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Rita Fior
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Molecular and Cell Biology of Cancer

When Cells Break the Rules and Hijack Their Own Planet

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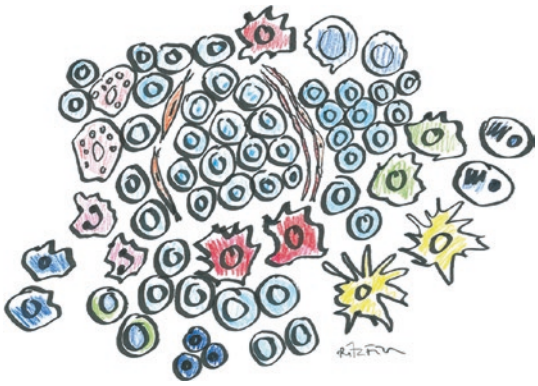
Preface



In this book, we have put together a series of lectures to take students on an expedition in cancer biology, where we mix developmental, evolutionary, and cell biology perspectives, to then wrap up with an integrated clinical approach in the form of case studies.

We look at cancer cells as outlaws, i.e., cells that break the rules by which the multicellular society is generated and maintained. Cells that not only disrupt their cell-autonomous control buttons but can also, through non-cell-autonomous interactions with normal cells, hijack and "corrupt" their host.

We start with an introductory chapter where all major concepts are broadly approached and connected in a developmental and evolutionary perspective, trying to open questions and tease curiosity for the chapters to come. Then we will explore the idea of cancer as a mass of somatic cells undergoing a microevolutionary process and how the same rules of Darwinian evolution may be applied to cancer but in a completely different time scale! The main signaling pathways deregulated in cancer will be revisited. Then, we will go through the main Hanahan and Weinberg "Hallmarks of Cancer," revising the topics of proliferation, apoptosis, genomic instability, DNA damage, and cancer metabolism, and then into hallmarks that illustrate how cancer cells hijack the host. Tumor cells not only avoid immune detection but also hijack and corrupt immune cells to work for them. Another form of hijacking the host is when tumor cells recruit blood vessels to feed the tumor and provide a highway to invade and migrate to other organs through the multistep process of metastasis. Then we will explore what is known about metastasis formation.

In most themes, we will go through historical experiments that led to key molecular discoveries or concepts, establishing a bridge between basic biology and biomedicine. Finally, all these concepts will be integrated in clinical studies where molecular diagnosis as well as various classical and modern therapeutic strategies will be addressed.

We would like to highlight that this book is not a thorough revision of each topic and in time some details will become outdated. Nevertheless, our goal is to provide essential concepts and a systemic and comprehensive overview of cancer. Designed for advanced undergraduates, master students, or even patients looking for a further understanding of the disease, we tried to use an easy-to-read language without underestimating the scientific accuracy. The editor rules of starting each chapter with an overview, defined learning objectives, and important concepts, and finishing with a take-home message, will surely help readers to an active understanding. Although there is a logical order in the

topics presented, each chapter can be read independently and used as a rapid revision concerning each specific theme. We hope this book gives a conceptual framework for the mass of information that is presently available about cancer but also stimulate readers to question and encourage further individual research.

We would like to thank all the authors that contributed to the writing of this book for their knowledge, expertise and dedication. Thank you **Lília Perfeito**, **Mariana L. Oliveira** and **João T. Barata**, **Irina S. Fonseca** and **Mónica Bettencourt-Dias**, **Ana Rita Carlos**, **Inês Castro**, **Vanda Póvoa**, **Ana Magalhães** and **Sérgio Dias**, **Hélia Neves**, **Mireia Castillo**, and **Joana Ribeiro**.

Specially, we would like to thank **Inês Amendoeira Cabral** (Instituto Gulbenkian Ciência) and **Ana Rita Carlos** for the beautiful illustrations based on the figures suggested by the authors.

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Cancer - when Cells Break the Rules and Hijack Their Own Planet

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1

What You Will Learn in This Chapter

In this Introductory chapter we will go through the major concepts that will be addressed in this book. We are going to look at cancer cells as outlaws, as cells that permit the existence of a healthy multicellular society. Tumor cells not only break their own internal controls (cell-autonomous traits) but are also able to hijack and sabotage the host (non-cell autonomous) to work for them, to nurture and help them thrive, ultimately to metastasize to distant territories.

We will also revisit some basic biology concepts to be able to move to a deeper knowledge of cancer in the next chapters.

Learning Objectives

After completing this chapter, students should be able to:

1. Think globally not locally i.e. have a broad perspective of cancer to then dive into the different mechanisms/questions/topics/issues of cancer, approached in each specific Chapter of the book.
2. Think about cancer, as cells that are breaking the rules that govern a healthy multicellular organism.
3. Refresh knowledge about evolution, to think about cancer as a microevolutionary process.
4. Define what are oncogenes and tumor suppressors.
5. Know and integrate the different hallmarks of cancer as cell-autonomous vs non-cell autonomous traits.

Important Concepts Discussed in This Chapter

- Germ cells vs somatic cells
- Cancer as microevolutionary process
- Clonal origin of cancer
- Tumor heterogeneity
- Oncogenes and tumor suppressors
- Drivers and passenger mutations in cancer
- Blockage of cell differentiation to induce cancer
- Hayflick limit – replicative senescence – telomere crisis
- Hanahan and Weinberg hallmarks of cancer

1.1 Multicellular Organism as a Society of Self-Sacrifice

Cancer refers to a large number of diseases characterized by the development of abnormal cells that divide uncontrollably and have the ability to infiltrate and ultimately destroy normal body tissue. To understand and fight cancer we need to understand what is "normal" and the basic rules or "laws" that allow the formation and maintenance of a healthy multicellular organism. These are the rules that are broken during cancer.

As the late Julian Lewis (1946–2014 – Developmental Biologist – FRS) would frame it, a multicellular organism operates as a society composed of cells that must organize themselves in a collaborative way. Furthermore, sooner or later, "all of the somatic cell lineages in animals are committed to die: they leave no progeny and instead dedicate their existence to the support of the germ cells, which alone have a chance of continued survival"

and transmit their genes to the next generation. Thus, each cell in the organism behaves in a "responsible manner", in a self-sacrificing mode, proliferating, differentiating *or dying* as required *for the "good"* of the society, to guarantee reproduction [1].

Somatic cells vs Germ cells: Germ Plasm Theory – August Weissmann

To have our basic concepts in place, let's go back to 1893, when Darwin and Lamark theories were being discussed. August Weissman proposed the germ plasm theory, an apparent very simple concept, however with many implications. Weissman postulated the existence of two different types of cells in some multicellular organisms: somatic and germ cells. But both cells come from one single cell: the egg. Thus – a simple question was raised – how do you make two cells different from one single cell? Weissman postulated asymmetric divisions, that now is a well-known molecular process that occurs in stem cells. But then he faced another conceptual problem: if the egg comes from the fusion of two germ cells then the germ cells must have half the "material" of the egg and somatic cells. He proposed reduction mitosis, a process that we now know as meiosis [2] (Fig. 1.1).

Weismann did a famous experiment, he chop-off the tails of 1500 rats, repeatedly over 20 generations, and reported that no rat was ever born without a tail. He stated that "901 young rats were produced by five generations of artificially mutilated parents and yet there was not a single example of a rudimentary tail or any other abnormality of the organ" [2]. The only way to affect the offspring is by changing the germ cells! So, his point was that the offspring does not inherit characteristics from the soma but from the germ cells. Germ cells are not influenced by the body that bears them! This had a huge impact on the evolutionary theories discussed at the time: Lamarck vs Darwin, and influenced also the way we think about cancer and how a living healthy organism is just

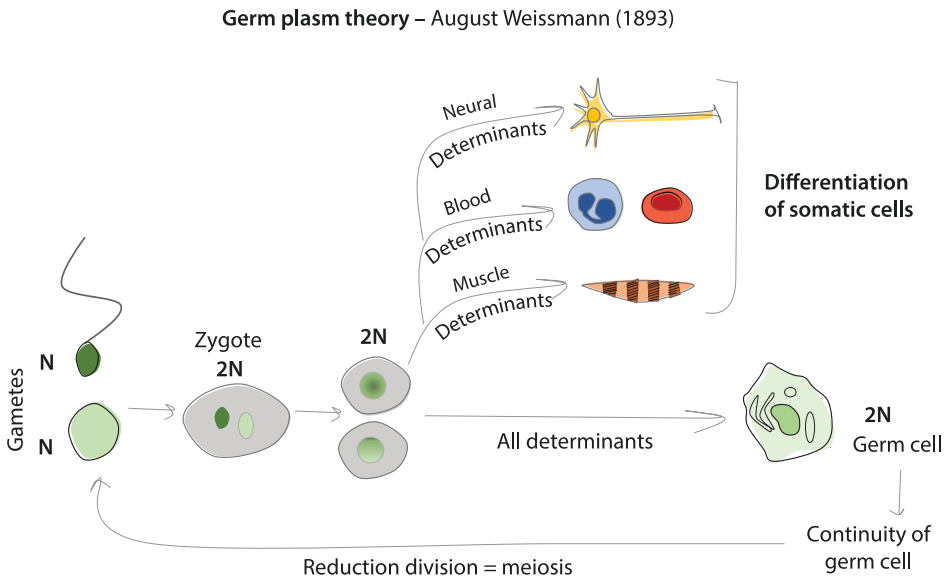


Fig. 1.1 Germ plasm theory – concept of germ cells vs somatic cells

a carrier and a guardian of the germ cells. We will discuss concepts of cancer evolution in ▶ Chap. 2.

Thus, in contrast to unicellular organisms where survival of the fittest by natural selection is the rule, in healthy multicellular organisms, cells must "self-sacrifice" for the success of the "whole cellular society".

1.2 Cancer as a Microevolutionary Process: Disruption of the Cellular Society

Molecular disturbances that disrupt these "social control buttons" lead to problems in a multicellular organism. What are these molecular disturbances? MUTATIONS – SELFISH mutations.

In a human body, billions of cells are subjected to mutations every day, with the potential to destroy these "social control buttons". Even in the absence of mutagens, mutations can never be completely avoided, because each time the genome duplicates there is a probability of error associated with the process itself [3]. It is estimated that each human being carries on average ~60 *new* point mutations that emerged in the germline of their parents. Also, the number of somatic mutations that occur during embryonic development and adult lifespan of each person is overwhelming – proliferative organs such as the intestine of a 60 year old individual, is estimated to have a mutation at almost every genomic site in at least one cell [4].

The good news is that most mutations either have a negative effect on fitness and are quickly eliminated or are neutral (see ▶ Chaps. 2 and 5).

With exception of some leukemias (liquid) and child cancers, in general, a single mutation is not enough to generate cancer. Nevertheless, a mutation may give one cell a selective advantage, allowing it to divide more than its neighbors becoming a founder of a new dangerous society, that can threaten the host.

Continual cycles of **mutation and natural selection** occurring in a population of **somatic cells** leads to cancer. Although this microevolutionary process occurs in a time frame of months/years in a population of cells in the organism, the same rules of Darwinian selection are applied [3] (◘ Fig. 1.2) (see ▶ Chap. 2).

1.3 Clonal Origin of Cancer-from Boveri to CLL

More than a 100 years ago, in 1914, the biologist Theodore Boveri proposed several hypotheses about the role of somatic genetic alterations in the development of cancer by studying the cell biology of sea urchin embryos [5]. His central hypothesis was that mammalian tumors are initiated by problems in mitosis that lead to alterations in chromosome numbers (aneuploidy) — this hypothesis was based on observations that defects in mitosis often led to abnormal embryonic development. This hypothesis was then extended to include many other concepts that are now well established, such as:

- Tumor cells are genetically unstable (▶ Chap. 5);
- Tumors come from one single cell;
- Tumor cells are insensitive to growth controllers due to gain or loss of critical chromosomes;
- Tumors may result from genetic modifications that do not involve entire chromosomes.

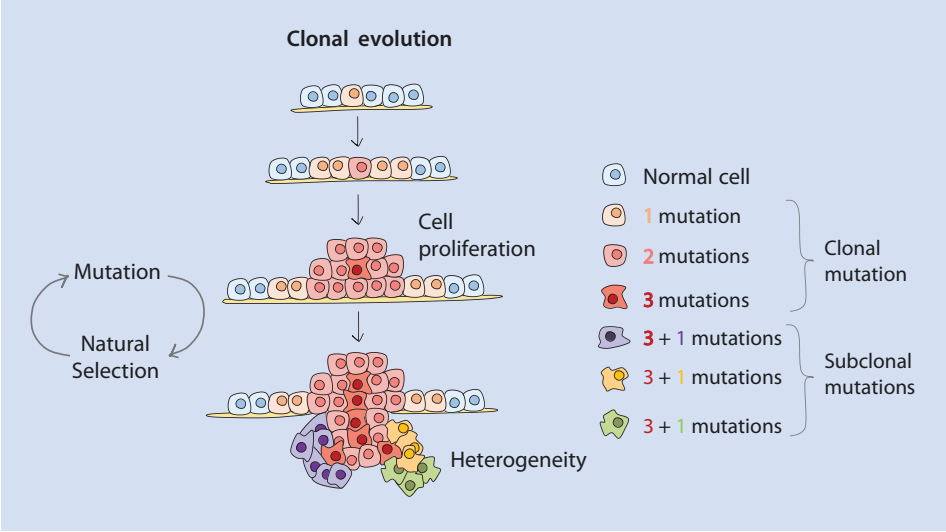


Fig. 1.2 Clonal origin of cancer. Cancer is the result of repeated cycles of mutation and natural selection in a population of somatic cells. Although tumors have a clonal origin they are not a homogeneous mass and are made of different subclonal populations. (Partially adapted from [3])

However, proof of Boveri theories were only confirmed with the dawn of molecular biology and more modern techniques. David Hungerford and Peter Nowell in 1960 described an abnormally small chromosome that was present in some blood cells of every patient with Chronic Myelogenous Leukemia (CML). Later it was shown that this abnormal chromosome – the Philadelphia chromosome (named after the city where it was discovered), is the result of a translocation between chromossome 9 and 22. This translocation leads to an oncogenic gene fusion between breakpoint cluster region (*BCR*- chromosome 22) with v-abl Abelson murine leukemia viral oncogene homolog (*ABL*) on chromosome 9 resulting in the Bcr-abl fusion protein. The Abl codes for a tyrosine kinase, but the Bcr-Abl mutant version is not regulated and it is always activated, resulting in the abnormal proliferation of myeloid cells that leads to CML (► Chap. 3). Besides activating cell cycle control genes promoting division, it also blocks DNA repair leading to genomic instability (► Chap. 5) [5]. This fundamental discovery led to the first and most successful target therapy – Imatinib (Gleevec) (see ► Chap. 3).

All these discoveries led Peter Nowell to propose a new perspective on cancer: **cancer as microevolutionary process**, driven by a stepwise increase in somatic cell mutations with several rounds of Darwinian selection, drawing a parallel with an asexually reproducing unicellular quasi-species [6]. The modern era of cancer biology and genomics has provided extensive support to this new cancer Darwinian perspective (see ► Chap. 2). In fact, some Cancer Centers have now Evolutionary Biologists working together with Medical doctors!

1.4 Tumor Heterogeneity

Although tumors have a clonal origin, they are not a homogeneous mass, on the contrary, with the recent genome profiling of multiple tumors, it has become clear that there is a huge genetic and non-genetic heterogeneity in cancer. This heterogeneity has been

observed not only between cancers (inter-tumor), but also within each cancer (intra-tumor) [7, 8]. The idea is that a founding clone can evolve to acquire different mutations that can be advantageous to that cell or not, however another clone can also acquire other mutations and competition between these different variants will define the dominant landscape, although most of the times other minor clones are still present (Fig. 1.2). These different clones can then have different characteristics and phenotypes contributing to diversity, an essential feature of evolution. Importantly, even identical colorectal cancer (CRC) cells, sharing the same genome, but different expression profiles, exhibit multiple functional traits (including distinct responses to therapies), implying that the basis for heterogeneity is not only genetic but can also be epigenetic (differential gene expression) or microenvironmental [9]. This diversity poses a major challenge to drug development and precision medicine, since even in biomarker-driven therapies response rates are not 100% [10].

1.5 Oncogenes and Tumor Suppressors Genes [7]

OK, so what are these "social control buttons" or "basic rules of behavior" that allow the formation and maintenance of a healthy multicellular organism, and if broken may lead to cancer?

There are two major classes of genes/buttons that can be altered in cancer (Fig. 1.3):

- **Oncogenes** – are mutated versions of genes – the proto-oncogenes – that generally code for proteins that when improperly activated can lead cells towards cancer i.e. when **activated** by mutations increase the selective advantage of that cell. By default, these are genes that when mutated in a single allele act dominantly, overriding the regulation of the *wildtype protein*/allele.
- **Tumor suppressor** genes, in contrast, are genes that code for proteins that their main function is to block/control cancer development. These are genes that generally act in a recessive manner i.e. both alleles must be lost to drive a cell towards cancer.

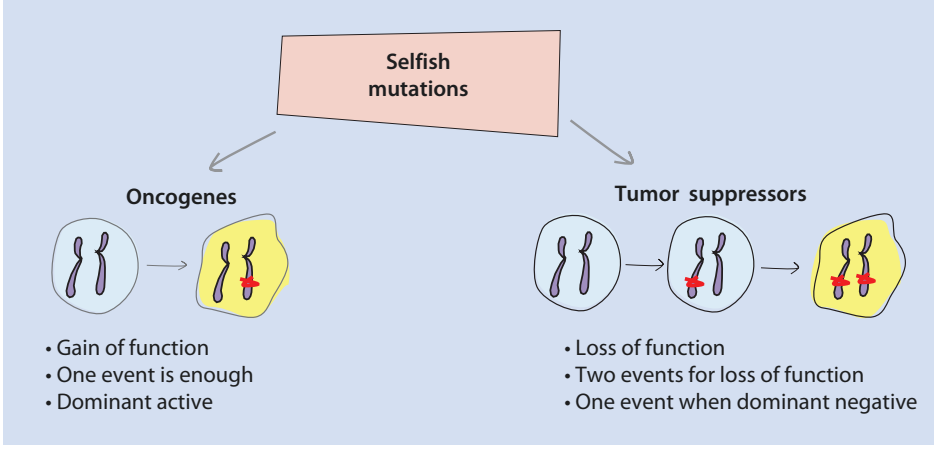


Fig. 1.3 Two classes of selfish mutations: Oncogenes generally code for proteins that when activated increase the selective advantage of that cell. Tumor suppressor genes, code for proteins that when inactivated increase the selective growth advantage of that cell

However, there are also examples of dominant-negative mutations i.e. mutations that lead to a protein that is able to block a wild-type-normal protein function. When **inactivated** by mutation, these genes increase the selective growth advantage.

Activating mutations of proto-oncogenes or inactivation of tumor suppressor genes may be achieved by:

- **Point mutations**:
  - Missense mutations may lead to hyperactive proteins versions, as it happens for instance when the regulatory subunit of RAS proteins is mutated
  - Nonsense mutations that can generate stop codons (nonsense mutations), that lead to truncated versions of a tumor suppressor, rendering the protein inactive, as exemplified by the case of a nonsense mutation in EphB2 tumor suppressor gene associated with prostate cancer [11]
- **Gene amplifications** that lead to protein overproduction - as the case of the MYC gene that is amplified in a significant number of epithelial cancers (breast, colorectal, pancreatic) [12, 13]
- **Deletions** that lead to a loss of function phenotype - as the case of hereditary retinoblastoma in which the tumor suppressor gene Rb on chromosome 13 has a deletion
- **Chromosome rearrangements**
  - That generate **fusion** proteins that can hyper-activate the protein, for example bcr-abl fusion
  - That **re-locate** in the genome new regulatory units that lead to overexpression of the proto-oncogene as in the case of Burkitt lymphoma, where a immunoglobulin highly active promoter on chromosome 8 was fused to MYC gene on chromosome 14 [14]
  - That lead to a **reduction of expression** of tumor suppressor genes or its inactivation, as it is the case of p53, PTEN, BRCA1 and BRCA2 that recurrently are truncated as a result of chromosome rearrangements in prostate cancer and show generally reduced expression [15].

OK. **If a clone has to accumulate different mutations, how many mutations do you think exist in tumors? Thousands or dozens? Are they all equally important? Do they have the same weight?**

We can find both to be true. The number of mutations present in cancer differs according to the type of tumor: lung cancer being the recordist on mutations-can harbour ~200 mutations per tumor; but in the other side of the spectrum, childhood and liquid cancers can have an average of 9.6 mutations per tumor [7]. The thousand part (of mutations) of the spectrum are either associated with potent mutagens like cigarette smoke and ultraviolet light (melanoma-skin cancer) or with mutations in the "caretakers" of the genome (detection/repair machinery-see ahead and ▶ Chap. 5). However, not all mutations found in cancer are **driver mutations** i.e. supply a selective growth advantage to the tumor cell, there are also many **passengers** mutations that do not confer any apparent selective advantage and are thought to be just marking the time that has passed between consecutive clonal expansions [7].

And how many driver mutations exist that can drive cancer?

With all the recent genome-wide sequencing studies it is thought that a number of ~125 driver mutations was reached, from which 71 are tumor suppressors and

54 oncogenes [7]. However, recent sequencing of noncoding regions, revealed that promoter regions can also be highly mutated and be driver mutations, by leading for instance to overexpression of oncogenes [16]. So this number can be underestimated…

How many driver mutations are needed to actually drive tumorigenesis?

It is thought that most tumors are caused by two to eight of these "driver gene" mutations, the remaining are passengers [7]. However, there are studies that show that the **order** of which these mutations occur can also be important, especially if there is a mutation affecting chromatin epigenetic modifications that then can change the expression of many other genes [17]….

To understand cancer and possibly even predict which genes and processes might be altered in cancer and therefore forecast potential oncogenes and tumor suppressors, it is important to step back and think about embryonic development and make "simple" questions:

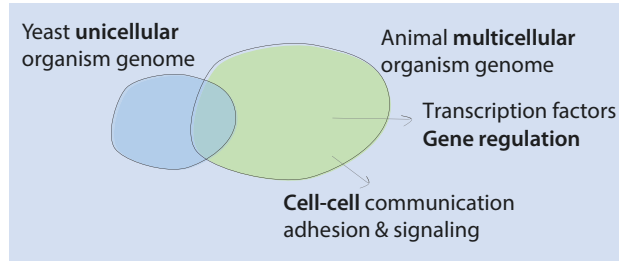
How do you go from one single cell to millions of different cells organized in complex tissues and organs that allow us to have a beating heart, a perfect circulatory system, walk, breath, think and all the important physiological functions in harmony?

Or let's simplify and go to the cellular level: if we compare the genome of a unicellular eukaryote like yeast with the genome of a multicellular organism, which genes must have been acquired by the multicellular organism?

If we think, cells in a multicellular organism must be different from one another. We have, neurons, skin cells, muscle cells, etc. … but the genome is identical in every cell of the organism. How do cells become different? Cells differ not because they contain different genetic information, but because they express different sets of genes. Thus, one class of genes that suffered an evolutionary explosion in multicellular organisms were **transcription factors** and **modulators of transcription**, allowing a multitude of different cell fates choices (◘ Fig. 1.4). Also, cells in a multicellular organism have to **communicate** with each other to coordinate their behavior [3]. Thus another class of molecules that emerged were the transmembrane adhesion and signaling proteins. Regarding the later, **signaling pathways** will be discussed in detail in ▶ Chap. 3, and we will see how most oncogenes and tumor suppressors are part of these signaling pathways (◘ Fig. 1.4).

Another important concept emerges when we think about development…

- How is it that the process of embryonic development is so precise, with a born baby after 9 months? If we incubate a chicken egg for 24 h at 37 °C, we'll have an embryo always at the same exact stage. Why is it that a pair of somites (precursor of the vertebrate skeleton) are formed every 30 minutes in zebrafish, 90 minutes in the chick or 2 h in mouse?
- How is it that cells in an embryo during morphogenesis "know" that have to turn right or left, stop or resume proliferation or simply die? How is this all so precisely controlled and reliable?



◘ **Fig. 1.4** Cartoon of a comparison of the genome of a unicellular eukaryote with the genome of a multicellular organism

The simple answer for this is **robustness,** which is achieved by redundancy and feedback. Like airplanes that have multiple controls – if one engine fails – another must quick in, in a cell, most essential processes are controlled by negative feedback and redundant players and safeguards. However, cancer cells not only are "professionals" at disrupting these controls but also unfortunately exploit them to achieve resistance to therapies! Do not forget this!

1.6 Disruption of Basic Social Control Buttons

Having this thoughts/concepts in place, what processes do you think cancer cells break?

We know that a cancer cell to form a tumor mass has to increase the number of cells. How does the cell achieve this? You can immediately guess that several strategies may be exploited:

- Increase proliferation
- Decrease cell death
- Block differentiation
- Become immortal

1.6.1 Increase Proliferation

To increase proliferation cells must de-regulate the **cell cycle** control buttons. Before entering proliferation, healthy cells must analyze whether they have conditions to proliferate, meaning if there are enough nutrients, growth factors, is their genome "safe and sound"? etc.… If not, the cell cycle breaks (named checkpoints) are ON and the cell will not engage into division. To start division, cells must receive proliferative signals that inhibit the cell cycle breaks, like for instance the CDK inhibitors or the Retinoblastoma protein (Rb), or increase cyclins production, a strategy often used in cancer. Most cancer cells have mutations to break the cell cycle control buttons, allowing uncontrolled proliferation. This topic will be discussed in detail in ▶ Chap. 4.

1.6.2 Decrease Cell Death

Another way to increase cell numbers can be simply to block cell death. Most times when a cell is in trouble, too much DNA damage/mutations for instance, cells just suicide i.e. enter **apoptosis** to avoid problems for the whole cellular society (Fig. 1.9). Therefore, once again most cancer cells acquire selfish mutations that allow them to overcome this safeguard mechanism. One of the most mutated genes in cancer is **p53**, the **guardian of the genome**, which senses DNA damage and stress and can trigger either repair, apoptosis or senescence. Also, many times cancer cells overexpress anti-apoptotic proteins such as Bcl2 family members to avoid entering apoptosis. This subject will be discussed in detail in ▶ Chap. 5.

1.6.3 Blocking Differentiation

To increase the number of cells, another strategy can be to block or change the differentiation program. How come? Most differentiated cells in our body are post-mitotic or

proliferate very little but are originated by undifferentiated proliferative progenitor cells or stem cells.

During embryonic development, the egg has to differentiate in all the different cells and tissues that form our organs in an organized and extremely orchestrated manner. And this highly regulated and robust program is controlled by a few signaling pathways such as [2]:

- Transforming growth factor β (TGFβ) family (nodal/BMP/TGFβ),
- Receptor tyrosine kinases family members (FGF/EGF/IGF/PDGF/Ephrins),
- Hedgehog (Hh),
- Wnt,
- Notch,
- Retinoic acid (RA),
- Hippo pathway

In the adult these signaling pathways are active in tissues that require cell-renewal to maintain tissue homeostasis. In these tissues, resident stem and progenitor cells have to go through most of the same signaling and cell-fate decisions that occur during embryonic development to generate all the different cell types that compose each organ. Therefore, is not a surprise that a mutation in a gene/pathway important for the differentiation program may render cells stuck in a stem or progenitor cell state with high proliferative capacity (Fig. 1.5)-a situation that provides a selective advantage for that cell to progress to cancer (Fig. 1.9).

The significance of Wnt/β-catenin signaling came to light with the discovery of frequent mutations in the Wnt signaling pathway associated with familial (germ-line) and sporadic colon cancers. Wnt signaling is essential for intestinal stem cell maintenance, and its downregulation is crucial for the differentiation program to proceed. Adenomatous Polyposis Coli (APC) loss of function or β-catenin gain of function mutations lead to Wnt signalling constitutive activation. When Wnt is constantly ON, cells are trapped in a stem/progenitor state unable to differentiate into the different intestinal cell types, proliferating uncontrollably (see Fig. 1.6 for details) [18].

Another example is in acute lymphoblastic leukemia (T-ALL), where Notch signaling can be always ON. Notch signaling is a cell-cell communication pathway involved in most cell fate choices that occur during development but also in adult tissues that need cell-renewal, like skin, gut, and blood for instance. The Notch receptor is nothing more than a membrane tethered transcription factor, that upon ligand binding (DSL family ligands) is subjected to a series of proteolytic cleavages that releases the Notch Intracellular Domain (NICD), which then is able to translocate into the nucleus and activate transcription of downstream target/effector genes [20]. All our blood cells, including white blood cells, red blood cells, and platelets derive from hematopoietic stem cells (HSC). HSCs divide to give rise to the Common Lymphoid Progenitor (CLP) and a Common Myeloid Progenitor (CMP) cells. The CLP then gives rise to T-cells, B-cells and NK-cells. The CMP, gives rise to erythrocytes, megakaryocytes, granulocytes and macrophages [21]. Notch signaling is involved in most of these cell fate decisions, in particular in the decision between B and T cells, favoring T-cell lineage development, proliferation and survival during the

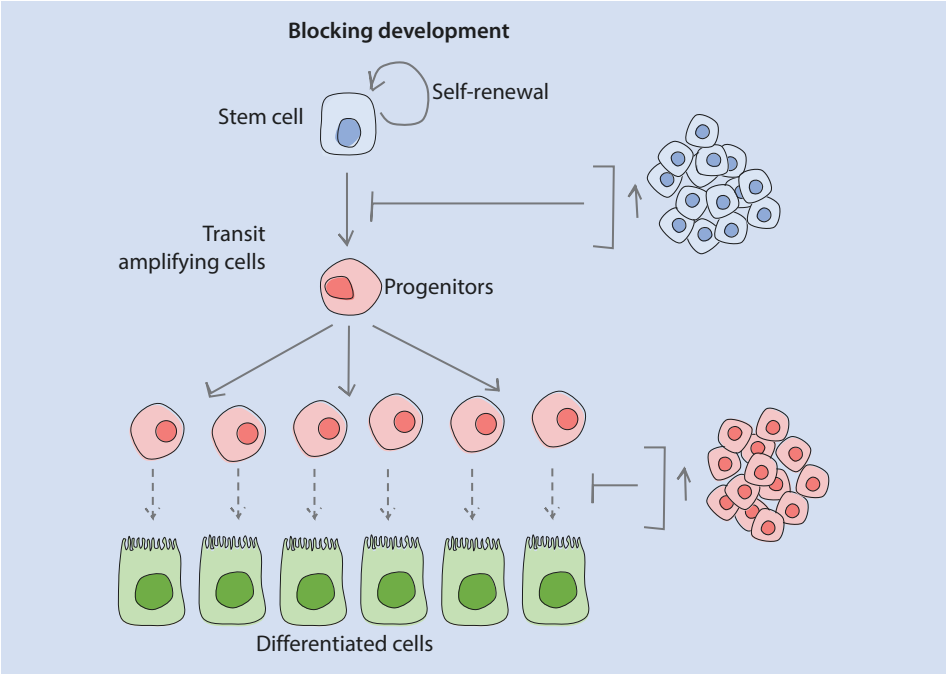


Fig. 1.5 Blocking Developmental programs can lead to increase in cell mass. When the transition from stem to transit amplifying progenitor is blocked, this can lead to an increase in the numbers of stem cells. When the block is further down the pathway i.e. in the transition from progenitor to differentiation it can lead to an increase of the numbers of progenitor cells

multiple stages of thymocyte development (Fig. 1.7). The first hint that Notch signaling could play a role in T-ALL was when a chromosomal translocation was identified in a T-ALL patient, which leads to a juxtaposition of the *TCR-β* locus with Notch1, resulting in constitutive active expression of NICD, and therefore Notch signaling is always ON [22]. Although this translocation is rare, ~60% of T-ALL patients present activating *Notch* mutations in different domains (reviewed in [22]), biasing the CLP towards the T-cell lineage and boosting proliferation of progenitor T-cells.

Notch inhibitors are now being tested in clinical trials to force cells to differentiate and in this way block cancer progression. However, blocking Notch systemically leads to other problems…so dealing with developmental programs is tricky since in some organs Notch may promote proliferation whereas in others may be a differentiation force; also, in one development time point may have one effect whereas in another step of the cascade it may have the opposite….

There are many other examples of embryonic developmental programs mutated in cancer…

After reading ▶ Chap. 3 you will have a better comprehension of the impact and importance of signaling pathways in cancer.

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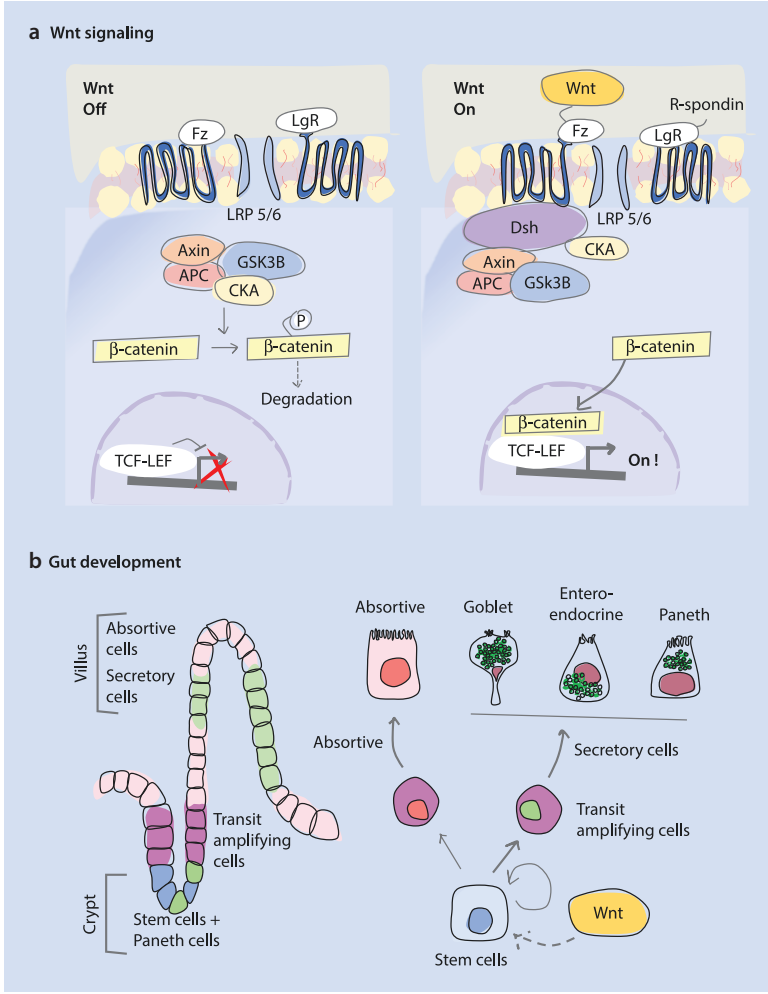


Fig. 1.6 Wnt signaling pathway and its role in gut development. **a** Cartoon of the Wnt signalling pathway. β-Catenin, is part of the adherens junction complex and therefore essential for the epithelial integrity of most tissues, however it can also translocate into the nucleus, but only in the presence of Wnt signaling activity. In the absence of Wnt, the cytoplasmic pool of β-Catenin is targeted for degradation by a destruction complex composed by: Axin, Adenomatous Polyposis Coli (APC), Glycogen Synthase Kinase 3 (GSK3) and casein kinase 1α (CK1α). Protein Phosphatase 2A (PP2A) also associates with the complex. Axin and APC are scaffolding complexes that put all these proteins in close proximity, allowing the sequential phosphorylation of β-Catenin by the Ser/Thr kinases- CK1 and GSK-3. Phosphorylated β-catenin is targeted for ubiquitination (by E3-ubiquitin ligase β-TrCP) and proteasome degradation and therefore is not able to translocate into the nucleus and activate transcription of downstream target genes. In the presence of Wnt, Wnt binds Frizzeled (Fz) and LRP5/6 receptors leading to the recruitment of Dishevelled (Dsh) that inhibits GSK3 and the destruction complex. This allows β-catenin accumulation and translocation into the nucleus and together with TCF/LEF (T-cell factor/lymphoid enhancing factor) transcription factors activate transcription of downstream target genes. [19]. **b** The cellular structure of the mammalian small intestine and the four types of terminally differentiated cells. Stem cells locate in the crypt base and nearby the Paneth cells (which provide Wnt ligands). On top of the stem cells are the transit-amplifying cells (dividing progenitors) which give rise to the post-mitotic differentiated cells of the absorptive lineage or the secretory lineage (goblet, enteroendocrine and paneth). Wnt signalling is essential for proliferation and maintenance of the stem cells. (Adapted from [18])

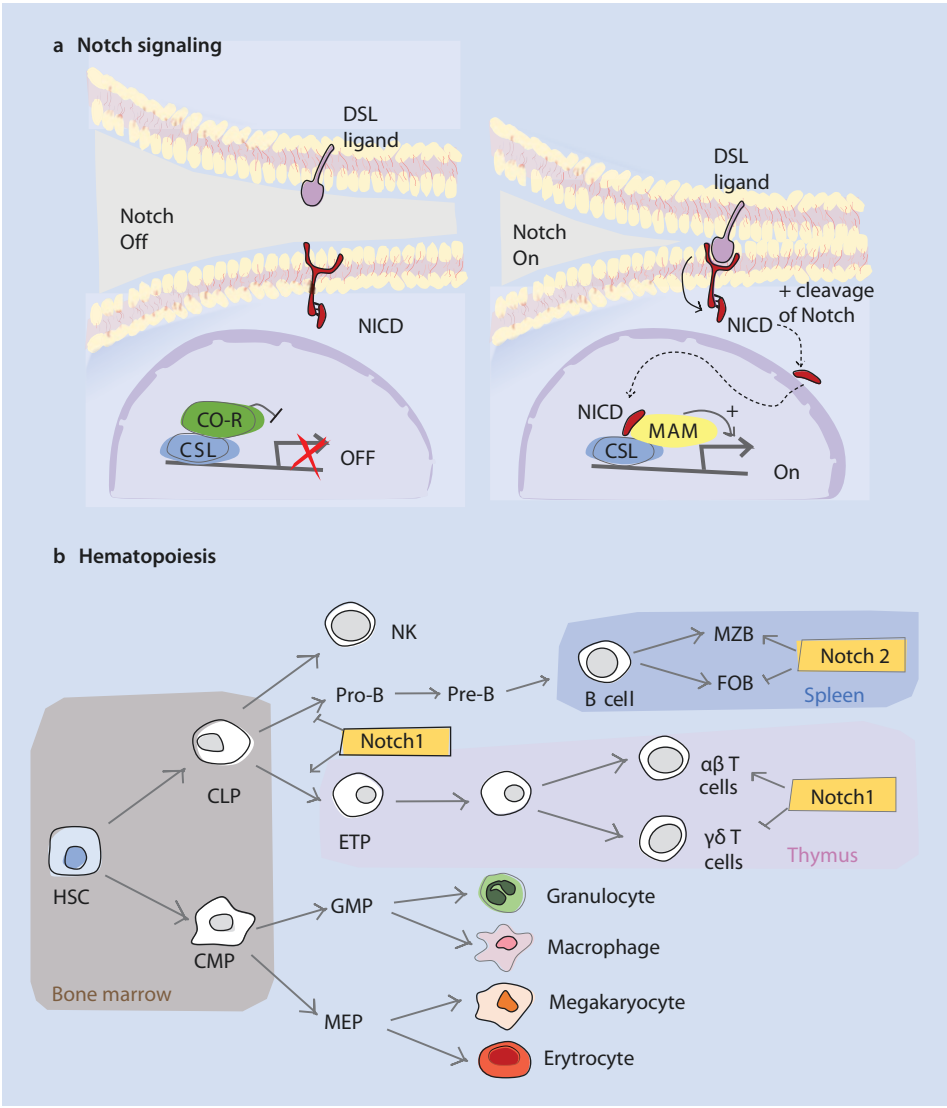


Fig. 1.7 **Notch signaling and some of its multiple roles during hematopoiesis. a** Notch is a large type-I transmembrane receptor that accumulates at the plasma membrane as a heterodimer, composed of the extracellular domain (NECD) and a membrane bound intracellular domain (NICD). The Notch ligands are part of the DSL family (Delta-Serrate-Lag2) of ligands and are also type-I transmembrane proteins. Upon ligand-receptor interaction, the Notch receptor undergoes successive proteolytic cleavages that lead to the release of NICD, which translocates to the nucleus. In the nucleus, NICD binds to the CSL transcription factor (an acronym for the mammalian CBF-1, *Drosophila* Suppressor of Hairless and *C. elegans* Lag-1) and leads to the displacement of CSL-bound co-repressors and recruitment of MAM and other co-factors, forming a different transcriptional complex with activator properties. Thus, the Notch target genes which were previously repressed now become transcriptionally active. **b** Hematopoietic stem cells (HSC) give rise to all our blood cells, the myeloid and the lymphoid lineages. Notch signaling is involved in most of cell fate decisions for example Notch1 is involved in the decision between B and T cells, favoring T and then later between γ/δ and α/β T cells. Notch2 is involved in the decision between 2 types of B-cells – the Marginal Zone B cells (MZB) and Follicular B cells (FOB). Notch is also involved, together with Wnt signalling in the maintenance of the HSC. (Adapted from [20, 21, 23])

1

1.6.4 Become Immortal

After acquiring mechanisms to proliferate, reduce cell death or inhibit differentiation to boost proliferation, cancer cells face another road block: the **Hayflick limit** or **replicative senescence** (RS).

What is RS?

Leonard Hayflick and Paul Moorhead, discovered that normal embryonic human cells could only divide a finite number of times in culture i.e. ~60 times and then would stop [24]. When we plate cells in culture they divide until they cover the flask's surface. When cells reach high confluency due to contact inhibition they stop dividing. But if you then dissociate cells and plate then in low concentration they re-initiate division, and you can do this platting and re-platting for several months. But ~60 divisions they completely stop dividing [25, 26]. This limit of cell replication was named "replicative senescence". Hayflick and Moorhead used in their experiments fibroblasts, but RS has been found in many other cell types, from embryonic tissues to adult-derived cells, and in cells taken from different animals. The exception being embryonic germ cells and most cell lines derived from tumors – these can divide indefinitely without reaching RS – they are considered "immortal" [26, 27].

Well, so if tumor cells to divide indefinitely and become immortal need to escape the Hayflick limit – how do they do that? A hint comes from thinking about cell division and the mechanisms of DNA replication. In the 70's, as the mechanisms of DNA replication were being revealed, it became evident that the 3′ end of linear DNA could not be replicated by DNA polymerase. James Watson coined this as the end-replication problem. Earlier, Hermann Muller and Barbara McClintock showed that the ends of chromosomes are protected by telomeres to prevent chromosome fusions. Alexey Olovnikov, in the 70′, also acknowledged Watson's problem. However, Olovnikov was visionary and proposed that the end-replication problem could lead to telomere shortening with division cycle, and that this mechanism could be the cause of RS [27]. Olovkikov's model proved to be correct. Telomere shortening is believed to be the main mechanism of RS and telomere length is the molecular clock that computes divisions; when telomeres become critically short it is said that the cell has reached the **telomere crisis**, which triggers RS [28] (Fig. 1.8).

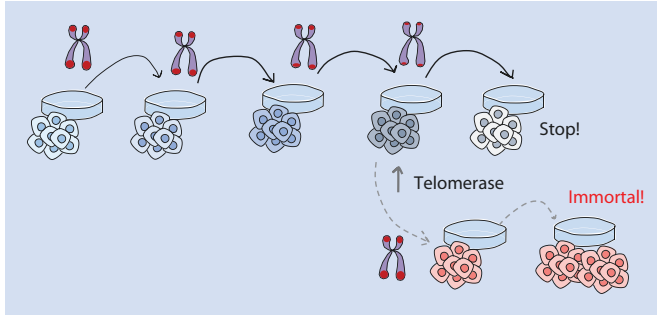


Fig. 1.8 The Hayflick limit or replicative senescence. When normal (non-tumorigenic) cells are plated in culture they can be platted and re-plated only a few limited times, until their telomeres become critically short. To have unlimited capacity of division i.e. become immortal, cells need to overcome this shortening of telomeres and one strategy often adopted by tumor cells is to overexpress the enzyme telomerase

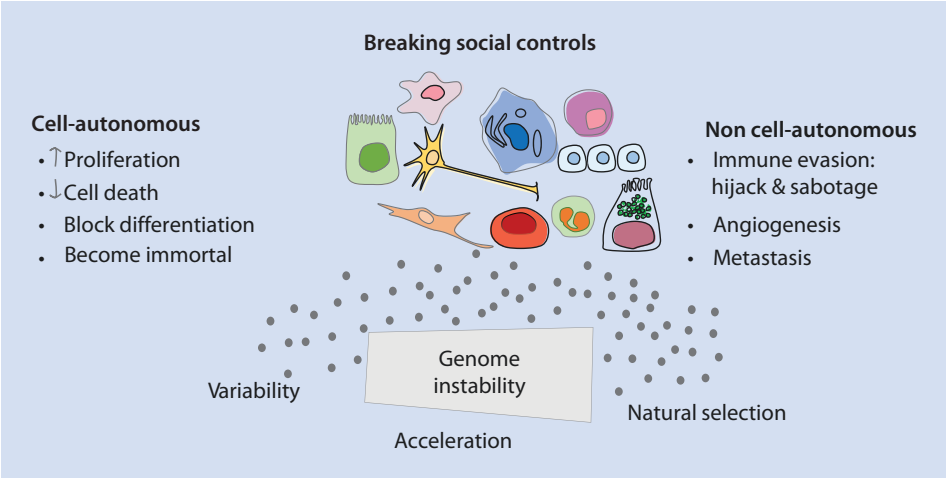


Fig. 1.9 Summary of the Hallmarks and enabling characteristics of tumorigenesis classified by cell-autonomous and non-cell autonomous traits

So how do tumor cells avoid this critical shortening, to become immortal? It has been shown that most tumors and tumor cell lines overexpress a critical enzyme for telomere synthesis – Telomerase Reverse Transcriptase (TERT). The definitive breakthrough came when it was shown that expression of hTERT in human cells avoids RS. In fact, one of the strategies in the lab to turn non-tumorigenic cells into immortal cells is to overexpress TERT [29]. Nevertheless, telomerase activity is not the only way to elongate telomeres, and other mechanisms have been described to elongate telomeres. Either way, with or without telomerase to become immortal, cells must stabilize their telomeres (reviewed in [27]).

1.7 Metabolic Switch

OK, so we have seen that for a tumor to grow, increase its number of cells; cells have to divide, avoid death, block differentiation and also become immortal. That is very good but what about the resources to do this?? Energy and nutrients must be used in a manner that these are not a limitation to growth. Amazingly, almost a 100 years ago Otto Warburg noticed that in contrast to normal cells, tumors metabolize glucose to lactate in aerobic conditions, this is known as the **Warburg effect**. You will learn in ▶ Chap. 6 that this is a major strategy of the cell not to produce more energy but to produce new proteins-biomass. Also you will see how the byproducts of this metabolic switch will have a major impact on the host, leading to immune suppression and angiogenesis, promoting tumor progression even further (Fig. 1.9).

1.8 Hijacking the Host

Until now we have seen that tumor cells need to break their own cell-autonomous controls and acquire different cell-autonomous traits to be able to form a tumor mass, but these are not the only ones. Tumor cells also break the "international social laws" and do not respect their "national borders" - they hijack and sabotage the host to help them expand and thrive in different territories.

1.8.1 Immune Evasion

Tumors reside in a host organism with healthy cells, which have an army to protect it from foreign threats, patrolling and surveilling the whole body. As we will see in ▶ Chap. 7, tumors cells develop mechanisms to escape detection by the immune system (immune evasion) to be able to progress in the host. However, tumor cells do more than that, not only escape detection but also hijack and corrupt the immune system to work for them; providing growth factors, inducing angiogenesis or helping tumor cells to metastasize….

1.8.2 Angiogenesis

Moreover, tumors to grow more than 1 mm of diameter are obliged to recruit blood vessels to be able to receive nutrients and oxygen to feed themselves. Thus it has been shown and will be discussed in detail in ▶ Chap. 8, how tumor cells have to established mechanisms to induce this angiogenesis process.

1.8.3 Metastasis

Finally, as you probably heard cancer kills patients because of its metastatic spread to vital organs. You will learn in ▶ Chap. 9, how the process of metastasis involves several steps: (i) invasion of surrounding tissues; (ii) intravasation into blood vessels; (iii) survival in the circulation; (iv) extravasation from the blood vessels and (v) survival and proliferation at a secondary site (i.e. colonization) [30, 31]. All these steps require different cellular processes that enable cells to perform efficiently these separate stages. Importantly, you will learn how tumor cells exploit the embryonic strategies (same genes and mechanisms) to migrate and invade distant sites. In general, tumor cells have to undergo an Epithelial to Mesenchyme Transition (EMT) in order to migrate and exploit the same developmental programs (genes) used, for example, during neural crest development and migration. Moreover, you will see that tumor cells although may travel throughout the whole body, they may "like" more some territories than others…this is the seed and soil hypothesis that was proposed by Paget in 1889. Nowadays there is a lot of evidence that this is indeed the case and that tumor cells may even secrete messages (exosomes) to prepare the soil for their arrival [32].

1.9 Genomic Instability

Last but not the least, a major fuel to sub-clonal diversity and therefore tumor evolution that nourishes the cell autonomous and non-cell autonomous traits is considered an enabling hallmark of cancer: genomic instability [33].

As referred before, most tumors, sooner or later acquire a p53 mutation – so this cell will not commit suicide, and will be able to continue to accumulate mistakes in its DNA. However, if this cell also acquires mutations in other "caretakers" of the genome

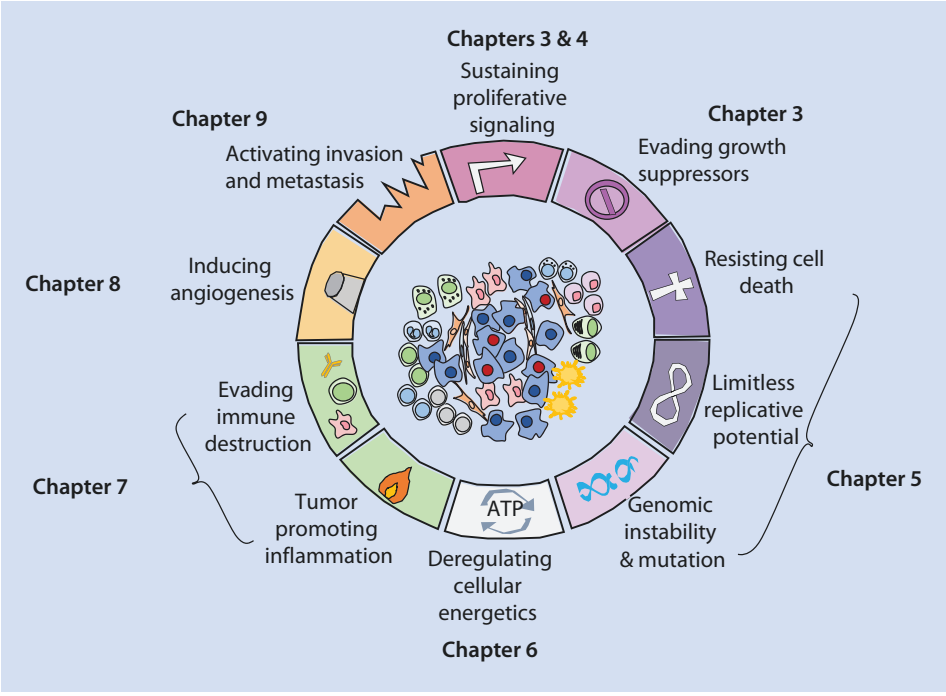
i.e. proteins that are responsible of detecting damage/activating the repair machinery or the repair machinery per se [34], then the probability of acquiring even more mutations increases exponentially and genome becomes genetically unstable. This increase in alterations in the genome, generates sub-clonal diversity; most will die, but the ones that generate an advantage to the tumor will survive and evolve, generally to very aggressive tumors. In other words it is though, that genomic instability accelerates and fuels the whole tumorigenesis process (◘ Fig. 1.9). For more details, read ▶ Chap. 5.

1.10 Hallmarks of Cancer

In 2000 and then in 2011 for an update, Douglas Hanahan and Robert Weinberg wrote a fundamental review article [33, 35], where they rationalized the major biological capabilities that allow cancer cells to thrive and produce a growing tumor and its metastatic dissemination. Most of these hallmarks will be addressed in this book in the following chapters (◘ Fig. 1.10).

Hanahan and Weinberg defined first 6 hallmarks:

1. Sustaining proliferative signaling (▶ Chaps. 3 and 4)
2. Evading growth suppressors (▶ Chap. 3)
3. Resisting cell death (▶ Chap. 5)



◘ **Fig. 1.10** Cartoon of the Hanahan and Weinberg Hallmarks of Cancer and corresponding book chapters where these concepts will be addressed. (Adapted from [33])

4. Enabling replicative immortality (▶ Chap. 5)
5. Inducing angiogenesis (▶ Chap. 8)
6. Activation of invasion and metastasis (▶ Chap. 9)
   and recently they proposed two more emerging hallmarks:
7. De-regulation of cellular energetics (metabolism) (▶ Chap. 6)
8. Avoiding immune destruction (▶ Chap. 7)
   And two more enabling characteristics:
9. Genome instability (▶ Chap. 5) and
10. Tumor promoting inflammation (▶ Chap. 7)

However, in this book we organized these hallmarks in a slightly different way: we first go into the Evolutionary perspective of cancer (▶ Chap. 2), then into general cell signaling and then enter in the cell-autonomous hallmarks to then finish in hallmarks that impact on the host and finally metastasis. After debating all these concepts we present several case studies written by Medical doctors, an Oncologist and a Pathologist that teamed up to try to integrate all this knowledge in the current management of patients. So, after connecting the different dots and getting a general perspective, let's go into the details – hope you enjoy!

Take Home Message

- Healthy multicellular organisms behave as a society of self-sacrifice/altruistic cells as opposed to cancer as a mass of selfish cells, harboring selfish mutations. These mutations disrupt the social controls and checkpoints that control the cellular society.
- Cancer can be viewed as a microevolutionary process, where rounds of mutation and selection drive cancer.
- Conceptually, selfish mutations are classified as oncogenes or tumor suppressors. Oncogenes are genes with activating mutations (gain of function) that are advantageous to the cell. In contrast tumor suppressor genes need to be inactivated in order to give advantage to cancer cells. These genes/proteins when functional provide protective mechanisms against cancer.
- In order for a cancer to grow- increase its mass (number of cells), cells must acquire cell-autonomous characteristics such as:
  - Increase proliferation
  - Decrease cell death
  - Block differentiation
  - Immortality
- In order to increase biomass, cancer cells must undergo a metabolic switch and engage on glycolysis followed by lactic acid fermentation, even in the presence of oxygen.
- In order to grow and thrive in the host, cancer cells must evade the immune system.
- Moreover, tumor cells can sabotage the host to work for them and feed them, can hijack the immune system to provide growth factors, and are also able to recruit new blood vessels to feed the tumor with nutrients and oxygen.
- Tumor cells can escape their original site and travel through the body and colonize distant organs – metastization. To achieve this, tumor cells exploit embryonic/developmental programs of cell invasion and migration. When cells arrive at critical organs (brain, lung, liver mostly) this means the end of multicellular society.

Questions

1. Define, explain and give examples of oncogenes and tumor suppressors.
2. Why do we refer to cancer mutations as selfish?
3. How do selfish mutations appear?
4. How do cancer cells become immortal?
5. How would you describe the Hayflick limit? To what does it lead?
6. Why is it important to understand cancer in an evolutionary perspective?
7. Explain how blocking differentiation programs can lead to cancer.
8. Define and explain briefly the 8 hallmarks of cancer.

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Cancer as an Evolutionary Process

Lília Perfeito

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2

What You Will Learn in This Chapter

We will discuss how natural selection is not restricted to populations of organisms but can in fact happen in cells and tissues. We will see how there is an antagonism between genes selected during somatic evolution and those that were selected during human evolution. We will glimpse at how the forces of natural selection shape tumors and their diversity. At the end of the chapter we will have an idea how each cancer is the result of a unique evolutionary process that depends on the host (patient) but also on the environment and on chance.

Learning Objectives

After reading this chapter students should be able to:

1. Name/describe the basic ingredients for evolution to occur.
2. Distinguish between somatic and germline evolution.
3. View cancer as an outcome of natural selection acting within tissues.
4. Understand that not all diversity is advantageous for the tumor, i.e., there are driver and passenger mutations.
5. Glimpse into the origins of the enormous diversity we observe in tumors.
6. Understand why each cancer is a unique disease.
7. Consider how we can use tools from evolutionary biology to fight cancer.

Important Concepts Discussed in This Chapter

- Mutation – change in the DNA sequence.
- Selection – differences in the ability of a cell or organism to reproduce.
- Fitness – measure of reproductive ability (selection). It is often equated to growth rate.
- Genetic drift – changes in the frequency of mutations caused by chance.
- Selective sweep – rapid increase in frequency of a mutation due to selection.
- Antagonistic pleiotropy – pleiotropy is the observation that one gene can affect multiple traits. It is antagonistic when those traits affect natural selection in opposite directions.
- Epistasis – the fact that mutations have different effects depending on the rest of the genome. It comes mostly from the interaction between proteins.
- Drivers and passengers – Driver mutations are mutations that cause a selective sweep. Passenger mutations are mutations that are present in the same genome as drivers and hence also increase in frequency. Usually they do not contribute to fitness.

2.1 Basic Ingredients of an Evolutionary Process

Evolution is inescapable in replicating biological systems: **whenever there are entities capable of self-replication, subject to mutations that cause phenotypic change, there will be evolution**. These entities can be organisms or groups of cells, including those that compose the somatic tissues of animals. In general, the changes that take place within somatic tissues are not represented in the germline. So, by definition, evolution within a somatic lineage is finite. However, the changes produced by somatic evolution may have terrible consequences to the individual.

In this chapter, we will review how somatic evolution can lead to cancer, particularly to very aggressive cancers. We will see how evolution can make each cancer unique and therefore not conform to a solution (therapy) that fits all.

2.2 Multi-cellular Organisms: The Clash of Two Evolutionary Processes

Throughout the lifetime of a multi-cellular organism, each cell goes through thousands or millions of cell divisions. In each division there is a high chance that at least one mutation will occur (for example, an average of eight mutations per cell division in human fibroblasts [1]). These mutations may be innocuous, i.e., have no effect on the behavior of the cell, or they can significantly alter its phenotype. Because of the latter type of mutations, eukaryotic cells have a number of checkpoints that make sure the cell cycle stops once a major error is detected. That error is then either repaired or the cell dies. Moreover, there are external mechanisms such as the action of the immune system which detect cells that are "misbehaving" and eliminate them. All of these systems are the result of natural selection acting at the level of the organism. They evolved and were maintained because they make sure all the cells behave in such a way as to maximize the survival and reproduction of the organism. Hence, the genome of a species is shaped by natural selection to maximize the survival of the germline!

Multi-cellular organisms thus evolve in two very different ways and at different timescales: **somatic evolution** at the time scale of days and **germline evolution** at the timescale of generations (decades in humans). Moreover, somatic evolution is self-contained. In general, whatever evolved within an organism dies with it. Curious exceptions have been found such as the rare transmissible cancers, as facial tumors of the Tasmanian devil [2] and a type of leukemia-like disease in clams [3]. In these two examples, cancer cells are transmitted horizontally from individual to individual, much like an infectious disease. But these are the exceptions that prove the rule. In general, somatic evolution is finite while germline evolution goes on forever unless the population goes extinct. In addition to the **difference in timescales**, there are also major differences on the **selective pressures** acting on soma and germline. In the somatic tissue, the cells that are over-represented will be those that proliferate faster. Fast proliferation can lead to organ-malfunction and cancer which shorten the life expectancy and the ability of the organism to leave progeny. Therefore, **mutations that increase cellular proliferation will be selected-for in the somatic tissue but selected-against in the germline**.

Due to this conflict between somatic and germline evolution, the germline acquired genes which reduce the probability of evolution within the soma. The most well-known are tumor-suppressor genes which you will read about in the other chapters of this book. These are genes which kill the cells when they behave abnormally. One notable example of the acquisition of tumor-suppressor genes in the germline is the Elephant. These are large animals and therefore it takes many somatic cell divisions to produce an adult. For this reason, there should be a general positive correlation between cancer incidence and body size. However, that is not the case, an observation known as **Peto's paradox** [4]. The solution that natural selection found for the Elephants was the acquisition of extra copies of the tumor suppressor gene p53 [5]. Moreover, during evolution, Elephants reactivated a gene that had been made silent (a pseudogene) in the Paenungulate ancestor and which also has tumor-suppressor activities [6]. There are other mechanisms such as physical barriers to proliferation. Since we know these mechanisms can be very effective, one can ask, "**why do animals still have cancer?**".

As stated above, adaptive evolution maximizes the probability of survival of the germline. However, once an organism has produced its offspring, there is no longer evolutionary pressure to keep it alive. Let's imagine an allele (a variant of a gene) that increases

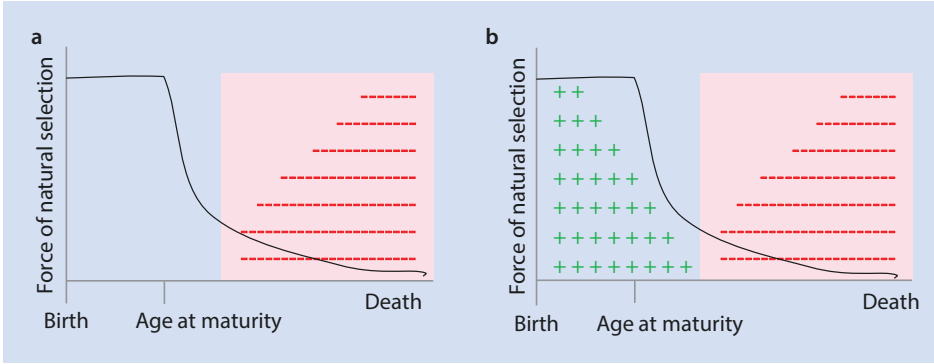

Fig. 2.1 Schematic representation of the strength of natural selection acting on phenotypes as a function of the age of the individuals expressing those phenotypes. In either panel the axes reflect there two measures and the line shows how natural selection is very strong up to the age of reproduction and becomes much weaker after that. Panel **a** represents a feature which is neutral early in life but deleterious later. Mutations causing this phenotype may remain in the population because natural selection is too weak to remove them. Panel **b** represents the antagonistic pleiotropy hypothesis whereby features which are beneficial early and deleterious late will be selected for because natural selection is strongest when they show their benefit. (Adapted from Fabian and Flatt [7])

the production of sperm. This allele will increase fertility, its carriers will have plenty of offspring and soon the allele will dominate the population. Now let's say that this allele, in addition to increasing sperm count, also leads to a moderate increase in mutation rate in skin cells. This increases the chances of skin cancer. But if the tumors only occur after the reproductive period, by the time the individuals die of skin cancer, their children are already part of the population. In other words, the allele that caused the increase in skin cancer already spread to the next generation and it does not matter that the individual dies as long as the germ cell has passed. It is due to this problem of time scale that genes that are deleterious late in life, but neutral or beneficial early in life, survive. Figure 2.1 is a schematic representation of this decrease in the strength of natural selection as time passes.

There are two types of mutations that escape natural selection because of the timing of their actions:

- Those that are neutral early in life but deleterious after reproduction;
- And those that are beneficial early in life but deleterious later on, as in our imaginary example above.

There are then two competing hypotheses:

- **Mutation-accumulation hypothesis** – that says most mutations that cause ageing-related diseases are of the first type
- **Antagonistic-pleiotropy hypothesis** – that says the second type is more prevalent.

As often, reality is probably a mixture of the two. In humans and even in other mammals, it is difficult to identify these types of mutations but there are a few hints that they might exist. In mice, two short alleles of the gene coding for TP53 are described to both confer resistance to cancer and reduce lifespan [8]. Similarly, a study in a human population

before access to contraception shows higher fertility in carriers of BRCA1 and BRCA2 mutations [9]. Both of these alleles are associated with increased rates of cancer. You will learn about the mode of action of BRCA as well as TP53 in other chapters of this book (▶ Chap. 5).

Taking all of this into account, it is easy to imagine that cancer is one of those diseases that was not counter-selected in our ancestors because it occurred well past the age of reproduction. This explains why humans are still plagued by it. It is nevertheless a very particular disease in that cancer in itself is an evolutionary process. It is as if each of us has in our body populations of cells that are fighting for survival, despite the fact that they will never be transmitted to the next generation of humans. Alas, natural selection is extremely short sighted.

