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THEME

CRISPR (Courtes répétitions en palindrome regroupées et régulièrement espacées) et le Cancer

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DECLARATION DE L'HONNEUR

Je soussigné, **Mr. BAHOUH Mohamed Wail**, déclare être pleinement conscient que le plagia de document ou d'une partie de document publié sous toute forme de support, y compris l'internet, constitue une violation des droits d'auteur ainsi qu'une fraude caractérisée. En conséquence, je m'engage à citer toutes les sources que j'ai utilisé pour écrire ce mémoire.

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Abstract:

CRISPR/Cas9 has become a powerful method for making changes to the genome of many organisms. First discovered in bacteria as part of an adaptive immune system, CRISPR/Cas9 and modified versions have found a widespread use to engineer genomes and to activate or to repress the expression of genes. As such, CRISPR/Cas9 promises to accelerate cancer research by providing an efficient technology to dissect mechanisms of tumorigenesis, identify targets for drug development, and possibly arm cells for cell-based therapies.

Here, we review current applications of the CRISPR/Cas9 technology for cancer research and therapy. We highlight the impact of CRISPR/Cas9 in generating organoid and mouse models of cancer.

Finally, we provide an overview of the first clinical trials that apply CRISPR/Cas9 as a therapeutic approach against cancer.

Résumé :

CRISPR/Cas9 est devenu une méthode puissante pour apporter des modifications au génome de nombreux organismes. Découvert pour la première fois chez les bactéries dans le cadre d'un système immunitaire adaptatif, CRISPR/Cas9 et ses versions modifiées ont trouvé un usage répandu pour l'ingénierie des génomes et pour activer ou réprimer l'expression des gènes. Ainsi, CRISPR/Cas9 promet d'accélérer la recherche sur le cancer en fournissant une technologie efficace pour disséquer les mécanismes de la tumorigènes, identifier des cibles pour le développement de médicaments et éventuellement des cellules de bras pour les thérapies cellulaires.

Ici, nous passons en revue les applications actuelles de la technologie CRISPR/Cas9 pour la recherche et la thérapie du cancer.

Nous soulignons l'impact de CRISPR/Cas9 dans la génération d'organoïdes et de modèles murins de cancer.

Enfin, nous donnons un aperçu des premiers essais cliniques qui appliquent CRISPR/Cas9 comme approche thérapeutique contre le cancer.

نبذة مختصرة :

أصبح CRISPR / Cas9 طريقة قوية لإجراء تغييرات على جينوم العديد من الكائنات الحية. اكتشفت لأول مرة في البكتيريا كجزء من جهاز المناعة التكيفي، وجدت CRISPR / Cas9 والنسخ المعدلة استخدامًا واسع النطاق لهندسة الجينوم ولتفعيل أو قمع تعبير الجينات. على هذا النحو، يعد CRISPR / Cas9 بتسريع أبحاث السرطان من خلال توفير تقنية فعالة لتشريح آليات تكوين الأورام، وتحديد أهداف تطوير الأدوية، وربما خلايا الذراع للعلاجات القائمة على الخلايا.

هنا، نراجع التطبيقات الحالية لتقنية CRISPR / Cas9 لأبحاث وعلاج السرطان.

نسلط الضوء على دور CRISPR / Cas9 في توليد نماذج فئران وOrganoïdesللسرطان.

أخيرًا، نقدم نظرة عامة على التجارب السريرية الأولى التي تطبق CRISPR / Cas9كنهج علاجي ضد السرطان.

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1. Introduction

Cancer is one of the main causes of disease-associated mortality, with a rising incidence worldwide. At the same time, progress has been made in the prevention and treatment of many cancers, leading to prolonged survival or even cures. A main pillar of innovation in cancer therapy has been an improved understanding of the underlying tumor biology.

Recently, genome engineering was greatly accelerated by the development of CRISPR/Cas9 technologies. Since its first use as a genome editing tool in 2013 in mammalian cells, the toolbox of CRISPR/Cas9 has been continuously expanded, enabling not only the modification of the genomic sequence of cells and organisms, but also the introduction of epigenetic and transcriptional modifications.

In this review, we describe how CRISPR/Cas9 opens new avenues for cancer research. In addition to its application as an effective screening method in functional cancer genomics, we outline how CRISPR/Cas9 can be used to explore the non-coding genome of cancer. Furthermore, we describe novel in vitro and in vivo cancer models that can be engineered by CRISPR/Cas9. Finally, we review the first clinical trials that apply CRISPR as a therapy against cancer (Yin, Xue, & Anderson, 2019) (Zhan, Rindtorff, Betge, Ebert, & Boutros, 2019)**.**

2. CHAPTER I: What is cancer?

a. Definition:

i. A cluster of related diseases:

Cancer is a genetic term used to describe a large group of diseases that can affect any part of the body and are characterized by the abnormal proliferation of miss-regulated body cells (World Health Organization, 2018) **&** (Davies & Lineweaver, 2011), which in contrast to normal cells that grow and divide to form new ones as the body needs them; and when they grow old or become damaged, they die, these abnormal cells grow beyond their usual boundaries and divide without stopping, forming in most cases lumps of cells called tumors, the miss-regulated cells can invade adjoining parts of the body and even spread to other organs in a process called metastasis, which is in fact the major cause of deaths from cancer (World Health Organization, 2018) **&** (National cancer institute, 2015)**.** From a biological point of view cancer could be described as a loss of multicellularity, where cells lose their specialization, value their existence over the organism's and ignore body signals that normally tell cells to stop dividing and/or ones that start a process known as apoptosis (programmed cell death) (Davies & Lineweaver, 2011) **&** (National cancer institute, 2015).

With the possible unique exception of the naked mole rat, almost all metazoans especially long living animals face the challenge of cancer, with some animals being more prone to it than others (Davies & Lineweaver, 2011), beyond animals there is an argument that other multicellular organisms i.e. plants suffer from cancer as well (Gaspar, et al., 1991), however, other authors beg to differ, they state that plants do not develop cancer (Doonan & Hunt, 1996). This quasi-ubiquity across almost all metazoan species enforces the idea that cancer mechanisms are deeply embedded in our evolutionary history, a conjecture that is in line with Genetics and Paleontology (Davies & Lineweaver, 2011) as dinosaur tumors have been documented several times (Rothschild, Tanke, & Helbling, 2003).

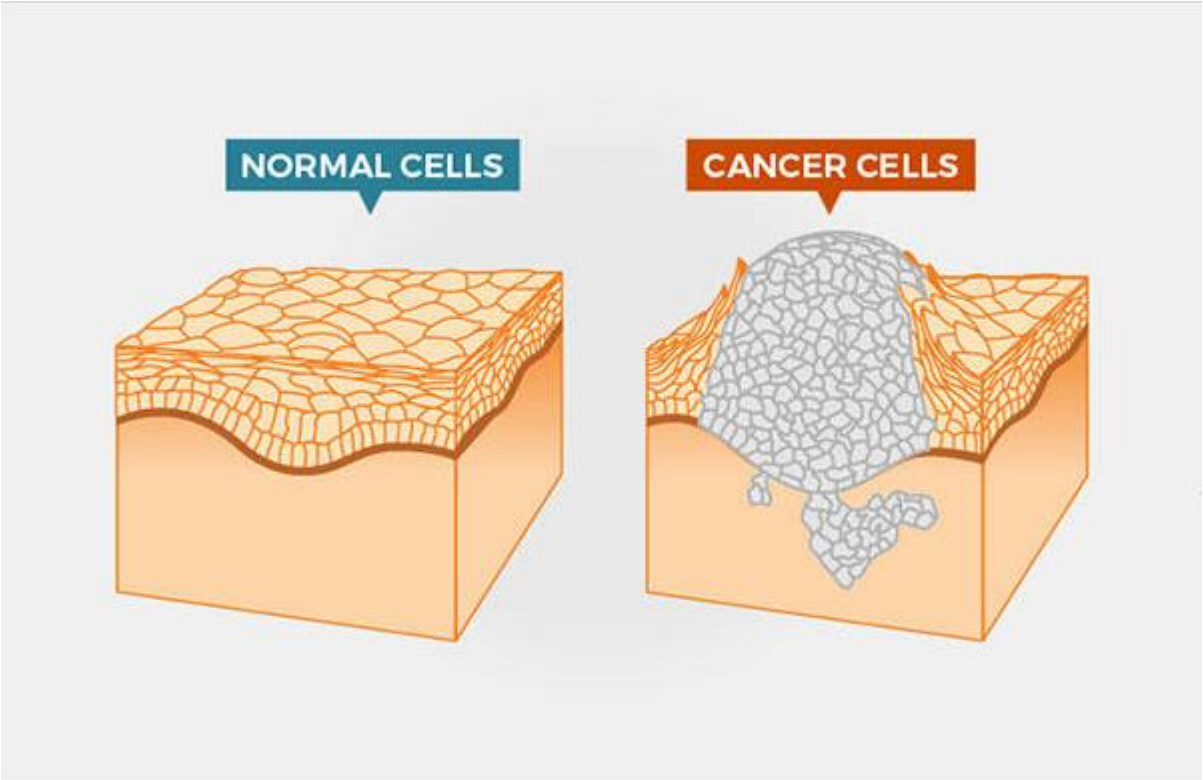

Figure 1: *Normal tissues and Cancerous ones*

(National cancer institute, 2015).

ii. Cancer Types:

Cancer can arise from almost anywhere in the body, which is comprised of trillions of cells. There are more than 200 types of cancer and most fall under one of three main categories: Carcinomas, Sarcomas, and Leukemias or Lymphomas. Carcinomas are malignancies of epithelial cells; the most prevalent category as they account for more than 90% of human cancer.

Sarcomas are malignancies of connective tissues like muscles, bones, cartilage and fibrous tissue, the rarest in human cancer, accounting for no more than 2%.

Leukemias and lymphomas, account for 8% of human cancer and arise from the blood-forming cells and from cells of the immune system, respectively (Geoffrey, 2000) (National cancer institute, 2015).

b. Cancer in animal cells:

i. How cancer arises & cancer drivers:

The question of "What causes cancer" has intrigued humanity for many generations and was among the hottest topics of science for centuries (Clarke , 2016). And today we know that the fundamental abnormality resulting in the development of cancer is the continual unregulated proliferation of cancer cells (Geoffrey, 2000). These cells arise from normal cells after they undergo a transformation process known as Carcinogenesis (oncogenesis or tumorigenesis) (Tessitore , et al.,

2014), this histopathological process is accompanied by the accumulation of genetic alterations to genes that normally control cell function, mainly ones that control how a cell grows and divides (National cancer institute, 2015)**,** (David, 2002). These genetic changes that contribute to cancer are called 'Cancer Driver Mutations' and tend to affect the following genes: proto-oncogenes, tumor suppressor genes and DNA repair genes (National cancer institute, 2015), the affected genes are known as 'Cancer Driver Genes' (Stratton, Campbell, & Futreal, 2009).

The alterations are usually somatic events, although some germ-line mutations can predispose a person to heritable or familial cancer and we must note that a central feature of today's molecular view of cancer is that cancer does not develop all at once, a single genetic change is rarely sufficient for the development of a malignant tumors, rather it is a multistep process of sequential alterations in several, often many of the cancer genes (National Institutes of Health, 2007) (Croce, 2008)**.**

- Proto-oncogenes encode proteins that control cell proliferation, apoptosis, or both. When these genes are altered in certain ways or activated, they may become cancer-causing genes or Oncogenes, allowing cells to grow and survive when they should not (Sarkar, et al., 2013)**,** (National cancer institute, 2015).
- Tumor suppressor genes are among the negative regulators of the cell cycle, they regulate cell growth and division, cells with certain alterations in tumor suppressor genes may divide in an uncontrolled manner (National cancer institute, 2015)**,** (Levine, Momand, & Finlay, 1991). Among these tumor suppressor genes is the P53 gene, alterations or inactivation of p53 through mutations or interactions with oncogenes are the most common genetic changes in Human cancer (Levine, Momand, & Finlay, 1991).
- Oncogenes and tumor suppressor genes may come in the form of MicroRNAs (MiRNAs) which are a family of small non-coding RNAs that regulate a wide array of biological processes including carcinogenesis. In cancerous cells these dysregulated miRNAs have been shown to affect the hallmarks of cancer, including sustaining proliferative signaling, evading growth suppressors, resisting cell death, activating invasion and metastasis, and even inducing angiogenesis (the process of forming new blood vessels) (Peng & Croce, 2016).
- DNA repair genes encode proteins involved in fixing damaged DNA. Cells with mutations in these genes tend to develop additional mutations in other genes facilitating the Carcinogenesis process (National cancer institute, 2015).

The driver mutations are the result of either intrinsic factors namely the evolutionary nature of metazoan cells and aging, or due to the interaction between a person's genetic factors and external agents (World Health Organization, 2018)**,** (Stratton, Campbell, & Futreal, 2009), these external agents are classified into 3 categories:

- Physical carcinogens, such as ultraviolet and ionizing radiation;
- Chemical carcinogens, such as components of tobacco smoke, arsenic (a drinking water contaminant);
- Biological carcinogens, such as infections from certain viruses, bacteria, or parasites (World Health Organization, 2018).

