Novel regulators of prostate cancer stem cells and tumor aggressiveness

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General Introduction

1. General Properties of Cancer

Cancer is considered one of the leading cause of morbidity and mortality worldwide, with 14 million new cases and 8.2 million cancer-related deaths registered in 2012 (1). The 5 most common cancers diagnosed in women in 2012 were breast, colorectal, lung, cervix and stomach cancer while in men these include lung, prostate, colorectal, stomach and liver cancer.

Carcinogenesis is a complex multi-step process that usually proceeds over several years and starts from one single cell. It progressively drives normal cell evolution into a cell with an increasingly abnormal neoplastic phenotype. This process is the result of a combination of genetic and epigenetic factors determined by individual variability caused by hereditary predisposition, life style and other variables like environmental influences, infectious agents, nutritional factors, hormonal and reproductive factors, and exposure to physical, chemical and biological carcinogens (2). Tumor formation and progression is driven by a sequence of essential alterations in cell physiology, cell homeostasis, randomly occurring mutations and epigenetic alterations of DNA. These events affect genes controlling different processes, such as cell proliferation, differentiation and survival, and bring cancer to acquire different malignant capabilities that together lead to malignant growth. The genetic abnormalities that contribute to cancer pathogenesis generally involve two main mechanisms: the inactivation of negative mediators of cell proliferation (tumor suppressor genes) and the activation of positive mediators of cell proliferation (proto-oncogenes) (3).

The definition of cancer, as established nowadays by the last advances in the tumor biology, includes multiple characteristics and aspects, that surprisingly are already present in the etymology of the word itself. The word “Cancer” originates from the ancient Greek word **“**καρκίνος” (*Karkìnos*, “crab”) credited to the Greek physician Hippocrates (460-370 BC). This word was probably chosen to describe the similarities of solid tumors with swollen veins and spreading projections reminding the shape of a “crab”. Strikingly, this description includes exactly all the elements that the modern biology of tumors ascribe to cancer; a primary tumor mass, the presence of new vessels and the spreading and invasion of the neighboring tissues. Today we learned that the biology of tumors should be investigated not only focusing on the traits of single cancer cells, but also considering the contributions of the tumor microenvironment, the interactions between tumor cells and the supportive stroma, the role of the immune system and the preferential tropism of spreading tumor cells for specific metastatic sites. These interactions are fundamental to understand the mechanisms that lead to the switch from a contained disease to the aggressive spreading and metastatic phase of tumors. These events and characteristics have been systematically outlined in 2000

by Hanahan and Weinberg as “Hallmarks of Cancer” (4) and more recently updated to include the supportive cellular and non-cellular microenvironment (5,6).

1.1. Hallmarks of Cancer

There is a certain series of events that have to occur to drive the transformation from a normal cell to a cancer cell. These events are part of a multistep process and all the steps involved in this process contribute progressively to the generation and development of cancer. The fundamental characteristics of cancer or the hallmarks of cancer represent the set of properties that a cell or a group of cells in general have to acquire to become a tumor and to interact with the surrounding stroma (5,6) (**Fig. 1**):

Sustaining Proliferative Signaling: normal cells are constantly proliferating as part of the physiological turnover present in every normal tissue. However, their proliferation is finely tuned and regulated by multiple growth factors to maintain a proliferative rate appropriate for the maintenance of the homeostasis of the tissues where they home. These growth factors are part of a paracrine signaling and their availability or signaling efficacy depends also on the capability of the “receiving” normal or tumor cells to properly react to these stimuli. In cancer, the tumor cells can instruct the supportive tumor-stroma to supply growth factors (7) or acquire a “self-sustained proliferative signaling” resulting in an autocrine and abnormal proliferative stimulation.

Evading Growth Suppressors: part of the regulation of the maintenance of the homeostasis of cells and tissue is determined by the suppression of the proliferation. Normal cells have to proliferate to generate new tissues and maintain the tissue integrity but also have to stop their growth to prevent abnormal hyper-proliferation. In cancer, tumor cells have to escape these suppressive mechanisms and have to circumvent the programs that negatively regulates cell proliferation. Typical alterations in tumor suppressor genes include the loss of function of critical “gatekeeper” of cell-cycle progression such as pRb (Retinoblastoma 1) and p53 which regulates apoptosis and is a stress responsive sensor.

Resisting to Cell Death: Programmed cell death, apoptosis, is one of the mechanisms in normal physiology that prevents cancer development and the afore mentioned p53 is one of the key player in this process. There are two main circuits that orchestrate apoptosis: one receives and process extracellular death-inducing signals (e.g. Fas and Fas ligand mechanism) and the other sense intracellular signals (intrinsic program). Both the machineries converge on the activation of a cascade of proteolytic cleavage events involving latent and effector caspases. Cancer cells have evolved a variety of

strategies to avoid this programmed death. The most common feature of tumor cells evading apoptosis is the loss of p53; other strategies include the suppression of pro-apoptotic factors (e.g. Bax, Puma, Bim) or the upregulation of anti-apoptotic genes (e.g. Bcl-2, Bcl-X_L).

