1.3

Cellular and Molecular Basis of Cancer

In this section we will learn that cancer is a genetic disease, involving huge levels of complexity and uncertainty in relation to the factors responsible for its initiation and development. Consequently, a combination of inherent genetic changes and a panoply of environmental factors, such as chemicals, radiation, and viruses, result in wide variations in cancer development and prognosis. In spite of this vast diversity of factors, it is now strongly appreciated that the tumour mass derives from clonal expansion of a cancer stem cell and involves the accumulation of chromosomal abnormalities [1,2].

In Section 1.2 we learned that, because organs and tissues are composed of a number of different cell types, a vast number of different malignancies can develop. From an anatomical and morphological perspective, cancers are a complicated integrated network of genetic and, subsequently, cellular changes.

From what we have seen so far, we are aware that many things can increase the possibility of cancer developing, and from an anatomical perspective cancer is multifaceted and involves many cellular processes and interactions. At face value this indicates that understanding the cancer development processes, and developing therapeutic strategies for its management, is very daunting. So, faced with this complexity and infinitely difficult process, should we just resign ourselves to never understanding cancer and accept that the task is beyond our intellectual capabilities? The answer is that maybe we should be thinking about cancer in a different way. Rather than looking at this topic from a global developmental perspective, the suggestion is that we approach our understanding from the other direction, that is, what drives its development, rather than what the final ‘product’ looks like. If we can understand the many similarities between cancers, it will allow us to appreciate the fundamental basis of cancer development (and the subsequent therapeutic management).

Despite the wide range of ‘causes’ and ‘types’ of cancer, and diversity amongst individuals, cancer fundamentally derives from molecular aberrations within susceptible cells. In principle, non-lethal genetic damage causes modifications to key pathways inside the cell, which, in turn, results in the lack of normal regulation of cell growth, conferring a survival advantage to the affected cells and the ability to invade neighbouring tissues and disseminate to other parts of the body.

Several types of alteration can affect the genome within these tumour cells, leading to cellular transformation and subsequently a selected growth advantage. The types of genes affected are roughly categorised into two groups: those which promote uncontrolled
cell growth (oncogenes) and those which inhibit tumour growth (tumour suppressor
gen genes). The contrasting balance in the activity and repression of these two opposing
gene categories drives the molecular evolution of cancer. In many cases, the perturba-
tion of this balance is an underpinning principle in the management of cancer, particu-
larly molecular targeted therapeutics, as discussed later in this book.

1.3.1 Oncogenes

An oncogene is a gene that, when expressed in a ‘normal’ cell, culminates in that cell
gaining a transformed phenotype (acquisition of altered growth properties characteris-
tic of cancer cells and neoplastic growth). Simplistically, oncogenes are analogous to the
accelerator of a motor car, which, if jammed (mutated to active form), will switch the car
from having the potential to move forward (normal gene function) to a situation where
the car is permanently moving forward.

So where do these oncogenes come from? Are they derived from ‘infection’ by an
external agent? Do we inherit these genes? Or are they silent and then activated when
cancer is initiated? In actual fact, to some extent, all of these origins are correct.

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Species</th>
<th>Virus</th>
<th>Functional oncoprotein target</th>
</tr>
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<tbody>
<tr>
<td>Abl</td>
<td>Mouse</td>
<td>Abelson murine leukaemia</td>
<td>Non-receptor tyrosine kinase</td>
</tr>
<tr>
<td>Akt</td>
<td>Mouse</td>
<td>Akt8 murine thymoma</td>
<td>Serine-threonine signalling kinase</td>
</tr>
<tr>
<td>Crk</td>
<td>Chicken</td>
<td>CT10 avian sarcoma</td>
<td>Modular signalling link</td>
</tr>
<tr>
<td>erbA</td>
<td>Chicken</td>
<td>Avian erythroblastosis</td>
<td>Thyroid hormone receptor</td>
</tr>
<tr>
<td>erbB</td>
<td>Chicken</td>
<td>Avian erythroblastosis</td>
<td>Epidermal growth factor receptor (EGF-R)</td>
</tr>
<tr>
<td>Fos</td>
<td>Mouse</td>
<td>FBJ murine osteogenic sarcoma</td>
<td>Activator protein 1 (AP1) complex</td>
</tr>
<tr>
<td>Jun</td>
<td>Chicken</td>
<td>Avian sarcoma-17</td>
<td>Activator protein 1 (AP1) complex</td>
</tr>
<tr>
<td>Kit</td>
<td>Cat</td>
<td>HZ feline sarcoma</td>
<td>Stem-cell growth factor receptor (SCF-R)</td>
</tr>
<tr>
<td>Mos</td>
<td>Mouse</td>
<td>Moloney murine sarcoma</td>
<td>Serine-threonine signalling kinase</td>
</tr>
<tr>
<td>Mpl</td>
<td>Mouse</td>
<td>Myeloproliferative leukaemia</td>
<td>Thrombopoietin receptor</td>
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<tr>
<td>Myc</td>
<td>Chicken</td>
<td>Avian myelocytomatosis</td>
<td>Transcription factor</td>
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<tr>
<td>Myb</td>
<td>Chicken</td>
<td>Avian myeloblastosis</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>pi3k</td>
<td>Chicken</td>
<td>Avian sarcoma virus 16</td>
<td>Phosphatidylinositol 3-kinase (Pi3K)</td>
</tr>
<tr>
<td>Raf</td>
<td>Mouse</td>
<td>Murine sarcoma-3611</td>
<td>Serine-threonine signalling kinase</td>
</tr>
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<td>H-Ras</td>
<td>Rat</td>
<td>Harvey sarcoma</td>
<td>GTPase</td>
</tr>
<tr>
<td>K-Ras</td>
<td>Rat</td>
<td>Kirsten sarcoma</td>
<td>GTPase</td>
</tr>
<tr>
<td>Sis</td>
<td>Monkey</td>
<td>Simian sarcoma</td>
<td>Platelet-derived growth factor (PDGF)</td>
</tr>
<tr>
<td>Src</td>
<td>Chicken</td>
<td>Rous sarcoma</td>
<td>Non-receptor tyrosine kinase</td>
</tr>
</tbody>
</table>
1.3.1.1 Viral Oncogenes

The initial discovery of oncogenes and, subsequently, the molecular alterations conducive of inducing tumour growth was obtained from viruses, with the limited size of the viral genome (often being <10 genes) being advantageous to identifying specific viral oncogenes [3]. The first type of virus identified with these abilities (in 1911) was the rous sarcoma virus (RSV), a retrovirus (an RNA virus from the same family as HIV) which caused sarcomas in birds and also transformed chick embryo cells in the laboratory (a major discovery, despite studying cancer in birds being a little unorthodox). The key oncogene was subsequently identified via comparison of the RSV genome with that of the closely related avian leukosis virus (ALV), a retrovirus that does not transform embryo cells [4]. These studies revealed that RSV contained a small additional gene that was not present in ALV. Although this gene did not itself drive viral replication, it was central to the ability of RSV to transform cells both in vitro and in vivo. The responsible oncogene was identified as src, named because of the ability of RSV to induce sarcomas [4]. Since the discovery of src, in excess of 30 viral oncogenes have been identified from similar transforming retroviruses, with three-letter nomenclatures related to the type of viral-induced tumour (e.g. mpl [myeloproliferative sarcoma]), the animal species targeted for viral infection (e.g. sis [simian sarcoma]), or the person who first identified the virus (e.g. abl [derived from name of Herbert Abelson and the fact it causes leukaemias]) [3] (Table 1.3.1).

In addition to the viral oncogenes identified in transforming (RNA) retroviruses, such as src and abl, several other virus classes have also been shown to express oncogenes, including DNA viruses [5]. Other viruses implicated in human cancer development include Epstein–Barr virus (EBV), hepatitis B and C virus (HBV, HBC), Kaposi’s sarcoma herpes virus (KSHV), human T lymphotrophic virus-1 (HTLV-1), and, most notably, human papillomavirus (HPV) [5]. The oncogenes in these viruses, such as BNLF-1, encode latent membrane protein-1 (LMP-1) in EBV, and E6 and E7 in HPV, which, in addition to cellular transformation, generally function to promote viral replication [5].

So the answer to the question ‘Are oncogenes derived from ‘infection’ by an external agent?’ would have to be ‘Yes, to a certain extent’ as infections are estimated to account for up to 20% of all cancer cases worldwide [5]. There is clear evidence supporting integration of viral oncogenes into the host cell genome, and their transfer to daughter cells, culminating in abnormal cellular growth, transformation and eventual development of a malignancy. Furthermore, there is now unequivocal evidence supporting a link between human papillomavirus (HPV) and the development of cervical cancers [5], with vaccination against this virus now widespread practice.

1.3.1.2 Proto-oncogenes and Cellular Oncogenes

The discovery of oncogenes in viruses, and the demonstration of the tumour-inducing properties of these factors, provided significant evidence of a molecular basis to cancer development. However, the majority of cancers are not caused by viruses (which we know account for approximately 20% of cancers) and therefore they must be the result of other factors inducing similar changes to the affected cells. Whatever the initiating source for the cancer, it is now without doubt that the development of cancer takes a
path that is mirrored at the molecular level by that illustrated by the viral oncogenic processes, a theory evidentially supported by the discovery of homologies between these viral oncogenes and the genetic material of cancer cells. This indicates that there must be key genetic targets which, when activated, arm the cell with similar tumourigenic capabilities to that provided by oncogenic virus infection. If correct, this implies that there are oncogenes already present within our cells. So, are we all walking around with a ticking time bomb of cancer in each of our cells? Are these oncogenes hidden within our genome and simply waiting for the right environment and conditions to occur before leaping out and initiating cancer, or are they more subtle, and is the situation actually more complex?

The answer is that all oncogenes are actually altered or overexpressed versions of normal genes associated with the regulation of cell proliferation, cellular communication, interaction with the cellular environment, or survival. This means that an oncogene is actually a normal gene which has either been abnormally switched on, or one for which the normal control mechanism for expression has gone astray. These ‘normal’ genes, with potential for driving malignancy, are termed proto-oncogenes (their discovery by Michael Bishop and Harold Varmus led to the award of the 1989 Nobel Prize in Physiology or Medicine ‘for their discovery of the cellular origin of retroviral oncogenes’).

An important principle in this regard is that proto-oncogenes are dominant from a genetic perspective, with activation of a single allele being sufficient to drive cellular transformation. The perturbation of these proto-oncogenes creates an alternative pathological form, with abnormal function and tumourigenic potential, which we then term an oncogene.

But what types of genetic mechanism convert proto-oncogenes into oncogenes? The answer is fairly straightforward if you consider that any change must increase the expression level or activity of a proto-oncogene: what is required is either a genetic change (mutation) in a proto-oncogene (which ‘switches-on’ and creates an overactive oncogene) or amplification of the particular proto-oncogene (potentially producing many hundred copies of the gene) and the subsequent overexpression of the otherwise normal protein [6]. In principle, the following changes are associated with conversion of a proto-oncogene to an oncogene:

1) point mutations, deletions, or insertions that lead to a hyperactive gene product,
2) point mutations, deletions, or insertions in the promoter region of a proto-oncogene that lead to increased transcription,
3) gene amplification events leading to extra chromosomal copies of a proto-oncogene,
4) chromosomal translocation events that relocate a proto-oncogene to a new chromosomal site that leads to higher expression, and
5) chromosomal translocations that lead to a fusion between a proto-oncogene and a second gene, which produces a fusion protein with oncogenic activity.