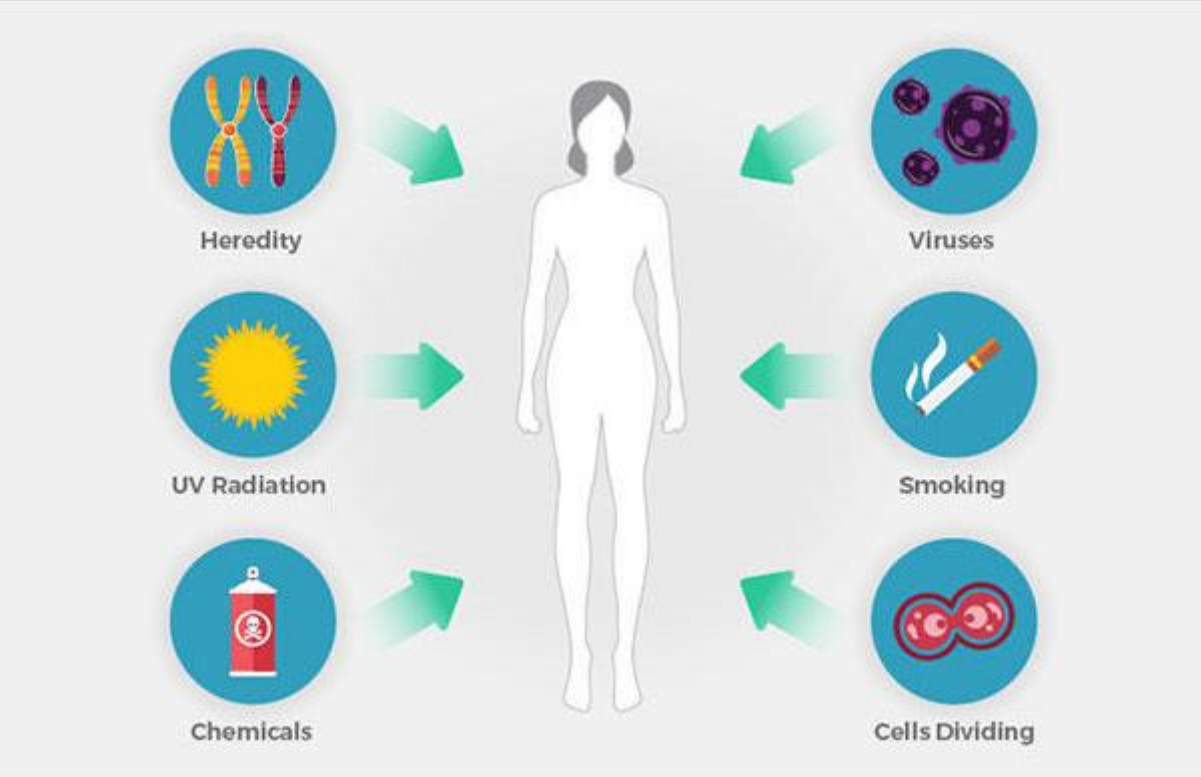

***Figure 2**: Cancer Carcinogens*

(National cancer institute, 2015)**.**

ii. Tumors and how cancer cells interact with their surroundings:

Cancer cells have an increased capacity for proliferation, survival and invasion, allowing them to survive when they should not, and to form new cells when they are not needed, the extra cells just like the original divide and multiply without stopping, they have also the ability to compete with normal cells, go unnoticed by the immune system and establish themselves in tissues forming in most cases growths called Tumors (National cancer institute, 2015) (Buder, Deutsch, Klink, & Voss-Böhme, 2019). A tumor is any abnormal proliferation of cells and almost all cancer types result in solid tumors with the exception of blood cancers like leukemias (Geoffrey, 2000) (National cancer institute, 2015)**.** Tumors can be benign or malignant (Geoffrey, 2000). A benign tumor does not spread into, or invade, nearby tissues but rather stay confined to its original location, and when removed this type of tumors do not usually grow back (National cancer institute, 2015) (Geoffrey, 2000). A malignant tumor on the other hand is capable of both invading surrounding tissues and of

spreading to distant organs throughout the body via the circulatory or lymphatic systems: metastasis.

In oncology only malignant tumors are properly referred to as cancer, and in fact the property of being metastatic is what makes this type of tumors so dangerous and is the reason for most deaths of cancer (Geoffrey, 2000). Contrary to most benign tumors elsewhere in the body, benign brain tumors are extremely dangerous and much like malignant tumors are life threatening. (National cancer institute, 2015).

Given the fact that tumors arise from the excessive and the non-stopping proliferation of cancer cells, it is a common misconception that they are lumps of clones of the same cells, tumors however much like organs are composed of multiple cell types with different functions and even an extracellular matrix, and despite being structurally and functionally abnormal a lot of tumor development aspects resemble processes seen in developing organs (Egeblad, Nakasone, & Werb, 2010). As tumors progress and develop they undergo dramatic morphological changes and much like normal organs this includes the stroma. Different components of the tumor stroma influence the progression and with varying influence rates as the stroma of the later stages is more supportive of tumor progression than the stroma of early stages. Cancer cells can also instruct surrounding tissues to undergo changes that promote malignancy, fibroblasts activated by the tumor microenvironment are largely responsible for tumor-associated changes in the Extracellular Matrix, mainly upregulating and extensive remodeling, this altered ECM then influences tumor progression by architectural and signaling interactions, further more normal fibroblast when in contact with their neighboring cells inhibit migration and adhere normal cells to each other, forming an orderly array of cells, cancer cells in contrast, move nonstop even after contact with their neighbors, migrate over adjacent cells, and grow in disordered, multilayered patterns.

Another fundamental aspect of the development and differentiation of tumors is the recruitment of vasculature. To assure growth, tumors recruit blood vessels in a process named the ‘Angiogenic switch’, which can occur at different tumor progression stages. In contrast to the normal vasculature, tumor blood vessels are irregular and can have dead ends resulting in an abnormal blood flow, as well as extravasation of excess fluid and proteins from the capillaries, the leaked fluids and proteins are then taken by the lymphatic system and reintroduced to the blood stream promoting lymphatic growth (Egeblad, Nakasone, & Werb, 2010) (Geoffrey, 2000).

The first step in the tumor development process is the initiation phase; it is the result of the original progenitor cell that gives rise to the tumor acquiring the genetic alterations that gives it the characteristics of a cancerous cell.

The promotion phase is next and it is a lengthy step characterized by the excessive proliferation and the outgrowth of a population of preneoplastic tumor cells. The next phase is a transitional phase

between the premalignant state and the development of invasive cancer: the progression phase, at this stage the proliferating cells may undergo more mutations at an accelerating rates, some of these mutations may give a selective advantage to the cell, such as an even more rapid growth, and the descendants of a cell bearing such a mutation will consequently become dominant within the tumor population, this causes a fast increase in the tumor size, some other mutations are theorized to have an invasive and metastatic potential that could promote the next phase which is Metastasis (Siddiqui, Sanna, Ahmad, Sechi, & Mukhtar, 2015) (Geoffrey, 2000).

iii. When Cancer Spreads 'Metastasis':

Considered a hallmark of cancer, metastasis involves the spread of tumor cells from the primary site to invade neighboring tissues and more importantly distant organs using both the circulatory and lymphatic systems, and the formation of secondary tumors throughout the body (Seyfried & Huysentruyt, 2013). Even in tumors that are sensitive to radiotherapy or chemotherapy, once cancer spreads throughout the body, it becomes a formidable foe even for the most sophisticated therapies as metastasis is the main reason of treatment failure, and is the primary cause of cancer morbidity and mortality (90% of cancer deaths), the best that people in this situation can hope for is to survive for a few more years (Fares, Fares, Khachfe, Salhab, & Fares, 2020) (Seyfried & Huysentruyt, 2013) (Qian, Mei, & Zhang, 2017) (Nature, 2020). Despite the high survivability only less than 0.1% of tumor cells acquire the ability to metastasize as these cells go through a series of sequential and interrelated stochastic events that first allow cancer cells to disperse and survive in distant sites and later to grow as secondary tumors. In order to complete the process which is termed metastatic cascade, cancer cells must detach from the primary tumor, break through supporting membranes; intravasate into the circulatory and lymphatic systems, hide from the immune system, extravasate at distant capillary beds, and finally infiltrate, invade and proliferate in distant organs. The adaptation to the microenvironment of the tissue in which the cells have landed which is invariably quite different, this may cause the metastatic cells to remain inactive at the distant site for a long time before proliferating again, if at all, when they do however, these cells establish a microenvironment much like the original tumor to facilitates angiogenesis and proliferation, resulting in malignant secondary tumors (Seyfried & Huysentruyt, 2013) (Weinberg, 2007) (National Cancer Institute, 2017).

Despite being the leading cause of deaths, metastasis remains the least understood process in cancer development as little is known about why it occurs, an early hypothesis suggests that it is an innate trait of cancer, caused by the genetic mutations, this conjecture however is not in line with evidence-based medicine as not a single gene has yet been identified as responsible for metastasis, also a number of embryonic cells during the development of any organism, naturally migrate over

long distances to their final location, without any genetic mutations. Other hypothesis state that metastasis is acquired, and induced by external factors like an adequate oxygen supply or Immune reaction/inflammation (Wang, Lu, & Fan, 2015).

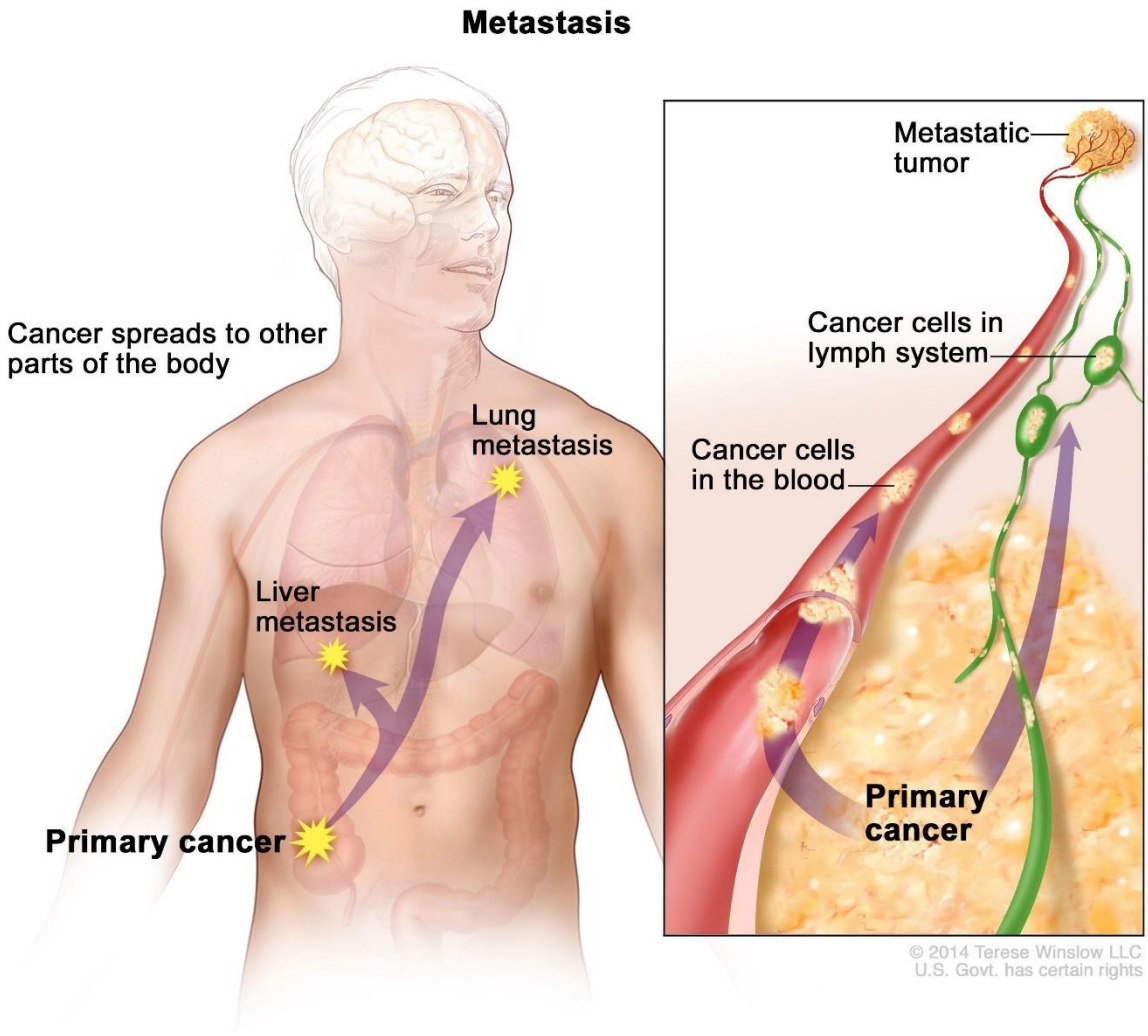

***Figure 3**: Metastasis: When cancer spreads*

(National cancer institute, 2015)

A new hypothesis suggests that Chromosomal instability induce the metastatic cascade. Chromosomal instability (CIN) is caused by continuous errors in chromosome segregation during mitosis, these errors induce the rupture of micronuclei and the secretion of genomic DNA into the cytosol, which subsequently activates cytosolic DNA-sensing pathways. The chronic signaling promotes transcriptional shift from a proliferative and highly metabolic state, ideally suited for primary tumor growth, to a mesenchymal state associated with upregulation of inflammatory pathways. On the light of these results a specific process comes to mind: The Epithelial-Mesenchymal Transition (EMT) (Bakhoum, et al., 2018). EMT is the trans-differentiation process through which transformed epithelial cells develop the ability to invade, resist stress, and

disseminate. It was shown that the various EMT stages possess diverse cellular characteristics, chromatin landscapes, and gene expression signatures which are regulated by distinct transcription factors and signaling pathways. Not only that but within a tumor, the various EMT stages are situated in diverse microenvironments and not in an anarchical manner, as metastatic cells with the most pronounced mesenchymal phenotype for example proliferate near endothelial and inflammatory cells. These tumor cells stimulate angiogenesis by releasing large quantities of chemokines and proteins to attract immune cells, thus promoting the development of a unique inflammatory and highly vascularized niche facilitating the Intravasation of metastatic cells into the blood and lymph streams. This hypothesis is further supported by the fact that tumor cells that express a mix of epithelial and mesenchymal phenotypes are more effective in circulation, colonization at the secondary site, and the development of metastasis (Fares, Fares, Khachfe, Salhab, & Fares, 2020) (Bakhoum, et al., 2018).