Enabling Replicative Immortality: normal cells in healthy tissues are capable of pass through only a limited number of division cycles. The mechanisms that limit the number of growth-and-division cycles are essentially two: senescence (nonproliferative but viable state) and crisis (cell death). Both are linked to the length of telomeric DNA that in a cell dictates how many cycles of division are still available before the cell enters in a phase of DNA instability (i.e. crisis). It is a remarkable properties of cancer cells their ability to proliferate indefinitely, escaping from this control. One of the mechanisms that drive immortalization in tumor cells is the presence of telomerase activity, responsible for the integrity of telomeric DNA, detected in up to 90% of spontaneously immortalized cells.

Inducing Angiogenesis: in every normal tissue, nutrients and oxygen are provided by a fully functional network of vessels, responsible also for the elimination of wastes and other products of the metabolism. In normal physiology, in an adult organism these vessels remain mostly stable and quiescent. In malignant tumors, vessel remodelling and new vessel formation occurs after the so-called “angiogenic switch” which cause the normal quiescent vasculature to sprout and produce new branches (neovascularization). This abnormal angiogenesis produces vessels that are histologically different from those formed during the physiological process. Moreover, highly invasive tumor cells can form fluid-conducting channels in a process defined as “vasculogenic mimicry” (8). The new vessels in the tumor have an aberrant morphology and are characterized by abnormal level of endothelial cells proliferation and apoptosis. In addition, the leakiness that characterize these vessels is one of the major effectors for the low efficiency in the delivery of therapies specifically to the tumor.

Activating Invasion and Metastasis: the invasion of the surrounding tissues, the intravasation in blood and lymphatic vessels and the formation of distant metastasis represent one of the critical problem in tumor progression. In this context transformed epithelial cells acquire a motile mesenchymal phenotype in a process referred to as “epithelial to mesenchymal transition” (EMT) (9). However, cancer cell migration is not restricted to singly migrating cells. Different patterns of cell migration include single-cell migration, multicellular streaming and collective cell migration, (reviewed in (10)). The impact of this process during the onset of metastatic spreading and its relevance in the

establishment of therapy resistance will be discussed more in details in the next paragraphs.

Interaction with the tumor stroma: tumor cells do not behave independently from the rich microenvironment where they are localized and that represents an important component during tumor initiation, growth and progression. During cancer progression the stroma co-evolves with the tumor and create a dynamic signaling network of paracrine signals that promotes cancer. The different stromal components involved in this process include: cancer-associated fibroblasts (CAFs), pericytes, endothelial cells, immune cells and the extracellular matrix (reviewed in (6)).

Metabolic reprogramming: In addition to the above mentioned specific characteristics, tumor cells can also adapt their metabolism and switch to the so called “aerobic glycolysis” converging their metabolism largely to glycolysis (i.e. Warburg-effect) (11). This is one of the basis of the non-invasive visualization of tumors based on positron emission tomography (PET) with radiolabelled analog of glucose as reporter. In proliferating (cancer) cells the mitochondrial metabolism is reprogrammed toward macromolecular synthesis to sustain multiple cell divisions, (reviewed in (12)). Moreover, oncogenic mutations in metabolic enzymes such as the cytosolic $NADP^+$-dependent isocitrate dehydrogenase 1 gene (IDH1) and the mitochondrial homolog IDH2 responsible for converting α-ketoglutarate to 2-hydroxyglutarate (2HG), a metabolite found only in reduced amounts in mammalian cells under normal conditions have been reported (12,13). Interestingly this has also effects on epigenetic mechanisms, resulting in altered histone methylation marks, hypermethylation at CpG islands and dysregulated cell differentiation(12).
Moreover, it is important to note the pro-inflammatory and immunosuppressive properties of cancer cells. Inflammation can sustain proliferative signaling and inhibiting cell death, activate extracellular matrix-modifying enzymes and support invasion and angiogenesis (14,15). Tumor cells can also secrete immunosuppressive factors or recruit immunosuppressive cells, blocking the action of cytotoxic lymphocytes or recruit tumor associated macrophages that can enhance tumor progression and metastasis and suppress antitumor immunity (16-18).