2.3 Intra-tumor Diversity and Evolution

Much like any other evolving population, tumors are very diverse within themselves. Decades ago, before the advent of large-scale sequencing, there was an idea that cancer resulted from the sequential take-over of clonal populations, much like in a population of bacteria. One cell would become mutated and increase its numbers, taking over the population in what is known as a **selective sweep**. Once that was done, a second mutation would come along and begin its own rise in frequency and so on. This turned out not to be the case, neither for cancer, nor for bacteria. In fact, several mutants may arise at the same time and begin increasing in frequency. Since they cannot recombine and be put in the same genome, they are destined to either **compete** (in which case only one will win) or **cooperate**. In either case, for some time that may be quite long, there will be co-existence of both variants. Now, given the large mutation rate, there can be several of these mutants. All increasing the growth rate and all either competing or cooperating, or both. Moreover, in addition to these mutations that increase the growth rate, there are also those that are either neutral or deleterious (decreasing the growth rate). Under normal circumstances, these mutations would either be purged or increase very slowly. However, when combined with mutations with strong positive effects they will hitchhike and become quite frequent as well. This is where the distinction between **driver** and **passenger mutations** comes in. Drivers are those mutations that cause the tumor to increase in size or in survival and passengers are those that do not do anything to the tumor. ◘ Figure 2.2 shows an example of how driver mutations compete and how passengers (or hitchhikers) can increase in frequency as well.

It is now known that there are many drivers and passengers within a tumor. However, successful therapies must target drivers and not passengers. Therefore, identifying the drivers is one of the most important yet challenging tasks of modern day's genetics. To make matters more complicated, passenger mutations can interact with drivers and with each other and contribute to tumor progression by conveying different phenotypic traits. For instance, imagine a tumor that has a drug efflux pump as a passenger mutation; while the tumor is growing, and the patient is not under therapy, the pump does not give any advantage. However, when therapy starts the clones that have this passenger mutation can become selected. Hence, the definition of driver and passenger, while very useful, it is not absolute.

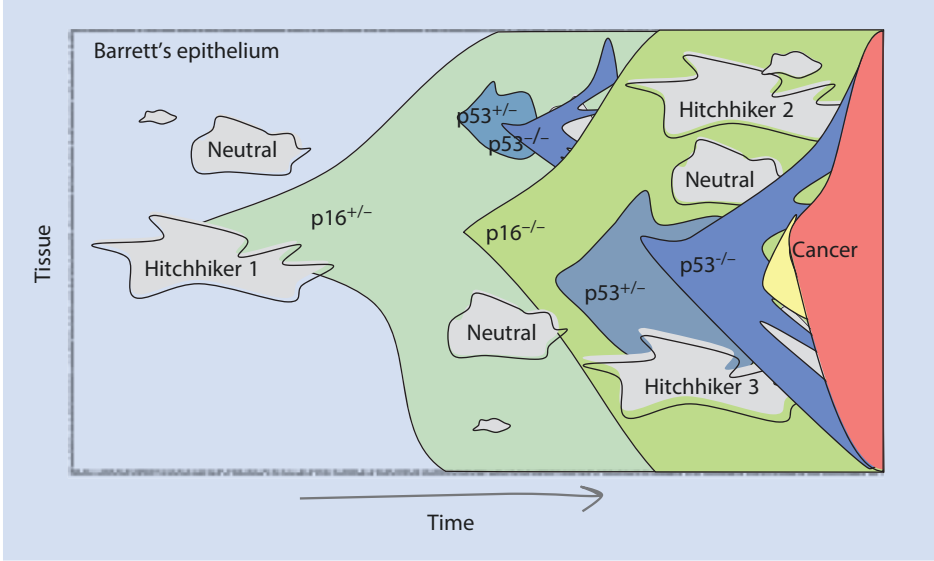

Fig. 2.2 Representation of the frequency of mutations in a tumor over time. The colors represent different mutations and the area shows their frequency at each time point. The grey bubbles are passenger mutations that appear and only have a chance of increasing in frequency if one of the cells carrying them also acquires a driver mutation. Note how certain mutations that are beneficial such as p53−/− appear and are lost if they have to compete against stronger mutations such as p16−/−. (Adapted from Maley and Reid [10])

2.4 Sources of Diversity and the Impact of Mutations on Fitness

Errors are inevitable in any replicating system. Mutations are errors in DNA which are not repaired by the cell. Indeed, cells have a number of mechanisms to repair errors in DNA (see ► Chap. 5). These include, for example, mismatch repair proteins, which detect mismatches between the two DNA strands and correct them. Still these systems are not perfect and allow a certain number of mutations to pass to the next cell generation. We call the mutation rate, the number of mutations that occur per cell, per generation. Mutation rates can be expressed per nucleotide or per genome. Mutation rates vary between cells and between organisms and it is not known what selects them to be a certain number. For the germline it is thought that the mutation rate is as low as it can be without compromising the normal functioning of the cell – if the cell spends all its resources in repair, it cannot do other essential functions such as metabolism. For somatic tissues, the mutation rate depends on the type of cell. Fibroblasts, for example, have a rate 100-fold higher than the germline [1]. The precise reason for this is not known. One trivial hypothesis relates to the **antagonistic pleiotropy** described above. Somatic cells need to replicate fast in order for development to occur. Moreover, processes such as wound healing require quick and efficient production of new cells. Diverting energy to speed means there is less time and fewer resources for repair. There has to be a fine balance between speed and repair. Any mutation happening in the germline or in early development will mean the death of the organism. However, mutations occuring in differentiated cells like adult fibroblasts will have little impact on reproduction and hence these cells have evolved to spend less resources on repair and more on proliferation. In addition to spontaneous mutations, the exposure of humans to chemicals, radiation or other external conditions which increase the DNA error rate also leads to increase in the mutation rate. The two most

well-known examples are smoking, which increases the probability of a number of cancers, partially due to an increase in mutation rate [11]; and skin cancer, where the appearance of tumors is clearly related to exposure to radiation from the sun, which also leads to an increased mutation rate [12].

2.5 Mutation Spectra and the Fitness Effects of Mutations

Independently of what causes them, there are different types of mutations. There are single nucleotide changes (SNPs – the 'P' stands for polymorphism and is reminiscent of a time when mutation rates were estimated from polymorphic DNA variants in populations), insertions and deletions, structural variants such as inversions, translocations, duplications and large deletions, whole chromosome gains and losses. Indeed, when looking at cancers, all of these mutations are visible [11].

Having more mutations is not in itself a good thing for the cancer cell. Being good, or bad as we saw earlier, depends a lot on the level of organization we look at. In this case, a mutation is good for cancer if it increases the survival and the growth rate of cancer cells. In other words, a mutation is good for cancer if it increases its fitness. Most mutations are neutral or deleterious, i.e., they do not affect fitness, or they decrease it. This is true even for cancer (or so we think). The rationale behind this statement is that when we make a random change to a functioning object, we very rarely make it better. Imagine hitting a functioning car with a very large hammer. What are the odds it will go faster? In addition to these arguments, there is also evidence form bacteria, yeast, *Caenorhabditis elegans* among others, which were grown in the lab in the absence of natural selection. Under these conditions their fitness decreases, indicating that most spontaneous mutations that affect fitness are deleterious. See for example Kibota, T. T., & Lynch, M. (1996) [13].

2.6 Constraints to Evolution/Genetic Drift

So far, I have equated evolution with adaptation. However, evolution is not always positive. **Evolution is a change in the frequency of genetic variants with time**. It does not mean that it has to be positive. And indeed, not all the diversity we observe is beneficial. In fact, we expect that some of the mutations we observe in cancer are deleterious (i.e., they decrease the tumor's fitness). These mutations may be passengers that increased in frequency because they happened to be in the same genome as a beneficial mutation; or they might have increased in frequency by chance, i.e., via the process of **genetic drift**. Any process that slows down natural selection will increase genetic drift. In general, genetic drift is the result of either a high mutation rate or of demographic constraints such as small population sizes or the presence of spatial structure. In the case of a high mutation rate, genetic drift becomes stronger than natural selection when deleterious mutations happen faster than natural selection can eliminate them. One approach to eliminate cancer is actually to increase its mutation rate [14]. The problem is that a moderate increase in mutation rate actually aids cancer by producing more driver mutations. Therefore, any such therapy must ensure the increase is large enough. The other way to increase genetic drift is by interfering with demography. One option is to reduce the number of cells that compose the tumor. If the number of cells is small enough, the chance of producing driver mutations is low and instead it is easier for deleterious mutations to increase in frequency. In the same way, the existence of spatial structure (e.g., in epithelia) also leads

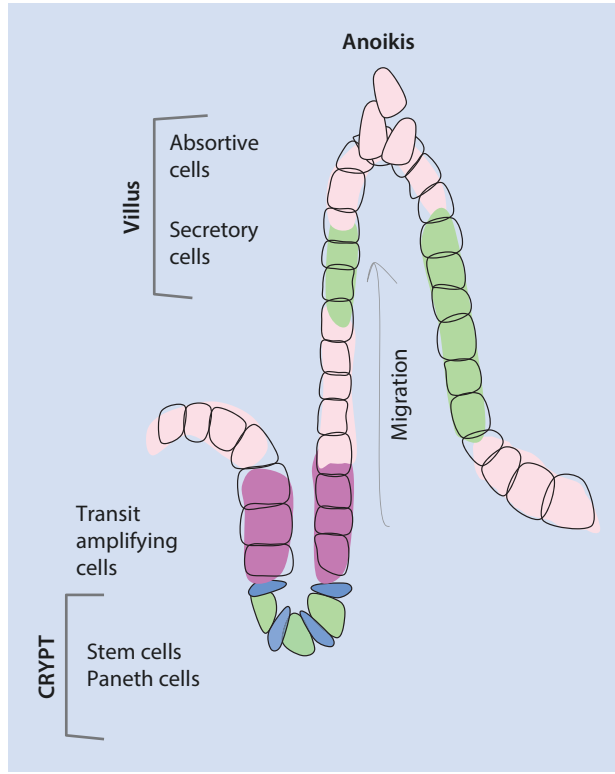

Fig. 2.3 The organization of the epithelial tissue. The only cells that remain in the tissue are located in the crypt. The rest are pushed away as these cells divide and eventually are shed into the external environment where they die (anoikis). Any mutation that occurs outside the crypt will be lost, even if it leads to increased proliferation. (Adapted from Barker [16])

to an increase in genetic drift. Any driver mutation that comes along will take longer to take-over the whole population because it can only compete with a few neighboring cells at a time. It is as if the total population size was smaller [15]. Many tumors come from epithelia. The most likely reason is the extent of cell turnover in these tissues, which probably leads to an increased mutation rate. Evolutionary theory predicts that, if not for the spatial structure, these tumors would be even more numerous and aggressive.

The role of spatial structure in preventing somatic evolution was likely co-opted by germline evolution to prevent tumors in highly proliferative tissues such as the intestinal epithelium. There, cell division is restricted to a small niche in the crypts. As cells divide, they migrate through the villi and are eventually shed. This means that any mutation acquired during this process will be pushed away and out of the tissue, even if it divides very quickly. Figure 2.3 shows a schematic representation of the intestinal epithelium and of the path cells take during normal tissue function.

2.7 Why Cancer Is Not a Single Disease and Why We Have Not Cured It: An Evolutionist's Perspective

Just as no two humans are alike, no two cancers are alike, even within the same patient. Cancer is the result of chance, selection, genetic and biophysical constraints. Whether or not a particular mutation contributes to cancer or not is highly dependent on the genetic

background (a phenomenon known as epistasis). Therefore, a tumor in one individual will have a different set of driver mutations than a tumor in someone else. In the same manner, chance events will be very different between any two patients. All of this points to a fact that medical doctors have been aware of for some time: each tumor is a unique disease that must be studied and treated individually. In fact, nowadays tumor genotyping is becoming common practice and new tumor biomarkers are helping to improve clinical outcomes by directing therapies. However, most biomarkers are not 100% prognostic. This is because we are still far from predicting the output of the interaction of the different mutations (plus the intronic mutations, epigenetic modifications, etc.), making it unpredictable to know which is, the best therapy for each individual patient. This is why different functional tests are being developed to challenge patient tumor cells with the different therapy options to select the best one, just like antibiograms for bacterial infections. These tests include xenografting patient tumor cells into animal models like zebrafish and mouse, generating organoids or cultivating tumor explants in the presence of the therapeutic options and then evaluating their response. Let's hope one of these tests work.

Take Home Message

This chapter covers the following points:

- Cells within tissues evolve in much the same way as populations of organisms. This is known as somatic evolution.
- Somatic evolution occurs in a shorter timescale than organismal (germline) evolution. Moreover, the phenotypes that are selected in one are often opposite. For example, somatic evolution may select for fast cell division and low repair mechanisms while the opposite will be selected for in the germline.
- Somatic evolution is finite, and the mutations selected during this process will die with the organism.
- Germline evolution is not able to eliminate all mutations that cause disease. Namely, for diseases that only happen after the age of reproduction (like cancer), natural selection is not very effective.
- Mutations may accumulate in the germline that make the individual prone to cancer, either because they have no effect on the reproductive output, or because they actually increase fertility early in life.
- A tumor is the result of a very complex evolutionary process where both natural selection and genetic drift are at play. Not all mutations found in a cancer contribute to the tumor.
- Each tumor is unique just as each human is unique. Therapies should take this into account.

Questions

1. What are the minimum requirements for evolution?
2. Give some example of genes that may be subject to antagonistic pleiotropy.
3. How would you identify mutations that are causative of a tumor?

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Cell Signaling in Cancer

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3

What You Will Learn in This Chapter?

After reading this chapter you should understand what cell signaling is, how it works, what it serves for and especially why it is important in the context of cancer. You should know the basics about three key signaling pathways:

- JAK/STAT,
- MEK/ERK and
- PI3K/Akt/mTOR,

and realize that they are very frequently involved in all different types of cancer. You should know, however, that many other signaling pathways exist, such as Notch, Wnt, Hedgehog, etc., that also partake in tumorigenesis. Importantly, you are expected to know in what consist the so-called "signaling therapies", which are their advantages, understand their potential and have some insight into the mechanisms that explain why they frequently fail. You should also understand some of the strategies aiming at overcoming resistance associated with signaling therapies and their possible caveats. Finally, we hope we will convey the need for a deep characterization of the cancer patient in order to devise the best targeted therapies.

Learning Objectives

After reading this chapter, students should be able to:

1. Understand the importance of signal transduction in both physiological and cancer contexts.
2. Enumerate the major post-translational protein modifications observed in signal transduction.
3. Recognize the main elements of the most common signal transduction pathways involved in cancer progression.
4. Pinpoint the reasons for hyper-activation of signal transduction cascades in cancer.
5. Identify the most commonly used therapeutic strategies to target the signaling machinery – "signaling therapies".
6. Recognize the mechanisms of resistance to signaling therapies, and possible strategies to overcome resistance.

Important Concepts Discussed in This Chapter

- Signal transduction and signal transduction pathways
- Microenvironmental and cell-intrinsic cues
- Post-translational protein modifications
- Protein phosphorylation
- Signaling players
- Oncogene addiction
- Signaling therapies
- Resistance mechanisms

3.1 What Signal Transduction Is and How it Works

Complex biological systems, such as mammals or birds (to name just two of many potential examples), engage mechanisms that allow them to perceive and cope with an ever-changing environment. This ability to respond to external cues in an appropriate manner is essential for their survival and adaptation. At a vastly smaller scale unicellular organisms, such as bacteria, do exactly the same – because this process of **perception-integration-**

response is as essential for their overall fitness as it is for multicellular organisms. So much so that any cell in a human being or in a mouse will have the same exact ability – of transforming signals, mainly from the outside of the cell, into a response (which we broadly call signal transduction). In fact, **signal transduction**, which occurs in each cell of the body, is largely required for the correct functioning of the whole organism [1].

How does signal transduction work in a cell? Like anything in biology, it is both very simple and very complex. Let's start by the simple before we move on to the more complex. To perceive the environment a cell needs a **receptor**, which will enable it to receive an "input". This receptor may come in many "flavors": it can be a cytokine receptor, a growth factor receptor, an adhesion molecule, a chemokine receptor and many other receptors that are located at the surface of any cell, in the plasma membrane. Or it can be cell type-specific, such as the T cell receptor, which as the name indicates is present only in T cells of the adaptive immune system; or the many odor receptors that are present only in olfactory neurons. It can even be a hormone receptor, which is not located at the cell surface but rather in the cytoplasm. Whatever the "flavor", the receptor will allow the cell to receive an "input" signal that triggers a response. This response is mediated by biochemical reactions that are usually referred to as **signal transduction pathways** or **signal transduction cascades**, since normally they follow a sequential pattern in which an upstream element impacts an immediately downstream element that subsequently affects another element further downstream. Below, we will describe briefly the biochemical reactions involved in signal transduction. At this stage it suffices to say that **protein kinases** are the most well-studied and prominent elements in these biochemical cascades. Finally, a certain response (an "output") should result. This response can range from cell cycle progression, to differentiation, to migration or apoptosis. It frequently involves the modulation of transcription factors and consequent transcriptional regulation of different genes, but in some instances it may not involve gene regulation and it may occur only at the protein level (as it happens with certain apoptotic stimuli that lead to release of cytochrome c from mitochondria and consequent activation of caspases) [1, 2].

In short, signal transduction is the biochemical process by which a cell perceives a certain cue, and triggers decision-making ultimately leading to a response to the initial cue (Fig. 3.1).

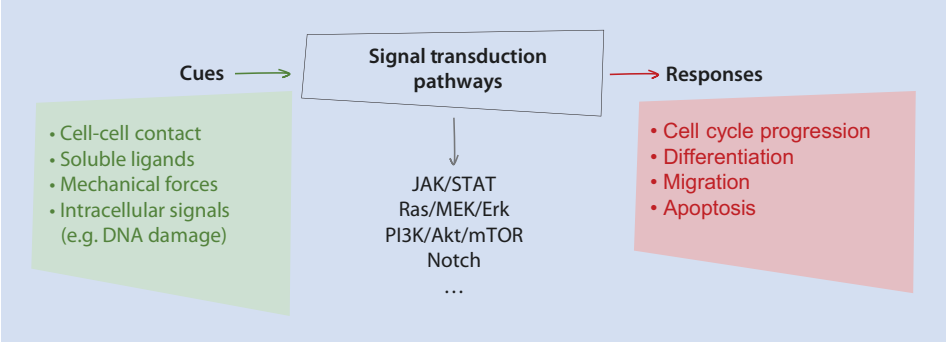

Fig. 3.1 The general organization of cell signaling. Cells have the ability to perceive and receive (environmental) stimuli (input signals, green arrow) via different kind of receptors and other sensors. This information is then integrated and processed to trigger appropriate response(s) (output signals, red arrow) mediated by complex intracellular signaling networks, called signal transduction pathways or cascades, such as JAK/STAT, Ras/MEK/Erk, PI3K/AKT/mTOR, Notch, etc. The cellular outputs may vary from cell cycle progression, to differentiation, to migration or apoptosis, and usually involve modulation at the gene and/or protein levels

Although the majority of the cues will expectedly lie outside of the cell, their nature can vary substantially and include not only cell-cell contact or soluble ligands, but also, for example, the sensing of mechanical forces. In addition, it should be underlined that input signals may originate also within the cell. A notable example is DNA damage, which activates signal transduction pathways that recruit the machinery required for the resolution of the damage and, in case of failure, eventually promote cell death (see ▶ Chap. 5).

3.2 Major Post-Translational Protein Modifications in Signal Transduction

Signal transduction pathways rely vastly on enzymes that catalyze reactions leading to **protein post-translational modifications**, including (◘ Table 3.1):

- ubiquitination,
- sumoylation,
- acetylation,
- methylation,
- O-glycosylation and
- phosphorylation.

The latter is, by and large, the most well-studied and characterized. This is not only because it is technically easier to analyze than many of the other modifications but also, and most importantly, because it is the one consistently involved in almost all of the main signal transduction pathways that have been characterized in higher organisms.

Protein phosphorylation is catalyzed by protein kinases, which transfer the terminal phosphate group of ATP to the hydroxyl moiety of an amino acid residue in a target protein. The products of this reaction are a phosphorylated protein and ADP (◘ Fig. 3.2). Since phosphate groups are highly negatively charged, phosphorylation has the ability to modify significantly the charge of the target protein, consequently altering its conformation and thereby affecting its function, association with other proteins and/or cellular localization [3].

◘ **Table 3.1** Main post-translational protein modifications in signal transduction

Protein modification	Description
Ubiquitination	Ligation of ubiquitin peptide (8.5 kDa) to a lysine residue
Sumoylation	Ligation of a SUMO peptide (12 kDa) to a lysine residue
Acetylation	Ligation of C_2H_3O to a lysine residue or N-terminus
Methylation	Ligation of CH_3 to a lysine or arginine residue
O-Glycosylation	Ligation of a sugar molecule to an oxygen atom in an amino acid residue
Phosphorylation	Addition of PO_4 molecule to serine, threonine or tyrosine residue

Roughly 2% of all eukaryotic genes are protein kinases, and the mammalian kinome includes more than 500 members.

Protein kinases acting within mammalian cells are classified into three categories:

1. Tyrosine-specific protein kinases – phosphorylate tyrosine residues on target proteins
2. Serine/threonine-specific protein kinases – phosphorylate serine or threonine residues
3. Dual-specificity protein kinases – are rare and display the capacity to phosphorylate simultaneously tyrosine and serine/threonine residues [4].

The vast majority of protein phosphorylation occurs on serine residues (80–95%), significantly less on threonines (4–17%) and less than 1% up to 4% on tyrosines. This does not mean phosphorylation of tyrosines is less relevant than of serines or threonines. On the contrary, **tyrosine phosphorylation** is frequently involved in the most upstream steps of signal transduction, with about half of tyrosine kinases being cell surface receptors and the remaining displaying activity mostly close to the plasma membrane.

Protein tyrosine kinases are subdivided into:

1. Receptor tyrosine kinases (RTKs), if they are cell surface receptors with intrinsic kinase activity, and
2. Receptor-associated or non-receptor tyrosine kinases, if they are cytosolic – normally associated with or recruited by cell surface receptors (Table 3.2) to propagate the signaling to downstream effectors [3, 4].

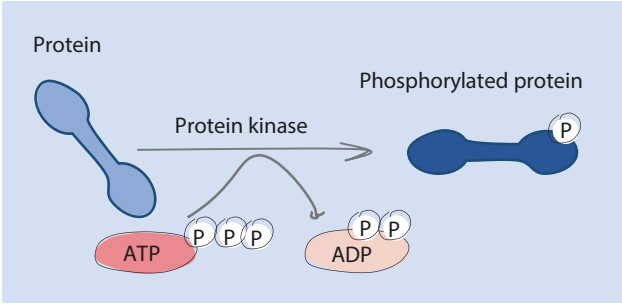

Fig. 3.2 Signal transduction occurs mainly through protein phosphorylation. Protein phosphorylation reaction is catalyzed by protein kinases, which convert ATP in ADP by transferring the terminal phosphate group of ATP to the hydroxyl moiety of an amino acid residue in a target protein

Table 3.2 Types of human protein kinases

Human protein kinases	Targets	Subtypes
Tyrosine-specific protein kinases	Tyrosine residues	Receptor tyrosine kinases (RTKs)
		Receptor-associated or non-receptor tyrosine kinases
Serine/threonine-specific protein kinases	Serine or threonine residues	–
Dual-specificity protein kinases	Both tyrosine and serine/threonine residues	–

Of course, **dephosphorylation** of proteins is also essential for signal transduction. In some cases to propagate the signal and in other cases (perhaps most often) to "reset" the system and allow a certain protein, and the pathway that it pertains, to go back to the pre-stimulus state, which is essential both to prevent pathway permanent activation (that, as we will see below, can put cells at risk for cancer transformation) and to allow for subsequent physiological re-stimulation. The reaction of protein dephosphorylation is catalyzed by **protein phosphatases** [5]. Although, for simplicity, we will not focus our attention on these in the next sections, it is important to keep in mind that **protein phosphatases are as essential as kinases** for the homeostasis of the cell as far as signal transduction goes [5].

3.3 Why Signal Transduction Is Important in Cancer?

At this point you should have a grasp of what signal transduction is but may be asking why one should spend time analyzing signaling in the context of cancer. There are several reasons. The most obvious is not difficult to guess, based on the type of cellular responses that are regulated by signal transduction pathways. These include:

- regulation of cell cycle and proliferation,
- migration,
- differentiation,
- viability versus cell death,
- metabolism, etc.

It is therefore not surprising that deregulation of signal transduction may have considerable impact on the homeostasis of the cell. For example, aberrant, constitutive activation of a pathway that promotes proliferation in a single cell may lead to a selective advantage that eventually leads to clonal expansion at the expense of other cells. So firstly, signaling pathways are important for cancer simply because they are essential regulators of all the different facets of normal cell physiology. Consequently, **signaling players** are often **oncogenes** (or, sometimes, **tumor suppressors**). An example: the first human proto-oncogene to be cloned, *RAS*, mutated in around one third of all cancers, encodes a small GTPase essential for activation of Ras/MEK/ERK signaling pathway. In fact, examples can be found in ALL classes of signaling players (Fig. 3.3) [2, 3]. Ligands such cytokines and growth factors (e.g. PDGF, VEGF, Wnt, IL-15) are often overexpressed and involved in aberrant autocrine/paracrine loops that promote malignancy. Their respective receptors, frequently RTKs, are well-known oncogenes (e.g. PDGFR, EGFR, IGFR, Notch, IL7R) often mutated and amplified in cancer cells. Of course, the examples of intracellular signaling constituents known to be altered in cancer are numerous (e.g. PI3Ks, JAKs, Ras, PTEN, etc). Finally, the transcription factors that are regulated by signaling pathways are also frequently oncogenes (e.g. MYC, TAL1) or tumors suppressors (e.g. P53).

Importantly, cancer cells frequently display functional dependence on activation of specific pro-tumoral signaling pathways in a process often referred to as "**oncogene addiction**". Why does this happen? Cancer cells are selected for their permanent activation of a certain pathway. This allows them to expand at the expense of their normal counterparts, but it also implicates that the malignant cells depend on the perpetual activation of that pathway for their maintenance [6]. This contrasts with what happens in normal cells that rely only transiently, after a certain physiological stimulus, on the activation of the same pathway. In other words, the "addiction" to a certain oncogenic signaling path-

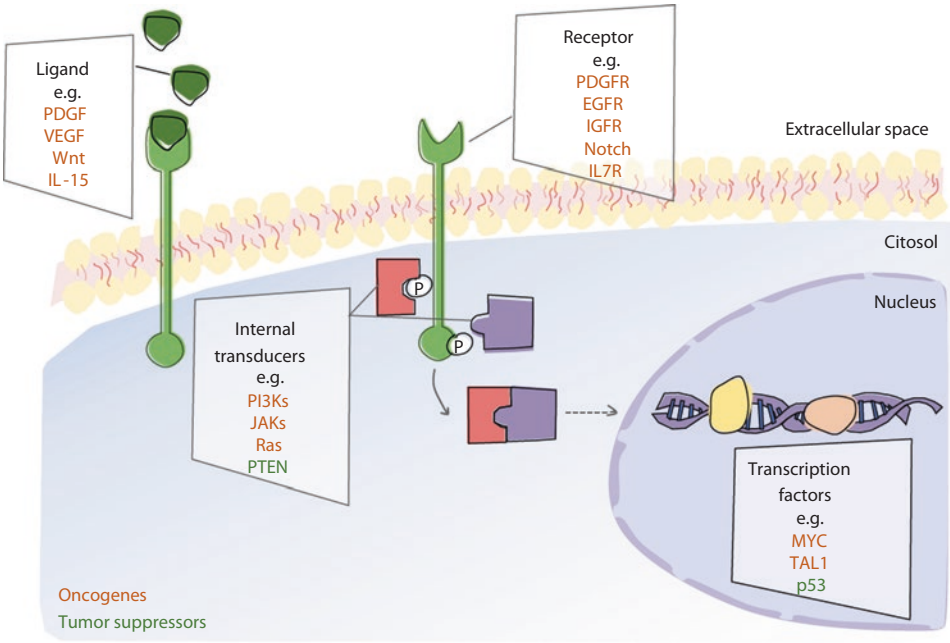

Fig. 3.3 Different signaling players can be targets for carcinogenic events. The following classes of signaling players can become oncogenes or tumor suppressors: ligands such cytokines and growth factors; receptors (frequently RTKs); intracellular signaling transducers; and gene regulating factors

way – a feature not shared by normal cells – constitutes an advantage to the cancer cell but it is its "Achilles' heel" as well. Evidently this also represents **a therapeutic opportunity**, because targeting a signaling pathway used by both cancer cells and normal cells may have substantially different consequences in each type – with normal cells expectedly being less sensitive to **signaling-specific drugs** than transformed, oncogene-addicted, cells.

This leads us to the third reason why signaling is important in cancer: signaling pathways constitute appealing molecular targets for therapeutic intervention, the actual practical reason being that kinases are easily "druggable" (using either ATP-competitive or allosteric inhibitors) and thus often excellent targets for drug development and clinical intervention [7].

Finally, there is a fourth reason placing signaling at the center-spot of tumorigenesis: the **crosstalk** between cancer cells and their microenvironment relies on the modulation of specific signal transduction pathways. It is now firmly established that **a cancer is a complex entity that includes not only the cancer cells themselves but also a multitude of normal cells** that include endothelial cells and pericytes, fibroblasts, and diverse immune cells. Therefore, cancer progression depends not only on lesions within the malignant cells but also on interactions between the cancer cells and their surroundings, in a manner that is ultimately beneficial for the tumor. This sort of dialogue occurs via, and is strictly dependent on, modulation of signaling pathways within the cancer cells and the tumor microenvironment [8].

Overall, the involvement of signaling pathways in cancer origin and progression, the dependence of cancer cells on certain pathways and the fact that signaling is also important for the pro-tumoral cross-talk between malignant cells and "normal" cells in their

surrounding microenvironment, all contribute to the notion that targeting signaling pathways in a rational manner constitutes a viable therapeutic strategy for cancer intervention. Of course, this requires a profound understanding of the biology and biochemistry underlying signal transduction activation in cancer cells.

3.4 Key Signaling Pathways in Cancer

The list of signal transduction pathways whose involvement in cancer has been demonstrated is growing (Table 3.3). Below, we will focus on three pathways: **JAK/STAT**, **Ras/MEK/Erk**, and **PI3K/Akt/mTOR**, the reason being that, in contrast to the remaining included in Table 3.3, they are very frequently activated in all types of cancer. Also, they are common effectors of different upstream lesions. For instance, different RTKs and cytokine receptors lead to the activation of STAT, PI3K and MEK signaling. Similarly, the fusion protein BCR/ABL, which is a hallmark of Chronic Myeloid Leukemia (CML), also activates these three signaling pathways. In short, these three critical signal transduction cascades are frequently hyper-activated in cancer as a consequence of gain-of-function mutations in their members, loss-of-function mutations or deletions in negative regulators or activation of upstream receptors. In the latter case, receptor activation can result from gene amplification or gain-of-function mutation, or from autocrine or paracrine aberrant stimulation due to ligand overexpression.

3.4.1 JAK/STAT

This is the pathway canonically activated by **cytokines**. There are four **Janus kinase (JAK)** family members (JAK1, JAK2, JAK3 and TYK2), which are tyrosine-specific protein kinases of around 110–130 kDa. With the exception of JAK3, which is largely restricted to hematopoietic cells, JAKs are ubiquitously expressed [9]. The other elements of the pathway are the **signal transducers and activators of transcription (STATs)**, which, as the name indicates, are latent transcription factors located in the cytoplasm. STATs are encoded by seven genes: *STAT1-4*, *STAT5A*, *STAT5B* and *STAT6*. STATs possess several important domains:

- an N-terminal domain used for instance for nuclear translocation,
- a coiled-coil domain involved in protein-protein interactions,
- a DNA-binding domain,
- a Src-homology 2 (SH2) domain required for binding to the receptor and subsequent STAT dimerization, and
- a C-terminal transactivation domain required for transcriptional activity. This transactivation domain is also where a critical tyrosine residue is located that is essential for STAT5 activity [9].

Some STATs (STAT-1, -3, -4 and -5) display one additional serine residue in the transactivation domain that is required for maximal transcriptional activity, which is obviously not a target of JAKs (given the fact that they are strict tyrosine kinases). This serine actually represents a good example of a phenomenon that is frequent in signal transduction: **cross-talk between different pathways.** In this case the serine residue is known to be phosphorylated by ERKs.

Table 3.3 Key signal transduction pathways implicated in tumorigenesis

Pathways	Activated by	Involved	Available clinical drugs
JAK/STAT	Cytokines (e.g. IL-3, IL-6 and IL-7)	Myeloproliferative neoplasms; breast and prostate cancers	Ruxolitinib; Tofacitinib
Ras/MAPK	Cytokines and mitogenic growth factors	Melanoma; thyroid, pancreatic and NSCL cancers	Vemurafenib; Trametinib
PI3K/Akt/mTOR	Insulin; growth factors; chemokines	Glioblastoma; breast, prostate, colon and endometrial cancer	Buparlisib; MK-2206; Rapamycin; BEZ-235
Nuclear hormone receptor	Steroid hormones (e.g. estrogen and progesterone)	Breast and prostate cancers	Tamoxifen; Enzalutamide
Notch	Delta-like (DLL) 1, 3 and 4; Jagged (JAG) 1 and 2	T-ALL; CLL; MCL; breast adenocarcinoma; SCC	γ-secretase inhibitors (RO4929097)
Wnt (β-catenin)	Wnt ligands	Melanoma; breast and colorectal cancers	LGK974; PRI-724
TGF-β (Smad)	TGF-β	Prostate, breast and pancreatic cancers	Fresolimumab; Galunisertib
NF-κB	Cytokines (e.g. TNF-α; IL-l)	Breast cancer; B-cell lymphomas; multiple myeloma	Bortezomib
Hippo	DCHS1, DCHS2	Breast cancer; familial and sporadic schwannomas	Verteporfin; Dobutamine
Hedgehog	Sonic (SHH), Indian (IHH), or desert (DHH) hedgehog	Glioblastoma; basal cell carcinoma; medulloblastoma; CML	IPI-926; Vismodegib
DNA repair signaling (ATM/ATR)	Single-stranded DNA (ssDNA) and DNA double-strand breaks (DSBs)	Breast, colorectal and lung cancers	Olaparib; MK-8776

For each pathway we provide the main examples of: activating physiological stimuli, the cancers in which their hyper-activation is involved, and available drugs in the clinic targeting them

How does JAK/STAT pathway work? It is actually very straightforward. The conformational changes triggered by the binding of a cytokine to its receptor, allow for the recruitment and/or activation of JAKs that associate with the cytoplasmic region of the receptor. The JAKs then transphosphorylate each other, leading to their maximal activity, upon which they phosphorylate tyrosine residues in the receptor itself that constitute binding sites for STATs via their SH2 domains. The recruitment of STATs to the vicinity of JAKs

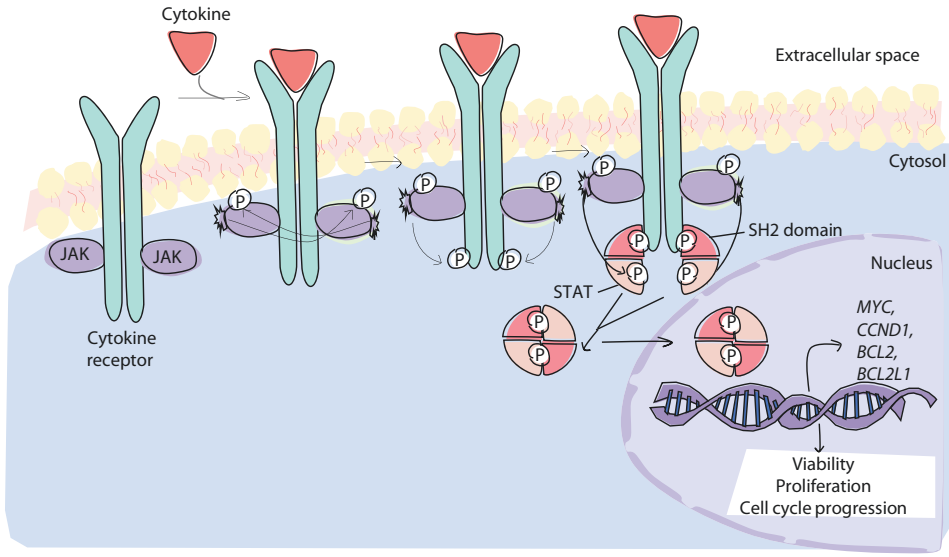

Fig. 3.4 Summary of JAK/STAT pathway. STAT proteins are recruited to the cytokine receptor and phosphorylated by JAKs, leading to STAT dimerization and nuclear translocation. In the nucleus, STAT dimers bind DNA and promote transcription of several target genes, such as *MYC, CCND1* and Bcl-2-family members *BCL2* and *BCL2L1*, which are positively involved in induction of cell viability, proliferation and cell cycle progression

allows the latter to phosphorylate them, promoting STAT homo- or hetero-dimerization and consequent translocation into the nucleus, where they are finally allowed to bind to the promoters of target genes and act as *bona fide* transcription factors (Fig. 3.4) [10]. Examples of STAT target genes include *MYC*, *CCND1* (encoding Cyclin D1), *BCL2* and *BCL2L1* (encoding BCL-X_L), which are positively involved in induction of proliferation and protection from apoptosis [11–13].

3.4.2 Ras/MEK/Erk

The MAPK pathway has three major signaling modules, but we are going to focus only on the classical extracellular signal-regulated kinase (Erk) pathway, or simply Ras/MEK/Erk pathway. This pathway is mainly activated by **mitogenic growth factors** and cytokines [14, 15].

It starts with activation of a Ras guanine nucleotide exchange factor (RasGEF) that triggers the release of GDP from Ras and its association with GTP. This event turns on the small GTPase Ras, which indirectly (through a rather complex mechanism) activates Raf and thereby transduces the signal via a cascade that contains three levels (Fig. 3.5):

- **First Level: Raf**, a MAPK kinase kinase (MAPKKK), phosphorylates and thereby activates MEK,
- **Second Level: MEK,** a MAPK kinase (MAPKK), phosphorylates and thereby activates Erk,
- **Third Level: Erk**, a MAP kinase [15], phosphorylates and activates several downstream targets, including the kinases $p90^{RSK}$ [16], Mnk [17] and Msk [18] and

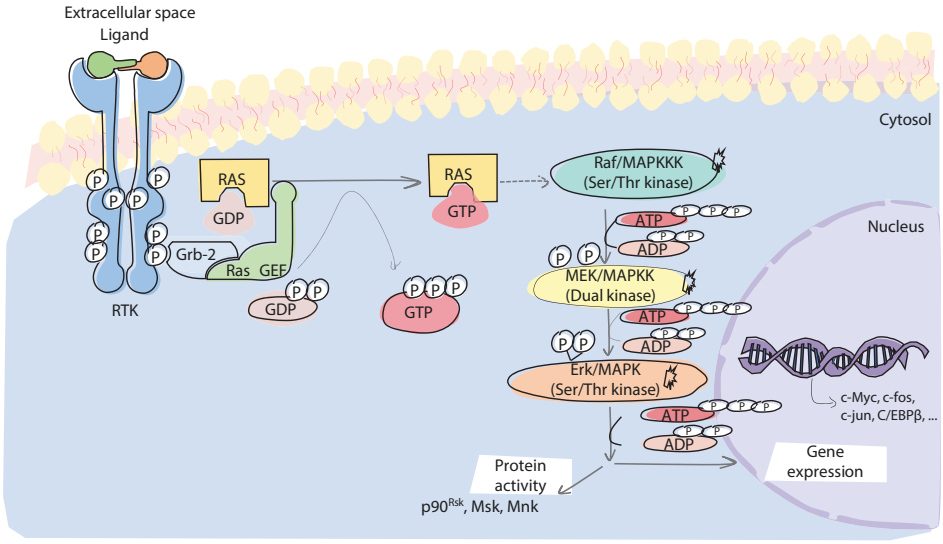

Fig. 3.5 Summary of Ras/MEK/Erk pathway. Upon ligand binding, Ras protein is turned on by association with a GTP molecule, thus leading to the activation of the phosphorylation cascade MAPKKK-MAPKK-MAPK. Erk1/2 (MAPK) activation leads to phosphorylation and activation of several downstream targets, including Rsk, Msk and Mnk, which are involved in mRNA translation. When translocated into the nucleus, Erk1/2 phosphorylates and regulates the action of different transcription factors such as MYC, FOS, JUN, C/EBPβ

transcription factors such as c-Myc, c-Fos, c-Jun and C/EBPβ [19]. It consequently promotes cell cycle progression by regulating the expression of cyclin D1, $p27^{kip1}$ and $p21^{cip1}$ [20].

Overall, this pathway promotes cell survival and proliferation and regulates differentiation.

3.4.3 PI3K/Akt/mTOR

The PI3K/Akt/mTOR pathway is a major cellular signaling module known to promote cell growth and survival, to inhibit apoptosis and to control metabolism [21, 22]. There are three different classes (I-III) of the phosphatidylinositol 3-OH-kinases (PI3K), but only the ones belonging to class IA are activated by RTKs.

Class IA PI3Ks form heterodimers of:

- a p85 regulatory subunit and
- a p110 catalytic subunit [23].

Upon activation, PI3K catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate (PIP_2) into the second messenger phosphatidylinositol-3,4-5-trisphosphate (PIP_3) [21–23]. This event triggers the recruitment of proteins containing a pleckstrin homology (PH) domain to the vicinity of the plasma membrane. Those proteins include the major downstream effector of the pathway, the serine/threonine kinase Akt/PKB (Protein kinase B), the serine/threonine 3-phosphoinositide dependent protein kinase-1 (PDK1) and the mammalian target of rapamycin complex 2 (mTORC2). Their co-localization elicits

Akt phosphorylation by PDK1 and mTORC2 (which acts as PDK2) and consequent Akt full activation. PDK1 phosphorylates T308, whereas mTORC2 phosphorylates S473 [21, 22]. Once activated, Akt is able to activate or repress, through phosphorylation, multiple downstream targets (Fig. 3.6). Without going into too much detail, Akt:

- inhibits the glycogen synthase kinase-3α/β (GSK3α/β), thus promoting both cell proliferation and viability;
- inhibits the forkhead box O family of transcription factors (FoxOs), known to promote the transcription of pro-apoptotic genes and cell cycle inhibitors;
- inhibits Bad and Caspase-9, proteins directly involved in apoptosis;
- leads to the activation of another complex involving mTOR (mTORC1), which is crucial for cell growth and metabolism [21, 23].

Downregulation of this pathway is mediated by the tumor suppressor PTEN (Phosphatase and tensin homolog), a lipid phosphatase that dephosphorylates PIP_3 into PIP_2 (Fig. 3.6) [21–23].

Since **mTOR** will be used to discuss some mechanisms of resistance to signaling therapies, we should go slightly deeper on how it works. As you have guessed by now, mTOR forms two distinct complexes:

- mTORC1, mainly composed by mTOR, the catalytic subunit, regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8) and proline-rich AKT substrate 40 kDa (PRAS40);

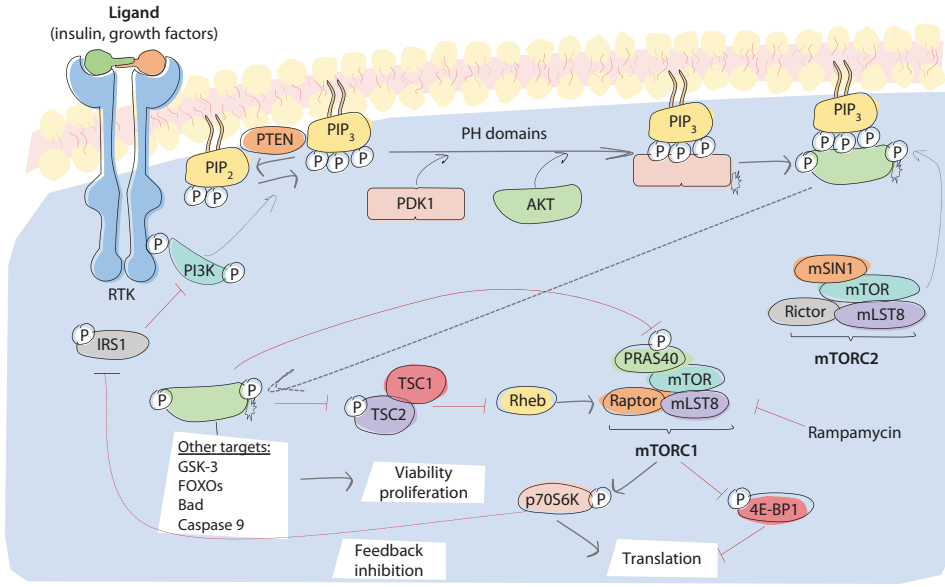

Fig. 3.6 Summary of PI3K/Akt/mTOR pathway. Activated PI3K phosphorylates PIP_2 into PIP_3, while PTEN antagonizes its action by dephosphorylation of PIP_3 into PIP_2. The generation of the second messenger PIP_3 leads to phosphorylation and activation of Akt by both PDK1 and mTORC2 (acting as PDK2). Activated Akt has several intracellular targets, such as GSK-3, FoxO family members, Bad, Caspase 9 and TSC1/2 complex. Inactivation of the TSC1/2 complex through phosphorylation leads to stabilization and activation of mTORC1, which in turn promotes protein translation via activation of p70S6K and inactivation of 4E-BP1. Further details of this pathway are described in the main text

- mTORC2, mostly formed by mTOR, rapamycin-insensitive companion of mTOR (Rictor); mammalian stress-activated protein kinase interacting protein (mSIN1) and mLST8 [24].

Importantly, mTORC2 phosphorylates and activates Akt directly, whereas Akt activates mTORC1 by two means: directly, via PRAS40 phosphorylation [25]; and indirectly, by phosphorylation of the tuberous sclerosis complex 2 (TSC2), which promotes destabilization of the heterodimer TSC1/2 and consequent activation of the small GTPase Rheb (Fig. 3.6) [26]. Upon activation, mTORC1 phosphorylates and inactivates the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) [27] and activates the p70 ribosomal S6 kinase (p70S6K) [28], leading to an increase in **protein translation at the ribosome**.

3.5 Targeted Signaling Therapies: The Pros and Cons

Now that we know the very basics of signaling we can better understand its potential to deregulate the balance in a cell towards an excessively proliferative state. In addition, one should recall that aberrant activation of cell surface receptors, such as RTKs, often leads to the concomitant activation of STATs, MEK/ERK and PI3K/Akt/mTOR signaling, amongst other pathways. In other words, the **oncogenic potential** of RTKs actually lies not on a direct detrimental effect on the cell but precisely on the ability of RTKs to activate downstream, potentially harmful, signaling players.

Of course, the knowledge that constitutive activation of certain signaling pathways is causal in cancer progression, maintenance and, quite often, resistance to conventional chemotherapies, makes them **highly attractive as targets for therapeutic intervention**. We have already mentioned the relative easiness in developing pharmacological inhibitors for protein kinases. So, one obvious strategy is to use **small molecule, cell-permeable inhibitors** that can directly target intracellular kinases [3]. We will mention this strategy repeatedly in the following sections. Yet, there is another obvious strategy if one refers to the most upstream elements in a signaling pathway, those that locate at the cell surface. In those cases, the plasma membrane does not constitute an obstacle and therefore **antibodies** can be used to neutralize and consequently inhibit the activity of the surface receptor, thereby preventing the activation of downstream signaling [3]. This strategy has the benefit of often also triggering an immune response against the (tumor) cells that express the receptor.

Contrary to conventional chemotherapy, these therapies targeting signaling elements **("targeted signaling therapies" or "signaling therapies")** have the advantage of being substantially more **selective** against the cancer cells and thus, at least to some extent, having the possibility to decrease toxicities towards normal cells and thus side-effects. This increased selectivity results from the fact that some signaling drugs inhibit targets expressed only by the tumor cells, such as the fusion protein BCR-ABL or mutant BRAF, which we will mention in more detail below. The other, less obvious, reason was already mentioned: oncogene addiction, the absolute reliance of cancer cells on a certain pathway in contrast to a normal cell. This does not mean that signaling therapies are bulletproof. Unfortunately, they do sometimes generate substantial side-effects. This, however, is not the real problem in most cases, but rather the development of resistance. In this regard, targeted therapies can be just as ineffective (if not worse at times) as conventional chemotherapies. Why this happens and how we can tackle this problem is what we will discuss next.

3.6 Why Do Cancer Cells Develop Resistance to Targeted Signaling Therapies?

There are many mechanisms of resistance to signaling therapies. We will briefly mention a few of the most well-known.

3.6.1 Mutational Activation of an Element in the Pathway Downstream from the Target

Let's start with a simple example. A group of RTKs well-known for their involvement in cancer is the epidermal growth factor receptor (EGFR/ErbB/Her) family, which has four members, the most tumorigenic of which appears to be EGFR2 (or Her2/Neu) [29, 30]. Our example, however, will rely on EGFR1. An effective treatment for colorectal cancer patients whose malignant cells express EGFR1, and consequently display constitutive activation of downstream signaling, involves the administration of a monoclonal antibody, named Cetuximab. It happens that the benefit of Cetuximab administration (in combination with conventional chemotherapy, as compared to conventional chemotherapy alone) is limited to patients with wild type, non-mutated, *KRAS* [31]. Why is this? Well, remember that *RAS*, no matter what isoform, is an oncogene and so mutated Ras in the context of cancer means ACTIVATED Ras. Now remember that the other name for signaling pathways is signaling cascades – one element activates the next which activates the next, and so on. Cetuximab inhibits EGFR1, which will no longer activate Ras, which will no longer activate Raf, which will no longer activate MEK, which will no longer activate ERK. Great! But wait a second now. How will Cetuximab inhibit Raf and MEK and ERK if Ras is constitutively activated as a consequence of a mutation and no longer requires EGFR to be turned on (Fig. 3.7)? A minor cancer clone displaying a *KRAS* mutation will eventually expand and drive resistance to Cetuximab. This notion that **a targeted therapy can become obsolete due to the mutational activation of a downstream effector of the target** is of utmost importance, and in fact sequencing for *RAS* mutations is now clinical practice for selecting patients for treatment with anti-EGFR therapies [32].

Of course, it is important to take into account the fact that there are other effector pathways downstream from EGFR. So, knowing that anti-EGFR targeting agents are not effective against all KRAS wild type cases begged the question: how about mutations in other signaling players (downstream from RAS or in other parallel pathways)? For example, what if PI3K is constitutively active? [32]. Although the relevance of mutations in PI3K family members has proved controversial and not generally implemented in the clinic to determine which patients should be treated with cetuximab and equivalent drugs, the fact is that this is another potential mechanism of tumor to escape to therapy. The corollary is that **one should have the best characterization of the patient's mutational profile in order to device the most effective signaling therapies** (Fig. 3.7).

Food for thought: there is evidence that JAK inhibitors such as the JAK1/JAK2-specific ruxolitinib may be useful to treat acute lymphoblastic leukemia patients, especially (but not only) those with gain-of-function mutations in the upstream receptor IL-7R or in JAK1 itself [33–35]. However, it is also known that some of these patients display STAT5B activating mutations [36]. How sensitive to ruxolitinib will the patients with *STAT5B* mutations be? Based on pre-clinical studies, the answer is: very little. However, the use of

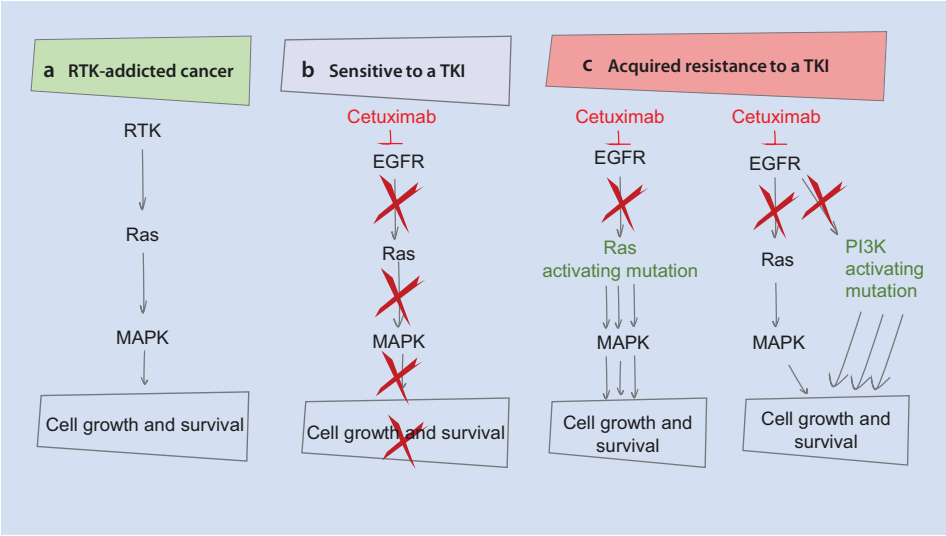

Fig. 3.7 Mechanisms of acquired resistance to receptor tyrosine kinase inhibitors such as Cetuximab, an anti-EGFR monoclonal antibody. **a** Cancers that are addicted to RTKs have activation of Ras/MAPK signaling, which promotes cell growth and survival. **b** Cancers expressing EGFR, and consequently displaying constitutive activation of downstream signaling, are sensitive to the tyrosine kinase inhibitor Cetuximab. Inhibition of the receptor leads to loss of Ras/MAPK signaling, blocking cell growth and survival and promoting apoptosis. **c** Cancers can become resistant by two means: first, by mutational activation of a downstream effector of the target (in this case Ras); and second, by mutation in other signaling players in other parallel pathways (e.g. PI3K activating mutations)

Bcl-2 antagonists may prove useful for those cases, given that STAT5 positively regulates anti-apoptotic Bcl-2 family members that can be inhibited by this class of drugs [35].

3.6.2 Release from a Negative Feedback Loop

So far, we have centered our attention on positive regulation of signaling pathways. However, as mentioned already, all pathways have control mechanisms to prevent permanent activation of signaling pathways for the homeostasis of the cell. We can use PI3K/Akt/mTOR pathway to illustrate how **negative feedback loop mechanisms** can turn into a problem in this context. You already know that mTOR phosphorylates p70S6K to regulate protein translation. What you may not know yet is that p70S6K is also involved in the negative regulation of PI3K-mediated signaling by phosphorylating and thereby hampering the function of IRS1, an adaptor protein that sometimes "links" cell surface receptors to PI3K and thus contributes to the full activation of PI3K (Fig. 3.6) [32]. To block PI3K/Akt/mTOR pathway activation in cancer cells one can use rapamycin (Sirolimus) or one of its analogs (Temsirolimus), which are highly selective allosteric inhibitors of mTORC1 [37]. This blocks the pathway on a downstream element, so it should be a very effective and smart way of making sure that the cascade is shutdown even if activating mutations occur in different upstream members. This is true. However, the allosteric inhibitors of mTORC1 have a downside: they can also inhibit the negative feedback loop. This means that PI3K and Akt are no longer under the control of this mechanism and therefore their

activity can actually increase. This would not be a problem if the pathway was absolutely linear – it would be inhibited at the mTORC1 level. But we know that Akt can regulate several other important players in cell cycle and apoptosis regulation and so easily bypass the effects of mTORC1 inhibitors. This problem can be overcome by the use of the so-called TOR kinase inhibitors, which inhibit mTOR catalytic site and thus affect both mTORC1 and mTORC2 (e.g. Torkinib (PP242), Ku-0063794), or by using dual inhibitors of mTOR and PI3K (e.g. PI-103, NVP-BEZ-235) [37, 38].

3.6.3 Mutations and Amplifications in the Target

Imatinib and related inhibitors constitute the most successful example of signaling therapies. Imatinib is used to treat patients whose malignant cells display BCR/ABL fusion proteins (ABL is a tyrosine kinase that becomes always ON as a result of this fusion) that arise from the reciprocal non-random translocation t(9;22)(q34;q11), and the consequent expression of a minute chromosome 22, called Philadelphia (Ph) chromosome. Virtually all patients with Chronic Myeloid Leukemia (CML) and a fraction of Acute Lymphoblastic Leukemia (ALL) patients are Ph-positive. The use of Imatinib has dramatically improved the survival and prognosis of these patients, especially those with CML [39]. However, resistance develops in some cases [40, 41]. Why is this? Imatinib binds close to the ATP binding site of BCR/ABL, locking it in a closed, self-inhibited conformation. Mutations exist in BCR/ABL itself that cause resistance to Imatinib by shifting its equilibrium toward the open or active conformation [41, 42]. There is also evidence that BCR/ABL locus amplification may occur, leading to higher expression of the protein that contributes to resistance to the inhibitor [42]. In any case, the strategy to overcome resistance normally involves the use of the so-called second-generation BCR/ABL inhibitors (such as Dasatinib), which in fact are less specific than Imatinib and affect also downstream targets of BCR/ABL such Src-family kinases [37, 43]. This means that even in the event of mutations occurring in BCR/ABL there is a safeguard mechanism of inhibition acting downstream. Of course, the potential downside is that **the less specific an inhibitor is the more likely it may affect normal cells and cause side-effects.**

Similar mechanisms of resistance have been described for another good example of targeted therapies: Vemurafenib – the specific $BRAF^{V600E}$ inhibitor, which demonstrated activity in melanoma patients with this specific mutation. In fact, paradoxical activation of MEK/ERK signaling due to the release from negative feedback loops is also at play in this case, as well as the occurrence (albeit infrequent) of activating ERK mutations.

3.6.4 Other Mechanisms of Resistance

Vemurafenib, inhibitor of $BRAF^{V600E}$ mutation, serves to exemplify other mechanisms of resistance. For example, via triggering of alternative mechanisms of activation of downstream members of the pathway. For example, gain-of-function mutations in MLK, a kinase able to phosphorylate MEK, lead to ERK activation without the requirement for any mutations in ERK itself or any other member of the canonical MEK/ERK pathway. Strange as it may sound given the fact that Ras is upstream of Raf, any event leading to Ras activation may also contribute to resistance to Vemurafenib. This is the consequence of Vemurafenib being a highly specific inhibitor of mutant B-Raf ($BRAF^{V600E}$). The problem

in this case is that there are other Raf isoforms (A-Raf, C-Raf) that can be activated by Ras and propagate the signaling even in the presence of Vemurafenib. Thus, if the cells display (or gain) activating mutations in Ras, or in upstream RTKs, or deletion or loss-of-function mutations in NF1 (a negative regulator of Ras), or aberrant autocrine loops, they will inevitably end-up driving resistance to the inhibitor. What are the strategies to deal with this challenge? Once again, one may try **combining drugs** – for instance, using MEK inhibitors (such as Trametinib) concomitantly with Vemurafenib to block putative activation of MEK/Erk pathway [44]. Besides the danger of increased toxicities this does not necessarily solve the problem, since some patients will display mechanisms of resistance that involve activation of PI3K/Akt/mTOR signaling rather than MEK/Erk – once again alerting to the necessity of thoroughly characterizing each patient's mutational profile.

Only **tailor-made therapies (i.e. adapted to the molecular characteristics of the tumor and the patient)** rationally-designed against actionable targets will have, most likely in combination with other strategies, namely immunotherapies, the potential to effectively cure cancer patients.

Take Home Message

The following points were covered in this chapter:

- Signaling pathways are essential for cellular homeostasis and their deregulation is often associated with cancer.
- Signaling pathways are excellent targets for therapeutic intervention in cancer. However, therapies targeting signaling players also have caveats – development of resistance being the most important.
- There are numerous mechanisms of resistance to signaling therapies, but also many rational strategies to overcome them.
- In the ongoing fight to minimize the likelihood of development of resistance, to decrease toxicities and to maximize anti-cancer effects, it is of utmost importance to have a deep knowledge of the mechanisms of signal transduction, and the molecular characteristics of the patient's tumor, so that rationally-designed therapies can be implemented and potential resistance predicted and managed.

Questions

1. Explain briefly what signal transduction is and why it is so important for the cells.
2. Give few examples of input signals and output responses.
3. List the main post-translational protein modifications in signal transduction and briefly explain the most characterized one.
4. List the main categories of human protein kinases and give an example in each category.
5. Why is signaling transduction so important in cancer? Indicate three reasons.
6. Indicate the three signaling pathways frequently activated in all types of cancer and what mainly contributes to their hyper-activation in cancer.
7. What are "signaling therapies" and their advantages?
8. Explain briefly one mechanism of resistance to targeted signaling therapies, using one example.
9. What are the common strategies to overcome resistance to signaling therapies?

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The Cell Cycle, Cytoskeleton and Cancer

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What You Will Learn in This Chapter?

The focus of this chapter is in the basics of the cell cycle and its deregulation in cancer. We begin this chapter by giving an historical perspective on the discovery of key component of the cell cycle, the cyclins and cyclin-dependent kinases (CDK) and the concept of checkpoints. Then, we review the eukaryotic cell cycle, starting with a brief description of the cell cycle phases. We provide detailed description of the main cell cycle regulators, the cyclins and CDKs, along with how they control the cell cycle. Next, we discuss the cell cycle checkpoints and DNA repair mechanisms. Later, we describe the cell cycle in cancer, and how tumor cells take advantage of cellular protective mechanisms to become malignant, focusing on the concept of tumor suppressor genes and oncogenes related to cell cycle progression. Finally, we focus on a particular structure, the centrosome, which is the major microtubule-organizing center in animal cells, and how its deregulation can promote tumorigenesis. We conclude this chapter by discussing therapeutic targets that are being used to eradicate cancer cells having abnormal centrosomes.

Learning Objectives

After completing this chapter, students should be able to:

1. Understand major concepts related to cell cycle progression and cell cycle regulators.
2. Explain how cyclin/CDKs regulate the cell cycle.
3. Discuss cell cycle checkpoints.
4. Understand centrosome structure, function and biogenesis.
5. Discuss how centrosomes can promote tumorigenesis.
6. Understand how cancer cells cope with centrosome abnormalities.
7. Discuss therapeutic targets aimed to destroy cancer cells having abnormal centrosome.

Important Concepts Discussed in This Chapter

- The concept of cell cycle regulators, CDKs and Cyclins – CDKs are the major kinases regulating cell cycle progression; Cyclins are the partners of CDKs that regulate their activity along the cell cycle.
- Cell cycle checkpoints – Regulatory control points during cell cycle progression
- Oncogenes and Tumor suppressor genes – The most common mutations seen in cancer that allow cells to evade protective mechanisms.
- The centrosome – The main microtubule (MT) organizing center in animal cells.
- Role of centrosome in cancer – By promoting aneuploidy and changes in cytoskeleton and signaling that lead to altered cell migration and invasive capacity.

4.1 The Cell Cycle

4.1.1 The Cell Cycle Review

The first illustration of cells was made in 1665 (353 years ago) by Robert Hook [1]. This illustration led to the first observation that cells are the structural base of life, and over the subsequent centuries, cells were discovered in every living organism. In 1958, Rudolf Virchow stated that cells are not only the basic structure unit of whole life but also the physiologic unit of life, arising from pre-existing cells – *Omnis cellula e cellula* – "All cells come from cells" [2, 3]. This occurs by the process of cell division, in which one cell gives rise

to two identical daughter cells. Cell division is essential for growth and replenishment of old cells, but also underlies the understanding of cancer. Ever since the nineteenth century (1858) scientists have observed cells dividing, but little was known about how they do it. The machinery that regulates cell cycle progression only started to be elucidated in the 80s.

In 2001, the Nobel Foundation awarded three cell biologists with the Nobel Prize in Physiology or Medicine for their outstanding contributions to the study of the cell cycle in eukaryotic cells. Sir Paul M. Nurse identified, cloned and characterized cyclin-depended kinases (CDKs) in yeast and showed that CDK's function is conserved in higher eukaryotes [4, 5]. CDKs are the major kinases regulating cell cycle progression. Richard Timothy Hunt received his award for the discovery of cyclins and for showing that cyclins are degraded during the cell cycle [6]. Cyclins are the partners of CDKs that regulate their activity along the cell cycle. Its/Their levels oscillate during the cell cycle, being synthesized only at specific stages in response to various molecular cues, and then degraded by ubiquitin-mediated proteolysis [7]. In contrast CDKs are constitutively expressed in cells. Finally, Leland Hartwell identified a particular class of genes that control the cell cycle, introducing the concept of checkpoints, i.e., regulatory control points during cell cycle progression [8].

In order to divide, a cell must complete several important tasks: grow, copy its genetic material (DNA) and physically split into two identical daughter cells. The cell cycle in eukaryotic cells consists of three phases (Fig. 4.1):

- Interphase (G1 + S + G2)
- Mitotic phase
- Cytokinesis.

In interphase, most somatic cells have two gap phases, G1 and G2, that precede and follow DNA replication (S phase), respectively.