A vast and ever-expanding number of activating mutations are known for a panoply of proto-oncogenes (far too many to describe here), with the identification of others rapidly accelerating as a result of the availability of the human genome sequence and improved sensitivities of sequencing methodologies (Figure 1.3.1) [6–8]. For instance, there are known to be in excess of 140 genes that, when altered by intragenic mutations, can promote and ‘drive’ tumourigenesis, of which at least 50 are known oncogenes (Figure 1.3.2) [6]. Because of this activating requirement and the need to ‘hit’ a particular
1.3 Cellular and Molecular Basis of Cancer

Region of the resultant protein, oncogenes are recurrently mutated at the same amino acid positions (Figure 1.3.2) [6]. In addition to the gain of activation mutations, the amplification of the particular proto-oncogene (potentially producing many hundred copies of the gene) and subsequent overexpression of the otherwise normal protein is another mechanism by which oncogenes can be created [6]. For example, the amplification of \( \text{erbB2} \) (commonly referred to as \( \text{HER2} \)) is detected in approximately 20% of breast cancers, an alteration which can be successfully exploited for therapeutic treatment of this cancer type (as discussed in Section 3.1). Similarly, amplification of the \( \text{myc} \)
oncogene is observed in approximately 30% of neuroblastomas, a change associated with a poor prognosis for this malignancy. Despite the underlying amplification or activation mechanism, increased activity of the resultant oncogenic protein is invariably associated with driving the malignant phenotype. To use an analogy from the movie Star Wars (sorry!), an oncogene is a Jedi knight – it has the potential to do good, but has crossed over to the Dark Side (i.e. Anakin Skywalker, aka Darth Vader).

There are now many examples confirming the links between particular cellular oncogenes and the development of specific cancers [9]. Although it has been postulated, it is by no means guaranteed that the activation of a single proto-oncogene will lead to the particular malignancy. However, the increased expression of cellular oncogenes has been found in many human cancers, supporting a central role for either the gene itself or the molecular pathway in which it functions. For example, the myc oncogene is over-expressed in neuroblastoma, multiple myeloma, and small cell lung cancers, amongst others [9,10]. Similarly, in specific types of leukaemia, the cellular abl oncogene (c-abl) is adjacent to the site of the chromosomal translocation that defines this malignancy (the Philadelphia chromosome, described in Section 3.3) [11].
In the case of viral-induced tumourigenesis, the viral oncogene effectively mimics the equivalent cellular oncogene and overrides the activity of the proto-oncogene, or initiates the inherent associated signalling pathway driving tumourigenesis. For example, the viral erbB oncogene product is the structurally related homologue of the cellular epidermal growth factor receptor (EGF-R) [12]. However, the resultant protein lacks part of the extracellular domain (including the EGF binding site) which, in combination with loss of a cytoplasmic phosphorylation site, causes the receptor to be permanently activated [12,13]. This ‘jammed accelerator’ drives growth-factor induced cellular proliferation independently of the actual growth factor, and thus satisfies a classical hallmark of cancer (see Section 1.3.6).

Since oncogenes are the driving force of tumourigenesis, with elevated expression and/or activity in all tumours, their inhibition or neutralisation is a major therapeutic strategy, particularly in the modern era of molecular targeted therapeutics. This approach and current clinically used drugs are discussed in detail in Sections 2.5 and 3.1.

1.3.2 Tumour Suppressor Genes

The opposite genetic function to an oncogene is that which normally prevents uncontrolled cellular growth. Such genes are termed tumour suppressor genes (see Table 1.3.2). The origin of tumour suppressor genes, unlike oncogenes, is clear in that these genes are naturally present within the individual’s genome, with their role being to inhibit potential tumourigenic processes, such as cellular proliferation or the acquisition of activating oncogenic mutations. The loss or inactivation of this type of gene allows the development of a transformed cellular phenotype. Consequently, because of the requirement to ‘inactivate’ these proteins, tumour suppressor genes are commonly mutated through protein-truncating alterations throughout their length, unlike oncogenes which are generally hit at consistent sites [6]. Within the set of genes (>140) that are reported to be modified by intragenic mutations and capable of ‘driving’ tumourigenesis, in excess of 70 are reported to be tumour suppressor genes [6]. Importantly, inactivating mutations in tumour suppressor genes are more prevalent than oncogene-activating mutations in many common solid tumours, with very few individual tumours containing more than one oncogene mutation relative to a multitude of changes to tumour suppressor genes [6]. Furthermore, since the loss (and not gain) of tumour suppressor genes is important in cancer, infection and introduction of these genes into a cell is not an option for cancer development (actually the converse!).

Using the analogy of cancer as a car, tumour suppressor genes can be viewed as the brakes: the loss of the full braking system (inactivation of tumour suppressor genes) results in the inability to stop the car moving, whereas damage to part of the brakes (genetic haploinsufficiency) may place the car in an unstable situation. If you then incorporate oncogenic activation in this situation, whereby oncogenes are activated and tumour suppressor genes are inactivated, the car then has uncontrollable acceleration with no means of stopping.

We now take the existence of tumour suppressor genes as fact, but the discovery of this type of gene revolutionised our understanding of the disease as, until this point, cancer was thought to be caused exclusively by oncogenes, with many believing external factors (such as viruses) were the causative agent. The defining study in this arena was
undertaken by the cancer clinician Alfred Knudson, who proposed the two-hit hypothesis and the concept that cancer is caused by two mutational events through study of the rare paediatric cancer, retinoblastoma [14]. This arose from his observations that hereditary retinoblastoma often presented with tumours in both eyes (bilateral) and typically before 5 years of age, whereas the sporadic form developed in only one eye (unilateral) and at a much later age. Knudson then suggested that hereditary tumours must have an inherited mutant copy of the retinoblastoma gene RB1 (passed down from an affected parent) (Figure 1.3.2), with the second gene copy mutated during the first few years of life. Since the inherited gene version would be present in all cells of the child’s body, they would be more prone to developing multiple tumours (in both eyes). In contrast, in the case of non-hereditary retinoblastoma, the individual is born with two normal RB1 gene copies, both of which need to be damaged and so the tumours develop later in life and only in the cells receiving the sporadic genetic insult [14]. It was thus concluded that these genetic hits must be recessive, as cancer only developed when both gene copies were affected, a theory proven by the observation that loss of heterozygosity (i.e. the inactivation of the remaining normal gene) led to cancer [15]. Thus, the existence of the tumour suppressor gene was born, alongside the concept that inheritance of mutated or inactivated versions of these genes can predispose an individual to tumour (and cancer) development. Furthermore, although in the majority of cases loss or damage to both allelic copies of these particular genes is required, this theory also indicates that inactivation of a single allele can place the cell in a precarious situation and, through the involvement of larger genetic networks, can position the cell at the precipice of cellular transformation.

There are now many examples of familial cancer types associated with inheritance of dysfunctional tumour suppressor genes, including the BRCA1 and BRCA2 genes, which predispose to breast and ovarian cancers (see Sections 3.1 and 3.6) [16]. Germline loss-of-function mutations (i.e. inherited) in the BRCA1/2 cancer susceptibility genes account for between 20% and 60% of breast cancer cases in families with multiple affected individuals (about 5% of cases) [17]. Normal BRCA1/2 genes produce proteins involved in genome surveillance, and sensing and repairing damaged DNA, thereby preventing transmission of putative mutations and potentially cancer development (i.e. tumour suppression) [17,18]. BRCA1 is now also believed to play a significant role in transcriptional control and cell cycle regulation, thereby increasing the significance of its dysregulation in cancer [17,18]. If an individual inherits a mutated version of these genes then this functionality is lost (or severely impaired) and the individual is placed at a higher risk of developing certain cancers. In this context, the presence of variants of the BRCA genes and a family history of breast cancer has led to many individuals (including the actress Angelia Jolie) opting for preventative surgery.

In another aspect, a prevalence of multiple benign tumours may arise in the ‘at risk’ tissue, as is the case with familial adenomatous polyposis (FAP), which is associated with inheritance of disabled versions of the adenomatous polyposis coli (APC) tumour suppressor gene and the subsequent predisposition to colonic carcinomas (Figure 1.3.3) [19,20]. This gene has a role in multiple cellular functions, including signal transduction of cell proliferation and cell polarity, mediation of intercellular adhesion, and stabilisation of the cytoskeleton [19]. As will be discussed later, APC is a major driver and exemplar for multi-step tumourigenesis in the development of colorectal carcinomas and malignancy.

1 Who sadly passed away in July 2016.
The conventional perception of tumour suppressor genes is that they are centrally involved in regulation of cellular proliferation, cellular death, and overall cellular survival. This class of tumour suppressor gene could be viewed as the antagonist to the effects of an oncogene’s over-activation, that is, the retinoblastoma tumour suppressor (RB), which is central to regulation of cellular proliferation as a ‘checkpoint’ within the cell cycle [21]. During tumourigenesis, functional inactivation of RB compromises the ability of cells to respond to signals that normally suppress cell proliferation, resulting in mis-expression of genes that drive cell division [21]. This anti-oncogene descriptor also supports the role of several other classical tumour suppressor genes and cell cycle regulator proteins, such as p16\textsuperscript{INK4A}, p15\textsuperscript{INK4B}, p18\textsuperscript{INK4C}, p19\textsuperscript{INK4D}, and p14\textsuperscript{ARF} [22,23].

However, a second mechanistic family of tumour suppressor genes also exists, exhibiting a ‘guardian’ phenotype. This class of tumour suppressor gene is responsible for detecting and responding to genomic damage, thereby protecting against the introduction of mutations or the overriding of normal cellular control functions. By sensing genomic damage (and the associated detrimental effects) these tumour suppressors activate pathways which halt cellular proliferation (often via signalling pathways linked to tumour suppressor genes involved in regulating cell division) or, if the damage is too severe and not amenable to just purely inhibiting proliferation (to allow the damage to be repaired), then these tumour suppressors can induce pathways which activate cell death. The archetypical tumour suppressor in this class is \textit{p53}, commonly termed the \textit{guardian of the genome}, the activity of which is mediated by the negative regulator MDM2 [24]; more than half of all human cancers have mutations in \textit{p53} [25,26]. The familial Li-Fraumeni cancer syndrome is also the result of the inheritance of a mutant
p53 allele. As a tumour suppressor, p53 is a central sensor of cellular stresses, including DNA damage, oncogene expression, nutrient deprivation, and an inadequate microenvironment for cellular division, causing a limitation of cellular propagation under adverse conditions [26,27]. In recent years, many more functions and roles for p53 have been identified and implied (Figure 1.3.4), including the modulation of processes such as metabolism, invasion, and metastasis, as well as communication within the tumour microenvironment [24,27].

Unlike the acquisition of an oncogene, mutation of these tumour suppressor genes does not conventionally lead to cellular transformation, as their loss is permissive of acquisition of other tumourigenic genomic mutations, and thus facilitating of tumourigenesis, rather than direct control of cell division or induction of cell death. This subsequent accelerated rate of tumourigenic mutation in oncogenes and other tumour suppressor genes in response to loss of the guardian functionality is commonly termed a ‘mutator phenotype’. Therefore, incapacitation of tumour suppressor genes, such as p53, removes the normal ability of the cell to respond to DNA damage by triggering repair or inducing cell death (apoptosis).

The greatest difficulty this poses for cancer management is that, despite tumour suppressor genes being predominant over oncogene changes in terms of number and diversity, drugs generally interfere with protein function and cannot replace the function of inactivated tumour suppressor genes. In these instances, therapeutic interventions (with the exception of gene therapy) target either stimulation of factors downstream of the dysfunctional tumour suppressor gene, or indirectly readdress the balance between oncogene activity and tumour suppressor control.

