		Biocompatible			
Carbon Nanotube	Carbon	Water-soluble (if modified)	Imaging	1. CNT-MTX	[27, 37, 38]
	Carbon cylinders made of graphene	Cytotoxic (without chemical modification)	Drug delivery	2. CNT-amphotericin B	
		Multifunctional			

Quantum Dots (QDs)

QDs are inorganic semiconductor nanocrystals that are the most widely studied nanoplatform for optical imaging applications due to their intrinsic fluorescence. They benefit from small size (as small as 2 nm), versatile surface chemistry, high quantum yields, and outstanding optical properties, including high resolution, high sensitivity, and high inherent photostability and brightness [5–7]. In addition, they have continuous absorption spectra that range from UV to near-infrared wavelengths, as well as long fluorescence lifetimes (>10 h), and large effective Stoke shifts [5–7]. Despite the potential for the toxic release of Cd from their core, *in vivo* studies have shown that they do not exhibit acute toxicity when their surfaces are properly coated. Thus, a number of *in vitro* and cell-based applications exist for QDs, including high-resolution cellular imaging, long-term *in vivo* cell tracking observation, diagnostic work, and tumor targeting [5–9].

Liposomes

Liposomes are self-assembling closed vesicles that have a spherical shape, in which natural phospholipid bilayers surround a central aqueous core that houses a therapeutic drug molecule. Liposomes are biocompatible, biodegradable, biologically inert, and weakly immunogenic. Liposomes are disintegrated by electrostatic, hydrophobic, and van der Waals forces, and can undergo opsonization, resulting in their rapid clearance from the body after their surfaces are coated with protein. As a result, they require steric stabilization, which is achieved by surface coatings with inert polymers, such as polyethylene glycol (PEG). The addition of PEG to the surface of liposomes has resulted in "STEALTH" technology, whereby the intact NP has been known to circulate throughout the body for days without clearance. This phenomenon is particularly useful for the delivery of therapeutic drug molecules, especially when combined with the capability of functionalizing liposome surfaces with targeting ligands or antibodies that assist in directing the nanoplatform to the target of interest (i.e. the tumor). Consequently, targeted liposomal vesicles are able to release the drug molecule in high concentrations at the cell membrane.

Dendrimers

Dendrimers are a unique class of polymeric macromolecules. They are treelike structures that are made up of multiple repeating perfectly branched polymeric monomers emerging from a central core. The branching units may be synthesized from the central core and emerge radially outwards (divergent method), or from the periphery inwards towards the core (convergent method). Depending on how the particular dendrimer is synthesized, it is possible to achieve precise control over its molecular shape, dimensions, density, polarity, flexibility, and solubility by

choosing different building/branching units and surface functional groups [10, 11]. In addition, the nanoparticle's chemistry permits that several modifications can take place to incorporate certain imaging agents, targeting ligands, and other components to increase tumor specificity.

Polymeric NPs

Polymeric NPs offer a surface with high potential for modification and functionalization with different targeting ligands. In addition, they are biocompatible, biodegradable, and demonstrate good pharmacokinetic control in the body [12]. Depending on the method of preparation, polymeric NPs can take different shapes, including nanoparticles, nanospheres, or nanocapsules. Nanospheres are spherical structures made up of a matrix-like system, in which the drug can be entrapped, adsorbed at their surface, or encapsulated in the matrix. Nanocapsules, on the other hand, are a vesicular system that is made up of a polymeric shell with a central cavity or core, which houses the drug [13–15]. The core may be made of solids, liquids, or gas, with the environment often being aqueous or oily [13].

Iron Oxide NPs (IONPs)

IONPs are the predominant class of inorganic nanoparticle being used for imaging tumors [16]. They offer an advantage over other nanoplatforms because they can be visualized by magnetic resonance imaging (MRI) due to their magnetic property and are an intrinsic contrast agent. In addition, IONPs can be guided towards the target of interest (i.e. the tumor) by an external magnetic field, or can be heated to provide hyperthermia for cancer therapy [17]. Several types of nanosize iron oxides have been studied, including those constructed of magnetite, Fe_3O_4, and maghemite,γ-Fe_2O_3. Magnetite is a common construction for the nanoplatform for medical applications as its biocompatibility has been well studied and proven [18]. With proper surface coatings, IONPs can be dissolved in solvents, which results in a homogenous suspension, called ferro-fluids [19]. In this state, the magnetic particles can be used for a number of different *in vitro* and *in vivo* applications, as they interact with an external magnetic field and if directed to a specific location, are able to facilitate medical diagnosis by MRI.

Gold NPs (AuNPs)

Gold NPs are another class of commonly employed inorganic metal NPs. The advantages of gold NPs are multifold: the gold core is relatively inert and non-toxic, making them biocompatible [20, 21]; they can be easily synthesized by simple, reliable, and low-cost methods, with core sizes ranging from 1 to 150 nm by changing

simple parameters; their surfaces can be easily functionalized by a number of biomolecules due to the presence of a negative charge; and their photophysical properties allow therapeutic drugs to be released in remote places [22]. Gold NPs are useful for medical applications due to their unique physicochemical properties, which include extremely small size, large surface area-to-mass ratio, intriguing optical properties, and superior surface reactivity. Although gold NPs include various physical dimensions and shapes, including nanospheres, nanorods, nanoshells, and nanocages, the most common type is the gold nanosphere, which has a bright red colour in aqueous solution [23]. In this review, the term "gold nanoparticle" will be used as a general term to describe the collection of gold nanoplatforms, unless a specific subtype is given.

Carbon Nanotubes

Carbon nanotubes are carbon cylinders that are made of graphene. They can be single or multi-walled, and have a hollow cage-like architecture [24]. Carbon nanotubes are completely insoluble in all solvents, resulting in toxicity concerns; however, when they are chemically modified, they can become water-soluble for introduction into the body [25]. Atoms may be trapped inside the cylinder, while their surfaces may be functionalized with a number of different active molecules, including peptides, antibodies, and therapeutic drugs [25]. In addition, carbon nanotubes are able to carry multiple covalent functionalizations on their sidewall and on the tips of the cylinders, giving them the advantage of being able to carry several molecules at one time.

Nanoparticles as a Platform for Nanocarrier Design

Nanoparticles benefit from a combination of different physical and chemical properties that facilitate their use with biological systems for real-time molecular imaging, intracellular uptake, and drug release. Specifically, their compact size and versatile surface modification strategies enable them to be a powerful platform for nanocarrier design.

Nanoscale Dimensions

NPs are synthetic structures on the nanometer scale. However, the different NPs used in nanomedicine vary in size, shape, and functionality. One of the most studied and prototypic NP is the QD, which consist of hundreds to a few thousand atoms, and possess a very small core size of only 2–10 nm in diameter, and are therefore,

one of the smallest platforms for NP-based drug delivery vehicle engineering [39]. Particles of this size offer advantages because they can be non-intrusively incorporated within larger drug delivery vehicles as tracers for imaging and monitoring intracellular trafficking and biodistribution, and they can be released from larger carriers, providing insight into the redistribution and eventual clearance of a drug or other NP component [39].

In practice, however, most nanocarriers are of a larger size, as they can better accommodate a wider range of materials, can provide more space for drug loading, and can integrate additional functionalities. For example, liposomes and dendrimers are popular drug delivery vehicles, and are "soft" and flexible NPs, that are able to penetrate biological membranes due to their flexibility [40]. Liposomes are made up of a lipid bilayer surrounding a water core hosting a drug, and they range in size from a minimal diameter of 30 nm to several microns [40]. Their advantage lies in their versatility: they have the capacity to carry diverse cargo; have proven to be stable in blood circulation; and they have on-demand drug release in response to intracellular or external stimuli [41–43]. Dendrimers, on the other hand, are the main polymeric architectures that are used in nanomedicine [40]. They are a unique class of repeatedly branched polymeric molecules with a nearly perfect 3-dimesional geometric pattern. Dendrimers range in size, and can be as small as 1.9 nm for a first generation dendrimer and 4.4 nm for a fourth generation dendrimer [44], where the generation number refers to the number of repeating branching units that are added during its synthesis. In addition, their chemistry permits several modifications to incorporate certain imaging agents, targeting ligands, and other components to increase tumor specificity.

Versatile Surface Chemistry

A number of different modifications to the surface of NPs facilitate specific targeting, such as to a tumor. These modifications complement the core of the NP and create a highly amenable platform for nanocarrier design.