4.1.1.1 G1 Phase

In the G1 phase, the cell synthetizes proteins and organelles (such as mitochondria and ribosomes), growing in size, guaranteeing that both daughter cells will inherit sufficient amounts (or number) of proteins/organelles (Fig. 4.1). Because of this, a G1 or G1/S checkpoint is established by the cell (Figs. 4.1 and 4.3a). The G1/S checkpoint is the main decision point for a cell. It represents a control mechanism to ensure that everything is ready for DNA synthesis in the following phase (S phase). At this point, the cell fate, to commit to divide or not, is determined. If the cell passes the G1 checkpoint, it enters the S phase, becoming irreversibly committed to divide. There are several internal and external conditions that the cell checks at this stage in order to proceed for DNA replication. Some of these conditions are:

- The cell size (to ensure that the cell is large enough to divide),
- Nutrients (if the cell has enough energy reserves or available nutrients to divide),
- Molecular signals (such as growth factors or other positive cues from neighboring cells),
- DNA integrity (to ensure that there is no DNA damage, before proceeding to next phase).

If the cell passes all these criteria, it moves to S phase. If not, it leaves the cell cycle, entering quiescence, also called G0 phase.

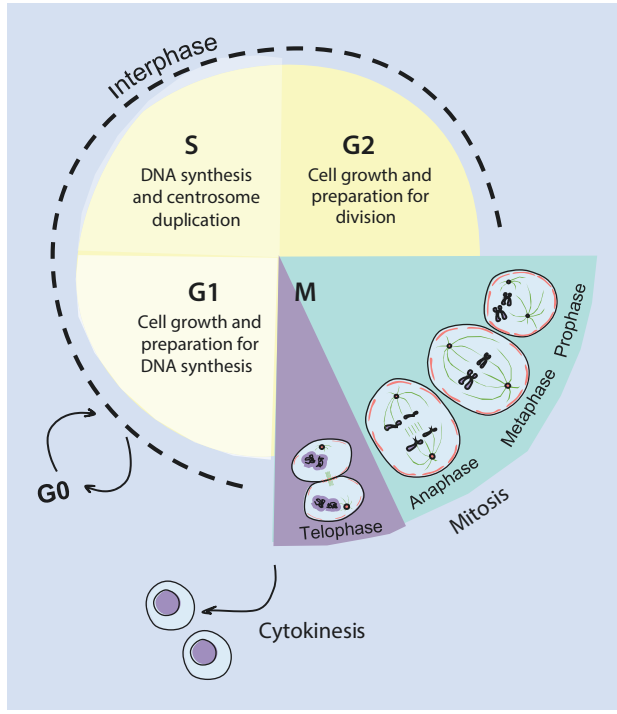


Fig. 4.1 The eukaryotic cell cycle. The cell cycle consists of Interphase, Mitosis and Cytokinesis. In the interphase, most somatic cells have two gap phases, G1 and G2, that precede and follow DNA replication (S phase), respectively. During the G1 phase, there is growth and preparation of the cell for DNA replication. In S phase, DNA synthesis and centrosome duplication occur. In the G2 phase, the cell prepares to enter mitosis, organizing the microtubules to form a mitotic spindle at the transition with mitosis. The mitotic phase is highly regulated, and the sequence of events has been traditionally divided into phases mostly based on the state of chromatin and position of chromosomes, known as prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, the chromatin condenses, the nucleolus disappears and the mitotic spindle begins to form, with the migration of centrosomes to the both poles of the cell. In prometaphase, the nuclear membrane breaks down and the kinetochore microtubules attach to kinetochores. In metaphase, all the chromosomes align in the "metaphase plate" and the mitotic spindle is formed. During anaphase, the sister chromatids separate from each other and are pulled towards the opposite ends of the cell. Finally, in telophase, the mitotic spindle is broken down, and two new nuclei forms, one for each set of chromosomes. The nuclear membrane and nucleoli reappear, and then, the chromosomes begin to decondense. Subsequently there is a physical separation of the cell membrane to form two new cells – Cytokinesis. The G0 phase represents a resting phase when cells leave the cell cycle temporarily or permanently. (*Adapted from Heng and Koh* [9])

4.1.1.2 S Phase

In S phase, DNA replication occurs. The DNA is duplicated, along with the major microtubule-organizing structure, the centrosome. The centrosome is important for many processes, including cell signaling, polarity and nucleation of microtubules to organize the mitotic spindle, and we devote a later section of this chapter to it (Fig. 4.1).

4.1.1.3 G2 Phase

The G2 phase (or second gap phase) starts after DNA synthesis. During this phase, the cell continues to grow, and prepares for mitosis, organizing the microtubules to form a mitotic spindle at the transition with mitosis (Fig. 4.1). The second checkpoint, the G2

or G2/M checkpoint ensures that the DNA was completely copied during S phase and with no damage, so that the cell can divide sister chromatids in two cells. If errors are detected, cells will be stuck in G2 until the errors are repaired; otherwise the cell may undergo programmed cell death (apoptosis) (◘ Figs. 4.1 and 4.3a).

4.1.1.4 Mitotic Phase

In the mitotic phase (M phase) the cell stops growing and divides into two daughter cells. During mitosis, DNA condenses and is pulled apart by the mitotic spindle (a specialized structure made of microtubules). M phase is highly regulated, and the sequence of events has been traditionally divided into phases mostly based on the condensation state and position of chromosomes in relation to the spindle, known as prophase, prometaphase, metaphase, anaphase, and telophase (◘ Fig. 4.1).

During mitosis, there is a spindle assembly checkpoint (SAC) or M checkpoint (◘ Figs. 4.1 and 4.3a). Here, the cell assesses whether the spindle has formed, and all the sister chromatids are correctly attached to the spindle microtubules before anaphase begins. Overlapping with the end of mitosis, the cytokinesis process occurs, where the cytoplasm, organelles and cytoplasmic membrane split, forming two new cells, often with equal shares of cellular components.

Cell division must be tightly controlled in order to prevent the development of disorders, such as cancer. Each of the phases discussed above is regulated by different cyclin/CDK complexes in animals (◘ Fig. 4.2a):

- G1 phase by CDK4/6 complexed with cyclin D,
- S phase by CDK2 complexed with cyclins E and A,
- G2 phase by CDK2 complexed with cyclin A,
- And mitosis by CDK1 complexed with cyclins B and A [10–12].

In a cell, each of the CDK/cyclin complex modifies a specific group of protein substrates. The proper phosphorylation of these substrates must also occur at particular times in order for the cell cycle to continue. Depending on the phase of the cell cycle, CDK/cyclin complexes can recognize multiple substrates and coordinate multiple events during each phase. For example, during G1 phase and beginning of S phase, G1-CDKs will catalyze the phosphorylation of proteins that prepare for DNA replication. Later on, during mitosis, M phase CDKs phosphorylate a wide range of other proteins. These include condensing proteins, which are essential for the condensation of mitotic chromosomes, and also laminin proteins, which will form a stabilizing network under the nuclear membrane that dissembles during mitosis. By phosphorylating the proteins that regulate microtubule dynamic and centrosome maturation, the M-CDKs also influence the assembly of the mitotic spindle.

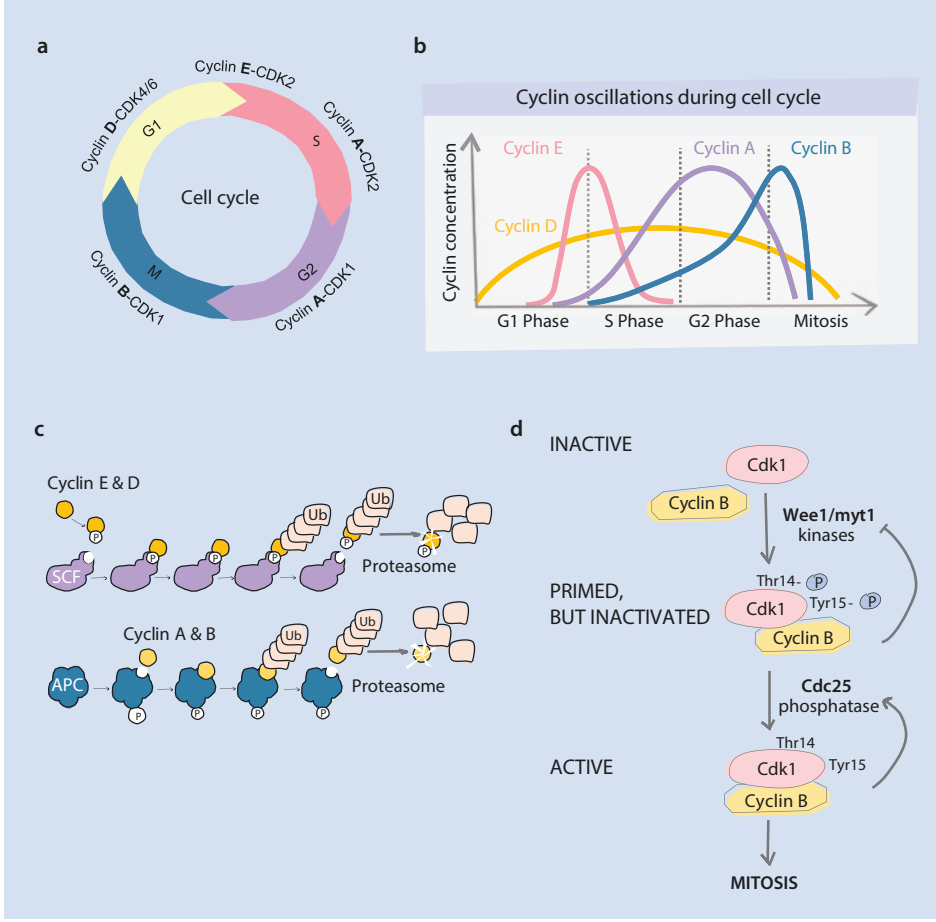
CDK's activity is regulated by many conditions, such as:

1. Cyclin availability – Cyclin availability is mostly regulated by their transcription and translation and then degradation (◘ Fig. 4.2b). Two major ubiquitin ligases are involved in cyclin degradation:
    1. The Skp1-Cullin-F-box-protein (SCF) complex that recognizes phosphorylated G1 cyclins (cyclin E and D) and targets it for degradation, and
    2. The Anaphase Promoting Complex (APC) that degrades mitotic cyclins (cyclin A and cyclin B) during mitotic progression [7, 10] (◘ Fig. 4.2c).

Failure of cyclin degradation leads to cell cycle arrest.

2. Activating and deactivating phosphorylation – CDK1/Cyclin B activity which drives G2/M transition is also regulated through kinases (for example Wee1), and phosphatases (as cdc25) [15] (Fig. 4.2d). During interphase, CDK1/Cyclin B complex is inactivated by phosphorylation of CDK1 at two residues, Threonine 14 (Thr14) and Tyrosine 15 (Tyr15), by Wee1 and Myt1 (Wee1/MYT1) kinases, thus blocking ATP binding and hydrolysis. During the G2/M transition, Myt1 and Wee1 are inactivated, while the phosphatase Cdc25 is activated. Cdc25 dephosphorylates Thr14 and Tyr15, allowing for activation of the CDK1/Cyclin B complex and entry into mitosis [16, 17]. Furthermore, CDK1/Cyclin B is also a negative regulator of Wee1 and Myt1, as both kinases are inactivated upon CDK1/Cyclin B phosphorylation.
3. CDK inhibitors – CDK's activity is further regulated by two families of inhibitors:
   1. INK4 proteins (composed of INK4A, INK4C and INK4D), and
   2. Cip/Kip family (including p21, p27 and p57) [11, 18].

Deregulation of CDK activity, for example through mutations or inactivation of CDK inhibitors or deregulation of cyclin levels is often observed in cancer [19–22]. These can drive cells prematurely into S phase and mitosis and cause genomic instability.



4.1.2 Cell Cycle Checkpoints, DNA Repair and Spindle Assembly

To avoid transmission of an altered genome to daughter cells, there are elaborate checkpoint pathways which arrest cell cycle progression and promote repair or, in case the damage is unrepairable, stimulate programed cell death – apoptosis. As described above, there are three known checkpoints (Fig. 4.3a):

- G1 checkpoint (the major checkpoint),
- G2/M checkpoint
- And spindle assembly checkpoint (SAC) or M checkpoint.

Along the cell cycle, each checkpoint ensures that the conditions of the cell are favorable to move from one phase to the next one. Sensor proteins detect and signal DNA damage to downstream effectors that, in turn, arrest cell cycle progression and promote repair.

4.1.2.1 G1/S Checkpoint

For example, in the presence of a DNA double strand brake (DSB), the Ataxia Telangiectasia Mutated (ATM) kinase is activated and triggers the G1 checkpoint by phosphorylating and activating the Checkpoint Kinase 2 (Chk2). In turn, Chk2 inhibits Cdc25A (a phosphatase that removes inhibitory phosphorylation of CDK2/cyclin A and CDK2/cyclin E complexes), preventing cells to proceed to S phase [23]. In addition, ATM induces the phosphorylation of p53, leading to p53 stabilization. Stabilized p53 induces $p21^{Cip/Waf1}$, which binds and inhibits CDK2/cyclin A and CDK2/Cyclin E complexes, DNA repair proteins and, if necessary, apoptotic cell death promoters [24, 25].

Fig. 4.2 The regulation of cell cycle through CDK/Cyclin complex. **a** In eukaryotic cells, there are multiple CDK/cyclin complexes that play specific roles at various phases of the cell cycle. The complex CDK4/6-Cyclin D stimulate the initiation of G1 phase. The progression towards the end of G1 phase is characterized by increasing levels of CDK2-Cyclin E, which in turn triggers the beginning of S phase. CDK2-Cyclin E levels are then degraded, and CDK2 -Cyclin A completes the S-phase and entry to G2 phase. In G2, cyclin A complexes with CDK1 until the beginning of M phase. At the end of G2 Cyclin B couples with CDK1 and their activity increases during mitosis and diminishes at the end of M phase. **b** Cyclin levels oscillate during the cell cycle, being synthesized only at specific stages in response to various molecular cues, and then degraded by ubiquitin-mediated proteolysis. **c** Cyclin degradation during cell cycle. Cyclins must be synthesized for the progression of the cell cycle, and then degraded immediately after. Two ubiquitin-ligase complexes are responsible for the degradation of cyclins: the skp1-cullin-F-box-protein (SCF) complex that recognizes phosphorylated G1 cyclins (cyclin E and D) and targets it for degradation, and the anaphase promoting complex (APC) that degrades mitotic cyclins (cyclin A and cyclin B) during mitotic progression. The SCF complex is active throughout the cell cycle and the degradation of its substrates depends on their phosphorylation status. On the other hand, the APC is activated at the onset of anaphase and degrades its substrates as cells exit mitosis. **d** Regulation of transitions in mitosis often operates through a series of positive and negative feedback loops. CDK-Cyclin B activity to drive G2/M transition and is regulated through kinases and phosphatases, such as Wee1 and cdc25, respectively. With low levels of cyclin B, Wee1 and Myt1 (Wee1/MYT1) kinases inactivate CDK1 by phosphorylating residues T14 and Y15, thus blocking ATP binding and hydrolysis. Once the concentration of Cyclin B increases exceeding a threshold, CDK1 becomes active. Cyclin B-CDK1 phosphorylates and activates the cdc25 phosphatase, allowing cdc25 to remove the inhibitory Thr14 and Tyr15 phosphorylations on CKD1 and thus, allowing the entry into mitosis. Furthermore, Cyclin B-CDK1 is also a negative regulator of wee1 and Myt1, as both kinases are inactivated upon Cyclin B-CDK1 phosphorylation. (*Adapted from Suryadinata et al.* [13], *Murray* [10] *and Deibler and Kirschner* [14])

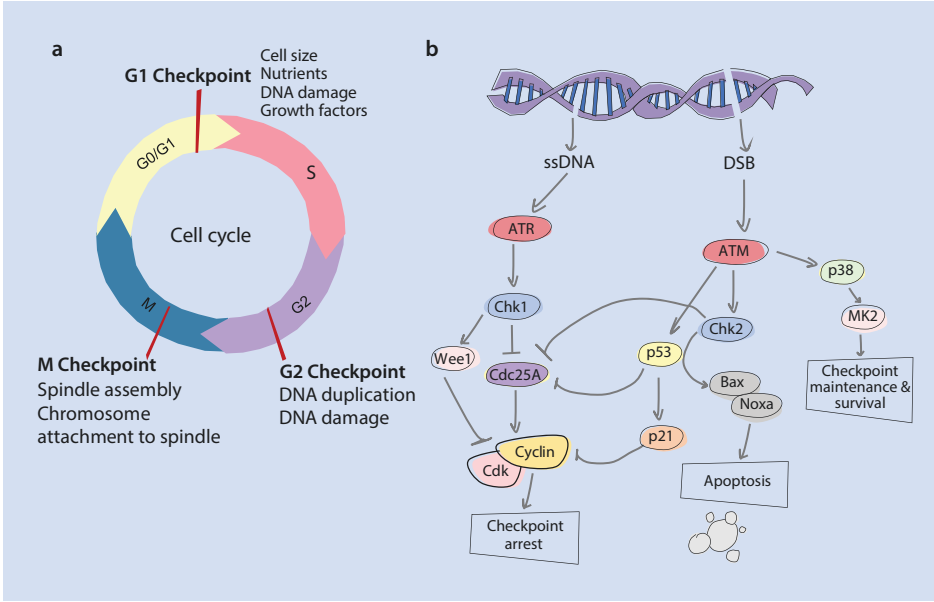


Fig. 4.3 Cell cycle checkpoints. **a** The cell cycle checkpoints: The G1/S checkpoint is the main decision point for a cell. It represents a control mechanism to ensure that everything is ready for DNA synthesis in the following phase (S phase). At this point, the decision to either divide or not is determined. If the cell passes all the criteria stablished, such as: cell size, nutrients, molecular signals, and DNA integrity, it moves into S phase. If not, it leaves the cell cycle, entering quiescence, also called G0 phase. The second checkpoint, the G2/M checkpoint ensures that the DNA was completely copied during S phase and with no damage, so that the cell can divide. If errors are detected, cells will be stuck in G2 until errors are repaired; otherwise the cell may undergo programmed cell death (apoptosis). In the M checkpoint, also known as spindle checkpoint, the cell assesses whether the spindle has formed, and all the sister chromatids are correctly attached to the spindle microtubules before anaphase begins. At this point, the Anaphase–Promoting Complex (APC) triggers the transition from metaphase to anaphase, by tagging specific proteins, such as securin, and M-cyclins (Cyclin A and B) for degradation. **b** Checkpoint activation signaling cascade related to DNA damage: DNA damage is sensed by diverse sensor and adaptor proteins, which in turn leads to activation of ATM and ATR kinases, allowing the establishment of a DNA-damage checkpoint. Chk1 and p53 are the major components activated during DNA damage response. Chk1 phosphorylates Cdc25 targeting it for degradation and activates Wee. Loss of Cdc25 prevents CDK-Cyclin activation leading to a cell cycle arrest. P53 is stabilized in the cell by multiple post-translational modifications, and increases the levels of p21, a CDK inhibitor, repressing the transcription of Cyclin B, contributing to CDK/Cyclin inhibition. Furthermore, cells also can activate Chk2 and p38/MK2 pathways, which are involved in apoptosis and checkpoint maintenance, respectively. (*Adapted from Suryadinata et al.* [13], *Murray* [10] *and Deibler and Kirschner* [14])

4.1.2.2 G2/M Checkpoint

On the other hand, when the DNA damage occurs in S and G2 phase, arising from a broader spectrum of DNA damaging lesions that generate single-stranded DNA (including nucleotide excision/repair process, stalled replication forks or as intermediates of DSB resolutions), the damage is sensed by Ataxia Telangiectasia and Rad3-related (ATR) kinases. ATR activates Checkpoint Kinase 1 (Chk1), which induces Cdc25A proteasomal degradation, blocking further progression through S phase (Fig. 4.3b). Moreover, ATR and Chk1 also trigger the G2/M checkpoint, phosphorylating p53 and preventing cells with damaged DNA to enter mitosis by inhibiting the CDK1/cyclin B activation [23].

In order to have an appropriate checkpoint, the response needs to be fast enough to prevent transition to the next phase with damaged DNA, and also durable enough to allow time for efficient DNA repair. The most well studied checkpoint protein is p53 and its transcriptional target p21. And in fact, both the G1 and G2 DNA damage control points are governed by the tumor suppressor p53 [23, 26, 27]. The *TP53* gene is the most frequently mutated gene in human cancers [28].

When the DNA damage checkpoint is triggered, the response will be according to the type of damage [29–31] (see also ▶ Chap. 5).

4.1.2.3 Single Strand Brakes

If the damage induces a single strand brake, the other strand will serve as a template to restore the sequence to the damaged strand. Two mechanisms exist to remove the damaged nucleotides and replace them with undamaged ones, complementary to that found in the undamaged DNA strand:

- Base excision repair (BER) – repairs **small, non-bulky DNA lesions** (nucleotides that have suffered relatively minor damage). The modified or damage base is removed by a DNA glycosylase, creating an apurinic or apyrimidinic (AP) site. Then, enzymes called AP endonuclease nick the damaged DNA backbone at the AP site. DNA polymerases remove the damaged region and correctly synthesize the new strand using the complementary strand as a template.
- Nucleotide excision repair (NER) [29] – repairs **bulky, helix-distorting damage**, such as the ones caused by UV light. The damaged site is recognized, then there is an excision of the damaged DNA both upstream and downstream of the damage by endonucleases, and then there is a re-synthesis of removed DNA region.

4.1.2.4 Double Strand Breaks

When both strands of the double helix are broken, thus leaving no strand template for repair, the cell can use one of two distinct mechanisms [32]:

- The Non-Homologous End Joining (NHEJ) repair, both strands are brought together and joined, however, this usually results in the loss of one or two nucleotides at the site of joining. NHEJ is active through the cell cycle, but it **occurs mostly in G1 phase**, before DNA replication, since there is no DNA template.
- Homologous Recombination DNA repair (HDR), requires an identical or nearly identical sequence to be used as a template for the repair. Therefore, in this pathway, a sister chromatid or a homologous chromosome is used as a template. This repair **usually happens in G2 phase**, after DNA replication. In this case, the repair machinery is able to transfer nucleotide sequence information from the intact double helix to the broken one [29, 33].

Because the detection of errors and repair mechanisms are crucial for cells, defects in the checkpoint mechanisms, either in the sensing or repairing of the DNA damage or in triggering apoptosis, can favor accumulation of mutations in genes essential for normal proliferation, leading to loss of proliferation control. Indeed, cancer cells are often defective in these checkpoint mechanisms, facilitating tumorigenesis. We can take as an example the SAC checkpoint, also referred as 'mitotic checkpoint' or 'M-phase checkpoint'. SAC prevents chromosome missegregation and aneuploidy by delaying cell division until accurate chromosome segregation is guaranteed. During mitosis, chromosomes must be correctly attached to the microtubule spindle apparatus via their kinetochores. If this attachment

is not done properly, the kinetochores activate the SAC network, which blocks cell cycle progression to anaphase [34].

The downstream target of the SAC is the APC, an E3 ubiquitin ligase that, as described above, targets several proteins for proteolytic degradation, including mitotic cyclins. Once all kinetochores are stably attached to the spindle, SAC is inactivated, alleviating the cell cycle block and allowing chromosome segregation and cell division [34, 35]. Dysfunction of SAC network has been implicated in aneuploidy and tumorigenesis [36], and in fact, there are therapies targeting SAC molecular mechanisms for some cancer treatments [37].

The excess of proliferation seen in cancer is often associated with a vicious cycle with a reduction in sensing signals that normally tell a cell to adhere, differentiate, or die. While normal tissues require signals before they can grow and divide, and the production and release of these signals is highly controlled in order to ensure a homeostasis in cell number; cancer cells however, deregulate these signals, becoming self-sufficient in growth signals.

In the next section, we will discuss more about the cell cycle in cancer, and mechanisms that are often deregulated, favoring cancer progression.

4.1.3 The Cell Cycle in Cancer

Control of the cell cycle is important to promote tissue expansion and differentiation in a regulated fashion. As discussed above, hundreds of genes intricately control the process of cell division, thus requiring a balance between the activity of genes that promote cell proliferation and those that suppress it. The most common mutations seen in cancer are mutations that generate either oncogenes with dominant gain of function, or tumor suppressor gene with recessive loss of function (see also ▶ Chap. 1). When a normal cellular gene which function is to encode proteins that stimulate cell division is mutated, it may become an oncogene, contributing to cancer progression [38, 39]. These oncogenes exhibit increased activity, thus leading to increased cell division, decreased cell differentiation and inhibition of cell death.

The first oncogene discovered was *src* in the 70s by Michael Bishop and Harold Varmus, who were later on awarded with a Nobel prize in Physiology or medicine. The superactivation of this oncogene leads to survival, angiogenesis, proliferation and invasion of cancer cells. Another example of these growth-promoting genes that become oncogenes includes RAS, which acts to stimulate cell growth and division. Therefore, instead of stopping to divide at the proper time, mutations on these genes lead a cell to continuously progress through further divisions. Additionally, mutation in cyclins D and E are also often seen in cancer [20, 21]. Uncontrolled CDK's activity, where they are constitutively activated, induces unscheduled proliferation, as well as genomic instability.

In contrast to oncogenes, other cancer-related mutations inactivate genes that suppress cell proliferation or that trigger apoptosis. These genes are known as tumor suppressor genes, as they normally function as a brake on proliferation/cell division or are involved in maintenance of cell cycle checkpoints or even promoting apoptosis. Once mutated, they no longer exert their role in the cell. The first tumor suppressor gene to be identified was *RB1,* in 1986 [40–43], which encodes the retinoblastoma protein (pRb). Its function relies on inhibiting the expression of genes required for progression into S phase of the cell cycle. Therefore, inactivation of Rb will allow uncontrolled cell division. Dysfunction

in Rb protein has been seen in several cancers, including retinoblastoma [44], sarcomas [45–47], glioblastomas [48], bladder [49, 50] and breast [51] cancers. Another example of a common mutation in a tumor suppressor gene often seen in cancer refers to mutations in the *TP53* gene [52]. p53 is a multifunctional protein that normally senses different cellular stresses, such as DNA damage, inappropriate proliferation, oxidative stress or hypoxia, and also acts as a transcription factor for the expression of checkpoint control genes [53–55]. As previously mentioned, p53 governs G1/S and G2/M damage controls. So, genetic alterations which inactivate p53 will inhibit the DNA damage response that prevents cell cycle progression.

For the inactivation of tumor suppressors both copies of the gene must be mutated in order for tumorigenesis to occur. If one copy of the gene is not mutated, it may provide sufficient activity for the cell to maintain proper growth and division. However, the mutated allele can also antagonize the WT allele, leading to a dominant negative function of the mutant protein [56, 57]. Despite this, such a heterozygous state is often transient as, for example, *TP53* mutations during cancer are frequently followed by loss of heterozygosity. This loss of heterozygosity is often seen when the WT allele is either mutated (point mutations) or deleted [28].

Cancer research has generated a rich and complex knowledge in the past decades showing that tumors are a complex tissue composed of multiple distinct cell types that participate in heterotypic interactions with one another. They are not an isolated cell mass. Therefore, the biology of tumors needs to acknowledge and embrace the contributions of the tumor microenvironment to tumorigenesis. In recent years, it has become clear that some, or perhaps all types of cancer cells acquire molecular, biochemical and cellular traits in order to have growth advantages. These traits were described as "The six Hallmarks of cancer" by Hanahan and Weinberg in 2000 [58], and later on, in 2011, updated with four new hallmarks [59] (See also ▶ Chap. 1).

Focusing more on the seventh hallmark, "Genome instability and mutation", which is relevant for this chapter: we know that in order to acquire the other nine multiple hallmarks, the neoplasm must depend in large part on a succession of alterations in its genome. Certain mutations will confer selective advantages and dominance of these cells in a local tissue environment. The defects in genome maintenance and repair are selectively advantageous for tumor progression if it accelerates the rate at which they accumulate more favorable genotypes, enables it to grow faster, survive longer and generates more offspring than the surrounding cells without that mutation. The genomic instability plays important role both in tumor initiation and progression, affecting also the overall prognosis of the affected patient. It can arise from different pathways, including telomere damage, epigenetic modifications, centrosome amplification, and DNA damage form endogenous and exogenous sources. Therefore, genome instability is described as an enabling characteristic that is causally associated with the acquisition of the hallmark capabilities.

These hallmarks have helped, until today, in the understanding the complex biology of cancer. Although cell cycle is highly regulated, it becomes dysregulated due to enabling genetic alterations that lead to cellular transformation and inactivating mechanisms that would otherwise prevent this.

In the next sections, we will focus in a particular structure, the centrosome, and its role in regulating the microtubule cytoskeleton, cell division and signaling, having also an important role in tumorigenesis.

4.2 Centrosome and Cilia

The Centrosome was first discovered in 1880s by Teodor Boveri [60] and Edouard Van Beneden [61] and was described as a vital organelle required for animal cells to divide. Centrosomes are the main microtubule (MT) organizing center in animal cells, being formed by a pair of orthogonally positioned barrel-shaped centrioles, called the mother and daughter centrioles, embedded in a proteinaceous matrix called the pericentriolar material (PCM), which confers the MT nucleation capacity [62] (Fig. 4.4). Besides the difference in age between the mother and daughter centriole (the mother centriole was built one cell cycle earlier that the daughter), they also differ structurally at their distal ends, with the older mother centriole containing distal and subdistal appendages required for MT anchorage and ciliogenesis, serving as the basal body for cilia and flagella formation (Fig. 4.4). Cilia and flagella are important structures involved in many processes, including sensing extracellular signals to moving fluid and for cell motility.

In addition to its role in cilia and flagella formation, the centrosome also participates in several other processes such as cell polarity and intracellular traffic in interphase, spindle pole organization during mitosis, and also in cell migration [63–66]. Centrioles are found in most eukaryotic cells with the exception of higher fungi, amoebas and higher plants [67]. Abnormalities in centrosome number and structure have been seen in many

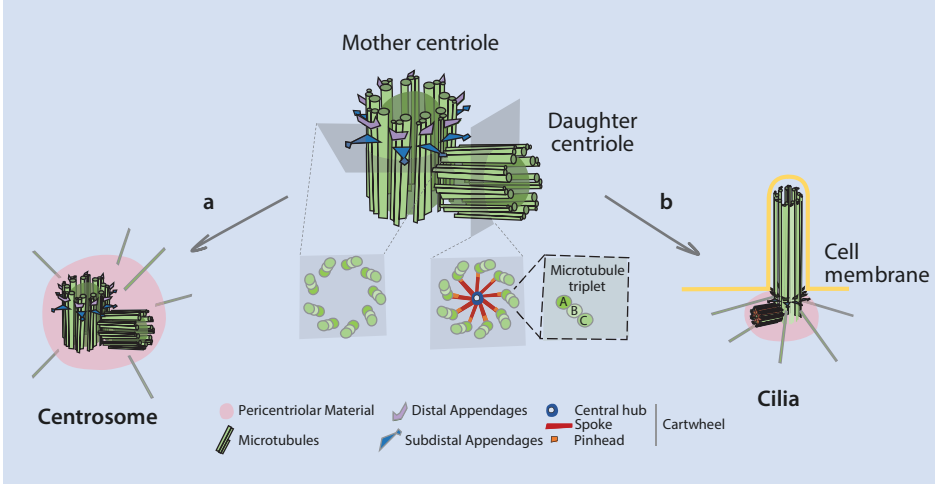


Fig. 4.4 Centrosomes and cilia are formed by centrioles. The centrosome is the major microtubule-organizing center in animal cells. The centrosome comprises two cylinder-shaped microtubule-based structures, the centrioles. Each centrosome is composed of a mother and a daughter centriole. The mother centriole possesses distal (purple) and subdistal (blue) appendages, necessary to anchor at the plasma membrane, serving also as cilia/flagella basal body. **a** Schematic representation of a pair of centrioles, which forms the centrosome, surrounded by an organized protein matrix cloud, the pericentriolar material (PCM - pink cloud). As observed, the mother centriole can be distinguished by the distal (purple stick) and subdistal (blue) appendages, and the daughter centriole by the possession of the cartwheel structure. The PCM nucleates and organizes microtubules, ensuring that the centrosome functions as the major microtubule-organizing center in many cells. During G1, the centrosome accumulates small amounts of PCM. At the end of G2, with the preparation to enter in Mitosis, the centrosome accumulates additional PCM, allowing them to organize many more microtubules during mitosis. **b** In some non-dividing cells, the centrosome migrates to the cell membrane and assemble cilia from the mother centriole, enabling cell movement and/or chemoreception

diseases, including cancer. **Centrosomes must thus be generated with high structural fidelity and rigorous number control.**

4.2.1 Centriole Biogenesis

Centrosomes can be formed *de novo* or through a centriole-guided mode:

- In *de novo* mode, centriole biogenesis occurs in the absence of pre-existing one, and occurs mostly in species that only form centrioles at certain phases of their life cycle (e.g. to form motile sperm in mosses [68]).
- In the centriole-guided mode, procentrioles form exclusively in association with a pre-existing centriole (new daughter centriole forms orthogonally to the existing 'mother' one). This is the major route of assembly in cycling cells and occurs in what is called the canonical centrosome duplication cycle.

Centrosome number and shape are tightly controlled through the **canonical centrosome duplication cycle**, which is coupled to the chromosome cycle. As cells enter G1 phase, they possess a single centrosome. During G1 the centrioles disengage but remain connected by a fibrous structure, known as interconnecting fibers. In late G1 and beginning of S phase the centrioles start duplicating in a semi-conservative manner, forming new centrioles perpendicularly to the existing ones. The daughter procentrioles then elongate and maturate, recruiting PCM. Following maturation of the centrosome at the G2 phase and entry into mitosis, the two centrosomes separate to opposite poles of the cell to help forming the mitotic spindle, contributing to appropriate chromosome segregation (Fig. 4.5). In this cycle the centrosome is duplicated **once and only once per cell cycle**, and upon cell division, each daughter cell inherits a single centrosome.

A key component for centriole biogenesis is the protein kinase Polo-Like-Kinase 4 (PLK4), which when in excess, triggers the formation of supernumerary centrosomes, whereas its depletion causes a reduction in centriole number [69, 70]. Downstream of it, several proteins play a role, including the coiled-coil protein Spindle Assembly Abnormal protein 6 (SAS-6), which is critical to define the nine-fold symmetry of the centriole and whose levels also determine centriole number. A variety of different proteins play a role in recruiting and stabilizing centriole microtubules [71–73]. Unlike other microtubules, centriole microtubules are very stable and resistant. The number (4 in mitosis), size (400–500 nm) and shape of centrioles is highly controlled. However, abnormalities in their number and size are often seen in cancer [74]. We will discuss now how centrosome amplification can be a double edge sword in cancer.

4.2.2 Centrosomes and Cilia in Cancer

Abnormalities in centriole number, structure and function cause different diseases, including microcephaly [75] and cancer [74, 76–78]. Over a century ago, Theodor Boveri was the first to propose that centrosome amplification (>2 centrosomes per cell) generates aneuploidy and promotes tumorigenesis. His theory was based on the observation of dispermic sea urchin eggs containing multiple centrosomes, which formed multipolar spindles, leading to asymmetric distribution of the genetic material. Supporting his hypothesis, abnormalities in centrosome number and structure have been observed in a

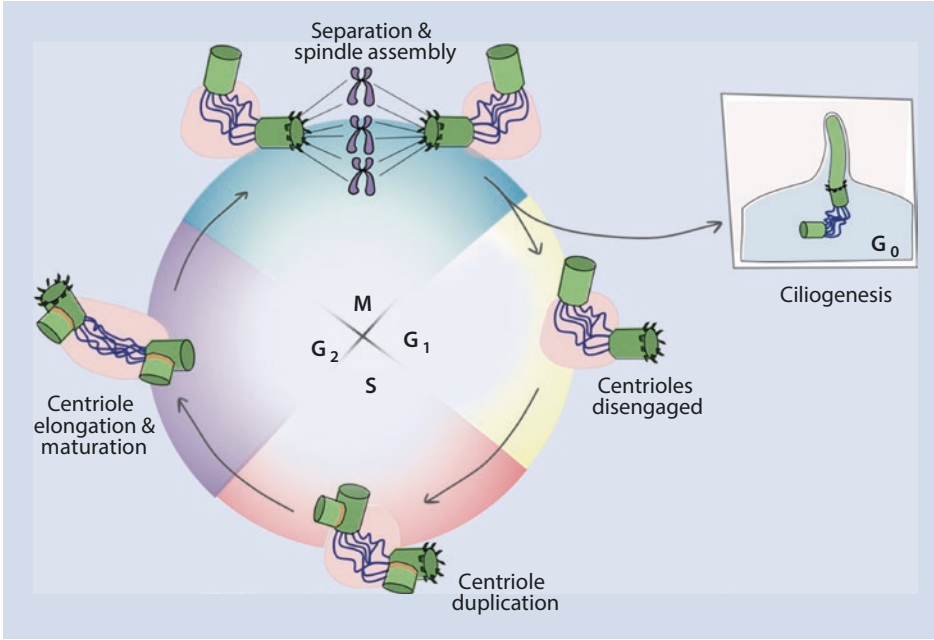


Fig. 4.5 Centriole biogenesis. Schematic representation of centrioles duplication cycle. The mother centriole can be distinguished by the presence of appendages. The centriole cycle can be described in four steps: (1) Centriole disengagement: During late mitosis and G1 phase the centrioles disengages but keep tethered. (2) Centriole duplication: in late G1/S, centrioles start duplicating in a semi-conservative manner, forming new centrioles (procentriole) perpendicularly to each mother centriole. (3) Centrosome elongation and maturation: in late S/G2, the procentriole elongates and mature, by recruiting PCM (pink cloud). (4) Centrosome separation and spindle assembly: at the onset of G2/M the two centrosomes separate, move to opposite poles of the cell and establish the mitotic spindle, contributing to appropriate chromosome segregation. When the cell exits the cell cycle and enters G0, centrioles can move to the plasma membrane, becoming basal bodies and assembling cilia

wide range of tumors [74, 79–83] and associated with genomic instability and poor patient prognosis [78, 84, 85]. By enabling the formation of multipolar spindles and chromosome missegregation, **centrosome amplification represents a mechanism leading to chromosomal instability and aneuploidy** [86]. Indeed, increased expression of the master regulator of centriole duplication, PLK4 [69, 70], was observed in several large cohorts of breast cancer patients [87, 88].

Only recently, more than 100 years after his proposal, was Theodor Boveri hypothesis directly tested. Experiments transiently overexpressing PLK4, and thus leading to centrosome amplification, induced tumorigenesis in WT mice and accelerated tumorigenesis in mice that lacked the tumor suppressor p53 [89–91]. Furthermore, in a 3D culture model, extra centrosomes can also promote invasive phenotypes and alteration of migration [92]. This invasive behavior is triggered by the centrosomal microtubule over-nucleation, leading to an increase in Rac1 activity, a small GTPase that leads to a disruption of cell-cell adhesion, thus promoting invasion and metastasis [92–94].

Several mechanisms, most relying on cell cycle deregulation, have been postulated to induce centrosome amplification: failure of cell division (cytokinesis failure), mitotic slippage, deregulation of the centrosome duplication machinery [84], *de novo* centriole

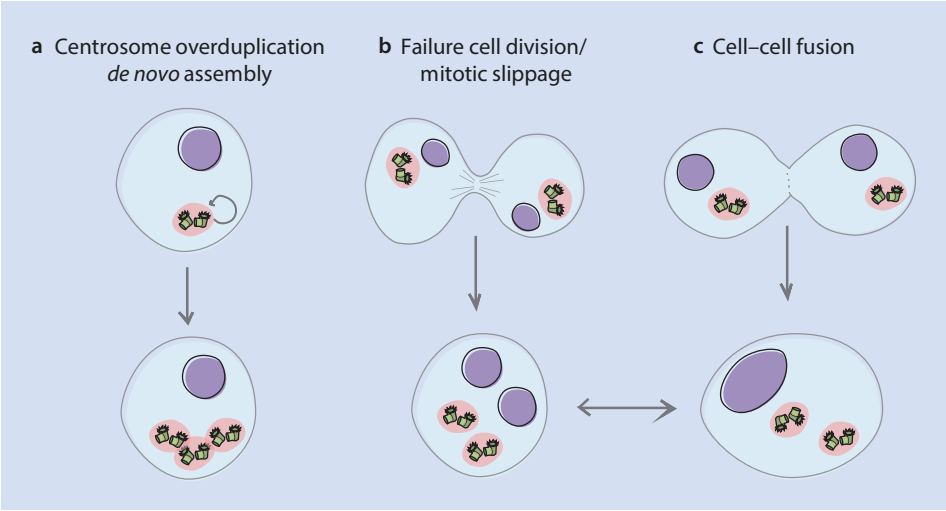


Fig. 4.6 Causes of centrosome amplification. **a** Centrosome amplification might be originated by deregulated centrosome duplication machinery, which duplicate centrosomes through several rounds within a single S phase, or through *de novo* centriole assembly. **b** Failure in cell division or mitotic slippage as a result of aborted mitosis, might also induce centrosome amplification. Depending on the cell cycle stage, the overcome of cell-cell fusion will originate different centrosome/genome ratios. Both cell fusion and failure in cell division/mitotic slippage will originate multinucleated cell, which then will form a single polyploid nuclei after subsequent mitosis. (*Adapted from Nigg* [95])

assembly and cell-cell fusion (Fig. 4.6). However, besides the increase in PLK4 in certain human cancers, little has been shown *in vivo* regarding the causes of centrosome deregulation.

How do cancer cells cope with supernumerary centrosomes?

Supernumerary centrosomes can be detrimental for cell proliferation for at least two reasons. First, the presence of both fewer and extra centrosomes activates the p53 signaling pathway in vertebrate cells, leading to either a G1 cell cycle arrest and a decrease in cell proliferation or directly induce apoptosis [55]. The mechanism by which this signaling is triggered is not yet well understood. Second, supernumerary centrosomes can lead to multipolar mitosis that can be catastrophic as cells inherit fewer chromosomes than what they need for viability.

However, cancer cells often can survive in the presence of multiple centrosomes. This is because they often do not have a functional p53 pathway. Moreover, they often also develop strategies to be able to divide, almost in a normal fashion with multiple centrosomes. To cope with extra centrosomes and overcome cell death, the malignant cell gains the ability to cluster centrosomes, where extra centrosomes remain close together through mitosis and still form a pseudo-bipolar spindle, facilitating chromosome segregation [76, 86, 96, 97] (Fig. 4.7a). It should however, be said that this process may still generate some aneuploidy. This is because the process of clustering promotes merotelic kinetochore-microtubule attachments [96] (Fig. 4.7b). Merotelic is a type of error in which single kinetochores attach to microtubules emanating from both spindle poles. This event is particularly dangerous, as it is poorly sensed by the spindle assembly checkpoint, and if not corrected, it may give rise to lagging chromosome during anaphase, leading

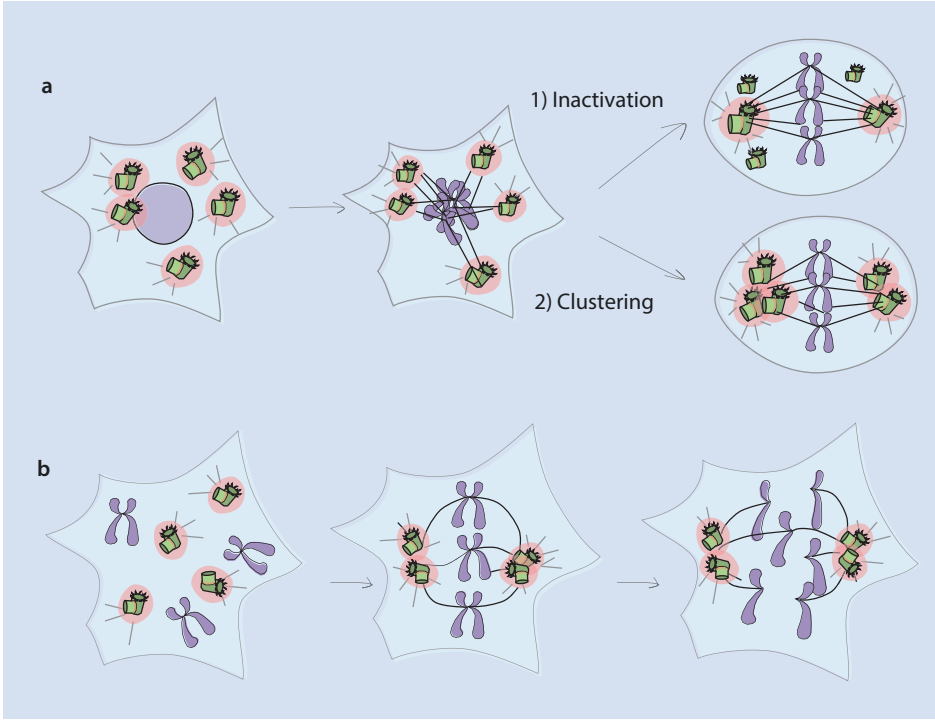


Fig. 4.7 Mechanism to suppress multipolar mitosis in cells with extra centrosomes. Centrosome amplification leads to aneuploidy. **a** To allow bipolar mitoses in cells with extra centrosomes, several distinct mechanisms are used: **a** Centrosome inactivation: by silencing MTOC activity of additional centrosomes, a bipolar mitosis can be successful achieved. Some extra centrosomes might lose PCM (pink cloud around centrosomes) and be incapable of function as MTOC. **b** Centrosome clustering: cells can combine their extra centrosomes into two groups in order to form a bipolar spindle. **b** Merotelic attachment caused by centrosome amplification. Extra centrosomes can give rise to merotelic attachments – one kinetochore attaches to microtubules that emanate from opposite spindle poles, due to altered spindle geometry, and lagging chromosomes and consequently, aneuploidy daughter cells. (*Adapted from Godinho et al.* [102] and *Rhys and Godinho* [103])

to chromosome missegregation [96, 98–101]. Moreover, besides centrosome clustering, other mechanisms have been shown to allow cells to cope with extra centrosomes, including inactivating centrosomes (Fig. 4.7a), centrosome loss and asymmetric segregation of centrosome during division [102, 103].

Deregulate ciliary signaling may also be an important event in cancer. Cilia are lost/compromised in multiple cancer types [104–106], including breast cancer [107], prostate cancer [108], melanoma [109] and pancreatic cancer [110]. As discussed above, primary cilium has an important function in regulating multiple signaling pathways. The Sonic Hedgehog (Shh) signaling is a cilia-dependent pathway, and has important functions in guiding embryonic development by regulating cell differentiation and proliferation [111–114]. Abnormal activation of Shh is observed in several types of cancer [105, 115, 116]. Moreover, the Wnt signaling is also critical to animal development and homeostasis, and regulation of Wnt signaling has also been linked to tumorigenesis, however, this is still controversial [104, 117]. Despite some associations between defective cilia and cancer, a

direct link of cilia in tumorigenesis is still unclear. Future studies will hopefully provide more evidences regarding this matter.

The knowledge on how centrosome abnormalities contribute to tumorigenesis is still a long way to go. Not only centrosome amplification, but also centriole loss and decrease in PCM recruitment might lead to alterations that could contribute to tumorigenesis [78, 118]. Focusing more on consequences of centrosome amplification in tumors, it is known that extra centrosomes can affect cells by promoting chromosome missegregation [96] and also by impairing asymmetric cell division in *Drosophila* neuroblasts [76], inducing the expansion of the neuronal stem cells, leading to tumors [76, 119, 120]. Moreover, centrosome amplification can also affect cilia signaling in interphase cells [106, 121, 122]. Additionally, extra centrosomes can affect cell polarity and signaling [85, 104, 106, 120], which can change the architecture of tumor tissue, favoring the tendency of tumors to metastasize. Furthermore, during interphase, extra centrosomes, which are clustered, recruits extra PCM, leading to increased microtubule nucleation [84], which can also alter the regulation of Rho GTPases, and thus, affect the migration and invasive properties of cells (Fig. 4.8).

4.3 Therapeutic Targets

As discussed above, cells with extra centrosomes can use unique survival strategies, including centrosome clustering. The use of drugs which **de-cluster extra centrosomes**, leading to multipolar mitoses, massive aneuploidy, causing cell cycle arrest and cell death, might stand an attractive target for a cancer treatment that does not affect normal cells (non-transformed). In fact, several drugs have been proposed to de-cluster extra centrosomes. Such drugs include:

- Griseofulvin [123, 124], a nontoxic antifungal, and a 2′-substituted derivative of griseofulvin, which has been seen to de-cluster centrosomes and selectively kill tumor cells (in concentrations non-toxic for normal cells).
- Phenanthrene-derived poly-ADP ribose polymerase (PARP) inhibitors have also been observed to de-cluster centrosomes, inducing multipolar spindles, mitotic catastrophe and death [125–127]. Non-transformed cells treated with the same PARP inhibitor (PJ-34) showed no spindle morphology changes and cell viability was maintained [128].

The discovery of critical players in centrosome clustering, such as the minus end directed kinesin KIFC1/HSET, looks promising for the cancer field. KIFC1/HSET, **a clustering inhibitor**, have been shown to have an non-essential function in most normal cells, inducing only multipolar divisions in a panel of cell lines with supernumerary centrosomes, impairing significantly the viability of these cells [129–132].

Because centrosome amplification can accelerate tumorigenesis, induce chromosome instability and promote cell invasion, effective treatments that eradicate these cells harboring abnormal centrosomes within the tumor could bring promisingly impact in cancer treatment. Drugs that also **prevent centrosome duplication**, like PLK4 inhibitors [133] (CFI-400945) are also in clinical trials, looking promising for therapeutics. However, the effectiveness of the treatments targeting centrosome in cancer cells *in vivo* is still unclear and further work is required to assess the validity of such strategy as a cancer therapy.

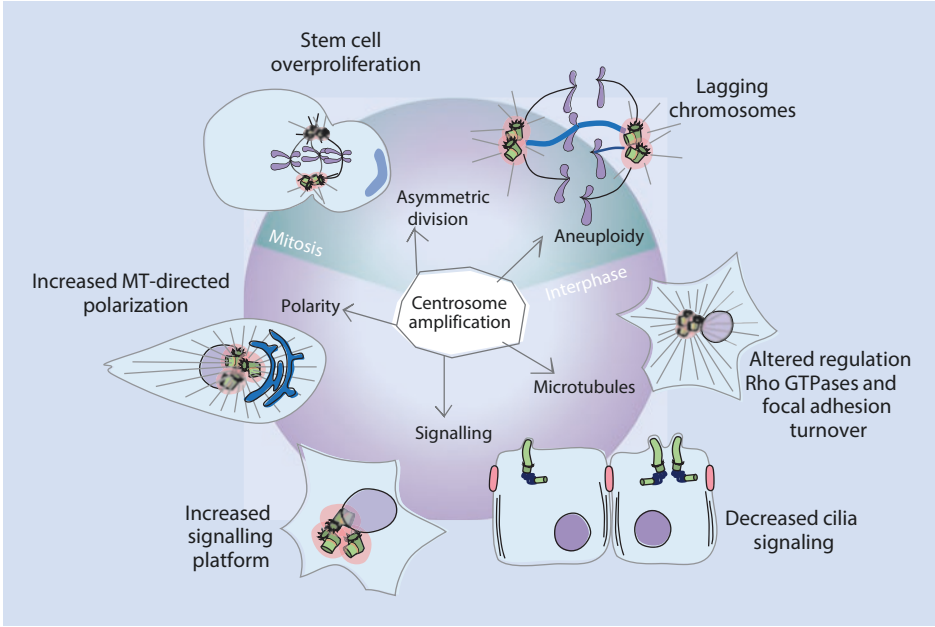


Fig. 4.8 Consequences of centriole amplification in cancer. A schematic representation of how centrosome amplification could contribute to tumorigenesis. Extra centrosomes can affect cells by promoting chromosome missegregation, leading to aneuploidy, and also by impairing asymmetric cell division in Drosophila neuroblasts (Basto, Renata et al. 2008 [76]). The role of extra centrosomes is not limited to mitosis. Centrosome amplification can affect cilia signaling in interphase cells. Moreover, increased microtubule nucleation in cells with extra centrosomes can alter the regulation of Rho GTPases and therefore affect the migration and invasive properties of cells. Furthermore, extra centrosomes could affect cell polarity and signaling. (Adapted *from Godinho and Pellman* [84])

Take Home Message

The following points were covered in this chapter:

- Each cell cycle phase is regulated by different cyclin/CDK complexes in animals.
- Checkpoints are control mechanisms that ensure that cells can move from one phase of the cell cycle to the next one.
- Defects in checkpoint mechanisms, which will not trigger DNA repair or apoptosis, will favor uncontrolled cell proliferation, and accumulation of different mutations, leading to tumorigenesis.
- The most common mutations seen in cancer are mutations that generate either oncogenes with dominant gain of function, or tumor suppressor genes with recessive loss of function that lead to the cell's ability to evade proliferation controls.
- Genomic instability plays an important role both in tumor initiation and progression, affecting also the overall prognosis of the affected patient.
- Abnormalities in centriole number and structure have been seen in many diseases, including cancer.
- Several mechanisms have been shown to allow cells to cope with extra centrosomes, including centrosome clustering, inactivating centrosomes, centrosome loss and asymmetric segregation of centrosome during division.

- One mechanism underlying chromosome instability and aneuploidy is through centrosome clustering, by promoting merotelic kinetochore-microtubule attachments.
- Extra centrosomes have also been shown to disrupt cilia signaling, and also promote alterations in the interphase cytoskeleton that facilitate invasion and alter cell migration.
- Treatments that eradicate cancer cells harboring abnormal centrosomes within the tumor could have an impact in cancer treatment.

Questions

1. What are the major cell cycle phases?
2. What is the role of checkpoints in the cell cycle?
3. How is CDK activity regulated along the cell cycle?
4. How do centrosome abnormalities induce tumorigenesis?
5. How do cells cope with extra centrosomes?
6. What kind of cancer treatments could you envisage to develop based on the fact that supernumerary centrosomes induce tumorigenesis?

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Genomic Instability: DNA Repair and Cancer

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What You Will Learn in This Chapter

This chapter explores how genomic instability can be generated and how this can impact in cancer. We start this chapter by summarizing key concepts, in particular genomic instability and DNA repair, which are the foundations to understand the remaining chapter. Next, we highlight the role of central proteins in the DNA damage response, such as the DNA damage signaling kinases ATM and ATR, and the tumor suppressor protein p53, known as the "guardian of the genome". We also explore the different cell fates that a cell may encounter upon damage in light of p53 function. Apart from the exogenous damage that a cell may encounter, endogenous damage also plays a central role in the generation of genomic instability. In this chapter we also describe the importance of maintaining the stability of telomeres, the protective structures present at the end of the linear chromosomes. We finish this chapter by explaining why, genomic instability is an important trigger for cancer progression and by giving some examples of well-known mutations that promote genomic instability.

5

Learning Objectives

After completing this chapter, students should be able to:

1. Define genomic instability.
2. Explain the role of DNA repair systems in preventing genomic instability.
3. Describe the cell cycle role on the decision of the pathway of DNA repair.
4. Understand the role of DNA damage response signaling during DNA repair.
5. Explain why p53 is considered the "guardian of the genome".
6. Acknowledge the presence of different cell fates upon damage.
7. Explain why telomeres can be seen as natural DNA breaks.
8. Describe how and why genomic instability can impact on cancer.
9. Give examples of mutations that promote genomic instability and cancer progression.

Important Concepts Discussed in This Chapter

- Genomic instability – accumulation of mutations in the genome, preventing the normal structure and function of the chromosomes, and which may lead to the generation of daughter cells with different genetic information.
- DNA repair machineries – diversity of mechanisms that aim at repairing different types of damage caused to the DNA.
- DNA damage signaling – activation cascade that goes from a sensor protein to an effector protein allowing for cell cycle arrest and onset of DNA repair.
- Cell fate – upon cell damage, the fate or destiny of a cell is determined by the type and severity of the damage and it can correspond to a complete repair of the damage and survival, to a senescent or quiescent state or to cell death.
- Telomere dysfunction – when the stability and availability of the protective structures present at the chromosome termini is compromised.
- Cancer hallmarks – central features that are acquired during tumorigenesis and allow cancer establishment.

5.1 Keeping Genomic Stability in Check

When we listen to the word "cancer" another word rapidly comes into our minds: "mutations". Even though this association between cancer and mutations is known, the mechanisms behind it require our understanding of the importance to maintain a

stable genome. But what is the genome? The term genome was first used by a German professor of Botany, called Hans Winkler, in 1920 to define the complete set of genetic material of a given organism and it consisted of blending two words: "gene" and "chromosome". Nowadays the term genome does not only include the coding information contained in the chromosomes, but all genetic material coding or non-coding, contained in the nucleus, mitochondria, chloroplasts, or even viruses, plasmids and transposable elements [1]. One can also use the terminology nuclear genome, mitochondrial genome or chloroplast genome to refer to the genetic information limited to each one of these cell compartments. According to the latest data the human nuclear genome contains approximately 19,000 coding genes [2], far from the 100,000 that were initially predicted. The human mitochondrial genome contains 37 genes and only 13 are protein coding, the remaining encode for ribosomal RNA (rRNA) and transfer RNA (tRNA) [3].

For most cases the genetic material that composes the genome consists of double-stranded (ds) DNA, but some exception are viruses that have single-stranded (ss) DNA or even RNA as genetic material. Overall the genome of a given cell represents its identity, which should be transmitted to the daughter cells. For that to occur cells underdo cell division. In prokaryotes, cell division comprises the replication of a single circular chromosome, cell growth and binary fusion, where two daughter cells arise containing each a single chromosome. In eukaryotes, cell division occurs through two main mechanisms: mitosis and meiosis, which are preceded by DNA replication and cell growth, similarly to prokaryotes. As described in ▶ Chap. 4, mitosis is a type of cell division that occurs mainly in somatic cells and allows one mother cell to generate two identical daughter cells, containing the same genetic information as the mother cell. Meiosis on the other hand, is a type of cell division that is linked to sexual reproduction and where the mother cell, which is diploid, gives rise to haploid cells, usually called gametes, and which contain only half of the chromosome of the original cell [4]. Taking mitosis as an example, apart from the mitotic phase, the cell cycle includes an interphase composed by G1, S and G2 phases. In order to be successful the cell cycle requires a highly regulated surveillance system that controls the different steps that lead to the generation of daughter cells, the so called cell cycle checkpoints (See ▶ Chap. 4 for more detailed information) [4]. Why is this surveillance system so important? Cell cycle checkpoints are critical to monitor, for example, problems that arise from replication issues, including replication fork stalling, DNA breaks and nucleotide mismatches, or from faulty mitosis, like incorrect chromosome alignment. The tight regulation of the cell cycle prevents DNA damage to be carried on from one phase of the cell cycle into the next and ultimately to the daughter cells.

The checkpoints that are specifically related to DNA damage are usually called DNA damage checkpoints and are defined as "biochemical pathways that delay or arrest cell cycle progression in response to DNA damage" [5]. There are three main DNA damage checkpoints:

- The G1/S checkpoint, which leads to inhibition of the initiation of replication thus ensuring that the DNA to be replicated does not display DNA lesions;
- The intra-S checkpoint, which is activated in response to damage that occurs during S phase, which can occur when the replication fork stalls, or to damage that has escaped the G1/S checkpoint;
- The G2/M checkpoint, which acts just before cells enter mitosis and is critical to ensure that there is no unrepaired damage that can be transmitted to daughter cells.

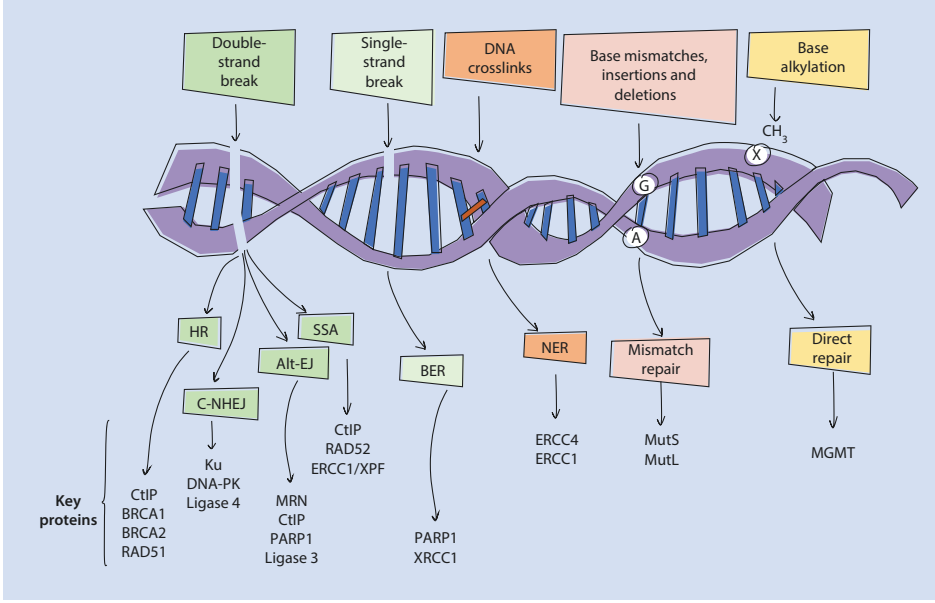
Failure to activate DNA damage checkpoints is one of the key sources of genomic instability, as defective cell cycle leads to generation of daughter cells with different genetic information and consequently to mutations. So, we connect the words "cancer" and "mutations" because failure to keep our genome under constant check leads to mutations, thus potentiating cancer.

5.2 Countering Genomic Instability

In the presence of DNA damage, cell cycle checkpoints lead to a pause in the cell cycle. But if a cell harbors damaged DNA how is it repaired? DNA is repaired due to the immediate activation of specialized mechanisms, which are collectively called DNA Damage Response (DDR). The DDR encompasses two main processes: the actual repair of the damaged DNA and the DNA damage signaling, which is critical for the activation of the cell cycle checkpoints. The importance of the genes involved in the DDR and their central role in protecting genomic stability has granted many of them the title of **caretaker** genes [6] (see ▶ Chap. 1).

5.2.1 Repairing DNA Damage

DNA damage is defined as any insult caused to DNA, which can be endogenous (e.g. Reactive Oxygen Species (ROS), recombination and replication intermediates) or exogenous (e.g. ultraviolet radiation and chemotherapeutic drugs) [5, 7] (◘ Fig. 5.1).



◘ **Fig. 5.1** DNA repair machineries. Depending on the type of DNA damage, different DNA repair machineries are activated. The figure represents different types of DNA damage and the corresponding pathways responsible for repairing them. (Adapted from Lord and Ashworth (2012) [8])

Depending on the source of DNA damage it can cause different DNA lesions, which include single stranded (ss) or double stranded (ds) DNA breaks, pyrimidine dimers, oxidized guanines, thymidine dimers, base mismatches, nucleotide(s) insertions or deletions, inter-strand crosslinks and stalled replication forks [5]. Different DNA lesions are recognized by particular DNA repair machineries. These machineries fall into six different categories:

- Double-strand break repair;
- Base excision repair;
- Nucleotide excision repair;
- Mismatch repair;
- Repair of inter-strand crosslinks;
- Direct repair.

Double Strand Breaks (DSB) are one of the most deleterious forms of DNA damage. Why? If one imagines that erosion of the DNA ends may occur as a consequence of the DNA break, how can a cell guarantee that no coding sequence is lost? Is there a DNA template? Are the two broken ends in close vicinity? These are just some of the challenges that a cell needs to overcome in order to ensure proper repair, which can occur via four different repair mechanisms [9] (Fig. 5.2):

- Homologous Recombination (HR);
- Classical Nonhomologous End Joining (C-NHEJ);
- Alternative End Joining (alt-EJ);
- Single-Strand Annealing (SSA).

Due to the importance of these repair mechanisms they are highly conserved across eukaryotes and in fact several of the initial studies where performed in the yeast *Saccharomyces cerevisiae* and only later homologous proteins have been identified in mammals [5].

Why does a cell need different DSB repair mechanisms? The first choice of the repair pathway is highly conditioned by the phase of the cell cycle.

HR is classically seen as an error-free DNA repair mechanism that relies on the sister chromatid for repair, and thus is the pathway of choice during S phase, also contributing to repair during G2 (Fig. 5.2). The MRE11-RAD50-NBS1 (MRN) complex is one of the first factors to be recruited to the DNA damage sites, playing an important role in the choice of the repair pathway [14]. This complex is involved in DNA resection together with other proteins such as CtIP, EXO1, DNA2 and the RecQ homologs WRN and BLM [14–16]. This resection is key for the choice of HR as the pathway of repair, since it allows generation of ssDNA tails that are required for the succeeding homology search and strand invasion. The ssDNA is rapidly coated by the ssDNA binding protein RPA, which also stimulates resection, but at the same time prevents ssDNA from degradation and inappropriate annealing [17]. RPA-coated DNA is required for the binding of RAD51, which is loaded onto the DNA by BRCA2 and forms a filament that is essential for homology search and strand exchange [18, 19]. The invading strand serves as 3′ end that allows extension by DNA polymerases. This leads to the formation of a four way DNA intermediate called Holliday junction, which is then removed by a resolvase, allowing proper chromosome segregation [20].