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1.3.3 Role of Epigenetics and Gene Promoter Regulation in Tumourigenesis

Cancer is a genetic disease, with mutations and genetic changes being the underlying cause of the resultant pathologies. This statement is hopefully correct as we have spent considerable time so far discussing it. Although cancer is, without doubt, a genetic disease, the reality is that cancer is not exclusively caused by changes in gene sequence. Genetic regulation actually involves a multitude of control factors, including epigenetic mechanisms. The term ‘epigenetics’ refers to heritable changes in gene expression determined by factors other than alteration of the primary DNA sequence (as is the case with mutations). In other words, the DNA provides the stored information relating to cellular phenotype (i.e. the ingredients) and epigenetics guides patterns of gene expression (i.e. the recipe book). Consequently, in the same manner that DNA mutations and chromosomal rearrangements facilitate gene overexpression (oncogenes) or inactivation (tumour suppressor), epigenetic changes can similarly control the expression and thus impact tumourigenesis. A clear illustrative example of a key role for epigenetics in cancer is again provided by the paediatric cancer retinoblastoma. Genetic analyses of this tumour type confirmed inactivation of both copies of the tumour suppressor gene RB1, but few other genetic changes [28,29]. However, this tumour type showed a high number of epigenetic aberrations, with changes in expression of several oncogenes associated with histone modifications and DNA methylation [28,29].

To appreciate the concept of epigenetics, and more importantly their significance for therapeutic management, it is paramount to first understand the principles associated with these regulatory processes. Epigenetic regulation is largely controlled by two processes: alteration in chromatin structure to regulate the access of the transcriptional machinery to the DNA (post-translational modification of histones) and direct epigenetic modification of DNA through methylation of specific nucleotides (DNA methylation).

In the promoter regions of genes, methylation occurs at dinucleotides comprising cytosines located prior to guanosine in the linear sequence of DNA bases. Regions of the genome with a high density of CpGs are termed CpG islands, with DNA methylation of these islands correlating with transcriptional repression and gene silencing [29,30]. This mode of silencing gene expression is essential to several normal long-term processes, such as inactivation of the X chromosome and genomic imprinting, and is a central mechanism in the regulation of cellular differentiation and ultimately cell identity and fate [31]. In cancer (or to be correct, tumourigenesis) there is global genome-wide hypomethylation,3 accompanied by hypermethylation of CpG islands (gene silencing) in the promoter region of many genes commonly involved with loss of tumour suppressor function. Many early stages of cancer, such as colorectal adenoma, demonstrate distinct patterns of localised DNA hypermethylation of gene-promoter regions, including the tumour suppressors p16INK4A and RB1 [29]. A central role for DNA methylation as an oncogenic factor is reinforced by the induction of growth arrest and cell death in haematological malignancies following exposure to inhibitors of DNA methyltransferases (azacitadine and decitabine), drugs which are now used clinically (see Section 2.1.8) [32].

3 The prefix hypo- refers to under (or less than normal); hyper- indicates over (or more than normal).
In terms of histone modifications, it is important to remember that DNA is packaged with these histone proteins to form chromatin, which undergoes a range of post-translational modifications to facilitate transcriptional and regulatory activity of DNA regions and individual genes [29,30]. The assembly of DNA into chromatin involves the wrapping of DNA around an octamer of histone proteins (tetramer of two histone H3–H4 dimers and a dimer of histone–H2), creating the nucleosome (the basic unit of chromatin). Subsequently, the positively charged histones interact with the negatively charged phosphate groups of the DNA backbone by electrostatic interactions, resulting in nucleosomal compaction. Ultimately, in combination with short linker DNA sequences, these long chains of tightly compacted nucleosomes (‘beads on a string’) form chromatin and ultimately chromosomes [33].

The degree to which the nucleosome aggregates are compacted (or relaxed) dictates transcriptional activity, causing expression or repression of specific genes. Essentially, tightly compacted nucleosomes cause ‘closed chromatin’ (heterochromatin) and subsequent transcriptional repression, whereas an ‘open’ chromatin structure (euchromatin) is characteristically transcriptionally active (Figure 1.3.5).

The modulation of nucleosomes, to facilitate active gene transcription, is controlled by the promotion or inhibition of the condensation of the DNA–histone complex, a process mediated predominantly by modifications of the N-terminal tails of the histone proteins [31]. These post-translational changes include methylation, acetylation, and phosphorylation, amongst others [31,34]. In the case of histone phosphorylation and acetylation, either a negative charge is added to histones (phosphorylation) or the degree of positive charges is reduced on the histones (acetylation), thereby repelling the DNA (negatively charged) from the histones, allowing the entry of the transcriptional machinery and thus facilitating gene regulation. Mechanistically, the complementary activity of the histone acetyltransferase (HAT) and histone deacetylase (HDAC) family of proteins adds and removes acetyl groups from key lysine residues within histone tails, respectively (Figure 1.3.5) [29,31]. Histone deacetylation (HDAC activity) increases the electrostatic attraction between the positive charges of the histones and negative charges of the DNA, ensuring tight binding and rendering promoter regions inaccessible to polymerases for gene transcription. The strong association between HDACs and gene activity, and regulated cooperation with HATs, supports the central importance of these enzymes in transcriptional regulation; HDACs act to remove acetyl groups added by HATs to facilitate transcriptional activity, providing a reset of chromatin structure for the subsequent round of transcription [35].

Cancer is linked to histone hypoacetylation, due largely to the overexpression of HDACs, a factor resulting in these enzymes being classified as oncogenes (a designation probably not applicable to all HDAC family members). With regards to the molecular association between HDACs and tumourigenesis, removal of acetyl groups from histones (by HDACs) leads to repression of several tumour suppressor genes, such as the cell cycle regulator locus CDKN2A (including p16INK4A and p14ARF) and the DNA repair gene, BRCA1 [35]. This association between HDACs and cancer, coupled to the targetability of these enzymes, led to significant efforts to develop therapeutic inhibitors with the objective of restoring the histone acetylation balance and inhibition of cancer growth [35,36]. To date, four HDAC inhibitors (vorinostat, romidepsin, belinostat, and panobinostat) have been approved for cancer treatment, specifically lymphomas and myelomas, and we will meet these drugs in Section 2.1.8 [35]. From a molecular perspective, HDAC
inhibition modulates and impinges upon many tumourigenic characteristics (hallmarks of cancer, discussed later), including proliferative capacity, response to cell death signalling, angiogenesis, and immune evasion.

Overall epigenetic regulation of gene expression is a dynamic process, with enzymes catalysing the addition of covalent modifications (writers), their removal (erasers), or recognition of previously deposited epigenetic marks (readers) to ‘landscape’ gene regulation (Table 1.3.2) [37]. Key writer enzymes include the histone acetyltransferases (HATs) and DNA methyltransferases (DNMTs), whereas key erasers include HDACs and DNA-demethylases. The third class within this group, the reader enzymes, possess the ability to recognise specific histones and their inherent methylation state, directing the epigenetic and transcriptional machinery to the appropriate site of action. Deregulation of epigenetic control mechanisms and dysregulation of the writer–eraser balance is a common feature of cancer, with clear involvements in multistage tumourigenesis (discussed later) and as a major force linked to several hallmarks of cancer (see Section 1.3.6).
In terms of the classification of genes associated with tumorigenesis, there are two well-defined genetic identifiers: the **oncogenes** and the **tumour suppressor genes** (as described earlier). These are further classified as to whether they are gene **drivers** or passengers for tumourigenesis. With the introduction of epigenetics into the equation, a further set of functional tumourigenic factors are thus identified: epigenetic modifiers, mediators, and modulators (Table 1.3.2) [29], the **modifiers** being those whose products modify gene expression through direct DNA methylation, post-translational chromatin modifications, or changes in chromatin structure (e.g. p53). Epigenetic **mediators** are those whose products are the targets of epigenetic modifiers (e.g. DNA methyltransferase 3A; DNMT3A). Lastly, the epigenetic **modulators** are those residing upstream from the modifiers and mediators in signalling pathways, linking cellular stresses to neoplastic change by increasing the likelihood that cancer will develop following acquisition of key (driver) mutations [29]. In all of these cases, the genes responsible for the epigenetic regulation can themselves be subject to genetic mutation, which adds a further level of complexity and the inter-relationships between molecular aspects of cancer. In essence, the regulator is modified and the modifier is regulated (and the modulator is somehow involved), further fuelling the ‘chicken and egg’ philosophy of molecular determinants.

<table>
<thead>
<tr>
<th><strong>Gene class</strong></th>
<th><strong>Definition</strong></th>
<th><strong>Examples</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genetic classification</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oncogene</strong></td>
<td>Activation by mutation or chromosomal rearrangement promotes tumourigenesis</td>
<td>Myc, K-Ras, PIK3CA, BRAF</td>
</tr>
<tr>
<td><strong>Tumour suppressor</strong></td>
<td>Inactivation by mutation promotes tumourigenesis</td>
<td>RB1, p53, APC, CDKN2A</td>
</tr>
<tr>
<td><strong>Selection classification</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Driver mutation</strong></td>
<td>Gene whose mutation is subject to tumourigenic selection</td>
<td>Myc, K-Ras, PIK3CA, RB1, P53</td>
</tr>
<tr>
<td><strong>Passenger mutation</strong></td>
<td>Gene mutated in cancer that does not drive tumourigenesis</td>
<td>Approximately 99% of all mutations in cancer</td>
</tr>
<tr>
<td><strong>Epigenetic classification</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Epigenetic modulator</strong></td>
<td>Gene that activates or represses epigenetic machinery in cancer</td>
<td>K-Ras, APC, P53, IDH1/2</td>
</tr>
<tr>
<td><strong>Epigenetic modifier</strong></td>
<td>Gene that modifies DNA methylation or chromatin structure in cancer</td>
<td>ARID-1A/-1B/-2, DNMT3A, PBRM1, BRD4</td>
</tr>
<tr>
<td><strong>Epigenetic mediator</strong></td>
<td>Gene regulated by an epigenetic modifier in cancer, increasing survival</td>
<td>OCT4, NANOG, SOX2, KLF4</td>
</tr>
</tbody>
</table>

APC, adenomatous polyposis coli; ARID, AT-rich interaction domain; BRD4, bromodomain containing 4; CDKN2A, cyclin-dependent kinase inhibitor 2A; DNMT3A, DNA methyltransferase 3A; IDH, isocitrate dehydrogenase; KLK4, Kruppel-like factor 4; PBRM1, polybromo 1; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit A; RB1, retinoblastoma 1; SOX2, sex-determining Y-box 2; STAT, signal transducer activator of transcription; VHL, von Hippel–Lindau tumour suppressor; WT1, Wilms tumour 1. Adapted from [29].
and the genetic–epigenetic relationship being somewhat like a hall of mirrors. Greater understanding of this area over the coming years will inevitably improve both the diagnosis and therapy of many malignancies.

1.3.4 Multistage Tumourigenesis

At the cellular level, tumourigenesis is commonly viewed as a multistep process involving a series of mutations and genetic (and epigenetic) changes, resulting in the persistence and survival of cells with a selective growth advantage, culminating in a transformed phenotype, malignancy and the ability to form metastatic deposits. The initial stage in tumourigenesis is termed initiation, the result of a genetic alteration of a single cell, primarily a carcinogen-induced gene mutation, leading to abnormal proliferation and persistence of a single cell. At this stage, this ‘initiated cell’ is neither a tumour nor a cancer and is purely a precursor lesion with a selective advantage. In theory this cell could be deleted or removed (such as by host defence against an ‘alien’ cell), but in terms of malignancy remains and is subject to further insults, although still being subject to Darwinian selection. The second stage in tumour development is termed tumour promotion, wherein increased cell division and further genetic (and epigenetic) changes lead to development of a proliferative tumour cell population. Additional mutations and epigenetic modifications over a period of time in this cell population, during which they gain a more aggressive and malignant potential, is the final stage of tumourigenesis, referred to as tumour progression. Overall, this continual acquisition of mutations, and genetic and epigenetic modifications will have both advantageous and deleterious effects on the cells, eventually leading to a resultant tumour cell population through clonal selection. This latter process is repeated multiple times during tumour progression, as the growing tumour cells gain genetic instability and the accumulation of mutations and further tumourigenic selection pressures accelerates. At the molecular level, tumour progression is associated with many steps and the gain of multiple alterations in several proto-oncogenes and tumour suppressor genes, accumulating independently in different cellular sub-clones, with ‘successful’ tumours eventually surviving. It is therefore important to realise that, despite cancer being monoclonal in origin (from a single ‘initiated’ cell), the ‘final’ cancer contains a mixed (heterogenous) collection of cells.