Polyethylene Glycol

PEG, a coiled polymer that is made up of repeating ethylene ether units, is typically added to the surface of any NP that is injected intravenously for tumor-targeting, as PEG assists in extending the circulation time of NPs *in vivo*. To increase steric stability *in vitro* (in buffers, for storage), and *in vivo* (longevity in biological circulation), the FDA-approved protective layer can be formed on the outer surface of NPs made from the hydrophilic PEG polymer layer, as solubility in buffer or serum increases due to the ethylene glycol subunits [45]. In addition, PEG prevents NP uptake by macrophages and the reticuloendothelial system (RES), and inhibits

their interaction with plasma proteins by reducing charge-based interactions that are typical of proteins or other small molecules [41, 45–47]. PEG is often the material of choice because it demonstrates increased hydrophilicity and flexibility [48]. The size and density of the PEG layer greatly influences NP circulation time and accumulation in tumors [40,45].

NP type is the most important consideration for stability and circulation time *in vivo*, and it is most affected by factors such as size, composition, and charge of the nanoplatform [45]. For example, NPs with positive surface charges and diameters >100 nm are rapidly cleared from the body despite careful strategies in PEG modification. In addition, PEG modification strategies vary between different NP types; PEG types suitable for liposomes cannot be successfully used for solid, metal-based NPs, as liposomes mimic naturally-occurring entities that circulate throughout the body better than other NP types, and therefore, require less strict PEGylation procedures [45]. As a result, liposomes are the most widely studied PEG-modified NP platform to date [45]. PEG-lipid conjugates can be incorporated into the lipid film of liposomes by several methods: during hydration of the liposome, PEG polymers can be incorporated directly into its lipid film; *post-conjugation*, which involves functionalized PEG being covalently attached to the pre-formed NP; and *post-insertion*, which requires that the pre-formed liposome be incubated with PEG-lipid conjugates in aqueous solution, resulting in micellar formation of the PEG-lipids due to the amphiphilic nature of PEG, and their subsequent insertion into the liposomes [49, 50].

Inorganic NPs, on the other hand, are subject to different methods of coating the NP surface with the layers necessary for colloidal stability. The NPs are frequently co-precipitated with a multitude of polymers or cross-linked polymers, which enhances the NPs monodispersity [51]. IONPs have been successfully coated with dextran, albumin, and PEG (MW 5000) for improved biocompatibility, which resulted in an improved $t_{1/2}$ by up to 200 min [51–53]. Gold NPs, on the other hand, are better suited for PEG grafting; thiol-PEG, bifunctional PEG, and sulfydrylated PEG have all been used in coating the NP surface for improving colloidal stability and biocompatibility [54]. In addition, PEG bidentate ligands (PEG-thioctic acid and PEG-dihyrolipoic acid) were recently shown to significantly improve the stability in biological media of both QDs and gold NPs [55]. Unfortunately, the effects of the size of PEG and the density of a layer are still unknown for inorganic NPs, particularly with respect to NP kinetics and accumulation in tumors, as inorganic NPs are significantly smaller than lipid-based NPs.

The efficiency of a PEG layer in improving steric stability of the NP depends on the arrangement of the individual PEG polymers on the NP surface [56–59]. PEG molecules are made up of one end that attaches to the NP surface (designated R1), while the other distal terminal end interacts with the solvent environment (designated R2). A number of ethylene glycol residues complete the space between R1 and R2 to create PEG of varying lengths. Poor PEG organization can impede NP interaction and uptake by tumor cells. While a denser PEG layer is achieved by incorporating a greater number of PEG-lipids, NPs bear a maximum molar percentage of PEG-lipids that can be incorporated into their lipid layer before their effects become unfavorable. For example, liposomal membranes are held together

by non-covalent bonds, which can cause the PEG-lipid conjugates to become dissociated from the lipid layer when PEG densities are not ideal [40]. Similarly, PEG conformation is important for it to carry out its proper function of increasing the NPs retention time by preventing its interaction with plasma proteins. A *brush*-like conformation, which occurs when the distance between the individual PEG-lipids, D, is less than the volume that each flexible PEG polymer cloud occupies, more commonly referred to as the Flory dimension, Rf, ($D < Rf$) is superior to the *mushroom* configuration, which occurs when the opposite is true ($D > Rf$) [60–63]. Therefore, increasing the concentration of PEG-lipids increases the PEG density, which subsequently decreases the distance, D, between each PEG molecule, and ultimately results in the PEG chains adopting the undesired mushroom shape. The brush configuration, which is a more linear conformation than the mushroom configuration, and therefore, allows for denser polymer coatings, is preferred because it produces greater protein repulsion and results in a longer circulation time [64].

Targeted Ligands

A targeting moiety can be added onto the surface of a NP to enable the selective recognition of the target of interest, such as a tumor cell. Without a targeting ligand on its surface, NPs are only able to recognize and interact with cell membranes non-specifically; such interactions can be insignificant when the surface of the NP is covered with PEG.

Cancer cells exhibit many of the same characteristics as healthy cells, which poses as a major difficulty in targeting these unhealthy cells with specificity. Thus, ligands are designed to target receptors that may be highly over-expressed on tumor cells, but that are often less prominently expressed on normal cells. The targeting layer, which is the most peripheral component of the NP, interacts primarily with cell membranes, and therefore, must exhibit the right conformation and a high affinity for its specific target in order to interact and possibly be internalized by the cell. Consequently, it is important that ligands be conjugated onto NPs in the most favorable way that allows them to preserve their high affinity for their targeting receptors.

Targeting moieties such as antibodies (full and fragmented), peptides, small molecules, and aptamers, have all been proven to facilitate NP targeting of cancer cells. For example, antibodies against receptors known to be over-expressed in tumors, such as the HER-2 receptor, transferrin receptor or epidermal growth factor receptor (EGFR), can be attached to NPs to provide cancer targeting. Similarly, small peptides with receptor specificity can be added onto NPs for targeting. For example, the arginine-glycine-aspartic acid (RGD) peptide, which has a high affinity for $\alpha_v\beta_3$ integrin receptors over-expressed on angiogenic vasculatures, is a common example of a short peptide sequence used to functionalize NP surfaces. Specific examples of these and other targeting approaches will be discussed in the subsequent sections on imaging and therapy.

Successful receptor targeting generally requires that ligands be affixed to the end of the PEG spacer. The purpose of the PEG spacer is to provide distance

between the solid NP platform and the targeting ligand, thereby providing the ligand with space and flexibility to interact with its receptor. In addition, the PEG chain length is an important consideration for effective targeting. A PEG spacer that has a longer chain than the PEG layer already present for improved colloidal stability can cause the ligand attached to its end to become buried within it, as it folds over to form a mushroom-like shape, rather than maintain its brush conformation [65]. As mentioned previously, the brush conformation is the desired configuration for PEG attached to a NP's surface, as it permits the ligand to extend past the NP core, and to better interact with proteins on the cellular membrane. Thus, ligands attached to long-chained PEG spacers are at risk of being less exposed to their target receptors than without the PEG spacer. For this reason, the ligand-conjugated PEG spacer should be of the same length as the non-conjugated free PEG chains.

While the presence of a PEG spacer improves targeting efficiency, the concentration of surface ligands is also important in the design of a targeting probe. A higher concentration of ligands attached to the surface of a NP is intuitively thought to increase the probability of interacting with and targeting receptors on cancer cells due to the positive effects of multivalency. However, the opposite may also be true, as higher concentrations of ligands may crowd out and negate the stabilizing effects of PEG, resulting in poorer NP delivery to the site of interest. NPs functionalized with high concentrations of ligands have been shown to accumulate in the liver and the spleen at faster rates and in higher concentrations than those NPs whose surfaces are conjugated with lower concentrations of ligands [66]. In addition, NPs with higher ligand densities localize in lower concentrations in tumor cells [66]. This indicates that high concentrations of non-PEG structures, such as targeting ligands, on NPs oppose the stabilizing effects of the PEG layer, and permit the unwanted recognition of those NPs by spleen-associated macrophages and plasma proteins.

Nanoparticles for Cancer Imaging

Many imaging modalities may be used to detect metastatic cancer and the use of NPs may improve the sensitivity and specificity for cancer imaging. Various nanoplatforms are available that allow for interaction with cancer biological targets either on the surface of or inside cells. Some NPs exhibit properties that provide them with intrinsic imaging capabilities, while others require surface functionalization in order for them to be used as imaging agents.