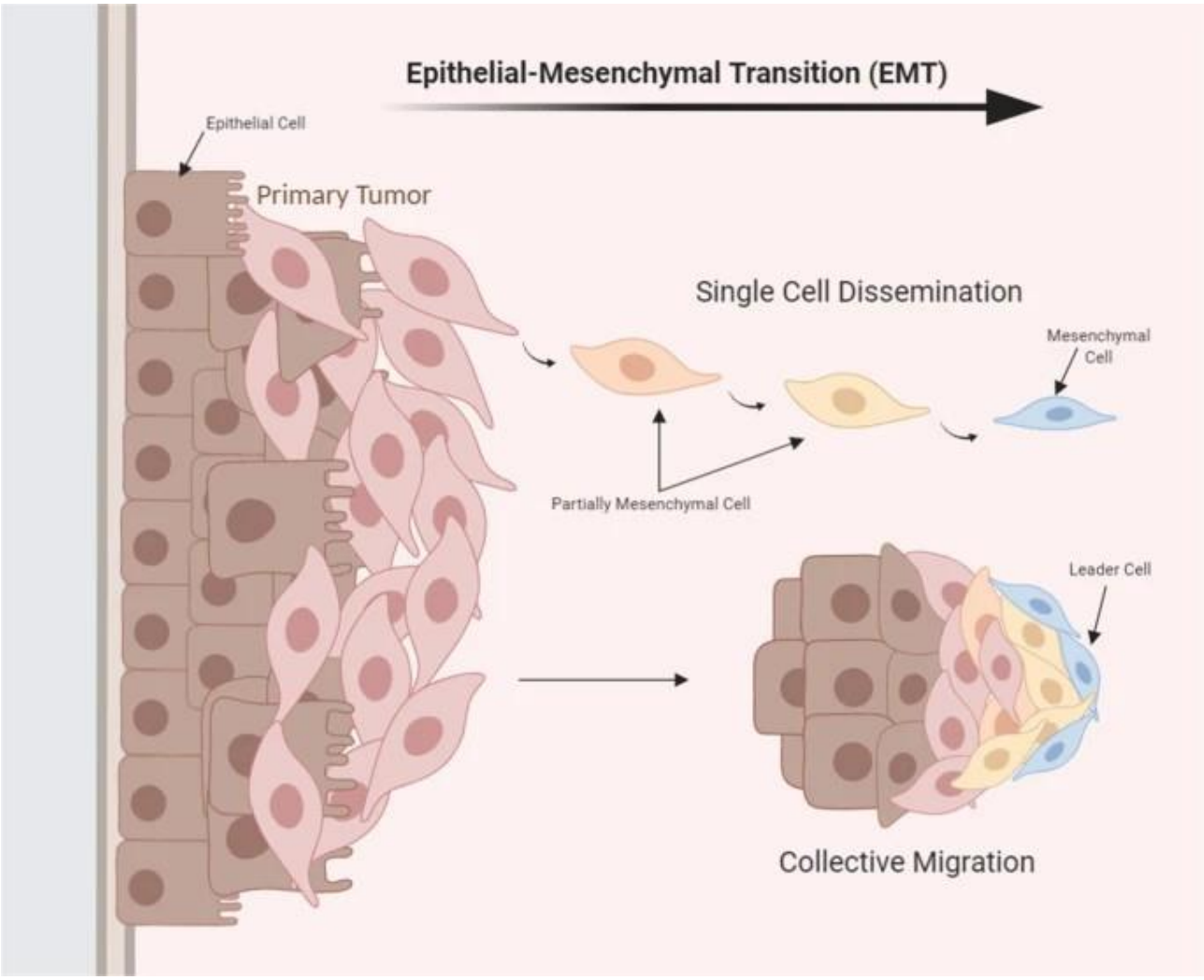

***Figure 4**: Mechanism of Epithelial-Mesenchymal Transition*

(Fares, Fares, Khachfe, Salhab, & Fares, 2020)

c. How the body deals with cancer:

Another question that always follows “What causes cancer?” is “How do cancer cells avoid destruction by the immune system?”.

The involvement of one’s immune system in tumor progression is well documented, weather be it inhibiting or enhancing to the development of tumors, this interaction is considered a hallmark of cancer (Vinay, et al., 2015)**,** (Gonzalez, Hagerling, & Werb, 2018).

In theory, the immune system by its adaptive nature should be able to control and stop tumors from developing, it is not the case however, as the tumor develops from neoplastic tissues to a clinically detectable tumors, cancerous cells develop mechanisms to go by unnoticed or ignored by the immune system and avoid tumoricidal attacks (Gonzalez, Hagerling, & Werb, 2018).

Carcinogenesis is a very conditional process, it is known that cells possess innate and effective defense mechanisms like DNA repair tools to deal with mutations, and when the damage is beyond repair they commit suicide or apoptosis, however in light of the fact that thousands of damage events affect the genome every day and the trillions upon trillions of cells where this could happen the chances of those mutations to go by infixed and to accumulate increase dramatically (Brown, 2002)**,** (National cancer institute, 2015)**,** most of the times though this isn’t an issue as the immune cells eliminate rather quickly any cell that displays cancerous behavior, but this is a numbers game, sooner or later some cells will develop the ability to elude from the immune system (World Health Organization, 2018) (National cancer institute, 2015).

This ability is termed Cancer immune evasion and it imposes a big obstacle in the development of effective cancer therapies (Vinay, et al., 2015).

i. Tumors and immunity:

Generally speaking, immune cells ($CD8^+$ and $CD4^+$) can suppress tumor growth through the production of interferon (IFN)-γ and cytotoxins, however several factors such as chronic inflammation may negatively affect this process.

Cancer cells possess tumor antigens, which facilitate their recognition and elimination by the immune cells, however due to the genetic instability the constant cell division can generate tumor cells with reduced immunogenicity that can evade immune elimination, this could create an “equilibrium” where the immune cells destroy tumor cells that keep dividing and accumulating mutational changes by chance or in response to immune-induced inflammation, this balanced state eventually favors the tumor as cancer cells develop the ability to impair the immune system capability to irradicate them, through immune suppressive effects or by loss of target antigen expression.

This phenomenon explains tumor dormancy, where tumors stay dormant for years in patients before remerging or reinitiating their proliferation (Vinay, et al., 2015).

1. Immune suppression:

In essence this happens mainly due to activated regulatory T cells (Tregs) or other regulatory cells in the tumor microenvironment suppressing other immune system components. Due to the dead ends in the tumor vascularization, a leakage of liquid and proteins happen at the site encouraging lymphatic formation this while tumor cells release chemokines to attract the Treg cells (Vinay, et al., 2015)**,** (National cancer institute, 2015).

2. Defective antigen presentation:

Tumors also evade the immune system through a complex down-modulating antigen processing that downregulate the major histocompatibility complex (MHC) I pathway, proteosome subunits latent membrane protein (LMP)2 and LMP7, transporter associated with antigen processing (TAP) protein, and tapasin, which leads to cytotoxic T lymphocytes (CTL) no longer recognizing target antigens on the tumor cells, allowing them to go by unnoticed (Vinay, et al., 2015).

3. Immune deviation:

Tumor cells exabit a behavior where they induce anergy in T cells by engaging the T cell receptor in the absence of costimulation, this happens because cancer cells in themselves fail to express costimulatory molecules.

Tumor cells evade immune attack by shifting the balance from Th1 to Th2 as well hence the name "deviation", they do this by releasing transforming growth factor (TGF)-β (Vinay, et al., 2015).

4. Apoptosis:

Apoptotic cells, themselves, are potent regulators of their cellular environment. Tumors, especially, exist in a dynamic balance of cell proliferation and cell death. In both normal and malignant tissues, apoptotic regulation is exerted through immune as well as non-immune mechanisms. Apoptotic cells suppress the repertoire of immune reactivities, both by attenuating innate (especially inflammatory) responses and by abrogating adaptive responses. In addition, apoptotic cells modulate multiple vital cell activities, including survival, proliferation, and growth (Ucker & Levine, 2018) (Vinay, et al., 2015)**.**

ii. Tumor heterogeneity:

As we stated earlier tumors much like organs present a wide range of different cells with different genetic alterations and states of differentiation, this is known as "Tumor heterogeneity", and is translated in tumor cells expressing a wide variety of antigens, an "antigen heterogeneity" that contributes to differences in behavior towards the immune system, also these antigens are unevenly distributed throughout the tumor's population and induce different immune responses to the same determinant, making the tumor more resilient (Vinay, et al., 2015)**,** (Croce, 2008)**.**

d. Oncology:

i. What is Oncology:

Oncology is a branch of science that deals with prevention diagnosis and treatment of cancer (Nature, 2020).

1. Prevention:

Involves any action taken to lower the risk of cancer, this includes maintaining a healthy lifestyle, avoiding exposure to known cancer-causing substances and cancer risk factors, keeping a healthy lifestyle, and taking medicines or vaccines that help the body prevent the occurrence of cancer (National cancer institute, 2015).

2. Screening:

Cancer screening is the action of checking for cancer (or for abnormal cells that may become cancer) in people who don't display any cancer symptoms.

The screening is carried out through a series of tests known as "Screening tests" and is of great importance, because one of the worst things about cancer is its ability to develop undetected, as such the screening helps doctors to diagnose and treat types cancer early on before reaching life threatening stages, where treating it might be difficult or even fail (National cancer institute, 2018) (Nature, 2020)**.**

Of the most effective tests we name:

- Low-dose helical computed tomography: used for the detection of the most predominant type of cancer: Lung cancer, showing great results as it helped in reducing deaths among heavy smokers.
- Pap test and human papillomavirus (HPV) testing: each alone or in a combination used for the early detection, diagnosis and prevention of cervical cancer as they allow doctors to spot abnormal cells before fully becoming cancerous.
- Colonoscopy, sigmoidoscopy, and stool tests (high-sensitivity fecal occult blood tests and stool DNA tests): These screening tests help in the early detection of colorectal cancer, colonoscopy and sigmoidoscopy help in spotting abnormal growths before turning into cancer.
- Mammography: Used for the detection of for breast cancer and has been shown to reduce deaths from the disease among women over age 50.
- Other screening tests include: Alpha-fetoprotein blood test, Breast MRI, CA-125 test, Clinical breast exams and regular breast self-exams, PSA test, Skin exams, Transvaginal ultrasound and Virtual colonoscopy (National cancer institute, 2019) .

3. Diagnosis:

Diagnosing cancer is not an easy task, due to its stealthy nature and having generic symptoms that can be confused with symptoms caused by other illnesses, doctors often use the following tests to better diagnose cancer (Nature, 2020) (National cancer institute, 2019) .

- Lab tests: includes any lab examination of tissue samples, blood, urine or other body fluids, to measure the levels of certain substances that could indicate the presence of cancer, these substances are dubbed tumor markers, which are any substance produced by tumor cells or by other normal body cells in response to the presence of cancer. Some of these lab tests provide good results about the state of the patient, other tests are done in addition to imaging tests for a more precise and better diagnosis (National cancer institute, 2019) (Nature, 2020).
- Cancer imaging: a term that covers an array of tests that are carried out using specialized machines to create images of areas inside of the body allowing doctors to check for the presence of tumors. Cancer imaging is also used to help guide surgical and radiographical treatments (National cancer institute, 2019) (Nature, 2020)**.** The imaging tests include:
    - CT scan: or Computed tomography, the patient lie down on a table that slides through a donut-shaped scanner which is linked to a computer, the scanner uses X-rays to take a series of images from different angles to construct a detailed 3D image of the inside of the body.
    - MRI: Much like the CT scan the patient lie down on a table but in this case, it slides into a long round chamber that uses powerful magnet and radio waves to produce detailed images that can show the difference between healthy and unhealthy tissues as well as the abnormal growths.

in some cases, when taking CT and MRI tests, the patient may receive a contrast material either by swallowing a dye or by injection to help highlighting certain areas and makes reading the images easier.

- Nuclear scan: Before this scan the patient takes radioactive substance known as “Tracer” which flows through the blood stream and collects in certain areas of the body, then the scanner measures the radioactivity in the body resulting of images of you the organs or bones where the traces has collected. The tracer then loses its radioactivity with time and is eliminated naturally from the body.
- Bone scan: falls under the nuclear scans type, it checks for abnormal areas in bones and Is used to diagnose bone cancer or metastatic cancer that has spread into the bone.
- PET: Positron emission tomography, also a nuclear scan, in this case however the tracer “radioactive glucose” is used to visualize and measure metabolic activities by tracking of where glucose is taken, cancer cells by nature take more glucose than normal cells giving 3d detailed pictures of the health and cancerous tissues.

- Ultrasound: The scanner “transducer” is passed on the skin of the body parts in question by a technician while the patient lies down on a table. The transducer uses high energy sound waves that runs through the tissues, then the machine captures the echo and uses it to produce the images which are dubbed “sonogram” (National cancer institute, 2019).

- Biopsy: in oncology biopsies are considered the cornerstone of malignancy diagnosis and are used to identify tumor histology, and confirm the presence of metastases for staging (Ziv, Durack, & Solomon, 2016). A biopsy test involves the observation of a tissue sample under the microscope to produce a complete “pathology report”, which contains details about the diagnosis. The sample is usually taken using a needle, through surgery or endoscopy (Colonoscopy and Bronchoscopy) (National cancer institute, 2019). In the past 2 decades, the introduction of imaging techniques into biopsy have enabled safe and accurate diagnosis of primary and metastatic cancer, and today percutaneous image-guided biopsy is one of the most commonly performed procedures in radiology departments (Marshall, Laberge, Firetag, Miller, & Kerlan, 2013). Another recent development in biopsy is the ability to detect, characterize and tumors using cancer biomarkers (circulating tumor cells, circulating tumor DNA and exosomes etc.…) circulating body fluids such as the blood, this approach is known as “liquid biopsy” and is set to revolutionize the early detection of cancer by allowing doctors to analyze the blood instead of a tissue sample as the synergy of multiple circulating biomarkers can reveal the specifics and of a cancer including status, origin and progression. Moreover, liquid biopsy helps characterize the immune response towards cancer facilitating immunotherapy (Alix-Panabières, 2020) (Nature, 2020) (Palmirotta, et al., 2018). Artificial intelligence plays a huge role in the standardization of liquid biopsy as the development of an algorithm that handles the information and classify the tumor (Alix-Panabières, 2020) (Nature, 2020). In fact, the development of an algorithm that can combine all the data liquid biopsy provides to obtain a precise tumor profile would usher a new era in cancer diagnosis, screening, and could provide a detailed orientation to guide treatment choices (Alix-Panabières, 2020).

4. Staging:

Cancer staging involves the classification of cancer by determining its state and extent of spread, determining the stage of cancer is indispensable in oncology as it helps doctors understand the state of the patient, the prognosis and the seriousness of the disease, and is a prerequisite to shape the optimal treatment plan (National cancer institute, 2015) (Brierley, Gospodarowicz, & O'Sullivan, 2016).

Several staging systems enable the determination of the stage of cancer, of which the TNM classification system is the most widely used.

- The TNM system classifies cancer according to the size and state of the primary Tumor (T) the

involvement of regional lymph nodes (N) and the presence metastasis (M) (National cancer institute, 2015) (Nature, 2020)**.**

Primary tumor (T):

TX: Main tumor cannot be measured.

T0: Main tumor cannot be found.

T1, T2, T3, T4: Refers to the size and/or extent of the main tumor. The higher the number after the T, the larger the tumor or the more it has grown into nearby tissues. T's may be further divided to provide more detail, such as T3a and T3b.