C-NHEJ is usually seen as a fast end joining process that brings together the two broken ends without requiring extensive resection, thus providing the means to rapidly repair DNA breaks, which might be one of the reasons why the process has thrived through

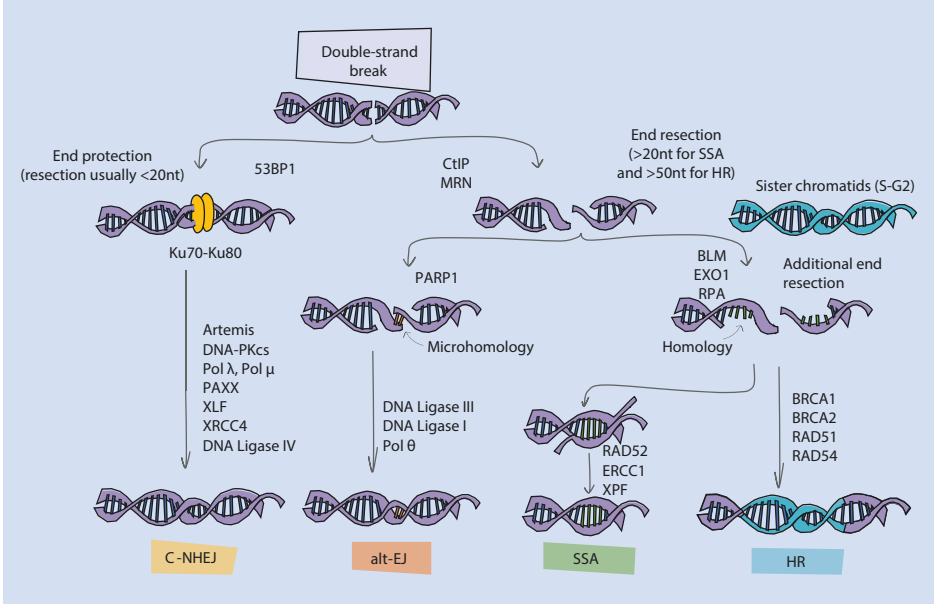


Fig. 5.2 Double strand break repair. As DSBs are highly deleterious several pathways have evolved to promote their repair, namely C-NHEJ, alt-EJ, SSA and HR. **C-NHEJ** is the predominant repair pathway in G1 and includes initial recruitment of 53BP1, which blocks resection [10]; end-recognition and protection by the Ku70/80 dimer and DNA-PK; limited resection by Artemis; and ligation via DNA ligase IV/XRCC4 [11]. **alt-EJ** requires resection, promoted by MRN complex and CtIP; PARP1 binding [9]; ligation via DNA ligase III/XRCC1 or ligase I; and fill in reaction by DNA polymerase θ [11, 12]. **SSA** requires extensive resection also mediated by MRN complex and CtIP; annealing of the two homologous sequences by RAD52; and flap removal via ERCC1/XPF complex [13]. **HR** requires an initial resection by the MRN complex, CtIP and EXO1 [14]; an extensive resection by EXO1, DNA2 and the RecQ homologs WRN and BLM [15, 16], which in followed by rapid coating of the ssDNA by RPA, thus preventing the ssDNA from degradation and inappropriate annealing [17]; homology search and strand exchange via the binding of RAD51, which is loaded onto the DNA by BRCA2 [18, 19]; extension by DNA polymerases; formation of a four way DNA intermediate called Holliday junction; and activation of a resolvase [20]. (Adapted from Chang et al. (2017) [11])

evolution despite its potential mutagenicity [9] (Fig. 5.2). In fact, in mammalian cells the ratio between C-NHEJ mediated repair and HR-mediated repair has been estimated to be 4:1 [21]. The choice of DNA repair pathway also relies on 53BP1, which is recruited to the chromatin due to histone modifications, and inhibits the extensive resection associated with other mechanisms of DNA repair [10]. 53BP1 has been also described to play an additional role which is to promote mobility of DNA ends and bridge distant ends together [10, 22]. If a cell has extensive damage how does it know which ends to ligate? This is a question that still requires further studies. What other proteins are involved in repair via C-NHEJ? For repair to occur via C-NHEJ it is required the sequential action of Ku heterodimer, which allows the recognition of the broken ends, DNA-PK catalytic subunit that when bound to Ku heterodimer becomes catalytically active recruiting the nuclease Artemis and promoting limited resection, and DNA ligase IV, which together with XRCC4 promotes the ligation of the broken ends [11]. Since C-NHEJ does not rely on a DNA template, this pathway can be activated at any point of the cell cycle, even though it has been shown to occur primarily in G1 [9]. A recent study also showed that

besides this fast resection-independent C-NHEJ, there is also a slow resection-dependent C-NHEJ [23], which relies on an initial resection step mediated by CtIP.

alt-EJ corresponds to other end-joining pathways that differs from C-NHEJ, and which gained more impact in the last decade (Fig. 5.2). In contrast to C-NHEJ, alt-EJ involves extensive resection, which requires MRN and CtIP, usually searching for a microhomology structure that may facilitate repair [11]. Following resection, it has been shown that the Poly(ADP-Ribose) Polymerase 1 (PARP1) acts as a platform that enables the recruitment of other factors required for microhomology search [9]. The latest steps of repair require the action of ligase III together with XRCC1, or alternatively ligase I, all leading to the ligation of the broken ends, and DNA polymerase θ that promotes the filling process of the resected DNA [11, 12]. Despite the fact that alt-EJ has gained more importance during the last decade, there is still a debate to understand if this is a mechanism always used by the cell to repair DSB or if this is a backup pathway that takes over when C-NHEJ is compromised [11]. Moreover, DNA breaks can occur during physiological processes, as for example, during the generation of immunoglobulins via V(D)J recombination and class switch recombination. Both V(D)J recombination and class switch recombination were first described as processes mediated by C-NHEJ, but further studies brought to light the importance of alt-EJ mechanisms [11, 24]. Although the absolute requirement of alt-EJ in these mechanisms is still not clear, one study has shown that cells lacking ligase IV have altered kinetics of class switch recombination and suggests that during these recombination events, C-NHEJ acts as a fast repair pathway and alt-EJ as a mechanism for end-joining [25]. Similar to C-NHEJ, alt-EJ can also account for mutations, since it usually involves resection of 2–20 bp which can be eliminated from the repaired DNA.

SSA is another DSB repair pathway that also involves loss of nucleotides [13] (Fig. 5.2). This mechanism of repair requires extensive resection (more than 20 bp), mediated by CtIP, in order to search for repeated sequences that are homologous in the two DNA chains. The annealing of the two homologous sequences is promoted by RAD52 and removal of the 3′ flaps by the nuclease activity of ERCC1/XPF complex [13]. Since alt-EJ and SSA require CtIP-mediated end resection, they occur primarily during S/G2 phases of the cell cycle, similar to HR, but unlike HR they do not require a sister chromatid to repair. The pathway choice between alt-EJ and SSA may rely on the extension of the resection. Overall, the distinct DSB repair mechanisms, their interplay and plasticity reflect how important it is for a cell to repair DSB.

In addition to DSB, other types of DNA damage are also promptly repaired by specific DNA repair mechanisms [5] (Fig. 5.1). For example, **base excision repair**, which is the pathway involved in the repair of oxidized guanines, is initiated by a DNA glycosylase that removes the damaged base to form an abasic site, subsequently repaired. **Nucleotide excision repair** requires the complete removal of the damage nucleotide and is the preferred mechanism for the repair of thymidine dimers. While base and nucleotide excision repair mechanism act primarily after exogenous damage, for example caused by UV light, **mismatch repair** is another pathway that surveilles DNA replication, preventing erroneous base pairing, and has as central players MutS and MutL, which are conserved from prokaryotes to eukaryotes [26]. **Inter-strand crosslinks** arise mainly due to chemotherapeutic agents and are repaired amongst others, by the ERCC1/XPF nuclease complex and the Fanconi anemia (FA) pathway. When not repaired in time, during DNA replication crosslinks lead to replication fork stalling and collapse and subsequently to the generation of DSB. There are also specific types of DNA damage which can be usually repaired by one single enzyme and for that reason are designated **direct repair** mechanisms [5]. This is the

case of O^6 -methylguanine DNA methyltransferase (MGMT), which allows the repair of alkylation adducts.

Single Strand DNA breaks (SSB) occur approximately three times more frequently than DSB and can arise due to sugar oxidation caused by ROS, upon base excision repair mechanisms or during replication, by the erroneous or abortive activity of DNA topoisomerase 1, which under normal circumstances acts by producing a DNA nick allowing DNA strand to relax during transcription and subsequent DNA replication [27]. A key protein in SSB repair is PARP1, which rapidly recognizes and binds to DNA breaks.

The multiplicity of DNA repair pathways is a strong barrier against genomic instability.

5.2.2 Sensing DNA Damage

So far, we have described the mechanism that allow DNA damage repair, now we will focus on how a cell signals for the presence of the damaged DNA and how this enables the activation of the DNA damage checkpoints. DNA damage signaling is heavily controlled by three kinases, the phophoinositide 3-kinase related kinases: Ataxia-Telangiectasia Mutated (ATM), ATM- and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) [28]. These kinases share several structural similarities, as well as substrates, being known to recognize preferentially serine or threonine residues that are followed by a glutamine, which are commonly referred to as S/T-Q. Upon activation, these kinases lead to signal transduction, which occurs mainly via a series of phosphorylation events that promote activation of DNA repair factors and ultimately cell cycle arrest. The recruitment of ATM, ATR or DNA-PK to DNA lesions requires prior generation of DNA repair intermediates, i.e., specific proteins that recognize and bind to the DNA breaks. One of the most studied examples is the MRN complex, which recognizes and processes DSB, generating a ssDNA region that provides the substrate for the activation of checkpoint responses mediated by ATM [28, 29]. In addition, the presence of ssDNA leads to the recruitment of RPA and the ssDNA-RPA complex provides the signal for ATR recruitment. Alternatively, and as previously discussed (see ▶ Sect. 5.2.1 "Repairing DNA Damage"), depending on the cell cycle stage, DSB can lead to the activation of DNA-PK, when Ku70/80 heterodimer is bound at the sites of break.

ATM is a key DDR factor that has been found to be mutated in patients suffering from Ataxia-Telangiectasia (A-T). A-T is a pleiotropic disease characterized by chromosomal instability, cancer predisposition, cerebellar degeneration, immunodeficiency, insulin resistance, high cellular ROS, ataxia and telangiectasia [28, 30]. Despite the fact that ATM is best characterized due to its role in DNA repair, ATM has also been shown to be essential to in oxidative stress response, hypoxia, insulin metabolism or mitochondrial maintenance (◘ Fig. 5.3), overall contributing heavily to maintain cell homeostasis [30].

ATM is promptly activated upon DNA damage through the binding to the MRN complex (◘ Fig. 5.4). ATM activation by autophosphorylation on Serine 1981 triggers a series of events that ultimately promote repair. ATM rapidly phosphorylates the histone H2AX (γH2AX) [31], allowing the damage signal to be spread to the chromatin surrounding the break. This signal is not only important to recruit more ATM molecules, but also other proteins that mediate the damage response (e.g. γH2AX RNF8, MDC1, MRN, 53BP1, CtIP, BRCA1) [28]. ATM activation is able to promote both HR and NHEJ, since it activates factors involved in both pathways. While γH2AX and 53BP1 activation promote

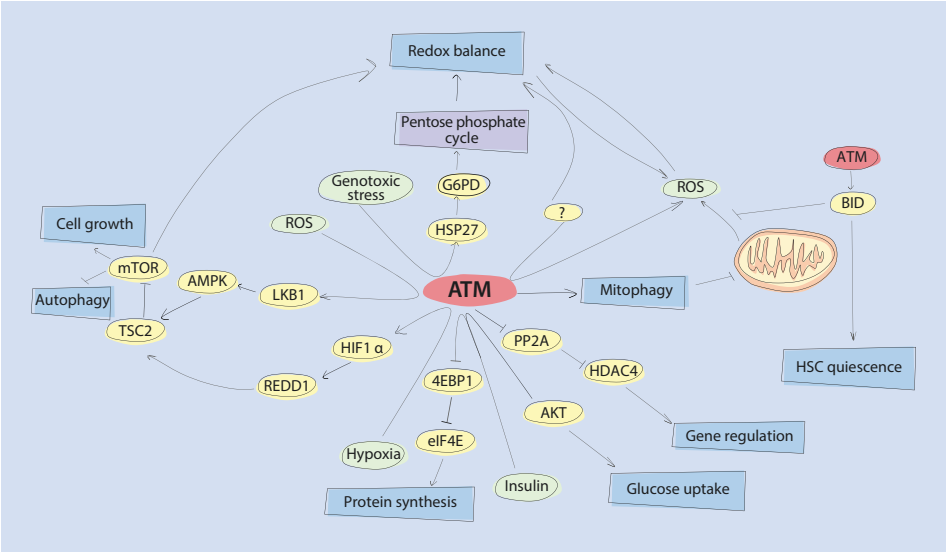


Fig. 5.3 Additional ATM roles. Apart from the critical role of ATM in DNA repair, this kinase has an extensive network of target genes, which are essential to maintain cellular homeostasis. These include genes involved in redox balance, autophagy, mitophagy, Hematopoietic Stem Cell (HSC) quiescence, cell growth, glucose uptake, gene regulation and protein synthesis [30]. (Adapted from Shiloh and Ziv (2013) [30])

chromatin bridging characteristic of NHEJ, CtIP and BRCA1 activation, promote the resection required for HR [28].

Additionally, ATM directly phosphorylates the checkpoint kinase-2 (CHK2) [32]. Upon phosphorylation, CHK2 phosphorylates CDC25 proteins leading to its inactivation. CDC25 proteins are phosphatases that dephosphorylate cyclin-dependent kinases (CDK), which are responsible for driving cells through the cell cycle. Thus CDC25 inactivation promotes cell cycle arrest and allows DNA repair. ATM also leads to the phosphorylation of the tumor suppressor p53 [28], either directly or via CHK2.

ATR was first identified as a DNA damage factor, being its preponderant role to maintain cell homeostasis. Nevertheless, ATR was also shown to have other cellular roles, in particular acting to maintain integrity of different cell structures and organelles, such as the nuclear envelope, centrosomes and the mitochondria, protecting them from different endogenous and exogenous insults [36].

ATR is promptly activated by DNA breaks upon binding of RPA to the ssDNA [28, 37] (Fig. 5.4). Since ATR only requires the RPA-ssDNA complex to be activated, this protein acts to repair not only DSB but also SSB, being particularly important in the response to replicative stress. In addition to ATR, the RPA-ssDNA complex also promotes the recruitment of ATR-interacting partners, such as ATRIP, TOPBP1 and the 9-1-1 complex, which are important for ATR activation. Like ATM, ATR is responsible for the phosphorylation of several targets including γH2AX, the Fanconi anemia proteins FANCD2, involved in inter-strand crosslinking repair, and CHK1. CHK1 phosphorylation by ATR, allows CHK1 to phosphorylate CDC25A targeting it for proteasome-dependent degradation. Inhibition of CDC25 leads to cell cycle arrest, preventing entry into mitosis [32]. ATR is also able to phosphorylate p53 directly or via CHK1.

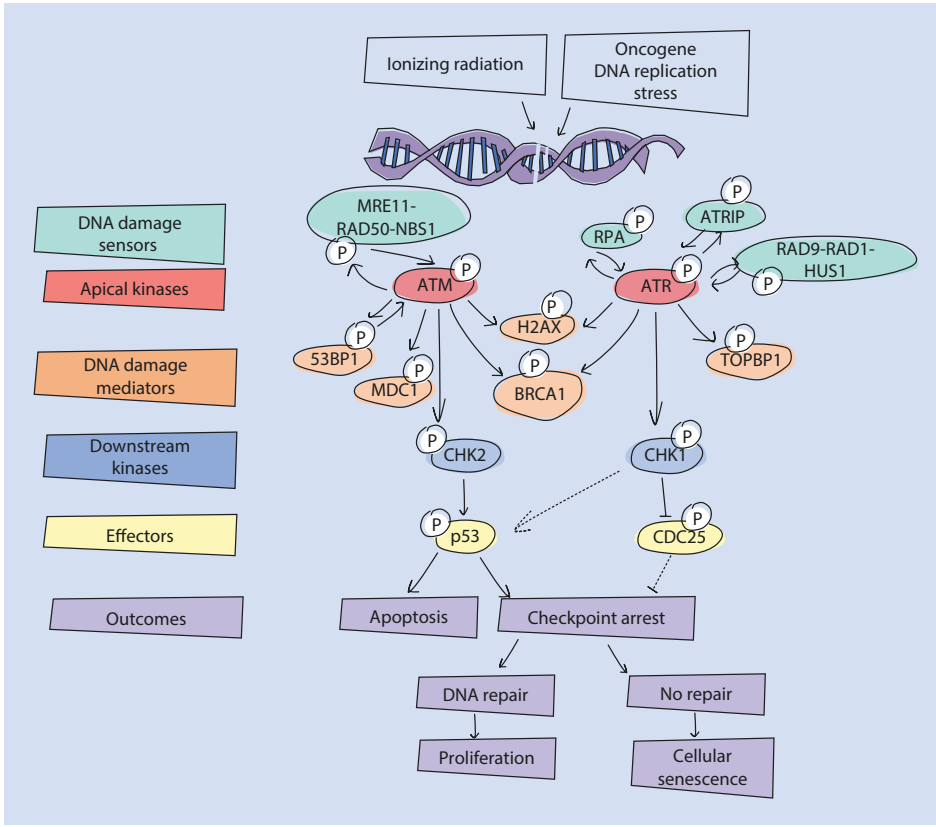


Fig. 5.4 DNA damage signalling cascade. Upon DNA damage several proteins are promptly recruited to the sites of damage. These proteins can be defined as DNA damage sensors. These sensors lead to the recruitment of the apical kinases ATM, in response to a DSB, or ATR, in response to SSB, which in turn initiate a phosphorylation cascade that leads to the activation of different proteins that act as DNA damage mediators, downstream kinases or effector genes [28, 29]. Depending on the extent of the damage different outcomes occur. A checkpoint arrest might be promoted leading to DNA repair, which allows cell proliferation to be resumed or in constrast repair may not occur and the cell enters cellular senescence. In the case of extensive damage, cells may undergo the activation of cell death via apoptosis [33, 34]. (Adapted from Sulli et al. (2012) [35])

Considering that not only ATM and ATR, but also the downstream kinases CHK2 and CHK1, respectively, are able to phosphorylate p53, this protein must play a critical role in the DDR.

Below we will explore the vital contribution of p53 in linking the DDR to cell fate and also why is p53 considered a "caretaker".

5.3 p53: "The Guardian of the Genome"

The vast majority of people in the scientific community have heard about p53, some due to its link to cancer, others in the context of DNA repair or cell death. But after all, why is it so important? What are p53 roles? The p53 protein was discovered almost 40 years

ago, by independent groups [38], as an interacting partner of the simian virus 40 (SV40) large T-antigen, a viral protein that acts as an oncogene, i.e., a factor that promotes the development of cancer, and which was already known at the time. Further studies showed that p53 was also present in non-viral promoted cancers, thus setting the stage for what is one of the most studied tumor suppressor genes, i.e. a gene that when mutated potentiates cancer development [38].

In normal cells p53, coded by the gene *TP53*, binds to its interacting partner MDM2 (or HDM2 in humans), which inhibits p53 activity, for example by targeting it for degradation. Upon different types of stress, such as DNA damage, p53 is promptly activated and the binding to MDM2 is lost [39] (Fig. 5.5). Thus, the modulation of the p53-MDM2 complex is key to assess cell's homeostasis. Moreover, this constant surveillance against insults to the cell, has granted p53 with the title of "guardian of the genome" [40].

p53 can be activated not only at post-translational level, for example through its phosphorylation, but also at transcriptional and post transcriptional levels [42]. Its activation and consequent release from MDM2 binding partner, allows p53 to transcriptionally activate specific target genes that contain p53 responsive elements (RE), including the cell cycle regulator p21. Additionally to its transcriptional activation role, p53 is also able

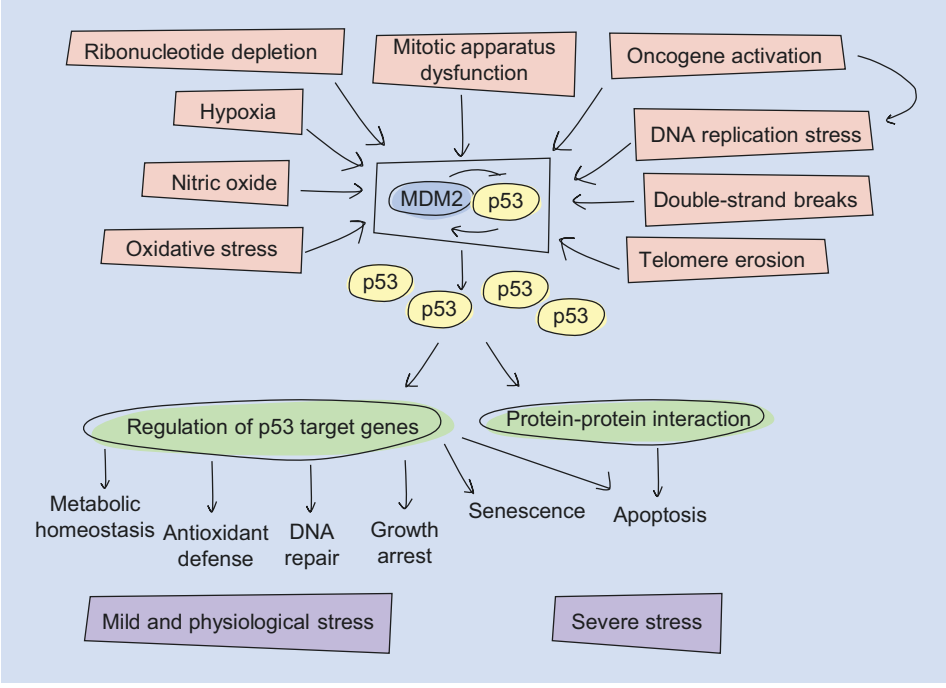


Fig. 5.5 Multiplicity of stress and damage cues activating p53. Apart from the well characterized role of p53 activation upon DNA damage, through its phosphorylation by ATM and ATR, or the downstream kinases CHK1 and CHK2, this tumour suppressor is also activated by other types of stress and damage including hypoxia, nutrient stress, oxidative stress, mitochondrial damage or oncogene activation [38, 41]. p53 activation leads to its release from MDM2 binding partner, allowing for its stabilization and subsequent activation of different transcriptional target genes, depending on the type and impact of the stress or damage. (Adapted from Levine and Oren (2009) [38])

to transcriptionally repress specific genes, as for example genes that can promote cancer development such as *Myc*, survivin or Vascular Endothelial Growth Factor A (VEGFA) [42]. The modulation of the transcriptome by p53 allows the activation of responses that promote cell cycle arrest, providing time for the cell to put in place mechanisms that allow it to repair any damage that might have been caused. If the damage cannot be repaired, p53 defines cellular fate, promoting cell death, e.g. apoptosis and necrosis, autophagy or senescence pathways [33, 34].

5.3.1 Apoptosis

How can p53 decide on the cell fate? How does it decide between cell cycle arrest and repair or cell death by apoptosis? The decision might relate with the affinity of p53 to bind specific promoters. While genes that are required for cell cycle arrest usually have high-affinity p53 response elements (RE), the ones involved in apoptosis harbor low-affinity sites [42]. This way the initial p53 response is likely to promote cell cycle arrest, but if repair is not possible and p53 accumulates, it will bind to low affinity RE, thus triggering apoptosis. Engagement with apoptosis, promotes p53 to activate pro-apoptotic genes including Bax, Puma, Noxa and Bid [41, 43] (Fig. 5.6). The onset of apoptosis is triggered by the mitochondrial outer membrane permeabilization, which requires the activation of several of these pro-apoptotic genes, including Bax and Bak. Pro-apoptotic proteins

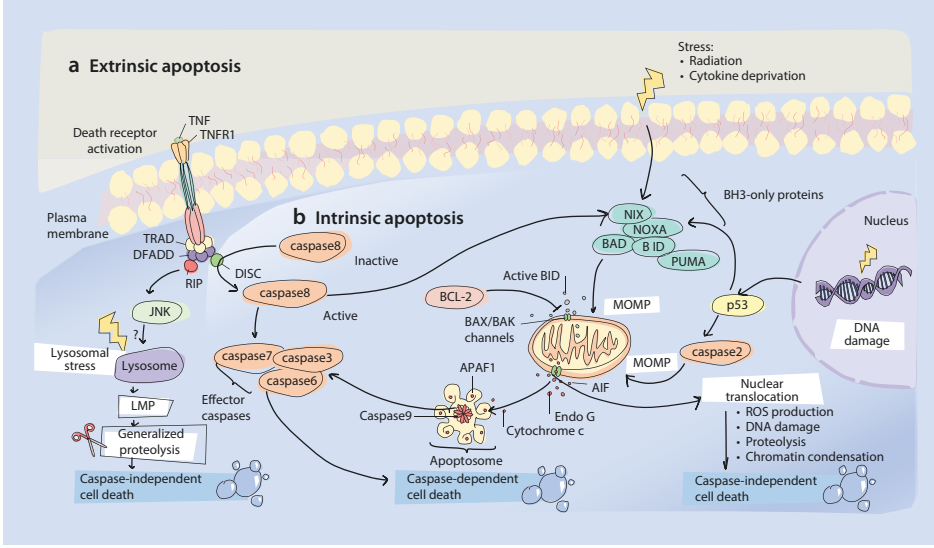


Fig. 5.6 Programmed cell death by apoptosis. There are two main pathways for death by apoptosis: the extrinsic **a** and the intrinsic apoptosis **b**. The two pathways differ primarily on the activation signal. While in the extrinsic pathway the first step is the activation of the death receptors, CD95 (or FAS), Tumour Necrosis Factor Receptor 1 (TNFR1) or TNF-Related Apoptosis-Inducing Ligand Receptor (TRAILR) by an external cue, in the intrinsic pathway the activation occurs upon formation of the Mitochondrial Outer Membrane Permeabilization (MOMP), due to the release of cytochrome C from the mitochondrial intermembrane space [43]. Both types of apoptosis lead to the activation of caspases, even though caspase-independent mechanisms of cell death have also been described [43]. (Adapted from Mariño et al. (2012) [43])

lead to cytochrome C release, which in turn promotes the formation of the Apotosome that also includes an initiator caspase, Caspase 9, and the Apoptotic Protease-Activating Factor 1 (APAF1) [43]. The activation of Caspase 9 allows the subsequent activation of other caspases, including Caspases 3, 6 and 7, which are effector caspases that promote apoptosis. Collectively caspases are proteases that are able to cleave after an aspartic acid. This cleavage is an activating signal that allows them to engage on a series of cleavage and dimerization events that finally lead to cell death.

Apart from this intrinsic apoptotic mechanism mediated by p53, there are other mechanisms intrinsic or extrinsic that trigger apoptosis and which are p53 independent. For example, Caspase 8 activation through ligation of the cell death receptor, also triggers the activation of the effector Caspases 3, 6 and 7 [43].

5.3.2 Senescence

Upon cell injury, namely DNA damage, p53 is rapidly activated and in turn activates a series of genes that promote cell cycle arrest, as for example the CDK inhibitor p21. When prolonged, activation of p53 may lead to cellular senescence [44, 45]. Alternative to p53-induced senescence, this form of growth arrest can also be mediated by the p16INK4a-retinoblastoma (RB) pathway [44, 45]. Cellular senescence is nearly irreversible and is characterized by the presence of several markers, including activation of the CDKN2A locus, transcription of p16INK4a and Alternative Reading Frame (ARF), hypophosphorylation of RB, Senescence-Associated Secretory Phenotype (SASP), Senescence Associated Heterochromatin Foci (SAHF) and senescence-associated β-galactosidase (SAβ-gal) [44, 45].

Having several pathways for cell death, why would senescence evolve? This mechanism of growth arrest has been highly studied for its role as tumor suppressor [45]. The fact that senescent cells remain metabolic active and able to signal neighboring cells, allows the surrounding tissue to activate surveillance mechanisms and so prevent tumorigenesis. Moreover, while mice harboring mutation in genes linked to apoptosis rarely develop tumors, mutations in senescence related genes are strongly linked to tumorigenesis [45]. In addition, senescence has been linked with another cellular feature: aging [45]. While in young organisms the expression of the senescence-related gene p16INK4a is barely detectable, in adult organism its expression increases, marking the increase of cell senescence. Moreover, if in one hand senescence acts as a tumor suppressor mechanism, in the other hand promotes aging and consequently age-related diseases [45].

5.4 Telomeres: "Natural DNA Double Strand Breaks"

So far, we have outlined different types of insults that a cell may face, highlighted the importance of DNA repair mechanisms and described different cell fates. What if a chromosome could itself be detected as DNA damage? In contrast to circular genomes, linear chromosomes have exposed ends. How can a cell identify that the end of the chromosome is a naturally occurring structure and not a DNA break? The answer to this question lies on the presence of telomeres. Telomeres are protective structures present at the chromosome ends, which are composed by a protein complex called shelterin and multiple repeats of telomeric DNA.

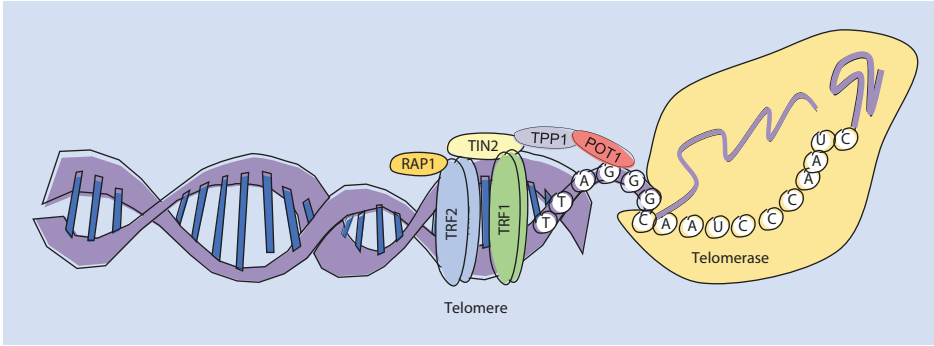


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Fig. 5.7 Telomeres and telomerase. Telomeres are protein-DNA protective structures present at the end of linear chromosomes. In mammals, they are TTAGGG repeats bound by the shelterin complex, composed by TRF1, TRF2, RAP1, TIN2, TPP1 and POT1 [46, 47]. In order to counter the end-replication problem, telomeres have a specialized reverse transcriptase enzyme, the telomerase [46, 47]. Telomerase is composed by an enzyme with reverse transcriptase activity (TERT) and a RNA template (TERC), which together allow the addition of new telomeric repeats even in the absence of a DNA template [46, 47]

5.4.1 Telomeres and Telomerase

In mammalian cells telomeres include six shelterin proteins (TRF1, TRF2, RAP1, POT1, TIN2 and TPP1) that bind to TTAGGG repeats and a series of interacting proteins, which have key functions in telomere maintenance [46, 47] (Fig. 5.7). Telomeres are highly conserved in eukaryotes and have arisen to guarantee the integrity of linear genomes, protecting them from the end replication problem [48]. For DNA replication to occur it is required an RNA primer, which provides the 3′ free end that is the starting point for replication. While the leading strand is able to be replicated continuously, the lagging strand requires several RNA primers. When replicating the telomeric DNA, the removal of the last RNA primer on the lagging strand leads to telomere shortening, as this space cannot be filled in. Despite the fact that telomeres shorten every cell cycle, the long stretches of telomeric DNA prevent loss of coding information contained in genome. While most somatic cells in the adult do not replicate and for this reason are not subjected to loss of telomeric DNA, during embryonic development or highly replicative cells, such as stem cells or germ line cells, need to be able to maintain their telomeres at a constant length in order to prevent the loss of genetic information. For that they activate a specific ribonucleoprotein called telomerase (Fig. 5.7). The telomerase holoenzyme comprises an enzyme with reverse transcriptase activity (TERT) and a RNA template (TERC), which allow the addition of new telomeric repeats even in the absence of a DNA template [46, 47]. Even though telomerase represents an important mechanism to prevent telomere shortening, the majority of mammalian cells do not have an active mechanism to maintain their telomere length and when telomeres become critically short, they impact on cell homeostasis triggering senescence or apoptosis.

5.4.2 Telomeres and DNA Damage

Telomeres are the chromosome termini and structurally the DNA ends highly resemble a DNA DSB. For this reason, when the integrity of the telomeres is compromised they can trigger a DDR [49]. Interestingly, several DNA repair proteins, including ATM, ATR, the MRN

complex and BRCA2/RAD51, have been shown to be required for telomere maintenance and biology, thus drawing a thin line between normal telomeric function and activation of DDR [49, 50]. Under homeostasis, shelterin components prevent DDR activation at the telomeres. In particular TRF2 has been described to prevent ATM signaling and activation of C-NHEJ, whereas POT1 was shown to inhibit ATR and repair via HR or alt-EJ [50, 51]. Spontaneous telomere uncapping, i.e., loss of telomere's protective structures, occurs in senescent cells due to erosion of telomeric repeats and in normally growing cells due to loss of protective structures during telomeric DNA replication in S/G2 [52, 53]. When the telomeres become dysfunctional they resemble DSBs and as such they trigger checkpoint activation and recruitment of DDR factors to the telomeres (e.g. ATM, γH2AX and 53BP1). For example, TRF2 removal from telomeres leads to the recruitment of factors such as MRN or Ku70/80, which bind to the telomeres and promote C-NHEJ [46, 49]. Repair by end-joining, and depending on the extent of the uncapping, can lead to several concatenated chromosomes end-to-end fused. If the checkpoint machinery is not fully functional, the cell may perceive this type of mechanisms as a valid DDR, even though these concatenated chromosomes pose a major challenge to mitosis. During mitosis the centromeres of the same dicentric or multicentric chromosome might be pulled apart, causing the chromosomes to break. In turn, the broken chromosomes may be repaired through end-joining, giving rise to a new dicentric or multicentric chromosome, being this process of constant fusions and breaks designated breakage-fusion-bridge (BFB) cycle [51]. Telomeric dysfunction and the subsequent BFB cycles are known to significantly contribute to the genomic instability observed in tumors.

5.5 Genomic Instability as a Trigger for Cancer

The idea that tumorigenesis is triggered by a single mutation and that *per se* is sufficient to cause cancer has been long abandoned. Analysis of human cancers showed that they display multiple mutations. This led to the proposal of the **mutator phenotype hypothesis** that postulates that the high mutation rates observed in human cancers can occur if one or more initial mutations allow to increase this mutation rate [54]. For example, mutations on DNA repair genes strongly potentiate the increase on the mutation rate, since DNA damage accumulates in cells leading to genomic instability. In turn, genomic instability is thought to underline the so-called cancer hallmarks [55, 56].

For the establishment of cancer, six hallmarks are thought to be necessary: "sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis" [55, 56] (See ▶ Chap. 1). These hallmarks are only possible to meet once the right mutations occur, which is the reason why genomic instability plays such a critical role, since it allows for multiple mutations, including insertions and deletions, chromosome rearrangement or aneuploidy (i.e. an abnormal number of chromosomes) [56].

While the mutator phenotype hypothesis seems indeed to be underlying tumor development in hereditary cancers, i.e. cancers that are linked to mutations in germline that are transmitted to the progeny (◘ Fig. 5.8), the impact of this hypothesis on sporadic cancers, or non-hereditary cancers, is still unclear. Moreover, high-throughput sequencing of human cancers, has revealed that primary sporadic tumors did not harbor increased number of mutations in DNA repair genes [56]. Instead, these tumors where characterized by mutations in genes, such as the tumor suppressors p53 and p16, or the oncogenes RAS and Epidermal Growth Factor Receptor (EGFR). Despite the preponderant role of p53,

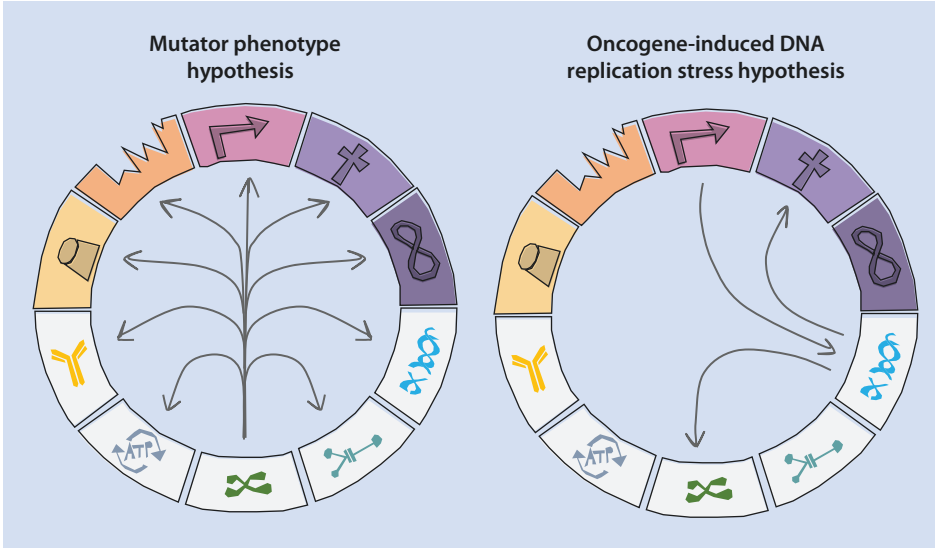

Fig. 5.8 Cancer hallmarks and drivers. How is cancer initiated is a question that is not clearly answered and gave rise to two hypothesis: the mutator phenotype hypothesis (left), where the genomic instability appears as the driving force for cancer, and the oncogene-induced DNA replication stress hypothesis (right), where the mutations in oncogenes are the starting point, causing growth deregulation, replication stress, and consequently promote DNA breaks that foster genomic instability [55, 56]. (Adapted from Negrini et al. 2010 [56])

the fact that some human precancerous lesions display genomic instability even before the mutations on *TP53* have been established, led to the proposal of the **oncogene-induced DNA replication stress hypothesis**, which postulates that genomic instability arises due to mutations in oncogenes causing growth deregulation, which in turn increases replication stress, thus promoting DNA breaks and consequently genomic instability [56].

Although both hypotheses differ on which are the initial mutations, they both consider genomic instability the key for tumor progression.

5.5.1 p53 Mutations in Cancer

The tumor suppressor p53 has long been considered the "guardian of the genome", which amongst other roles, has an essential part in preventing cancer development. In fact, *TP53* is one of the most commonly mutated genes in cancers and even when p53 is not mutated, its levels are usually compromised, for example through mutations in its interacting partner MDM2 [57].

Mutations on p53 often lead to the expression of a protein that has a dominant negative effect, that is, the function of the mutant p53 prevails over the wild-type form. These mutations may lead to a mutant p53 that acquires the ability to activate or repress the transcription of genes that are not target genes of the wild-type p53, either by direct binding to new promoters or by acting as a co-factor [57]. Other p53 mutations may also influence p53 binding to proteins, creating new binding partners or inhibiting previous protein interactions. This way a p53 mutant has the ability to modulate several pathways promoting their deregulation. These important changes in p53 function are often trig-

gered by a specific change in one single amino acid, as for example the replacement of an arginine by a histidine, due to a single nucleotide modification in the position 175 [57].

The central role of p53 as genome keeper, together with the presence of specific hot spots on *TP53* gene, which have increased mutation rate, justify the prevalence and the relevance of p53 mutations in cancer.

As mutations tend to accumulate with age, an interesting conundrum has puzzled scientist for several years: why elephants, which have a lifespan around 60 years, do not develop cancer? The answer to this question seems to be linked to the number of copies of the *TP53* gene. While humans only have one copy of this gene, elephants have approximately 20, perhaps compensating for mutations occurring in one or more copies [58]. This reinforces the idea of p53 as the "guardian of the genome".

5.5.2 Telomerase Reactivation in Cancer

Apart from mutations in DNA repair genes, oncogenes or tumor suppressor genes, mutations in telomeric components also potentiate genomic instability [51]. In addition, to telomere dysfunction, also telomere lengthening plays a critical role during cancer progression.

One of the hallmarks of cancer is to enable **replicative immortality**. In order to reach immortality, cancer cells need to maintain their telomere length, otherwise, the high replicative rate of these cells and the consequent telomere shortening, which leads to **telomere crisis**, may promote loss of coding sequencing and cell death or senescence. Most cancer cells maintain their telomere length due the reactivation of telomerase, which is usually silenced in adult somatic cells [51]. As telomeres are such important structures for cell viability, is not surprising that approximately 90% of cancers are characterized by telomerase reactivation. Telomerase mutations will favor the survival of cancer cells that reached the telomere crisis point (See ▶ Chap. 1), allowing them to maintain telomere length and enabling replicative immortality. The ones that do not acquire this feature maintain their telomere length through distinct mechanism called Alternative Lengthening of Telomeres (ALT) [51]. Despite these mechanisms that maintain telomere length, telomere dysfunction still persists even after telomere crisis. Reactivation of telomerase or ALT leads to the presence of heterogeneous chromosomes, harboring different chromosome lengths. While the longer ones regain their capping structures and functionality, the shorter ones might still undergo erroneous repair mechanism, thus still generating new mutations and further promoting genomic instability [51].

Even though the debate regarding the clear importance of telomerase reactivation in cancer has emerged, suggesting that this reactivation in 90% of cancers may result from an *in vitro* artifact due to multiple passaging of the cell lines, is clear that mechanisms that allow telomere maintenance including ALT, play an important role in tumor progression.

5.5.3 Towards Therapy: BRCA2, PARP1 and the Synthetic Lethality

We have described why DNA repair genes are so important in maintaining genomic stability. Has one would expect mutations in DNA repair genes is a common feature in cancer and has been particularly well characterized for hereditary cancers. Amongst these mutations, probably some of the best characterized are the ones that compromise the function of the homologous recombination proteins BRCA1 and BRCA2, which are well-known caretaker genes.

These mutations where identified in patients with breast or ovarian cancer, which is the reason why they are designated breast cancer 1 and 2 (BRCA1 and 2). Understanding the role of these proteins and how their absence promotes cancer has been a great propulsor for the development of different therapies. Studies that aimed at developing therapies against these cancers have focused on identifying factors that, when inhibited, are **synthetic lethal** with BRCA1 or BRCA2 [59]. When a mutation, or inhibition of one gene, is able to promote the death of cells with an initial mutation or inhibition of another gene, this is called synthetic lethality. One well studied example is the BRCA2 and PARP1 synthetic lethality [59]. PARP1 is a central player in the response to SSB. In the absence of PARP1, for example through its inhibition, SSB are not repaired and often are converted into DSB during DNA replication. If the HR machinery is compromised by mutations in genes such as BRCA2, then these DSB that arise predominantly during S/G2 phases are also not repaired, accumulate in cells, and become lethal to them (Fig. 5.9). In contrast, cells that have one of these pathways functional will thrive.

There are some examples of PARP1 inhibitors that have already reached the clinical trials, including Olaparib (AZD2281, AstraZeneca/KuDOS) and Iniparib (BiPAR/Sanofi-Aventis), both with some promising results, but not effective against all BRCA mutations. This fact has emphasized the importance of knowing exactly, which are the mutations present in each cancer type so that a more effective and patient-specific treatment can be applied.

Future Directions

Although several advances have been made to understand how is generated the genomic instability that underlies cancer progression, it is important to develop new studies that

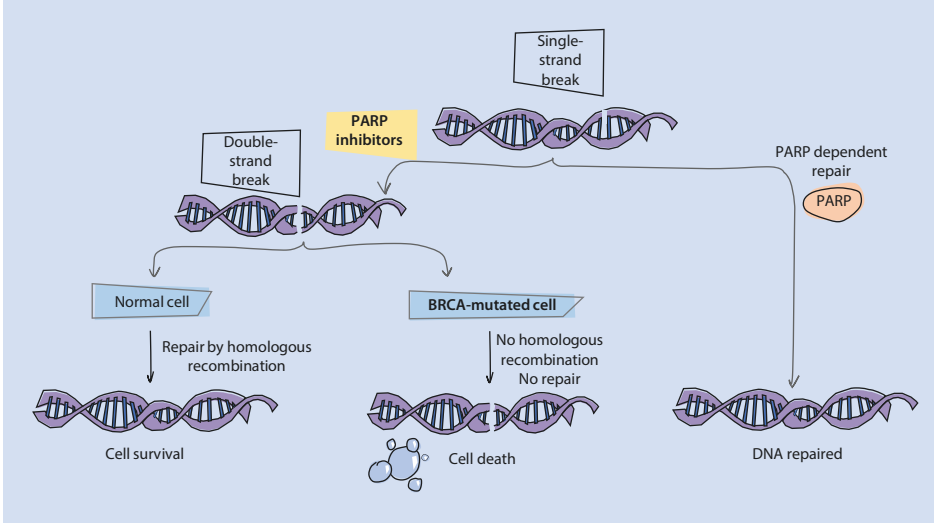

Fig. 5.9 The BRCA-PARP1 synthetic lethality. When occurs a SSB PARP1 binds to the DNA promoting repair. The use of PARP inhibitors prevent DNA repair and the SSB gives rise to a DSB, most often due to replication fork stalling and collapse [59]. In a normal cell, this DSB is recognized and repaired by the HR machinery, allowing the repair of the break. However, in the context of BRCA mutations and use of PARP inhibitors both repair mechanisms are impaired (HR and PARP), thus the DSB persists leading to cell death – synthetic lethality. Together this means that PARP inhibitors promote the elimination of cells with BRCA mutations that are undergoing proliferation, that is, leads to the death of cancer cells [59]. (Adapted from Sonnenblick et al. (2015) [60])

will further identify which are the factors involved in the generation of this deleterious instability and how do they act. This will help to find new target genes and design new drugs that target specifically cells that have high genomic instability.

Take Home Message

This chapter aims at conveying the following messages:

- The key role of DNA repair mechanisms is to prevent the generation of genomic instability.
- The decision of which DNA repair mechanism acts to repair the DNA damage, relies primarily on the type of damage and on the cell cycle phase, which determines the presence or absence of a homologous chromosome.
- Along with DNA repair mechanisms there is also the activation of DNA damage response signaling, which promotes cell cycle arrest, allowing time for the cell to repair.
- The tumor suppressor p53 has earned his title of "guardian of the genome" not only for its contribution to DNA damage signaling and consequent repair, but also for its role in determining cell fate in response to a variety of stresses. If a cell is able to repair the damage it resumes its normal functions, otherwise p53 is able to promote the engagement with other cell fates including senescence and apoptosis.
- Telomeres are the protective structures present at the end of linear chromosomes. Being the termini of the chromosomes telomeric DNA can be perceived as a DNA break, highlighting the important of their protection.
- Genomic instability is a central phenomenon that underlies cancer establishment.
- Hypothesis underlying the role of mutations in triggering cancer: the mutator phenotype hypothesis, which predicts that the high mutation rates observed in human cancers can occur if one or more initial mutations allow to increase the mutation rate; and the oncogene-induced DNA replication stress hypothesis, which proposes that genomic instability arises due to mutations in oncogenes causing growth deregulation, that in turn, increase replication stress, thus promoting DNA breaks and consequently genomic instability.
- Synthetic lethality is a strategy often used to target cancer cells. It is based on the fact that inhibiting a specific protein or pathway may specifically kill cells harboring a particular mutation. This is the case of BRCA2 cancer cells, which can be target using PARP1 inhibitors.

Questions

1. Explain the concepts of genome and genomic instability.
2. Which of the following pathways act primarily in S/G2 phases of the cell cycle: classic non-homologous end-joining or homologous recombination?
3. Explain, what are the differences between classic and alternative end-joining, emphasizing the extent of resection and their consequences on DNA repair.
4. What is the role of ATM and ATR kinases during DNA damage response?
5. Discuss the other ATM and ATR cell functions apart from their role during DNA damage.

6. Describe how is p53 modulated in normal cells and how, when activated, it can trigger different pathways.
7. Summarize the differences between senescence and apoptosis.
8. Explain why the telomeres can be considered "natural double strand breaks".
9. What role has telomerase in a cell?
10. Discuss the impact of genomic instability during cancer establishment and progression.
11. Describe the "mutator phenotype hypothesis" and "oncogene-induced DNA replication stress hypothesis" in light of their contribution to establish genomic instability.
12. Explain the concept of synthetic lethality, giving as an example BRCA2 and PARP1 genes.
13. Discuss how the different number of copies of *TP53* gene between humans and elephant's impact on the incidence of cancer.
14. Explain, what is the role of telomere lengthening in overcoming telomere crisis.

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Cell Metabolism in Cancer: An Energetic Switch

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What You Will Learn in This Chapter

Over the years, cancer has been viewed as a genetic disorder caused by an unbalance in proliferation. However, recent evidence has suggested that cancer is also a metabolic disease. Growing tumors rewire their metabolic programs to meet and even exceed the bioenergetic and biosynthetic demands of continuous cell growth. The metabolic profile typically seen in cancer cells includes increased consumption of glucose and glutamine, high levels of glycolysis, changes in the use of metabolic enzyme isoforms, and an enormous amounts of lactate secretion. Reinforcing the idea that cancer is, indeed, a metabolic disease, oncogenes and tumor suppressors have been shown to have roles in cancer-associated changes in metabolism as well. This chapter discusses recent research in the field of cancer metabolism, looking to find answers for the following questions: What characterizes the metabolic signature of a cancer cell? Why do cancer cells shift their metabolism? Are these changes a consequence or a driver of cancer progression? Can cancer metabolism be targeted to benefit patients?

In addition, we have organized this chapter around known cancer-associated metabolic changes such as: (1) the Warburg effect, (2) the role of glycolysis/TCA cycle intermediates for biosynthesis and NADPH production, (3) the importance of nutrient acquisition in cancer cells due to the increase demand for reduced carbon and nitrogen sources (4) alterations in metabolite-driven gene regulation, and (5) metabolic interactions with the microenvironment. While few tumors display all these metabolic features, most display several. The specific metabolic hallmarks [1] exhibited by an individual tumor may ultimately contribute to determine potential metabolic vulnerabilities to be targeted therapeutically.

Learning Objectives

After completing this chapter, students should be able to:

1. Understand the basic concepts of cell metabolism.
2. Describe the metabolic differences between a normal cell and a cancer cell.
3. Explain the Warburg effect, how this observation changed over the years and how it contributed for the development of medicine.
4. Understand which are, the crucial nutrients for a cancer cell to survive and propagate.
5. Describe how glycolysis is the ideal metabolic solution for a cancer cell.
6. Explain how some of the most common oncogenes affect the metabolism of a tumor cell.
7. Describe how a metabolic state of a cell can affect its own fate and the fate of other cells in its vicinity.

Important Concepts Discussed in This Chapter

- The Warburg's effect.
- Cancer cell's glucose and glutamine addiction.
- Tumor cells-associated metabolic switch.
- The importance of nutrient acquisition in cancer cells.
- The need of reduced carbon and nitrogen sources for cancer progression.
- Oncogene-driven metabolic changes.
- The role of glycolysis in cancer.
- The macromolecular biosynthetic demands of proliferating cells.
- The impact of the microenvironment on tumor development.

6.1 Cell Metabolism: Basic Concepts to Retain

Reprogramming of cellular metabolism is fundamental to tumorigenesis and it can be seen as a direct or an indirect consequence of oncogenic mutations. In order to understand cancer pathology, it is mandatory to fully comprehend the metabolic pathways and regulation of a cancer cell. However, before we start, let's review a few basic concepts of cell metabolism.

Metabolism is the sum of all chemical reactions that take place within a cell or organism and through which energy is produced. There are two major metabolic pathways that allow mammalian cells to obtain energy (Fig. 6.1a):

- Lactic fermentation or anaerobic glycolysis – In lactic fermentation, which occurs exclusively in the cytosol, glucose is initially converted to pyruvate through glycolysis and then, pyruvate is reduced to lactate, which is excreted to the bloodstream. Altogether, this process yields two adenosine triphosphate (ATP) per glucose molecule and usually occurs in the absence or low oxygen conditions, such as intense exercise – the lactate is the responsible for you muscle pain!
- Aerobic respiration – aerobic respiration, is a much more complex energy generating process, where glucose and other substrates are completely oxidized to carbon dioxide (CO_2) and water (H_2O). It comprises glycolysis, Tricarboxylic Acid (TCA)/Krebs cycle and Oxidative Phosphorylation (OXPHOS). The last two pro-

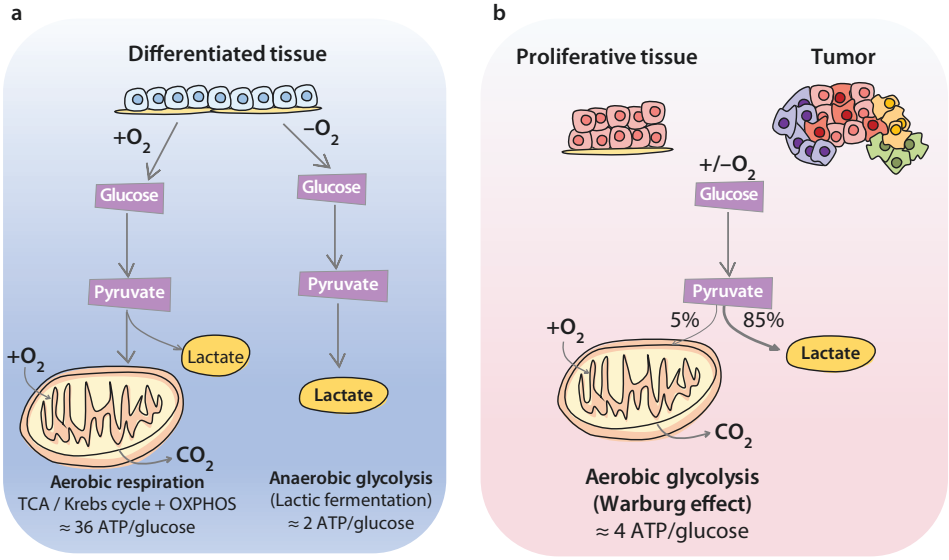

Fig. 6.1 Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect). **a** In the presence of oxygen, nonproliferating tissues first metabolize glucose to pyruvate via glycolysis which is then completely oxidized in the mitochondria to CO_2 through a process known as oxidative phosphorylation. Oxygen is the final electron acceptor to completely oxidize the glucose, making it essential in this process. When oxygen is limiting, cells can redirect the pyruvate away from mitochondrial by generating lactate (anaerobic glycolysis). **b** In cancer, Otto Warburg reported that cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis). Mitochondria remain functional and oxidative phosphorylation is still happening. (Figure adapted from Vander Heiden et al. [3])

cesses occur inside mitochondria. Aerobic respiration has a much higher energy yield (36 ATPs) than lactic fermentation but it can only occur in the presence of O_2, which functions as electron acceptor, whereas fermentation usually occurs in its absence [2].

In a normal and healthy cell, the metabolic program serves to maintain homeostatic processes through ATP production. However, to sustain the uncontrolled growth rate, cancer cells have adopted a different yet unconventional mechanism to generate energy.

6.2 Otto Warburg's Discoveries

Cancer cells metabolize glucose to lactate even in the presence of oxygen, a process known as **aerobic glycolysis** or **Warburg effect**.

The initial observation that cancer cells exhibit a completely different metabolic signature, can be traced to the pioneering work of Otto Warburg in the first half of the twentieth century. In addition, he also observed that highly proliferating cells are mainly glycolytic, heavily dependent on glucose and have what is described as a "sweet tooth", meaning that they generally take up more glucose from outside than do normal cells (Fig. 6.1b).

Otto's important discovery granted him the Nobel prize in physiology in 1931, but surprisingly the glycolytic pathway was never really used as a potential target for drug discovery. Instead, Otto's findings gave rise to the most commonly used imaging technique for diagnosing metastasis and response to therapy – the Positron Emission Tomography (PET) [4]. The way this technique works is rather simple. Deoxyglucose is an analog of glucose that is transported into the cell by glucose transporters (GLUT1), which are highly produced in cancer cells. However, this analog is not metabolized in the glycolytic pathway, so it accumulates. Attached to it there is a positron-emitting radionuclide, most commonly fluorine-18 (18-FDG), that allows for the metabolite to be visualized. The increased uptake of 18-FDG indirectly measures the glucose import of cancer as well as healthy proliferating cells; therefore, it is used as an imaging biomarker for glucose metabolism. So if a patient has cells spread over their body the different metastatic foci can be detected, correlating with a poor cancer prognosis [5].

6.3 Metabolic Shift: Why?

So far it is clear that cancer cells have a very particular metabolic profile. They are heavily addicted to glucose, they consume an enormous amount of it and they rather prefer to generate less energy and convert it to lactate, than to go through a much more energy efficient metabolic process (Fig. 6.1).

The next obvious question is, why? Why do tumor cells undergo this dramatic shift?

One compelling idea to explain the Warburg effect, is that this alteration in metabolism confers a selective advantage for survival and proliferation in a unique tumor microenvironment. As the tumor expands, the local blood supply becomes limited, leading to hypoxia. In this context, a decreased dependence on aerobic respiration becomes advantageous. But, wouldn't hypoxia stimulate blood vessels formation? Yes, angiogenesis is an important consequence of tumor grow. However blood vessels architecture becomes very disorganized, blood is not effectively delivered and as a consequence hypoxia is not

completely alleviated (reviewed in [5] and see ▶ Chap. 8). The oxygen levels vary within a tumor, resulting in fluctuating oxygen levels that potentially select for tumors that constitutively upregulate glycolysis.

Obviously, based on what we just mentioned, the tumor microenvironment selects for a deranged metabolism but, in the presence of O_2 why not use the mitochondria and get more energy out of each glucose molecule?

Initially it was wrongly thought that cancer cells would mainly rely on fermentative glucose due to mitochondrial impairments. However, it has been shown that mitochondrial respiration persists in most cancer cells [6, 7], so it would make perfect sense to use them once O_2 is available. Except that they don't.

The idea that proliferating cells are highly dependent on energy levels is wrong. In order to engage in replicative division, a cell must duplicate its genome, proteins and lipids and assemble the components into daughter cells. In short, it must become a **factory for biomolecular biosynthesis**. These activities require that cells take up extracellular nutrients like glucose and glutamine and allocate them into metabolic pathways that convert them into biosynthetic precursors that are then used for biomolecule production, among which are fatty acids and cholesterol, nucleotides and non-essential amino acids (◘ Fig. 6.2).

The growth and persistence of tumor cells depends extensively on extracellular nutrients, on finding new ways to acquire them and on reaching the perfect metabolic compromise. In the next sections, we will see how a simple metabolic process such as glycolysis can become the ideal metabolic solution to cope with these extraordinary biosynthetic demands and next how cancer cells manage to get the nutrients that they need.

6.4 Glycolysis and Biomolecular Biosynthesis

Proliferating cells must first transform acquired nutrients into a diverse pool of structural intermediates, and then focus on producing *de novo* important biomolecules such as, fatty acids, cholesterol, nucleotides, and nonessential amino acids.

Glycolysis is a major provider of structural elements for macromolecules biosynthesis (**anabolism**). The whole **catabolic** process of glucose is highly interconnected with several other metabolic pathways that use glycolytic intermediates as substrates for the *de novo* synthesis of several biosynthetic molecules ([8]; ◘ Fig. 6.2). In addition, many of these important biosynthetic anabolic reactions are reductive by nature and thus require a source of reducing power, which is provided by glycolysis, making it the ideal metabolic solution for a proliferating cell with high biosynthetic demands (◘ Fig. 6.3).

To make it clearer, let's analyze glycolysis in more detail.

Once glucose enters the cell it is immediately converted into glucose-6-phosphate (G6P), (◘ Fig. 6.1). A part of it, is then oxidized by the pentose phosphate pathway (PPP) to generate ribose-5-phosphate (R5P) and NADPH. R5P is an important structural component of nucleotides, essential for DNA and RNA replication. NADPH contributes to the cellular defence mechanisms against oxidative stress and is a well-known donor of reducing equivalents that has a particularly critical role in supporting de novo fatty acid synthesis. Accordingly, it makes sense that a proliferating cell allocates a portion of its carbon substrates to be used in NADPH production.

Continuing along the glycolytic pathway, the remaining part of G6P is converted into Frutose-6-phosphate (F6P), which can leave glycolysis and become a substrate of

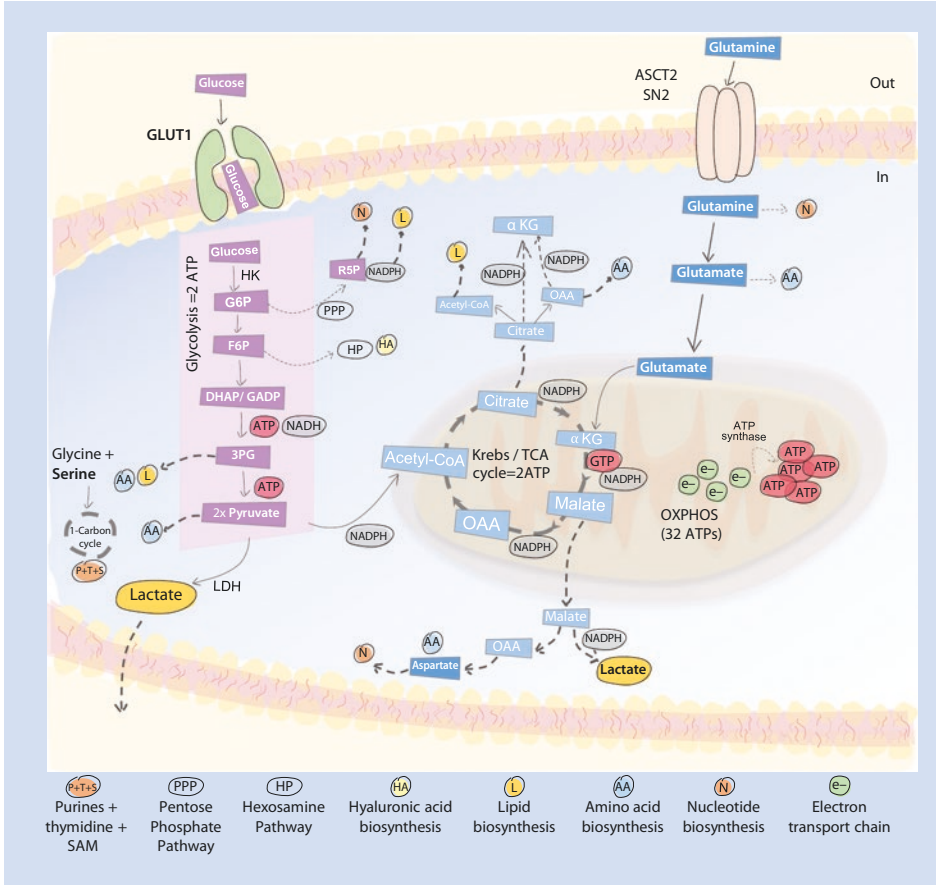

Fig. 6.2 Proliferating cells must satisfy three metabolic demands: (i) bioenergetics, (ii) macromolecular biosynthesis, and (iii) redox maintenance. The metabolic program of tumor cells is characterized by an increased uptake of glucose and glutamine. The preferential catabolism of glucose to lactate results in a less efficient way to generate energy (2 ATPs vs 36 ATPs of the oxidative phosphorylation), but at the same time it allows proliferating cells to generate various glycolytic intermediates (purple boxes) that are being sent into parallel branching anabolic pathways that support additional metabolic requirements. Glutamine serves as the most important nitrogen source for the biosynthesis of nucleotides and various nonessential amino acids. Additionally, glutamine is also an important carbon source. In a cancer cell, the amount of acetyl-CoA that is generated from pyruvate is rather small and so the TCA cycle's activity becomes limited. In this context, glutamine becomes fundamental for the replenishment of TCA cycle intermediates (anaplerosis, blue boxes), which are diverted into various anabolic pathways during proliferation. Glutamine associated anaplerosis is sufficient to maintain both mitochondrial integrity and residual ATP production despite the low levels of TCA cycle activity. AA amino acid biosynthesis, αKG α-ketoglutarate, DHAP dihydroxyacetone phosphate, F6P fructose-6-phosphate, G6P glucose-6-phosphate, GADP glyceraldehyde-3-phosphate, L lipid biosynthesis, N nucleotide biosynthesis, OAA oxaloacetate, 3PG 3-phosphoglycerate

the hexosamine biosynthesis. The hexosamine pathway provides substrates for important cellular glycosylation reactions as well as hyaluronic acid biosynthesis, an important contributor to tissue hydrodynamics, movement and proliferation of cells [9].