Molecular Imaging

Molecular imaging is a non-invasive method of characterizing biological processes and measuring their changes at cellular and molecular levels. In nanomedicine, NPs decorated with targeting ligands, imaging labels, or therapeutic drugs can be monitored in real-time as they move through a biological organism, and therefore,

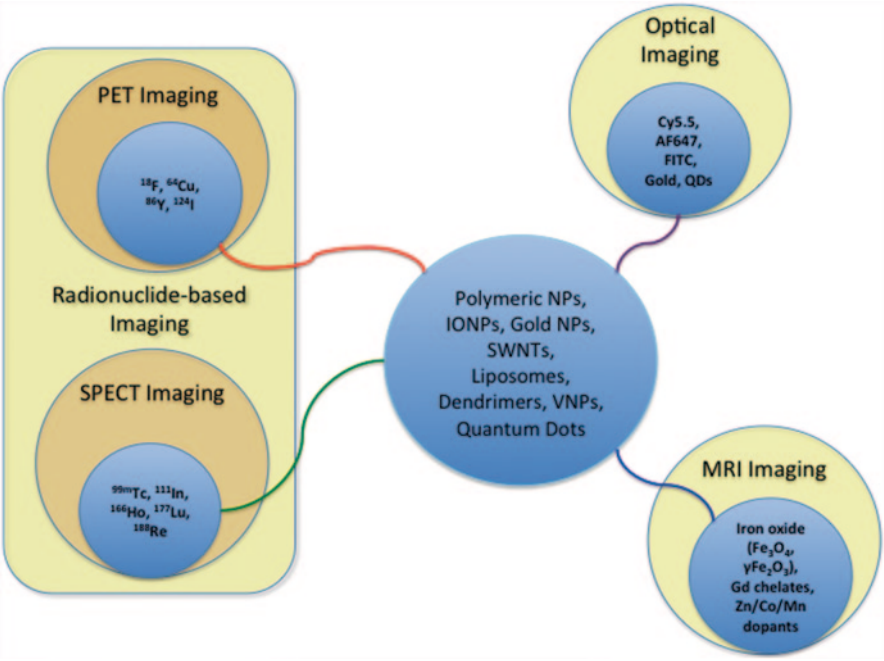

Fig. 10.1 A schematic illustration of the different nanoplatforms used for multimodality imaging. Tools used for imaging are attached to the nanoparticle core through a linking molecule, such as a spacer, peptide linker, or antibody

provide a comprehensive understanding of the delivery, administration, and effects of a drug nanocarrier. A number of different imaging modalities exist for use with NPs, and each of which depends upon different surface modifications (Fig. 10.1).

Optical Imaging

Optical imaging is a technique used to characterize a biological system using non-ionizing radiation and the spectral properties of photons. Most commonly, fluorescence is utilized, whereby excitation light is directed to the target of interest and the emission light is collected at a shifted wavelength, which produces an image. The benefit of using optical imaging is that it reduces patient exposure to harmful radiation, and is therefore, a safer method of imaging tumors. In addition, it produces images faster, and can therefore be applied to lengthy and repeated procedures to monitor the progression of a particular disease. On the other hand, optical imaging is limited by poor tissue penetration, and therefore, the imaging information is surface-weighted [67]. As a result, the imaging modality is generally not quantitative, and is used almost entirely to examine shallow lesions, and subcutaneous or surgically exposed tumors [23, 67].

QDs are the most well studied platform for optical imaging applications. Their natural fluorescence in two spectral windows at near-infrared wavelengths, 700–900 nm 1200–1600 nm [68], enables them to be used as a direct measure of brightness, and therefore, provides them with the potential to be a superior near-infrared fluorescence (NIRF) imaging probe. The use of QDs *in vivo* requires that they reach their specific target of interest without alteration; therefore, their surfaces are often outfitted with a variety of targeting molecules that assist in their delivery. However, specific targeting and imaging with QDs is difficult due to their relatively large size (>20 nm in hydrodynamic diameter) and their short circulation half-life. In one report, the size of QD705-RGD (approximately 20 nm in diameter) prevented efficient extravasation, and thus, mainly targeted tumor vasculature instead of tumor cells [69].

Specific targeting with QDs *in vivo* is possible by functionalizing their surfaces with peptides as targeting ligands, and the peptide-conjugated QDs can subsequently be tracked and imaged [70]. Cai et al. has studied QDs for biomedical applications extensively, and demonstrated the *in vivo* targeted imaging of tumor vasculature using QDs [69]. One of the most common examples of tumor-targeting with QDs is the use of the tripeptide sequence RGD to target integrin $\alpha_v\beta_3$, a cell adhesion molecule that is over-expressed on activated endothelial cells of tumor neovasculature and some tumor cells but that has limited expression on mature endothelial cells [71]. Studies performed by Cai et al. showed that peptides containing RGD that were conjugated to QD705, which has a maximum emission at 705 nm, were able to bind integrin $\alpha_v\beta_3$ specifically with high affinity *in vitro* and *ex vivo*, and that imaging with NIRF was successful [69].

In addition to peptide-conjugated QDs, antibodies can be conjugated to the NPs to create a nanoplatform that targets tumor cells specifically. A study by Yu et al. revealed that an anti-alpha-fetoprotein antibody, a marker for hepatocellular carcinoma cell lines, could be added to QD surfaces and be used to image tumors *in vivo* [72]. Tada et al. used high-resolution intravital confocal fluorescence microscopy to monitor tumor targeting by antibody-conjugated QDs in real-time [73]. The study demonstrated that vascular transport, extravasation, binding to cancer cells, and cellular internalization in a living mouse could be observed at a single NP level [73]. In addition, quantitative determination of nanocarrier transport kinetics was possible, which enabled the rate-determining step of nanoparticle drug delivery to be identified [73].

Radionuclide-Based Imaging

Radionuclide-based imaging, also commonly referred to as nuclear imaging, includes single photon emission computed tomography (SPECT) and positron emission tomography (PET). It uses internal radiation that is introduced through a targeted molecule that is labeled with a radioisotope at doses far below those that may cause pharmacologic effects. Nuclear imaging offers greater sensitivity than other

imaging modalities with no tissue penetration limits [74], and it is quantitative. Greater sensitivity from nuclear imaging is the result of advancements in hardware development and improvements in image-processing algorithms [75]. However, despite its clear advantages, the disadvantage is that both SPECT and PET result in lower resolution images than other modalities. For this reason, radiolabeled NPs are used almost exclusively for studying pharmacokinetic properties of new devices or nanoparticle vehicles [76]. Most radiolabeled NPs are made up of a core, a targeting biomolecule, and the radioisotope. The most commonly employed radioisotopes used with NPs for PET imaging are ^{18}F ($t_{1/2}$=109.8 min), ^{68}Ga ($t_{1/2}$=68.1 min), and ^{64}Cu ($t_{1/2}$=12.7 h), and those used in SPECT imaging are ^{99m}Tc ($t_{1/2}$=6 h), and ^{111}In ($t_{1/2}$=2.8 days).

PET Imaging

^{64}Cu-labeled NPs

To this point, QDs, IONPs, single-walled carbon nanotube (SWNT) and gold NPs, have all been radiolabeled with ^{64}Cu ligands for PET/MRI or PET/NIRF imaging [77]. Combining two different methods of imaging permits the same molecular target to be imaged in order to obtain additional information, such as anatomical and molecular information, which would not normally be achieved by a single modality. Thus, the combination of modalities used to study the same target enhances diagnostic accuracy due to improved sensitivity and greater resolution [77].

QDs have recently been reported for use as a PET/NIRF probe. QDs have been conjugated with the RGD peptide sequence and 1,4,7,10–tetraazacyclododecane-N, N'N'', N'''-tetraacetic acid (DOTA) chelator for integrin $\alpha_v\beta_3$ imaging, as well as with VEGF and DOTA for VEGF imaging [78, 79]. In both cases, a strong correlation between the *in vivo* PET images obtained and the *ex vivo* NIRF images of uptake of both the ^{64}Cu-DOTA-QD-RGD and ^{64}Cu-DOTA-QD-VEGF probes was observed. In addition, it was demonstrated that the ^{64}Cu-DOTA-QD-RGD probe targets the tumor vasculature through RGD-integrin interaction, and with little extravasation [78]. It was concluded that this dual-modality probe is superior to solely using optical imaging, as there was little observed toxicity and as it was able to overcome the tissue penetration limits found in optical imaging.