Regional lymph nodes (N):

NX: Cancer in nearby lymph nodes cannot be measured.

N0: There is no cancer in nearby lymph nodes.

N1, N2, N3: Refers to the number and location of lymph nodes that contain cancer. The higher the number after the N, the more lymph nodes that contain cancer.

Distant metastasis (M):

MX: Metastasis cannot be measured.

M0: Cancer has not spread to other parts of the body.

M1: Cancer has spread to other parts of the body (National cancer institute, 2015).

5. Treatment:

The history of cancer treatment dates back to antient Egyptian and Greek civilizations, although radical and primitive, surgery and Cauterizations were practiced to remove the abnormal growths, these were in most cases ineffective and even caused deaths, but it was a start, a start to a bumpy history with ups and downs, not only to the ineffectiveness and side effects but also to raising hope and facing the harsh reality of failure (Falzone, Salomone, & Libra, 2018) (Arruebo, et al., 2011).

Nowadays, oncology and cancer treatment branch of into three categories: Medical oncology, which is based on the use of chemotherapy, immunotherapy hormonotherapy, and several drugs to treat cancer, radiation oncology which includes the use of radiation to treat the disease, and surgical oncology which rely on surgical approaches to remove tumors and deal with cancer, chemotherapy, surgery and radiotherapy remain the most common and widely used cancer treatments (Nature, 2020) (Arruebo, et al., 2011)**.**

Being a complex disease, most cancer cases are treated with a combination of two or more treatment approaches rather than one.

Another concept that has always been involved with cancer treatment is the management of side effects. Because cancer is essentially a mall function and an abnormal proliferation of cells, most

treatments that deal with it tend to harm healthy tissues as well, which in some cases would have sever implications on the patient's health (National cancer institute, 2015).

ii. Common treatment approaches:

1. Surgery:

The oldest trick in the book regarding the treatment of cancer, although quite different and evolved than early approaches in ancient civilizations in essence cancer surgery still involves the removal of the tumor or cancerous cells from the body. The classical surgery approach remains the most used in cancer treatment, technological advances however drove several approaches to surgically treat cancer:

- Cryosurgery: For some early stage cancer types like skin cancers this approach is used, it involves the use of liquid nitrogen or argon gas to destroy tumorous cells by exposure to extreme cold.
- Lasers: Unlike classical surgery which depends in sharp tool to cut the tissues this approach uses focused light beams which increases the accuracy dramatically.
- Hyperthermia: it refers to exposure of the tumor cells to high temperatures for their destruction or to make them more sensitive for other treatments like chemotherapy and radiotherapy (National cancer institute, 2015) (Arruebo, et al., 2011) (Falzone, Salomone, & Libra, 2018)**.**

2. Chemotherapy:

Chemotherapy relies on the use of drugs to stop or slow the fast growth of cancer cells; the substances are known as cytostatics and they fall under different types and often are used in combination with each other. The medication is usually introduced into the body through infusion into a vein (IV) or in some cases as tablets. In systemic treatments the drugs circulate in the blood stream and affect the whole body meaning they could deal with undetected cancer cells or tumors; however, this causes these cytostatics to act on and harm healthy tissues. In some types of cancer local chemotherapy is used and the medications are introduced not to the blood stream but rather injected directly into the affected part of the body. For people requiring chemotherapy treatment over a long period of time a port is installed under their skin, which is a small container that is connected to a large vein and remain in the body for as long as the course of treatment lasts enabling the infusion to be connected directly to it without having to look for a vein and puncture it for each treatment.

- Chemotherapy cycles: Most chemotherapy treatments are given in repeating cycles with specific intervals, the nature of the drug (how long the effect lasts and side effects), the state of the patient (how much it takes for the body to recover), and the overall length of the treatment influence the number of cycles and the intervals (Institute for Quality and Efficiency in Health Care, 2012) (National cancer institute, 2015)**.**

3. Radiotherapy:

In oncology radiation therapy plays a big role in dealing with cancer as 50% of all cancer patients receive radiation therapy at some point during their course of cancer treatment, and it contributes towards 40% of curative treatment. Radiation could be used as a cure for cancer as well as palliative treatment to relieve patient of the symptoms, and in a lot of cases is used in combination with surgery, chemotherapy and immunotherapy.

Considered a physical agent (photons, protons, electrons or neutrons), the radiation used in this approach form electrically charged ions hence the name "ionizing radiation", and deposit energy into the cells of the tissues it passes through. The radiation acts by damaging genetic materials thus blocking and striping the cells of the ability to divide, multiply and proliferate, this however is true for all cells and radiotherapy is harmful for both healthy and cancerous tissues. Normal cells are more efficient at repairing themselves compared to cancerous ones, still with the aid of technological advances the aim of this type of therapy is to maximize the radiation doses to abnormal cells while minimizing exposure to normal cells even in tumor neighboring tissues.

In most cases radiation is delivered as external radiation by specialized machines that aim high energy beams to the tumor, in some cases like prostate cancer that requires routine treatment or when retreatment is indicated, internal radiation is used instead, dubbed "brachytherapy" is done by sealing a radiation source directly to the tumor site, other cases require a third type known as "Radioisotope therapy" which involves the injection of a liquid form of radiating substances that are designed to target specific tissues like thyroid.

Given that radiotherapy is very harmful to healthy tissues, innovation and technological advances are of great importance for optimal treatment process this drives different techniques for radiotherapy:

- Fractionation: In a nutshell it involves the deliverance of different radiation regimes depending on the type, stage and state of the tumor as well as the sensibility of the tissues surrounding it.
- 3D Conformal radiotherapy (3DCRT): guided by CT imaging this technique allows for accurate localization of tumor for the deliverance of radiation as well as normal organs for shielding.
- Intensity modulated radiation therapy (IMRT): Guided and controlled by a computer, this approach allows the oncologist to control the intensity of the radiation enabling irregular-shape radiation doses, enabling better targeting for tumors and avoidance of healthy tissues.
- Stereotactic body radiation therapy (SBRT): Relying on the previous technological advances this approach is based on the precise deliverance of very high individual doses of radiation over only a few treatment fractions to small, well-defined primary and oligometastatic tumors anywhere in the body.

Radiotherapy falls under two types depending on the type of radiation used:

Photons radiation (X and Gamma rays) which is the most widely used and Particles radiation (Protons, Electrons and Neutrons) (Baskar, Lee, Yeo, & Yeoh, 2012) (Jaffray & Gospodarowicz, 2015) .

Radiation therapy other than as a cure, it is commonly used as adjuvant treatment following surgery, or after chemotherapy when by itself is not expected to be enough of a cure. Another revolutionary use for radiotherapy is when associated with chemotherapy in what is known as "radio-chemotherapy", as cytostatics have effects that activate when combined with radiation, in this case it is local chemotherapy as it only has an effect where radiation was applied (Institute for Quality and Efficiency in Health Care, 2012) (Jaffray & Gospodarowicz, 2015).

4. Immunotherapy:

Surgery, chemotherapy and radiotherapy have few things in common of which the wide use in oncology and that they are not specific therapies, in contrast cancer immunotherapy represents a standing example of precision oncology, in essence, this type of treatment could be defined as helping or tailoring the immune system to deal with cancer. The idea of using the immune system to fight off cancer originated in the nineteenth century when scientists noticed spontaneous regression of tumors following the development of superficial skin infection, the idea reemerged again in the twentieth century but it remained a hard to control and to manipulate approach, until recent years with better understanding of the process of immune surveillance, by which innate immune cells eliminate cancer ones and thanks to technological advances it revolutionized the field of oncology in terms of survival and quality of life (Waldman, Fritz, & Lenardo, 2020) (Liu & Guo, 2018) (Esfahani, et al., 2020)**.** Immunotherapy is built on 4 main pillars, immune checkpoint blockade, cytokine therapy, cellular therapy and therapeutic vaccines.

- Immune checkpoint blockade: One of the main ways cancer cells evade the destruction by the immune cells is through immune suppression by calling for regulatory T cells, this approach involves inhibition of the immune system's intrinsic regulatory mechanisms, thus, driving the activation of a better anti-cancer immunological responses (Christofi, Baritaki, Falzone, Libra, & Zaravinos., 2019).

- Immunotherapy with Cytokine Therapy: cancer cells influence and activate their microenvironment (stroma, blood vessels and immune cells), this influence has a positive feedback on the development of cancer and is indispensable for tumor progression and metastasis, this happens through an orchestrated signaling crosstalk between tumor-associated neutrophils (TANs), tumor-associated macrophages (TAMs), innate lymphoid cells (ILCs), MDSCs, mast cells, T cells and NK cells that produce various factors such as enzymes, chemokines and cytokines which can increase angiogenesis and drive cancer progression. This therapeutic approach relies on the

disruption of this dynamic by the calculated injection of cytokines that stimulate the immune cells to attack and destroy the cancerous ones (Christofi, Baritaki, Falzone, Libra, & Zaravinos., 2019).

- Cellular Therapy (redirected T cells): a new and very promising approaches have surfaced in the last few years which include the isolated Tumor-infiltrating lymphocytes (TIL) from an existing tumor mass, and co-culturation with IL2 to grow and proliferate ex vivo, then the reintroduction of the subpopulation of the proliferating cells into the tumor site, the high numbers of TILs are reinfused to the patient to provoke in vivo immune response, this is known as the adoptive cell transfer (ACT) and despite being promising and showing great results this approach however works only on specific cancers and not all types, hence the focus on the development of ACT strategies using modified patient-specific T cells that carry genetically engineered antigen receptors in the form of either native engineered T Cell Receptor (TCRs) or chimeric molecules (CARs). The idea for the TCRs approach arose from the fact that not all patients have T cells that have already recognized their tumors due to defective antigen presentation or other factor, thus taking T cells from patients, but instead of just activating and expanding the available anti-tumor T cells like with the classic ACT strategy, here the T cells can also be equipped with a new receptor that enables them to target specific cancer antigens enabling targeted therapy. The CARs approach on the other hand revolves around equipping the T cells (and in newer trials Natural killer cells 'NK') with synthetic receptors to overcome the biological limitations of the antigens needing to bounded by the major histocompatibility complex (MHC) to be recognized by the immune cells (Christofi, Baritaki, Falzone, Libra, & Zaravinos., 2019) (Cancer research institute , 2020).

- Therapeutic Vaccines: vaccination against tumor initiation-involved viruses can provide a precautionary measure against cancer. This is known as "preventative vaccines" that help in the prevention of cancer, the revolutionary approach however, involves engineered vaccines that stimulate an immune response against cancer "Therapeutic Vaccines". By acting against both tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs) these engineered vaccines can provoke an immune response by stimulating the immune system to recognize and destroy tumor cells presenting said antigens. Another promising and novel approach revolves around the activation of anti-Treg cells (anti-regulatory T cells), this enables the immune system to bypass the immune suppression induced by the cancerous cells in the tumor microenvironment (Christofi, Baritaki, Falzone, Libra, & Zaravinos., 2019) (National cancer institute, 2015).

iii. Why conventional treatments fail:

Despite coming along way, despite all the technological advancements, and despite all the efforts of oncologists, research centers and philanthropists, cancer therapeutics remain the treatment with the lowest clinical trial success rate of all major diseases, this is in one part to the inability of conventional treatments (Surgery, chemotherapy and radiation) to completely irradicate cancer, even if few tumor cells survive their nature gives them the ability to proliferate again and cancer will reemerge, even though immunotherapy shows great promise to deal with this promise the fact of the matter is the approaches in this type of treatment are in the early stages and more clinical trials are needed for the incorporation of this treatment method (Alberts, et al., 2002) (Waldman, Fritz, & Lenardo, 2020) (Esfahani, et al., 2020) (Liu & Guo, 2018). To make matter worse, his survivability issue is amplified by the fact that tumors possess cytogenetically different clones that arise from the initial transformed cell after undergoing further genetic alterations, this heterogeneity results cancer cells behaving differently and vary widely in their response and sensitivity to radiation and to the different kinds of cytotoxic drugs, making clinical management difficult (Croce, 2008) (Alberts, et al., 2002).

Due to their genetic instability cancer cells can develop treatment resistance and it gets even worse as in a lot of cases it is a "multidrug resistance" even for drugs these cells have never been exposed to, this is correlated with the amplification of a part of the genome that contains a gene called Mdr1. This gene codes for a plasma-membrane-bound transport ATPase, which if overproduced can prevent the intracellular accumulation of certain lipophilic drugs by pumping them out of the cell (Alberts, et al., 2002).

Another big reason and one of the most treatment failure causing properties of cancer is its metastatic nature, metastasis renders almost all local therapies useless and furthermore the ability to metastasize in a stealthy manner hinders the accuracy of diagnosis and the ability to determine the right treatment plan (Chakraborty & Rahman, 2012).

The search of cancer cures is very difficult but the situation is not entirely hopeless, our understanding of cancer increases by the second and new technological advances are always on the horizon, one in particular carries with it a great promise to revolutionize cancer treatment field as well as plethora of other biology and translational medicine: the "CRISPR-CAS" system (clustered regularly interspaced short palindromic repeats – CRISPR associated proteins).

3. *CHAPTER II: What is CRISPR:*

a. Introduction into gene editing:

i. What is gene editing:

Gene editing involves inserting, deleting, modifying and replacing DNA in the genome of living organisms, it started in the 1970s, when scientists bombarded plants with radiation to cause random mutations. Gene editing started to show great promise with the incorporation of engineered gene-editing nucleases (Zink finger, Transcription Activator-Like Effectors, Meganucleases and CRISPR-Cas systems) which introduced the concept of targeted genome editing. The nucleases induce Double-Stranded breaks (DBS) into the targeted genomic locus. The nuclease-induced DSBs can be repaired by one of two different pathways that operate in nearly all cell types and organisms: nonhomologous end-joining (NHEJ) and homology-directed repair (HDR). The NHEJ pathway involves the cell's own repair system joining the DNA ends of a DSB, this process is prone to errors and can lead to the efficient introduction of insertion/deletion mutations (indels) of various lengths, which can disrupt the function of the gene. The HDR pathway relies on recombination of the DSBs ends and a homologous piece of DNA present in the nucleus, this system can be used to introduced precise and desired mutations to the target genome (Sander & Joung, 2014).