The next glycolytic intermediate involved in macromolecular synthesis is dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GADP), which is converted into 3-phosphoglycerate (3PG, Fig. 6.2). 3PG is important for the biosynthesis of several

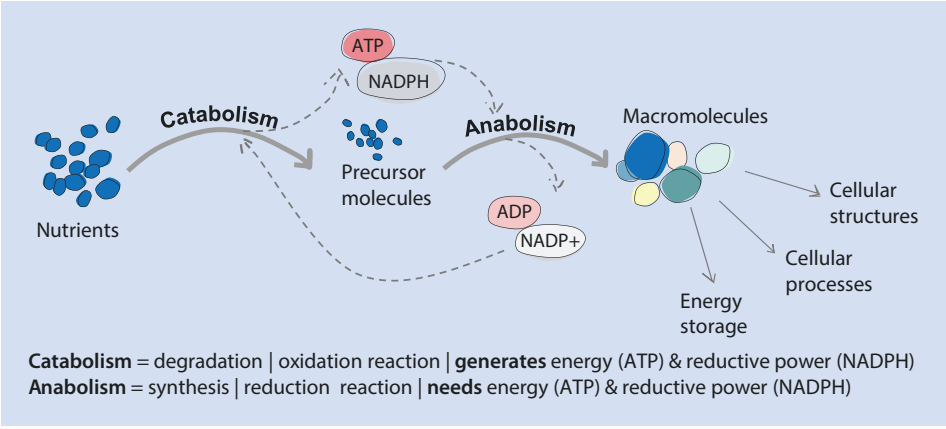

Fig. 6.3 Sketch of basic metabolism reactions. Metabolism includes two major parts: anabolism and catabolism. Catabolism comprises the metabolic processes that break down large molecules, generating energy, precursor molecules (building locks) and reductive power necessary for building macromolecules (anabolism)

phospholipids and triacylglycerols, two major structural components of cellular membranes. 3-PG also provides backbone carbons for multiple nonessential amino acids, such as glycine, through its flux into the serine biosynthesis pathway (Fig. 6.2) [8].

Curiously, cancer cells utilize as much as 50% of glucose-derived carbon in **serine biosynthesis** and its subsequent catabolism [10] (Fig. 6.2). Why? Because of its crucial role in the generation of reducing agents. Serine has a unique metabolic role in the cell as a major substrate for the so called **one-carbon, or folate, cycle**. Serine's carbons can be transferred to a carrier molecule, tetrahydrofolate (THF) generating 5, 10-methylene-THF and glycine. 5,10-methylene-THF can then undergo a series of oxidative-reductive transformations, creating a battery of one-carbon-THF species [11]. One-carbon-THF species are utilized as substrates for the biosynthesis of purines, thymidine, as well as in the production of S-adenosylmethionine (SAM), a principal substrate for cellular methylation reactions. In addition, step wise oxidation of one-carbon-THF species was recently shown to produce up to 50% of all cellular NADPH [12] (Fig. 6.2).

Downstream of glycolysis, pyruvate can enter the tricarboxylic acid (TCA) cycle and contribute to the production of mitochondrial citrate which, upon its export to the cytoplasm, can then provide the precursors acetyl-CoA and NADPH, (Fig. 6.1) [13].

Reducing agents such as NADPH are important co-factors of reductive biosynthetic reactions and to maintain the redox balance (Fig. 6.3). Normal proliferating cells often accumulate electron transport flux that exceeds the capacity of the ATP synthase, resulting in the formation of excess reactive oxygen species (ROS; [14]). In tumor cells, the situation is slightly different. Although mitochondria are not the main source of ATP, they are still active and able to respire, so ROS are still important side products that need to be taken into account.

In general, high levels of ROS sound like trouble. Proteins and lipids are more likely to become oxidized and dysfunctional, DNA mutations are more likely to occur and oncogene-induced cellular senescence, which is a phenomenon associated with a profound oxidative damage and irreversible growth arrest, is also more like to be triggered [15].

However, contrary to what one could expect, ROS accumulation can also constitute an important signaling input that contributes to the maintenance of the tumorigenic state [16]. In fact, it has been shown that ROS are able to oxidize and inhibit protein phosphatases such as PTEN, which is an inhibitor of the PI3K/Akt pathway [1]. In a very similar fashion, ROS can also act as activators of important kinases families such as Src and MAPK, important players on cellular transformation and oncogenic activity [17–19]. Furthermore, increased ROS levels facilitate the activation of HIF1α and NRF2 transcription factors, thus promoting transcriptional programs that further contribute to tumorigenesis [20, 21]

In conclusion, glycolysis is utilized by proliferating cells as a versatile production line that generates metabolic intermediates for numerous biosynthetic processes. In a very interesting and complex way, cancer cells use glycolysis to maintain their macromolecule requirements completely fulfil, to generate substances such as hyaluronic acid that can give them survival advantages and to generate NADPH, and essential co-factor for many biosynthetic reactions and an important anti-oxidant molecule necessary to maintain redox balance.

6.5 A Greedy Nutrient Acquisition

Ok, but then how can tumor cells have access to so many nutrients to support this biomass production? They need to fuel glycolysis with glucose, but cells also need a source of nitrogen for protein production and redox players, as well as fatty acids for lipids for all the new membranes they need. How can they get all their supplies? We will see how cancer cells have gathered many mechanisms to achieve their goal and these strategies are in fact metabolic hallmarks of cancer.

6.5.1 Glucose and Glutamine

The two main nutrients that support survival and biosynthesis in mammalian cells are glucose and glutamine. Through the catabolism of glucose and glutamine, a cell maintains pools of diverse carbon intermediates, which are utilized as building blocks for the assembly of various macromolecules.

■ Glucose

Despite the fact that glucose is highly available in the nutrient-rich plasma and the extracellular fluid, healthy cells are not able to import nutrients as they please. Quite the contrary, glucose intake is not driven by the immediate bioenergetic needs of a cell, instead is strictly regulated by extracellular signals that trigger growth factor signaling pathways that end up importing glucose [22] (◘ Fig. 6.4).

Warburg's observations have shown that cancer cells suffer from glucose addiction and so aiming to survive, tumor cells had to find new ways to make glucose always available. In order to do that, cancer cells accumulate oncogenic alterations that impact on multiple growth signaling nodes that become aberrantly activated. These mutations share an ability to facilitate cellular access to glucose and confer to cancer cells a significant degree of independence from the external requirements [23, 24].

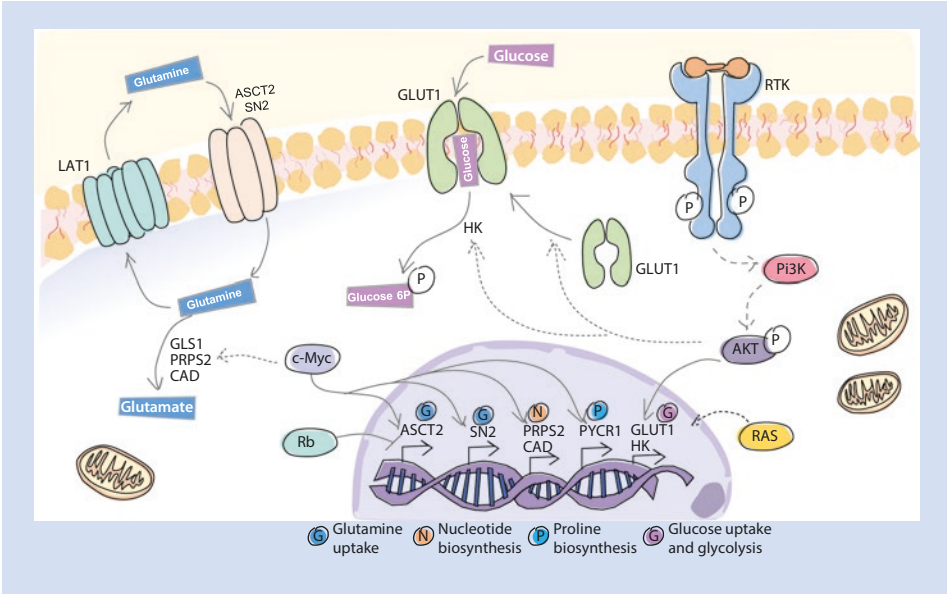

Fig. 6.4 Cancer cells exhibit a deregulation of glucose uptake and amino acids. In contrast to their normal counterparts, which require growth-factor-driven signaling inputs to proliferate, cancer cells accumulate aberrantly activated oncogenes and loss of tumor suppressors that convey to them a significant degree of independence from these external requirements. (Figure adapted from Pavlova and Thompson [1])

Mutations that lead to the activation of PI3 kinase (PI3K)/Akt pathway lead to a significant increase in glucose uptake and metabolism in several cancer types (Fig. 6.4). This growth factor signaling pathway lies downstream of receptor tyrosine kinase (RTK) activation and is a master regulator of glucose intake. PI3K/Akt signaling promotes both the expression of glucose transporter *GLUT1* mRNA and the translocation of GLUT1 protein to the cell surface (Fig. 6.4) [25, 26]. In addition, Akt increases the activity of the hexokinase (HK) enzyme, which phosphorylates glucose molecules and prevents their efflux back to the extracellular space (Fig. 6.4) [27, 28]. As expected, the 18-FDG-PET signal intensity in tumors correlates closely with the level of PI3K/Akt pathway activity; and PI3K and RTK inhibitors have been shown to attenuate it [29, 30].

Akt signaling is crucial for glucose uptake under highly proliferative demanding conditions but is not alone in facilitating the access to glucose. *Ras* genes are the most common oncogenes in human cancer [31] and they are also able to upregulate *GLUT1* mRNA expression and increase cellular glucose consumption [32]. For a cancer cell that needs to proliferate it is mandatory to have ready available the proper metabolic resources. In order to assure this, aberrantly activated oncogenes can make sure that cancer cells are able to constitutively scavenge all the available glucose, which, in turn, enables their uncontrolled proliferation.

Glutamine

Glutamine is the most abundant free amino acid found in human serum and serves as the principal way in which reduced nitrogen is transported between cells [1].

The amide group glutamine is an indispensable donor of nitrogen for the generation of purine and pyrimidine bases, essential in the biosynthesis of nucleotides. Building a guanine base depends on three molecules of glutamine, cytosine and adenine require two and uracil and thymine production cost only one glutamine molecule. Accordingly, glutamine levels have been shown to be rate-limiting for cell cycle progression, and glutamine deprivation leads to cell cycle arrest in S phase in certain cellular contexts [33, 34].

Tumor progression and proliferation is extremely dependent on glutamine, so it is not surprising to see important oncogenes deeply involved in the regulation of glutamine levels.

One good example is the Rb tumor suppressor family of proteins. Glutamine uptake is under the negative regulation of some Rb family members whose inactivation have been shown to play a role in the pathogenesis of human cancer [35]. The downregulation of these proteins has been shown to upregulate the uptake and utilization of glutamine via the E2F-dependent upregulation of glutamine transporter ASCT2 (Fig. 6.4) [36].

Another perfect example is the transcription factor c-Myc. This factor is the principal driver of glutamine utilization and is frequently targeted by amplification in various tumor types [37, 38]. c-Myc promotes the transcriptional activation of glutamine transporters ASCT2 and SN2 and promotes the expression of glutamine-utilizing enzymes responsible for converting glutamine to glutamate (Fig. 6.4) [39–41]. The resulting glutamate cannot exit the cell through glutamine transport, and so it accumulates [42]. Glutamine-derived glutamate serves as a donor of nitrogen to produce a number of nonessential amino acids via transamination [42].

Interestingly, c-Myc not only facilitates glutamine uptake but also promotes its utilization in the biogenesis of purine and pyrimidine bases. It orchestrates nucleotide biosynthesis by upregulating the expression of enzymes such as phosphoribosyl pyrophosphate synthetase 2 (PRPS2), which catalyzes a first step of purine biosynthesis, and carbamoyl phosphate synthetase II (CAD), which initiates the pyrimidine ring-building cascade [39, 41, 43] (Fig. 6.4).

Most proliferating cells in culture require an exogenous supply of glutamine. However, some cell types, such as embryonic stem cells, are capable of proliferating in the absence of glutamine in the culture medium, indicating that in some cellular contexts, glutamine can be produced de novo [44, 45]. In fact, glutamine synthetase (GS) has been found to be overexpressed in some cancers [46], yet its role and its mode of activation remain to be fully understood.

The role of glutamine in cancer metabolism goes beyond the nitrogen requirements described above, it is also an important carbon and NADPH source (Fig. 6.2).

The TCA cycle contains intermediates that may act as substrates in various biosynthetic and NADPH-generating pathways. The majority of cancer cells, due to mitochondria's limited function, have reduced catabolic TCA cycle activity and the levels of intermediates metabolites becomes limited. The only way for highly proliferating cells to maintain the TCA cycle is by replenishing these depleted intermediates through parallel chemical reactions that form these intermediates-a process called **anaplerosis** [6].

Glutamine is an important carbon source for anaplerosis in many proliferating cells. Glutamine associated anaplerosis is sufficient to maintain both mitochondrial integrity and ATP production despite the low levels of TCA cycle activity. How? After being deamidated into glutamate, glutamine-derived glutamate is then converted into α-ketoglutarate (αKG) and enters the TCA cycle (Fig. 6.2). Once αKG enters the TCA cycle it contributes to mitochondrial citrate production, which is then exported to the cytoplasm, and

converted to acetyl-CoA and oxaloacetate (OAA). Acetyl-CoA continues its fate into fatty acid synthesis or protein acetylation. OAA can be further metabolized in a multistep process to re-generate αKG and NADPH (Fig. 6.2) [6], or it can be transaminated to aspartate, which can then be used as a carbon source in nucleotide biosynthesis. Finally, glutamine-derived malate, the following metabolite of the TCA cycle, can exit the mitochondria and be converted into lactate, again with concomitant production of NADPH [47].

6.5.2 Other Reduced Nitrogen Sources

Arginine

Arginine is also an important nitrogen provider. Carrying four nitrogen atoms, arginine serves as a precursor of several nitrogenous compounds, such as polyamines, creatine, and pyrroline-5-carboxylate, a precursor for the biosynthesis of proline [1]. Arginine is a non-essential amino acid that can become somehow essential in some tumorigenic contexts. Indeed, some tumor cells choose to obtain arginine mainly from external sources rather than depending on its de novo production [48]. Why?

One possible explanation is that the inactivation of the urea cycle allows cancer cells to accumulate ornithine, which is then utilized in the production of polyamines, a class of nitrogen-containing molecules that have been shown to inhibit apoptosis and promote tumor growth and invasion [49, 50].

Another possible reason may be related to the fact that the suppression of the de novo arginine synthesis leads to an accumulation of aspartate, which can then be applied toward nucleotide biosynthesis. Once again, another example of how a cancer cell can modulate a metabolic pathway towards the creation of new biomass and biomolecules.

Proline

In addition to its effects on glutamine metabolism, c-Myc also orchestrates the metabolism of proline [51]. The production of the principal enzyme in proline biosynthesis, pyrroline-5-carboxylate reductase (PYCR1), is upregulated by c-Myc [52]. In fact, a meta-analysis of metabolic enzyme expression across diverse tumor types identified *PYCR1* as one of the most commonly overexpressed genes in tumors [53] (Fig. 6.4). Concomitantly, proline oxidase (POX), which mediates proline degradation, is negatively controlled by c-Myc [54, 55].

We still don't know exactly the contribution of altered proline metabolism to tumorigenesis, however it is possible that the elevated proline pool may facilitate the production of collagen which in turn facilitates tumor invasion.

Taken together, it is clear that similarly to carbon, tumor cell's dependence on nitrogen sources, particularly glutamine, is extremely high. The metabolism of nitrogen is still under active investigation and apparently it also undergoes complex reprogramming during tumorigenesis.

6.5.3 Alternative Ways for Nutrient Acquisition

Tumors are heavily dependent on nutritional sources and typically the vascular supply is generally insufficient, so it is common for tumor cells to find themselves under conditions of nutrient scarcity [56]. In order to survive, it is interesting to see how certain tumors acquire mutations that activate alternative ways of obtaining such precious nutrients.

Macropinocytosis

Plasma and interstitial fluids of tissues are rich in soluble proteins, yet they are not generally used as a nutritional source. Aiming for those valuable proteins, cancer cells activate a process known as macropinocytosis, in which bulk extracellular fluid is taken up into giant vesicles [57]. Fluid-filled macropinosomes are trafficked into the interior of the cell, where they fuse with lysosomes and the engulfed proteins are subjected to proteolytic degradation, resulting into free amino acids. Mutations in *Ras* or *c-Src* alleles stimulates actin-driven cytoskeleton remodeling and allows cells to recover free amino acids through macropinocytosis [58].

Entosis and phagocytosis of dead cells

Free amino acids can also be recovered from the engulfment and digestion of entire living (entosis) or dying cells [59, 60]. Cancer cells that harbor a mutant *KRAS* allele are more prone to promote entosis, meaning they are more likely to eat neighboring cells than to be eaten [61]. This property is very handy; not only these cells have a nutritional advantage over the surrounding neighbors as they can also actively eliminate the latter. This can potentiate the emergence of more aggressive and adapted cell populations by promoting cell competition within the tumor.

Fatty acid pursuit and recovery

The lack of vascularity in a tumor can compromise several biosynthetic reactions that require molecular oxygen as an electron acceptor. For example, hypoxia limits the introduction of double bonds into the de novo produced fatty acids, creating a deficit of unsaturated fatty acid species. Cancer cells always find new ways to deal with their own limitations and to acquire the components that are fundamental for their survival. Accordingly, while some tumors develop mechanisms that allow them to recover fatty acids directly from plasma, others induce the release of stored lipids from the neighboring cells. As an example, upregulation of FABP4, a long chain fatty acid-binding protein, on the surface of metastatic ovarian cancer cells allows them to acquire fatty acids directly from the adipocytes of the omental fat [62].

In summary, tumor cells always find ways to assure nutrient acquisition that allows them to survive and proliferate in inhospitable conditions. These new mechanisms generally involve an ability to reach normally inaccessible nutrient sources, as well as to recover ready-made molecules when their cellular synthesis has been compromised.

6.6 Alterations in Metabolite-Driven Gene Regulation

Up until this point we have pretty much established that reprogramming of cancer cell metabolism, drives tumorigenesis by activating growth and survival signals, enabling increased nutrient acquisition and allowing for massive biomolecular biosynthesis.

However, cancer deregulated metabolic networks are not merely passive recipients of growth signals, they directly transmit the information about the cellular metabolic state to a series of regulatory enzymes, including the ones that mediate the **deposition** and **removal** of **epigenetic marks from chromatin**, which then can dramatically impact on gene expression and therefore tumorigenesis [63].

Acetylation

Acetyl-CoA is a very common epigenetic mark and it accumulates in cancer cells. The deposition of acetyl marks on histones is associated with the increased accessibility of

the genomic DNA for the assembly of transcriptional complexes. **Histone acetylation** is extremely sensitive to alterations in the cellular nutritional and signaling status [64, 65]. The levels of glucose, as well as activation of oncogenic signaling, increases total histone acetylation, which, in turn, promotes the enhanced and broader gene expression [66].

■ Methylation

Another example of a common epigenetic mark is the methyl group. The deposition of methyl marks on histone tails, cytosine methylation on DNA, and adenosine methylation on mRNA use SAM as a donor of methyl groups. SAM is a product of the folate cycle and, as previously described is fueled by serine catabolism. Histone and DNA methylation is sensitive to alterations in SAM levels, so it is intimately regulated by the metabolic status of a given cell [67–69].

■ De-acetylation & De-methylation

However, the cellular metabolic state can also guide the opposite reactions i.e. the removal of acetyl and methyl marks. For instance, the removal of acetyl marks from histone and nonhistone proteins by sirtuins, a class of deacetylases, utilize NAD^+ as a cofactor. Similarly, lysine-specific demethylase LSD1 uses FAD as a cofactor [70, 71]. Sensitive to changes in NAD^+ or FAD availability, these enzymes orchestrate global posttranslational and epigenetic changes that can impact deeply in tumor development [72].

Another important group of posttranslational modifications in the cell are carried out by the members of a large class of αKG-dependent dioxygenases: the TET (Ten–eleven translocation) family of DNA demethylases, the Jumonji C family of histone demethylases, and a family of prolyl hydroxylase (PHD) enzymes. Accordingly, the intracellular levels of αKG can directly influence the activity of these enzymes. In addition, αKG-dependent dioxygenases are prone to the inhibition by their reaction product, succinate, as well as by fumarate, the downstream product of succinate degradation in the TCA cycle [1].

Interestingly, there are several tumors that, although affecting different parts of the body, exhibit very similar phenotypical characteristics, due to accumulation of succinate [73, 74]. Tumors that accumulate this particular metabolite share features that are consistent with dioxygenase inhibition, among which is a characteristic global increase in DNA methylation [75].

6.7 Metabolic Interactions with the Microenvironment

The metabolic state of a cell can have a huge impact on the fate of that particular cell (cell-autonomous) but, more importantly, it can also influence the fate of other cells in its vicinity i.e. have a non-cell-autonomous effect on the tumor microenvironment (TME).

The tumor microenvironment consists of malignant cells, immune cells, non-cancer cell stroma's, fibroblasts as well as the vasculature and lymphatics of the tumor [76].

As we saw before, in glycolytic tumors, lactate levels of cancer cells are remarkably elevated [77, 78] and are highly correlated with cancer aggressiveness and poor survival [78]. Historically, lactate has been considered as the toxic 'end of the road' of aerobic glycolysis, with no major interest. However, recent findings have demonstrated that the accumulation of extracellular lactate has been shown to affect significantly the tumor microenvironment. For instance, increased lactate levels promote the emergence of an immune-permissive microenvironment by attenuating dendritic and T cell activation as

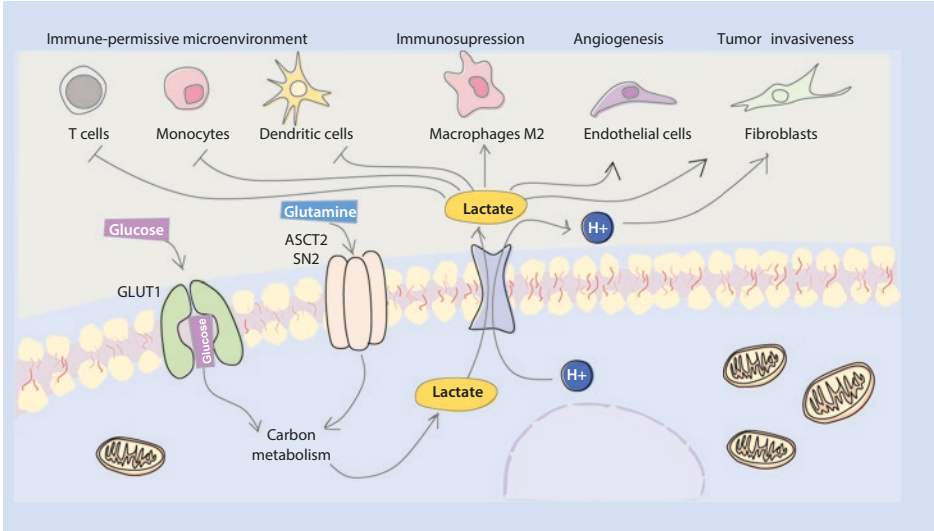

Fig. 6.5 Cancer cells alter the chemical composition of the extracellular milieu, which exerts pleiotropic effects on the phenotypes of normal cells that reside in the vicinity of the tumor, as well as the extracellular matrix. Reciprocally, the microenvironment affects the metabolism and signaling responses of cancer cells themselves. (Figure adapted from Pavlova and Thompson [1])

well as monocyte migration (Fig. 6.5) [79–81]. Additionally, lactate also promotes the polarization of resident macrophages to an M2 state [82, 83], potentiating an escape from the immune system and favoring proliferation (see ▶ Chap. 7).

Furthermore, lactate stimulates angiogenesis by stabilizing HIF1α, activating PI3K/Akt signaling in endothelial cells, and inducing the secretion of a proangiogenic factor VEGF from tumor-associated stromal cells (Fig. 6.5) [84–86]. Finally, high levels of lactate also stimulate hyaluronic acid production by fibroblasts, which as we already mentioned, may contribute to tumor invasiveness (Fig. 6.5) [87].

A typical characteristic of tumor microenvironment is its acidic nature, which seems to be key for the interaction and signaling of the different players involved in carcinogenesis. The secretion of lactate into the extracellular space via the monocarboxylate transporter MCT1 is coupled to the cotransport of H^+, promoting the acidification of the cellular microenvironment. In addition, the extra CO_2 generated by mitochondrial decarboxylation reactions contributes to the extracellular acidification as well [88]. Increased extracellular acidification stimulates the proteolytic activity of matrix metalloproteinases (MMPs) and cathepsins, that promote the degradation of the extracellular matrix components that can enhance tumor invasion and therefore promote metastasis [89, 90].

Whereas lactate accumulation and extracellular space acidification may have been seen as collateral damage of cancer-specific metabolic reprogramming, this could be in fact a very interesting way to promote the emergence of an immune-permissive microenvironment around them. On the other hand, the conditions within the tumor microenvironment can also impose profound alterations on the metabolism of a cancer cell. As discussed earlier, tumors are often faced with nutrient – and oxygen-poor surroundings. Tumors are pushed to develop various nutrient-scavenging strategies and hypoxia selects for glycolytic cells. The lack of oxygen also impedes the ability of cells to carry out reactions that require oxygen, disrupts the redox balance and affects cellular signaling and transcriptional programs.

In summary, reciprocal interactions between cancer cells and their microenvironment impose a selective pressure that shapes cancer cell metabolism and contributes to the emergence of a more aggressive cell population.

6.8 Cancer Metabolism: Therapeutic Target?

Proliferating cancer cells exhibit considerably different metabolic requirements to most normal differentiated cells. Metabolic pathways must therefore be rewired in such a way that balances biosynthetic processes with sufficient ATP production to support cell growth and survival. As all cancer cells are dependent on this change in metabolism, these altered pathways represent attractive therapeutic targets [91, 92]. However, because normal proliferating cells have the same metabolic requirements as cancer cells (Fig. 6.2), finding a therapeutic window between proliferating cancer cells and proliferating normal cells remains a major challenge in the development of successful cancer therapies targeting metabolic pathways.

Nonetheless, a therapeutic opportunity that has been recently explored is the small-molecule inhibition of key enzymes involved in metabolic pathways such as glycolysis and fatty acid synthesis. In most cases, preclinical studies have suggested that this strategy might have great potential, however it remains to be determined whether the potential coming from these laboratorial results will remain durable in clinical studies [93]. For example, the glucose analogue 2-deoxyglucose (2DG) is a glycolytic inhibitor that has been previously tested as an anticancer agent in phase I clinical trials. Although sufficient amounts of 2DG can potentiate cancer cell arrest and/or death by limiting glucose catabolism, the dosing necessary to achieve such effects in patients resulted in adverse toxicity [93].

So far, our knowledge of how pathways are regulated to facilitate cell proliferation is incomplete [94]. Once we improve our knowledge on how cells regulate nutrient uptake and utilization, we will then be ready to target cell metabolism. Understanding tumor cell metabolism requires the use of specific methods that are not often used in cancer drug discovery. However, similar to what was done for antibiotics, targeting the biosynthetic processes that are unique to microorganisms, the possibility of selectively targeting the biosynthetic processes of cancer cells holds promise as a strategy for improving cancer therapy.

Cancer metabolism has come a long way. Warburg's observations stood the test of time, its importance in cancer diagnosis is unquestionable but it has not been fully explored as a potential source of new molecular targets. However, through recent years, scientific evidence has been confirming that alterations in metabolism is an important driver of mutations, suggesting that exploring metabolic enzymes as therapeutic targets might be a very interesting road to follow when aiming to cure cancer.

Take Home Message

The important elements in this chapter are summarized here:

- Reprogramming energy metabolism is an emerging hallmark in deregulation of cell proliferation;
- The Warburg effect, or aerobic glycolysis, is the observation that most cancer cells produce energy through a high rate of glycolysis followed by lactic acid fermentation, even in the presence of oxygen;

- The metabolic ecology of tumors enables component cells to generate ATP, undertake biosynthesis, and generate enough reducing equivalents to cope with the reductive nature of the majority of biosynthetic reactions;
- The two main nutrients that support survival and biosynthesis in cancer cells are glucose and glutamine;
- Cancer cells accumulate metabolic alterations that allow them to gain access to conventional nutrient sources as well as to unconventional ones;
- Aberrantly activated oncogenes and loss of tumor suppressors deregulate the import of glucose and amino acids into cancer cells;
- Through the catabolism of glucose, a cell maintains pools of diverse carbon intermediates, which are utilized as building blocks for the assembly of various macromolecules;
- A proliferating cancer cell must synthesize a number of nitrogen-containing molecules de novo, including nucleotides, nonessential amino acids, and polyamines. Therefore, the cell increases the consumption of carbon and concomitantly elevates the cellular demand for reduced nitrogen.
- The information about a metabolic state of a cell affects not only its own long-term decision-making but also has the potential to influence the fate of other cells in its vicinity.

? Questions

1. Define cell metabolism referring the difference between catabolism and anabolism.
2. Describe briefly the different methods through which a cell produces its energy.
3. What are the main metabolic differences between a normal cell and a cancer cell?
4. How would you describe the Warburg effect?
5. In what way did the Warburg effect contributed to the development of Medicine?
6. Explain the metabolic shift observed during tumorigenesis.
7. In what way does the catabolism of glucose and glutamine contributes for the development of tumors?
8. Describe how the oncogene c-Myc regulates the uptake of glutamine.
9. What is entosis and which gene, when mutated, promotes this phenomenon in cancer cells?
10. What is the main designated donor of reducing equivalents in a cell?
11. Describe the importance of the serine biosynthetic pathway in a cancer cell.
12. Discuss why it is so important for a cancer cell to maintain its reducing power.
13. Name two important sources of reduced nitrogen in a cell.
14. Name an important donor of methyl groups in a cell.
15. Describe how different tumors, affecting different cells or organs, may exhibit similar phenotypical characteristics.
16. Discuss how the accumulation of lactate might affect the tumor microenvironment and vicinity.

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Cancer Immunoediting and Hijacking of the Immune System

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What You Will Learn in This Chapter?

In this chapter we present an historic overview of the relationship between cancer and the immune system. We will see how it was not always clear that the immune system was able to recognize and fight cancer and how different theories have evolved, from immunosurveillance to the recent immunoediting theory. We will see how the tumor microenvironment is extremely rich in different immune cell populations. Then we will broadly revise, the main components of the immune system and how it roughly works (immunology in a very small nutshell). This will help understand how cancer cells not only evade the immune system to remain undetectable and sustain within the host but how they can also hijack immune cells to help cancer progression. The molecules and cells that are major players in these processes will also be addressed. We then conclude this chapter by describing the several new revolutionary approaches to fight cancer using the patient's own immune system.

Learning Objectives

After reading this chapter, students should be able to:

1. Describe the immunosurveillance concept.
2. Describe the immunoediting process.
3. Explain the differences between innate and adaptive immunity.
4. Discuss how the immune system controls cancer cells.
5. Describe the cell players that constitute both arms of immunity and their general function.
6. Describe the major cellular components of TME.
7. Discuss the immune evasion mechanism, providing some examples.
8. Describe the use of immunotherapy for cancer treatment and discuss why some treatments work so well in some patients while not at all in others.

Important Concepts Discussed in This Chapter

- Cancer Immunosurveillance – cells and tissues are constantly monitored by the immune system, which in the early stages is able to eliminate the first cancer cells but then it becomes entangled in a cross-interaction with tumor cells that corrupts their initial surveillance role.
- Cancer Immunoediting – is the result of the cross-interactions between the anti-tumor response of the immune system and the tumor cells, leading to the selection of immune-resistant clones/variants.
- Cancer Immune Evasion – is a strategy used by cancer cells to escape the host immune response, increasing its probability to thrive in the immune competent host.
- Cancer Immune Suppression – is a reduction of activation of the immune system functions.
- Immunotherapy – is a type of cancer treatment that enhances the patients' natural defenses to fight cancer.
- Immune Checkpoint Blockers – are the "brakes" of the immune system, that normally act to avoid auto-immune responses.

7.1 Cancer Immunoediting and Hijacking of the Immune System

All living organisms are hosts for other species, establishing different types of symbiotic interactions. However, all organisms and all cells in a multicellular organism need to defend themselves from dangerous invaders like bacteria, viruses, fungi or larger

parasites which have not evolved a positive and/or neutral relationship. In parallel, dead cells resulting from normal tissue homoeostasis also need to be cleared. Vertebrates have two major armies of defense: the innate and adaptive immune system, which perform these functions.

7.1.1 Cancer Immunosurveillance Hypothesis

A hundred years ago Paul Ehrlich (1909) put forward an hypothesis in which the immune system could recognize and destroy tumor cells, a concept that was further developed by Burnet and Thomas (1957) as the **cancer immunosurveillance hypothesis** [1]. This theory proposed that cells and tissues were constantly monitored by a vigilant immune system, and such immunosurveillance was responsible for recognizing and eliminating the majority of incipient cancer cells [2].

This immunosurveillance hypothesis was supported by the observations of the "father" of immunotherapy – William Coley (1891). Coley was a surgeon that noticed that some cancer patients who got infections after surgery had their tumors regressed more efficiently than patients who didn't get infections. He hypothesized that infection had stimulated the body's "resisting powers" [3]. At first Coley injected live bacteria into tumors, but later he used heat-killed bacteria to induce a high temperature (fever) without causing a real infection. However, not all patients responded well to this immune activation and also the advent/introduction of radiotherapy lead to Coleys's approach to be mostly forgotten until the recent advent of immunotherapy [3].

The immunosurveillance theory was supported by other later observations such as:

- Increase of certain cancers in immunodeficient individuals, like immunosuppressed organ transplant recipients and HIV/AIDS patients [4].
- In mice, tumors were rejected when transplanted into syngeneic hosts (genetically identical) whereas normal tissues grow with no constrains, suggesting existence of tumor-specific antigens [5].
- Patients with high number of intratumoral lymphocytes and Natural Killer (NK) cells in different types of cancers show increasing survival rates [6].
- Effectiveness of the bacterium *Baccillus Calmette-Guérin* (BCG) vaccine in the treatment of superficial bladder cancer [7].

However, there were also several other contradicting observations that suggested a positive role for the immune system in cancer development. For example:

- Thymus excision after birth correlated to a reduction of breast cancer prevalence [8] and immune reconstitution restored susceptibility to cancer [9].
- Immunosuppressed patients present less chance to develop breast carcinomas compared with immunocompetent individuals [10].
- Low incidence of human cancers in leprosy and sarcoidosis diseases, which are characterized by immunosuppression [11].

Therefore, the concept of cancer immunosurveillance has been shaped with debate until the early '90s, where finally the development of new immunodeficient mouse models made it possible to address these questions in a more reliable manner [12].

7.1.2 The Cancer Immunoediting Concept

Using a chemical carcinogen, Schreiber and colleagues, found that 58% of RAG2$^{-/-}$ mice (which lack adaptive immunity, i.e., T and B cells) developed tumors, contrasting to only 19% in the wild-type (WT) strain. A striking finding was that, when these tumors were transplanted into WT immunocompetent recipients (from the RAG2$^{-/-}$ or WT donors), the tumors derived from RAG2$^{-/-}$ were more immunogenic as a group i.e. had a higher rate of rejection in comparison to the tumors derived from the WT strain (Fig. 7.1). These results showed that tumors that had developed in the WT immunocompetent host were subjected to a selective immune-related pressure (**editing process**) whereas the others did not [2].

In summary, these and other experiments led to the idea that highly immunogenic cancer cell clones are routinely eliminated in immunocompetent hosts, leaving behind only weakly immunogenic variants (poorly recognized by the immune system) to grow and generate "immunoedited" tumors. These weakly immunogenic cells can then colonize very efficiently in immunocompetent recipients. In contrast, when arising in immunodeficient hosts (RAG2$^{-/-}$), the immunogenic cancer cells are not selectively depleted and can prosper in an immunodeficient recipient (unedited). However, when transplanted into an immunocompetent host they are no longer able to thrive because they were not previously "edited" (negatively selected), they are now recognized and eliminated. These results show that the immune system not only protects the host against tumor formation, but also edits/selects tumor immunogenicity.

These and others studies led to the current view of **cancer immunoediting**, which integrates the **paradoxical anti- and pro-tumoral roles** of the immune system [13].

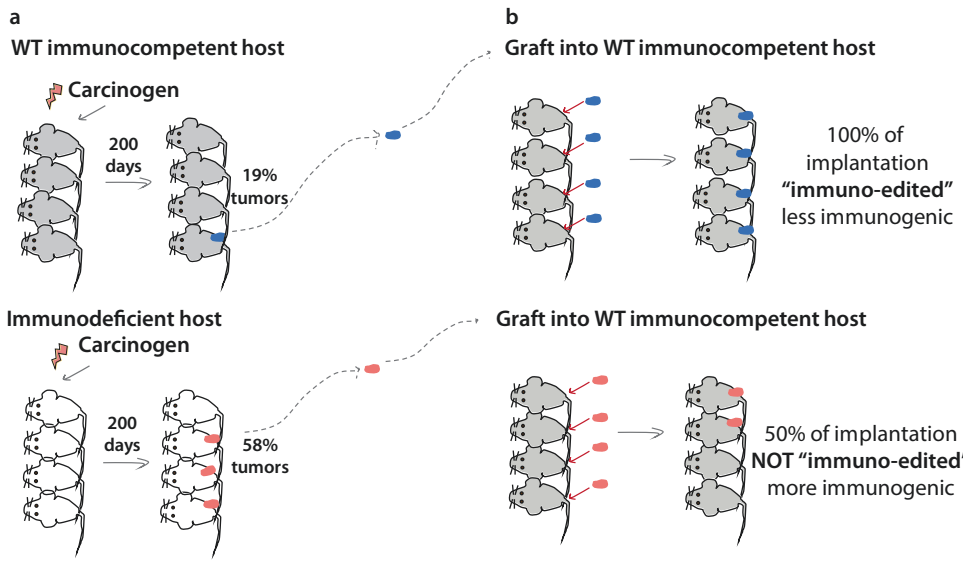

Fig. 7.1 Tumor immunoediting. **a.** Immunodeficient mice (Rag2$^{-/-}$) were more susceptible to carcinogen-induced tumors formation (58%) when compared with WT immunocompetent host (19%). **b.** WT-derived tumors when transplanted into WT recipients implant with 100% efficiency (blue). In contrast, tumors derived from mice lacking adaptive immunity (red) are more immunogenic (were not previously eliminated/edited) and only 50% were able to implant in WT recipients. (Adapted from Schreiber et al. [2])

Schreiber and colleagues developed the concept of "cancer immunoediting", a dynamic process composed by three phases [12]:

1. Elimination – in which transformed cells are killed by the action of innate and adaptive immunity.
2. Equilibrium – a state of equilibrium between immune and tumor cells.
3. Evasion/escape – which concludes with the appearance of clinically detectable tumors.

During cancer immunoediting, the host innate and adaptive immune system interact dynamically with the tumor, determining its progression. In this dynamic process, some tumor cells variants have the capacity to evade and/or suppress the immune system and in this way, are immunologically sculped by Darwinian selection, expanding in an uncontrolled manner in the immunocompetent host [12].

In this chapter we will discuss some of the known molecular mechanisms involved in this process of **immune evasion** but also how cancer cells can **hijack the immune system** to help tumor development and therefore explain why in some contexts the immune system plays a positive role in tumor development. However, before we enter into the molecular details, we need to take a detour and look at the components of the tumor microenvironment and also into the basics of immunology.

7.1.3 The Tumor Microenvironment (TME)

Tumors have been recognized as complex entities, where during the course of tumorigenesis they build their own microenvironment, which can contribute to tumorigenesis *per se* (Fig. 7.2).

More than just cancer cells, the cellular TME can be composed by (Fig. 7.2) [14, 15]:

- Cancer-associated fibroblasts (CAFs)
- Endothelial cells (ECs)
- Pericytes (PCs), that surround ECs

and a variety of immune-related cells types, all derived from hematopoiesis:

- Myeloid lineage
  - Macrophages – tumor associated macrophages (TAMs)
  - Neutrophils – tumor associated neutrophils (TANs)
  - Mast cells (Mcs) – granulocytes rich in histamine and heparin
  - Dendritic cells (DCs)
  - Myeloid – derived suppressor cells (MDSCs)
- Lymphoid lineage:
  - T cells, including $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ T helper cells (Th), regulatory T cells (T-Regs) and γδ T cells
  - B cells
  - Natural killer cells (NK)

7.1.4 Immunology in a Very Small Nutshell

As evident by this list of possible TME cellular components, the immune system is highly represented in the tumor ecosystem. As referred before, the immune system can be divided

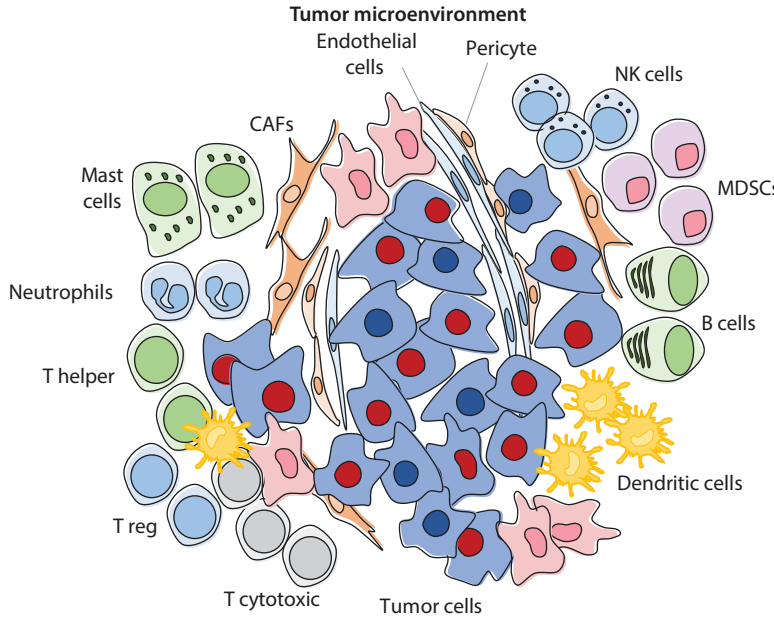

Fig. 7.2 Cellular components of the tumor microenvironment. The TME mainly includes CAFs, endothelial cells and pericytes as well as many bone marrow-derived cells as immune cells from myeloid and lymphoid origin, which can be present in different stages of their differentiation state

in two categories: the innate and the adaptive immunity which dynamically interact to defend the host (Fig. 7.3).

7.1.4.1 Innate Immunity

Innate immunity is considered the basic defense mechanism, acting as a first-line of response to infection and disease and is not specific for a given pathogen or antigen (Ag). However, innate immunity is essential to call in the highly specific adaptive response allowing both armies to work together to eliminate the threats [16, 17].

The innate arm includes:

- The **complement system** – consists of ~30 interacting soluble inactive-proteins produced in the liver that go into circulation and can get activated by three types of pathways: classical pathway (antibodies), lectin pathway (lectins) and alternative pathways. Once activated, all pathways converge in the activation of the potent anaphylatoxins C3a and C5a and in the formation of the membrane attack complex – MAC (composed by C5b, C6, C7, C8 plus several C9) – a pore complex responsible for cell and pathogen lysis. During this process several small peptides are generated by cleavage, recruiting immune cells to assist the fight.
- **Macrophages** and **granulocytes** (ex. neutrophils) – are phagocyte cells which are able to engulf and kill invading pathogens by a combination of strategies involving degrading enzymes (lysozymes), antimicrobial peptides and oxygen-derived toxic molecules (superoxide, oxygen peroxide and hydroxyl radicals). In addition, they express Pattern Recognition Receptors (PRRs) that bind specific Pathogen-Associated Molecular patterns (PAMPs) and Danger-Associated Molecular patterns (DAMPs). Activation of PPRs triggers the inflammatory response (secretion of cytokines, chemokines, prostaglandins, NF-Kβ signaling, interferon response).

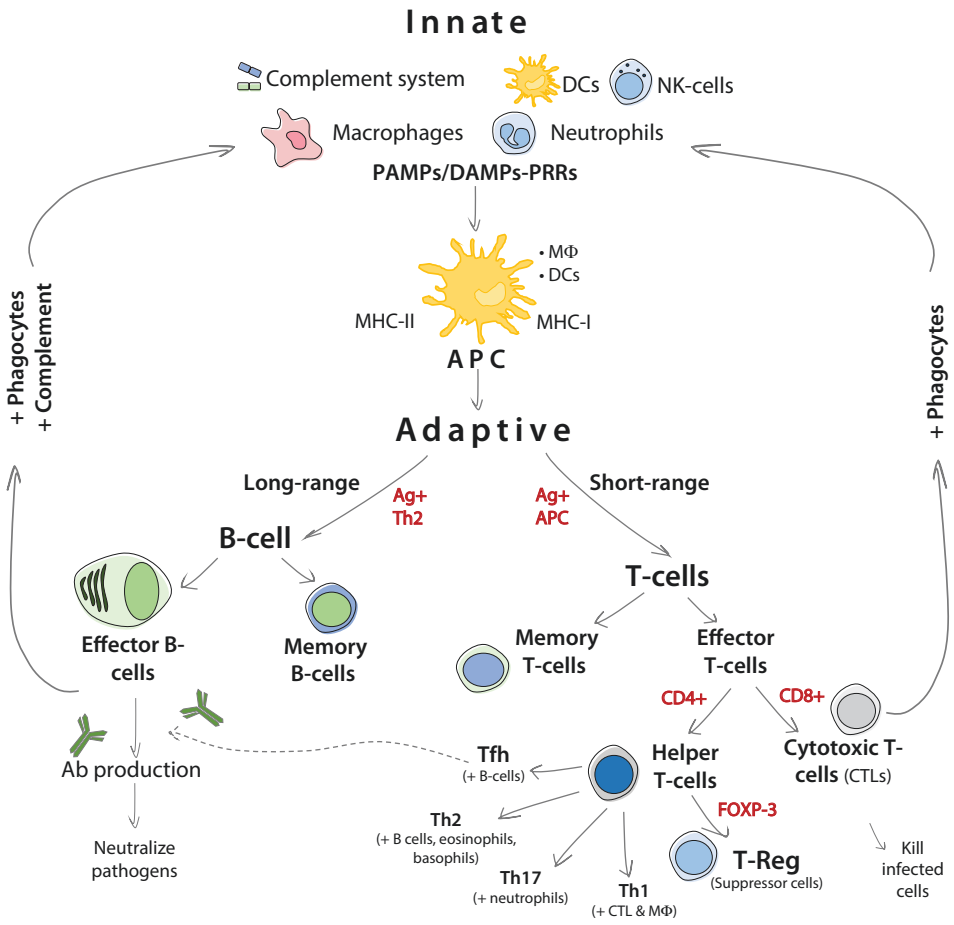

Fig. 7.3 Immunology in a very small nutshell. The immune system comprises the innate and adaptive arms. Innate immune mechanisms are the first line of defense and is not specific. Innate responses include the action of soluble factors (complement, chemokines and cytokines) as well as activities mediated by cellular components, mainly myeloid cells (neutrophils, macrophages, etc…) as well as NK and γδ T cells (lymphoid lineage). These innate cells express Pattern Recognition Receptors (PRRs), which are an alarm system that recognizes Pathogen-Associated Molecular Patterns (PAMPs – present in microbial pathogens) and Damage-Associated Molecular Patterns (DAMPs – molecules that are released/expressed by damaged or dying cells). Activation of PRRs leads to release of inflammatory cytokines and activation of the complement system. Antigen Presenting Cells (APCs-DC and macrophages mainly), after phagocytosing the pathogens/debris, present antigens through MHC molecules to the adaptive T-cells, constituting a direct link between innate and adaptive immunity. The cells of adaptive immunity, B and T lymphocytes express specific receptors. T cells recognize the antigens through the TCRs and MHC bound peptides at the surface of APCs. MHC class I is presented to CTLs ($CD8^+$) that kill infected cells, and MHC class II to T helper cells ($CD4^+$). $CD4^+$ helper cells differentiate in secondary lymphoid tissues into T-Reg, Th1, Th2, Th17 and Tfh (follicular T helper cells). Th1 assist activation of CTLs and macrophages while Tfh help in the differentiation of B cells. B cells proliferate and may differentiate into effector cells, termed plasma cells (upon release of cytokines by Tfh cells), which are short-lived cells that secrete specific antibodies (Ab) against pathogens. Others may differentiate into memory cells that help mount an effective response in a second exposure to the antigen. The net result of activation of antibodies and effector T cells ends with a positive feedback of activation of the innate immune cells (phagocytosis). T cells secreted chemokines and cytokines and recognition of the antigen bound to the antibodies leads to activation of the complement, or the direct activation of macrophages through recognition of the Fc portion of the Ab by the Fc receptor present in macrophages

- **Dendritic cells (DCs)** – are the most important antigen presenting cells (APC) and the main link between innate and adaptive immunity. DCs are specialized at presenting antigens, small peptides and proteins, to activate naïve T cells (adaptive immunity), and therefore are also known as **professional Antigen Presenting Cells** (**APCs**). The activated dendritic cells cleave proteins of the "pathogen" into small peptides, that then bind to newly synthesized MHC proteins, which carry the fragments to the cell surface. Activated/matured DCs express co-stimulatory markers (CD40, CD80, CD83, CD86) and MHC class I and II molecules. Then they migrate to lymph nodes where they present the peptide-MHC complexes to T-cells of the adaptive arm, starting the adaptive response.
- **Natural killer cells (NKs)** – Although belonging to the lymphoid lineage, they mediate innate immune responses. NK cells patrol the body and are able to kill tumor cells and virus infected cells by inducing apoptosis. This apoptosis can be mediated by granzymes and perforin or via expression of Fas ligand and TRAIL (TNF-related apoptosis-inducing ligand). However, their killing activity is dependent on the **balance between activating and inhibitory receptors** on the NK cell surface. These inhibitory receptors bind MHC class I molecules – explaining why **NK cells preferentially kill cells that express low levels of MHC class I** and do not kill normal healthy cells (that express MHC-I). Downregulation of MHC-class I is a strategy employed by virus to avoid being detected by T cells. Nevertheless, we will see that cancer cells also use this strategy. NK cells seem to have evolved as a response to this adaptation – so virus infected cells and tumor cells cannot hide from the NK cells! Moreover, NK cells secrete cytokines such as IFN-γ and TNF-α, which act on other immune cells like macrophages and dendritic cells to enhance the immune response.

7.1.4.2 Adaptive Immunity

Adaptive immunity is a defense mechanism that requires a sophisticated gene recombination strategy to generate antigen specific receptors (TCRs and BCRs) and antibodies that identify **specific targets** and **remember them** (immunological memory), generating a **very precise** way to **recognize** and **kill foreign threats**, even if they come back later in time. This recombination strategy allows adaptive immunity to respond to millions of different foreign antigens in a highly specific manner [17]. It is amazing!

Adaptive immunity can be subdivided in two classes of immune responses:

- **Antibody responses** (humoral immunity) – mediated by **B cells** that are activated to secrete **antibodies** which circulate in the blood stream and therefore act over **long distances.** Antibodies neutralize pathogens (blocking their binding to specific cell receptors) or by marking pathogens to be dwelt by innate immunity through phagocytes (that recognize these Ab through Fc receptors), NK cells or the complement system.
- **T-cell mediated responses** (cellular immunity) – in general may require cell-cell contact and therefore act over **short distances** at the lymphoid organ (activating B cells) or at the site of infection. **T cells** recognize foreign Ag bound to MHC molecules on the surface of the APC, such as dendritic cells, macrophages or B cells. T cells act either by directly killing the infected cells (CTLs) or by stimulating phagocytes or B cells to help fight infection (Th).

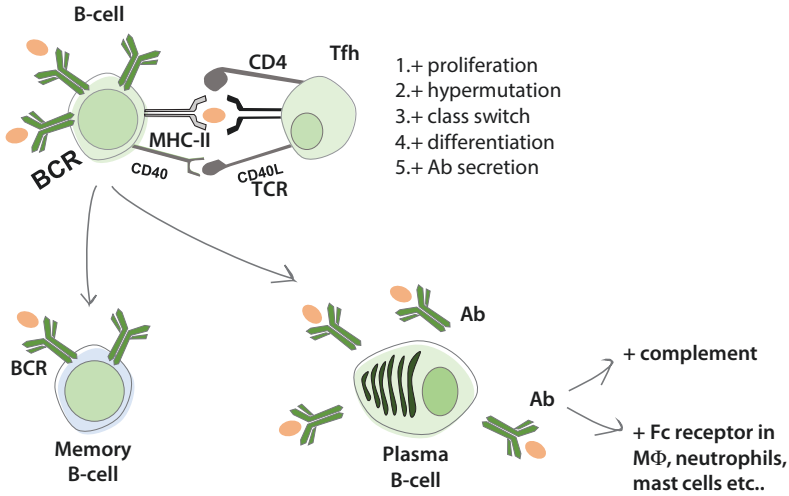

Fig. 7.4 B cell differentiation in the lymphoid follicles. B cells are produced and undergo maturation in the bone marrow (BM) expressing BCR on their surface. After they leave the BM, they circulate through blood and peripheral lymphoid organs. If they recognize an antigen, they will endocytose both BCR and the antigen. Then the antigen will be processed and presented as a small peptide through MHC-II to follicular helper T-cells (Thf) (co-stimulated by CD40/CD40L). Tfh in turn, promote B-cell proliferation, somatic hypermutation, class switch recombination, differentiation into memory B cells and differentiation into plasma cells that abundantly secrete antibodies. The order of these processes does not occur necessary in sequence and differentiation/maturation of B-cells in germinal centers is still under intense investigation

B Cells

B cells are the cells that produce and secrete antibodies. However, B cells first synthesize the antibodies which are immunoglobulins (Igs) in a membrane bound form – the B cell receptors (BCRs). These BCRs are produced in billions of arrangements, each with a different amino-acid sequence, with a unique binding site, by a process of somatic recombination called V(D)J recombination [18]. B cells only start secreting antibodies after antigen recognition. Antigen binding to the BCR together with co-stimulatory factors, provided by follicular helper T cells (Tfh), activate B cells to proliferate and differentiate into either memory B cells (long-lived) or antibody-secreting effector cells, which are called plasma cells (short-lived cells) [17, 19] (Fig. 7.4).

Box 7.1
V(D)J recombination – somatic mechanism of DNA recombination to generate diversity of antigen receptor genes during B and T lymphocyte development. B cells generate the innumerable antibodies and T cells generate the TCRs. It is directed by two enzymes: the Recombination Activating Gene 1 (RAG1) and RAG2 that bind and cleave genomic DNA at specific recombination signal sequences next to antigen receptor gene segments [20].
Somatic hypermutation (SHM) – recombination process that generates diversity of the variable regions of the immunoglobulin genes during B cell differentiation/maturation being fundamental for the development of high-affinity antibodies [21].
Class-switch recombination (CSR). Another recombinational process that replaces the immunoglobulin heavy chain constant region Cμ (which encodes the Fc portion of IgM) for that of the constant region of IgG, IgA or IgE, (Cγ, Cα or Cε respectively) [22].

T Cells

All T cells express T-cell receptors (TCRs), which are cell surface antigen receptors, encoded by genes that are assembled by multiple gene segments during T cell development and also generated by a V(D)J genetic mechanism of recombination.

Besides memory T cells, there are four main classes of effector T cells:

- **Cytotoxic T-cells** (**CTLs**) are characterized by expressing αβTCRs and CD8 co-receptors and kill infected cells by inducing cells to undergo apoptosis. CTLs activate apoptosis either by activating the Fas pathway or through cytotoxic proteins (granzymes and perforin) that lead to the activation of the caspases cascade.
- **Helper T-cells** (**Th** cells) are characterized by expressing αβTCRs and CD4 co-receptors and are responsible for:
  1. Secreting cytokines
  2. Activating $CD8^+$ T cells
  3. Activating B cells to proliferate and differentiate to become Ab secretory, start hypermutation and class switch
  4. Activating macrophages, granulocytes and effector cells
- **Regulatory T-cells** (**T-Regs**) are characterized by expressing CD4 co-receptors and the master transcription factor FOXP3. T-Regs suppress the activation, development or function of most other types of immune cells by secretion of immune suppressive cytokines like TGFß and IL10 and inhibitory proteins like CTLA-4 and PD-1.
- **γδ T-cells** are characterized by expressing γδTCRs but with reduced diversity, do not express CD4 and CD8 co-receptors and are activated in an MHC-independent manner. Upon activation produce cytokines, chemokines, induce cytolysis (due to secretion of perforin, granzymes and TRAIL) and interact with other immune cells. Similar to NK, exhibit features of innate and adaptive immune system and are abundant in epithelial barriers like in the gut mucosa, skin and uterus. Therefore, are referred as innate lymphoid cells [23].

T and **B** lymphocytes **continuously circulate** between the different peripheral lymphoid organs **via** the **lymph** and **blood stream** and only when lymphocytes expressing their unique cell-surface antigen receptors (BCR and TCR) encounter their matching antigen (presented in the peripheral lymphoid organs), they engage proliferation and differentiation into **effector** and **memory** cells.

As referred before TCRs recognize peptide fragments displayed in MHC proteins on the surface of APCs. APCs present Ag to cytotoxic T cells through MHC-class I molecules and CD8 co-receptors, whereas helper T cells receive Ag by MHC-class II molecules and activation is mediated by CD4 co-receptors. Besides the Ag binding, lymphocytes need co-stimulation by other molecules: B cells depend on Tfh cells to provide co-stimulatory molecules such as CD40L and T cells depend on co-stimulatory reactions between the CD28 receptor and the B7 molecules expressed at surface of APC (Fig. 7.5) [24].

During maturation of B and T cells, mechanisms to ensure that B and T cells do not react against the host's own cells and molecules had to evolve – a process called **immunological self-tolerance**. In other words, during the maturation process, cells with BCRs and TCRs that recognize self, are eliminated or diverted to regulatory pathways by several mechanisms. Over-activation of the immune response also has to be regulated and several checkpoints and negative feedback loops ensure that the massive cellular expansion and

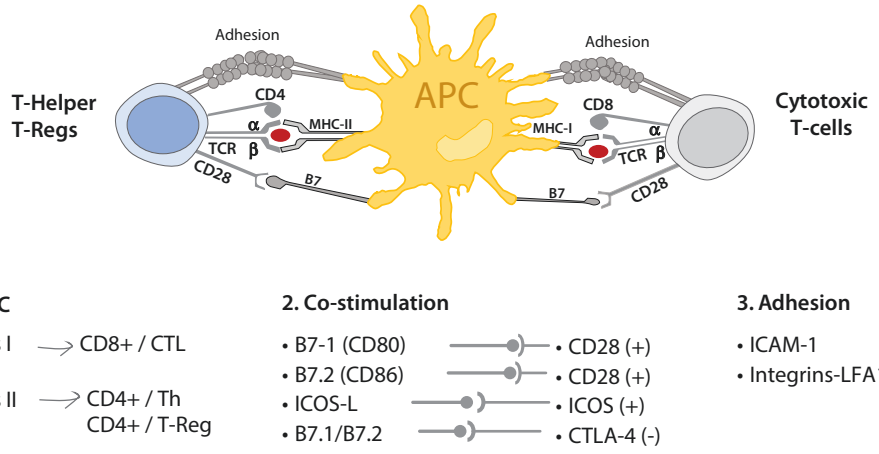

Fig. 7.5 Interactions between APCs and T cells. APCs present Ag through MHC molecules that will bind to the TCR of T lymphocytes. CTLs ($CD8^+$ cells) recognize an Ag bound to MHC class I whereas Th cells (CD4+) associates with MHC class II molecules. Co-stimulatory molecules such as B7.1 (CD80) and B7.2 (CD86) are present on APC's which interact with CD28 on T-cells to mount an immunological response. Also for a full functional immunological synapse APCs must bind T-lymphocytes through adhesion molecules such as intracellular adhesion molecule (ICAM) and integrins

cytokine storm that accompany the immune response do not overwhelm the host and do not incorrectly destroy healthy cells (autoimmune reaction)!

We will see straightaway how cancer cells exploit exactly these defense mechanisms, that dampen the immune response (to avoid autoimmunity) to their own benefit. Tumor cells can evade/escape immunity through several mechanisms; they can become invisible, kill and suppress the immune system or even sabotage and hijack the immune cells to work for them to fuel tumorigenesis instead of fighting it! This is one of the reasons why it is so difficult to fight this devastating disease – the police is corrupted!

7.2 Immune Evasion Mechanisms

Evasion – "the act of physically escaping from something, an opponent or a pursuer or an unpleasant situation". During tumor immunoediting, high immunogenic (highly reactive) clones get eliminated, while low immunogenic clones remain (i.e., get selected because they are the ones that are not eradicated). This selection allows the survival of these tumor cell variants in an immunologically unrestricted manner [25]. Many mechanisms have been reported that enable cells to pass undetected and evade the immune system (Fig. 7.6). Keep in mind that the examples given below are not an exhaustive review.

7.2.1 Mechanisms to Become "Invisible" – The "Harry Potter" Invisibility Cloak

- Downregulation of the MHC class I, through alterations in the expression of MHC molecules or in the processing or presentation of tumor-associated antigens

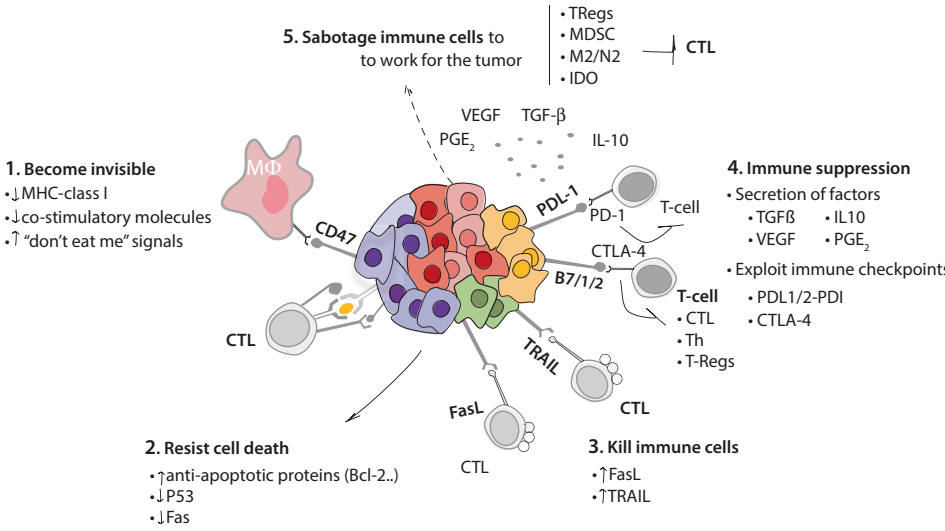

Fig. 7.6 Tumor escape mechanisms. An illustration of different key factors governing tumor immune evasion. (1) Tumor escape can occur through cell-contact-dependent mechanisms, in which tumor cells have acquired strategies to become undetectable by the immune system. (2) Tumor cells develop apoptotic resistance. (3) Capacity to kill immune cells via TRAIL or FasL upregulation that will lead to activation of caspase pathway in immune cells. (4) Tumor cells may present aberrant expression of cell-surface ligands that downregulate T-cell activity, such as PD-L1/CTLA-4. In addition, tumor cells also employ cell contact-independent mechanisms like secretion of tumor-derived factors like VEGF, IL10, ROS, IDO, PGE2 and TGF-β. (5) Finally, tumor cells can also manipulate various myeloid and lymphoid cells to contribute to tumor growth

(TAA), allows tumor cells to become invisible for CTLs and can only be recognized by NK cells [26].

- Expression of "**don't eat me signals**" like CD47, a cell surface molecule that inhibits the phagocytic activity of macrophages and DCs. CD47 molecules seem to function as a negative innate immune checkpoint and a marker of self to ensure that healthy cells are not inappropriately phagocytosed during inflammatory conditions. Once more, tumors exploit this mechanism for their own benefit avoiding being phagocytosed by macrophages and DCs [26].
- Lack/reduction of expression of co-stimulatory molecules necessary for proficient T-cell activation (B7 family) [26].

7.2.2 Mechanisms to Resist Cell Death (See Chap. 5)

- Upregulation of anti-apoptotic proteins such as **Bcl2** and **Bcl-xl**
- Downregulation or loss of pro-apoptotic factors such as **P53** and **Fas receptor**. Fas-mediated killing is an important defense mechanism during the effector phase of the immune reaction. Thus tumor cells that express high levels of Fas receptor will get killed, remaining the Fas^{low} resistant variants – this is an example of the sculpting action of the immune system [27].