IONPs have magnetic properties that have allowed them to be extensively employed as direct contrast agents in MRI. A dual-modality PET/MRI probe, ^{64}Cu-DOTA-IONP-RGD (diameter 45 ± 10 nm), was created to image integrin $\alpha_v\beta_3$expression [80]. Each IONP probe was made up of 35 RGD peptides and 30 DOTA chelators [80]. In addition, superparamagnetic iron oxides (SPIO) were functionalized with cRGDfC and 1, 4, 7-triazacyclononane-N, N', N''-triacetic-thiol and labeled with ^{64}Cu, resulting in strong tumor-targeting capability and tumor contrast in U87MG cells when imaged by PET/MRI [81].

The biodistribution of SWNTs functionalized with PEG-DOTA, RGD, and subsequently labeled with ^{64}Cu was studied by Liu et al. (2007) by PET, *ex vivo* biodistribution, and Raman spectroscopy. The results indicated that these SWNT probes were highly stable *in vivo*, and that when efficiently PEGylated, they have a relatively longer circulation half life (~2 h) and are subject to lower uptake by the RES [82]. In addition, it was found that the ^{64}Cu-DOTA-PEG-SWNT-RGD probe was able to target integrin $\alpha_v\beta_3$-positive tumors in mice with high efficiency, resulting in high tumor accumulation (~7% at 1 h) and that there was limited retention in the kidneys and efficient renal clearance [82].

^{18}F-labeled NPs

Fluoride-18 is the most widely used PET isotope and numerous examples of ^{18}F-labelled NPs have been reported. Devaraj et al. synthesized and studied a modified ^{18}F-labeled trimodal IONP (^{18}F-CLIO). The particles were made up of a SPIO core shell that was cross-linked with dextran molecules, and functionalized with ^{18}F in high concentration by means of azide-alkyne cycloaddition click chemistry. *In vivo* characterization of the probe determined that it was suitable for PET, NIRF, and MRI. When applied for *in vivo* PET/MRI imaging of lymph nodes for cancer metastasis detection, the probe was able to identify small lymph nodes as well as the precise anatomical information [83]. Analogously, Jarrett et al. designed a probe using IONPs coated with dextran sulfate that could be labeled with ^{18}F for PET/MRI to target vascular inflammation [84].

Recently, Zhu et al. reported on ^{18}F-labeled gold NPs that were also modified to contain PEG chains and thus have improved water solubility suitable for *in vivo* use [85]. Through the addition of the cyclic peptide octreotate, this NP system has potential for imaging of neuroendocrine metastasis by targeting the somatostatin receptor.

SPECT Imaging

Integrin $\alpha_v\beta_3$, which is a marker of angiogenic vascular tissue, has been targeted and imaged with ^{99m}Tc-labeled RGD peptides conjugated to gold NPs [86]. Specifically, binding assays performed in C6 glioma cancer cells, which overexpress $\alpha_v\beta_3$ receptors, proved that the ^{99m}Tc-AuNP-RGD specifically recognized the receptors [87]. The gastrin-releasing peptide (GRP) receptor, which is overexpressed in prostate cancer and breast cancer, has been targeted with ^{99m}Tc-Lys^3-bombesin [88, 89]. For example, ^{99m}Tc-AuNP-Lys^3-bombesin was shown to have specific recognition for GRP receptors in PC3 cancer cells [90]. Studies with ^{99m}Tc- and ^{111}In-labeled carbon nanotubes [91–94], ^{125}I-labeled silver NPs [95] and ^{99m}Tc-labeled IONPs [96] for SPECT imaging have also been reported.

Molecular MRI

MRI is a non-invasive method of imaging biological organisms by placing the subject in a magnetic field, and is based on the interaction of particular nuclei, such as protons, with one another and with molecules found in the tissue of interest [97]. The image that results from an MRI scan is quite detailed, and enables the different tissues to be visualized and studied with a high degree of accuracy. Each of the different tissues is subject to different relaxation times after the magnetic field has been removed, which can produce endogenous contrast. In addition, exogenous contrast agents, such as gadolinium chelates, can be applied for some procedures in order to further increase the contrast by selectively correcting for the optimal T1 (longitudinal) or T2 (transverse) relaxation time [98, 99]. Recently, compounds using particular NP designs have been applied as novel contrast agents, as they have larger relaxivities, such as paramagnetic gadolinium-containing liposomes/micelles and SPIO nanoparticles [98, 100]. Unlike radionuclide-based imaging, MRI has the advantage of not using radiation to arrive at an image as well as a higher spatial resolution. However, it is less sensitive than other modalities, which can only be partially corrected for by working at higher magnetic field strengths, applying exogenous contrast agents, and/or imaging for a longer period of time. The need for a high concentration of a targeted MRI contrast agent in order to provide meaningful signal, gives the potential for a pharmacological response to the agent and additional care must be taken in the toxicological evaluation of the imaging agent during development.

Non-Targeted MR Contrast Agents

Iron-oxide nanoparticles (IONPs) are one of the most common nanoplatforms used as MR contrast agents. They have been shown to be detectable at such low concentrations that single-particle detection has been proven effective [101, 102]. In addition, non-targeted IONPs are usually used to image the liver [103, 104], the spleen [105, 106], and the lymph node [107, 108] because their small size causes them to be nonspecifically taken up by the RES [109], and to accumulate in lymphatic tissue, which surrounds those organs. Furthermore, IONPs have been found to accumulate in tumors due to the leaky vasculature that arises at tumor sites, and therefore, they have been used to image brain tumors successfully [110]. Another important approach to creating NPs for MRI is to incorporate gadolinium onto the surface of the NP through the addition of a metal chelator. For example, Milne et al. reported a water soluble AuNP functionalized with Gd-DOTA metal complexes and found this nanoplatform to be suitable for preclinical *in vivo* MRI [111]. In addition, chemical exchange saturation transfer (CEST) is believed to be a promising approach for MRI contrast. Endogenous or exogenous compounds containing exchangeable protons or molecules, such as amide, amine, or hydroxyl groups, are selectively saturated and subsequently detected with enhanced sensitivity [112,

113]. For example, Castelli et al. created liposome-based CEST probes, which when stimulated by endogenous (variation in pH) and externally applied (nonfocused ultrasound) stimuli, successfully imaged the selective release of material from the nanoparticle [114].

Molecular MRI of Integrin Expression

The most well studied target of molecular MRI is integrin $\alpha_v\beta_3$ [115–117]. The first study in which integrin $\alpha_v\beta_3$ expression was imaged by MRI used antibody-coated paramagnetic liposomes that contained Gd^{3+} ions [118]. Paramagnetic liposomes coated in LM609, a mouse anti-human integrin $\alpha_v\beta_3$ monoclonal antibody, were targeted to the angiogenic vasculature of squamous cell carcinomas in rabbit [118], resulting in successful imaging by molecular MR.

Integrin $\alpha_v\beta_3$-targeted magnetic NPs have been shown to improve imaging sensitivity and quality by increasing MR signal significantly. For example, when used to image a Vx-2 squamous cell carcinoma model at 1.5T, MR signal improved in the tumor periphery at 2 h post-injection [119]. Interestingly, these NPs were able to enter into the leaky tumor neovasculature, but did not reach the interstitium. In another model, athymic nude mice with human melanoma xenografts were successfully imaged using $\alpha_v\beta_3$ integrin-targeted paramagnetic NPs [120].

Molecular MRI of Other Targets

NP size and magnetic properties are important characteristics that influence MR signal. A comparison was made between two types of NPs, manganese-doped magnetism-engineered iron oxide (MnMEIO) and cross-linked iron oxide (CLIO), both conjugated with trastuzumab, an anti-HER2 monoclonal antibody [121, 122]. Fluorescent-activated cell sorting analysis was performed and demonstrated that both NPs had similar targeting abilities *in vitro*. However, MnMEIO was found to be far superior in imaging small HER-2 positive tumors implanted in mice than CLIO. It is believed that the reasons for CLIO-trastuzumab conjugates resulting in lower MR contrast enhancement when compared to conjugates with MnMEIO are two-fold: CLIO is a larger particle than MnMEIO, and therefore, leads to poorer extravasation from the leaky tumor vasculature, and also it has poorer magnetic properties, which hinders MR contrast significantly, especially at low concentrations [121, 122].