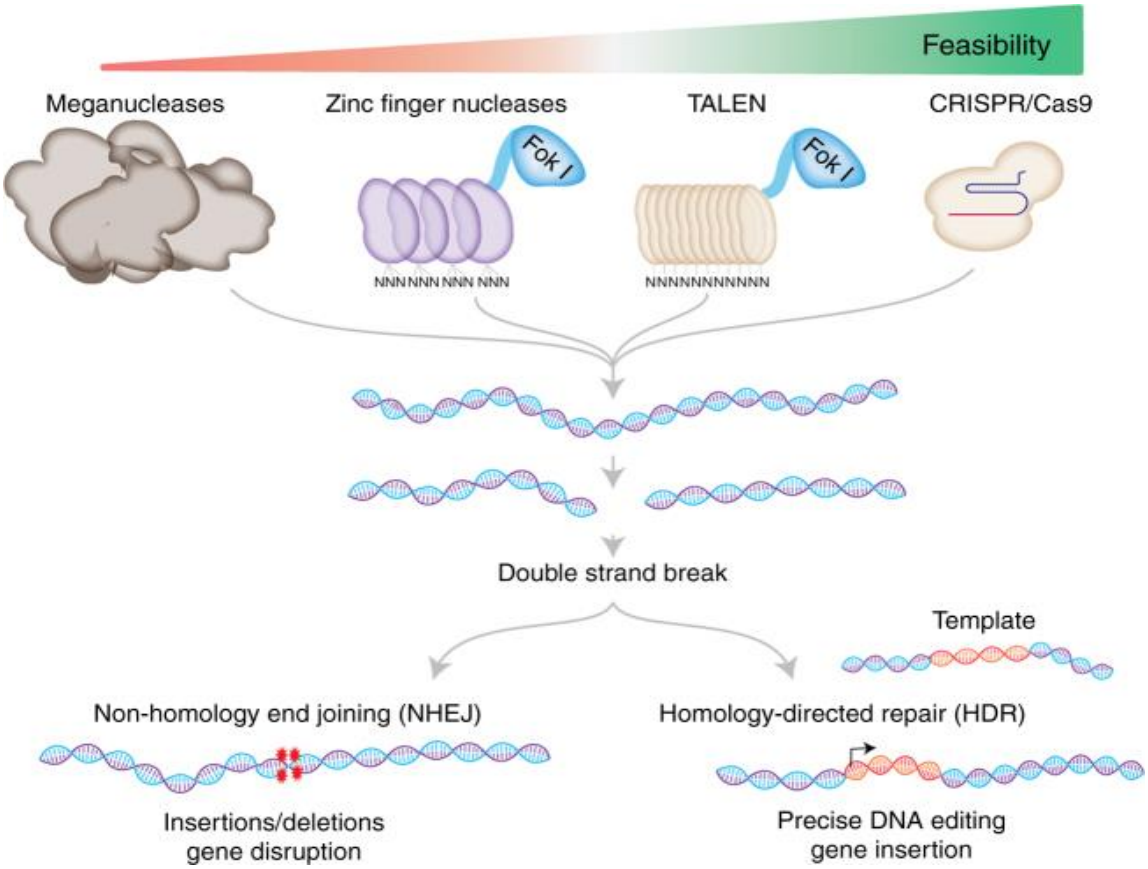

Figure 5*: The basic working principle of major genome-editing technologies*

(Adli, 2018)**.**

ii. Gene editing before CRISPR:

1. Zinc-Finger Nucleases:

ZFNs are fusions between Cys2-His2 zinc-finger protein and the cleavage domain of the Fok I restriction endonuclease that have been adopted as gene-targeting tools, in essence they are targetable DNA cleavage reagents (Carroll, 2011) (Gaj, Sirk, Shui, & Liu, 2016). Their discovery came when Chandrasekaran S. realized that the natural restriction enzyme, Fok I, has physically separable binding and cleavage activities and the cleavage domain could be guided and the cutting could be redirected by replacing the recognition domains with alternative ones (Carroll, 2011). Of these alternatives the Cys2His2 zinc fingers (ZFs) were the most useful, usually 3 to 4 zinc fingers are fused to the Fok I cleavage domain, of which each finger is comprised of 30 amino acids residues and interacts with three base pairs of DNA with occasional overlap from an adjacent domain. In order for the Fokl domain to cut DNA it needs to dimerize (a chemical reaction that joins two molecular subunits, resulting in the formation of a single unit known as dimer) and the dimer interface is weak, and to solve this problem, achieve better specificity and the best way to achieve cleavage is to construct two opposed sets of fingers directed to neighboring sequences, when both sets are bound to their recognition sites, high local concentration facilitates the dimerization process and cleavage, resulting in a double-strand break (DSB) in the DNA (Carroll, 2011) (Gaj, Sirk, Shui, & Liu, 2016). Despite the great promise the difficulty of constructing the Zinc fingers hindered the widespread of this approach as it remains really challenging to create zinc-finger domains that can effectively recognize all DNA triplets outside specialized laboratories (Gaj, Sirk, Shui, & Liu, 2016).

2. TALE Nucleases:

TALEs (transcription activator-like effectors) are bacterial proteins that in 2009 scientists discovered the code behind TALEs' capability to recognize DNA dp, enabling the creation of custom TALE nucleases which are comprised of an amino-terminal TALE DNA-binding domain fused to a carboxy-terminal Fok I cleavage domain, enabling the editing of nearly any gene. Much ZFNs the Fokl domain is responsible of the dimerization and the cutting, unlike zinc fingers, which recognize DNA triplets, each TALE repeat recognizes only a single bp, and typically TALEs are assembled to recognize from 12 to 20 dps with more bases typically leading to higher genome-editing specificity. TALEs have a highly repetitive structure, making their delivery to the cell (through lentiviruses or a single adeno-associated virus) less efficient however have been reported to show improved specificity and reduced toxicity compared to some ZFNs, and engineering TALE arrays is much easier reducing the amount of time and experience needed to assemble a functional nuclease (Gaj, Sirk, Shui, & Liu, 2016) (Boch, et al., 2009).

3. Meganucleases:

The final member of the targeted nuclease family, also known as homing endonucleases, are highly specific DNA cleaving enzymes that have been engineered to be used for applications that require targeted gene modification (Stoddard, 2014). Discovered in the 1970s, these enzymes recognize and cleave long DNA sequences after make extensive sequence-specific contacts with their DNA substrate and show superlative specificity. Their biggest limitation however is that the recognition and cleavage domains are not separable and thus their repurposing and engineering is challenging, and their utility is limited (Gaj, Sirk, Shui, & Liu, 2016) (Stoddard, 2014)**.**

b. CRISPR:

i. What is CRISPR:

CRISPR are distinct arrays of 29-nucleotide repeat sequences separated by various 32-nt spacer sequences, these sequences were discovered in 1987 when they were observed in some bacteria, and then found to be in 40% of all sequenced bacterial genomes and in nearly 90% of archaea. Although scientists have hypothesized on the function of the CRISPR sequences for years, the nature of that function has been elucidated only recently, in 2005 it was observed that the spacer sequences actually originate from phage genomes, and that these bacteria and archaea were immune to viruses with genomes identical to the sequences carried in the CRISPR arrays (Makarova, et al., 2011) (Song, et al., 2016) (Bolotin, Quinquis, Sorokin, & Ehrlich., 2005) (Mojica, Díez-Villaseñor, García-Martínez, & Soria, 2005).

Independently, several types of Cas genes were found adjacent to CRISPR sequences, when analyzed in detail the adjacent sequences found to contain domains that are characteristic of several nucleases, a helicase, a polymerase and various RNA-binding proteins, these proteins were initially thought of to constitute to a novel DNA repair system in prokaryotic cells, however with the observation that these Cas genes always neighbored the CRISPR array and the spacer sequences being identical to fragments of viral genome, a new hypothesis emerged, the CRISPR-Cas system serves as a critical immune system to protect bacteria and archaea from pathogen invasions (Makarova, et al., 2011) (Song, et al., 2016)**.** This hypothesis was confirmed in 2007 when it was shown that that integration of a short phage-specific sequence into the CRISPR array of Streptococcus thermophilus conferred resistance to the cognate phage, and a complete loss of immunity towards the virus with the existence of a single mismatch between the CRISPR spacer and the target virus sequence (Barrangou, et al., 2007).

ii. Classification and Associated proteins:

Given the fact that CRISPR is a fairly new technology, several of its properties and functions are only recently understood or are yet to be elucidated, this is also the case for its associated proteins Cas (CRISPR-Associated proteins), as such new classifications are out every few years. The current classification includes 2 classes, 6 types and 33 subtypes.

- Class one: use a complex of multiple Cas proteins to degrade foreign nucleic acids, includes the Types I, III and IV, and is comprised of 16 subtypes.
- Class two: use a single large Cas protein to induce DNA degradation, includes the Types II, V and VI, and 17 subtypes.

The subtypes are characterized by a "signature gene" found almost exclusively in the category (Makarova, et al., 2019).

iii. How does the CRISPR-Cas system work?

The process in which the CRISPR–Cas systems mediate immunity against foreign genetic elements is divided into three stages “Adaptation, expression and interference” that fall under two subsystems; the first subsystem is highly conserved and is known as the “information processing” subsystem, includes the adaptation stage, and the proteins involved in it (Cas1 and Cas2) are conserved between most prokaryotic beings. The “‘executive’” subsystem is second and includes the expression and interference stages, and unlike the first the proteins in this subsystem vary greatly between different organisms (Makarova, et al., 2011).

- The adaptation stage revolves around the integration of the short pieces of virus DNA sequences or plasmid into the CRISPR loci. This insertion prosses is known as “spacer acquisition” and is triggered by viral invasions where a single virus-derived resistance-conferring spacer, with a characteristic length of approximately 30bp, at the leader side of a CRISPR locus, this is accompanied by the duplication of a repeat sequence creating a new spacer–repeat unit (Makarova, et al., 2011) (Barrangou, et al., 2007) (McGinn & Marraffini, 2018).

The adaptation stage starts off with the recognition of foreign nucleic acids or “protospacers”, this step is of great importance for the prokaryotic cells to avoid the integration of self-targeting spacers from the host’s own chromosome into CRISPR which leads to autoimmunity and even cell death. To avoid the self-targeting, the CRISPR system employs various mechanisms that are bias to only acquire foreign genetic elements, CRISPR-Cas exploits the nature of the viral genome to stimulates spacer acquisition from double-strand breaks, this is carried out by relying on DNA repair machinery of the host, this only binds to free ends of double stranded DNA to perform end resection during homologous recombination, this creates a biased recognition for viral DNA, as the bacterial chromosome is circular and lacks free DNA ends. Furthermore, the actions of the DNA repair system are inhibited by the presence of chi sites, which are eight nucleotide sequence motifs, the

concentration of these Chi sites is much higher in the host's genome than is in the invader's DNA, this constrains spacer acquisition from the host genome and differentiate self from non-self-nucleic acids. It remains unresolved if and how the DNA captured by the repair system could be used for spacer integration, with the leading hypothesis being that Cas1 and Cas2 physically associates with the DNA repair component to directly uptake degradation products (McGinn & Marraffini, 2018) (Levy, et al., 2015)**.** Another important aspect of the recognition step is that the CRISPR-Cas system must select protospacers that can be functional spacers under type specific targeting requirements, this is to ensure the cleavage of foreign DNA and to prevent the cleavage of the spacer sequence in the CRISPR array during the interference stage. Different types present different requirements, in type I and II the acquisition machinery preferentially samples genes that are neighbored by "protospacer-adjacent motif" however the two types achieve this differently, in type I the Cas1–Cas2 complex has direct, sequence-specific interactions with the PAM that bias acquisition to PAM-adjacent protospacers, in type II however this selectivity for PAM adjacent is the PAM-interacting domain of Cas9 which interacts directly with the Cas1–Cas2 complex (McGinn & Marraffini, 2018). In type III systems, the discrimination between self and non-self is achieved via the 5′ tag of the mature crRNA, which must not base pair with the target to enable degradation by the complex (Hille & Charpentier, 2016).

- The expression stage involves the transcription process of the CRISPR locus to generate RNA-protein guides, all systems transcribe the CRISPR locus, and generate the long primary transcript pre-crRNA which depending on if the CRISPR has repeats or not may contain a series of secondary structures (hairpins), from here the pre-crRNA is processed down and catalysed by endoribonucleases into short crRNAs, the endoribonuclease either operate as a large complex like "Cascade"(CRISPR associated complex for antiviral defence) in the case of type I CRISPR-Cas system in Escherichia coli or as a single enzyme such as Cas6 in type III CRISPR-Cas system of the archaeon Pyrococcus furiosus.

In the case of the Type I systems the complex remains associated with the Mature crRNA and in the Type III systems the Cas 6 passes the crRNA to a complex (a Type III complex) to undergoes a ruler-based sequence-unspecific processing and trimming on the 3' end, yielding a mature crRNA with a defined 5′ end and variable 3′ end (Makarova, et al., 2011) (Rath, Amlinger, Rath, & Lundgren, 2015). On the other end of the spectrum there are Type II systems, which employ a very different approach for crRNA biogenesis, the maturation process is carried out by the host RNase III in the presence of a trans-encoded RNA (tracrRNA) that base pairs with the pre-crRNA, this process is stabilised by the Cas9 protein and yields an intermediate form of crRNA, which is further processed by unknown mechanisms to reach the mature crRNA (Rath, Amlinger, Rath, & Lundgren, 2015) (Hille & Charpentier, 2016)**.**

- The interface stage starts with mature crRNAs, where in Type I CRISPR systems use a Cascade like complexes to bind the foreign DNA then stimulate the Cas3 protein to carry out the degradation, while Type II rely on a single protein, the Cas9 to induce the immunity. The Cas9 protein guided by the Trans-crRNA duplex scans the cell and when it encounters the matching nucleic acid it performs a double strand break at a very specific site. Type III CRISPR systems are able to target both DNA and RNA, by relying on Cas10-Csm (types III-A and III-D) and Cas10-Cmr (types III-B and III-C) complexes, where the Cas10 cleaves the DNA while Csm3 and Cmr4 cleave the transcribed mRNA in type III-A and type III-B CRISPR-Cas systems, respectively (Hille & Charpentier, 2016).