The selective growth advantages endowed upon the tumour cell are the result of driver mutations and other driver genetic changes (translocations etc.), each of which in physiological terms arms the cell with a marginal survival advantage (Table 1.3.2) [6,38]. Over time, despite individually only providing a small selective growth benefit, this slight advantage will culminate in a large malignant mass [6]. In addition to the gain of these key genetic changes, the tumour will also receive many more passenger mutations which, although having no defined effect on malignant development, may be silent, non-functional, or facilitate a driver change [6,38]. By way of explanation, driver gene mutations in the APC tumour suppressor result in truncation within the protein N-terminal (the start of the protein) affecting several interactions of this protein, whereas missense mutations later in the gene or modifications which cause truncations within the C-terminus are classified as passenger gene mutations [6].

The inclusion of passenger mutations in reported genome analyses explains why a colorectal cancer in a geriatric patient has a significantly greater number of mutations
in comparison to a morphologically identical colorectal tumour in a middle-aged patient. Similarly, the detection of fewer mutations in pancreatic and brain tumours (glioblastomas) relative to colorectal tumours supports the driver and passenger mutation concept as, unlike colorectal epithelia, both pancreatic epithelia and glial cells are predominantly quiescent (non-proliferative) [6].

A major hindrance to cancer treatment is the development of progressive resistance to chemotherapy, often evident with tumour recurrence. This acquired resistance to chemotherapy is a consequence of either the selection of tumour sub-clones carrying a pre-existing resistance mutation (acquired during tumour promotion and progression), or the development of a resistance mutation arising in cells that survived the initial therapy (indicative of continual tumour progression and gain of further mutations), a concept exemplified by inhibitors of epidermal growth factor receptors (see Section 2.5) [39].

1.3.4.1 Multistage Tumourigenesis in Colorectal Cancer

The pathogenesis of colorectal cancer illustrates the multistage development of human cancer, through the acquisition over time of several mutations and epigenetic changes as the tumour develops from an initiated cell to a full-blown malignancy. Although the initiating cell (the cause of which can be one of many carcinogenic insults) is invariably unidentifiable, it leads to increased proliferation of the colonic epithelium and allows the cells to become a clone armed with a selective growth advantage. The most common (and thus earliest) mutation observed in colorectal carcinoma is found in the tumour suppressor *APC* gene [40]. As described previously, the loss of this gene is the underpinning facilitator of FAP.

Tumour development is promoted in the resultant slow-growing small adenoma through gain of a second mutation in another gene, such as the K-Ras oncogene, which leads to an acceleration of growth and increased adenoma size [40,41]. This tumour promotion and progression then continues through the mutation (or genetic and epigenetic alteration) of genes such as the *PIK3CA* oncogene or the tumour suppressor genes *p53* and *SMAD4* [40,41]. Finally, the tumour becomes malignant and gains the potential to spread to regional lymph nodes and metastasise to other organs. Although described in a linear fashion (Figure 1.3.6), following the initiating *APC* mutation, it is the accumulation of genetic changes and modulation of key pathways which leads to the malignancy, with the order of their change largely irrelevant.

1.3.5 Oncogene Addiction

It is fully accepted that tumourigenesis is a multistep process initiated and progressed by a series of driver mutations and changes in oncogenes and tumour suppressor genes. Despite disruption of many pathways throughout this process, the reversal of a few of these abnormalities can profoundly retard the malignant phenotype, a phenomenon referred to as oncogene addiction [42,43].

A clinical example of oncogene addiction is exemplified by chronic myeloid leukaemia (CML), involving the *BCR-ABL* oncogene. Addiction of CML to BCR-ABL was demonstrated
in patients through the exquisite clinical response achieved with the kinase inhibitor imatinib (targets BCR-ABL) [44]. Furthermore, patients with CML who initially responded to imatinib, but then relapsed (and no longer showed a response to the drug) were shown to express a mutation in the kinase domain of BCR-ABL, which eliminates the response to imatinib [43]. This selective pressure in CML indicates the dependence of this tumour type on a specific oncogene, in this case BCR-ABL, and the ‘addiction’ towards a particular gene within the tumourigenic process. Further examples of oncogene addiction are provided through therapeutics targeting oncogenic kinases, which demonstrate single-agent efficacy against their respective tumour types, as indicated in Table 1.3.3. In these particular cases, the specific target kinase has multiple roles in many complex interacting pathways, with its inactivation affecting multiple pathways and thus being the Achilles heel of multiple key driving factors of tumourigenesis [43,44]. The implications of oncogene addiction and its application for molecular targeted therapeutics is further addressed in subsequent chapters.

1.3.6 Hallmarks of Cancer

Just to recap, cancer encompasses a range of diseases, characterised by the loss of regulated cell growth, leading to the growth of a cell mass with the potential to invade surrounding tissues and migrate to new sites via a process known as metastasis. Multiple successive genetic alterations caused by various external or internal processes gradually lead to the loss of the cells’ ability to maintain normal cellular functions and regular growth patterns; a process termed tumourigenesis. In principle, this description of cancer is fundamentally correct but, although it indicates key oncogenes and tumour suppressor
genes, it does not offer explanations regarding the molecular characteristics, or indeed the actual mechanisms, of cancer development. In essence, it lacks the detail required to allow us to understand cancer development and, importantly, strategies for therapeutic intervention.

Over the last few decades many oncogenes and tumour suppressor genes have been discovered, with relationships and dysfunctions often identified across several cancer types (as we described earlier in this chapter). This ever-increasing mountain of knowledge led to the concept that there are common affected traits between cancers, resulting in the suggestion that fundamental physiological and cellular changes must exist in the process of tumorigenesis. Based on this, it was proposed that cancers require a manifestation of six essential alterations, known as the hallmarks of cancer (Figure 1.3.7) [46]:

1) **limitless replicative potential**
2) **self-sufficiency in growth signals**
3) **insensitivity to anti-growth signals**
4) **evasion of cell death**
5) **development of sustained angiogenesis**
6) **ability to invade and metastasise.**

A decade later **two** further hallmarks were added (Figure 1.3.7) [47]:

1) **de-regulation of cellular energetics**
2) **ability to avoid destruction by the immune system.**

As were **two enabling characteristics** [47]:

3) genome instability
4) tumour-promoting inflammation.

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**Table 1.3.3** Examples of targets of oncogene addiction and the associated targeted therapy (adapted from [44,45]).

<table>
<thead>
<tr>
<th>Oncogene target</th>
<th>Cancer type</th>
<th>Therapeutic agent</th>
</tr>
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<tbody>
<tr>
<td>c-Kit</td>
<td>Gastrointestinal stromal tumour</td>
<td>Imatinib</td>
</tr>
<tr>
<td>VEGF</td>
<td>Breast, colorectal, kidney</td>
<td>Bevacizumab</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Kidney</td>
<td>Sorafenib</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>Chronic myeloid leukaemia</td>
<td>Imatinib</td>
</tr>
<tr>
<td>HER-2</td>
<td>Breast</td>
<td>Trastuzumab</td>
</tr>
<tr>
<td>EGFR</td>
<td>Non-small cell lung cancer</td>
<td>Gefitinib, erlotinib</td>
</tr>
<tr>
<td>EGFR</td>
<td>Head and neck, colorectal</td>
<td>Cetuximab</td>
</tr>
<tr>
<td>EGFR</td>
<td>Pancreas</td>
<td>Erlotinib</td>
</tr>
<tr>
<td>BRAF</td>
<td>Melanoma</td>
<td>Vemurafenib</td>
</tr>
<tr>
<td>RET</td>
<td>Medullary thyroid</td>
<td>Vandetanib</td>
</tr>
</tbody>
</table>

c-Kit, stem cell factor receptor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; HER-2, human EGFR-2; EGFR, epidermal growth factor receptor.
Despite the complex nature of cancer, it is often simplistically characterised as the uncontrolled growth of cells with the consequent development of a tumour. However, uncontrolled cell growth is far from simple and results from the deregulation of a number of closely controlled features. These include the prevention of replication limiting features, the development of cellular insensitivity to anti-growth signals, and the evasion of programmed cell death.

1.3.6.1 Limitless Replicative Potential

In non-tumourigenic cells, the number of cellular divisions each cell can undergo is normally restricted. This limited replicative capacity is a feature regulated by chromosomal shortening, associated with cellular growth. Chromosomes are capped by specialised DNA (and DNA-binding proteins) known as telomeres, which are degraded progressively each time the cell undergoes replication. Once telomeres reach a specific shortened length, the inherent DNA repair machinery detects them, leading to cell cycle arrest and senescence. Furthermore, the ensuing genomic instability associated with shortened telomeres and the concomitant activation of repair mechanisms also hinders mitosis, which eventually culminates in mitotic catastrophe and further initiation of cell death processes.
Cancer cells must be capable of renewing telomeres in order to ensure their continued reproductive capacity. The enzyme telomerase extends the telomeric DNA at the very tips of linear chromosomes by the addition of a short (G–T)-rich repetitive DNA sequence, thus preventing the loss of genetic material in proliferating cells. Under normal circumstances, telomerase activity is progressively downregulated during embryogenesis. However, in cancer this enzyme becomes re-activated, resulting in the maintenance of telomeres and overriding of the normal physiological limitation on cellular divisional lifespan [48,49]. Although telomerase activity is present in the majority of human cancers, approximately 10% of tumours do not show telomerase activity. In this latter case, telomere length is maintained via a recombination-based mechanism (alternative lengthening of telomeres, ALT) [48–50]. The central involvement of telomerase in human cancer development subsequently led to strategies to develop inhibitors of telomerase activity, which, unfortunately, have had little success to date [49,51,52].

Several attempts have been made to link telomerases into the car analogy of cancer, with limited success, but our attempt would be that, if an oncogene is the accelerator and tumour suppressor genes are the brakes, then telomerase activation is the addition of indestructible tyres (i.e. they never need replacing, allowing the car to constantly keep going).

1.3.6.2 Self-sufficiency in Growth Signals

Cancer cells employ several strategies to drive proliferation and overcome insensitivities to normal growth regulation, with the ultimate aim being to move all cells into, and then through, the cell cycle, culminating in production of new daughter cells. To appreciate the molecular characteristics of this hallmark, it is important to appreciate the normal cell cycle and its high-level regulation and control. This cell replicative process is stimulated by a wide range of external parameters, including growth factors (e.g. epidermal growth factor; EGF), the growth environment (adequate nutrients and oxygen etc.), and the extracellular matrix (ECM) (e.g. cellular integrin interactions). The cell cycle (Figure 1.3.8) is divided into four phases: the gap 1 phase (G₁), the synthesis phase (S), during which DNA is synthesised, the gap 2 phase (G₂), and the mitotic phase (M), during which the cell undergoes mitosis. Each cell cycle phase is dependent upon the correct activation and completion of the preceding one. At key phase intersections in the cell cycle, specifically G₁–S, and G₂–M, checkpoints exist to confirm integrity and evaluate the suitability of the cellular environment. During tumourigenesis, dysregulation of the cell cycle checkpoints, through mutations or genetic aberrations, accelerate the accumulation of further mutations, leading to oncogene activation and tumour suppressor gene inactivation.