7.2.3 Mechanisms to Suppress the Immune System

7.2.3.1 Tumor Cells Secrete Immunosupressive Factors

Tumor cells **secrete factors** that have multiple repressive effects on the immune system affecting all sort of cells of the immune system:

- **Transforming growth factor-β (TGFβ) has been shown to** [28]:
  - Inhibit $CD8^+$ CTL clonal expansion and inhibit transcription of key genes such as perforin, granzymes, blocking the "cytotoxic program"
  - Induce FOXP3-T-Reg cell differentiation
  - Inhibit B-cell proliferation and Ab secretion
  - Inhibit proliferation and function of NK cells
  - Promote pro-tumoral macrophages and neutrophils and mediate the immune suppression function of MDSCs (see ahead).
- **Prostaglandins (PGE2)** have multiple and paradoxical effects. It is critical to start the inflammatory response, promoting local vessel dilatation and attraction/activation of neutrophils, macrophages, and mast cells. However, it also shuts down this early response by directly suppressing the production of several pro-inflammatory cytokines (IL2, IL12, IL15 for example) and promoting the production of suppressive IL10, leading to a general immune suppression affecting both innate and adaptive immunity at multiple molecular and cellular levels. PGE2 suppresses the innate cells (NK, macrophages, neutrophils to perform their function, i.e., call in CTLs) and suppresses adaptive immunity (inhibit activation and expansion of CTL and inhibit Th1 favoring Th2), but also promotes the development and activity of suppressive cells (T-Regs, MDSC and pro-tumoral macrophages-M2-like) [29].
- **Interleukin-10 (IL10)** is an anti-inflammatory cytokine with also paradoxical roles in cancer. Some studies point to an immune suppressive pro-tumoral role whereas others show exactly the opposite. IL10 can directly modulate innate and adaptive immunity. IL10 is thought to inhibit MHC class I on tumor cells and MHC class II and co-stimulatory molecules B7-1/B7-2 expression on APC (monocytes/macrophages) reducing production of pro-inflammatory cytokines (IL1α and β, IL6, IL12, IL16, and TNF-α) and chemokines. IL10 can also act directly on helper T-cells $CD4^+$, inhibiting proliferation and production of pro-inflammatory cytokines (IL2, IFN-γ, IL4, IL5 and TNF-α). These direct effects on monocytes/macrophages and Th cells are thought to lead to an overall reduction of T cell activation and differentiation in the lymph nodes and decrease of pro-inflammatory responses in tissues. However, IL10-deficient mice and humans develop inflammatory bowel disease (IBD) and are more susceptible to cancer. Moreover, tumor cells overexpressing IL10 are rejected! Also, treatment with IL10 can induce tumor rejection. Therefore, although in many reviews you will find that IL10 is a immunosuppressive molecule that helps tumors escape the immune system, you need to be cautious because there are conflicting reports showing that IL10 can actually activate T-cells [30–32]. Definitely we need to learn more!
- **VEGF** inhibits pro-inflammatory reactions within the tumor microenvironment by promoting the expansion of MDSC and impairing DC maturation and activation [25].

7.2.3.2 Exploit Immune Checkpoints of Self-Tolerance

As referred before the immune system has evolved mechanisms to control overactivation, preventing for instance auto-immune diseases – these are called mechanisms of self-tolerance i.e. mechanisms that dampen the immune response – the immune-checkpoint pathways. It is now clear that tumors co-opt these pathways as a major mechanism of immune resistance, particularly against T cells.

- **Programmed Death receptor-1 (PD-1)** is found expressed on T, B and myeloid cells. PD-1 receptor interacts with its ligands PD-L1 (also termed B7-H1) or PD-L2 and leads to blockage of T cell proliferation and cytokine production. The inhibitory effects of PD-1 were initially observed when PD-1-deficient mice developed autoimmune diseases. PD-L1 is strongly expressed on a variety of tumors cells as well as DC's and macrophages present on the TME and inversely correlates with patient prognosis [33].
- **Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4)** is constitutively expressed in T-Regs. CTLA-4 is induced after T cell activation as a negative feedback mechanism that competes with CD28 for B7 ligands inhibiting T cell proliferation and IL2 secretion [34].
- **Indoleamine 2,3-dioxygenase (IDO)** is a heme-containing enzyme that catalyzes the first and rate-limiting step in the kynurenine pathway (NAD^+ production from tryptophan). IDO has been shown to be expressed in tumor cells, endothelial or innate immune cells such as DC, MDSCs and macrophages of the TME [35], generating **2 major effects:**
  - Production of soluble factors (kynurenine and downstream metabolites) that bind and activate the aryl hydrocarbon receptor (AhR), which in turn activate T-Reg differentiation and push dendritic cells (DCs) and macrophages to an immunosuppressive phenotype [7]. Thus, when IDO is active, APCs which in normal conditions would produce inflammatory cytokines such as IL12, instead express IL10 and TGFβ inhibitory cytokines [35].
  - IDO can also create a local suppression of effector T cells by metabolic depletion of tryptophan and production of the catabolite kynurenine [7]. Thus, IDO upregulation can alter the phenotype of the APC itself, activate T-Regs, and induce the production of suppressive cytokines, changing the whole local ecosystem from immunogenic to tolerogenic. Overall the main mechanism of IDO pathway mediated immune suppression is to reduce T cell infiltration in TME.
- **Depletion of intratumoral T cells - tumor cells kill immune cells!** Tumors can induce T cell death by:
  - Upregulation of ***Trail* expression** which binds TRAIL-receptor (TRAIL-R1) positive CTLs cells, leading to their apoptosis [36].
  - Upregulation of **Fas ligand** (FasL) that expressed or released by tumor cells in tumor-derived exossomes, activating Fas in T cells inducing also their apoptosis [37].

7.2.4 Immune "Sabotage" and "Hijacking" Mechanisms

Tumor cells can manipulate and hijack the cells themselves (lymphoid or myeloid immune cell populations) to work for them by sabotaging normal defense mechanisms of immune tolerance i.e., inducing immune suppressive cell phenotypes that then contribute to tumor escape and progression [15]. In both mice and humans, a number of immune suppressive

cell sub-types have been identified, including T-Regs, myeloid-derived suppressor cells (MDSCs), pro-tumoral macrophage (M2-like) and neutrophils (N2-like).

7.2.4.1 Regulatory T Cells (T-Regs)

T-Reg cells occur naturally and act to inhibit autoimmune responses but can also suppress the generation of tumor-specific T-cell responses, possibly through similar mechanisms [26]. Increased numbers of T-Reg cells have been found in the peripheral blood of different cancer types [38]. T-Regs can suppress effector T cells and thus prevent the development of anti-tumor immunity by four basic "modes of action" [26]:

- Expression of inhibitory cytokines, like TGF-β, IL10 and IL35
- Directly kill CTLs by expression and release of granzymes and perforin
- Indirectly kill CTLs by cytokine deprivation: by expressing high affinity IL2-Receptor α (CD25), T-Regs scavenge IL2, decreasing its levels in the TME leads to CTLs death
- Blocking DCs maturation or function

7.2.4.2 Myeloid Derived Suppressor Cells (MDSC)

Tumor progression evolves with the accumulation of inhibitory myeloid cells, designated as Myeloid Derived Suppressor Cells (MDSC). MDSCs are expanded in several pathological conditions, not only in cancer. MDSCs are not a defined subset of myeloid cells, but a heterogeneous population of myeloid progenitor cells and immature myeloid cells (IMCs) that have been blocked from fully differentiating into mature cells. In the steady healthy state, IMCs lack suppressive activity and are present exclusively in the bone marrow and found in secondary lymphoid organs only in pathological conditions. When activated, in these pathological conditions, MDSC can suppress anti-tumoral immune functions [39].

It has been shown that several tumor-derived factors can induce expansion and activation of these MDSC, which migrate from the bone marrow to the lymphoid organs and to the tumor site.

These expansion factors include: PGE2, granulocyte macrophage CSF factor (GM-CSF), M-CSF, stem-cell factor (SCF), vascular endothelial growth factor (VEGF), IL10 and IL6 [40]. Most of these molecules activate signaling pathways that activate Janus kinase (JAK) family members and STAT3 (see Chap. ► 3) that promote the expansion of the MDSCs. For activation of the immune suppressive activity it has been shown the involvement of other signaling molecules such as: IFN-γ, ligands for Toll-like receptors (TLRs), IL4, IL13 and TGFβ that ultimately lead to activation of STAT1 and NFKβ [41].

A number of studies have implicated these MDSCs in immunosuppression, mainly through [25, 39, 41]:

- Secretion of immune suppressive cytokines **TGFβ and IL10**
- As a major source of **PGE2** (they highly express cyclooxygenase 2 – COX-2)
- Production of **arginase 1**, which leads to arginine depletion, inhibiting T-cell proliferation
- Reduction of local **tryptophan** levels due to the activity of IDO
- Production of inducible Nitric Oxide Synthase (**iNOS**) that results in the generation of Reactive Oxygen Species (**ROS**) including nitric oxide (NO) and peroxynitrite ($ONOO^-$), which ultimately alter T-cell signaling, activation and survival (TCR nitrosylation)
- Expression of inhibitory **PDL-1** in MDSC in the TME
- **T-Reg cells** induction (lymphoid organ) and attraction to the TME

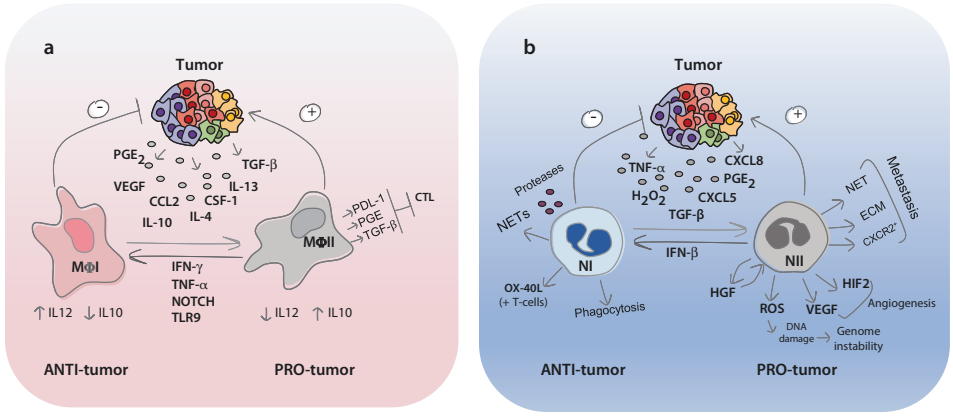

Fig. 7.7 Dynamic states of anti- and pro- tumoral macrophages and neutrophils. **a**. Anti-tumor macrophages (M1-like) and pro-tumoral macrophages (M2-like) phenotypes and signaling involved. IFN-γ and TNF-α have been reported to induce activation of the M1 phenotype. M1-like macrophages produce high levels of IL12 and low levels of IL10 cytokines and can contribute to tumor control. In contrast, the M2-like phenotype has an $IL12^{low}$, $IL10^{high}$ cytokine profile and a pro-tumorigenic role. IL4, IL13, TGFβ, PGE2, VEGF, CCL2, and CSF1 can induce M2-like macrophages. **b** Anti-tumor (N1-like) and pro-tumoral (N2-like) phenotypes and signaling molecules involved in neutrophil behavior. Besides being "killing" machines (N1), neutrophils can revert to a pro-tumorigenic role and impact on angiogenesis (activation of VEGF) and metastasis by remodeling the extracellular matrix via matrix metalloproteinases (MMPs) for example [43, 45, 46]

MDSCs can be found in the lymphoid organs and in the TME, where they engage on different mechanisms of immunosuppression. In peripheral lymphoid organs, immunosuppression by MDSC is contact dependent, mainly antigen-specific whereas in TME suppression is more potent and non-antigen-specific, nevertheless both rely on activation of the two key enzymes: **arginase1** and **iNOS** [39].

7.2.4.3 M2-Like Macrophages and N2-Like Neutrophils

Within the myeloid-derived cell compartment, the Tumor Associated Macrophages (TAMs) and Neutrophils (TAN) can either adopt an anti-(M1/N1-like) or pro-tumoral (M2/N2-like) phenotype (Fig. 7.7), which can be reverted and modulated by tumor-derived signals. We would like to highlight that the terms M1/N1 M2/N2 is for the sake of simplicity, since nowadays researchers are realizing that there are many different subtypes and "states" of these cells and that these are highly dynamic. **IL10**, **IL4**, **IL13**, **CCL2**, and **CSF-1** secreted by tumor cells can drive TAMs into pro-tumoral M2-like macrophages (Fig. 7.7a). M2 exert a pro-tumoral role through several mechanisms of immunosuppression (PDL-1, PGE2, TGF-β, IL10, CCL2, etc), that can block anti-tumor T cell activity and interferon type I responses (IFN) [42–45]. In contrast, N2-like neutrophils are more involved in promoting angiogenesis and metastasis than immune suppression *per se* but nevertheless also contribute to tumorigenesis [43, 46].

In summary, it appears evident that tumors develop a parasitic relationship with its host to take control of both myeloid and lymphoid compartments to further prolong tumor growth and progression. Many times corrupting the "police force", by turning the good cops (M1/N1-like) into bad cops (M2/N2-like) and recruting T-Regs and MDSC that block the action of the CTLs. It is almost like promoting a civil war between immune cells, generating an immunosuppressive environment that enables the uncontrolled tumor growth.

7.3 Immunotherapy

The great advances in understanding this dynamic cross-talk between tumor cells and immunity has led to the emergence of immunotherapy as a transformative approach to cancer treatment. Immunotherapy aims at unleashing the patient's own defense mechanisms to fight cancer and is giving hope to the most mortal types of cancer like melanoma and renal cell carcinoma.

To date, cancer therapies such as conventional chemo- and radiotherapy fail to obtain long term responses, probably due to the escape of resistant sub-clones. So, if we are able to block the immunosuppressive mechanisms and turn tumor cells visible for the immune system, the innate and adaptive armies will be able to find these small hidden clones, no matter where they are and eradicate the disease before it reaches vital organs – or even after dissemination – this is hope for cure…. Nevertheless, unleashing the immune system can also have adverse effects similar to auto-immunity – there is a delicate balance between activation and inhibition of immunity to fight cancer but at the same time do no harm to the normal cells….

There are several approaches to boost the immune system to fight cancer, which we describe below:

7.3.1 Administration of Cytokines

Administration of cytokines like Interleukin-2 (IL2) and IFN-α, boost the activity of the anti-tumor immune response. **IL2** administration was the first method to show that immunotherapy – exploiting the body's own immune system to kill cancer – could actually work (if we don't take in account Coley's early work). After 66 failed attempts (different patients), Dr. Steven Rosenberg and colleagues were finally able to induce a complete remission of a metastatic melanoma patient [47]. This was the first cancer patient to respond to IL2 infusion and to demonstrate that modulation of the immune system, by stimulation of T cells, could mediate complete destruction of cancer. From then on many melanoma and renal-cell carcinoma patients were treated with IL2 with an overall response rate of ~15% [47]. Although, other types of cancer do not respond to IL2 treatment, IL2 had a profound impact on the development of cancer immunotherapy. IL2 allowed the *in vitro* expansion of T-cells, permitting the development of another type of immunotherapy: adoptive cell therapy also pioneered by Rosenberg and colleagues [47, 48].

7.3.2 Adoptive Cell Therapy

Adoptive cell therapy (ACT) involves isolation of tumor-specific T cells from patients and their expansion *ex vivo* to increase the number of these cells in order to infuse them back into patients to fight cancer [48]. IL2 is used not only to grow T cells *in vitro* but is also administered together with the infused cells to support their growth and survival in patients [47].

Exome sequencing of tumor mutations has showed that Tumor-Infiltrating Lymphocytes (TILs) are able to recognize unique tumor mutations – named neo-antigens or Tumor-Associated Antigens (TAA). This explains why tumors with high mutational

burden, like melanoma, smoking-induced lung cancer or tumors with mismatch repair mutations may have a better chance of response to immunotherapy [47], i.e. these tumors are more immunogenic! The larger the number of mutations, the higher is the probability to generate neo-antigens that will exhibit a strong binding to a MHC molecule for tumor recognition! So, ACT can be coupled with tumor sequencing to identify the tumor neo-antigens to then engineer or select T-cells capable of targeting more specifically and efficiently the tumor cells of that particular patient.

There are numerous forms of adoptive T cell therapy used for cancer treatment:

- Expansion of **TILs**
- **CAR-T** cells – T cells engineered to express Chimeric Antigen Receptors (CARs) that recognize cancer-specific antigens, rendering them more efficient in recognizing and attacking specifically tumor cells. The process of generating CAR-T cells involves extracting patient's T cells, transfecting them with a gene for a **chimeric antigen receptor** and reinfuse them back into the patient. In 2017, CAR T-cell therapies targeted to CD19 were approved for children with Acute Lymphoblastic Leukemia (ALL) and for adults with advanced B-cell lymphomas [49, 50].

7.3.3 Immune Checkpoint Therapies

This strategy aims at removing inhibitory pathways that block anti-tumor T cell responses in the tumor microenvironment (Fig. 7.8). These therapies use monoclonal antibodies against specific molecules that modulate the immune repressive mechanisms, that in normal conditions refrain the immune system to avoid autoimmunity [7].

As mentioned earlier, T cell activation depends, not only on direct contact with APCs, which present Ags through MHC molecules to the corresponding TCR, but also depends on the interaction of co-stimulatory molecules such as CD28 and B7 that are mandatory for full activation (Fig. 7.6). However, to avoid catastrophic over activation of the immune system, T cell activation is highly regulated and subjected to feedback regulation by inhibitory checkpoints and T-Regs.

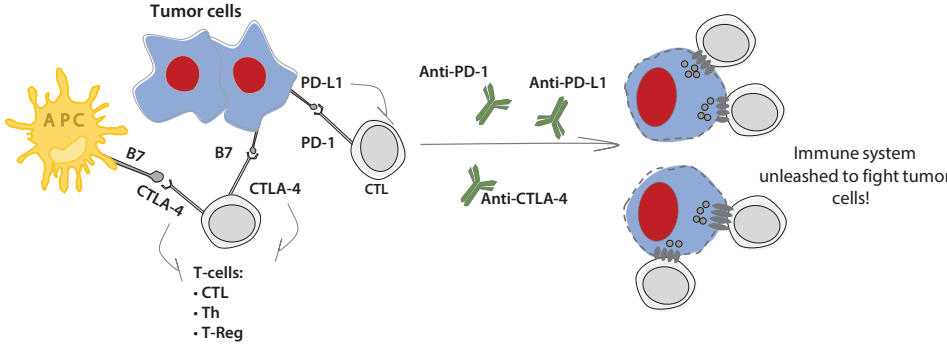

Fig. 7.8 Mechanism of action of immune checkpoint inhibitors. When activated T-cells encounter a PD-L1-expressing tumor cell, PD-1 receptor is activated in T-cells leading to T-cell exhaustion. CTLA-4 competes with CD28 for B7 ligands (CD80/CD86) decreasing T cell activity. Therefore, blocking PD-1/PD-L1 and CTLA-4 activity with immune checkpoint antibodies blocks immune suppression and stimulates effector T cells, boosting anti-tumor responses

Seminal work by James Allison, Padmanee Sharma and colleagues showed that CTLA-4, which is constitutively expressed in T-Regs and induced after T cell activation, competes with CD28 for B7 ligands with much more affinity, inhibiting proliferation and IL2 secretion by T cells (abrogating its anti-tumor response). It was also shown that CTLA-4 blocking antibodies could treat tumors in immune competent animal models and later in clinical trials showed very promising results. In 2011, FDA (Food and Drug Administration) approved the first anti-CTLA-4 antibody – Ipilimumab – to treat metastatic melanoma [51, 52].

Another immune checkpoint molecule expressed by activated T cells to suppress activation is PD-1 (Programed Death receptor 1) and it has been shown that PD-L1 (ligand) expression can be exploited by many tumors to evade immune attack. Antibodies blocking the PD-1 and PD-L1 inhibitory axis can unleash activated tumor-reactive T cells and have very encouraging results [7, 51]. Anti-PD-1 (Nivolumab) and anti-PD-L1 (Pembrolizumab) were also recently FDA approved for metastatic melanoma and advanced/metastatic non-small cell lung cancer. The combination of anti-PD-1/PD-L1 with complementary checkpoint inhibitor CTLA-4 has also been shown to have promising results in many other types of cancer [51]. James Alison for CTLA-4, together with Tasuku Honjo for the PD-1/PDL-1 therapies, just got the *2018 Nobel Prize* in Physiology or Medicine!

In the clinic, the presence of TILs and PD-L1 expression correlates with patient survival/better prognosis. This hint, of a "hot" tumor microenvironment, indicates that the patient will benefit with either TILs or anti-PD-L1 therapy. In contrast, if the tumor microenvironment is "cold", anti-CTLA-4 should be administered to drive T cells into the tumor and induce PD-L1 expression, in order to be responsive to combinatorial therapy.

7.3.4 Combinatorial Immunotherapy

The efficiency of the immune checkpoint blockade with monoclonal antibodies in cancer treatment is remarkable and has durable effects. However, only a fraction of patients benefit from this therapy. Therapeutic intervention often fails because tumor cells are not immunogenic enough i.e. they do not express sufficient Ag to be recognized and presented to T cells or they may face other suppressive mechanisms present in the TME. To enhance and broaden the anti-tumor activity of immune checkpoint inhibition it is possible to combine other agents [7, 53]. For example:

- Chemotherapy or radiotherapy – have been shown to expose tumor antigens and therefore aid recognition of tumor cells by the activated T cells
- IDO inhibition – IDO when expressed in the TME either by tumor or host immune cells, leads to immunosuppression by increasing T-Regs and decreasing proliferation of effector T cells. Combination of IDO inhibition and immune checkpoint blockage are currently under clinical investigation.

7.3.5 Cancer Vaccines

Although most cancer vaccines are employed as therapeutic rather than preventive agents, there is one paradigm that revealed to be a huge achievement – the Human Papilloma Virus (HPV) vaccine that protects women against cervical cancer (ovarian). All other cancer vaccines, in general have a therapeutic action and involve administration of TAAs

in the form of either peptides, recombinant proteins, DNA or even whole cells to stimulate the immune system to attack cancer cells. The stimulation of immunity can be either direct, i.e. directly administrated to patients, or the tumor antigens can be presented to immune cells *ex vivo* (*in vitro*) to expand them and to then re-infuse the activated/selected cells into patients (Dendritic cell vaccines) [7, 26]. For now, in humans, the majority of vaccines are only being used in clinical trials.

Many more immunotherapies are being developed, some focusing also in the innate cell compartment. For example, the inhibition of CSF-1R (receptor of macrophage colony stimulating growth factor) reduces the frequencies of TAMs and increases IFN production, confining tumor progression. Additionally, this therapy can also synergize with anti-PD1 or anti-CTL4 antibodies [54]. Another strategy reported is the use of antibodies that block "don't eat me" signals, to unleash the phagocyte activity of macrophages [55].

In conclusion, this new approach to fight cancer using the patient's own immune system, just like Coley originally proposed, is giving hope to finally manage or even cure this shattering disease. However, not all patients respond, so there is still a long way to go in research to understand all the strategies cancer cells employ to avoid and suppress the immune system to make immunotherapy a reality for all patients.

Check out these movies:

- ▶ https://youtu.be/3hlGq-3F1uQ
- ▶ https://youtu.be/K09xzIQ8zsg

Take Home Message

- Immune system evolved to protect the host against diseases – innate and adaptive immunity work together to eliminate possible threats
- Immunosurveillance is the first step in preventing and fighting cancer
- Tumors are edited and immunologically sculpted over time leading to detectable cancers
- There is an active dialogue between cancer and immune cells in the tumor microenvironment that influences immune anti- or pro-tumoral function
- Tumors are able to circumvent immune attack employing immunosuppressive mechanisms and mechanism of death resistance
- Inhibitors of mechanisms responsible for tumor escape could restore anti-tumor immune responses in cancer patients
- Cancer Immunotherapy as a pillar of cancer therapy – can be combined with other types of therapies to enhance its efficiency for long term

Questions

1. What are the differences between innate and adaptive immunity? Who are the players of each type of immune response?
2. Explain the concept of cancer immunoediting referring briefly the three processes underlying it.
3. Which types of immunity can be provided by the adaptive immune system? Describe the main cells involved in both responses.
4. Describe how the innate interacts with the adaptive system and how they work together to protect the body against cancer.

5. What are the major components of the tumor microenvironment?
6. What are the relevant immune evasion mechanisms that cancer cells employ to circumvent immune response? Which molecules act on those processes?
7. Provide some examples of adoptive cell therapy.
8. Explain the mechanism of immune checkpoint therapies.

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Angiogenesis – Vessels Recruitment by Tumor Cells

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What You Will Learn in This Chapter?

In this chapter you will learn that tumor blood vessels play important roles in tumor progression and that targeting the tumor vasculature is an alternative cancer therapy. Blood vessels are formed by endothelial cells organized in a monolayer, attached to a basement membrane and covered by pericytes. In general, endothelial cells are adherent to each other through molecular complexes that form the adherens and tight junctions. Such adhesion complexes guarantee the barrier function of the endothelial monolayer, crucial for the regulated transport of molecules and cells between the blood and the tissue and vice-versa. Blood vessels are essential for cell survival as they bring oxygen and nutrients to cells and also participate in the transport of waste products away from tissues. In that sense, as tumors grow, through the rapid proliferation of tumor cells, they require the formation of a new vasculature that irrigates the cells. Without a new vasculature, tumor cells are too far away from a vascular bed, which results in hypoxia and nutrient starvation. These are believed to be the main triggers of new blood vessel formation. The formation of a new blood vessel from a pre-existing one is called angiogenesis and is the main type of blood vessel formation that takes place in tumors. However, other ways to ensure blood deliver to tumor cells have been described. Angiogenesis also occurs in physiological conditions such as during development and wound closure and several cellular and molecular mechanisms that govern it have been elucidated. The most common experimental models used to study physiological angiogenesis are the mouse retina, the transparent zebrafish and human endothelial cells cultured in *in vitro* systems. The vascular Endothelial Growth Factor (VEGF) is the main signaling molecule that regulates angiogenesis, although several other pro- and anti-angiogenic stimuli exist. In tumors, due to an imbalance of such stimuli, angiogenesis is abnormal, resulting in a disorganized and leaky vascular network. The abnormal nature of the tumor vasculature plays critical roles in cancer progression, in particular its inadequate ability to ensure the barrier function of the vessels has several pathological consequences and is an obstacle to the delivery of therapeutic drugs to tumors. Abnormally permeable blood vessels allow the uncontrolled movement of molecules, extracellular vesicles and cells, in and out of the tumor. This is the starting point for cancer to become a systemic disease. Through leaky blood vessels, tumors send signals to distant tissues, recruit and control immune response and invade the blood through a process called intravasation. Targeting blood vascular formation in tumors or its interaction with tumor and immune cells, or instead, normalize the otherwise abnormal tumor vessels, are alternatives for anti-cancer therapies.

8

Learning Objectives

After reading this chapter students should be able to:

1. Describe Judah Folkman's discoveries that led to the tumor angiogenesis concepts.
2. Explain the formation of a new vascular network and molecules involved.
3. Delineate the cellular and molecular mechanisms involved in tumor angiogenesis.
4. Characterize the features of the tumor vasculature.
5. Establish the relationship between the immune system and tumor vasculature and how it impacts on tumor development.

Important Concepts Discussed in This Chapter

- Tumor growth is compromised by lack of blood flow within tumors.
- To sustain growth, tumors developed ways to ensure their blood perfusion.
- A well-known mechanism to ensure perfusion is tumor angiogenesis: the growth of blood vessels from pre-existing ones.

- The main molecular drivers of angiogenesis are the VEGF family of proteins.
- VEGF production in tumor cells are induced by hypoxia and nutrient deprivation.
- VEGF binds to VEGF receptors on endothelial cells inducing angiogenesis.
- Tumor angiogenesis is abnormal.
- Cancer therapies can target endothelial cells.
- Anti-angiogenic therapies have been developed and are currently used in the clinics as part of some cancer treatments.
- Anti-angiogenic drugs can also be used to "normalize" the tumor vasculature and facilitate chemotherapy delivery.
- There are other ways apart from angiogenesis that allow tumor perfusion.
- Metastasis (the growth of tumor cells at distant sites from the primary tumor) occur by dissemination of tumor cells through the circulatory system.
- The entry of tumor cells in the circulatory system is called intravasation.
- The abnormal tumor vasculature affects intravasation.
- Tumors have evolved ways of avoiding the entry of immune cells via inhibition of the interaction of immune cells with blood vessels.
- Tumor vasculature puts cancer cells in contact with all systems of the organism and therefore is crucial for cancer to become a systemic disease.

8.1 Angiogenesis

Tumor growth depends on angiogenesis, the formation of new blood vessels that bring oxygen and nutrients to highly proliferative tumor cells. This theory is the consequence of many decades of research, which culminated in 1971, when Judah Folkman published (in the "New England Journal of Medicine") the hypothesis that tumor growth could be angiogenesis-dependent and that inhibition of angiogenesis represents a novel way to control tumor growth [1, 2]. Since then the cellular and molecular mechanisms that regulate the formation of new blood vessels in tumors have been extensively studied and revealed the existence of a complex network of molecular signals produced by tumor cells, cells that form the blood vessels and cells from the tumor microenvironment. These different elements work in an orchestrated manner in physiological situations, but also contribute towards the production of new, although abnormal blood vessels in tumors. Based on such knowledge, therapeutic interventions to block angiogenesis have been developed and hundreds of thousands of patients have benefited from it. However, the anti-angiogenic therapies have largely shown limited efficacy mainly due to the appearance of alternative mechanisms for angiogenesis and resistance to the drugs [3]. New therapeutic alternatives, such as interfering with endothelia cell metabolism are now under scrutiny [4]. Moreover, the conclusion that blood vessels in tumors grow abnormally and have a limited ability to properly allow blood flow, led to a current hypothesis: that will be therapeutically more efficient to normalize blood vessels in tumors, in order to facilitate the delivery of intravenous chemotherapies, instead of inhibiting angiogenesis per se [5, 6]. What has also become clear upon more than 40 years of research on tumor angiogenesis, and on physiological angiogenesis, is that blood vessels are much more than just channels transporting oxygen and nutrients. Blood vessels are also the vehicle that brings immune cells into the tumor, the vehicle that place tumor-produced factors in circulation (being cytokines and extracellular vesicles or exosomes an example), and finally the vehicle for tumor cells to enter circulation and invade other tissues. In other words, blood vessels

are the gatekeepers that allow cancer to become a systemic disease. Finally and not less important, the cells that form blood vessels, endothelial cells in particular, are now clearly known to play active roles in determining tissue regeneration versus tumor onset and progression, through the production of "angiocrine" factors [7]. Here we will present a review of the cellular and molecular events that take place during the formation of new blood vessels within a tumor. In particular we will review the research on how angiogenic blood vessels contribute to the immune cell exclusion and immune privileged environment of tumors [8] and how the tumor vasculature regulates the entry of tumor cells into the blood, in the early steps of metastasis formation.

8.2 The Formation of a New Vascular Network

Angiogenesis, the formation of new blood vessels through sprouting from pre-existing ones is an essential physiological process for embryologic development, normal growth, and tissue repair [3, 9]. Angiogenesis is believed to be the main way to sustain vascularization in tumors; however other ways allowing blood circulation on tumors have been proposed and are summarized in ▶ Sect. 8.2. The cellular and molecular mechanisms involved in angiogenesis have been studied using several models such as cell culture, the mouse retina and zebrafish [9]. **Hypoxia** (lack of oxygen) and **nutrient deprivation** are believed to be the main underlying "reasons" for the onset of angiogenesis. Rapid cellular division during tumor growth enhances both oxygen and nutrient demand due to increased metabolism and therefore induces angiogenesis. Cellular and molecular responses to such triggers coordinate themselves in order to form a molecular signature of pro- and anti-angiogenic factors that regulate the formation of a new vascular network.

To make this complex system easier to understand, the main cellular events that take place during angiogenesis can be grouped in three phases:

1. Sprouting and the definition of tip cells
2. Vessel assembly and lumen formation take place
3. Blood vessel maturation and vascular remodeling close the process.

Such events, which are regulated by particular sets of signaling molecules, will be discussed next and are summarized in ◘ Fig. 8.1.

8.2.1 Sprouting – VEGF and Notch Signaling Pathways

Blood capillaries consist of a monolayer of endothelial cells interconnected by adhesion molecules and surrounded by pericytes or mural cells. Endothelial cells and pericytes at rest produce a common basement membrane that sustain blood vessels and contribute to their integrity. In healthy adult capillaries, endothelial cells are believed to be mainly quiescent (not dividing), due to the action of pericytes and also the absence of external proliferative factors. Angiogenesis starts from the response of the quiescent endothelial cells to angiogenic signals, such as vascular endothelial growth factor A - VEGFA. VEGFA, commonly known simply as VEGF, is the master regulator of new blood vessel formation and its production within a tumor is induced by hypoxia and nutrient deprivation in ways that have been identified in detail [10–14].

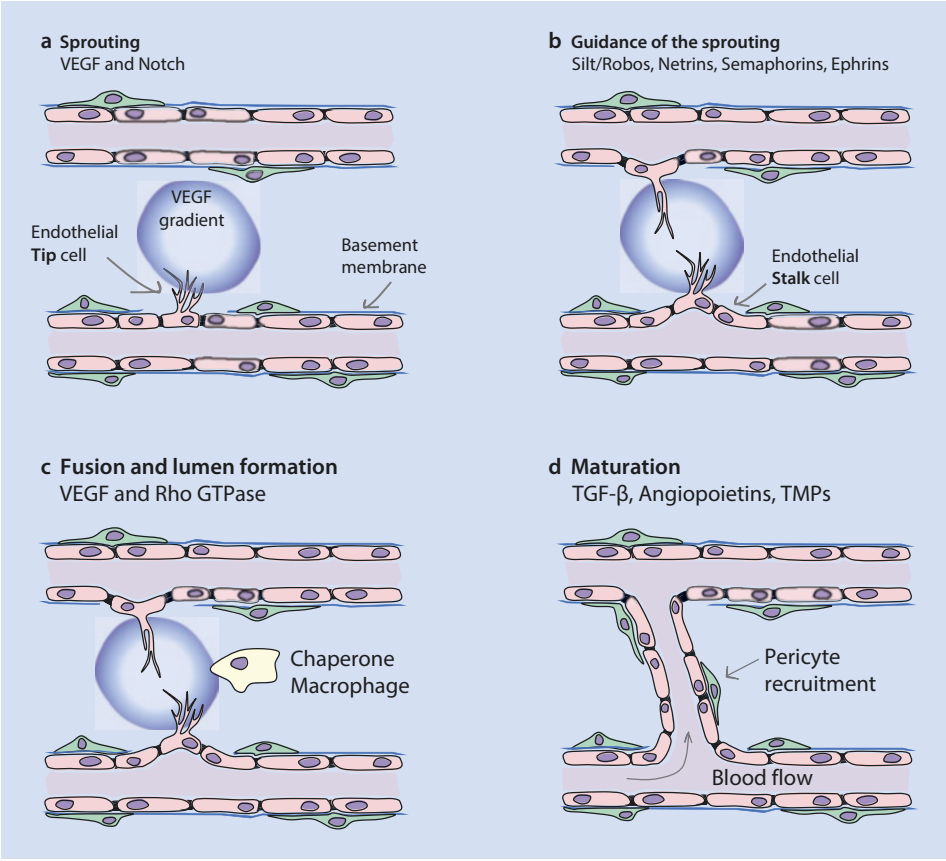

Fig. 8.1 Cellular events and key molecular players of angiogenesis. **a** Angiogenesis, the formation of a new blood vessel from a pre-existing one can be divided in several cellular events. First, there is the morphological alteration of an endothelial cell from a quiescent vessel in response to a VEGF gradient. This cell is called the tip cell, and their neighbors the stalk cells. This first step also requires the detachment of pericytes and degradation of the basement membranes. **b** The tip cell leads the formation of a new branch in response to guidance cues. **c** At the same time stalk cells divide and the fusion of endocytic vesicles allow lumen formation. Finally, there is the ligation of the two tip cells that is chaperoned by macrophages. **d** The angiogenic process culminates in the formation of a new branch that allows blood flow and eliminates the VEGF gradient. To ensure proper blood flow the new blood vessel requires a maturation process that consists in the re-attachment of pericytes, repositioning of a basement membrane. (Figure adapted from Carmeliet, P. & Jain, Nature 2011 [3])

In tumors, VEGF is produced by tumor cells, but also cells from the tumor microenvironment, such as macrophages. The main VEGF receptor that translates the VEGF signal into the formation of a new blood vessel branch is VEGFR2 (also known as KDR or FLK1) [15, 16]. Binding of VEGF to VEGFR2 on endothelial cells from a mature vessel, leads to reduced adhesion to their neighboring cells and their migration towards the VEGF gradient. This cell is called tip cell, as it is the cell that will lead the formation of the new branch, the ones that are left behind are called stalk cells (Fig. 8.1a). The tip cell/stalk cell dichotomy is regulated by the Notch signaling pathway, which is firstly activated on tip cells by VEGF [17]. VEGF binding to VEGFR2 leads to the upregulation of the Notch ligand Dll4

on tip cells. Binding of tip cell Dll4 to Notch of neighboring endothelial cells, defines the neighbors as the stalk cells. VEGF belongs to a family of signaling molecules that also participate in angiogenesis, this includes Placental Growth Factor (PLGF), VEGFB, VEGFC and VEGFD [18]. VEGFC, a ligand of the VEGFR2 (FLK1) and VEGFR3 (FLK4) receptors, activates blood-vessel tip cells and is also a major regulator of lymphangiogenesis (formation of lymphatic vessels from pre-existing lymphatic vessels) [19–21].

8.2.2 Vessel Assembly – Guidance Molecules

In physiological angiogenesis endothelial cell sprouting continues in a highly directional and regulated manner. This occurs similarly to neuronal sprouting in a way that involves the response to axon guidance cues such as Slits/Robos, Netrins, Semaphorins and Ephrins [22] (Fig. 8.1b). Similar to what happens in neuronal sprouting, these molecules act as attractive or repulsive cues to guide tip cells during angiogenesis.

- **Attractive cues**

Endothelial Robo4 maintains vessel integrity and barrier function as it counteracts the permeability-inducing effects of VEGF [23]. These effects have been suggested to be mediated by binding of Robo4 to UNC5B, another guidance receptor that also binds Netrins [24, 25]. In tumors, Slit2 has been shown to be expressed by tumor cells, in a center to periphery gradient, and to attract endothelial cells through the interaction with Robo1 [26].

- **Repulsive cues**

Semaphorins bind to Plexins and Neuropilins and have a suppressive role in vascular growth [27]. In tumors, binding of Sema3A to NRP1 is required for vascular development, while Sema3F acts as a repellant for endothelial cells [28, 29]. Tumor vascularization is also induced by SemaD4D, which has been shown to be produced also by cells from the tumor microenvironment [27, 30, 31]. Finally Ephrins and their receptors are regulators of cell-contact-dependent signaling and generate mostly repulsive signals [32]. Ephrin-B2 regulates VEGFR2 and VEGFR3 internalization and therefore proper VEGF signaling [33, 34].

Together, these "attractive" and "repulsive" cues guarantee proper vessel growth during angiogenesis. In tumors, aberrant expression of attractive or repulsive ligands and receptors may account for some of the vascular defects observed.

Sprout elongation is followed by vessel fusion, which occurs when tip cells encounter other vascular branches. The fusion of migrating tip cells is called anastomosis and is mediated by chaperone macrophages [35] (Fig. 8.1c). Once the contact between tip cells is established, VE-cadherin-containing junctions consolidate the connection.

8.2.3 Lumen Formation, Maturation and Remodeling

Lumen formation occurs in stalk cells through the fusion of pinocytic vesicles and is, similar to lumen expansion, regulated by VEGF and Rho GTPases [36]. The onset of blood flow in the new lumen also shapes and remodels vessel connections [37]. This is in part regulated by responses to shear stress such as those conveyed by the shear stress-

responsive transcription factor Krüppel-Like Factor 2 (KLF2) [38]. The mechanisms of lumen formation during sprouting angiogenesis have been shown to depend on the vascular bed or type of vessel (vein, artery, capillary) formation. The end of the process consists in blood vessel maturation and the return of endothelial cells to a quiescent state (◘ Fig. 8.1d). This strongly involves the interaction with pericytes and signals such as Platelet-Derived Growth Factor B (PDGF-B), Angiopoetin 1 (ANG-1), Transforming Growth Factor-β (TGF-β), ephrin-B2 and Notch [39–43]. At this stage the deposition of basement membrane also occurs and involves protease inhibitors known as Tissue Inhibitors of Metalloproteinases (TIMPs) and Plasminogen Activator Inhibitor-1 (PAI-1). In parallel, junctions are re-established to ensure optimal flow. At the end vessels regress if they are not perfused; the networks formed by vessel sprouting subsequently undergo extensive remodeling to form a functional and mature vasculature. This remodeling includes distinct processes of trimming of the vascular network, also called vascular pruning, or the regression of selected vascular branches [44]. At the cellular level it mainly involves apoptosis and cell migration, processes that are once again regulated by VEGF, but also by the WNT, Ang/Tie2 and Notch/Dll4 signaling pathways. What is however still not known is what are the initial triggers that select the particular branches for vessel regression and whether this is dictated by active signaling pathways or if it results from the absence of survival factors (including VEGF). In all these processes, blood flow and mechanosensory systems also play important roles [45]. In tumors, the process of blood vessel maturation is seriously compromised, resulting in abnormal vessels in shape and in function. This will be further discussed in ► Sect. 8.3.

8.3 Alternative Ways to Generate a Neo-Vasculature in Tumors

Angiogenesis defines the formation of a new blood vessel through sprouting from a pre-existing one. Although this seems to be the prevalent way of creating blood vessels in tumors, other processes have been described (◘ Fig. 8.2).

■ Intussusception

Vessels have been shown to be able to split by a process known as intussusception, giving rise to daughter vessels [46].

■ Recruitment of bone-marrow-derived cells

Tumors can promote the recruitment of bone-marrow-derived cells (BMDCs) including endothelial progenitor cells that become incorporated into the endothelial lining and help in the repair and expansion of the tumor vasculature. This process happens in a similar way to what happens during the formation of blood vessels in development and as such is known as postnatal vasculogenesis. Two particular sub-types of bone-marrow derived cells contribute to postnatal vasculogenesis and have been shown to be present in tumors:

- VEGFR2 expressing endothelial progenitors
- and VEGFR1$^+$ hematopoietic precursor cells [47]

■ Co-option and vascular mimicry

Tumor cells have been shown to hijack the existing vasculature in a process called co-option (particularly important in the case of brain tumors, or gliomas); this is a process

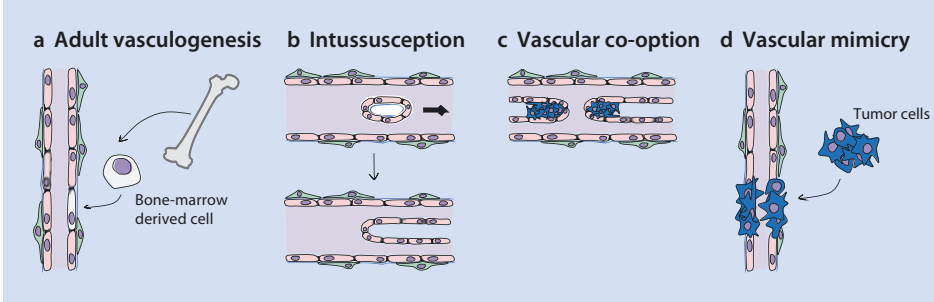

Fig. 8.2 Alternative ways to generate a neo-vasculature in tumors. In addition to angiogenesis, other cellular processes have been described that ensure blood flow inside tumors, describing well all the plasticity that occurs in tumors. Examples are: **a** Adult vasculogenesis, that consists in the incorporation in the vasculature of endothelial progenitor cells recruited from the bone marrow; **b** Intussusception, the division of one blood vessel in two; **c** Vascular co-option, the hijacking of the existing vasculature by tumor cells and **d** Vasculogenic mimicry, the organization of tumor cells as tubes that allow blood flow. The relevance of each one of these processes is not fully understood, but it seems clear that it is tissue-dependent. (Figure adapted from Carmeliet, P. & Jain, Nature 2011 [3])

by which tumor cells grow along pre-existing blood vessels prior to the occurrence of an angiogenic switch. Tumor cells may also differentiate and form "tube-like" structures, or channels, able to accommodate blood flow – a phenomenon known as vascular mimicry (particularly evident in the case of melanoma and ovarian cancers [48–50] (Fig. 8.2)).

8.4 Consequences of Tumor Angiogenesis

Unlike the vasculature of normal organs, which has an organized and regular branching order, the tumor vasculature is described as chaotic and tortuous. It is also irregular in lumen diameters, highly permeable and deficient in pericyte coverage. This is believed to be mainly due to the imbalance of pro- and anti-angiogenic signaling within tumors, and the lack of sufficient cues to temporarily and spatially coordinate the process [6, 51]. An important consequence of this abnormal vasculature is an increase in interstitial fluid pressure, which results in **heterogeneous tumor perfusion**. Such feature results in an inefficient delivery of chemotherapeutic agents into tumors [52] and access of antitumor lymphocytes may also be impaired [8]. These observations gave rise to a new hypothesis, that, the administration of **normalization agents** of the tumor vasculature, together with anti-tumor drugs, could be a therapeutic possibility. Such hypothesis was supported by the fact that the combination of anti-VEGF therapy with conventional chemotherapy, improved survival in cancer patients compared with chemotherapy alone [53]. As **VEGF production is believed to be excessive in tumors**, compared with situations of normal angiogenesis such as during wound healing, VEGF could be the main responsible for the aberrant vascularization of tumors.

In fact, preclinical studies have shown that anti-VEGF therapy changes tumor vasculature towards a more "mature" or "normal" phenotype characterized by:

- attenuation of hyperpermeability,
- increased vascular pericyte coverage,
- normalized basement membrane, and
- reduction in tumor hypoxia and interstitial fluid pressure [6, 51].

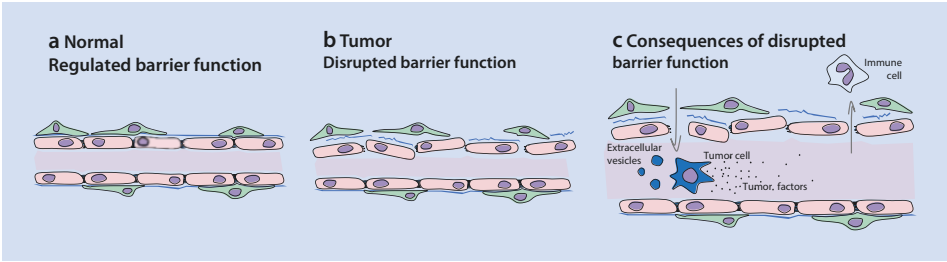

Fig. 8.3 Tumor blood vessels are abnormal and leaky. **a** One of the main functions of blood vessels is to ensure the regulated flux of molecules and cells from the blood and the tissues and vice-versa. **b** In tumors, this barrier function is disrupted due to less pericyte coverage, breaks in the basement membrane and disruption of the endothelial cell junctions. **c** As such, leaky vessels that form within tumors lead to the unregulated extravasation of tumor-produced soluble factors, such as chemokines, the release of tumor-derived extracellular vesicles into the blood stream, and the entry of tumor cells in circulation. Finally, abnormal tumor blood vessels also regulate the infiltration of tumors by immune cells

With the goal of finding "normalizing" agents to be used in combination with anti-tumoral therapies, genetic and pharmacological approaches began to unravel some key regulators of vascular normalization apart from VEGF, such as proteins that regulate tissue oxygen sensing (PHD2) and vessel maturation (PDGFRβ, RGS5, Ang1/2, TGF-β) [5]. Nevertheless, this seemingly impaired vasculature is however functional enough to provide not only the nutrients for a growing tumor, but also to allow the infiltration of immune cells, the exit of tumor-produced factors such as exosomes and even the requirements for metastatic dissemination of the escaping tumor cells (Fig. 8.3).

8.4.1 Vascular Permeability

One of the main functions of endothelial cell monolayers is to form a barrier to the free passage of molecules and cells from the blood into the tissue and vice-versa. For this, quiescent endothelial cells express specialized cell-cell communication molecules such as adherens and tight junction molecules including VE-cadherins, occludins and claudins [54]. In order to form new vessels, angiogenic endothelial cells, loose their junctions turning blood vessels more permeable but also less able to perfuse blood. The decreased attachment that occurs between adjacent cells in tumor blood vessels is regulated by angiogenic factors such as VEGF [54, 55]. In fact, VEGF was firstly identified a vascular permeability factor [12, 13]. Since then, the molecular mechanisms involved have been identified in detail. It has been shown that binding of VEGF to VEGR2 activates Src and Rho GTPase signaling, that culminates with the endocytosis of VE-cadherin at cell:cell junctions, opening gaps between cells and consequently increase permeability [56, 57]. Additionally, ROS production and the displacement of the Rho GEF Syx from junctions have also been shown to promote adherens junction disassembly [58]. Several phosphorylation events on VEGFR2 that take place upon VEGF binding have been identified and a recent study identified a phosphorylation event specifically associated with disruption of junctions and vascular leakage, as well as metastasis formation [59]. Apart from VEGF, the de-localization of VE-cadherin from cell-cell contacts can also be achieved by inflammatory molecules highly expressed in tumors such as TNF-α and leukocyte adhesion

molecules such as ICAM and VCAM. Also, besides low levels of cell:cell adhesion molecules localized in between neighboring endothelial cells, sparse pericyte and basement membrane coating also contribute for increased permeability of blood vessels in tumors [5]. Taken together, the **highly permeable blood vessels within tumors** have several consequences for tumor progression and as such, may be considered as a possible target for therapy. Examples of such consequences are the deregulated traffic of immune cells in and out of the tumor, and the exit of tumor cells from the primary tumor – the onset of metastasis. These two events will be discussed in more detail next.

8.4.2 Intravasation of Tumor Cells

In order to form metastasis via hematogenous dissemination, tumor cells need to cross blood vessels at least twice – to enter and to exit circulation. It is believed that tumor cells exit blood vessels in a similar way as leukocytes do, in a process called **transmigration** [60]. Several cellular and molecular mechanisms that allow the exit of tumor cells from circulation – **extravasation**, have been identified [60]. The entry of tumor cells in circulation – **intravasation**, is in contrast, one of the less studied processes of the metastatic cascade. To understand intravasation it is crucial to take into consideration the abnormal nature of the intra-tumoral vasculature and the highly leaky environment. Modern live-cell imaging in animal models is offering important insights into the process of intravasation and the role of angiogenic vessels in such process. As such, the most commonly used models are the CAM in the chicken embryo [61], the transparent zebrafish [62] and the mouse. In the mouse, a recent study in which tumor cells are implanted in the mouse ear (an organ of easy access for live cell imaging), suggested that intravasation events mainly take place in the interior angiogenic core of tumors [63]. Intravital multiphoton imaging of genetically induced mammary tumors, has also allowed visualizing that often tumor cells intravasate in close contact with tumor associated macrophages that are characterized by having a perivascular location and high expression of Tie2 and VEGF [64, 65]. Once again, the majority of such events were visualized around the center of the tumor, that also had increased blood vessel density [65]. The specific role of blood vessel cells in this process has however not been investigated. The recent use of genetic approaches to specifically target certain signaling pathways on endothelial cells is starting to unravel that endothelial cells play an active role during intravasation. Apart from molecules that form adherens and tight junctions, other proteins concentrate at the intercellular borders of endothelial cells and are implicated in cell-cells interactions and blood vessel integrity. Examples are VCAM1, and endoglin (CD105). Loss of endoglin has been shown to promote intravasation in a model of breast cancer [66].

Recently it has also been shown that sustained Notch-1 activation in the tumor vasculature facilitates intravasation and metastasis in a model of breast cancer, most likely through the upregulation of the adhesion molecule VCAM1 [67]. In agreement to this finding, repression of Notch signaling on endothelial cells due to binding of AES (transcriptional regulator amino-terminal enhancer of split) expressed by tumor cells, prevents colorectal cancer cell intravasation [68].

Another report demonstrated that the reduced expression of *Shb*, an adaptor protein downstream of VEGFR2, can result in vascular leakiness and increased metastasis [69].

Finally, tumor endothelial cells have been shown to behave different from normal endothelial cells, affecting the metastatic outcome [70]. Indeed, a recent report showed that the co-implantation of endothelial cells isolated from highly metastatic tumors with

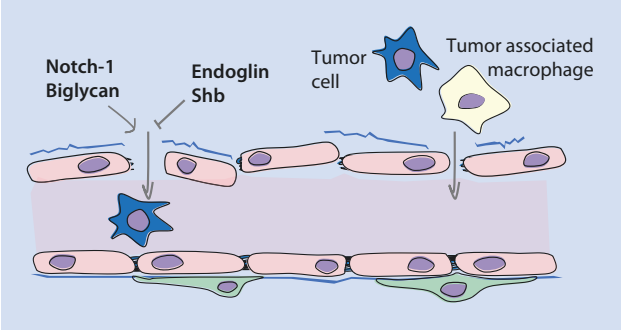

Fig. 8.4 Tumor blood vessels regulate the intravasation of tumor cells. Tumor blood vessels regulate the intravasation of tumor cells in the blood and some molecular players produced by endothelial cells have been identified. Loss of endoglin has been shown to promote intravasation, while Shb, an adaptor protein acting downstream of VEGFR2 has been shown to suppress intravasation. Endothelial Notch and biglycan, in turn, have been shown to promote the entry of tumor cells in circulation. Finally, tumor cells have often been seen to follow tumor-associated macrophages to enter blood vessels, the endothelial contributions to this, have however not been identified

tumor cells from low metastastic tumors accelerated lung metastases of the later. Further investigation led to the identification of biglycan, a proteoglycan secreted by endothelial cells that activates tumor cell migration [71] (Fig. 8.4).

8.4.3 Immune Response against Tumors

The vessels are also a door and highway for the immune system, therefore blood vessels are highly involved in the regulation of the immune cell response against tumors. Through cell:cell contacts, tumor vessels modulate immune cell extravasation, intravasation but also their identity. Moreover, tumor cells have found ways of regulating the communication between blood vessels and immune cells in a way that contributes for their escape from immune control. Blood vessels regulate the immune response in a tightly controlled manner that has been thoroughly investigated and that culminates with the transmigration of leukocytes (lymphocytes, monocytes, and granulocytes) through inflamed blood vessels. In detail, in response to inflammatory stimuli such as TNFα, endothelial cells express at their apical side (luminal side of the vessel) leukocyte adhesion molecules such as ICAM1, VCAM1 and Selectins. Such molecules trap circulating leukocytes, that ultimately transmigrate through endothelial cells and the basal membrane in the direction of the inflammatory source [72] (See Fig. 8.5a). A similar mechanism is believed to take place in tumors although tumor-produced factors, such as angiogenic factors, have been shown to inhibit the expression of adhesion molecules in vitro [73–75] and more recently also in mouse tumor models [76, 77]. Such phenomenon consisting on the lack of endothelial response to inflammatory stimuli that takes place in tumors is called **endothelial anergy** [74]. A number of pre-clinical studies have shown that various antiangiogenic therapies, including tyrosine kinase inhibitors and inhibitory monoclonal antibodies against VEGF and VEGFR2, may help to increase tumor infiltration by lymphocytes (reviewed in [8]). Selective extravasation of different leukocyte subsets may also be mediated by endothelial cells such as through CLEVER-1/stabilin [78] and Fas ligand (FasL) [79] (Fig. 8.5b).

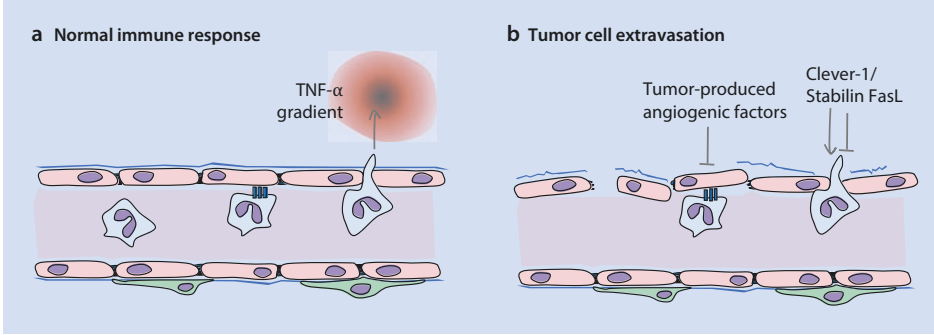

Fig. 8.5 Tumor blood vessels regulate the extravasation of immune cells. **a** In a normal situation, in response to inflammatory stimuli, such as mediated by a chemokine gradient (TNF-α for example), circulating leukocytes are arrested by endothelial cells, through the binding to adhesion molecules such as ICAM, VCAM and Selectins. This is followed by the transmigration of leukocytes through the endothelial monolayers (extravasation) in the direction of the inflammatory stimuli. In tumors, tumor-produced angiogenic factors have been shown to inhibit this inflammatory response of endothelial cells, compromising the immune response against the tumor. **b** In addition, endothelial cell molecules, such as Clever-1/stabilin, or the FAS ligand have also been shown to regulate the selective extravasation of different types of immune cells to the tumor and as such regulate the immune response against tumor cells. (Figure adapted from Carmeliet, P. & Jain, Nature 2011 [3])

To summarize, and as you could appreciate, the vasculature plays pivotal roles during tumor progression: it allows tumor growth by bringing oxygen and nutrients but it's also the way tumor cells use to escape the primary tumor and metastasize and finally it puts the tumor in communication with the rest of the body through secreted molecules, vesicles and circulating non-tumor cells such as immune cells. The vascularization of the tumor is therefore the critical step for the establishment of cancer as a systemic disease that communicates with several of the body systems. Finally, and not less importantly the vascular system is the way used by clinicians to deliver drugs and try to kill tumor cells. For all such reasons understanding how tumors are perfused is critical for the improvement of cancer therapies.

Take Home Message

The following topics were covered in this chapter:

- Angiogenesis is the formation of new blood vessels from pre-existing ones.
- Angiogenesis is the main process for tumor vascularization.
- Angiogenesis also occurs in several physiological processes.
- Blood vessels bring oxygen and nutrients to tumor cells.
- Tumor blood vessels are essential for tumor progression.
- Other ways to ensure blood flow in tumors exist.
- Hypoxia and nutrient starvation are the main triggers for angiogenesis.
- Vascular endothelial growth factor (VEGF) and VEGF receptors are the main regulators of angiogenesis at the molecular level.
- Due to an imbalance of pro and anti-angiogenic stimuli angiogenesis in tumors is abnormal.
- Abnormal angiogenesis has pathological consequences during tumor progression.
- Abnormal angiogenesis turns blood vessels more leaky.

- Leaky blood vessels lead to an "easy access" of tumor-derived factors and tumor cells to systemic circulation – consequently metastasis formation.
- Leaky blood vessels result in a limited efficacy of delivering drugs to tumors.
- Tumor blood vessels regulate the anti-tumoral immune cell response.
- Several anti-angiogenic drugs, targeting the VEGF signaling pathway, have been developed and used in the clinics as a cancer therapy. However, their efficacy was limited mainly due to development of resistance.
- Anti-angiogenic drugs in combination with chemotherapy resulted in a more efficient therapeutic alternative.
- Normalizing tumor blood vessels may be an alternative approach to improve drug delivery into tumors.
- Targeting the interaction between blood vessels and tumor cells during intravasation may be a therapeutic alternative.
- Understanding the role of tumor blood vessels in the anti-tumoral immune response may give rise to new therapeutic approaches against cancer.

Questions

1. What is the definition of angiogenesis?
2. Why tumors require angiogenesis to grow?
3. Name three alternative ways through which angiogenesis allow blood flow in tumors.
4. What is the main molecule that regulates angiogenesis?
5. What are the two signaling pathways that define the tip/stalk cell dichotomy?
6. Name at least two axon guidance molecules that play roles in tip cell guidance during angiogenesis.
7. Name at least two molecules involved in blood vessel maturation.
8. What are the features of tumor blood vessels (at least two)?
9. What is the main component of adherens junctions?
10. Name adhesion molecules that allow binding of leukocytes to blood vessels during transmigration (at least two).
11. What are the designations for the processes of entering and exiting blood vessels by tumor cells, during the metastatic cascade?

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Tumor Niche Disruption and Metastasis: The Role of Epithelial-Mesenchymal Transition (EMT)

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What You Will Learn in This Chapter

In this chapter you will learn that, besides the initial oncogenic mutations that trigger tumorigenesis, cancer cells must have additional molecular changes and morphological modifications in order to metastasize and become malignant. One of the mechanisms that tumor cells may undergo to achieve this state is the biological process of Epithelial-Mesenchymal Transition (EMT). After giving a global perspective of what EMT is and of the main EMT activating factors, the particularities of EMT in tumors are discussed. We begin by addressing the role of EMT in cancer and describing the stages of tumor progression that lead to metastasis formation. A relevant point of cancer EMT is addressed – the tumor cell heterogeneity –, and that is revealed by the fact that not all tumor cells will experience EMT simultaneously, and that most cancer cells where EMT is activated often go through an 'incomplete' or 'partial' EMT. Next, we discuss the molecular mechanisms that underlie the tumor EMT phenotypic plasticity.

Finally, we point out the contribution of EMT studies for the development of a new generation of cancer therapies aiming at EMT targets and confronting them with the fact that EMT is also related with cancer cell drug resistance.

Learning Objectives

After completing this chapter, students should be able to:

1. Know the difference between a benign and a malignant tumor. Define metastasis and its consequences.
2. Describe the main cellular morphological changes in EMT.
3. Know the main signaling pathways and transcription factors (EMT-TFs) that are involved in the EMT process.
4. Explain the role of EMT-TFs and give examples.
5. Discuss the role of EMT in pathogenesis namely in the formation of metastasis of certain carcinomas. Justify why tumors may require EMT to progress.
6. Describe the steps of metastasis formation based on EMT program.
7. Indicate the layers of regulation of EMT and provide specific examples.
8. Relate the emergence of cancer stem cells CSCs with EMT.
9. Discuss the basics of tumor plasticity.
10. Argue the principles for the development of EMT based drugs as a promising approach in cancer therapy. Debate the fundamentals of EMT-related cancer cell drug resistance.

Important Concepts Discussed in This Chapter

- Metastasis is the first malignant transformation, the mortal phase of cancer progression.
- Metastasis formation involves the detachment of cancer cells from their original territory (niche – primary tumor) to invade and proliferate at distant tissues/organs – the secondary site.
- One of the mechanisms responsible for metastasis formation is a common embryonic cellular program – EMT – which is triggered in different biological contexts.
- Mesenchymal-epithelial transition (MET) is another precondition, in some scenarios, for successful metastasis.
- The role of tumor microenvironment and tumor-stroma crosstalk in the establishment of successful metastasis.
- Aberrant activation of the embryonic EMT program was proposed as the key step for tumor cells to acquire the ability to delaminate, migrate and invade new

territories, offering cancer cells the capacity to overcome physical and biological barriers that would hamper metastasis progression.
- EMT is also associated with generation and maintenance of cancer stem cells (CSC).
- EMT in cancer is regulated by different gene expression mechanisms: transcriptional regulation, epigenetic modifications, alternative splicing, non-coding RNAs (ncRNAs).
- Tumor populations are highly heterogeneous. Not all cancer cells are able to undergo EMT and at the same time, and not all cells that have activated an EMT program become competent to metastasize.
- Tumor heterogeneity is further increased by the existence of epithelial/mesenchymal hybrids (E/M) in the highly metastatic circulating tumor cells (CTCs) and CSCs – tumor cell's plasticity.
- Cellular plasticity is essential for the acquisition of both invasive capabilities and stemness traits.
- The inherent cell plasticity due to EMT programs may contribute to chemoresistance.

9.1 Malignant Phenotype – Metastasis Formation – Co-option of EMT

Malignant cancers, as well as benign tumors, are characterized by uncontrolled cell growth. However, there is a clear difference between the two: **malignant tumors can acquire metastatic properties** disrupting their natural environmental boundaries. Metastasis formation is responsible for more than 90% of cancer mortality, being the major barrier to cancer patient survival. In fact, metastasis is the deadliest phase of cancer progression. For metastasis to be formed, cells must detach from their original niche (primary tumor) to invade and proliferate at distant tissues/organs – the secondary site [1, 2]. The process of metastasis involves several steps [2, 3] (Fig. 9.1):

1. Cell delamination from the primary tumor and invasion of surrounding tissues
2. Intravasation (entrance into circulation)
3. Survival in circulation
4. Extravasation from the lymphatic and/or blood vessels
5. Survival and proliferation at a secondary site (i.e., colonization).