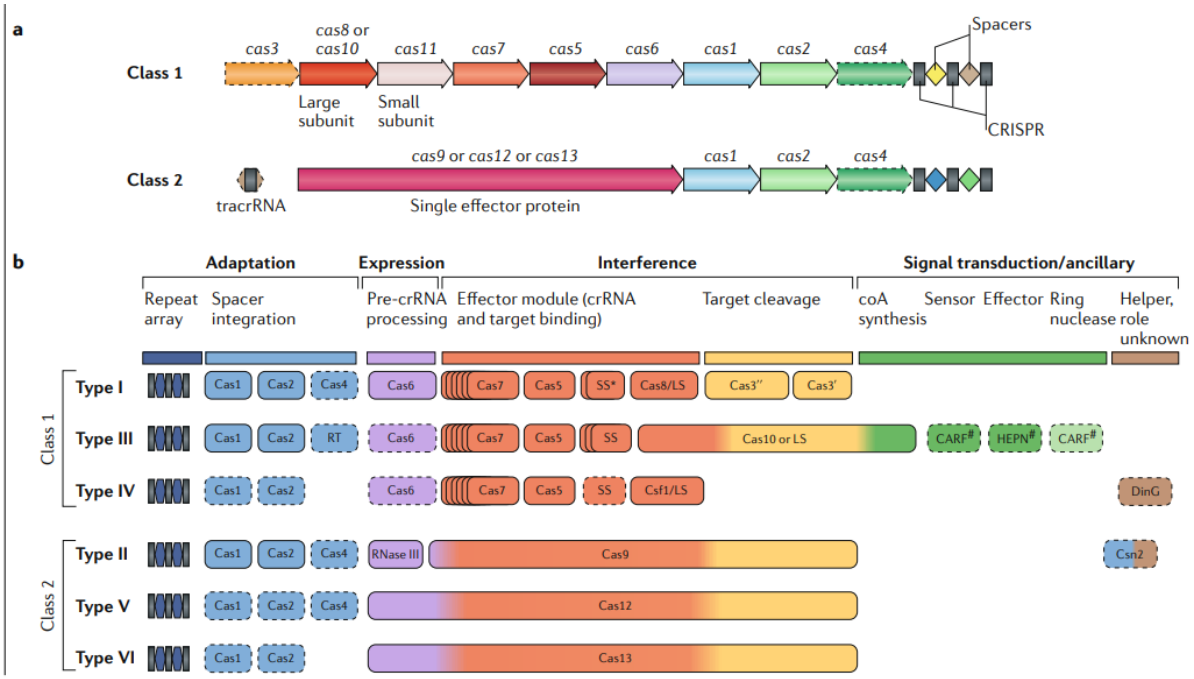

***Figure 6**: The two classes of CRISPR–Cas systems and their modular organization*

(Makarova, et al., 2019)

iv. How it is used in gene editing:

1. Different CRISPR systems in gene editing:

The one common theme among all CRISPR systems is their ability to induce target specific DNA changes, weather degradation or double stranded breaks; this qualify almost all of these systems to be used in genome editing, however some systems are too complex for practical use. Nowadays the Type II CRISPR-Cas9 system is the most widely used due to the simple "NGG" PAM sequence requirements (Adli, 2018).

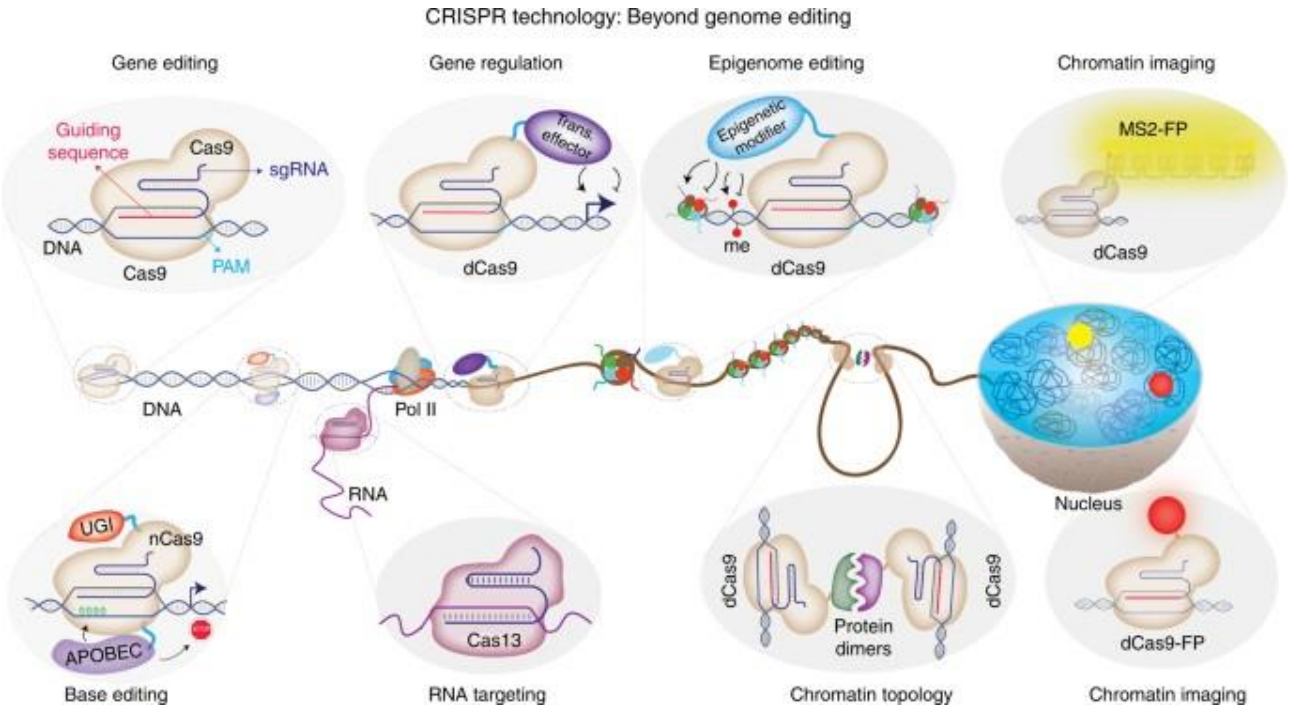

Figure 7*:Major application areas of CRISPR-Cas-based technologies beyond genome editing* (Adli, 2018).

2. The CRISPR/Cas9 system in gene editing:

The brilliance of the Type II CRISPR-Cas9 system lies in in the mixture of versatility and simplicity it provides, this type relies on the Cas9 endonuclease of Streptococcus pyogenes (spCas9) to cleave the target DNA; the Cas9 protein is comprised of two cleaving domains, the HNH domain and the RuvC-like nuclease domain. The union of crRNA and tracrRNA "gRNA" guides the Cas9 to the target site to perform sequence-specific cleavage by simple interaction of crRNA by base pairing, target sites must lie immediately 5′ of a PAM sequence that matches the canonical form 5′-NGG.This flexibility allows scientists to easily direct the Cas9 nuclease cleaving activity to any DNA sequence of the that is comprised of 18-24nt followed by NGG, simply by altering the first 18-24 nt of the gRNA (Rodríguez-Rodríguez, Ramírez-Solís, Garza-Elizondo, Garza-Rodríguez, & Barrera-Saldaña, 2019) (Sander & Joung, 2014). Since 2012 when it was shown that the CRISPR-Cas9 system could be programable with chemical RNAs that retain the

properties of the gRNA, the system has been implemented successfully for gene editing, and for the control of different types of biological systems, ranging from bacteria to in vitro human cancer cells and human pluripotent stem cells to whole organisms like the zebrafish, all while reducing the cost and time necessary for such alterations; as targeting of a new genomic locus for gene deletion, mutation and targeted insertion can be done rapidly through the generation of a gRNA, making CRISPR a powerful tool for research and drug development. However, being an acquired evolutionary arm that gives immunity mostly against viruses which undergo mutations quite frequently, a slightly less specific CRISPR system would be more advantageous to the host, and as such the CRISPR-Cas9 system the system allows cleavage at genomic locations partially complementary to the gRNA, this hinders the accuracy of the system and remains among the biggest issues that in some cases results in undesired consequences (Rodríguez-Rodríguez, Ramírez-Solís, Garza-Elizondo, Garza-Rodríguez, & Barrera-Saldaña, 2019) (Adli, 2018) (Sander & Joung, 2014)**.**

Several approaches have been taken to increase the specificity of the CRISPR-Cas9 system, through the re-engineering of the existing spCas9, the first proof of concept came in a study where it was shown that a specific point mutations has significantly increased the specificity of SpCas9, another approach is the replacement of the traditional SpCas9 with a mutant variant, the nSpCas9 which cuts a single strand through the inactivation of a nuclease domain RuvC or HNH, in this case and much like the ZFN and TALEN methods, two nCas9 are needed to target opposite strands of DNA in close proximity with each nCas9 guided by its own sgRNA reducing the off-target activity by 50 to 1,500 times. Furthermore, the fusion of the dCas9 (catalytically inactive Cas9) with the DNA cleavage domain of the Fok I, much like the previous approach the presence of two distinct sgRNAs substantially reduces the off-target activity (Adli, 2018) (Rodríguez-Rodríguez, Ramírez-Solís, Garza-Elizondo, Garza-Rodríguez, & Barrera-Saldaña, 2019) (Kleinstiver, et al., 2016). Away from the re-engineering of the Cas9 protein, efforts also focused on modifying the sgRNA which interestingly enough proved that both increasing and decreasing the length of the sgRNA guiding sequence by a few base pairs have enhanced the targeting specificity (Cho, et al., 2014) (Fu, Sander, Reyon, Cascio, & Joung, 2014).

3. CRISPR-Cas9 beyond gene editing:

Due to its ease of use and flexibility, the CRISPR-Cas9 provides a versatile tool for applications that are revolutionizing many genetic studies beyond gene editing. Soon after the discovery of the dCas9 and the ability to acquire catalytically inactive Cas9 that strongly binds to the DNA target sequence, interfering with the activity of other DNA binding proteins and without initiating any cleavage process and, scientists have tried to exploit this property to regulate gene expression, mediated epigenome editing and screening.

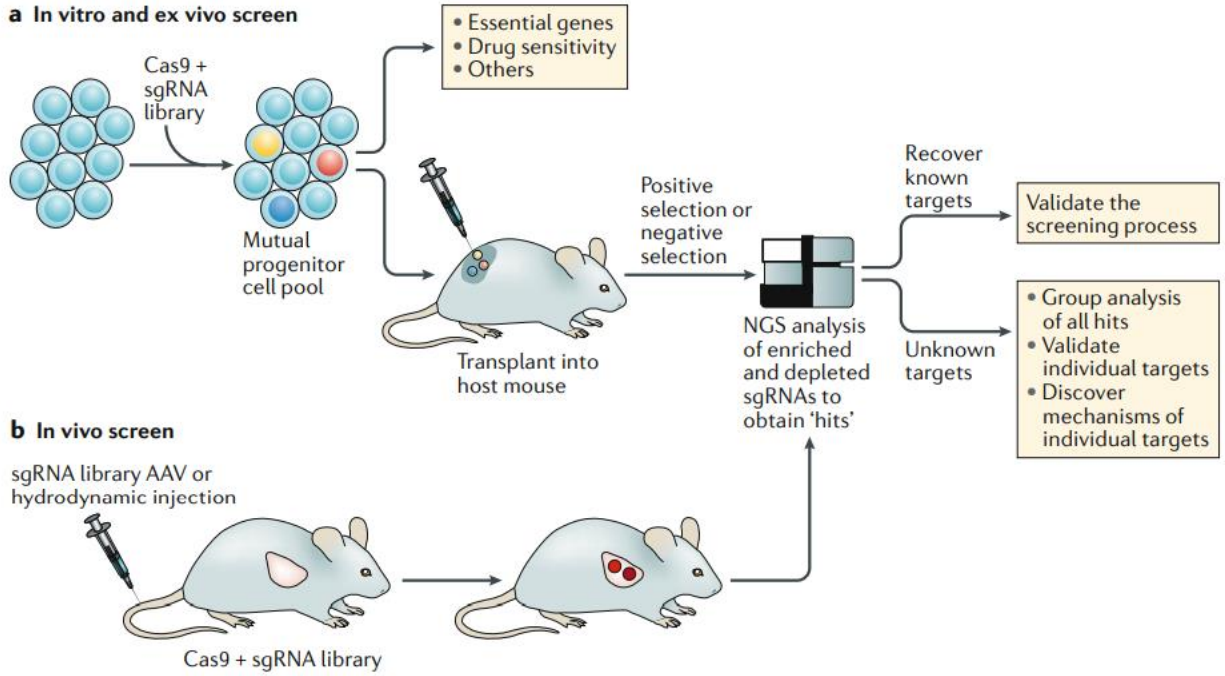

***Figure 8**: CRISPR for genetic screening*

(Yin, Xue, & Anderson, 2019)

Another use of the dCas9 is in live cell chromatin imaging; The organization of chromatin structure in the 3D nuclear space plays a critical role in regulating lineage-specific gene expression, as such the dCas9 is used in fusion with fluorescent proteins or labels to target specific and repetitive regions enabling their imaging. Non-repetitive regions imaging is more challenging due to interference of the background fluorescence signals (free-floating fluorescently labeled dCas9 proteins), for this, transfection of as many as 26–36 unique sgRNAs is typically required to achieve live cell imaging of a non-repeat genomic region (Adli, 2018). Chromatin imaging allows for a better understanding of their 3D structure, but beyond imaging one of the most exciting applications for the CRISPR-Cas9 system is Chromatin topology, which involves engineering of artificial chromatin loops between regulatory genomic regions provides a means to manipulate endogenous chromatin structures to understand their function and contribution to gene expression. An elegant way to achieve this is through the fusion of two dimerizable protein domains ABI1 and PYL1

(which are taken from the plant-based abscisic acid signaling pathway) with two dCas9, this forces chromatin loop formation between distal enhancer and promoter regions, allowing for targeted chromatin topology (Morgan, et al., 2017) (Adli, 2018).