The cell cycle and integral checkpoints are very tightly controlled, with highly regulated and sequential molecular mechanisms. The cycle is regulated by two distinct classes of protein, the cyclins and the cyclin dependent kinases (CDKs), which are concerned with orderly progression through the cell cycle. The cyclins (named because of the cyclic nature of their production and degradation) are expressed sequentially from the G₁ through to the M phase (in the order cyclins D, E, A and then B) and form defined complexes with specific CDKs, which then phosphorylate (and activate) regulatory proteins associated with the proliferative process. For instance, the key protein involved in the G₁–S phase transition is retinoblastoma protein (a tumour suppressor,
There is an additional level of cell cycle regulation within normal cells through the activity of proteins called CDK inhibitors (CDKIs), which work to restrict the activity of the CDKs. These proteins demonstrate either broad selectivity for CDKs (e.g. p21\textsuperscript{WAF1} and p27\textsuperscript{kip1}) or are selective for certain CDKs, such as the selectivity of p16\textsuperscript{INK4A} for cyclin-CDK4 and cyclin-CDK6.

In normal cells, anti-proliferative signals, such as TGF-\(\beta\), serve to maintain cells within a quiescent state. These extracellular signals lead to the inhibition of transcription factors via the retinoblastoma protein, thereby activating the G\(_1\) checkpoint and halting the progression from the G\(_1\) to the S phase of the cell cycle (Figure 1.3.8).

During G\(_1\) the cell undergoes a period of preparation for DNA synthesis. At the end of this stage the cell reaches the G\(_1\)–S checkpoint, at which the decision is made whether to progress into S phase or withdraw from cellular replication and enter into quiescence (G\(_0\) phase). This is an important decision point (and rate-limiting step) within the cell cycle, as progression past the G\(_1\)–S checkpoint places the cell in an irreversible commitment to DNA replication. This checkpoint therefore serves as a quality control step to

**Figure 1.3.8** Molecular regulation of the cell cycle. The cell cycle comprises four (or five, if G\(_0\) is included) phases, each regulated by the concerted activity of specific cyclins and CDKs. Binding of cyclin D to CDK4/CDK6 progresses the cell through the initial gap phase (G\(_1\)) in preparation for DNA synthesis. Through G\(_1\), cyclin D–CDK4/6 levels diminish and levels of cyclin E–CDK2 increase. The cell then arrives at the G\(_1\)–S checkpoint, where an assessment of ‘readiness’ and DNA integrity is completed, prior to progression into the DNA synthesis phase (S phase). Degradation of cyclin E and release of CDK2 initiate S phase. Progression through S phase is achieved by cyclin A–CDK2, thereafter the cell enters the second gap phase (G\(_2\)) involving cyclin A–CDK1. The cell reaches a second checkpoint (G\(_2\)–M) to verify successful DNA synthesis and mitotic readiness. The level of cyclin B increases at the start of mitosis and diminishes at end of the M phase, with the inactivation of CDK1 due to decreasing cyclin B triggering completion of the cell cycle. Throughout the cell cycle, the activity and function of CDKs is further kept in check by endogenous inhibitors (p16\textsuperscript{INK4A}, p27\textsuperscript{kip1} etc.). After the cycle is completed, the cell either undergoes another replicate cycle or withdraws and enters quiescence (G\(_0\) phase).
assess the integrity of DNA and availability of the required building blocks and materials before DNA replication commences. If DNA damage is detected, a signal is transmitted via a number of protein kinases to trigger DNA repair mechanisms, with failure to progress through the G1 checkpoint allowing the cell an extended time in which to undergo pre-replicative repair of DNA. However, if the extent of DNA damage is too great, the cells are triggered to undergo programmed cell death (apoptosis) or exit the cell cycle and into a permanent G0 quiescent phase. The decision point primarily involves the guardian of the genome p53 tumour suppressor protein, a transcription factor and central regulator of a wide range of cellular processes, including DNA repair, senescence, cell cycle arrest, and cell death, as discussed above [24,26,27].

DNA integrity is further monitored on reaching a second cell cycle rate-limiting step after DNA synthesis, the G2–M checkpoint [54]. At this point, the integrity and success of DNA replication is assessed (as well as the suitability of cellular environment) and a decision is made as to whether or not the cell can safely enter mitosis, undergo cytokinesis, and create two new daughter cells. Conversely, the presence of DNA damage or errors also triggers a cell cycle arrest and potentially initiation of cell death [54], again regulated by p53 and analogues [24,26].

The tumour suppressor, p53, is a key regulator of both the G1–S and G2–M checkpoints; p53 is, however, inactivated in over half of clinical cancers, allowing cells with damaged DNA to replicate and produce daughter cells carrying these uncorrected DNA modifications [24,26,27]. Consequently, the mutation of p53 reduces the rate of DNA damage-induced cell death, whilst increasing the likelihood of the transmission of DNA faults through an imbalance in p53-mediated functionality [24,26,27]. This, however, raises the question as to who regulates the regulator, and how p53 receives its mutations; does it not self-check? Alternatively, is the persistence of mutations in p53 a consequence of another errant regulatory process in tumourigenic cells?

A core principle in the pathophysiology of cancer is uncontrolled cellular proliferation (analogous to a car accelerator and brakes), mediated through activation of CDKs and concomitant inactivation of inhibitory processes, promoting increased (unregulated) cell-cycle progression [53,55]. The most common clinical alterations target inactivation or overriding of the G1–S checkpoint, with amplification of the CDK4 and CDK6 genes and overexpression of cyclin D evident in several tumour types [55,56]. Furthermore, inactivating mutations of the CDKi are also common tumourigenic drivers, with p16INK4A disabled in many human malignancies [57]. As a consequence, therapeutics targeting CDKs (analogous to a CDKi), especially CDK4 and CDK6, are a major strategy in the drug pipeline, with several under clinical evaluation [53].

1.3.6.2.1 Independence from Growth Factor Regulation

Cellular proliferation is managed through the receipt of growth factors and subsequent stimulation of signalling pathways, with the tyrosine kinase class being the predominant players in this area (e.g. EGF, VEGF etc.). Under normal circumstances, the binding of these factors to the specific receptor on the cell surface (e.g. EGFR) provides transient receptor activation, with the signal thereafter being transduced from the cell membrane to the nucleus via a series of signal transduction molecules (e.g. RAS and the mitogen-activated protein kinase (MAPK) pathway). These molecular signals then activate and initiate DNA transcription through proteins such as MYC, culminating in activation of the cell cycle and cellular division.
Under normal circumstances, the vast majority of proliferation-inducing growth factors are synthesised by a particular cell type within the respective tissue, acting in a **paracrine** fashion to stimulate proliferation of another cell type. This affords a further level of control and proliferative security as the cell providing the growth factor does not normally express the respective receptor, and vice versa. However, a major mechanism by which cancer cells acquire self-sufficiency is through gaining the ability to both synthesise and respond to a particular growth factor, creating an **autocrine** loop. In a second tumourigenic mechanism, some cancer cells interact with their microenvironment and falsely stimulate normal cells to produce growth factors and subsequently promote tumourigenesis. In each scenario, cells gain the ability to respond to elevated production of the growth-inducing factor. In terms of the cancer car scenario, this is equivalent to the car gaining the ability to make its own fuel and thus become independent of the filling station.

Further mechanisms associated with this cancer hallmark (self-sufficiency in growth signals) include the gain of an activating mutation (creating an oncogenic protein) in the receptor or the increased expression of the receptor (gene duplication or chromosomal translocation). In these situations, cells either override the normal proliferative control mechanisms (and gain independence from the external growth factor) or are rendered hyper-responsive to levels of the growth factor that would not normally trigger proliferation. A clear example of these mechanisms is provided by the ErbB superfamily, including EGFR (ErbB1) HER2/NEU (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). EGFR is overexpressed in over 50% of colorectal cancers, 30–50% of pancreatic cancers, and the vast majority of lung cancers (Figure 1.3.9). Similarly, HER2/NEU has elevated expression in approximately 30% of breast, lung, and ovarian cancers. These tumours are exquisitely sensitive to EGF, with a small concentration causing a disproportionally large proliferative response. Furthermore, elevated expression of this receptor family associates with a poor prognosis in several cancer types [58].

A further common mechanism associated with autonomy to growth signals is through genetic alterations or epigenetic modifications of genes linking growth factor receptors to the effector mechanisms of mitogenesis in the nucleus. Such changes activate the downstream signalling pathway, effectively mimicking the growth-promoting effects of growth factor pathway activation. Key examples in this context are activation of the oncogenic protein BRAF, mutated in the vast majority of melanoma, and mutation of the **RAS** proto-oncogene to oncogenic **RAS** (the driver in over 30% of all cancers), which stimulates downstream regulators of proliferation by both the PI3K/AKT and MAPK pathways, the common pathways of tyrosine kinase-mediated proliferation [59]. In essence, **RAS** mutations fix the protein in an active conformation and the cell in a continually proliferating state.

The final stage of the mitogenic signal transduction pathway is the stimulation of nuclear transcription factors, leading to the regulated expression of genes related to orderly cell cycle progression. Ultimately, this final process is dysregulated in cancer and the cell cycle is continuously stimulated. Autonomy of growth can also be due to mutations in transcription factors, such as Myc, Jun, and Fos, which regulate expression of cyclins, CDKs, and other cell cycle related genes [60]. For example, Myc is often amplified in breast, colon, and lung cancer, amongst others [60].
1.3.6.3 Insensitivity to Anti-growth Signals

In the same way cells receive signals to promote proliferation and progress through the cell cycle, they also must receive messages to inhibit, retard or leave the cell cycle (enter G<sub>0</sub>). Effectively these signals are provided by tumour suppressor genes (opposing effect to the oncogene-mediated promotion of proliferation), with their disruption making the cells refractory to their growth inhibitory activity and theoretically making them mimic the effects of oncogene activation. Consequently, the description of this hallmark and its implications are principally the same as those discussed for a tumour suppressor gene, exemplified by retinoblastoma and p53.

In normal cells there are several mechanisms by which these anti-growth signals function, but ultimately they all prevent progression past the G<sub>1</sub>–S or G<sub>2</sub>–M checkpoint in the cell cycle. Central to regulation of transit from the G<sub>1</sub> to the S phase is the retinoblastoma protein (Rb), a DNA-binding protein that exists as either an ‘inactive’ hyperphosphorylated state or an ‘active’ hypophosphorylated state. As discussed earlier regarding the cell cycle, initiation of DNA synthesis (entry into the S phase) is mediated principally by cyclin E/CDK2. At the beginning of G<sub>1</sub>, Rb is hypophosphorylated and bound to the E2F transcription factor (which is held away from DNA and other interacting proteins). Progression through this phase of the cell cycle is mediated by cyclin D bound to CDK4 and CDK6, which phosphorylate Rb,
1.3 Cellular and Molecular Basis of Cancer

releasing E2F to induce the genes required for cell cycle progression. The Rb protein is then dephosphorylated as cells move through mitosis, regenerating hypophosphorylated Rb ready for subsequent entry to a further cell cycle. This unequivocally shows that Rb is centrally important and the linchpin of the control of the cell cycle. Therefore, damage or loss of Rb (or one of the factors driving its activity, such as cyclin D or CDK4) permits cells to enter the S phase unchallenged, driving tumourigenesis and acquisition of further mutations and genetic changes. In effect, loss of the Rb tumour suppressor regulatory capacity equates to the insensitivity of antigrowth signals, analogous to loss of the cancer car-braking system.