In many studies to date, *ex vivo* analyses have not been performed to determine if the targeted NPs are actually targeting the desired tumor vasculature and/or cells, or if they are accumulating nonspecifically in the interstitial space. Due to the large overall size of the targeted IONPs (>20 nm in diameter), which includes surface polymer coating and targeting ligands, they tend to travel as far as the vasculature. However, with the development of smaller NPs bearing longer circulation times, NPs used as MR contrast agents are showing improved extravasation from the leaky tumor vasculature. In addition, surface conjugation with peptides or small

molecules instead of antibodies may also improve the ability of these NPs to reach their target, due to the possibility of modifying the NP surface with more targeting ligands, while maintaining a smaller overall size. However, the primary issue that arises when imaging with MR is inherent low sensitivity. Therefore, the ideal NP design would be a MR contrast agent that provides improved imaging sensitivity and that has a targeting mechanism for tumor specificity.

Multimodality Imaging

Each individual imaging modality has its strengths, and therefore, is used for specific applications; however, no single modality is perfect or capable of sufficiently gaining all of the necessary information. Therefore, multimodality imaging exists and has been used extensively to study the movement of engineered NPs *in vivo*. A single NP that has been functionalized at its surface can be imaged using different modalities. For example, Xie et al. developed a triple functional IONP probe for use with PET imaging, NIRF imaging, and MRI [123]. In this study, IONPs were modified with dopamine to provide them with moderate polarity, and they were subsequently inserted into human serum albumin (HSA) matrices. The HSA-IONPs were then labeled with Cy5.5 dye and ^{64}Cu DOTA chelates to be used as imaging agents using NIRF and PET, respectively. The inherent magnetic property of IONPs enables them to serve as contrast agents in MRI. Thus, the trimodality probe was developed and its pharmacokinetics were investigated in xenograft U87MG tumors (Fig. 10.2). The combination of multiple imaging modalities within a single nanoplatform offers synergistic advantages over a single modality alone, as it is possible to gain more detailed and accurate information from the resulting image.

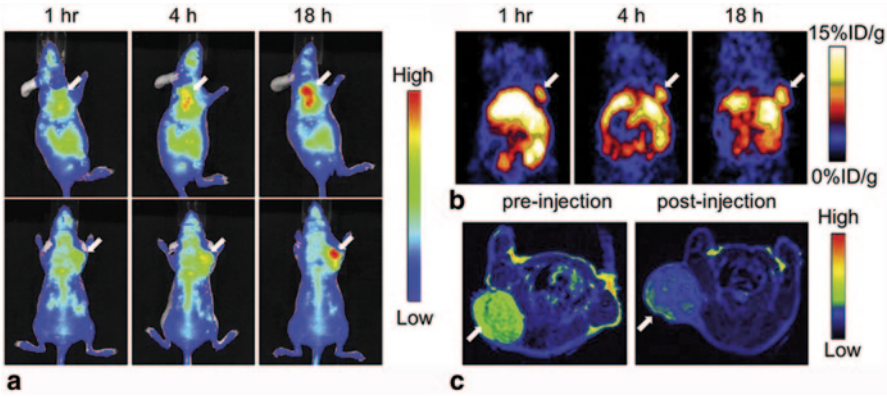

Fig. 10.2 *In vivo* optical-PET-MR trimodality imaging of IONP probe after 1, 4, 18 h post-injection in a mouse model. **a** *in vivo NIR image* of mice. **b** *in vivo PET images* of mice. **c** *in vivo MR images* of mice acquired pre-injection and 18 h post-injection. *White arrows* indicate xenograft U87MG tumors. Reproduced with permission from Xie J, Chen K, Huang J, Lee S, Wang J, Gao J, Li X, Chen X (2010) PET/NIRF/MRI triple functional iron oxide nanoparticles. Biomaterials 31(11):3016–3022. Copyright © 2010, with permission from Elsevier

Targeted Cancer Therapy

Upon administration to a patient, classical anticancer agents are dispersed throughout the whole body and are unable to distinguish between normal cells and tumor cells. Therefore, the unwanted delivery of an anticancer drug to healthy cells can result in significant adverse effects and toxicity. In addition, rapid clearance from the body, enzyme degradation, nonspecific delivery of the drug, and suboptimal accumulation of the drug in the targeted tissue require that the dosage of the drug be increased significantly, which is neither economical nor medically safe, as it leads to further adverse effects and systemic toxicity. Nanomedicine presents an improved approach to administering anticancer therapeutics, as it is able to provide a targeted mechanism for cancer therapeutics and a controlled release of a therapeutic. Those nanoparticles that are capable of drug loading, and that have optical, magnetic, or photothermal properties, provide a strong framework for therapeutic treatment.

Drug Delivery

Currently, a number of different nanoplatforms exist for use as drug delivery vehicles. They each have a different architecture, and are of different sizes, shape, and material. As a result, they have different drug loading capabilities (by encapsulation, surface attachment, or entrapment), drug release rates, targeting abilities, and vary in their *in vivo* stability. The use of nanoparticles as drug delivery systems is highly advantageous. Firstly, their submicron size allows them to penetrate across barriers into small cells, thereby enabling them to arrive and accumulate at the target site. As a result, toxic side effects in nonspecific areas of the body are reduced [124]. In addition, NPs offer a scaffold for targeted delivery of a drug, as its surface can be modified with stabilizing and targeting molecules. Furthermore, NPs are often used because they offer superior drug stabilization in the body, including improved bioavailability, increased aqueous solubility, decreased degradation, and are capable of producing a prolonged release of the drug. This chapter focuses on only a few of the nanoplatforms currently being employed.

Polymer-Based Drug Nanocarriers

Polymer-based drug nanocarriers include polymeric NPs, micelles, and dendrimers. Depending on how the nanoplatform is prepared, the drug can either be physically entrapped inside, or covalently bound to the NP surface, producing structures that are capsules (polymeric NPs), amphiphilic cores (polymeric micelles) or hyperbranched structures (dendrimers) [125].

Polymeric NPs

The use of biodegradable materials in polymeric NP preparations is a strategic means of allowing sustained drug release at the target site for a longer duration. For example, biodegradable NPs prepared from D, L-lactide co-glycolide (PLGA) and polyactide have been studied for drug delivery [15, 126]. Recent studies have been done using NPs formulated with paclitaxel. For example, paclitaxel-loaded PLGA NPs demonstrated greater and sustained anti-proliferative activity in HeLa cells [127]. In this study, PLGA NP-encapsulated paclitaxel was shown to be a promising controlled drug-delivery system after they were found to increase apoptosis of HeLa cells. In addition, the use of naturally occurring polymers, such as albumin, chitosan, and heparin, has been explored as drug conjugates for the delivery of oligonucleotides, DNA, protein, and drugs. For example, NP serum albumin-bound paclitaxel (Abraxane) has been used in the clinic in order to treat metastatic breast cancer, non-small cell lung cancer and metastatic adenocarcinoma of the pancreas [33].

Polymeric Micelles

Polymeric micelles are made up of a hydrophobic core, which houses hydrophobic drugs, and a hydrophilic shell, which stabilizes the structure and enables their interaction with the liquid environment surrounding the micelle structure. As a result, they are water-soluble, and are ideal for the delivery of water insoluble drugs by intravenous injection [128, 129]. Drugs can be loaded by either physical encapsulation [130] or covalent attachment [131]. Polymeric micelles formulations of paclitaxel have recently been studied. PEG-poly(D, L-lactide)-paclitaxel (Genexol-PM) is a cremophor-free micelle-formulated paclitaxel, which has been applied in phase I and pharmacokinetic clinical trials in patients with advanced refractory malignancies [132]. It is believed that multifunctional polymeric micelles that contain targeting ligands as well as imaging and therapeutic agents will become an important model of micellar formulations [133].