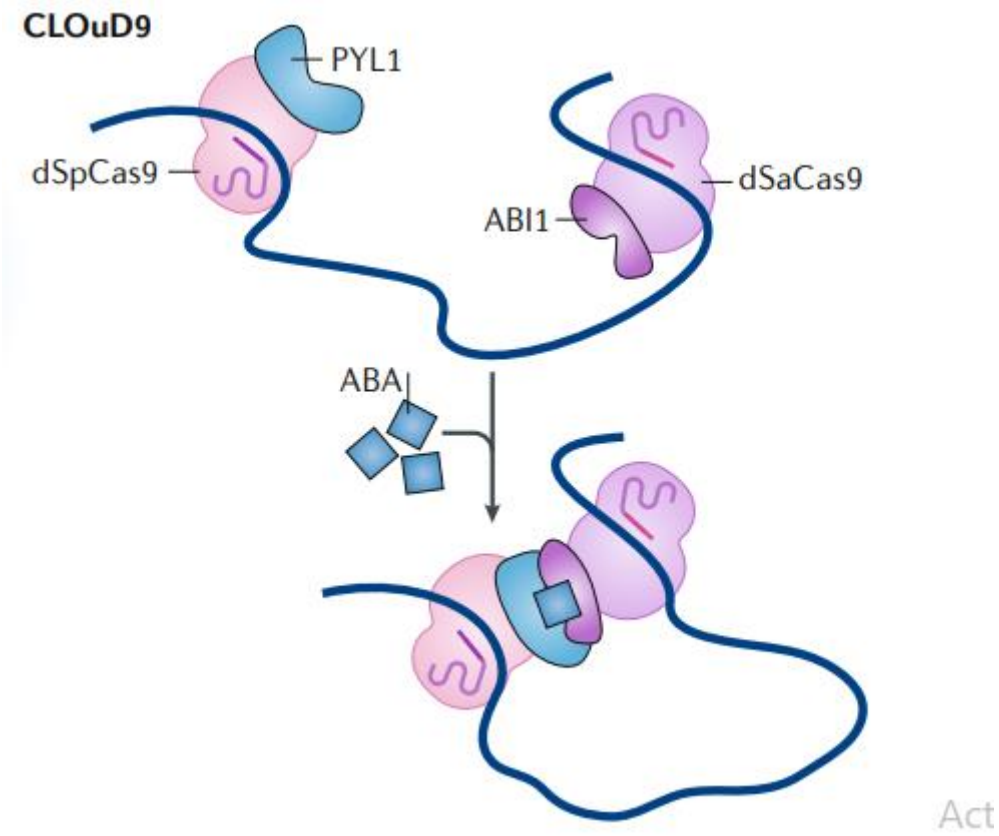

***Figure 9**: CRISPR use in Chromatin topology*

(Pickar-Oliver & Gersbach, 2019)

4. CHAPTER III: CRISPR and Oncology:

Given its extreme versatility and ease of use, the CRISPR-Cas9 system presents an extraordinary therapeutic potential for treating different diseases that originate from known genetic dysfunctions, as well as helping with understanding ambiguous aspects of complex genetic diseases through the creation of cell or animal models. Current preclinical research on genome editing primarily concentrates on viral infections, cardiovascular diseases (CVDs), metabolic disorders, primary defects of the immune system, hemophilia and muscular dystrophy, however we can't mention genetic diseases and their therapeutic trials without addressing Cancer (Li, et al., 2020) (Yin, Xue, & Anderson, 2019)**.** Ever since it was discovered that cancer originate from DNA changes, scientists have been attempting to find ways to comprehend and correct those mutations, although several technological advancements have been made regarding gene editing and regulating over the years, none of them were satisfactory enough, up until the conception of CRISPR in 2013. The system has taken cancer research by storm, revolutionizing several fields of oncology from Cancer modeling and screening to immunotherapy and treatment (National cancer institute, 2020).

a. CRISPR for cancer modelling:

To treat cancer, we have to conceptualize it first, the identification of the genes and mutation that initiate carcinogenesis and drive tumor progression in genetically tractable models is of a great importance for the development of clinically effective therapeutics; the whole-genome sequencing of cancer cells reveals the multiple point drive mutations and as such cellular and animal models can be established using these data in order to understand the molecular mechanisms underlying tumorigenic responses. For the longest part this was a lengthy and costly process which required laborious gene targeting, hindering the advancement of cancer research. However, his limitation is now alleviated by the CRISPR-Cas9 system, which in recent years have been extensively used to produce cancer models (Yin, Xue, & Anderson, 2019) (Ratan, et al., 2018).

i. Cellular Modeling:

Because it relies on the gRNA rather than protein–DNA interactions, the CRISPR-Cas9 system allows for a fast yet cheap modelling of mammalian cells with single genetic alteration, this accelerates the pharmacological studies of targeted therapies substantially, especially regarding the validation of gene function and their involvement in tumorigenesis (Yin, Xue, & Anderson, 2019), one big example comes to mind when speaking of this subject is "the MELK controversy": Maternal embryonic leucine zipper kinase (MELK) is an enzyme that is encoded by the MELK gene, it was believed that the MELK enzyme is essential for cancer proliferation, however through the CRISPR-mediated silencing of the MELK gene, it was found to have no effect on cancer cells

growth and is fully dispensable (Yin, Xue, & Anderson, 2019) (Lin, Giuliano, Sayles, & Sheltzer, 2017) (Huang, et al., 2017). Another example involves the gene with the most mutations in human cancer the p53 tumor suppressor gene, CRISPR-Cas9 was used to validate the p53-reactivating molecules nutlin and RITA, which were both initially identified as compounds that exhibit an antiproliferative activity in tumor cells with an active p53, but not in p53-mutated or p53-deleted cells, nutlin was confirmed to inhibit tumor proliferation via a p53-dependent mechanism, but the activity of RITA was found to be p53-independent (Yin, Xue, & Anderson, 2019) (Wanzel, et al., 2015). Beyond the validation of certain genes identified in cancer research, CRISPR-cas9 system could be used to disrupt functional genes or induce mutations to identify drug resistance properties in cultured cells. Furthermore, scientists use the CRISPR-Cas9 system to manipulate multiple genes in a cell in order to explore the genetic complexity of malignancies (Yin, Xue, & Anderson, 2019). But it doesn't stop there, the system's versatility allows for the study of tumor biology and their interaction with its micro environment, through genome edited organoids, which are tiny, self-organized three-dimensional tissue cultures, derived from stem cells. These organoids and through the use of the CRISPR-Cas9 system can be modelled to replicate much of the complexity of an organ, or to express selected aspects of it like producing only certain types of cells (STEMCELL Technologies , 2020) (Yin, Xue, & Anderson, 2019).

ii. In vivo modelling:

To fully understand how cancer operates it is indispensable to study the interactions in whole organisms. The establishment of the CRISPR-Cas9 system in organism modeling facilitated the study of cancers in living organisms, as the ability to edit the genome of somatic cells to induce driver mutations is more practical and cost efficient by margins than the manipulation of germline cells used before CRISPR. Moreover, the genetic manipulation of somatic cells resembles the naturally occurring driver mutations. The CRISPR-Cas9 system is introduced to the somatic cells through viral or plasmid delivery systems. The viruses used in the viral delivery system are:

- Lentivirus or Retrovirus: Lentiviral vectors enable stable expression of Cas9 and sgRNA and can therefore enable efficient gene editing in vivo8. Lentiviral vectors have been widely used to deliver CRISPR locally in the target organs of interest in order to create animal models of brain, breast, colon, lung or pancreatic cancer. Despite their utility, lentiviruses present some limitations which include the fact that their delivery is surgery dependent in some tissues like the brain and the need d to account for the random genomic integration of lentivirus in order to exclude lentivirus-induced off-target effects.
- Adeno-associated virus and adenovirus: due to their broad- ranging tissue tropisms, AAS and adenoviruses have seen a great share of use for genome editing to generate animal models of

cancer. These viruses can be delivered to the lungs quite easily with a simple intranasal or intratracheal injection. They present the downside of their cargo capacity which is smaller than other viruses used for the delivery hindering their ability to deliver, another limitation is their ability to generate a strong immune response in the liver which might compromise the disease phenotype.
- Hydrodynamic injection or electroporation of plasmids: a high volume and pressure injection of the tail vein, is a well-established method of delivering plasmids to the liver in rodents. This approach doesn't require any viruses for the delivery and, and it has been shown to reliably model point mutations of tumor suppressor genes and oncogenes in the liver. However, this approach presents the lowest efficiency rate and the risk of liver damage (Yin, Xue, & Anderson, 2019).

b. CRISPR and Immunotherapy:

For decades surgery, chemotherapy and radiotherapy have been the treatments of choice when it comes to cancer. However, in the last decade the focus has been changing slowly but surely towards immunotherapy. CRISPR stands at the center of the technological advances that are helping in the shift from general therapies to precise, targeted and immune induced therapies (Schirrmacher, 2018) (Yin, Xue, & Anderson, 2019) (Waldman, Fritz, & Lenardo, 2020) (Liu & Guo, 2018) (Esfahani, et al., 2020). CRISPR systems are being incorporated and adapted to improve the efficacy of immunotherapies through enhancing potency, reducing toxicity and manufacturing cost and facilitating the discovery of new immunotherapeutic strategies (Yin, Xue, & Anderson, 2019). The CRISPR-Cas9 system has been used to establish the most promising approaches in cancer immunotherapy, for instance in the Immune checkpoint blockade approach, scientists have relied on CRSIPR screening to identify checkpoint mediator genes and their functional expression, and as such the screening was used to point out the mechanisms by which the tumor cells avoid immune cells, this enabled a more effective engineering of the native T Cell Receptor (TCRs) or Chimeric Antigen Receptors (CARs) (Khalaf, et al., 2020) (Yin, Xue, & Anderson, 2019).

c. Direct Tumor targeting with CRISPR-Cas9 system:

This approach could be described as going back to the basics of CRISPR-Cas9 gene editing capabilities. Cancer cells present specific genes which are usually the product of the driver mutations, that are absent in normal cells, this sparks the idea of using the CRISPR-Cas9 system to induce DSBs by relying on a pair of Cas9 nickases targeting two neighboring cancer-specific genes, after that a suicidal gene (encoding a prodrug converting enzyme) with homology to the sequences surrounding the breakpoints is delivered with an adenoviral vector to enable introduction into the genome via HDR (Chen, et al., 2017) (Yin, Xue, & Anderson, 2019).

5. Conclusions:

Since its development into a genome editing tool, the CRISPR/Cas9 technology has revolutionized biology by providing a simple and versatile method to manipulate the genome and epigenome across a broad range of organisms. The potential of CRISPR/Cas9 for both basic and translational cancer research is yet beginning to unfold. In the future, pooled CRISPR screens will provide a comprehensive set of essential genes across most cancer cell lines. This resource, combined with the already available information on the genetic and epigenetic characteristics of cancer cell lines, will enable the extensive identification of synthetic lethal interactions and facilitate the discovery of novel drug targets. The future use of CRISPR/Cas9 in translational medicine will largely depend on the ability to develop Cas9 variants with minimal or no off-target effect and novel methods to improve the yet inefficient engineering of precise genetic changes by homology directed repair. Furthermore, future improvements of viral and non-viral delivery methods will be necessary to improve the in vivo application of CRISPR/Cas9, laying the ground for the therapeutic use of CRISPR in the future. In summary, the development of the CRISPR/Cas9 technology has and will greatly accelerate cancer research in many areas.

References:

1. Adli, M. (2018). The CRISPR tool kit for genome editing and beyond. *Nature communications*. doi:10.1038/s41467-018-04252-2
2. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter., P. (2002). *Molecular Biology of the Cell. 4th edition.* New York: Garland Science.
3. Alix-Panabières, C. (2020). The future of liquid biopsy. *Nature*. doi:https://doi.org/10.1038/d41586-020-00844-5
4. Arruebo, M., Vilaboa, N., Sáez-Gutierrez, B., Lambea, J., Tres, A., Valladares, M., & González-Fernández, A. (2011). Assessment of the evolution of cancer treatment therapies. *Cancers*. doi:10.3390/cancers3033279
5. Bakhoum, S. F., Ngo, B., Laughney, A. M., Cavallo, J.-A., Murphy, C. J., Ly, P., . . . Chadalavada, &. a. (2018). Chromosomal instability drives metastasis through a cytosolic DNA response. *Nature*. doi:10.1038/nature25432
6. Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., . . . Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science*. doi:10.1126/science.1138140
7. Baskar, R., Lee, K. A., Yeo, R., & Yeoh, K.-W. (2012). Cancer and radiation therapy: current advances and future directions. *International journal of medical sciences*. doi:10.7150/ijms.3635
8. Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., . . . Bonas., U. (2009). Breaking the Code of DNA Binding Specificity of TAL-Type III Effectors. *Science*. doi:10.1126/science.1178811
9. Bolotin, A., Quinquis, B., Sorokin, A., & Ehrlich., S. D. (2005). Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology*. doi:10.1099/mic.0.28048-0
10. Brierley, J., Gospodarowicz, M., & O'Sullivan, B. (2016). The principles of cancer staging. *Ecancermedicalscience*. doi:10.3332/ecancer.2016.ed61
11. Brown, T. (2002). *Genomes. 2nd edition.* London: Oxford: Wiley-Liss. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK21114/
12. Buder, T., Deutsch, A., Klink, B., & Voss-Böhme, A. (2019). Patterns of Tumor Progression Predict Small and Tissue-Specific Tumor-Originating Niches. *Frontiers in Oncology*. doi:10.3389/fonc.2018.00668
13. Cancer research institute . (2020, January). *How Cellular Immunotherapies Are Changing the Outlook for Cancer Patients*. Retrieved from Cancer research institute : https://www.cancerresearch.org/immunotherapy/treatment-types/adoptive-cell-therapy#tcr
14. Carroll, D. (2011). Genome engineering with zinc-finger nucleases. *Genetics*. doi:10.1534/genetics.111.131433

15. Chakraborty, S., & Rahman, T. (2012). The difficulties in cancer treatment. *Ecancermedicalscience*. doi:10.3332/ecancer.2012.ed16

16. Chen, Z.-H., Yu, Y. P., Zuo, Z.-H., Nelson, J. B., Michalopoulos, G. K., Monga, S., . . . Luo, J.-H. (2017). Targeting genomic rearrangements in tumor cells through Cas9-mediated insertion of a suicide gene. *Nature Biotechnology*. doi:10.1038/nbt.3843

17. Cho, S. W., Kim, S., Kim, Y., Kweon, J., Kim, H. S., Bae, S., & Kim, J.-S. (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome research*. doi:10.1101/gr.162339.113

18. Christofi, T., Baritaki, S., Falzone, L., Libra, M., & Zaravinos., A. (2019). Current Perspectives in Cancer Immunotherapy. *Cancers*. doi:10.3390/cancers11101472

19. Clarke , B. B. (2016, February 10). Historical review of the causes of cancer. *World Journal of Clinical Oncology*. doi:10.5306/wjco.v7.i1.54

20. Croce, C. M. (2008). Oncogenes and Cancer. *The New England Journal of Medicine*. doi:10.1056/NEJMra072367

21. David, S. (2002). EMERGING MOLECULAR MARKERS OF CANCER. *Nature reviews cancer*.

22. Davies, P. C., & Lineweaver, C. (2011). Cancer tumors as Metazoa 1.0: tapping. *Phys. Biol. 8 015001*. doi:10.1088/1478-3975/8/1/015001

23. Doonan, J., & Hunt, T. (1996). Why don't plants get cancer? *Nature*.

24. Egeblad, M., Nakasone, E. S., & Werb, Z. (2010). Tumors as organs: complex tissues that interface with the entire organism. *Developmental cell*. doi:10.1016/j.devcel.2010.05.012

25. Esfahani, K., Roudaia, L., Buhlaiga, N., Del Rincon, S. V., Papneja, N., & Miller, W. H. (2020). A review of cancer immunotherapy: from the past, to the present, to the future. *Current oncology*. doi:10.3747/co.27.5223

26. Falzone, L., Salomone, S., & Libra, M. (2018). Evolution of Cancer Pharmacological Treatments at the Turn of the Third Millennium. *Frontiers in pharmacology*. doi:10.3389/fphar.2018.01300

27. Fares, J., Fares, M. Y., Khachfe, H. H., Salhab, H. A., & Fares, Y. (2020). Molecular principles of metastasis: a hallmark of cancer revisited. *Signal Transduction and Targeted Therapy*. doi:10.1038/s41392-020-0134-x

28. Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M., & Joung, J. K. (2014). Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nature Biotechnology*. doi:10.1038/nbt.2808

29. Gaj, T., Sirk, S. J., Shui, S.-L., & Liu, J. (2016). Genome-Editing Technologies: Principles and Applications. *Cold Spring Harbor perspectives in biology*. doi:10.1101/cshperspect.a023754

30. Gaspar, T., Hagège, D., Kevers, C., Penel, C., Crèvecoeur, M., Engelmann, I., . . . Foidart, J.-M. (1991). When plant teratomas turn into cancers in the absence of pathogens. *Physiologia Plantarum*.