Although the principles of this hallmark and its relationship to cell cycle mechanistic regulation are fairly straightforward, there are also several external biological factors that provide growth inhibitory signals. A prime example in this context is TGF-β, a potent inhibitor of proliferation through activation of dedicated TGF-β receptors. Stimulation of this pathway causes transcriptional activity of CDKi which inhibit the activity of the CDKs and thus prohibit passage of cell-cycle checkpoints. In terms of cancer, mutations to proteins within the TGF-β pathway are a common occurrence, often in either the receptor itself or the SMAD signalling proteins that convey the growth inhibitors signal from receptor activation to the cell-cycle regulation [61]. For instance, SMAD4 inactivation is a common occurrence in colorectal cancer (Figure 1.3.6).

Interestingly, although TGF-β clearly demonstrates tumour suppressive activity in the earlier stages of cancer, current evidence suggests that perturbation of this pathway may also play a role in promoting tumour progression and metastasis in later stages of tumourigenesis [61,62]. This conflicting activity within the same pathway illustrates the complexity of tumourigenesis and the care that needs to be taken when considering and approaching cancer diagnosis, development, and therapy in a linear fashion [62].

An alternative mechanism by which cells moderate growth promotion and inhibition is through a process termed contact inhibition, whereby cell–cell contacts in confluent (complete cell coverage) populations act to inhibit further cell proliferation. In cancer, this contact–inhibition mechanism is non-functional, allowing cells to continue growing beyond the physical cellular boundary (evidenced in the laboratory wherein cancer cells continue growing and pile up on top of one another). Although the actual mechanism of contact inhibition is not yet fully resolved, it is known to be initiated by cadherin intercellular junctions and the transmembrane E-cadherin protein, culminating in active recruitment of CDKi (e.g. p16INKA and p27kip1) and induced expression of tumour suppressors (e.g. p53 and p27kip1) [47].

1.3.6.4 Evasion of Programmed Cell Death

Inappropriate proliferation of cells can be controlled by the tightly regulated process of programmed cell death, a term known as apoptosis (Figure 1.3.10). In this context, under normal physiological conditions, molecular signals can induce signalling complexes that, in turn, bring about nuclear fragmentation, cell membrane blebbing, and formation of apoptotic bodies. These pathways are initiated as a response to extracellular stresses such as limitation of growth factors, oxygen or nutrients, or intracellular stresses such as DNA damage or telomere shortening [63]. In cancer cells, defects in these pathways confer resistance to apoptosis, thereby allowing unchecked cellular replication.
Figure 1.3.10 Extrinsic and intrinsic pathways of apoptosis. Cellular stress (e.g. DNA damage by radiotherapy/chemotherapy) activates the intrinsic pathway via p53; pro-apoptotic Bax and Bak subsequently permeabilise the outer mitochondrial membrane, resulting in efflux of cytochrome c, which binds to the adaptor Apaf-1 to recruit the initiator procaspase 9 into a signalling complex termed the apoptosome. Activated caspase 9 then cleaves and activates the effector caspases 3, 6, and 7 to trigger apoptosis. Cytotoxic immune cells produce pro-apoptotic ligands such as TNF-related apoptosis-inducing ligand (TRAIL), which binds to the pro-apoptotic death receptors (DR4 and/or DR5) on the surface of a target cell. Ligand binding induces recruitment of an adaptor protein and the initiator caspases 8 and 10 as pro-caspases, forming a death-inducing signalling complex. This eventually triggers the activation of the effector caspases 3, 6, and 7. (Constructed using protein structures from the PDB, IDs: 1NQL, 1TUP, 2BID, 2M0B, 2ITX, 4NBL, 4S0P, 5CIR, 5I9B, 5ITD, 5IY5, and 5FMJ.)
As you may expect, programmed cell death has to be a tightly controlled and regulated process. If it wasn’t so highly regulated, we would all be subject to inappropriate loss of cells, possibly ending up full of holes and becoming a bag of jellified mess! However, it is equally important that any damaged cells are removed from the body before they can do further damage, such as development of cancer, so alternatively we can’t survive without programmed cell death.

There are two apoptosis pathways, responding to **external (extrinsic)** and **internal (intrinsic)** signals, which both culminate in activation of degradative enzymes (termed caspases) and orderly dismantling and deletion of the cell [63]. The **extrinsic** pathway is mediated by cell-surface receptors, predominantly of the tumour necrosis factor receptor (TNF-R) family, such as Fas (CD95), TNF-receptor 1, and the TNF-related apoptosis-inducing ligand receptors (TRAIL-R1/death receptor 4 [DR4] TRAIL-R2/DR5) [63,64]. In normal cells, activation of the extrinsic pathway is central to maintaining T-cell homeostasis and deletion of unrequired T-cell clones [64]. Alternatively, the **intrinsic** pathway responds to many cellular stresses, such as cellular injury and replicative mistakes (e.g. DNA damage), cellular stresses (e.g. hypoxia), and biochemical insult (e.g. presence of reactive oxygen species). This pathway is centrally mediated through the mitochondria, with the integrity of the mitochondrial membrane being a site of regulation for this pathway [63,65]. Loss of integrity causes the release of cytochrome c, a protein involved with cellular respiration and located between the inner and outer mitochondrial membrane, which acts as the initiating protein for the intrinsic pathway. Gates in the mitochondrial membrane permitting cytochrome c to escape are regulated by specific door-keepers belonging to the Bcl-2 family [63]. Mechanistically, pro-apoptotic members (e.g. Bax) form these pores, with their action counteracted by anti-apoptotic family members (e.g. Bcl-2, Bcl-XL), a fine balance which can both promote and prevent the initiation of apoptosis [65–67]. Once released, cytochrome c activates a chain of molecular events involving a cascade of proteolytic caspases, which converges with the extrinsic pathway at caspase-3 and ultimately orderly cell disassembly. With regards to cancer development, there are several sites within these pathways which are common sites for tumourigenic intervention, not least overexpression of anti-apoptotic proteins (as is the case with B-cell lymphomas [66,67]), down-regulation of cell-death receptors, or up-regulation of the many inhibitory proteins within these pathways. In the context of cancer treatment, multiple molecular therapeutic strategies are centred around these pathways, such as pro-apoptotic mimetics, inhibitors of anti-apoptotic proteins, and activators of cell surface death receptors [66,67]. Therefore, in the car analogy, programmed cell death can be perceived as the service warning light, with cancer cells ignoring this warning and refusing to have a mechanical service or to go to the scrapyard.

### 1.3.6.5 Angiogenesis

Up until this point, we have learned that cancer grows – both in size and aggressiveness – due to increasing its rate of division, avoiding inbuilt limitations on cell lifespan, becoming self-sufficient towards growth-promoting factors, lacking responsiveness to growth inhibitory pathways, and, finally, ignoring signals toward programmed cell death (apoptosis). Effectively the cancer car is **accelerating fast**, has **no brakes**, is fitted with **indestructible tyres**, **doesn’t need a driver**, and isn’t being **mechanically serviced**.
However, despite all of these factors the car may still be sitting in the garage as none of these hallmarks facilitate mobility. For the same reasons a car needs fuel and regular access to a filling station, all cells need access to nutrients and oxygen and removal of waste products to exist and function.

For cells to achieve (i.e. have plenty of food, drink and oxygen, and be able to remove waste), they must be within close proximity of a blood (or lymphatic) vessel, to allow maximal diffusional delivery or clearance of these factors. Like normal cells, this concept is also applicable to cancer, as tumours cannot grow to a diameter larger than 1–2 mm unless they have a vascular supply [68,69]. The difference that exists with cancer is that the ever-increasing tumour mass subsequently needs a larger and larger blood supply to support it. To fulfil this requirement, the tumour mass has to gain the ability to initiate something called angiogenesis, which is where new blood capillaries sprout from pre-existing blood vessels [70]. The resulting tumour vasculature, due to rapidity of tumour growth, is, however, abnormal, poorly organised, chaotic, and leaky [70]. Furthermore, in addition to facilitating and supporting tumour growth and expansion, the newly developed neovasculature increases the escape routes for tumour cells and, by default, increases tumour malignancy and the spread of tumour cells to other parts of the body (metastasis). Therefore, with regards to the cancer car, angiogenesis is analogous to the creation of an ever-increasing fuel supply and provision of filling stations (including lavatories to get rid of the waste!). In other words, it is assumed that as the cancer car increases in speed, it simultaneously consumes more fuel and thus needs more and more filling stations. Furthermore, because the route the car is taking is random, there will need to be filling stations on all of the roads the car could travel (some roads will be dead ends, while others may have difficulties with access, and other roads may lead back to the starting point). So how does the tumour develop this new blood supply? Is it just due to inquisitive blood vessels or does the tumour beckon the blood vessels towards it? The angiogenic process (both physiological and in tumours) involves a number of stages, controlled by a fine balance between pro-angiogenic and anti-angiogenic factors [70]. The ensuing hypoxia developing in the tumour as it reaches the critical size limited by diffusional restraints, prevents destruction of the oxygen-sensitive transcription factor, hypoxia-inducible-factor 1 (HIF-1α), which subsequently relocates to the nucleus and initiates expression of VEGF. Therefore, until the tumour mass reaches the critical size associated with supply and demand there is no requirement for further blood vessels. Once the tumour hits this critical size the angiogenic switch is triggered, allowing the balance of pro-angiogenic factors to exceed anti-angiogenic factors [70].

The elevated production and secretion of VEGF from tumour cells and the surrounding microenvironment activates VEGFRs on the endothelial cell surface, leading to increased permeability and vasodilation, loosening of endothelial cell contacts, and finally formation of endothelial fenestrations [70]. Simultaneously, increased levels of VEGF (and other pro-angiogenic factors, such as basic fibroblast growth factor, bFGF) stimulate the release of proteolytic enzymes, including the serine protease plasmin and several zinc-dependent matrix metalloproteinases (MMPs). The cleared pathway created by these proteases permits the migration of the proliferative endothelial cells in the direction of the tumour mass, up a gradient of chemokinetic factors released from the tumour, forming migration columns and primitive blood vessels [70,71]. Completion of the column between the established vessel and the tumour mass leads to endothelial cell
differentiation, involving morphological alterations and resultant adherence of these cells to form the lumen of the vessel. Finally, the newly developed vessels are stabilised via recruitment of pericytes (smooth muscle-related cells) to the external surface of the endothelium. However, in tumour angiogenesis there is a decreased association of pericytes with the newly formed and immature vessels (compared to mature normal blood vessels), providing an explanation for the observed irregularly structured, leaky blood vessels and continually proliferating endothelial cells observed in tumours [70].

1.3.6.6 Metastatic Potential

Uncontrolled cell growth is not necessarily hazardous to the wellbeing of the patient. Indeed, benign tumours grow without the ability to invade neighbouring tissues or relocate from their resident location to another site within the body. However, by definition, malignant cancer cells exhibit the ability to spread, show invasive potential, and, ultimately, metastasise to distant regions of the body [47], As we have described, metastasis is the most important cause of cancer mortality and, if present, dramatically worsens the prognosis for the patient compared to if the cancer was diagnosed prior to metastatic spread. Unfortunately, it is often the case that metastasis has already begun to occur by the time the primary cancer has been diagnosed (lung cancer [Section 3.4] is a good example of this).
In effect, cancer metastasis is the ultimate finale, comparable to the open road in the cancer car analogy. In reality, it is probably a lot more severe than providing the tumour with quaint country roads, and is more comparable to giving the cancer car access to go anywhere, only limited by physical access (and angiogenesis, i.e. fuel and filling stations). Effectively metastatic capability and the access anywhere potential of the cancer car can be summed up by the quote by Dr Emmett Brown in the 1980s film *Back to the Future*: ‘Roads? Where we’re going, we don’t need roads.’