Dendrimers

Dendrimers are treelike structures that are made up of multiple perfectly branched polymeric monomers emerging from a central core. Dendrimers bear advantageous properties for drug delivery, including monodisperse size, ease of surface functionality, multivalency, water solubility, and drug-loadable central core [44]. Dendrimers are a multifunctional platform, as their surfaces can be conjugated with several molecules, including imaging agents, targeting ligands, and therapeutic drugs [44]. An early example of dendrimers in drug delivery involved complexing cisplatin (Fig. 10.3a), an antitumor drug, to the surface of a polyamidoamines (PAMAM) dendrimer, which resulted in slower release and higher accumulation in

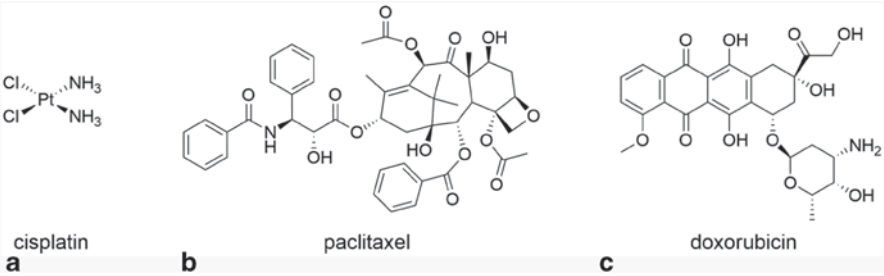

Fig. 10.3 Chemical structures for three chemotherapeutic drugs commonly administered with nanoparticles

solid tumors, and lower toxicity when compared with the free drug [32]. Doxorubicin has been covalently attached to a poly(ethylene oxide)-dendrimer through a hydrazine linkage, allowing for release of the drug in the more acidic environment of lysosomal compartments [134].

Lipid-Based Drug Nanocarriers

Liposomes

Liposomes may be classified by the number of bilayers they have—unilamellar systems have an aqueous core for encapsulation of water-soluble drugs, and multilamellar systems encapsulate lipid-soluble drugs. Liposomal anticancer drugs were the first to gain approval for use in cancer therapy, and are typically applied in the multilamellar system for the transport of lipid-soluble drugs. For example, liposomal formulations of the anthracyclines doxorubicin, which can be either PEGylated (Doxil in the United States and Caelyx outside of the United States) or non-PEGylated (Myocet), and PEGylated liposomal daunorubicin (DaunoXome) are approved for the treatment of metastatic breast cancer [30, 28, 135]. After extravasation, Doxil liposomes disintegrate and doxorubicin is released. It has been reported that the drug accumulation is 10 fold greater in tumor tissue when delivered by a targeted NP than when it is administered on its own by conventional methods [136]. DaunoXome demonstrated delayed opsonization and avoided rapid RES clearance, resulting in increased plasma circulation [137].

Metal-Based Drug Carriers

Gold Nanoparticles

Gold NPs can be synthesized as very small particles (2–50 nm in diameter), which enhances their ability to carry higher drug dosages due to their relatively large surface

area-to-mass ratio. Gold NPs also offer a number of advantages for cancer therapy, including simple and reliable methods of synthesizing the NP, easy functionalization by various biomolecules due to their negative surface charge, biocompatibility, and low toxicity (if greater than 3 nm in diameter) [21]. In drug delivery, gold NPs conjugated with oxaliplatin were synthesized and studied by Brown et al. and shown to enhance cytotoxicity in all cell lines that were tested, and to penetrate the nucleus in lung cancer cells [138]. In addition, functionalized gold NPs have demonstrated efficient drug delivery to drug-resistant tumor cells when 3-mercaptopropionic acid capped gold NPs efficiently delivered drugs to drug-resistant leukemia K562/ADM cells [139]. Thus, functionalized gold NPs are a potential platform to inhibit multidrug resistance in targeted tumor cells.

Other Methods of Drug Delivery

Image-Guided Drug Delivery

Drug delivery vehicles must be able to successfully accumulate in the tumor at concentrations above a therapeutic threshold, and be distributed evenly throughout the tumor. However, a challenge in drug delivery arises when several of its fundamental determinants, including vessel density, permeability, and expression of the tumor markers, differ among tumor regions, disease stages, and in patients. For this reason, imaging tools can be used to guide drugs to the targeted site, enabling individualized treatment.

Image-guided drug delivery is used to monitor the biodistribution, blood circulation, and tumor accumulation of administered drugs, resulting in greater knowledge of a tumor's response to treatment. To accomplish this, drugs are loaded onto or into NPs that can be used for medical imaging purposes. SPIOs are a popular choice, as they offer excellent MRI contrast and biocompatibility, and can be easily loaded with chemotherapeutic drugs, such as doxorubicin and paclitaxel [133, 140–142] (Fig. 10.3b). Dextran-coated SPIOs conjugated with siRNA, and with a NIR dye, Cy5.5, attached have also been employed to image *in vivo* siRNA delivery using MRI/NIRF dual modality imaging [143].

Magnetic Drug Targeting

Magnetic NPs act like nanosized magnets, and therefore, experience a force when placed in a magnetic field. The force depends on the magnetic moment of the NP and the gradient of the magnetic field [144]. Therefore, by carefully planning the conditions of the magnetic field, magnetic NPs that carry anticancer drugs can theoretically be guided towards the tumor [145, 146].

The first use of magnetic targeting *in vivo* was carried out with magnetic albumin microspheres in the 1970s, in which magnetite particles of 10–20 nm diameter were clustered with albumin [147]. In the early 1980s, the same magnetic particles were used to deliver doxorubicin (Fig. 10.3c) to Yoshida sarcoma tumors in a rat model,

and resulted in complete remission of the disease [148]. In addition, MTC-DOX, another magnetic microparticle, proved successful in animal models; however, it failed to reach clinical trials in 2004 [149]. Studies were later refocused to involve magnetic NPs of < 10 nm diameter, as they offer superior biocompatibility, biodistribution, and deeper tumor penetration than microparticles. A number of magnetic NPs, including micelles, liposomes, and SPIOs, have been reported to have successful magnetic targeting of chemotherapeutic drugs to tumor sites in animal models [140, 150].

Thermal Therapy

Cancer cells are more vulnerable to treatment at elevated temperatures, as local heating is able to increase tumor vasculature extravasation of drug carriers. For this reason, combining chemotherapy treatments with thermal therapy is believed to provide a synergistic effect for cancer intervention [151]. Metal NPs, such as gold NPs, and those NPs with magnetic properties, are highly effective for thermal therapies, and are also suitable for loading drugs into their interior, thereby providing the benefit of both drug-delivery and thermal therapy combinations [151].

As heat can potentially degrade the anticancer drug, or the NP itself, controlled drug release is important for thermal therapy. The drug molecule is stored in a heat-responsive structure, which triggers the slow release of the drug at the target site when local heating is exerted. Hu et al. demonstrated that local heating of NPs made up of magnetite and silica cores in an oscillating magnetic field was able to break the magnetic shell, releasing the drug molecule at the tumor [152]. Thomas et al. further reported the rapid release of the anticancer drug, doxorubicin, from zinc-doped iron oxide nanocrystals encapsulated in mesoporous silica under focused hyperthermic conditions [153].

Avoiding the Reticuloendothelial System (RES)

Unmodified NPs typically demonstrate short circulation times in the body due to their small size and surface properties. They are generally removed from circulation by the RES, with the greatest accumulation in the liver, spleen, and lungs [13]. In order to extend circulation times, NPs should be as small as possible; studies have demonstrated that NPs of 100 nm in diameter or less have the longest circulation times. In addition, NPs with hydrophilic surface coatings have been shown to remain in the circulatory system longer than those that are more hydrophobic. Hydrophilic polymers placed at the NP surface, such as PEG (described in Sect. 3.2.1), are able to repel plasma proteins and other blood macrophages [154]. Unlike hydrophobic NPs, which are the prime targets of the RES, hydrophilic NPs were revealed to have less than 1 % uptake by the spleen and liver, with 8–10 % still circulating in blood 8 h post-injection [155].

Passive Targeting

While normal tissue vasculature is lined with endothelial cells that are tightly aligned, which prevents extravasation of NPs, tumor vasculature grows at an uncontrollable rate and the endothelial cells that line it adopt an abnormal shape, resulting in a leaky and imperfect architecture that is hyperpermeable to small particles [156–159]. In addition, tumor vessels exhibit poor lymphatic drainage, which when coupled with rapid vascularization, results in the preferential accumulation of NPs in the tumor interstitial space. This concept is referred to as the enhanced permeability and retention (EPR) effect. The EPR effect is the primary means of carrying out passive NP tumor targeting, as it facilitates the movement of macromolecules, such as drug-loaded NPs, into tumor tissues.

Active Targeting

Active targeting differs from passive targeting in that it does not rely on the natural accumulation of particles at the desired tumor site. Instead, targeting is achieved by functionalizing the particle surface with a targeting moiety that facilitates its delivery to the site of interest. Thus, active targeting is based on the specific interactions of a ligand to a receptor, such as antibody-antigen or peptide-receptor interactions.