The very first clue to start to unveil metastasis formation was a morphological observation: tumor cells at early-stages of tumor progression continue to display epithelial characteristics whereas in more advanced stages of highly aggressive primary tumors, cells often display a mesenchymal phenotype (reviewed in [4, 5]). Moreover, most cell migration tricks observed in carcinogenic tumors mimic migratory strategies observed during embryonic development [6]. These changes in cell behavior suggested that besides the growth advantage conferred by oncogenes, other genetic modifications could awaken **an embryonic developmental program** - the **epithelial-mesenchymal transition** (**EMT**) program. By **co-opting** this development process, carcinoma cells acquire the capacity to delaminate, migrate and successfully form metastasis in a new territory [7–10]. At the same time, EMT is a highly dynamic phenomenon, often interconvertible with the opposite process, the **Mesenchymal–Epithelial Transition** (**MET**) that, in cancer, is responsible for colonization and effective establishment of cancer cells in a secondary organ (reviewed in [11, 12] (Fig. 9.1)).

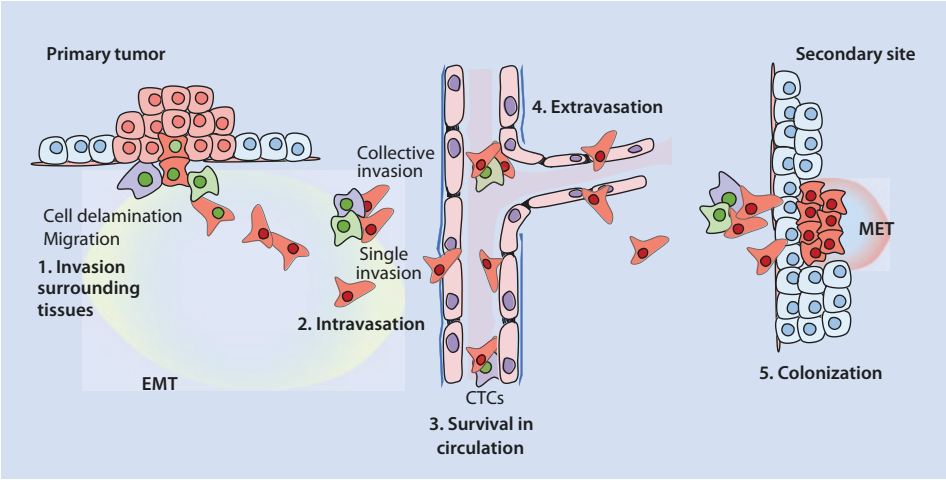


Fig. 9.1 Main steps involved in the metastatic cascade. During metastasis formation a subpopulation of epithelial cells at the invasive edge of a heterogenous primary tumor may undergo EMT, i.e., lose epithelial traits and acquire mesenchymal properties. In these early steps of metastasis, in response to EMT-promoting signals supplied by the microenvironment, tumor cells get the proficiency to detach from the tumor bulk, break down the basement membrane, perform a single or collective invasion of the stromal niche (1), disseminate through the vascular system (intravasation) (2), survive in the circulation (3), extravasate from vessels (4) and finally colonize a distant organ (5). In this secondary site, disseminated cells are exposed to signals distinct from those of the primary tumor, and the mesenchymal state may confer survival advantages to single neoplastic cells or alternatively may support long-term dormancy. When the appropriate contextual signals become available, reinforced through autocrine and paracrine signals, disseminated cells may undergo MET and gradually reacquire epithelial properties to outgrow as micrometastasis

In recent years, several studies have highlighted the central role of EMT in the biology of tumors with epithelial origin (**carcinomas**), such as tumors of the lung, colon, breast, pancreas, prostate, bladder, ovary, kidney, and liver. The particularities of EMT support in part, as you will soon see, the diversity of processes in tumors and malignancy grades. But before going into the specificities of EMT in cancer let's make a quick review of what EMT is and their biological roles.

9.2 What Is the Epithelial-Mesenchymal Transition Process?

EMT is a transient process that occurs between epithelial and mesenchymal cellular states. The morphological description of epithelial and mesenchymal cells goes back to the late XIX century and this inter-conversion between both cell states was first described by Frank Lillie in 1908 (reviewed in [13]). Yet, EMT as a fundamental process for vertebrate embryonic development was first identified by Betty Hay [14]. Further studies revealed that this phenomenon also occurs in other physiological processes, such as wound healing, and in pathological conditions such as fibrosis and cancer. While in EMT a polarized epithelial cell undergoes molecular modifications and loses its adhesion properties, altering its morphology and behavior to acquire motile mesenchymal properties, in MET occurs the reverse: a transition from motile, multipolar or spindle-shaped mesenchymal cells to stationary, planar and polarized epithelial cells.

9.2.1 Phenotypic Changes in EMT

The trademarks of the EMT program are (reviewed in [15, 16]):
- cell morphological transition from an epithelial to a mesenchymal phenotype,
- cell gain of motility and
- cell migration.

The first phenotypic evidence of EMT is the **loss of epithelial morphology** by the elimination of several epithelial structural features, such as cell-cell junctions and apical–basal polarity. An epithelium is characterized by polarized sheets of cells held together by junctional complexes, which confer strong cohesive cellular arrangements. The most common types of cell-cell junctions are (Fig. 9.2):

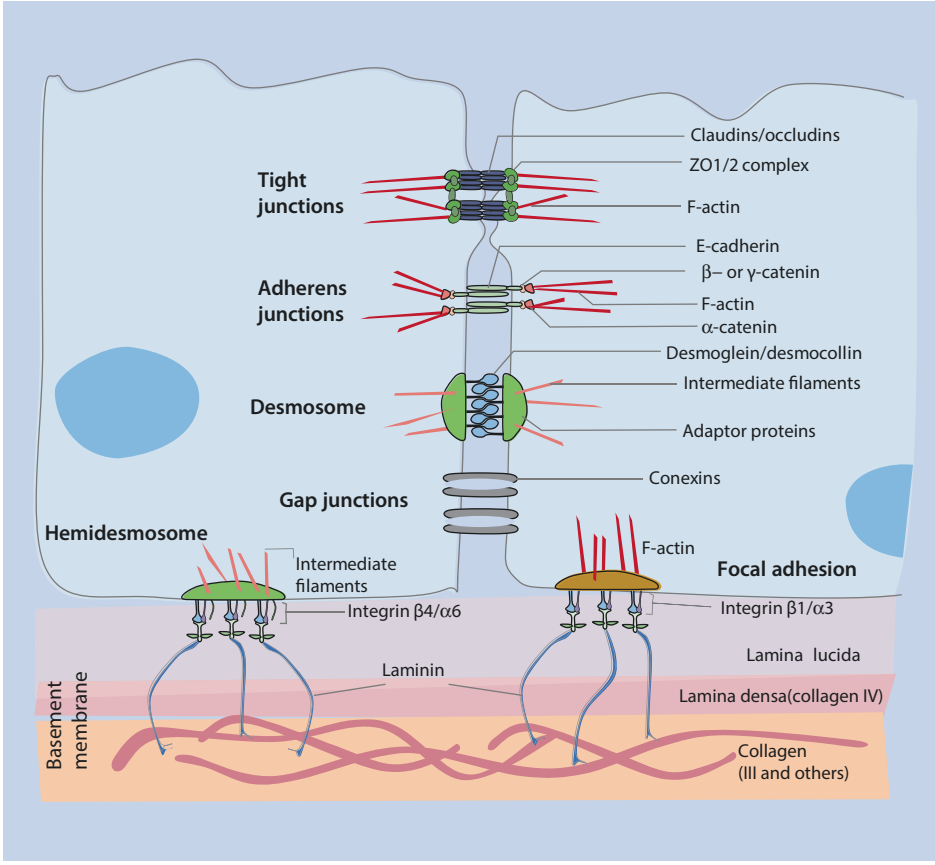


Fig. 9.2 Epithelial cell features. Epithelial cells are characterized by an apical-basal polarization with structured junctional complexes, that are responsible for cell-to-cell interactions: tight junctions, E-cadherin-based adherens junctions, desmosomes and gap junctions. Tight junctions and E-cadherin-based adherens junctions are connected to the actin microfilaments, while desmosomes are connected to the cytokeratin intermediate filament of the cytoskeleton. Epithelial cells also have specialized junctions responsible for cell adhesion to the laminin-rich basement membrane: the hemidesmosomes and focal adhesions, which are connected to the cytoskeleton via intermediate filaments and microfilaments, respectively

- tight junctions,
- adherens junctions and
- desmosomes

In addition, the epithelial cells rest in the Basement Membrane (BMb) which anchors the cells to the connective tissue. The BMb connection to the epithelial cell through hemidesmosomes (via-intermediate filaments of the cytoskeleton) and focal adhesions (via actin microfilaments of the cytoskeleton) contributes to the epithelial apical-basal polarity. Together, all these cellular junctional complexes are essential for epithelial phenotype and its elimination results in the loss of epithelial integrity and polarization (Fig. 9.2).

One of the critical events of EMT is the decreased synthesis of E-cadherin, a molecule that contributes for epithelial cohesion and relative cell immobility by maintaining lateral contacts via adherens junctions [17–19]. Other hallmarks of EMT include the reduction of proteins involved in tight junction proteins, such as zona occludens 1 (ZO1), occludin and cytokeratins, and also an increase in mesenchymal markers (reviewed by [20] (Fig. 9.3)).

In fact, progression towards a **mesenchymal morphology** is determined by the temporal up-regulation of several proteins including neural cadherin (N-cadherin), vimentin, and fibronectin [19, 21, 22]. While the loss of cellular junctions triggers cytokeratin network disassembling, vimentin up-regulation promotes the reorganization of the cortical actin network [23]. This new cytoskeleton arrangement alters cellular polarity originating spin-

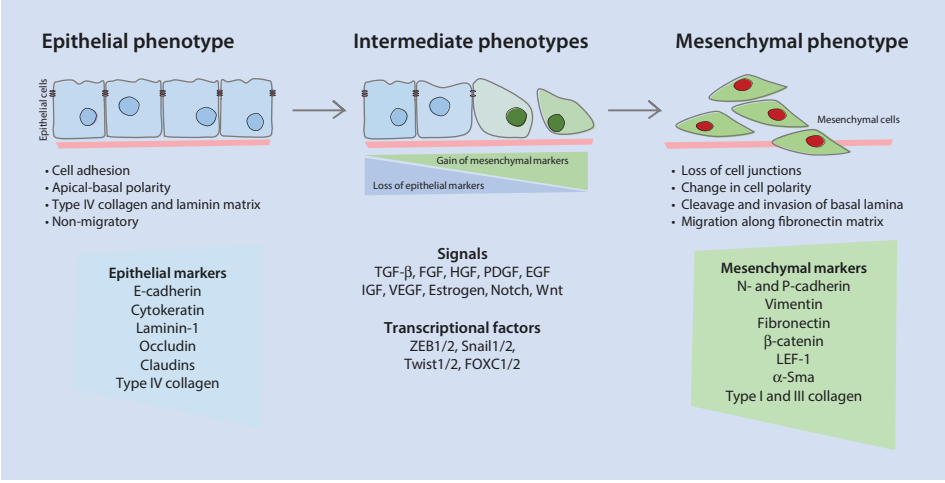


Fig. 9.3 Phenotypic characterization of EMT. EMT involves a functional transition of polarized epithelial cells into mobile and ECM component-secreting mesenchymal cells. The epithelial and mesenchymal cell markers generally used to characterize cell phenotypes during EMT are listed. Co-localization of these two sets of distinct markers defines an intermediate phenotype of EMT, indicating cells that have passed only partly through an EMT. Signals from the environment prompting EMT initiation and EMT-TFs responsible for acquisition of mesenchymal traits are indicated. α-Sma alfa smooth muscle actin, EGF epidermal growth factor, FOXC1/2 forkhead box C1 and C2, FGF fibroblast growth factor, HGF hepatocyte growth factor, IGF insulin-like growth factor, LEF lymphoid enhancer binding factor, N- and P-cadherin neural and placental cadherin, PDGF platelet-derived growth factors, TGF-β transforming growth factor β, VEGF vascular endothelial growth factor, ZEB1/2 zinc-finger E-box-binding 1 and 2. (Adapted from [20])

dle-shaped cells (reviewed by [20] (◘ Fig. 9.3)). The cells **gain of motility** and **migration** during the transition to a mesenchymal state is then facilitated by the formation of new membrane protrusions (such as filopodia e lamellipodia) [23, 24] but also by the production of matrix metalloproteinases (MMPs) (see ◘ Box 9.1) [25]. Protrusive structures appear on the leading edges of migrating cells to guide and assist the direction of the migrating cells. MMPs-dependent degradation of BMb and extracellular matrix (ECM) helps cellular **delamination** and the **invasive behavior** of mesenchymal cells, respectively [3, 24, 26].

Throughout this chapter you will realize that malignant tumor cells not only show similar morphological properties and migratory behavior to embryonic cells, but also display a common gene expression pattern [27–29] (◘ Fig. 9.4). In point of fact, the **touchstone of the EMT co-option process** has its foundations **in the overlap of many Transcription Factors (TFs)** that ensure a robust EMT program during embryonic development and that are also re-used by malignant cancer cells - the EMT-TFs [28, 30]. The very first precedent for this molecular association was the observation that the Snail family EMT-TF, originally described in mesoderm formation during gastrulation and on delaminating Neural Crest (NC) cells from the neural tube, was also identified in cancer cells [31]. Indeed, this data became the kick start for research that helped to unveil the role of EMT in diseases, such as metastatic cancer.

9

Box 9.1
Important activities should be assigned to MMPs:
1. MMPs release cell-surface and matrix-bound latent growth factors and cytokines, such as EGF family ligands, Tumor Necrosis Factor-α (TNF-α), Vascular Endothelial Growth Factor (VEGF) and receptor activator of Nuclear Factor Kappa-B Ligand (RANKL), that can act in an autocrine or paracrine manner to influence cell growth, survival and inflammation [79].
2. MMPs can mediate the proteolytic cleavage of E-cadherin, generating extra-cellular E-cadherin fragments that increase motility [85].

Now, under this "TFs *umbrella*" perspective, let's proceed with the EMT general molecular features. You will see that this process starts with EMT-TFs activation, which are pivotal in controlling the synthesis of proteins involved in the multiple steps of EMT.

9.2.2 EMT Activating Signals

The activation of the EMT process may begin in response to various microenvironemental EMT cues and to **inducing agents** such as, hypoxia, obesity, alcohol, nicotine and ultraviolet light, that trigger several **signaling pathways** (reviewed in [29] (◘ Figs. 9.5)). Such signals may act alone or in combination, as well as their corresponding pathways, in a cell context-dependent manner [33]. The downstream response converges on the EMT-TFs (see ▶ Sect. 9.2.3), which are the keys for the EMT programs.

The most frequently described signaling pathways inducers of EMT are (see ◘ Fig. 9.5 for more details and ▶ Chap. 3):
- Transforming Growth Factor Beta (TGF- β)/Bone Morphogenetic Protein (BMP)
- Fibroblast Growth Factor (FGF)

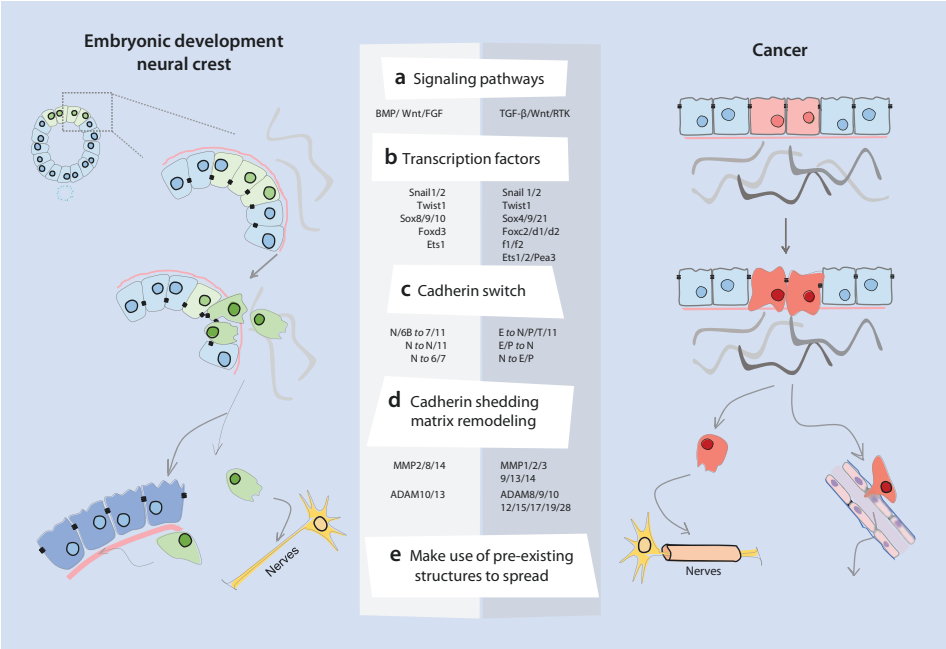
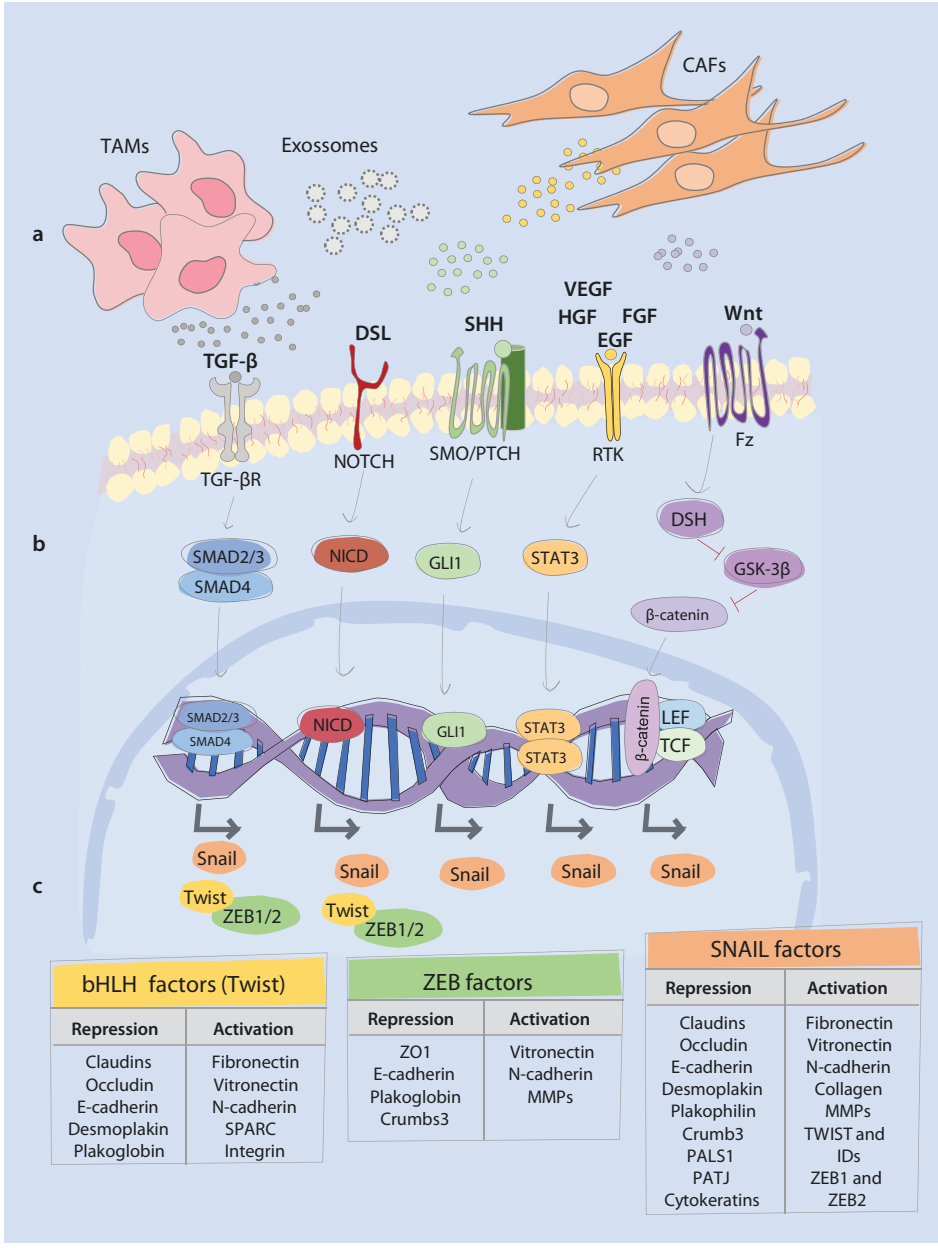


Fig. 9.4 The biological processes of NC cells migration (zoom in from the right side of the roof plate) and metastasis formation exhibit striking similarities. **a** Pre-migratory NC cells and benign tumor cells share similar activation signals of signaling pathways, such as TGF-β/BMP, Wnt and FGF/RTK. **b** These pathways activate the expression of several TFs of the Snail, Twist, SoxE, FoxD, and Ets families that trigger EMT. These TFs are essential for proper NC development and are upregulated in many cancers. **c** Among the phenotypic and molecular changes happening during EMT, the cell-cell adhesion properties are modified allowing NC cells and malignant tumor cells to separate from their original tissue. It should be noted the expression switch between different cadherin types during EMT. **d** In addition, NC cells and tumor cells express various proteinases of the MMP and ADAM families that further contribute to the modification of cell adhesion properties by promoting remodeling of the ECM and shedding of cell surface molecules including cadherins. It is worth noting that in cancer cells the synthesis of theses proteinases is often associated with high invasive potential and poor prognosis. **e** NC cells and tumor cells migrate in a solitary or collective fashion and make use of pre-existing structures to help them in the process. Both rely on nerves but while NC cells also interact with the basement membrane of epithelia, tumor cells interact with the basement membrane of blood vessels. In addition, they both respond to external signals controlling directional migration and homing into specific tissues and organs. See main text for details and references. ADAM a disintegrin and metalloproteinase, BMP bone morphogenetic protein, E-, N-, P- and T-cadherin epithelial-, neural-, placental-, heart-cadherin, Ets E26 transformation-specific, FGF fibroblast growth factor, FOXC1/2 forkhead box C1 and C2, MMPs matrix metalloproteinases, NC neural crest, RTK receptor tyrosine kinase, Sox Sry-related HMG box, TGF-β transforming growth factor β, ZEB1/2 zinc-finger E-box-binding 1 and 2. (Adapted from [32])

- Epidermal Growth Factor (EGF)
- Wnt protein ligands
- Notch signaling ligands

TGF-β signaling is the most well-characterized pathway known to induce EMT and is typically activated by the TGF-β superfamily of ligands which activate a panoply of receptors. The signaling cascade at the cell surface drives the phosphorylation and the formation of SMAD ("Small", "Mothers Against Decapentaplegic") signal transducers complex

(reviewed in [34]). Once inside the nucleus, the SMAD complex can bind directly to the promoter of *Snail*, one of the classical EMT-TFs, to induce its transcription. SMAD complexes and Snail, in turn, can suppress the expression of the genes needed to be downregulated in the first steps of EMT (genes encoding E-cadherin and occludin) [35, 36] (Fig. 9.5). The Notch signaling pathway was also shown to activate the expression of the classical EMT-TFs (reviewed in [34]). In addition to the direct effects mediated by its intracellular domain, Notch also indirectly regulates EMT through the crosstalk with various signaling



bHLH factors (Twist)

Repression	Activation
Claudins	Fibronectin
Occludin	Vitronectin
E-cadherin	N-cadherin
Desmoplakin	SPARC
Plakoglobin	Integrin

ZEB factors

Repression	Activation
ZO1	Vitronectin
E-cadherin	N-cadherin
Plakoglobin	MMPs
Crumbs3	

SNAIL factors

Repression	Activation
Claudins	Fibronectin
Occludin	Vitronectin
E-cadherin	N-cadherin
Desmoplakin	Collagen
Plakophilin	MMPs
Crumb3	TWIST and
PALS1	IDs
PATJ	ZEB1 and
Cytokeratins	ZEB2

pathways [37] (Fig. 9.5). In several tumors, Wnt signaling is inappropriately active and directly induces classical EMT transcription factors (reviewed in [34] (Fig. 9.5)).

In conclusion, an array of extracellular signals (both soluble and matrix/cell-bounded) and overlapping pathways are involved in EMT, suggesting a myriad of EMT programs dependent on specific cellular microenvironment or cell states, in a temporally defined manner.

9.2.3 EMT-TFs Signaling

The EMT-TFs belong to different super-families, including the (reviewed in [38]):

- zinc-finger binding transcription factors Snail1 and Snail2 (also known as Slug)
- zinc finger E-box–binding homeobox 1 (ZEB1) and ZEB2
- basic Helix-Loop-Helix (bHLH) factors such as Twist1 and Twist2
- forkhead box proteins FOXCs (FOXC1, FOXC2)

Fig. 9.5 Correlation between EMT triggering signals, activated signaling pathways and roles of the major EMT-TFs responsible for tumor progression. **a** The multifactorial contribution of some microenvironment players, soluble factors and cells, to the activation of EMT. Basically, all the factors have the ability to direct a specific molecular program through the activation of downstream signaling pathways that support and influence the activation or maintenance of the EMT-TFs network. Of note are the signaling proteins TGF-β, FGF, HGF, EGF and VEGF, originated from various stroma-derived autocrine or paracrine signaling networks that can activate intracellular signaling factors. Other players able to promote the activation of the EMT program, are different types of cells like TAMs and CAFs, as well as exosomes. TAMs act by creating a cancer stem cell niche through juxtacrine signaling, while exosomes function as cargo of molecules that drive cells toward an aggressive phenotype more prone to EMT. **b** Representation of the main signaling pathways involved in EMT. In response to extracellular cues, cell surface receptors trigger different signaling pathways that can cooperate to induce EMT responses. TGF-β, via TGF-βR, activate the TGF-β signaling pathway that, via activation of SMADs, leads to the transcription of several EMT-TFs (Snail, ZEB1/2 and Twist). Virtually, activated SMAD2 and SMAD3 complex with SMAD4 and the trimeric SMAD complex enter the nucleus promoting EMT-TFs expression. Notch receptors can be activated by binding to Delta and Jagged ligands (DSL). After activation, NICD is released through a cascade of proteolytic cleavages and goes to the nucleus to activate several EMT-TFs. In Sonic Hedgehog (SHH) signaling, PTCH1 receptors activate the SMO and GLI family of transcription factors that induce Snail expression. Several growth factors such as EGF, FGF, HGF and VEGF, can induce EMT through RTKs, another type of receptor. Wnt signaling promotes EMT, inhibiting the destruction complex containing GSK-3β, through DSH, to stabilize β-catenin, which is translocated to the nucleus. EMT responses can be increased through crosstalk and cooperation between distinct pathways. For instances, TGF-β signaling can also increase EMT responses initiated by growth factors such as FGF or EGF. **c** Roles of the major EMT-TFs. EMT is driven by Snail, ZEB and basic bHLH transcription factors, like Twist, that repress epithelial marker genes and activate genes associated with the mesenchymal phenotype. Their specific actions in EMT are listed. Post-translational modifications regulate their activities, subcellular localization and stability (ex. the Wnt and RTKs pathways modulate Snail nuclear transport and degradation through GSK3b). bHLH basic helix–loop–helix, CAFs cancer-associated fibroblasts, DSH Disheveled, E- N-cadherin epithelial- neural- cadherin, EGF epidermal growth factor, FGF fibroblast growth factor, GLI glioma-associated oncogene homolog, GSK-3β glycogen synthase kinase 3 beta, HGF hepatocyte growth factor, ID inhibitor of differentiation, MMPs matrix metalloproteinases, NICD Notch intracellular domain, PALS1 protein associated with Lin-7 1, PATJ PALS1-associated tight-junction protein, PTCH ligand binding to Patched, RTKs receptor tyrosine kinases, SMAD SMA ("small" worm phenotype) and Drosophila MAD ("Mothers Against Decapentaplegic") and 2/3 indicates SMAD2 and SMAD3, SMO smoothened, STAT3 Janus kinase (JAK)-signal transducer and activator of transcription, is signal transducer and activator of transcription 3, SPARC secreted protein acidic and rich in Cys, TAMs tumor-associated macrophages, TCF/LEF T-cell factor/lymphoid enhancer-binding factor, TGF-β transforming growth factor-β, TGF-βR TGF-β receptors, VEGF vascular endothelial growth factor, ZO1 zona occludens 1, ZEB zinc-finger E-box-binding

The T cell factor (TCF) transcription factor family member called Lymphoid Enhancer binding Factor-1 (LEF-1), many times downstream of Wnt signaling, can also directly induce EMT [39].

The EMT-TFs Snail1, Snail2 [40], ZEB1 and ZEB2 [41] were shown to bind to E-box sequences in the promoters of cell-cell adhesion, such as E-cadherin, and repress its transcription (see ◘ Fig. 9.5 for details). These TFs are sometimes referred to as E-cadherin repressors due to the critical role of E-cadherin loss in EMT [16]. On the other hand, Twist1 and Twist2 repress the expression of E-cadherin through the induction of Snail, in both *Drosophila* and humans [42, 43]. This transcriptional modulation is not solely mediated by Twist. BMI1, a polycomb-group repressor complex protein, acts in a concerted fashion with Twist to repress E-cadherin and the cell cycle inhibitor $p16^{INK4\alpha}$ [44]. It is, however, important to bear in mind that reduction of E-cadherin can also occur in other non-related EMT biological contexts, and that the solo increased E-cadherin protein levels is insufficient to revert the fibroblastic phenotype [45].

Twist1, in turn, also promotes the induction of filopodia-mediated matrix degradation during EMT and ZEB1 and ZEB2 also increase the expression of genes encoding MMPs, implicating these TFs in various matrix remodeling mechanisms associated with EMT [46].

The Snail and ZEB TFs can also downregulate proteins of the tight junction complex, which include the occludin, claudins, ZO1, and connexins JAM1/A [47, 48].

Globally, these are a few examples on how the EMT-TFs elicit the EMT program by repressing the epithelial phenotype and promoting mesenchymal traits. The regulation of EMT-TFs involves complex interactions, particularly regarding TFs synergistic actions and the use of common pathways (reviewed in [49, 50]). Therefore, multiple phenotypes may be observed while carrying out EMT programs in distinct cellular contexts.

9.3 EMT in Different Cellular Contexts

During our life time, EMT programs are activated in a variety of biological contexts, from healthy processes such as embryogenesis and wound healing to disrupted physiological events like fibrosis and cancer. Although, sharing many common features, the distinct EMTs also have some functional differences enabling their classification in three different types [20] (◘ Fig. 9.6):

- type 1 identifies the developmental EMT
- type 2 denotes those related to wound healing, tissue regeneration, and organ fibrosis
- type 3 associated with cancer

9.3.1 EMT in Embryonic Development: Type 1

Developmental EMT, or EMT type 1, helps cell shape changes during animal development. The paradigm example is the gastrulation process that involves the formation of an embryo with three germ layers: the ectoderm, the mesoderm and the endoderm. These layers derive from a single layer of pluripotent cells, the epiblast. During gastrulation the epithelial-shaped epiblast cells undergo EMT resulting in the disruption of

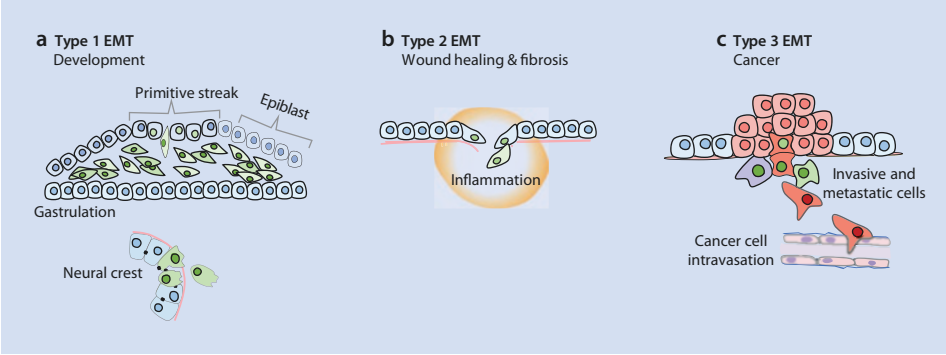


Fig. 9.6 Classification of EMT according to different cellular contexts. **a** Type 1 EMT, is a regulated developmental process and is associated with embryonic implantation, gastrulation, neural crest migration and organ formation. The primitive epithelial layer gives rise to a mesenchymal tissue via an EMT. These mesenchymal cells can be re-induced to form a secondary epithelia by a MET program. Consecutive rounds of EMT and MET occur during development. **b** Type 2 EMT occurs in wound healing and is important for tissue regeneration. Unlike the type 1 EMT, the type 2 EMT is associated with an inflammatory response and can result in organ fibrosis if the primary inflammatory insult is not removed or attenuated. **c** Type 3 EMT, is involved with malignant cell transformation, cancer progression and with cancer stem cell properties. The epithelial cells undergo EMT and acquire invasive metastatic cellular properties. (Adapted from [20])

cell-cell adhesion contacts and ingression of mesenchymal-shaped cells at the primitive streak [51–54] (Fig. 9.6a). Other example of EMT type 1 occurs during neural crest formation and migration. Neural crest cells are a transient embryonic population specified at the border of the neural plate and the non-neural ectoderm (reviewed in [55]). For neural crest formation, precursor cells undergo EMT and delaminate from the roof plate of the neural tube. The mesenchymal-shaped neural crest precursors migrate to different embryonic territories and differentiate into varied cell types (Fig. 9.6a). The developmental EMT can be extended to other transitory morphogenetic events in the embryo (reviewed in [56]).

9.3.2 EMT in Wound Healing, Tissue Regeneration and Organ Fibrosis: Type 2

The EMT process is also an essential component to adult tissue homeostasis. In particular, EMT has an active role in physiologic tissue repair and pathologic fibrosis of tissues and organs. This EMT type 2, unlike type1 EMT, is **associated to an inflammatory response** [20, 57]. In type 2 EMTs, the program is activated after a trauma and inflammatory injury, as part of a repair mechanism that normally generates fibroblasts and other related cells to restore tissue function. As an example, during cutaneous wound healing the keratinocytes at the wound edge lose their intercellular adhesions and migrate across the wound to restore the epidermal barrier (Fig. 9.6b). Actively migrating keratinocytes present elevated Snail2 synthesis that regulates keratinocyte motility during re-epithelialization by repressing E-cadherin which leads to decreased cell-cell adhesion [58–60].

In normal wound healing, the type2 EMT program cease once inflammation is attenuated and once re-epithelialization is complete with many myofibroblasts undergoing apoptosis and disappearing [61, 62]. However, if the primary inflammatory insult is not removed or attenuated, the EMTs program continues leading to tissue fibrosis and eventually to organ destruction. Tissue fibrosis is a permanent form of wound healing due to persistent inflammation (reviewed in [20]).

9.3.3 EMT in Tumors: Type 3

EMT in tumors, called type 3, occurs in neoplastic cells that have previously undergone genetic and epigenetic changes, especially in genes that favor clonal expansion and local tumor growth (Fig. 9.6c). In the simplest terms, the triggers of type 3 EMT are genetic changes that affect mostly oncogenes and tumor suppressors that, acting together with a variety of EMT players lead to malignant transformation (reviewed in [20]). Apart from the above described EMT general cellular modifications detected in types 1 and 2, a new set of cellular features lead to a complex succession of steps termed 'tumor progression' that enables metastatic cell migration through circulation, from primary to metastatic sites. What is then the **role of EMT in cancer progression**? To answer this question, we will give a broad view of the main events and players involved in the acquisition of this particular phenotype along with the respective consequences.

9.4 Cellular Events and Stages of Tumor Progression and Metastasis Formation

As previously referred, during tumor progression, a malignant cell follows a sequence of steps that may conduct to metastasis formation (reviewed in [5] (**see** ▶ Sect. 9.1 and Fig. 9.1)). EMT, as a possible mechanism of metastasis formation, follows a similar pattern as those described in the physiological EMT programs. However, it is important to emphasize the high degree of genetic (different clones) and non-genetic (epigenetic) differences within a single tumor i.e. its **heterogeneity**, that results in cell diversity and therefore in an unequal activation of EMT in tumor cells [63, 64]. Moreover, depending on the **cell location within the tumor,** different subsets of tumor cells may unfold an EMT program to various extents. This is particularly evident in cells at the tumor invasive front that display focal events of EMT, while others keep their physical associations with the main tumor bulk that stays largely epithelial [65].

Another tumor feature contributing to the global heterogeneity of the malignant carcinoma, is that EMT in cancer is not defined by a "one way" course: individual tumor cells may undergo reversible transitions between cell states displaying partial to full mesenchymal phenotype [66] (Fig. 9.3). Not only this means that in several tumors TFs favoring EMT may be active in the 'leader' cell, that still remains attached via *adherens* junctions to the cells just behind [67] but also, above all, this implies that tumor cells consist of a miscellany of distinctive differentiation cell states [68]. In other words, tumor cells can exist in a partial mesenchymal state and exhibit several combinations of epithelial and mesenchymal features. It is unclear how many cellular intermediate states

exist – a spectrum of transitory cells goes from those that have ceased to differentiate while others correspond to stages of tumor progression. Importantly, these **hybrid epithelial-mesenchymal cells (E/M)** highlight an intrinsic and relevant characteristic of tumors – their **phenotypic plasticity** [8, 20, 69]. Phenotypic plasticity, defined as the ability of cells to differentiate and change their phenotype, in some cases repeatedly, is the reflex of the back-and-forth transitions between more or less differentiated cell states [70, 71] (Fig. 9.3). In cancer cells, cell plasticity reaches its maximum exponent. Even if the main agent of cancer are driver mutations profiles (see ▶ Chap. 2) and altered signaling networks, the key molecular players for cell plasticity are EMT-TFs which curiously, are rarely mutated in cancer. Indeed, for interconversion of cells between these alternative states, a complex network of interactions between different EMT-TFs, ubiquitous TFs and epigenetic regulators, is required. All these actions may be transient or stable due to inherited chromatin configurations or autocrine signaling loops that last for multiple cell generations [66].

The bottom line is that tumor heterogeneity and cell plasticity allow cancer cells to adapt and be selected to changing conditions, specially when travelling to their secondary site and when on therapy [72]. This is why it is so difficult to eliminate metastasis: the possibility of a survivor cell is always there.

This being said, we cannot neglect that EMT in cancer context starts and happens in a particular tumor microenvironment (TME). So, shall we visit the basis?

The role of the microenvironment

As in the other the EMT types, in cancer EMT the focal event involving only part of the tumor mass is responsive to local microenvironment and can adopt multiple patterns of invasion from collective to single cell invasion [73–75] (Fig. 9.1). Besides cancer cell genetic background, in each metastatic step, multiple cells and factors are involved, such as:

- diffusible factors, in particular hormones, cytokines and growth factors,
- insoluble factors, like extracellular matrix molecules,
- stromal cells such as Cancer-Associated Fibroblasts (CAFs) and Tumor-Associated Macrophages (TAMs),
- endothelial cells and pericytes,
- immune cells (natural killer, T-, B- lymphocytes),
- Bone-Marrow-Derived Cells (BMDCs).

These cells and factors are concentrated and/or recruited to cooperate and establish a context **tumor-stroma crosstalk** that regulate EMT. This can occur not only at the primary tumor, but also in the secondary niche [76]. As in other systems, migration and targeting to specific tissues are controlled by **external cues**. Let's see some examples:

- The recruitment of pro-angiogenic immune cells may lead to vascular leakiness helping metastatic dissemination [77];
- TAMs have been shown to promote single cell dissemination in carcinomas by local production of TGF-β or helping out on local intravasation by its association with cancer cells and endothelial cells [78];
- CAFs and TAMs, can produce different MMPs that degrade the BMb and the ECM providing the physical pathway for metastatic cancer cells to start moving away from

the primary tumor site [79, 80]. Indeed, the expression of different types of MMPs is associated with a worse prognosis in several cancers, including ovarian [81], breast [82], gastric [83] and colorectal cancers [84].

However, most importantly, the **TME is the essence of the "seed and soil" hypothesis** proposed by Paget [86]. Paget proposed that the organ-preference patterns of tumor metastasis are the product of favorable interactions between metastatic tumor cells (the "seed") and their organ microenvironment (the "soil") (see ▶ Chap. 1). In fact, the significance of the role of TME emerged upon the observation that, either in clinical samples as in experimental models, some tumor cells – even derived from the same organ as the breast – can preferentially go to lung, while others go to the liver or to the brain [87]. Why does this happen? Why some tumor cells "prefer" to invade some organs instead of others? Do they go there and got expelled because are unable to comunicate, or simply do not survive the "ride"? Or are they not attracted at all to the niche while their "kinsman" are? Beyond the genetic and microenvironment factors above mentioned, it was recently discovered that tumor cells use **exosomes to promote the formation of the pre-metastatic niche by "educating" BMDCs** for a pro-vasculogenic and pro-metastatic phenotype. Tumor-derived exosomes, are small membrane vesicles that are shed into circulation, and may contain mRNAs, microRNAs and proteins i.e., messages that can be used to transfer information to target cells in a systemic way. Exosomes recipient cells, mainly BMDCs, undergo genetic and epigenetic changes, that lead to the mobilization of some of these cells to the future metastatic site. Here, they establish an intercellular communication with local stromal cells, preparing a pre-metastatic niche for the arrival of the tumor cells [88–90] and (reviewed in [91])! Illustrating this, is the case of pancreatic tumor-derived exosomes that are able to recruit liver macrophages to establish a pre-metastatic niche in this organ, resulting in an increase hepatic macro-metastatic burden [92].

■ Intravasation and extravasation

After delamination from a primary tumor and local invasion, cells need to intravasate into the blood or lymphatic circulation (see also ▶ Chap. 8). The intravasation process is facilitated by the tortuous vasculature and specially by the local and systemic secretion of several factors that render vessels more permeable [3, 93, 94]. Nonetheless, further molecular changes are needed to help tumor cells to cross the blood/lymphatic vessel barrier.

Cells that were able to go into the vasculature or lymphatics and are carried around the body in circulation are known as **Circulating Tumor Cells** (**CTCs**) [95]. CTCs have to cope with shearing forces, anoikis and immunosurveillance while surviving the harsh conditions in the bloodstream. A related pool of CTCs known as **Disseminating Tumor Cells** (**DTCs**) was shown to settle only in the bone marrow [96]. Intravasated CTCs are the seeds for the subsequent growth of metastasis in vital distant organs [95]. But to do so, they must leave the vessels (extravasation) in order to infiltrate and anchor into another tissue or organ where they form micrometastasis and finally colonize to macro-metastasis (◘ Fig. 9.1) [1, 12]. Again, extravasation is possible because tumor cells can increase the permeability of the vasculature, especially at the site of extravasation where normal endothelial cells are tightly aligned.

■ Colonization… still EMT?

So far, we have seen the multiple actions of the EMT program to support metastatic cancer cells since their release from the primary tumor up to their colonization in a secondary site. Yet, this last stage seems to be the least efficient in the multistep cascade of the metastasizing process. Indeed, infiltrated cancer cells should undergo a continuous proliferation process, which likely depends on their adaptation to a hostile, or at the best, different tissue microenvironment [2].

Having said that, what also seems far-fetched is the role of the EMT program in the macrometastasis development and the appearance of a clinically recognizable tumor. In fact, for this to happen, cells must switch from the migratory to the proliferative mode and regain, at least in part, some of the epithelial features through the reverse process i.e., MET [12, 97, 98].

Although the MET program in the secondary tumor site was questioned for many years, recent *in vivo* observations and *in vitro* studies, encourage EMT/MET hypothesis of metastasis formation. For example:

1. Bone marrow-derived monocytes recruited to the lung stimulate the MET process in metastatic cells by attenuating the levels of phosphorylated Smad2; this results in increased cell proliferation and metastasis growth [99];
2. The frequent detection of E-cadherin-positive metastatic cells derived from E-cadherin-negative breast tumors suggests the presence of MET-stimulating factors at secondary tumor sites [100].

Actually, metastasis found in patients with carcinoma often display the epithelial features, at the molecular and cellular levels, and spatial organization similar to their original tissue [96]. Conceivably, the metastatic population of cells regain heterogeneity, as in the primary tumor cells, due to cellular plasticity [13, 101]. It is this plasticity that allows carcinoma cells to complete the last step of the metastatic cascade: colonization [102, 103].

Wrapping up this topic on 'cellular events and stages of tumor progression and metastasis formation', an important concept to take into account is that **invasive properties and metastatic potential are not equivalent functional terms**. Why is that so? Well, an invading cell may not be able to metastasis. And why is that? Because, after invasion, and in order to become metastatic, a cell must intravasate, survive in circulation, extravasate and colonize the secondary site. These are the reasons why perhaps, from 10,000 tumor cells that enter the circulation, only one is capable of developing a macroscopic metastasis! The plasticity of carcinoma cells is therefore of upmost importance in escaping death during the different stages of tumor progression.

9.5 Uncovering the Molecular Mechanism and Consequences of Metastasis Formation Through EMT

At this point you should have a good understanding of what EMT is, and its contribution to tumor progression and malignancy. Now, we are going to analyze some of the molecular mechanisms of tumor EMT program to realize that this adopted program by neoplastic cells presents some atypical traits.

Keep in mind however, that some clinical data obtained from patients' tissues diverge from experimental data, feeding conflicting views about the role of EMT in cancer biology [5, 104, 105]. The reasons for this can be: (1) the EMT complexity; (2) the use of different experimental models (transgenic mice xenograft implantation and *in vitro* work) [106–108]; (3) distinct methodologies to interpret data. Moreover, data concerning the complete extent of tumor niches involved in EMT induction, as well as previous observations of co-migration of both epithelial and mesenchymal cells and of hybrid epithelial-mesenchymal (E/M) tumor cells phenotype, were not fully taken into consideration.

To tackle these incongruities and foreseeing clinical applications based on EMT, different experimental approaches burst and allowed clarification of some molecular and cellular aspects of EMT during tumor progression. From these we will focus on the:

1. Pleiotropic functions of EMT-TFs in cancer biology
2. Layers of regulation of EMT-MET regulation and metastasis formation

9.5.1 The Pleiotropic Role of EMT-TFs

The diversity of cellular contexts imply that a specific **combination of different EMT-TFs** may be executed. This means that in different tumor types, as in embryonic EMT, specific EMT programs may depend on the tissue of origin. For instance, it was demonstrated that Snail triggers metastasis in breast cancer [109], whereas it has no effect on metastasis in a pancreatic cancer model [105]. Conversely, ZEB1 favors metastasis in pancreatic cancer [110]. Members of the same EMT-TF family can even have antagonistic functions. In melanoma, ZEB1 is tumor-promoting while ZEB2 reduces tumor aggressiveness [111, 112]. Despite the fact that activation of EMT is a common denominator of EMT-TFs, additional properties and functions:

1. In **favoring the acquisition and maintenance of Cancer Stem Cells** (CSCs) (see ◘ Box 9.2). EMT has been associated with increased number of CSCs in several types of cancer [73, 113]. Some authors even propose that stemness of tumor cells may arise as a consequence of the EMT [1, 2]. Based on these, EMT together with stemness can explain tumor initiation in various cell types and tissues, which could hardly be explained only by the classical EMT features. The link between EMT-TFs and CSCs generation is better understood, for instances, when we correlate the high expression of EMT-TFs ZEB1 [114] Snail1 and Snail2 [115] in cancer cells, with the onset of stemness factors, such as Sox2, BMI1 and OCT4 [114, 116]. Clinically, the acquisition of stem-like properties, is associated with aggressiveness of neoplastic cells.
2. As crucial mediators of **cellular plasticity**, that allow the adaptation of cancer cells to the changing and often adverse environmental conditions (see ▶ Sect. 9.4) [38].
3. In **immune evasion**, by upregulating the expression of pro-inflammatory and immunosuppressive cytokines in cancer cells, that alter the composition of the TME creating a chronic inflammatory TME prone for cancer risk [66] (see ▶ Chap. 7).
4. In the **double-strand DNA repair system**, by helping cells to escape from senescence and inducing an anti-apoptotic and pro-survival phenotype favoring tumor development (see ▶ Chap. 5). These repair actions, and respective consequences, are particular evident under various types of stress conditions [38].

5. In the **cancer biology of central and peripheral nervous system tumors** and certain **mesenchymal tumors** (for example, sarcomas). In fact, the term EMT often associated with carcinomas, is shifting because EMT-TFs are frequently expressed and have profound effects in many non-epithelial tumors. These include glioblastomas, melanomas, different sarcoma types and even leukemia [121].

Box 9.2
Cancer Stem Cells (CSCs) are a subpopulation of neoplastic cells that have self-renewal and proliferative properties and therefore are able to initiate and maintain tumor growth (reviewed in [117]). They were identified within leukemias and solid tumors contributing to initiation, maintenance of the tumor growth, metastasis and resistance to conventional (and new) therapies [72]. This population has been characterized by [118, 119]:
- Cellular surface antigens (CD133, CD34, $CD44^{high}$, $CD24^{lo}$, ABCB5)
- Pluripotent markers (SSEA-1, Oct-4)
- Hoechst 33342 dye-efflux capability and
- Aldehyde Dehydrogenase (ALDH) enrichment

Although normal stem cells and cancer stem cells share similar properties they behave differently. Some of the CSCs, as circulating tumor cells, often display other features such as immune evasion, invasiveness, and resistance to different treatments [120]. CSCs are thought to exist in primary tumors from the very early stages of tumorigenesis and may be the oncogenic derivatives of normal-tissue stem or progenitor cells. Metastatic CSCs eventually enter the blood stream and seed a secondary organ. It was shown that a large portion of bone marrow DTCs have stem cell properties and are in a quiescent state.

9.5.2 Layers of EMT-MET Regulation and Metastasis Formation

In agreement with the hallmarks of cancer, the paradigm proposed for the origin of tumors involves the accumulation of mutations and chromosomal genetic changes (such as chromosomal translocation or deletion). These mutations can occur at different times, they are not static and are variable. The after effect is that they are not only responsible for the initiation of carcinogenesis, but also may have an impact on the progression of EMT regulating the development of different types and grades of metastatic cancers [122]. Nevertheless, these genetic changes are quite irreversible and hence did not explain the reversible phenomena of EMT and MET. How can then the EMT/MET reversibility and the cell's transitory states observed during metastasis formation be explained? As we have been claiming, molecular changes occurring in EMT/MET rely on profound changes in gene expression, being regulation by EMT-TFs generally considered the master step in EMT. Yet, besides transcription, multiple layers of reversible and dynamic regulation have been described, namely:
- epigenetic alterations [68]
- microRNAs networks [123]
- long non-coding RNA regulation [124]
- post-transcriptional RNA processing [125]
- translational control and post-translational modifications [50]
- protein stabilization [126]

Epigenetic alterations

Fundamental during the EMT onward process are epigenetic alterations, i.e., the reversible covalent **modifications of histones or DNA residues** (like DNA methylation and DNA hydroxymethylation) that modulate gene expression (Fig. 9.6). By acting in a two-way mode, by default and at any time, these modifications cause back-and-forth stepwise changes, which reinforce the gradual but not simultaneously process of metastasis, and that result in different degrees of cancer metastasis [122]. A molecular "stamp" of human cancer is the inactivation of tumor suppressor genes due to their promoter hypermethylation [127, 128]. Other targets for epigenetic changes include signaling pathways that regulate apoptosis, autophagy and microRNAs [129]. For example, in metastatic ovarian cancers epigenetic suppression of TGF-β signaling was observed [130] as well as methylation of the TGF-β receptors 1 and 2 genes in high grade esophageal squamous cell carcinoma [131].

Globally, the impact of epigenetic changes can be inferred by the observation that they induce pro-cancer characteristics even in mutation-free cells (reviewed in [132]).

MicroRNAs networks

As you know, miRNAs are implicated in the regulation of a variety of cellular processes. In cancer, miRNAs can function either as oncogenes or tumor suppressors [135, 136] (Fig. 9.7a). For example, the miR-200 family members (miR-200 s) are known as **tumor suppressive miRNAs** that inhibit EMT and control stemness by directly silencing the transcription repressor ZEB1/2 and the polycomb repressor complex protein (ex. Bmi1 and Suz12) [137]. However, in different types of cancer the expression of miR-200 is selectively downregulated, by other miR, like miR-22, releasing ZEB1/2 inhibition and therefore activating the EMT program (Fig. 9.7b) [138, 139].

Long non-coding RNA regulation

Other noncoding RNAs, such as long noncoding RNAs (lncRNA), have also been shown to have a role in regulating EMT (reviewed [140]) (Fig. 9.7a). For example, lncRNA H19 is under epigenetic control by hypomethylation (which increases its expression), suppressing E-cadherin [141] in bladder cancer and leading to metastatic cancer progression [142].

Post-transcriptional RNA processing

Post-transcriptional events can strongly contribute to the fine-tuning of EMT. More particularly, in response to developmental or environmental cues, **Alternative Splicing** (AS) of specific genes can give rise to different protein isoforms that affect cell-cell contacts, polarity and cytoskeleton, modulate the balance between self-renewal and differentiation, thus contributing to EMT plasticity during malignant transformation [143].

How is AS induced? An archetype of this, is given by the family of Epithelial Splicing Regulatory Proteins 1 and 2 (ESRP1 and ESRP2) that act as transacting factors, coordinating epithelial cell-type-specific AS programs [144]. During EMT, ESRP1 activity is reverted due to its transcriptionally inactivation. ESRP1 is among the most downregulated genes in multiple EMT model systems [145].

Somehow predictable, dysregulation of splicing factor's function occurs frequently in human tumors, which also suggests the importance of AS regulation for cancer cells to acquire unique features that confer advantages over surrounding cells and sustain tumor malignancy [146].

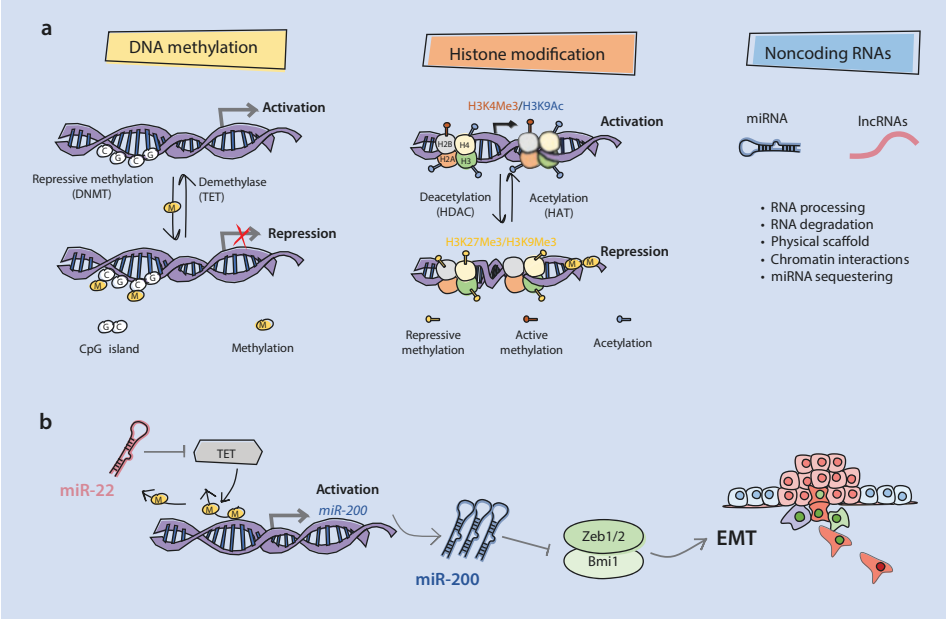


Fig. 9.7 Main epigenetic and non-coding RNA mechanisms involved in regulation of EMT. **a** EMT-TFs are able to recruit methyltransferases (DNMTs), that methylate DNA leading to gene repression, or DNA demethylases, like TET (Ten eleven translocation), that allow gene activation. A large number of histone-modification enzymes like histone acetyltransferases (HATs) and histone deacetylases (HDACs), have been identified in complexes with EMT-TFs affecting locus accessibility or chromatin compaction during gene regulation, respectively. Further specific histone acetylations will mark this locus for gene activation. Regulation of EMT by noncoding RNAs include two main types of regulatory RNA molecules: microRNAs (miRNAs) of size 21–25 bp (base pairs) and long noncoding RNAs (lncRNA), with transcripts > than 200 bp. Both types control EMT by either directly affecting the expression of EMT-TFs, epigenetic modifiers and chromatin-remodeling complexes. miRNAs specifically lead to mRNA degradation, translational blockage, or deadenylation. lncRNA mode of action includes direct transcriptional regulation, interference with RNA processing, modulation of miRNA expression, and act as a scaffold molecule for the formation of protein complexes. **b** Model for the role miR-22 during EMT. Epigenetic inactivation of the tumor repressive miR-200 occurs through directly targeting of the TET family of methylcytosine dioxygenases that can specifically erase existing methylation marks. This family of enzymes initiate DNA demethylation by converting the modified DNA base 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC). miR-22, specifically, decreases the level of 5hmC by negatively regulating TET family. When this happen the *mir-200* promoter will keep its methylation marks and its expression is silenced. Consequently, no anti-metastatic action of miR-200 is exerted promoting EMT and stemness toward metastasis. miR-22 showed to be a crucial epigenetic modifier in breast cancer patients leading to poor clinical outcomes. (**a** Adapted from [133]; **b** Adapted from [134])

9.6 EMT Therapeutic Targets and Cancer Drug Resistance

The identification of EMT program opened the door for therapies aiming at EMT targets [147–149]. Some examples are given in Table 9.1.

Besides these specific targets, the development of epigenetic drugs (such as histone deacetylase (HDAC) inhibitors) was also expanded. It appears that, these drugs enhance the action of conventional therapeutics by demethylating tumor suppressor genes, allowing their re-expression and subsequent inhibition of cancer progression [150, 151].

Table 9.1 Inhibitors of EMT markers in clinical phase trials

Therapy	Mode of action	Target	Stage	Cancer
ADH-1	Vascular-targeting; disruption of tumor vasculature through N-cadherin blocking	N-cadherin	II	Mela-noma
Sorafinib	Small inhibitor of several tyrosine protein kinases	TGF-β, VEGFR, PDGPR, Raf	III	Hepato-cellular
Batimastat	Angiogenesis inhibitors; acts as a m MMP inhibitor by mimicking natural MMPI peptides	MMP	I	Hepato-cellular
Pictilisib	PI3k inhibitor	PI3K	II	Breast
Metformin	Antihyperglycemic agent	ZEB, SNALL2, TWIST. Vimentin	III	Pancreatic

Adapted from [149]

Another valuable diagnostic and anti-cancer treatment is based on the oncogenic alternative splicing-variants specifically expressed during tumor EMT and in CSC [116, 152, 153]. In fact, optimization of antisense oligonucleotides-based approaches are being developed to selectively control splicing switches [154–156]. However, in 1992 Sommers et al. suggested that chemotherapy may lead to activation of EMT [157]. Since then, it has been observed that upon similar treatments, different types of cancer display an increased number of cells expressing mesenchymal markers accompanied with cancer drug resistance and clinical relapse [158, 159]. The simplest explanation is that mesenchymal-like cells are better adapted to cell deformation, resisting shear stress and being more resistant to drugs. Other plausible mechanisms to confer chemoresistance is the cellular plasticity underlying the EMT-MET processes [96, 160–162], or the alleged CSC pool composed by multiple phenotypic sub-populations within a single tumor [72].

In conclusion, although elucidation of the exact steps and molecular regulation of EMT have been helping in the development of improved anti-metastatic therapies that may be useful against circulating metastatic cancer cells and chemoresistant cancer cells, targeting EMT effectors did not appear to be a successful strategy due to the inherent transience of each phase of the metastatic progression [163]. Until now it was observed that EMT inhibitors are most effective on the EMT-associated outcomes and not so much on tumor cell proliferation and survival. This suggests that EMT inhibitors will need to be utilized in combination with established anti-tumor drugs [148]. Nevertheless, hope exists for the identification of EMT biomarkers that will help develop therapeutic strategies to fight metastasis initiation, relapse and to predict responses to specific treatment regimens in personalized cancer medicine, turning a fatal metastatic cancer into a chronic condition [164]. The identification of other developmental mechanisms active in cancer could also provide new targets to reduce tumor and metastasis progression besides outdoing therapeutic resistance.

9.7 Final Remarks and Future Directions

For cancer biologists, the greatest interest in EMT programs derives from their association with the processes of invasion and metastasis. Although, collectively, all the findings related to EMT and cancer offer a perspective of applicability in prognosis [66] several questions are still pending due to biased results, different biological contexts and the inherent complexity of this disease. Nevertheless, it is generally accepted that the EMT program is important for the progression of metastatic disease, but manifests differently in distinct cancer types [105, 106, 165].

Advanced technologies in genomic and cellular imaging, better experimental models and clinical oncology knowledge of the different metastasis arrangements will allow a more exhaustive tracing and characterization of the EMT mechanisms underlying the formation of metastatic tumor cells and will definitely conduct to a better understanding of the fate and vulnerability of tumor cells at distinct stages of disease progression and during treatment.

Take Home Message

This chapter aims at conveying the following messages:

- The EMT program may represent the first step in the metastatic cascade of malignant epithelial cancers to more aggressive stages.
- The EMT program is activated in some cancer cells of a tumor allowing them to break down the BMb, invade into the stroma (local invasion), enter and exit the blood and lymph circulation (intravasation and extravasation) and manage to survive before they can be kept at a secondary organ (metastatic site), and grow (colonization) into clinically detectable metastasis.
- Metastasis formation includes both EMT and the reversible process of MET to revert mesenchymal-like cells to an epithelial-like state and colonize a new destination.
- Exosomes derived from the primary tumor can act as "facilitators" to prepare the pre-metastatic niche.
- Activated EMT-TFs are pivotal in controlling the expression of proteins involved in several processes responsible for EMT cellular phenotype.
- In carcinoma cells, EMT is regulated by diverse molecular mechanisms mainly, tumor microenvironment cues, molecular networks of signaling pathways, transcription factors, epigenetic modifications, alternative splicing and microRNAs.
- Tumor populations are highly heterogeneous. Not all transformed cancer cells are able to undergo EMT at the same time and not all cells that have activated an EMT program become competent to form metastasis since during dissemination they must overcome several physical and chemical barriers.
- Most cancer cells where EMT is activated often go through an 'incomplete' or partial EMT giving rise to of epithelial/mesenchymal hybrids.
- Invasive properties and metastatic potential are not equivalent functional terms.
- In cancer, EMT is linked to metastasis formation, as well as, CSC generation and maintenance.
- Epithelial-mesenchymal plasticity allows cancer cells to undergo functional adaptations during the invasion-metastasis cascade.
- Cellular plasticity in cancer can lead to chemotherapy resistance.

Questions

1. Define epithelial-mesenchymal transition.
2. Why carcinomas may require EMT to progress?
3. Indicate the progressive steps of metastasis formation mediated by EMT.
4. Characterize the tumor microenvironment indicating their main cellular and soluble components.
5. What are the signaling pathways that define the EMT process?
6. Give an example where is evident a functional molecular network between tumor microenvironment cues, signaling pathways and transcription factors regulating a specific EMT event in carcinoma cells.
7. In what sense the roles of the TF involved in EMT are pleiotropic?
8. What are the main molecular mechanisms that regulate EMT? Give two examples.
9. Explain the molecular fundamentals of the 'seed and soil' hypothesis.
10. How do you define phenotypic cell plasticity? Why does tumor cell plasticity favor progression to metastasis formation?
11. How is EMT related with possible new therapies? And what is the basis for drug resistance due to EMT programs in cancer?

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Case Studies: Molecular Pathology Perspective and Impact on Oncologic Patients' Management

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What You Will Learn in This Chapter

In this chapter you will have a medical perspective of cancer, how it is diagnosed and treated. In particular we focus on the crucial role of pathology assessment for diagnosis/staging and guide patient treatment/management. We will give an historical view of pathology, how it evolved through the centuries from the standard histopathology methods to this new era of molecular analysis. Also, we will generally describe the different treatment options available. Then, through the discussion of three case studies we will illustrate how pathology evaluation (classical and molecular) gives a window to tumor biology, providing a framework for patient treatment and follow-up.