The process of metastasis is a series of rate-limiting steps that must occur in order for cancer to disseminate to sites other than the primary tumour. Tumour metastasis follows a set pattern of processes:

i) The primary tumour forms an avascular mass acquiring its nutrients from the host tissue. Tumour expansion increases hypoxia within this mass, a major driver of several elements of the metastatic process [72], leading to activation of the angiogenic switch. The resultant vascularisation of the tumour increases its scope for expansion and, ultimately, malignancy.

ii) Next, as the tumour grows it acquires further genetic aberrations which provide it with the potential and tools to gain mobility and often trigger a conversion and transition from an epithelial to mesenchymal (EMT) phenotype. The EMT is a complex pathway during which epithelial cells begin to lose their differentiated characteristics and acquire mesenchymal features, which include motility, invasiveness, and resistance to apoptosis [47,73]. Currently, it appears that EMT-inducing transcription factors can coordinate the majority of steps in the invasion/metastatic cascade [47,73].

iii) The acquisition of mobility and the ability to invade through tissues eventually results in the tumour cell(s) breaching the local basement membrane and transiting towards blood and lymphatic vessels.

iv) Tumour cells can then intravasate from the invaded tissue into the circulation (or lymphatic system), wherein they adhere to endothelial cells and platelets, and eventually transit through the vasculature until becoming trapped or deposited in capillaries of another tissue, whereby they *extravasate* out of the blood vessel and into the new host tissue.

The consequent tumour deposit (now termed a micrometastases) can remain dormant at this new site until their growth is triggered, the host environment becomes conducive to re-growth, or the angiogenic-switch is triggered. As we have discussed previously, the growth environment and suitability rely heavily on the seed and soil theory, the principle being that the new host tissue must have a permissive environment for tumour growth, including an appropriate extracellular matrix, growth factors, and supportive cellular network. Therefore, if the new home for the tumour cells is acceptable, the tumour begins to grow and forms a secondary tumour.

From a molecular perspective, this hallmark is effectively two capabilities grouped together: the initial ability of tumour cells to move and invade into neighbouring tissue (local invasion) and the ability to move from one site and disseminate to another (metastasis).

### 1.3.6.6.1 Molecular Basis of Tumour Cell Invasion

For tumours to invade, the basement membrane and ECM must be proteolytically degraded to allow the tumour cells to traverse towards blood (and lymphatic) vessels. Similarly, at the other end of the metastatic process the tumour cell must have the ability...
to exit the vasculature and then ‘reverse invade’ back into the recipient tissue. As such, tumour invasion is an active and multi-staged process. The first stage in invasion is for the tumour cells to break-free from their neighbours, a process commonly involving downregulation or mutation in the genes for the tumour suppressor protein E-cadherin [74]. The loss of E-cadherin, in addition to releasing the cell, also triggers EMT, as we have described above [74].

Once the tumour cell has gained freedom, the next molecular step is the degradation of the ECM and basement membrane through release of proteolytic enzymes from both the tumour cell and indirectly via tumour-cell-triggered induction of stromal cells (tumour fibroblasts and supporting cells). Although many protease families are implicated in this process, the MMPs are known to possess the ability to degrade most, if not all, components of the ECM and the basement membrane, contributing to the formation of a microenvironment that promotes tumour expansion and metastasis [75]. A number of MMPs have been associated with tumour cell invasion, as evidenced by the correlation between local tissue penetration and increased MMP levels [76]. However, the view of MMPs in cancer being ‘bulldozer’ enzymes, clearing a way through the ECM to allow cancer cells to metastasise (and angiogenesis to progress, as described above), is grossly over-simplistic. MMPs (and several other proteases) are now known to effect cancer progression by the creation of a favourable microenvironment through activation of several factors involved in signalling pathways that control cell growth, inflammation, and angiogenesis [77].

The last step in the process, once cells have freed themselves and have gained the equipment to invade and breakthrough physical cellular barriers, is the ability to move and migrate freely through tissues. This motile phenotype involves a multitude of receptors, ECM proteins and their proteolytic metabolites, integrins, and normal cellular motility factors, such as hepatocyte growth factor (HGF). Historically, it was believed that tumour cells gained these progressive traits, effected invasion through paracrine interactions with the local cellular environment, and subsequently moved through the tissue and ECM toward their metastatic escape route of blood and lymphatic vessels. It is now known that this is too simplistic, with the microenvironment of the tumour being a major contributor to successful migration and invasion [78]. The non-tumour cells coexisting with malignant cells include fibroblasts, endothelial cells, immune cells, and others such as adipocytes or myocytes, which collectively are classified as purely the tumour stroma [79]. Depending on the situation, these cells and their functional activities can promote or prevent tumour survival, cellular mobility, and, ultimately, malignancy. However, during the earlier stages of malignancy, including local invasion, evidence suggests that the protective constraints of stromal cells are often overruled with the environment functioning in a tumour-promoting fashion, influencing local invasion [78,79]. Consequently, there are many additional factors, notwithstanding the tumour cells themselves, which contribute to this complex invasive process. It may well be that the most aggressive tumours are those with the greatest relationships and command over their neighbourhood, with the ability to co-opt and convince stromal cells to help. Simplistically, these tumour cells could be the flashiest car!

1.3.6.6.2 Molecular Basis of Tumour Cell Dissemination and Metastasis

The transit of the cells through the stroma and ECM is followed by vascular dissemination of these cells to other sites. Although the most common route for metastatic dissemination
is the haematogenous circulation (bloodstream), many tumour cells will travel to their destination via the lymphatic system, but for the purposes of explanation we will focus on the former route. Following intravasation, the tumour cells (now armed with an invasive portfolio of skills) enter the big bad world of the circulatory system, where they need to evade immune detection and subsequent destruction. Fortuitously, the majority of tumour cells do not survive this challenge and are eliminated before getting to their new site [79]. The transit of tumour cells through the circulatory system commonly involves formation of tumour emboli, consisting of the tumour cell aggregating with platelets and other leucocyte cells, which, by default, cloak the tumour cells and thereby avoid immune surveillance mechanisms. If it can adapt to survive within the new environment of the circulatory system, the cell must then arrive at its target organ, extravasate back out of the blood vessel and into the new host cellular environment, and then persist and eventually re-grow into a tumour, with each of these stages being inefficient [79].

The eventual site of tumour metastasis is often a consequence of the location of the primary tumour and its vascular (or lymphatic) drainage (as discussed previously). For instance, colon carcinoma often metastasises to the liver, wherein it forms secondary tumour deposits. However, in several cases this predictable route of dissemination is not straightforward and the rule is disobeyed. One such example is metastasis of lung carcinoma to the brain, uphill from the lungs; similarly, prostate cancer commonly spreads to the bone, which again is not the initial predictable site. The reason for this is the aforementioned seed and soil theory; from a molecular perspective, the seed can have a preferred site for growth encoded through acquisition of genetic mutations and alterations through its development. Unfortunately, it is still the case that the precise route taken and the eventual localisation of metastases are not predictable for any cancer type. Apparently, tumour cells have satellite navigation with a multitude of route options – all for an unspecified location.

So, how do cancer cells find their way to the site for metastatic deposition and growth? Well, this specified directional movement of cancer cells towards the ‘promised land’ is mediated by chemokines and their receptors. Normally, the chemokines orchestrate the inflammatory response and facilitate the directed movement of pro-inflammatory cells to their required location (a process known as chemotaxis), but in cancer this process is hijacked to control site-specific migration of metastatic-competent cells [79,80]. For instance, the chemokine receptor CXCR4 is expressed at high levels in metastatic human breast cancer cells, but in normal breast tissue it is low or even absent. Concomitantly, major sites for breast cancer metastasis (lung, liver, lymph nodes, and brain) express high levels of CXCL12, the chemotactic ligand for CXCR4 [80]. Similarly, the spread of pancreatic ductal adenocarcinomas to neural systems is supported by the expression of CX3CR1 on the tumour cells and the reciprocal ligand on peripheral neurons [79,81].

Despite metastasis being a devastating outcome of cancer, it is now accepted that in many cases the disseminated tumour cells may lie dormant at their new site for many years. In several cases, it is the presence of the metastatic deposit(s) which is detected and confirms the presence of cancer in a patient; in other cases metastatic seeding may have occurred several years before diagnosis of a primary tumour. The metastatic microenvironment obviously contributes and retards the development and regrowth of the disseminated tumour, with either progressive genetic aberrations in the tumour or
perturbations in this cellular host environment being the trigger for establishment of the clinically evident metastasis. Successful tumour metastasis is therefore heavily reliant upon the tumour to adapt and respond to its surroundings at each stage of the invasive and metastatic process: the primary tumour site, invasive environment, systemic circulation, and final metastatic location.

1.3.6.7 Reprogramming Energy Metabolism

To survive and adapt, the tumour must modify and respond to the surrounding environment; this includes how it derives energy and utilises the available nutrients to this effect. The metabolic phenotype of cancer cells is comparable to that observed in other rapidly proliferating cell scenarios, with the exception that in cancer these changes are a consequence of genetic alterations and subsequent cell-autonomous signalling, rather than a response to the conventional exogenous growth-factor-mediated signalling pathways. In terms of metabolic changes, tumour cells demonstrate increased glucose uptake, elevated glutamine uptake and enhanced lipid and nucleotide biosynthesis. The high glucose consumption and increased production of lactate by cancer cells, through aerobic glycolysis, was identified almost a century ago and is termed the Warburg effect (a concept which led to Otto Warburg winning the Nobel Prize in 1931). It is now recognised that the Warburg effect is not limited to cancer, but is actually common across many situations associated with high proliferative capacity, indicating a fundamental role for this concept in supporting cellular growth.

Normally cells generate energy (in the form of adenosine triphosphate, ATP) from glucose by mitochondrial oxidative phosphorylation, producing CO$_2$ as an end product. However, energy production in cancer cells occurs mainly by aerobic glycolysis of glucose, even when there is sufficient oxygen and the cells have fully functional mitochondria. Relative to oxidative phosphorylation, which produces 36 molecules of ATP per molecule of glucose, aerobic glycolysis is an inefficient means of generating ATP, with only two molecules of ATP produced per glucose molecule. Conversely, the rate of glucose metabolism in aerobic glycolysis is significantly greater than in oxidative phosphorylation, with lactate being produced up to 100 times faster in glycolysis. Subsequently, the two processes counterbalance one another and the amount of ATP produced is comparable between oxidative phosphorylation and aerobic glycolysis [82]. In light of the poor efficiency of aerobic glycolysis, and the higher rate of glucose breakdown, tumour cells need to uptake a considerably high level of glucose in order to meet their energy requirements. This fundamental increase in glucose uptake is exploited clinically in the visualisation of tumours using PET-imaging strategies, as discussed in Section 1.2.

Most cancers do not exhibit impairment of mitochondrial energy production, supporting the concept that high aerobic glycolysis is not a consequence of the failure of normal metabolic processes nor a bystander in cancer pathogenesis, but rather a specific and deliberate adaption that supports tumour cell growth [82]. Despite the central role for the Warburg effect in cancer development, several hypotheses and molecular mechanisms have been provided for its involvement, but as yet no definitive function for the Warburg effect in cancer has been confirmed. It is, however, clear that changes promoting adoption of the aerobic glycolysis are a common phenomenon in tumour cells, with reprogramming of energy metabolism now a defined hallmark of cancer.
1.3.6.8 Evasion of the Immune System

The ability of tumour cells to avoid identification and removal by the immune system is now considered a fundamental hallmark of cancer. Since tumour cells are not, in effect, ‘foreign’, the vast majority of cancers avoid immunosurveillance and therefore naturally evade immune detection [83]. Similarly, because tumour cells are fundamentally ‘self’, any autoimmune response is, generally speaking, prevented. The tumour cells which do raise an immune response are generally associated with the expression of immunogenic tumour antigens [84], but several mechanisms have evolved to allow tumours to escape immune detection or eradication in this regard. A good example is that tumour cells can form emboli with platelets in the circulatory system to hide from normal immune surveillance. In addition, there are also many other mechanisms that can feed into this particular hallmark, primarily focused around the reduction in the presentation of tumour immunogenic markers and the subsequent invisibility to the immune system [83,84]. Additionally, some tumours have evolved to turn the immune system against itself by causing the death of the immune cells through an activation-induced cell death mechanism that normally functions to limit the immune response under physiological conditions [84]. Ultimately, the immune system is tricked into failing to detect or recognise tumour cells, or to down-regulate itself and drop below a level for adequate immune activation [83,84].