Tumor-Specific Targeting

Overexpression of receptors on tumor cell surfaces makes targeting of these tumor environments possible. Therefore, one plausible mechanism for tumor cell targeting is through the conjugation of specific ligands, such as antibodies, peptides, small molecules, etc., onto the surfaces of NPs to recognize and selectively bind the tumor cell with the overexpressed receptor on its surface. However, targeting effectiveness depends on the NPs ability to reach the cell surface after extravasation across the tumor vasculature endothelium. Recently, it was shown that NPs with similar characteristics, such as size and surface charge, can have different extravasation behavior *in vivo* [160], suggesting that there is little true understanding of conditions under which the extravasation of NPs occurs. For example, Smith et al. determined that QDs extravasate in LS174T tumors significantly better than SWNTs, while SWNTs extravasate better in U87MG tumors than QDs, but that neither QDs nor SWNTs demonstrate extravasation in xenograft SKOV-3 tumors [160]. In addition, NPs must overcome a number of biological barriers following extravasation, including high interstitial fluid pressure and a dense collagen matrix [161], and must travel tens to hundreds of micrometers before reaching and binding the tumor cell surface [162]. Thus, targeting the tumor cell surface with functionalized NPs faces many challenges. For example, the use of antibody targeting of lipid-based NPs demonstrated greater targeting capability *in vitro*, but showed little improvement over

non-targeted NPs in reaching solid tumors *in vivo* [163]. However, other examples demonstrate successful *in vivo* targeting and appear to be dependent upon the NP platform being used and the specific targeting mechanism employed. Vapreotide (VAP)-modified core-shell liposome NPs targeting the somatostatin receptor and containing VEGF siRNA as well as paclitaxel, were reported to significantly inhibit tumor growth in a murine model as compared to a non-targeted control NP [164].

Targeting the Tumor Vasculature

Tumor angiogenesis is the rapid and uncontrolled formation of new vessels from the pre-existing vasculature. In normal tissue, the formation of new blood vessels is controlled by the release of anti-angiogenic molecules, such that angiogenic stimulatory molecules are in equilibrium with the inhibitory molecules. However, during tumor neovascularization, this balance is interrupted, causing the increased secretion of the stimulatory molecules, and ultimately, results in the spread of new blood vessels that cause the tumor mass to grow uncontrollably. Consequently, tumors characteristically have poorly vascularized areas with large amounts of necrosis, tumor vessels that are highly branched and abnormal in shape, and a leaky vasculature due to abnormalities and holes in the basement membrane. As a result, molecules are able to move more freely through the blood vessel wall into the interstitial space surrounding the tumor cell.

Molecules that are selectively overexpressed on the tumor vasculature that are involved in angiogenesis are thought to be potential targets for treatment. For example, the VEGF/VEGFR signaling pathway is important for vasculature development in both normal and diseased tissues [165]. Similarly, integrins, which are a family of cell adhesion molecules, are involved in interactions between cells and the extracellular matrix, and can therefore, control cell migration and survival during angiogenesis [166]. Therefore, recent efforts have been directed towards inhibiting tumor growth during angiogenesis. For example, Feng et al. developed a core-shell type NP co-encapsulating VEGF targeted siRNA (siVEGF) and paclitaxel, with VAP targeting peptides on their surface for interaction with somatostatin receptors, referred to as VAP-PLPC/siRNA NPs [164]. The nanocarrier was designed to produce a synergistic inhibition of tumor growth due to RNA interference, which assists in down regulating VEGF, and the simultaneous delivery of the drug, paclitaxel, into cells. Their study demonstrated that the targeted NP had stronger drug distribution in tumor tissue and was more efficient at inhibiting tumor growth and neovascularization *in vivo* [164]. In another study, Jiang et al. showed that integrin-mediated poly(trimethylene carbonate)-based stealth NPs, c(RGDyK)-NP, loaded with paclitaxel had greater tumor penetration, accumulation, and growth inhibitory effect than other conventional NPs [167].

Challenges and Future Outlook

NPs have a number of potential cancer applications, particularly in personalized oncology, which allows the detection, diagnosis, and treatment to be tailored to each individual case, as well as in predictive oncology, which allows molecular markers to be used as guides for disease development, progression, and clinical outcomes [3]. Recent advances in nanotechnology have demonstrated the promise of NPs in cancer research, such as in the delivery of siRNA, overcoming drug resistance, and applications in multivalent targeting; however, there is still a great need for concentrated effort to improve the field and applications of nanotechnology, and to help guide it towards a future of increasing clinical relevance.

Drug resistance is one of the major obstacles that restricts efficient cellular delivery of chemotherapeutic agents. Several mechanisms exist at the cellular level that are responsible for drug resistance. The most well known example is membrane bound P-glycoprotein (P-gp), a drug efflux protein on the cell membrane, which decreases the intracellular concentration of chemotherapeutic drugs [168–170]. In addition, P-gp may be present on the nuclear membrane, which can limit the transport of drugs into the nucleus [171]. Moreover, the drugs that do gain intracellular access are often trapped in cytoplasmic vesicles and then degraded, or are externalized directly from the cell by exocytosed vesicles [172].

Several strategies exist to overcome P-gp mediated resistance. For example, agents that inhibit P-gp, such as verapamil and cyclosporine, are administered simultaneously with a cytotoxic drug. It is believed that NPs can evade recognition by the P-gp efflux pump by being engulfed in an endosome upon entering the cell, resulting in greater drug concentration inside the cell [173]. Therefore, NPs can be loaded with both a cytotoxic drug and a P-gp inhibiting agent for simultaneous delivery into the cell [174]. Furthermore, ligand-targeted strategies are useful overcoming P-gp mediated resistance, as the ligands are internalized due to receptor-mediated endocytosis. For this reason, NPs functionalized with ligands and PEG polymers have the potential to deliver elevated drug concentrations to the plasma membrane, thereby saturating the plasma membrane and reversing the effects of P-gp [169]. A transferrin-conjugated NP loaded with paclitaxel [175] and a folate-receptor targeted, pH-sensitive polymeric micelle that contained doxorubicin [176] were shown to have greater ability to inhibit drug resistance in MCF-7 cells and xenografts than their non-targeted counterparts.

Despite novel designs for cancer-targeted NPs, very few NP-based chemotherapeutics are currently used in the clinic, as current NP technologies fail to universally improve the efficacy of a drug. The reasons for this are multifold:

i. Tumor vasculature targeting results in low absolute tumor accumulation of the NP (typically < 10 % ID/g) when compared with accumulation in the liver (> 20 % ID/g) [161]. It has been widely accepted and proven that the nanoplatform's size, chemical composition, surface charge, and density of PEG and targeting ligands affect its biodistribution and accumulation at the site of interest; however, general rules governing the design of NPs for vasculature targeting

have yet to described [161]. It has been suggested that a side-by-side comparison of *in vivo* tumor vasculature targeting be conducted with different nanoplatforms of different size, composition, ligand density, etc., as no summary of this kind exists in literature [161].

ii. The therapeutic drug is often poorly loaded onto the NP for delivery to the tumor, with the drug making up < 5 % of the NPs weight. This runs the risk of having the drug concentration be too low to be pharmacologically active upon delivery, or the need for NP concentrations to exceed pharmacologically safe limits, resulting in unwanted side effects and high toxicity levels.

iii. Drugs encapsulated in NPs, or adsorbed onto their surface, are susceptible to being released prematurely or too quickly (termed "burst release") upon administration. Consequently, a large portion of the drug is often released before reaching its target in the body, which results in lower activity at the tumor, and ultimately, can produce greater side effects.

iv. The risk for toxicity and side effects exists with the use of NPs for tumor targeting with imaging agents and therapeutic drugs. Toxicity can result from NP introduction into the body by adsorption through the skin, inhalation, and ingestion [76]. This can result in the accumulation of NPs in unintended organs, potentially causing adverse effects. In addition, NPs may not be cleared from the body by hepatic and/or renal pathways at a rate that escapes toxic build-up. Similarly, the body must be able to respond quickly enough to dispose of the NP in order to evade toxicity and adverse side effects [76]. Moreover, there are concerns about toxicities resulting from the NP material itself, such as CdSe and CdTe in QDs [9].