31. Geoffrey, C. (2000). *The Cell: A Molecular Approach 2nd Edition.* Sinauer Associates Inc. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK9839/

32. Gonzalez, H., Hagerling, C., & Werb, Z. (2018). Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev.* doi:10.1101/gad.314617.118

33. Hille, F., & Charpentier, E. (2016). CRISPR-Cas: biology, mechanisms and relevance. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. doi:10.1098/rstb.2015.0496

34. Huang, H.-T., Seo, H.-S., Zhang, T., Wang, Y., Jiang, B., Li, Q., . . . Dhe-Paganon, S. (2017). MELK is not necessary for the proliferation of basal-like breast cancer cells. *eLife*. doi:10.7554/eLife.26693

35. Institute for Quality and Efficiency in Health Care. (2012, February 9). *How does chemotherapy work?* Retrieved from InformedHealth.org: https://www.ncbi.nlm.nih.gov/books/NBK279427/

36. Jaffray, D. A., & Gospodarowicz, M. K. (2015). Radiation Therapy for Cancer. In H. Gelband, P. Jha, R. Sankaranarayanan, & S. Horton, *Cancer: Disease Control Priorities, Third Edition (Volume 3).* Washington DC: The International Bank for Reconstruction and Development . doi:doi: 10.1596/978-1-4648-0349-9_ch14

37. Khalaf, K., Janowicz, K., Dyszkiewicz-Konwińska, M., Hutchings, G., Dompe, C., Moncrieff, L., . . . Kempisty, B. (2020). CRISPR/Cas9 in Cancer Immunotherapy: Animal Models and Human Clinical Trials. *Genes*. doi:10.3390/genes11080921

38. Kleinstiver, B. P., Pattanayak, V., Prew, M. S., Tsai, S. Q., Nguyen, N. T., Zheng, Z., & Joung, J. K. (2016). High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*. doi:10.1038/nature16526

39. Levine, A., Momand, J., & Finlay, C. (1991). The p53 tumour suppressor gene. *Nature.* doi:10.1038/351453a0

40. Levy, A., Goren, M. G., Yosef, I., Auster, O., Manor, M., Amitai, G., . . . Sorek, R. (2015). CRISPR adaptation biases explain preference for acquisition of foreign DNA. *Nature*. doi:10.1038/nature14302

41. Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., & Zhao, X. (2020). Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. *Signal Transduction and Targeted Therapy*. doi:10.1038/s41392-019-0089-y

42. Lin, A., Giuliano, C. J., Sayles, N. M., & Sheltzer, J. M. (2017). CRISPR/Cas9 mutagenesis invalidates a putative cancer dependency targeted in on-going clinical trials. *eLife*. doi:10.7554/eLife.24179

43. Liu, M., & Guo, F. (2018). Recent updates on cancer immunotherapy. *Precision clinical medicine*. doi:10.1093/pcmedi/pby011

44. Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J., Charpentier, E., Horvath, P., . . . Koonin, E. V. (2011). Evolution and classification of the CRISPR–Cas systems. *Nature Reviews Microbiology*. doi:10.1038/nrmicro2577

45. Makarova, K. S., Wolf, Y. I., Iranzo, J., Shmakov, S. A., Alkhnbashi, O. S., Brouns, S. J., . . . Koonin, E. V. (2019). Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants. *Nature Reviews Microbiology*. doi:10.1038/s41579-019-0299-x

46. Marshall, D., Laberge, J. M., Firetag, B., Miller, T., & Kerlan, R. K. (2013). The changing face of percutaneous image-guided biopsy: molecular profiling and genomic analysis in current practice. *Journal of vascular and interventional radiology*. doi:10.1016/j.jvir.2013.04.027

47. McGinn, J., & Marraffini, L. A. (2018). Molecular mechanisms of. *Nature reviews microbiology*. doi:https://doi.org/10.1038/s41579-018-0071-7

48. Mojica, F., Díez-Villaseñor, C., García-Martínez, J., & Soria, E. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution*. doi:10.1007/s00239-004-0046-3

49. Morgan, S. L., Mariano, N. C., Bermudez, A., Arruda, N. L., Wu, F., Luo, Y., . . . Wang, K. C. (2017). Manipulation of nuclear architecture through CRISPR-mediated chromosomal looping. *Nature Communications*. doi:10.1038/ncomms15993

50. National cancer institute. (2015, February 9). *About Cancer*. Retrieved from National cancer institute: https://www.cancer.gov/about-cancer/understanding/what-is-cancer

51. National cancer institute. (2015, December 23). *Causes and Prevention*. Retrieved from National cancer institute: https://www.cancer.gov/about-cancer/causes-prevention

52. National Cancer Institute. (2017, February 6). *Metastatic Cancer*. Retrieved from National Cancer Institute: https://www.cancer.gov/types/metastatic-cancer#:~:text=In%20metastasis%2C%20cancer%20cells%20break,cancer%20as%20the%20primary%20tumor.

53. National cancer institute. (2018, April 9). *Cancer Screening*. Retrieved from Nationa cancer intitute: https://www.cancer.gov/about-cancer/screening

54. National cancer institute. (2019, July 17). *How Cancer Is Diagnosed*. Retrieved from National cancer institute: https://www.cancer.gov/about-cancer/diagnosis-staging/diagnosis

55. National cancer institute. (2019, January 16). *Screening Tests*. Retrieved from National cancer institute: https://www.cancer.gov/about-cancer/screening/screening-tests

56. National cancer institute. (2020, July 27). *How CRISPR Is Changing Cancer Research and Treatment*. Retrieved from National cancer institute: https://www.cancer.gov/news-events/cancer-currents-blog/2020/crispr-cancer-research-treatment

57. National Institutes of Health. (2007). *Understanding Cancer*. Retrieved from The National Center for Biotechnology Information: https://www.ncbi.nlm.nih.gov/books/NBK20362/

58. Nature. (2020). Cancer diagnosis. *Nature*. doi:https://doi.org/10.1038/d41586-020-00840-9

59. Nature. (2020, November 8). *Cancer imaging*. Retrieved from Nature: https://www.nature.com/subjects/cancer-imaging

60. Nature. (2020). *Oncology*. Retrieved from Nature: https://www.nature.com/subjects/oncology

61. Palmirotta, R., Lovero, D., Cafforio, P., Felici, C., Mannavola, F., Pellè, E., . . . Silvestris, F. (2018). Liquid biopsy of cancer: a multimodal diagnostic tool in clinical oncology. *Therapeutic Advances in Medical Oncology*. doi:10.1177/1758835918794630

62. Peng, Y., & Croce, C. (2016). The role of MicroRNAs in human cancer. *Signal Transduction and Targeted Therapy*. doi:10.1038/sigtrans.2015.4

63. Pickar-Oliver, A., & Gersbach, C. A. (2019). The next generation of CRISPR–Cas. *Nature Reviews Molecular Cell Biology*. doi:10.1038/s41580-019-0131-5

64. Qian, C.-N., Mei, Y., & Zhang, J. (2017). Cancer metastasis: issues and challenges. *Chinese journal of cancer*. doi:10.1186/s40880-017-0206-7

65. Ratan, Z. A., Son, Y.-J., Haidere, M. F., Uddin, B. M., Yusuf, M. A., Zaman, S. B., . . . Cho, J. Y. (2018). CRISPR-Cas9: a promising genetic engineering approach in cancer research. *Therapeutic advances in medical oncology*. doi:10.1177/1758834018755089

66. Rath, D., Amlinger, L., Rath, A., & Lundgren, M. (2015). The CRISPR-Cas immune system: Biology, mechanisms and applications. *Biochimie*. doi:https://doi.org/10.1016/j.biochi.2015.03.025

67. Rodríguez-Rodríguez, D. R., Ramírez-Solís, R., Garza-Elizondo, M. A., Garza-Rodríguez, M. D., & Barrera-Saldaña, H. A. (2019). Genome editing: A perspective on the application of CRISPR/Cas9 to study human diseases (Review). *International journal of molecular medicine*. doi:10.3892/ijmm.2019.4112

68. Rothschild, B., Tanke, D., & Helbling, M. (2003). Epidemiologic study of tumors in dinosaurs. *Naturwissenschaften*. doi:https://doi.org/10.1007/s00114-003-0473-9

69. Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature Biotechnology*. doi:10.1038/nbt.2842

70. Sarkar, S., Horn, G., Moulton, K., Oza, A., Byler, S., Kokolus, S., & Longacre, M. (2013). Cancer Development, Progression, and Therapy: An Epigenetic Overview. *International journal of molecular sciences*. doi:10.3390/ijms141021087

71. Schirrmacher, V. (2018). From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment. *International journal of oncology*. doi:10.3892/ijo.2018.4661

72. Seyfried, T. N., & Huysentruyt, L. C. (2013). On the origin of cancer metastasis. *Critical reviews in oncogenesis*. doi:10.1615/critrevoncog.v18.i1-2.40

73. Siddiqui, I., Sanna, V., Ahmad, N., Sechi, M., & Mukhtar, H. (2015). Resveratrol nanoformulation for cancer prevention and therapy. *Annals of the New York Academy of Sciences*. doi:10.1111/nyas.12811

74. Song, G., Jia, M., Chen, K., Kong, X., Khattak, B., Xie, C., . . . Mao, L. (2016). CRISPR/Cas9: A powerful tool for crop genome editing. *The Crop Journal*. doi:https://doi.org/10.1016/j.cj.2015.12.002

75. STEMCELL Technologies . (2020, July). *CRISPR-Cas9 Genome Editing of Human Intestinal Organoids*. Retrieved from STEMCELL: https://www.stemcell.com/crispr-cas9-genome-editing-of-intestinal-organoids-using-arcitect-and-intesticult.html

76. Stoddard, B. (2014). Homing endonucleases from mobile group I introns: discovery to genome engineering. *Mobile DNA*. doi:10.1186/1759-8753-5-7

77. Stratton, M., Campbell, P., & Futreal, P. (2009). The cancer genome. *Nature*. doi:10.1038/nature07943

78. Tessitore , A., Cicciarelli, G., Del Vecchio, F., Gaggiano, A., Verzella, D., Fischietti, M., . . . Alesse, E. (2014). MicroRNAs in the DNA Damage/Repair Network and Cancer. *International Journal of Genomics*. doi:10.1155/2014/820248

79. Ucker, D. S., & Levine, J. S. (2018). Exploitation of Apoptotic Regulation in Cancer. *Frontiers in immunology*. doi:10.3389/fimmu.2018.00241

80. Vinay, D. S., Ryan, E. P., Pawelec, G., Talib, W. H., Stagg, J., Elkord, E., . . . al., A. A. (2015). Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Seminars in Cancer Biology*. Retrieved from https://doi.org/10.1016/j.semcancer.2015.03.004

81. Waldman, A. D., Fritz, J. M., & Lenardo, M. J. (2020). A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nature reviews. Immunology*. doi:10.1038/s41577-020-0306-5

82. Wang, R.-A., Lu, Y.-Y., & Fan, D.-M. (2015). Reasons for cancer metastasis: A holistic perspective. *Molecular and clinical oncology*. doi:10.3892/mco.2015.623

83. Wanzel, M., Vischedyk, J. B., Gittler, M. P., Gremke, N., Seiz, J. R., Hefter, M., . . . Stiewe, T. (2015). CRISPR-Cas9-based target validation for p53-reactivating model compounds. *Nature chemical biology*. doi:10.1038/nchembio.1965

84. Weinberg, R. A. (2007). Is metastasis predetermined? *Molecular oncology*. doi:10.1016/j.molonc.2007.07.001

85. World Health Organization. (2018, September 12). *Cancer*. Retrieved from World Health Organization: WHO: https://www.who.int/news-room/fact-sheets/detail/cancer

86. Yin, H., Xue, W., & Anderson, D. G. (2019). CRISPR–Cas: a tool for cancer research and therapeutics. *Nature Reviews Clinical Oncology*. doi:10.1038/s41571-019-0166-8

87. Zhan, T., Rindtorff, N., Betge, J., Ebert, M. P., & Boutros, M. (2019). CRISPR/Cas9 for cancer research and therapy. *Seminars in Cancer Biology*. doi:https://doi.org/10.1016/j.semcancer.2018.04.001

88. Ziv, E., Durack, J. C., & Solomon, S. B. (2016). The Importance of Biopsy in the Era of Molecular Medicine. *Cancer journal*. doi:10.1097/PPO.0000000000000228