Learning Objectives

After reading this chapter you should:

1. Have an idea of why pathology, and more specifically molecular pathology, is important for better understanding the molecular alterations that occur during tumor initiation and progression, and how this can impact in the clinical management of oncological patients.
2. Know how the specialty of pathology has evolved from its beginnings, which are the main fields comprised in this discipline, and which are the different techniques currently used in the oncological clinical setting, with their advantages and limitations.
3. Recognize the nomenclature used to describe different types of neoplasms (e.g. benign versus malignant, depending on tissue type) as well as the staging systems for classification of malignant neoplasms and their implications in patient prognosis and election of specific therapeutic strategies.
4. Understand the usefulness of applying molecular biology techniques in the oncology setting using human specimens (both cells and tissue samples) to obtain predictive and prognostic markers to guide patient management.
5. Learn the different therapeutic approaches that are currently available for cancer patients, depending on their disease.

Important Concepts Discussed in This Chapter

- Determination of the tumor stage is essential to assess the prognosis of the patient and to select the most suitable therapeutic approach.
- Molecular advances have allowed a better understanding of the disease, improvement in diagnosis accuracy and development of new targeted therapies.

10.1 Cancer

Lifetime risk of cancer diagnosis, according to 2018 GLOBOCAN database in humans is about 20% in male and 16% in female [1]. Approximately one-in-eight men and one-in-eleven women will die from cancer. Autopsies of individuals dying of other causes reveal many covert malignant cancers and pre-malignant carcinomas in prostate, breast, kidney, thyroid and other sites [2–4]. For both sexes, the leading cause of death was cancer of the lung (18.4%). For men, the 2nd most common cause was cancer of the colon-rectum and for women breast cancer [1].

10.2 Basic Nomenclature

Before starting to talk about Pathology, we need to have a good understanding about the meaning of different terms that we will use throughout this chapter. **Anatomy** is a branch of science that studies the structure of organisms, establishing the position, relations, structure, and function of the different organs. This discipline is based in gross examination of the organs after dissection of the organism. **Histology** explores the microscopic structure of tissues that form the different organs, and as its definition implies, microscopic visualization is mandatory to perform histological analyses. Tissues are composed by cells and extracellular components, and the branch of science concerned with the structure of cells is called **Cytology**. Finally, **Cell Biology** is the study of cell structure and function, including its physiological properties, metabolic processes, signaling pathways, chemical composition and interactions with the environment. The field of cell biology is based in the fact that the cell is the fundamental unit of life, as described by Virchow (see ► Sect. 10.3).

When we observe abnormal tissues under the microscope, we refer at this specialty as **Histopathology**, derived from the combination of two terms: "Histology" and the Greek word "páthos", which means disease. Similarly, **cytopathology** is the branch of pathology created by **Georgios Nikolaou Papanikolaou** that studies diseases by looking at free cells (for example from vaginal smears, bronchial secretions, urine, etc).

In the past decades, a new discipline within pathology emerged, **Molecular Pathology**, which is focused in the study and diagnosis of disease through the examination of molecules (e.g. proteins, DNA, RNA) within tissues, cells or body fluids, which in turn provides more information.

10.3 History of Pathology

Pathology is a medical discipline that has evolved enormously since it was developed. The origins of academic pathology observations are dated back to the XVI and XVII centuries in Italy, where autopsy procedures were systematically performed to elucidate the cause of diseases. **Antonio Benivieni** (1443–1502) carried out many autopsies and correlated the anatomic alterations with the cause of death, for which he is considered the father of pathologic anatomy. It was some centuries later that **Giovanni Battister Morgagni** (1682–1771), considered the father of modern anatomic pathology, introduced the anatomo-clinical method in medicine by correlating clinical symptoms and signs in patients with anatomic findings during autopsy [4]. This descriptive pathology was mainly based on anatomic gross findings, and it was later succeeded by the French and English tissue-based pathology schools, whose main representative is the physiologist and pathologist **Marie-François Xavier Bichat** (1771–1802), considered the father of histology. Although he did not use the microscope for his evaluations, he was able to distinguish up to 21 different types of tissues, which by different combinations formed all the organs of the human body. With the use of the microscope to visualize tissues, pathologists from the German school led by **Rudolf Ludwig Carl Virchow** (1821–1902) described that tissues were constituted by small units, named cells. He studied human diseases following the "cell theory", developed in conjunction with physiologist **Theodor Schwann** (1810–1882) and botanist

A. Steps of sample preparation

1. Chemical fixation
2. Grossing
3. Processing
   - 3.1 Dehydration (ethanol)
   - 3.2 Clearing (xylene)
   - 3.3 Impregnation (paraffin)
4. Embedding-block generation
5. Block sectioning
6. Slide generation and staining
7. Histopathological assessment

Fig. 10.1 Processing of tissue specimens. Summary of steps for processing of the "fresh" specimens to obtain tissue sections for histological evaluation and illustration of the most important steps

Matthias Jakob Schleiden (1804–1881) who defined that all living organisms are made up of cells, and that these cells are the basic unit for structure, function and reproduction [5]. His theory defended that alterations in the cells were the bases for understanding disease. During the next centuries, different schools of pathologists have described and classified diseases, based in microscopic evaluation, including cancer. Technology has incredibly evolved since then, but current Departments of Pathology worldwide are still using the same basic principles for histological evaluation, including Hematoxylin and Eosin staining (see ▶ Sect. 10.4) developed at the beginning of the twentieth century. The main objective of anatomic pathology is to give a diagnosis after observing the histological features of the specimens that have been excised from a patient; if it corresponds to a neoplastic lesion, pathologists also analyze additional parameters (such as tumor staging and immunohistochemical results, described below) which provide oncologists with information about patient prognosis and can guide therapeutic approaches.

In the next section we will describe the methods that are routinely used in all pathology services with very few variations, which comprise several steps since the pathologist receives the surgical specimen until he has the tissue slides to analyze under the bright-field microscope (Fig. 10.1A).

10.4 Standard Histology Methods

Surgical specimens, from skin biopsies to large resection specimens such as total colectomy (resection of all the large intestine) are resected from the patients and the surgeons send them to the pathologists to be processed to obtain a histological diagnosis. Usually biopsies are performed when a lesion has been identified in a person (for example in a patient who has undergone a colonoscopy, if a polyp is identified in the mucosa) to get a histopathological diagnosis and know if it is malignant or not. On the other side, surgical specimens are resected when a patient has a lesion for which surgery is the best primary

therapeutic approach; pathologists then will be able to get more information from this surgical specimen, such as the stage, molecular features of the tumor and prognosis (see below ▶ Sect. 10.5). When specimens are obtained in the operating room, without any fixation they are called "fresh tissues", since they have not been processed yet. Other than for clinical diagnosis of the patient's disease, these "fresh" specimens may be used for research purposes, for example to generate primary cell lines or patient derived xenografts. Also, specific pieces of tissue can be frozen immediately after surgery for further analyses such as DNA/RNA sequencing. It is very important that a pathologist or a trained technician observes the samples to give a piece of tissue to the researcher, exactly knowing what this specimen is (e.g. tumor tissue, adjacent non-tumoral tissue). In order to generate slides from these "fresh" specimens, tissues need to be processed following several steps, which include fixation, grossing, tissue processing, paraffin embedding, block generation, block sectioning and slide staining (◘ Fig. 10.1A, see video from biogenex [6]).

A critical process that needs to be done, by observing the surgical specimen and collecting different pieces for further analyses is called **grossing**. During this process, the characteristics of the surgical specimen will be extensively described, including the aspects that are more important for the pathologist to give an accurate histological diagnosis. For example, for oncological specimens, it is very important to describe the size and location of the tumor, the depth of invasion, the distance to the surgical resection margins, etc. (◘ Fig. 10.1A.2). Grossing can be done when the specimen is "fresh" or after it has been put into a fixative. **Fixation** is a critical step in the processing of the "fresh" specimens that ensures the preservation of the tissues avoiding decay due to autolysis. Fixation needs to be done as soon as possible after the specimen is taken out from the patient, to reduce to the minimum de so-called "ischemia time" (time since the organ does not receive arterial blood until it is fixed). In this context, it is also important to know that "ischemia time" is one of the limiting parameters for obtaining high quality molecules both from "fresh" and fixed specimens [7, 8]. Fixation will permit the preservation of tissue structure, but most importantly, it will ensure the preservation of all the intracellular components, from organelles to proteins and nucleic acids, allowing further molecular analyses on the fixed specimens. In most pathology departments, fixation is done with 10% buffered formaldehyde, but there are other fixatives that may be used for different purposes such as 4% paraformaldehyde, ethanol, glutaraldehyde, etc. During grossing, representative pieces of tissue (with a specific size of no more than 2 cm × 1.5 cm, with a maximum thickness of 0.3 cm to allow optimal tissue fixation by penetration of the fluids) will be collected and put in plastic cassettes (◘ Fig. 10.1A.2). Tissue will then undergo **processing** and **paraffin-embedding**, nowadays performed in automated processors which include a set of chemicals for dehydration, clearing and impregnation (with melted paraffin, ◘ Fig. 10.1A.3). Once the tissue has been processed, it is taken from the cassette and a solid paraffin block is generated by using melted paraffin and allowing it to solidify in a cold plate (**block generation**, ◘ Fig. 10.1A.4). After the block is ready, we can cut it in thin slices using a microtome and put them on top of a glass slide (**block sectioning**, ◘ Fig. 10.1A.5). Sections for histological analyses usually have a thickness of 5 μm. This slide with the unstained tissue section is called "blank slide" and although usually it is stained with different dyes for microscope evaluation, it can also be used for other purposes such as nucleic acid extraction. Last step of processing consists in **slide staining,** nowadays performed in most pathology services using and automated system (◘ Fig. 10.1A.6).

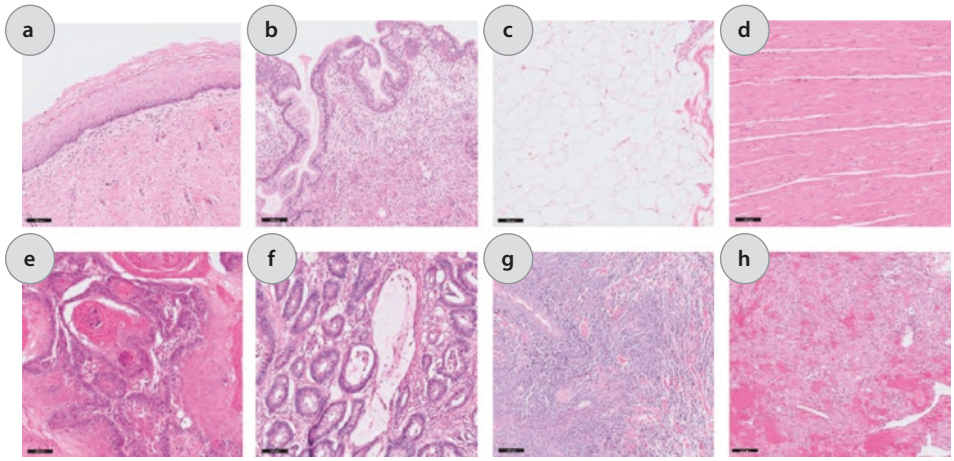


Fig. 10.2 Histological images stained with H&E. **a** Normal histology of a squamous stratified epithelium of the exocervix of the uterus (epithelium on top, stroma on the bottom); **b** Normal histology of the glandular cylindrical epithelium of the endocervix of the uterus (epithelium on top, stroma on the bottom); **c** Normal histology of adipose tissue. Note that the cells have a wide clear cytoplasm due to the disappearance of the fatty acids by processing, and only the cytoplasmic membranes and small nuclei are evident; **d** Normal histology of smooth muscle. Note the cells with spindle shaped nuclei and organized forming fascicles; **e** Representative histological appearance of keratinizing squamous cell carcinoma, constituted by solid nests of atypical cells, with large cytoplasm and production of cytokeratin pearls (eosinophilic material); **f** Representative histological appearance of adenocarcinoma, constituted by atypical cells that form glandular structures with different sizes and shapes; **g** Representative histological appearance of a lymphoproliferative lesion, constituted by round cells with little cytoplasm; and **h** Representative histological appearance of an Kaposi sarcoma, which is a malignant proliferation of vessels, constituted by spindle shaped cells which leave some spaces between them filled with red blood cells (reddish anuclear areas)

Many different coloration methods exist, but the standard staining used in anatomic pathology is Hematoxylin and Eosin (H&E), which is a dual color staining procedure that allows visualization of the cellular compartments. The principle of the staining is based in the fact that the acidic components of the cell (such as nucleic acids in the nucleus) have affinity for basic dyes such as Hematoxylin, whereas basic components (such as most proteins in the cytoplasm) will have affinity to the acid dye Eosin. Hematoxylin after oxidation stains in blue whereas eosin is pink, and the different mixtures of these two dyes gives rise to the standard histological image of a tissue specimen. Figure 10.2 shows a set of H&E images, both from normal and tumor specimens, where it is evident that cell nuclei are stained in dark purple, whereas the cell cytoplasm and extracellular components vary between different intensities of pink to light purple. Other stains can be used to detect specific components of the cells, such as Periodic acid–Schiff (PAS) staining for glycogen and certain mucins, Alcian blue for acidic mucins, Oil Red O for lipids, etc.

Depending on the characteristics of the cells (shape, size), cell organization (forming glands or not) and other extracellular components, pathologists are able to recognize histological patterns of the normal tissues in the different organs as well as pathological patterns to classify different types of neoplasms. In the next section we will explain how pathologists classify neoplasms and which is the most commonly used staging system.

10.5 Histopathological Analyses of Tumor Specimens: Importance of Staging

A standard nomenclature in pathology is used for classification of neoplasms arising from the different tissues, with few exceptions (■ Table 10.1). Benign neoplasms are named using the suffix "-**oma**", whereas malignant neoplasms are named using the suffix "-**carcinoma**" if they are derived from epithelial tissues or "-**sarcoma**" if they derive from mesenchymal tissues. Also, when tumors are characterized by formation of glands, they have the prefix "adeno-". For example, if we have a benign tumor derived from a glandular epithelium of the colon, we call it "aden**oma**", but if it is malignant, we will call it "adeno**carcinoma**". Similarly, if we have a benign tumor derived from fat tissue, we will call it "lip**oma**", whereas the malignant counterpart will be named "lipo**sarcoma**". As usual, exceptions exist, and for example a lymphoma is a malignant tumor derived from the lymph nodes although its name ends in -oma.

Another very important feature in oncological diseases that comes out from the histological evaluation (including the grossing characteristics) is the **staging** of a neoplasm. The staging system is mainly used to assess the prognosis of the patient, but also to determine which therapeutic approach is recommended (for example, a patient with localized disease to an organ and no distant metastases could undergo surgery as first approach to control his/her neoplasm, whereas a patient with metastatic disease will need to be given systemic therapy, see ▶ Sect. 10.7).

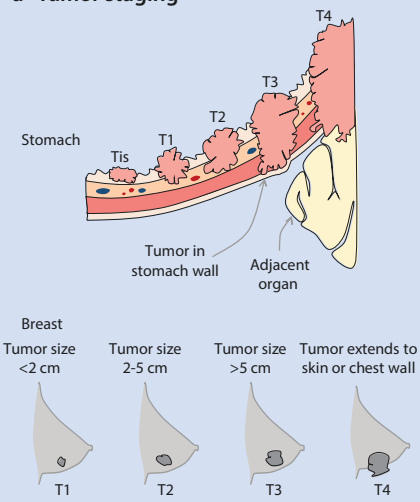
The most widely used system of staging is the **TNM classification** [9], which considers:

- the status of the tumor (T),
- regional lymph nodes (N) and
- metastasis (M).

■ **Table 10.1** Neoplasms nomenclature

Type of tissue	Benign neoplasia	Malignant neoplasia
Epithelial	**-oma**	**- carcinoma**
Glandular epithelium	adenoma	adenocarcinoma
Squamous epithelium	Squamous cell papilloma	squamous cell carcinoma
Mesenchymal	**-oma**	**- sarcoma**
Connective tissue		
Adipose tissue	lipoma	Liposarcoma
Smooth muscle tissue	leiomyoma	leiomyosarcoma
Endothelium and other		
Blood vessels	hemangioma	Angiosarcoma
Lymphatic vessels	lymphangioma	lvmohangiosarcoma
Other tissues		
Lymphoid tissue		Lymphoma
Hematopoietic cells		Leukemia

a Tumor staging



b Prognostic stage groups (colon)

When **T** is …	and **N** is …	and **M** is …	the **stage** group is …
Tis	N0	M0	0
T1. T2	N0	M0	I
T3	N0	M0	IIA
T4a	N0	M0	IIB
T4b	N0	M0	IIC
T1 – T2	N1/N1c	M0	IIIA
T1	N2a	M0	IIIA
T3 – T4a	N1/N1c	M0	IIIB
T2 – T3	N2a	M0	IIIB
T1 – T2	N2b	M0	IIIB
T4a	N2a	M0	IIIC
T3 – T4a	N2b	M0	IIIC
T4b	N1 – N2	M0	IIIC
Any T	Any N	M1a	IVA
Any T	Any N	M1b	IVB
Any T	Any N	M1c	IVC

Fig. 10.3 TNM classification of neoplasms. **a** Tumor staging may depend on the depth of invasion of the organ wall (e.g. in stomach) or tumor size (e.g. breast); **b** Summary of prognostic stage groups for colon adenocarcinoma, which is determined by putting together the TNM. (Adapted from reference [9])

Staging can be done clinically (named cTNM), by imaging (named iTNM) or histopathologically (named pTNM), the three of them being useful for patient management and therapeutic decision-making.

Tumor status in some of the organs depends on the depth of invasion (for example all hollow organs such as the urinary bladder, esophagus, stomach or small and large bowel), but in other organs the size of the tumor determines the T stage (for example in the breast, lung or kidney) (Fig. 10.3a). T status can go from T0 to T4. N status depends on the number of lymph nodes that are invaded by neoplastic cells and it goes from N0 (no lymph node metastases) to N3. Finally, M status depends on the absence (M0) or presence (M1) of distant metastases. The combination of TNM scores defines the "prognostic stage group" which goes from stage 0 (no invasive tumor identified) to stage IV (advanced metastatic disease) (Fig. 10.3b).

10.6 Methods in Molecular Pathology

Histological analyses of H&E stained slides most of the times is enough to get a diagnosis of a tumor, but in the last decades with the development of molecular pathology, the advance of different techniques such as immunohistochemistry (IHC), cytogenetics, analysis of DNA/RNA content and other genetic assays have added valuable content to cancer diagnosis [10]. These methods, particularly IHC, have significantly enhanced our ability to define the lines of differentiation of human tumors. Molecular pathology aims to identify the alterations involved in the development and progression of neoplastic diseases. Clinically relevant objectives of molecular pathology include the establishment of a

definitive diagnosis and classification of tumors based on unique molecular aberrations that occur in specific tumor types, rendering prognostic information through the assessment of molecular predictors of outcome and assistance in the selection of individualized treatment regimens depending on tumor features.

In the next sections we will shortly describe some of the most commonly used technologies in current molecular pathology.

10.6.1 Immunohistochemistry (IHC): Immunofluorescence (IF)

Immunohistochemistry (IHC) is an auxiliary technique routinely used in most pathology laboratories to define phenotype through the analysis of molecular expression in cells and tissues of all their components. Compared to other protein analysis techniques, such as Western Blot, IHC has the great advantage of being able to reveal exact protein location within the examined tissue, and even more importantly its subcellular location (e.g., nucleus, cytoplasm, cell membrane, or even intracellular compartments such as Golgi apparatus, nucleolus). In addition, it allows for assessment of functionality, since active (e.g., phosphorylated protein isoforms) or inactive (e.g., deglycosylated proteins) molecules can be defined at microanatomical detail. Today it represents an important tool for scientific research as well as a fundamental complementary technique in the elucidation of differential diagnosis in the clinical settings which are not determinable by conventional H&E analyses.

The immunostaining procedure is based in the use of primary antibodies that recognize specific cell and tissue structures, such as protein epitopes (antigens), in histological specimens or cells. Following a set of steps, the final visualization of the reaction between the antibody and the antigen can be accomplished in different ways. For chromogenic IHC, a secondary antibody binds to the primary antibody and then a high-affinity molecule is conjugated to an enzyme, such as peroxidase or alkaline phosphatase, which in turn catalyzes a color-producing reaction that can be then visualized in the cells or tissue using a bright-field microscope (Fig. 10.4a, **left panel**, see video from biogenex [11]). Currently, in most pathology laboratories IHC is performed in an automatized way, using specialized equipment that increases reproducibility and allows for better quality control of the procedures. As an alternative, in immunofluorescence (IF) staining, the antibody (primary or secondary) or the high-affinity molecule can be labelled with a fluorophore, which can be then visualized using a fluorescent microscope with the specific corresponding filters (Fig. 10.4a, **right panel)**. Advancement in the past years in antibody generation, in IF protocols, and in microscope systems has allowed the development of "multiplex immunofluorescence", which permits the quantitative analyses of several antibodies labelled with different fluorophores in the light spectrum (from 350 nm to 700 nm) stained in the same slide using a multispectral camera [12].

10.6.2 *In Situ* Hybridization (ISH): Fluorescent ISH (FISH)

Similar to IHC that is able to identify proteins in tissues or cells, *in situ* hybridization (ISH) serves to identify nucleic acids, allowing spatial information about gene expression and specific genetic *loci*. The principle of ISH is based on the complementary pairing of

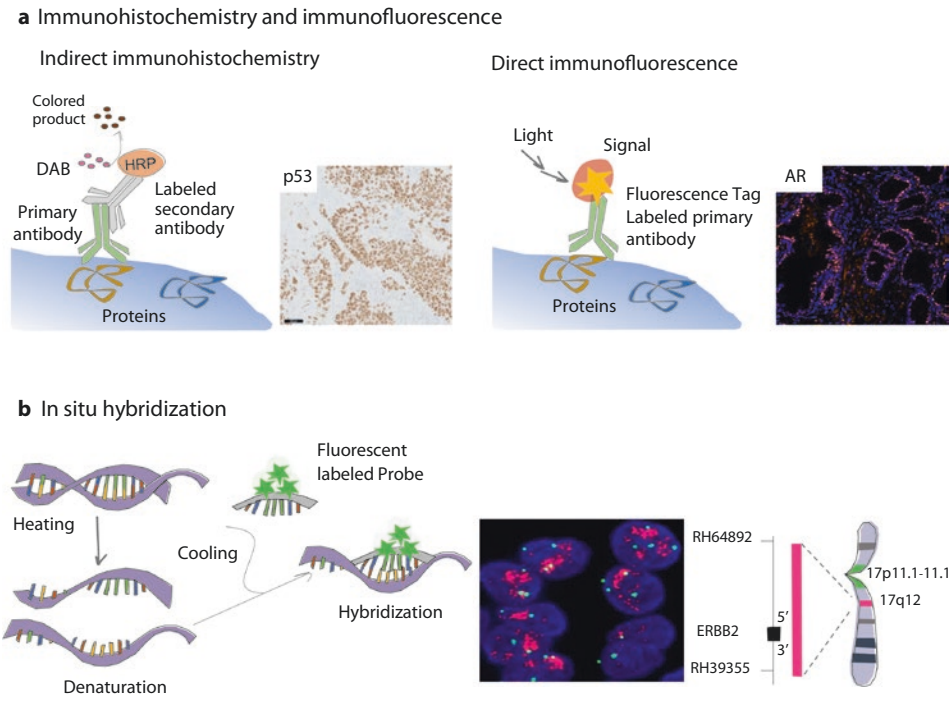


Fig. 10.4 Immunohistochemistry/Immunfluorescence procedures and *in situ* hybridization (fluorescent *in situ* hybridization). **a** Schematic illustration of the technical procedure for immunohistochemistry (IHC) and immunofluorescence (IF). Representative images obtained by the different techniques. Left: p53 IHC. Note that standard immunohistochemistry is done using diaminobenzidine as a substrate which gives a brown color to the areas that express the protein of interest; Right: androgen receptor IF. Note the nuclear expression in a prostate cancer tumor in orange color; **b** Fluorescence *in situ* hybridization (FISH) methodology. Schematic representation of the Her2Neu locus specific probe in red and the centromeric probe (CEP17) in green and FISH image showing an amplification of the Her2Neu locus in a breast cancer tissue. Note that nuclei are counterstained with Hematoxylin for IHC and with DAPI for fluorescently labeled techniques

labelled DNA or RNA probes with nucleic acid sequences in intact chromosomes, cells or tissue sections (Fig. 10.4b, see webpage from Scitable, a Collaborative Learning Space for Science [13]). Also similar to IHC, there are two basic ways to visualize RNA and DNA targets *in situ*, chromogenic (CISH) or fluorescence (FISH) detection, which can be visualized by bright-field and fluorescent microscopy, respectively. FISH is routinely used in pathology services to study different genetic aberrations, by using different sets of probes, which can be specific for the genes of a locus of interest or chromosomic centromeric probes. We can analyze gene amplification (e.g. in breast cancer, Her2Neu amplification is a prognostic marker, and it can guide the use of specific targeted therapies, Fig. 10.4b); gene fusions (e.g. some lymphomas and sarcomas have specific gene fusions which are used to determine the final diagnosis); gene loss (less commonly used, due to the fact that tissue sections do not have the complete nucleus because the thickness of the section (5 μm) is sometimes smaller than the nucleus itself. It is then mandatory to count plenty of cells to ensure that the specific locus is lost, comparing the number of locus signals to the number of signals of the centromeric probe).

10.6.3 Comparative Genomic Hybridization (CGH)

This technology was first developed for cancer in the early 1990s to study DNA copy number imbalances in cancer cells [14, 15]. The methodology consists in the DNA extraction from the cells of interest and comparison with a "reference" DNA by labelling the two DNAs with two different fluorochromes by nick-translation (usually the test DNA is labelled with a red fluorophore (e.g. Cyanine 5) and the reference DNA sample is labeled with a green fluorophore (e.g. Cyanine 3)) (Fig. 10.5a). Equal amounts of labelled DNA are hybridized on a slide with normal cells in metaphase, and then chromosomes can be visualized in a fluorescence microscope. If tumor cells show loss of specific chromosomal sites, these will be seen as green (because the reference DNA (green) will bind to that DNA region, with no red-DNA binding); on the contrary, if there are gains of chromosomal regions they will be seen as red; finally, if tumor DNA is similar to reference DNA that chromosomal region will be seen as yellow (mixture of green and red fluorophores). One of the main problems of this technology is the difficulty of the analysis, because it requires the recognition of the different chromosomes by G-banding to be able to perform the fluorescence analyses. To overcome this problem, a Comparative Genomic Hybridization array (aCGH) was developed, which consists in a DNA microarray (different DNA probes representing all or specific chromosomal regions) where test DNA and reference DNA are hybridized as described above (Fig. 10.5a, [15]). Array CGH is an automated system with greater resolution than CGH (it can detect alterations in regions as small as 100 kb), it requires much less DNA, and is less time consuming. However, a great limitation of CGH and aCGH is the fact that they cannot detect structural chromosomal aberrations with no copy number variation, such as inversions, balanced translocations. This may be one of the reasons why this technology is not widely used in the oncological clinical setting, but it provides essential information for diagnosis and prognosis in patients with hematologic malignancies, such as lymphomas, and may be useful in solid tumors too.

10.6.4 Gene Expression Microarrays

Opposite to CGH, which studied structural DNA *status*, gene expression microarrays were developed to study the transcriptional activity of a biological sample based on RNA, since it is well known that genes have their effect through expression. The advantages of this technique reside in the fact that we can make a large-scale study of several biological processes measuring the activity in a cell at a certain time point. The technology is very similar to CGH and is also based in hybridization of the specimens in a DNA microchip were several thousands of probes are attached. RNA extracted from the samples of interest is retro-transcribed into labelled cDNA, which is then hybridized and analyzed similar to the aCGH (Fig. 10.5b, [16]). This methodology has been widely used for research in pathology and medical oncology for the past decades, and gene expression arrays allowed sub-classification of diverse oncological diseases such as breast cancer and diffuse large B-cell lymphomas with very important prognostic implications [17, 18]. Currently, with the development of more sophisticated and cheaper RNA sequencing technologies, gene expression arrays are not as widely used neither in research nor in the clinical setting.

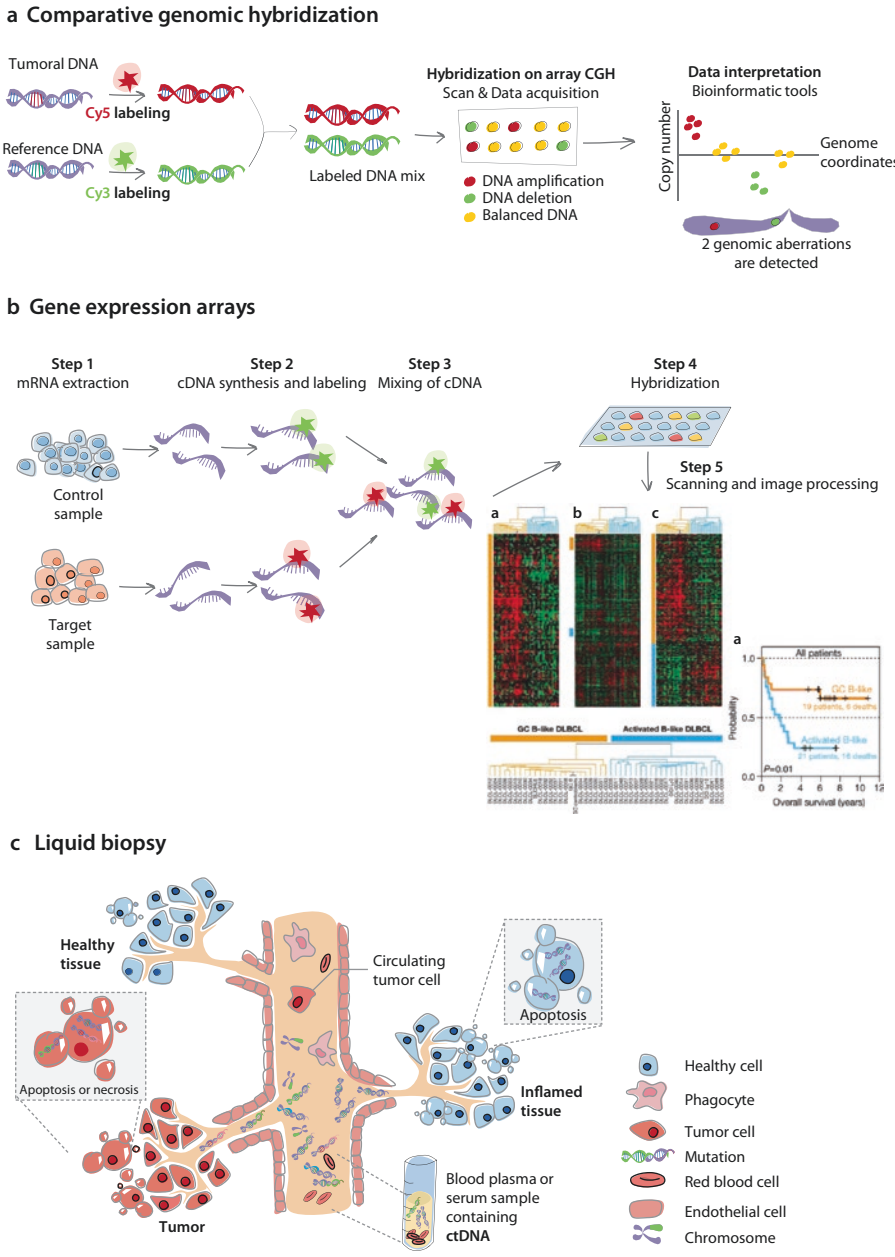


Fig. 10.5 Comparative Genomic Hybridization, Gene expression microarrays, and Liquid biopsy. **a** Schematic illustration of the technical procedures to perform CGH, and **b** gene expression arrays, and **c** the principles of liquid biopsy. Gene expression arrays allowed for a better sub-classification of diffuse large B cell lymphomas (**b**, **lower panel**) with significant prognostic implications. (**c** Adapted from Reference [21])

10.6.5 Next Generation Sequencing

One of the greatest revolutions in science of the twenty-first century came with the first publication of the human genome in the journal Nature in 2001 [19]. The Human Genome Project was an international scientific project that started in 1990 and was finished in 2003, mainly funded by the United States Government through the National Institutes of Health (NIH), with the idea of determining the sequence of nucleotides of the human DNA, to identify all the genes and mapping them physically and functionally. This adventure has accomplished the sequencing of approximately 92% of the human DNA sequence and has provided huge information in the field of molecular medicine. After the Human Genome Project, in 2005 the United States government through the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) started funding the next sequencing project: The Cancer Genome Atlas (TCGA). One of the first tumor types to be analyzed was glioblastoma multiforme [20] a very aggressive cerebral tumor. This analysis has been now extended to up to 33 human cancers, most of them solid tumors. Due to the great amount of data generated by all these high throughput technologies, many web pages exist where researchers can search and download information regarding genetic data related to cancer. The most convenient are:

- ► https://portal.gdc.cancer.gov/. It is the TCGA database.
- ► https://dcc.icgc.org/. It is the International Cancer Genome Consortium (ICGC) database, a collaborative effort of the world's leading cancer researchers.
- ► http://www.cbioportal.org/. It is a web page developed at the Memorial Sloan-Kettering Cancer Center (MSKCC) in the United States as a user-friendly open-access resource with data from different sources, including the TCGA and the ICGC databases.
- ► https://portals.broadinstitute.org/ccle. It is the Cancer Cell Line Encyclopedia (CCLE), developed at the Broad Institute in the United States, which provides public access of diverse genetic analyses for over 1000 cancer cell lines.
- ► https://www.proteinatlas.org/. This webpage was initiated as "The Human Protein Atlas" in Sweden in 2003 with the aim to map all the human proteins in cells and tissues, and lately with RNA data.

10.6.6 Liquid Biopsy

Finally, we will discuss the importance of liquid biopsy in the management of oncologic patients, which has opened new horizons in the way we diagnose and follow-up patients. Liquid biopsy is a term used to describe a blood test to identify neoplastic cells or any circulating tumor-derived material such as DNA, RNA, exosomes and proteins amongst other (◘ Fig. 10.5c, [21]). It is a non-invasive test which is useful for diagnosis, prognosis, follow-up of treatment and identification of tumor recurrence and minimal residual disease. Although in the past years there has been great interest in this technology, liquid biopsy started back in 1948, when circulating tumor DNA was identified in peripheral blood of cancer patients [22]. There are many advantages of liquid biopsy compared to tissue analyses obtained by biopsy or surgical resection such as a lower cost, lower patient risk and easiness of longitudinal collection during time. Moreover, another advantage is

the capability of analyses of several products from a single sample, such as circulating tumor cells, tumor DNA, microRNAs, exosomes etc. Some studies have described that liquid biopsy permits analysis of the whole tumor heterogeneity, whereas biopsy specimens may only represent specific tumor clones, but there is a lot of controversy on this, because there may be irregular shedding of cells depending on tumor clones characteristics. Notably, there are some significant limitations in this technique, mainly residing in the lack of standardization of procedures amongst laboratories, high and expensive downstream methodology to get data, and lack of information in control patients. Currently one of the tumor types in which liquid biopsy has been more widely implemented in the clinics is non-small cell lung cancer, used to identify tumor mutations during follow-up of patients [23].

10.7 Cancer Treatment

10.7.1 Aims of Cancer Treatment

When a patient with a new diagnosis of cancer is seen at the clinic, the first assessment to be made is regarding the staging of the disease. As mentioned in ▶ Sect. 10.5, staging refers to the extent of disease at loco-regional and distant sites and the TNM system is the most widely used cancer staging system. There is a clear distinction between treatment of localized and metastatic disease (◘ Table 10.2). In the former, disease is limited to the organ of origin or associated lymph nodes. In this case, the intention of treatment is curative, and options are made to improve the chance of cure. In this setting both loco-regional and systemic approaches of treatment are essential. In the metastatic setting, spread to other organs or throughout the body has already occurred. At this stage the disease is incurable, and the intent of treatment is palliative, being the goals to prolong life and relieve cancer associated symptoms leading to an improvement in the quality of life. In this clinical setting, surgery plays a minor role in treatment and systemic therapy comprises the mainstay of treatment.

◘ **Table 10.2** Goals and types of cancer treatment according to the stage of disease

Early stage cancer I-III	Metastatic cancer IV
Curative intent Improve the chance of cure	**Palliative intent** Prolong life, relieve cancer associated symptoms leading to an improvement or stability in quality of life
Local treatment Surgery Radiotherapy	**Systemic treatment** Chemotherapy Hormone therapy Molecular targeted therapy Immunotherapy
Systemic treatment Chemotherapy Hormone therapy Molecular targeted therapy	**Local treatment** More limited role: surgery and radiotherapy for palliation

10.7.2 Types of Cancer Treatment

Surgery and radiotherapy are two forms of **loco-regional therapy**. Historically, surgery was the only method used for cancer treatment. With the development of anticancer drugs and ionizing radiation, treatment rapidly evolved and nowadays cancer treatment involves the integration of several therapeutic options both for primary and metastatic tumors. The timing of each modality of therapy must be carefully considered for every patient in multidisciplinary team meetings.

Surgery may be considered for treatment of primary cancer, metastases or with cytoreductive or palliative intent. The cardinal principle of surgical cure for primary cancer is the total removal of neoplastic tissue that includes obtaining a complete margin of normal tissue around the primary tumor. Surgical treatment for metastatic disease must consider several elements namely tumor histology, location, size and extent of metastatic disease. Most cases in which this form of therapy is considered comprises patients with oligometastatic disease. Cytoreductive surgery [24], performed usually before systemic treatment in advanced stages comprises the removal of as much visible tumor as possible. In clinical studies the benefit of this approach has been limited to pediatric solid tumors, lymphoma and carcinoma of the ovary. Palliative surgery aim is to relieve symptoms and applications include relief of intestinal obstruction or pain and control of hemorrhage.

Radiotherapy is a form of loco-regional treatment based on the biologic effects of radiation in tissues. As radiation travels through the patient it deposits energy. These interactions set electrons in motion that produce ionizations. This ultimately leads to cell death due to chemical bonds disruption and damage to molecules and structures within the cells. If this damage occurs in cell's critical structures the effect of the accumulation of radiation damage will be cell death. Clinical types of radiation therapy include teletherapy (e.g. treatment from a cobal-60 source), external beam X-rays (from a linear accelerator) and brachytherapy (using a source of radiation inserted or implanted into the patient). Most centers currently use linear accelerators for general radiation therapy. Other modalities of radiation treatment include brachytherapy (interstitial or intracavitary), systemic targeted radionuclide therapy or particle radiation therapy (neutron and proton therapy). As with surgery, radiotherapy can be used in the adjuvant setting, to reduce the risk of loco-regional recurrence (e.g. breast cancer) or in the palliative setting (e.g. control of pain from bone metastases or hemorrhage from bleeding inoperable lesions).

Systemic treatments modalities comprise chemotherapy, hormone therapy, biologic/molecular targeted therapy and immunotherapy.

Chemotherapy involves a large group of cytotoxic drugs that interfere with cell division and DNA synthesis. Most of these agents were developed prior to 1975. At this time, the development of therapeutics was not yet informed by detailed knowledge of the genetic and biochemical mechanisms of cancer pathogenesis. This class of agents take advantage of the vulnerabilities that cancer cells have once they have discarded critical checkpoints controls operating during the normal cell cycle. Classic cytotoxic agents comprise alkylating agents, antimetabolites, anthracyclines, platinum agent, topoisomerase inhibitors, anti-microtubules. These agents are directed against specific targets (e.g. thymidylate synthase or tubulin) that, unlike molecular targeted agents, do not characterize the process of tumor progression and transformation but participate in physiologic process such as DNA synthesis or mitosis and therefore may affect healthy cells.

Cells actively undergoing cell division (like germ cells) are considered as being clearly more sensitive to most chemotherapy agents than resting cells. This explains, in part, why testicular germ cell tumors (TGCTs) are highly curable. TGCTs are the most common type of cancer in young males below 40 years old and even in the metastatic setting most patients (more than 70%) are expected to be cured. These high cure rates are due to their extreme sensitivity to chemotherapy since they are highly proliferative and have reduced heterogeneity. For example, Lance Armstrong, the famous cyclist, had testicular cancer; and although having brain and lung metastasis, survived cancer after testis surgical excision and systemic treatment with cisplatin-based chemotherapy!

Hormonal therapy involves drugs that interfere with growth signaling through hormone receptors on cancer cells. These agents are mainly used in breast and prostate cancer. Unlike the previous example – with TGCTs – endocrine therapy is mainly used in tumors with an indolent behavior i.e. in which cells are not actively undergoing cell division. In fact a low proliferation rate is a predictive biomarker of sensitivity to endocrine therapy.

Biologic/Molecular targeted agents (MTA) refer to a specific treatment strategy directed against well-defined molecular targets considered to be involved in the process of neoplastic transformation. The development of these agents has been prompted by the modern advances in molecular biology that lead to the identification of specific molecular abnormalities that characterize certain types of cancer in a unique and repeated manner. In this way MTA are capable of selectively target the specific cancer-cell population while minimizing the hazardous effect to normal cells. The key alterations of cancer cells described in the seminal article by Weinberg and Hanahan [25], and already discussed in the previous ▶ Chap. 1, are often used as a framework to classify MTA. A possible classification according to the molecular mechanism modified by the agent maybe be as follows:

- Self-sufficiency in growth signals → Cell-cycle inhibitors and signal transduction modulators (e.g. Cyclin –dependent kinase 4/6 inhibitor)
- Insensitivity to antigrowth signals → Cell-cycle inhibitors and signal transduction modulators
- Sustained angiogenesis → Antiangiogenic and antivascular agents (e.g. Antiangiogenic agents – Bevacizumab, Sunitinib)
- Tissue invasion and metastasis → Anti-invasive agents
- Evading apoptosis → Apoptosis modulators
- Limitless replicative potential → Anti-telomerase and telomere

Immunotherapy comprises a class of agents that target the induction or augmentation of anticancer immune responses and can be classified according to 5 major classes: vaccine-based strategies, immunomodulators, monoclonal antibodies, oncolytic virus and cellular immunotherapy.

- Vaccine-based strategies: are designed to stimulate the patient's own immune system against tumor antigens inducing tumor-specific or tumor-reactive immunoreactivity *in vivo*. Cancer vaccines can be broadly categorized in:
  1. Peptide-based (consisting of immunogenic epitopes, usually from tumor-specific or tumor-associated antigens),
  2. DNA based (plasmids containing cDNAs encoding tumor antigens are administered to the patient so that the patient begins to express these antigens and provides immunity and T cell response against them) or

3. Dendritic cell (DC)-based. Sipuleucel is a DC-based cancer vaccine approved for the treatment of metastatic castration-resistant prostate cancer based on the overall survival benefits seen in the IMPACT trial [26]. It is composed of autologous peripheral-blood mononuclear cells (PBMCs), including antigen-presenting cells (APCs), that have been previously activated *ex vivo* with a recombinant fusion protein PA2024. PA2024 consists of a prostate antigen, prostatic acid phosphatase, that is fused to granulocyte–macrophage colony-stimulating factor, an immune-cell activator.

- Immunomodulators are agents that enhance the body's immune response in a cancer non-specific way. Examples include intra-vesical administration of Bacillus Calmette-Guerin (BCG) or cytokines (interferons and interleukins) administration. BCG, an attenuated strain of mycobacterium originally developed as a vaccine against tuberculosis, is effective in the treatment of early-stage bladder cancer. The BCG treatment is delivered intra-vesically and it functions through its ability to attract a variety of immune cells, namely $CD4^+$Thelper and $CD8^+$Tcytotoxic lymphocytes, macrophages and Natural Killer (NK) cells, that are able to induce a localized inflammatory response.
- Monoclonal antibodies (mAbs) are the most commonly used and approved cancer immunotherapy method in clinical practice. mAbs are produced against a single epitope of the antigen and can be chimeric, humanized or human antibodies. The main types of cancer targeted by mAbs are breast, colon and lymphomas. The efficacy of mAbs is based on three main mechanisms that include:
  1. Inhibition of the targets (antigen) by antibody binding. These targets can be factors and receptors that activate signal pathways used by cancer cells in division and angiogenesis for example;
  2. Antibody-dependent cellular cytotoxicity (ADCC); the Fc portion can be recognized by Fc receptor present in NK cells and phagocytes, which then release cytotoxic factors that induce cell death of the targeted cell.
  3. Complement-dependent cytotoxicity (CDC) by complement activation upon antibody binding.

Examples of mAbs with ADCC properties include rituximab used in lymphomas, Trastuzumab and Pertuzumab in breast or gastric cancer and Cetuximab in colorectal cancer. mAbs with CDC properties include Rituximab, Alemtuzumab, Cetuximab or Ofatumumab. Recently a novel class of monoclonal immune-targeted antibodies have demonstrated remarkable efficacy and durable responses in a wide variety of tumors (e.g. melanoma, lung and bladder cancer). Their target are molecules involved in the inhibition of T cell immune responses (e.g. CTLA4, PD-1 or PD-L1). Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is expressed on both CD8 and CD4 T cells and behaves as an attenuator of an immune response. Ipilimumab is a fully human anti-CTLA4 mAb used in melanoma. Nivolumab blocks the Programmed cell Death-1 (PD-1) immune checkpoint molecule. PD-1 is an antigen-independent co-receptor and plays an important role in modulating immunological responses. It is widely used in the treatment of melanoma, non-small cell lung cancer, renal cell carcinoma and Hodgkin's lymphoma.

- Oncolytic virus are new class of therapeutic agents that promote anti-tumor responses through a dual mechanism of action that is dependent on selective tumor cell killing and the induction of systemic anti-tumor immunity. These viruses are genetically modified to lack virulence against normal cells. Selective tumor cell

killing is mediated by the ability to invade and lyse cancer cells which have sacrificed many of their normal anti-viral cellular defenses to amplify their growth potential. Further attack by immune system is stimulated by a plethora of tumor antigens released by lytic destruction [27]. The first oncolytic virus approved by the FDA is named "T-VEC" and it's used in to treat advanced melanoma. T-VEC is a herpes simplex-1 virus (HSV-1), modified to express GM-CSF which further stimulates proliferation of immune cells. T-VEC is injected directly into areas of melanoma that a surgeon cannot remove.

- Cellular immunotherapy or Adoptive T Cell Therapy is based on the infusion of large numbers of tumor-specific T cells that can be derived from:
  1. The tumor environment (e.g. tumor-infiltrating lymphocytes (TILs));
  2. Peripheral blood (CAR T cells)
  3. Or can be genetically modified to express a high affinity anti-tumor T cell receptor (TCR).

In 2017, CAR T therapy was approved by FDA for the treatment of children with acute lymphoblastic leukemia and for adults with advanced lymphomas. CAR T cell therapy requires isolation of patients T cells. The T cells are then genetically engineered to produce Chimeric Antigen Receptors (CAR). In adult lymphomas T cells are genetically modified to target CD19. CD19 is a transmembrane glycoprotein that is expressed at all stages of differentiation of normal B cells. As a target, CD19 is expressed in over 95% of B-cell malignancies including chronic lymphocytic leukemia, B-cell NHL and acute lymphoblastic leukemia being thus an attractive target for immunotherapeutic approaches. The final step is the infusion of the CAR T cells into the patient after "lymphodepleting" chemotherapy regimen. The engineered cells further multiply and with guidance from their engineered receptor, recognize and kill cancer cells that harbor the antigen on their surfaces.

10.8 Case Study 1

We are showing here the cases of two middle-aged women, who developed right colon adenocarcinoma and underwent right hemicolectomy. After histological analyses, both tumors showed similar features, being moderately differentiated adenocarcinomas (Fig. 10.6), with no lymphovascular invasion, with perineural invasion and no lymph node metastases, with a pathological stage after surgery of pT3pN0. From these findings, we could conclude that these two women have similar tumors with similar prognosis. Nevertheless, this was not the case. Mismatch Repair Proteins status is currently assessed as part of routine diagnosis in patients with colorectal carcinoma. Patient 1's tumor showed loss of MLH1, MSH6 and PMS2 expression by immunohistochemistry (Fig. 10.6, **top middle panels**). This tumor is considered to have microsatellite instability (MSI). Patient 2's tumor did not show any loss in the mismatch repair proteins, thus being classified as microsatellite stable (MSS). The prognosis of these two patients is very different, since MSS tumors have worse outcomes when compared to MSI tumors at the same stage [28]. On the other hand, and even more importantly from the patient management point of view, patient 1 will not receive any adjuvant chemotherapy because patients with MSI tumors have not shown any survival benefit after this treatment, whereas patient 2 will probably benefit from receiving adjuvant chemotherapy, improving her clinical outcome.

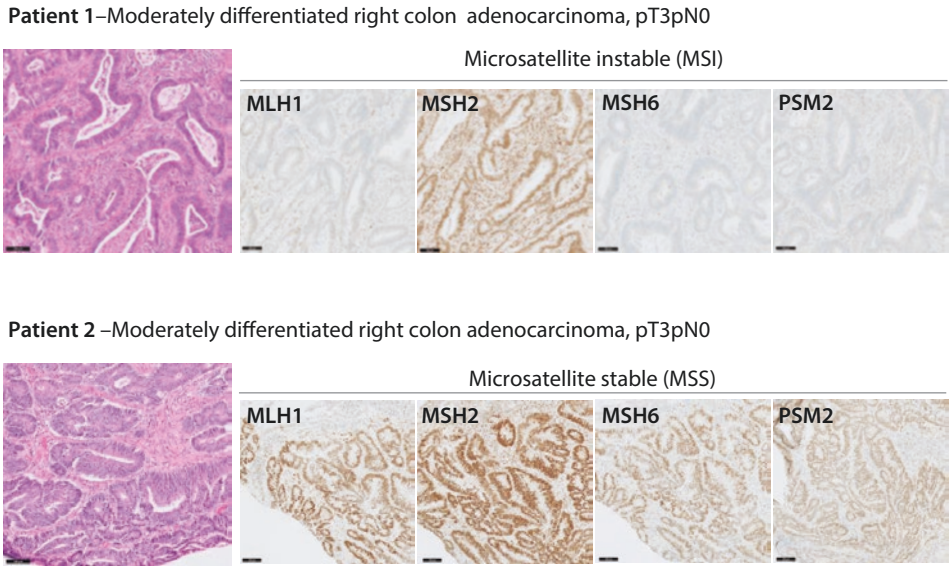


Fig. 10.6 Case study 1. Left panels display representative H&E stained sections of adenocarcinoma of the right colon from two different patients with similar histological features. Patient 1's tumor shows loss of immunohistochemical expression for MLH1, MSH6 and PMS2, whereas there is nuclear expression of MSH2 in the tumor cells. This tumor is considered to harbor microsatellite instability. Note that the non-tumoral cells show nuclear expression of all the mismatch repair proteins. Patient 2's tumor is characterized by immunohistochemical expression of MLH1, MSH2, MSH6 and PMS2, for which it is considered microsatellite stable

10.9 Case study 2

This clinical case corresponds to a heavy smoker middle-aged man, who presented in November 2013 at the moment of diagnosis, a lung adenocarcinoma with bone metastases to the lumbar spine (all metastatic patients are stage prognosis group IV, which usually entails a very bad prognosis, Fig. 10.7a). He first received standard chemotherapy with cisplatin, but the tumor did not show any clinical response by imaging. The tumor was assessed for Epithelial Growth Factor Receptor (EGFR) mutations as routine diagnosis for non-small cell lung carcinomas, and this analysis revealed an exon 19 mutation, which is one of the most common mutations in the EGFR gene. This finding and the lack of response to chemotherapy made the patient amenable to be treated with Erlotinib, a Tyrosine Kinase Inhibitor (TKI) approved for this type of patients. Under this therapy, he had stable disease from November 2013 until March 2016, when new sub-pleural nodules with multiple bone metastases and a supraclavicular lymph node were detected by Positron Emission Tomography (PET) (Fig. 10.7b). A biopsy of the lymph node confirmed the presence of metastatic adenocarcinoma of the lung and new mutational analyses revealed a novel EGFR 790 M mutation, which is well-known for providing resistance to some TKI. Treatment was then changed to Osimertinib, a TKI specific for tumors with this mutation. Unfortunately, the patient developed disease progression after 6 months of treatment with Osimertinib, due to resistance to this therapeutic agent driven by amplification of the *MET* gene, encoding a tyrosine kinase (Fig. 10.7c). Patient was then pro-

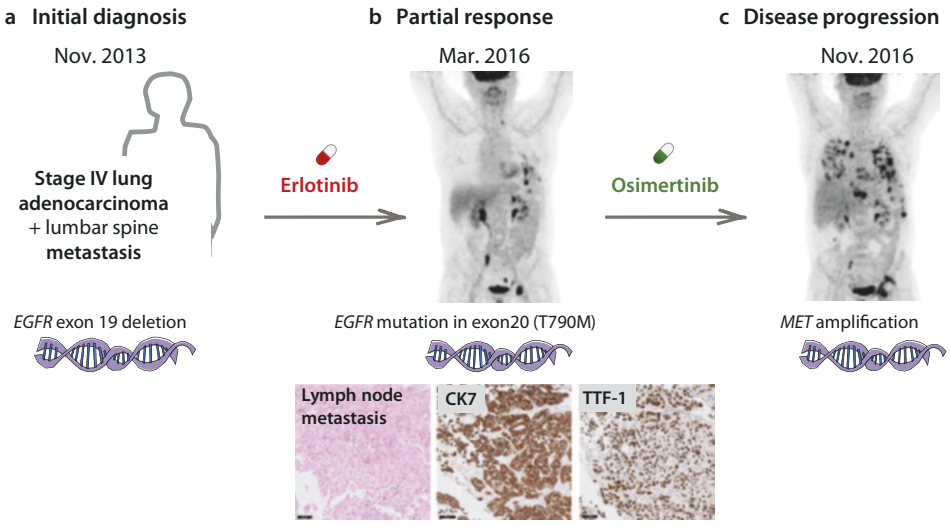


Fig. 10.7 Case study 2. Schematic representation of the follow-up of a patient with a diagnosis of lung adenocarcinoma, which presented several EGFR mutations that evolved in time and defined the best therapeutic choice at each moment

posed to start Crizotinib due to this new genetic alteration. This case is presented to show that targeted therapies, such as TKIs, have changed completely the prognosis and clinical outcomes of patients with oncological diseases: in this case, the patient had a very bad prognosis at the moment of diagnosis due to his advanced stage, nevertheless, he survived in quite good condition more than 3 years due to the use of these novel therapeutic approaches.

10.10 Case Study 3

Clinical case of a 58 years old, post-menopausal patient without any relevant past medical history or family history of cancer. The patient self-palpated a lump in the left breast in April 2014. In June 2014, a diagnosis of inflammatory left breast cancer was made. The breast ultrasound showed a suspicious nodule with 80 × 55 mm in the left breast, suspicious axillary lymph nodes in the left and right side. Pathology exam showed a lobular invasive breast cancer, grade 2, estrogen receptor (ER), progesterone receptor (PR) and cerbB2 positive and with a proliferative index determined by Ki67 expression of 10%. A macrometastasis on the ipsilateral and contralateral axillary lymph node was detected. For systemic staging of the disease a CT scan of thorax-abdomen–pelvis was performed. In addition to the bilateral axillary lymph node, mediastinal lymph nodes were also detected. No other lesions were described. Bone scan was negative for osteoblastic bone disease. This patient was staged as a T3N1M1 – Triple positive (estrogen receptor, progesterone receptor and HER2 positive) breast cancer. Disease was considered metastatic due to contralateral axillary and mediastinal lymph node involvement. First line metastatic treatment with Docetaxel and dual anti-HER2 blockade (Trastuzumab and Pertuzumab) was

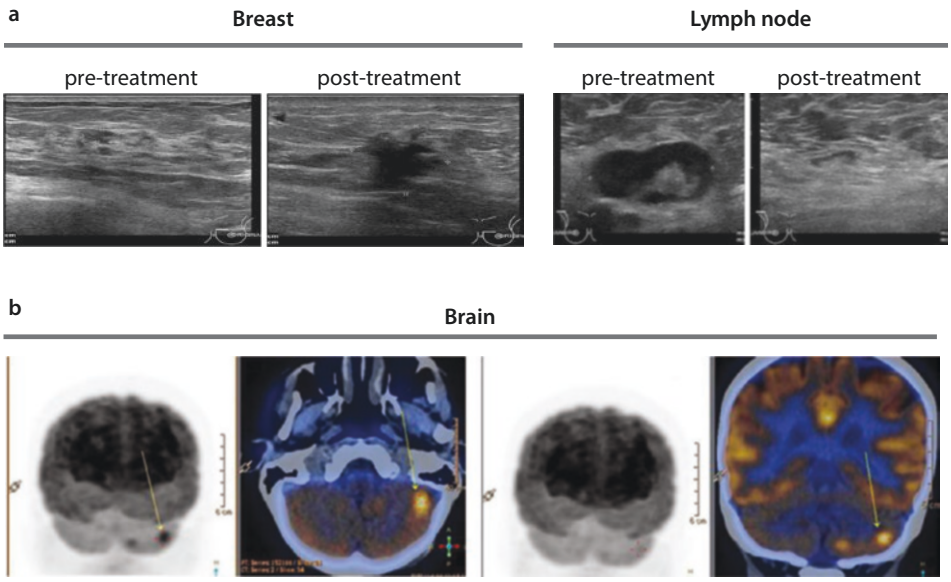


Fig. 10.8 Case study 3. **a** Ultrasound evaluation before and after 4 cycles of therapy in the breast (left panels) and axillary lymph node (right panels); **b** Brain metastases seen in PET-CT scan (2 distinct lesions are shown)

started. After 4 cycles of treatment a partial response (decrease >20% in target lesions) was achieved (Fig. 10.8a).

PET-CT (Computed Tomography) was performed and no evidence of metabolic active disease in the body was detected. The patient underwent additional 4 cycles of therapy. At this time (December 2014), there was no evidence of systemic disease and surgery of the breast and axilla was performed – left mastectomy with limited bilateral axillary dissection. A complete pathologic response was achieved, which means that there was no evidence of cancer in the breast or lymph nodes. The patient performed radiotherapy to the chest wall and locoregional lymph nodes and systemic therapy with Trastuzumab and Pertuzumab was maintained. As the disease was endocrine responsive (ER and PR positive) therapy with oral aromatase inhibitor was started at this point. No toxicities or progression of disease was seen until January 2016. At this time several lesions in the brain were diagnosed (Fig. 10.8b). The patient was asymptomatic, and PET-CT did not showed disease in other organs. Whole brain radiotherapy was performed. The use of radiotherapy or surgery for disease in the central nervous system is required due to the low rate of penetration of systemic therapy – either chemotherapy, endocrine therapy or biologic therapy – through the Blood-Brain-Barrier (BBB). We kept the systemic therapy (Letrozol and Trastuzumab) because no evidence of disease in other organs was seen. In April 2016, the patient achieved a partial response in the brain. However, in October 2016, the disease progressed once again in the brain (no disease in other organs). Neurosurgery and radiotherapy evaluation confirmed the absence of indication for any kind of locoregional treatment due to the number of lesions (12). At this time, we changed systemic therapy to Lapatinib and Trastuzumab. Lapatinib is a TKI and it is suggested that due to

its 'small molecular weight it as improved CNS penetration. Unfortunately, the patient presented severe toxicity with diarrhea – grade 3 – and therapy was stopped. Another line of therapy with T-DM1 (antibody drug conjugate Trastuzumab plus Emtasine, a cytotoxic compound) was done and patient presented a partial response. Treatment is ongoing. We are presenting this case to underline several important concepts. The first concept relies as in the previous clinical cases on the importance of biology knowledge in driving treatment choices. Breast cancer with HER2 amplification clearly represents a specific subset of breast cancer with a distinct prognosis and a particular therapeutic approach. The basis of treatment relies on anti-HER2 targeted therapy (Trastuzumab, Pertuzumab, Lapatinib) alone or combined with chemotherapy or endocrine therapy.

The *erbB* gene (also known as *erbB2, neu,* or *HER2* – from now on *HER2*) was first discovered in the genome of avian erythroblastosis virus and later found to be present in increased copy number in the DNAs of human stomach, breast, and brain tumor cells. In 1987, amplification of the *HER2* gene, was reported in many breast cancers with a strong correlation between increase in gene copy number (more than five copies per cancer cell) and a decrease in patient survival provided a strong indication that this gene, amplified, was causally involved in driving the malignant growth of breast cancer cells. The observed amplification of the *erbB2/ HER2* gene was correlated with an increased expression of its encoded protein and elevated signaling, which has been shown to promote division and protection from apoptosis.

This discovery changed forever the way we perceived breast cancer, from this moment on, as a collection of distinct diseases in which identification relied on pathology.

In addition, the clinical case presented also illustrates a typical example of host-dependent drug resistance mechanism driven by anatomic drug barriers (also called tumor sanctuaries) most noticeable present in the brain and testis. The BBB acts physiologically to protect the brain tissue from the contents of the circulation. However, in cancer patients undergoing systemic treatment this can lead to several disadvantages namely the decrease concentrations of drugs reaching brain metastasis. Another mechanism by which BBB can negatively impact cancer patient's outcomes is by blocking the effective surveillance by the immune system shielding it form routine monitoring by cells of the immune system.

Finally, in this clinical case we could also learn how new drugs – namely antibody-drug conjugates (T-DM1) are being used in cancer treatment. This new class of drugs combines the desirable properties of monoclonal antibodies (in this clinical case Trastuzumab) to specifically target tumor cells with highly potent killing activity of a cytotoxic drug (in this case DM1), a payload that is too toxic for systemic administration. In this way, these novel drugs act by reducing systemic toxicity and increasing the therapeutic benefit for patients.

As we have seen, cancer treatments nowadays take into account the cellular and molecular mechanisms discovered in basic and translational research that are evaluated mainly by pathology. Diagnosis and disease progression are highly monitored by imaging (CT scans, PETs etc) to adjust treatment options. In the future, hopefully, tumor cells will be tested *in vitro* (organoids) [29] or in *in vivo* models (zebrafish) [30] to choose the best therapeutic option for each individual patient, towards a truly personalized treatment.

Take Home Message

This chapter aims at conveying the following messages:

- Pathology is an old medical specialty that although evolved enormously with the advancement of microscopic and molecular technology, still maintains similar basic techniques for diagnosis.
- Tissue specimens obtained from patients both through biopsy or surgery need to be processed following a series of well-established steps before pathologists can look at the sections under the microscope to give a diagnosis.
- Pathology is a medical discipline that studies disease in cells (cytology) and tissues (histology) and in the setting of oncology it provides the basis for treatment of patients by medical oncologists.
- Molecular pathology techniques provide information which increases diagnosis accuracy, with prognostic implications and determines best therapeutic approaches for specific patients.
- There is a whole variety of techniques that comprise molecular pathology, some study proteins, such as immunohistochemistry, and other nucleic acids, such as microarray expression arrays (RNA), *in situ* hybridization (RNA and DNA).
- Liquid biopsy has opened a new possibility of studying tumor alterations in a less aggressive manner and at different time points.
- Presently there are 5 main types of therapy: surgery; radiotherapy; chemotherapy; biological /molecular targeted agents and immunotherapy
- We are starting an era of precision therapy, where molecular biomarkers are guiding treatment options.

Questions

1. Who was the pathologist who is regarded as the father of modern pathology for his cell theory?
   (a) Rudolf Virchow
   (b) Giovanni Battista Morgagni
   (c) Marie François Xavier Bichat
   (d) James Ewing
   (e) Thomas Hodgkin

2. What is currently the most frequent molecular technique used in pathology in the clinical setting?
   (a) Whole exome sequencing
   (b) Immunofluorescence
   (c) Fluorescence *in situ* hybridization (FISH)
   (d) Immunohistochemistry
   (e) Gene expression analysis

3. Which of the genomic alterations is targetable with current therapeutics in cancer?
   (a) 9q chromosomal deletion
   (b) p53 mutation

(c) Her2Neu amplification
(d) t(14;18) chromosomal translocation
(e) None of the above

4. Which clinical impact has molecular pathology provided in oncology?
   (a) Improvement in tumor classification
   (b) Better understanding of altered pathways involved in tumor initiation
   (c) Advance in tumor prognostication
   (d) Identification of genomic alterations that can be targeted
   (e) All the above

5. What is one of the benefits of liquid biopsy compared to tumor tissue analyses?
   (a) Possibility of tumor genetic analyses
   (b) Easier longitudinal studies
   (c) Less aggressive for the patient
   (d) a and c
   (e) b and c

6. Oncologic treatment in the metastatic setting mainly relies in:
   (a) Surgery of the primary tumor
   (b) Systemic treatment (chemotherapy, targeted therapy)
   (c) Radiotherapy to the metastatic lesions
   (d) None of the above

7. HER2 positive breast cancer is mainly characterized by:
   (a) High expression of hormone receptors
   (b) Mutation of the *HER2* gene
   (c) Amplification of the *HER2* gene
   (d) Deletion of the *HER2* gene

8. All of the following are considered loco-regional forms of treatment except:
   (a) Cytoreductive surgery
   (b) Radiotherapy
   (c) Hormone therapy
   (d) Palliative surgery

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