Therefore, greater efforts need to be directed towards better and safer technologies for optimizing loading capacities and release of challenging therapeutic drugs from the different nanoplatforms. In addition, there is a need for improving *in vivo* targeting efficiency in order to determine the optimal conditions for tumor targeting, and to decrease the unwanted accumulation of drugs in the RES, thereby improving the tumor to liver ratio. Similarly, improved design strategies in order to develop NPs with enhanced clearance properties are also required.

Multifunctional NPs have the potential to be an exciting future direction in nanomedicine. Important factors include imaging (i.e. single- vs. dual-modality), therapy (i.e. single drug or combination of drugs) and targeting (i.e. one or more targeting ligands). With each additional NP functionalization, NPs can be used for novel applications due to their added capabilities.

NPs that carry multiple contrast moieties already exist and are currently being employed as multimodality imaging agents using different modalities for efficient signal enhancement or for comparing *in vivo* to *ex vivo* specimens. In addition, NPs can be designed to use a single platform to combine therapeutics and imaging for personalized patient management ("Nanotheranostics") [177], such that a single NP may be capable of identifying and targeting cancerous cells by way of a specific targeting ligand on its exterior, visualizing their location in the body in real-time, delivering chemotherapeutic drugs in a controlled manner with minimal adverse

side effect, and finally, monitoring the treatment in real-time. Some nanoplatforms that have intrinsic imaging capabilities, such as IONPs, gold NPs and QDs, can be advantageous in developing new strategies for multifunctional NPs, as the composition of the particle itself produces imaging contrast. Similarly, NPs with inherent therapeutic properties may decrease the need for as complex modifications to the particle. For example, magnetic NPs (i.e. IONPs, gold NPs, SWNT NPs) have thermoablative properties, which can lead to carcinoma destruction when localized heat, from sources such as infrared lamps, ultrasound or lasers, is applied [77]. Unfortunately, it is difficult to contain the heat in a localized area; however, the problem can be avoided with NPs that are designed to absorb NIR light, such as that emitted from laser irradiation. When NIR light is absorbed by these NPs, the target site increases in temperature from about 40 to 50 °C, potentially resulting in irreversible selective cellular destruction [178].

Another aspect of multifunctional NPs focuses primarily on the delivery of multiple therapeutic drugs simultaneously. Most of the NP platforms studied to date, including liposomes, micelles, and dendrimers, are incapable of loading and releasing multiple drugs due to the complexities of ratiometric delivery and synchronized release of multiple drugs from a single NP scaffold [179–182]. Liao et al. successfully synthesized the first example of a NP platform that is capable of controlled loading and synchronized release of precise molar ratios of three chemotherapeutic drugs, doxorubicin, camptotheticin, and cisplatin [183]. In delivering multiple drugs simultaneously, it is important to consider the toxicity profiles of the individual drugs, in order that the drugs do not adversely interact with one another. These three drugs were chosen because their toxicity profiles do not overlap with one another [184, 185]. In this study, they present a novel strategy wherein convergent methods were applied for the synthesis of the NP platform, such that the drug molecules are used as building blocks for the particle itself. In doing so, complexities that arise from drug conjugation and encapsulation reactions are eliminated. In addition, it was believed that the concentration of each drug at (or near) the maximum tolerated dose would lead to maximum therapeutic index. The study demonstrated that the three-drug-loaded nanoplatform with concentrations of each drug in multiples of each drug's maximum tolerated dose successfully outperformed their one- and two-drug-loaded NP counterparts *in vitro* with ovarian cancer cells [183]. Although *in vivo* studies have yet to be performed, the approach carried out in this study is not believed to be limited by the number and ratio of molecular species that could be built into particles. As a result, this first example of triplex drug delivery provides the framework for future combination drug studies. Combining this approach to drug delivery along with a cancer specific targeting mechanism would fully utilize the capabilities of the NP platform and has great potential for targeted anti-cancer therapeutics.

Nanomedicine would also benefit from the use of different nanoplatforms, particularly those that are already developed in nature. Viral nanoparticles (VNPs) are naturally occurring bio-nanomaterials that have recently gained much attention because they offer numerous advantages over synthetic platforms: they are made of biocompatible and biodegradable materials; they are derive from

bacteriophages and plant or animal viruses, which can be infectious or non-infectious; they are deemed safe for human application; they can be produced quickly and in large quantities; they can be modified and functionalized with ligands with high specificity and accuracy because their structures are known to atomic resolution; and finally, functionalization can be carried out by way of genetic engineering and chemical bioconjugation, resulting in relatively flexible construction [186, 187]. VNPs derived from plant viruses and bacteriophages are of particular interest because they are the least pathogenic in humans, and therefore, are believed to produce less adverse side effects [186].

Efficient therapy requires that the drug is able to avoid interaction with normal cells, and that it accumulates in the tumor. Targeted NPs have been shown to have affinity for particular receptors in diseased tissue, and therefore, reduced accumulation in nontargeted tissue. Cowpea mosaic virus (CPMV) is a plant-derived VNP that has a natural affinity for and is internalized by mammalian endothelial cells *in vivo* [188]. Animal toxicity studies have revealed that CPMV show no clinical symptoms, and therefore, can be applied in therapeutic drug delivery [189]. Studies have shown that CPMV possess a lysine residue with enhanced reactivity in each asymmetric unit [190] (Fig. 10.4).

Steinmetz et al. synthesized and characterized a novel multifunctional VNP using CPMV for the detection and visualization of human prostate cancer. In this study, CPMV was modified to display the targeting peptide bombesin, PEG polymers, and the NIR dye Alexa Fluor 647 (AF647) for specific targeting and imaging of GRP receptors, which are over-expressed in prostate cancer [187]. Unlike previous studies of this kind, the bombesin peptide was attached at the outer ends of the PEG chains, distal to the VNP core. It was found that the inclusion of the hydrophilic PEG linker in the hydrophobic bombesin peptide increased the solubility of the particle, and improved its stability, without resulting in aggregation. The CPMV-PEG-bombesin

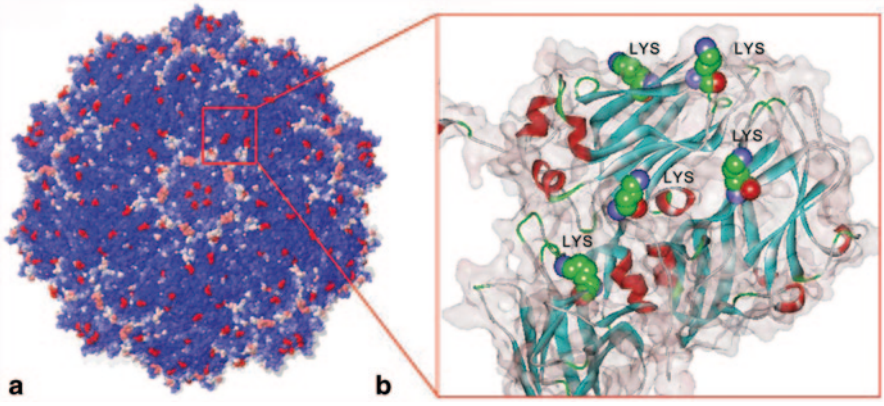

Fig. 10. 4 **a** Molecular model of CPMV nanoparticle generated by the VIPER website from protein data bank file 1NY7. Lys sidechains are highlighted in *red*. Image was created with Jmol. **b** A closer view of a *Lys-rich* region. Lys residues are shown as 'Ball and stick' and colored by Atoms. Image was created with Ds 3.5 client

targeted particle was shown to accumulate in a human PC-3 tumor model, and its uptake in the tumor was visualized and quantified in real-time [187]. In addition, the targeted particle demonstrated more uniform uptake throughout the tumor and was retained at significantly higher levels in the tumor as compared with its nontargeted analogue (CPMV-PEG) [187].

The versatility of NPs and their applications in nanomedicine make for promising tools in the medical world. The combination of their applications in nanoscale drug delivery systems and the development of nanoscale imaging indicates that the potential exists for future multifunctional, multivalent nanoplatforms that can be used in personalized oncology. Furthermore, there is the possibility of new nanoplatforms that can be applied for simultaneous *in vivo* imaging and treatment of the disease. While nanomedicine still requires a great deal of maturation before it can be routinely applied in a clinical setting, considerable progress has already been made with respect to cancer research, and therefore, the use of different NPs in individualized cancer treatment may soon become an exciting reality.

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