1.3.6.9 Enabling Characteristics: Genome Instability

Whereas the hallmarks of cancer (Figure 1.3.7) identified by Hanahan and Weinberg [47] define the phenotypic attributes of cancer resulting from genetic mutations and epigenetic regulation of a range of oncogene and tumour suppressor genes, these are underpinned by an enabling requirement for their appearance. Despite cells throughout the human body being exposed to a panoply of putative mutagenic factors, the rate and extent of cancer development is significantly lower than that predicted by these encounters. This is because, under normal conditions, cells are well equipped to both detect and repair DNA damage, with severe damage triggering cell removal via initiation of apoptosis [85]. In contrast, cancer cells are often deficient in a normal DNA repair function, with this deficiency allowing the tumour to develop genomic instability. In the presence of this deficiency, the tumour cell becomes more susceptible to disruption of tumour suppressor genes, generation of oncogenic fusion genes, and chromosomal aberrations, which subsequently progress and accelerate the tumour towards a more malignant state, thereby enabling development of subsequent cancer hallmarks.

The importance and significance of DNA repair as an enabling characteristic in cancer is evident from inherited forms of cancer, in which genes encoding DNA repair proteins are defective or absent [85]. One such example is xeroderma pigmentosum, associated with increased dermal tumours following exposure to the sun (ultraviolet [UV] light), a consequence of inherited loss of key DNA repair genes. In this condition, UV light initiates DNA crosslinks, preventing normal DNA synthesis. Such repair is normally effected by the nucleotide excision repair system, with loss of members of this signalling pathway being associated with development of this inherited cancer syndrome.

Other inherited syndromes which exemplify the cancer-enabling characteristic of defects in DNA repair genes include hereditary nonpolyposis colon cancer (HNPCC),
which is associated with loss of genes involved in coding the DNA mismatch repair enzyme. Typically, genomic instability arises when both copies of the respective gene are lost, with a few promoting cancer in the presence of loss of heterozygosity or haploinsufficiency.

### 1.3.6.10 Enabling Characteristics: Tumour-promoting Inflammation

Although inflammation has long been associated with tumour development, the involvement and presence of this condition is now believed to be a protective response toward the cancer, which enables malignancy. Commonly, an inflammatory component is present in the microenvironment of the vast majority of solid malignancies. This cancer-related inflammation involves cells of both the adaptive and innate immune system, including the infiltration of leukocytes, predominantly tumour-associated macrophages, and several inflammatory mediators, such as tumour necrosis factor-α (TNFα), interleukin-6 (IL-6), and chemokines, such as CXCR4 and CXCL12.

The presence of inflammatory cells within a tumour was initially perceived as being related to an immune reaction and an attempt by the host to destroy the tumour. Although this may provide a contribution towards explaining the presence of inflammatory cells within the tumour, it is now known that growth factors, chemokines, and other cellular mediators can act to promote several tumourigenic processes, such as tumour proliferation, the invasive tumour phenotype, angiogenesis, and tumour chemotaxis and motility.

An alternative involvement for an inflammatory response in predisposing to cancer is offered by those tumours associated with either persistent chronic inflammation or an autoimmune response. For instance, gastric carcinoma is associated with *Helicobacter pylori* infection, and subsequent inflammatory response. Similarly, autoimmune disorders, such colitis-associated cancers, also involve an inflammatory response, with the release of several cellular factors. In all cases there is compensatory proliferation of cells to repair tissue damage, involving several growth and cellular regulatory factors. The persistent presence of these factors has a direct effect upon the tumourigenic cells, placing the cells at risk of acquiring further detrimental mutations or exogenous growth factor pressures.

### 1.3.7 Principles of Cancer Treatment

The treatment of cancer is strongly dictated by the nature of the disease being treated. As detailed right at the beginning of this section, cancer is a complex and heterogenic genetic disease, involving an almost infinite combination of molecular changes and consequent cellular effects. Before we go on to discuss the scientific basis behind current cancer treatments and the evolution of molecular targeted therapeutics exploiting the hallmarks of cancer, one point that you must embed within your thoughts is that we must never lose sight of the fact that we are dealing with people with a potentially life-threatening disease. This should be used to contextualise both the benefits and limitations of current clinically utilised strategies, and, similarly, those therapeutic options still under development.
Following diagnosis, several options are available for treatment: in all cases, the initial goal is, of course, to cure the patient using single or multi-modal treatment. However, it is also important to remember that not all cancers can be cured, and in these cases the goal of treatment would be to prolong the patient’s life, whilst maintaining or improving their quality of life. The principle for cancer therapy, and more specifically the type of treatment, is based on several factors. In terms of the patient, these include the efficacy of the approach under consideration and their performance status (more on this in Section 3, where we consider the treatment of specific cancers). In terms of choice of cancer treatment itself, this is determined by the type of cancer, its location, its molecular phenotype (oncogenes, tumour suppressor genes, and hallmarks of cancer), the degree to which it is confined to the originating organ, and conversely the extent to which it has spread to other parts of the body. Of course the other, and perhaps most important, factors when reaching a treatment decision are the views and wishes of the patient.

The first line of attack in the treatment of any cancer is to consider removing it surgically. The use of surgery to cure cancer was first suggested by the Scottish surgeon John Hunter (1728–1793), who stated that if the tumour had not invaded the neighbouring tissue and was ‘moveable’, then there was no reason not to remove it. Although this was a clear milestone in cancer treatment, it should be noted that anaesthesia was not developed until about a century later! The success of this surgical approach is largely determined by the site and location of the tumour and the ability of the surgical team to remove all the cancer cells. With regards to this approach, it is worth remembering that under normal circumstances the tumour isn’t coloured differently and doesn’t have flashing lights to distinguish itself from the surrounding tissue, making it often difficult for the surgeon to identify all the cancer tissue. However, in recent years there have been significant advances in cancer imaging, which have now facilitated much more of an image-guided approach to surgical resection. Several medical imaging techniques are now indispensable tools for the diagnosis and monitoring of cancer in the clinic, including computerised tomography (CT), magnetic-resonance imaging (MRI), positron emission tomography (PET), and various other optical techniques (see Section 1.2). The full description of such techniques in terms of cancer treatment are outside the scope of this book, but if you are interested in finding out more, Sharma and colleagues have written an excellent review on the subject [86]. Nevertheless, whether image-guided or not, the surgeon must be confident they have removed as much of the tumour as possible, and consequently will also invariably remove a band of normal healthy tissue (visually at least) from around the tumour.

Another line of treatment involves the use of radiation to damage and destroy the cancer cells. The use of radiation as a therapeutic strategy, similar to surgery, is primarily used for the treatment of localised cancers. The advantage of radiation over surgery is that it can also target cancer cells locally disseminated from the tumour mass, beyond the scope and feasibility of surgery alone. In some cancer types radiation therapy is often the preferred option; this can be due to either the tumour being inaccessible to surgical resection or the focused nature of radiation therapy being advantageous for tissue preservation. Furthermore, radiation is commonly used in combination with surgery to additionally eliminate cancer cells invading into adjacent normal tissue. Such an approach can be beneficial in limiting the extent of surgery and significantly reducing the risks caused by any remaining cancer cells following resection.
The concept behind radiotherapy (targeted destruction of cancer cells via radiation) is to use high-energy radiation (X-rays, gamma rays etc.) or beams of particles (e.g. electrons) to ionise atoms as the radiation beam passes through the biological tissue and destroys cancer cell structure. Biologically, the radiation beam causes the production of reactive chemical species within the cancer cells, which in turn damage the DNA and other biologically susceptible molecules. Radiotherapy-induced cellular damage is, however, non-selective and consequently will affect any proliferative cell within the vicinity of the targeted beam, causing several – and often severe – adverse effects, for example nausea, vomiting and diarrhoea (caused by damage to the gastrointestinal tract), skin damage and hair loss (caused by damage to proliferative hair follicles), anaemias (damage to bone marrow and blood-forming cells), and sterility (damage to the reproductive cells).

There have been significant advances in both surgical and radiotherapeutic treatments of localised cancer, which in many cases have had a significant impact on improving quality of life (by reducing cancer-related symptoms) and increasing survival. Despite this, many cancers are not detected until later in the pathogenesis process when localised treatment is not possible. As we described earlier, unlike many other diseases, cancer in its early stages is often asymptomatic. At this point, the malignant tumour is either too small to be noticed by the patient, is not interfering with normal tissue and organ functions, or is undetectable by standard physical assessments available to the general practitioner. Consequently, there is no reason to look for something that the patient is unaware is present, analogous to finding a needle in a haystack. However, earlier diagnosis is becoming more common through the array of screening programmes and initiatives launched to identify neoplasms in at-risk populations. Unfortunately, in many cases a positive cancer diagnosis is made when the cancer has disseminated to other sites within the body (occasionally it is the secondary cancer that gives rise to the symptoms that cause the patient to seek medical attention). In cases such as this, surgery or radiotherapy may not be possible, feasible or appropriate; this, then, brings us on to the use of chemotherapy in the management of cancer.

1.3.7.1 Evolution of Molecular Targeted Therapeutics

The basic principle of cytotoxic chemotherapy was eloquently described in 1909 by the Nobel Laureate Scientist Paul Ehrlich:4

“In order to pursue chemotherapy successfully we must look for substances which possess a high affinity and high lethal potency in relation to the parasites, but have a low toxicity in relation to the body, so that it becomes possible to kill the parasites without damaging the body to any great extent. We want to hit the parasites as selectively as possible. In other words, we must learn to aim and to aim in a chemical sense. The way to do this is to synthesize by chemical means as many derivatives as possible of relevant substances.”

4 Paul Ehrlich and Ilya Ilyich Mechnikov won the Nobel Prize in Physiology or Medicine in 1908 ‘in recognition of their work on immunity’.
Despite this principally relating to his involvement in the discovery of the first effective medicinal treatment for syphilis (arsphenamine), this effectively initiated the concept of chemotherapy, and is equally pertinent to the chemical treatment of cancer. As we shall see in Sections 2 and 3 of this book, many agents still used in the clinical management of cancer are cytotoxic and function by targeting nucleic acid replication or synthesis, with many being approved for clinical use decades ago. Mechanistically, these agents do not exclusively target cancer cells, and will also attack any rapidly proliferating cell type, such as those in the digestive tract or bone marrow, which results in the development of a vast array of adverse effects, some of which can be life threatening to the patient. Over the past decade or so, however, we are pleased to report that the increased understanding toward the molecular basis of cancer has greatly advanced anticancer therapy into an era of targeted molecular therapeutics (Figure 1.3.12) [87,88]. A good example of this is the kinase inhibitors (Section 2.5). These new targeted therapies focus their activity towards...
the hallmarks of cancer (rather than rapidly proliferating cells) and consequently have a
different spectrum of anticancer activity compared to traditional cytotoxic chemother-
apy. Indeed, the kinase inhibitors have good activity towards certain cancers in certain
individuals, but, as we will see in Section 2, the targeted approach does not necessarily
mean these agents are devoid of adverse effects. To continue the fight against this com-
plex, burdensome disease, it is crucial that we continue to understand the molecular
basis behind cancer and use this information to inform our approach to drug design and
development.

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