All the aspects discussed above depend, to a large extent, on genomic alterations in neoplastic cells. Different cells can gain different alterations and the combination of several alteration together will produce a cancer cell, capable of outgrow and gain a local dominance over other neoplastic and/or normal cells. In this perspective, tumor progression is characterized by the expansion of different heterogeneous clones. In this process, only those clones capable of gaining all the hallmarks of cancer will succeed in

generating a malignant tumor. The issue of intra-tumor and inter-tumor heterogeneity and its impact on resistance to current therapies will be discussed in the next paragraphs.

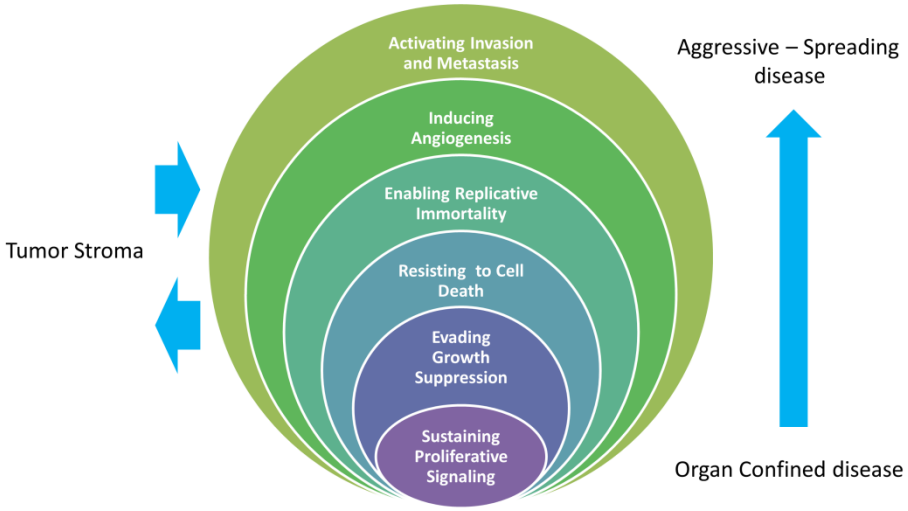

Figure 1. Schematic representation of the hallmarks of cancer, their correlation with cancer progression and reciprocal interaction with the tumor stroma.

1.2. Tumor Heterogeneity

There are two main levels of complexity in tumor heterogeneity. If we consider the tumor mass as an independent entity, one level consists of the differences *between* different cancer types or different patients affected by the same cancer and is defined as *inter-tumor heterogeneity* (**Fig. 2**). Another level of heterogeneity encompasses the cellular differences *within* the same tumor (e.g. multiple cell clones with different properties, dispersed within the same tumor mass of the same patient) and is defined as *intra-tumor heterogeneity* (**Fig. 2**). Despite the fact that, overall, the evolution and progression of these tumor can be similar (e.g. onset of primary tumor, progression from benign to malignant growth, neo-angiogenesis, invasion of surrounding tissues, and formation of distant metastasis) there are intrinsic differences that distinguish one cancer from the other and, within the same cancer family, one cancer subtype to another (e.g. hormone-naïve or androgen-independent prostate cancer). These differences are part of the so-called inter-tumor heterogeneity and reflect the differential responsiveness to specific therapeutics and not to others. Additionally, the tropism for specific metastatic sites (e.g. osteotropism in prostate cancer), is also characteristic of certain malignancies and can be ascribed to the inter-tumor heterogeneity.

The second level of complexity comprises intra-tumor heterogeneity. As already discussed, cancer formation is a multistep process that starts from one single cell; on the other hand, tumors are tissues and therefore are constituted by a variety of different tumor- and other-cell types. As established in the last decade by high-resolution genome-wide studies, the formation and progression of tumors is characterized by a continuous “Darwinian-like” evolution of branches of specific clones (19). This process of “clonal evolution” results also in the construction of a supportive tumor microenvironment, which is continuously being remodelled during the tumor progression. Different cell types contribute to increase the complexity and the heterogeneity of this environment. As previously mentioned, these cell types include, among the others, also non-malignant cells, such as immune and inflammatory cells, endothelial cells, pericytes, and cancer-associated fibroblast. For the purpose of this thesis we will mainly focus on the cancer cells and discuss the different cell types and subpopulation that are represented within the tumor. Macroscopic tumors are constituted of different subpopulation of malignant cells. Depending on environmental stimuli and stochastic processes, or depending on their alterations, such as mutations and epigenetic changes, these clones can acquire a dominant phenotype with clinically relevant characteristics (e.g. resistance to therapy). In this respect, the intra-tumor heterogeneity is one of the relevant problems in the identification of therapeutic strategies capable to eradicate completely all the different cancer cell subpopulations

and clones that maintain the cancer. Similarly, this also has an impact on studies that approach tumor biology without distinguishing the different cell types dispersed within the tumor.

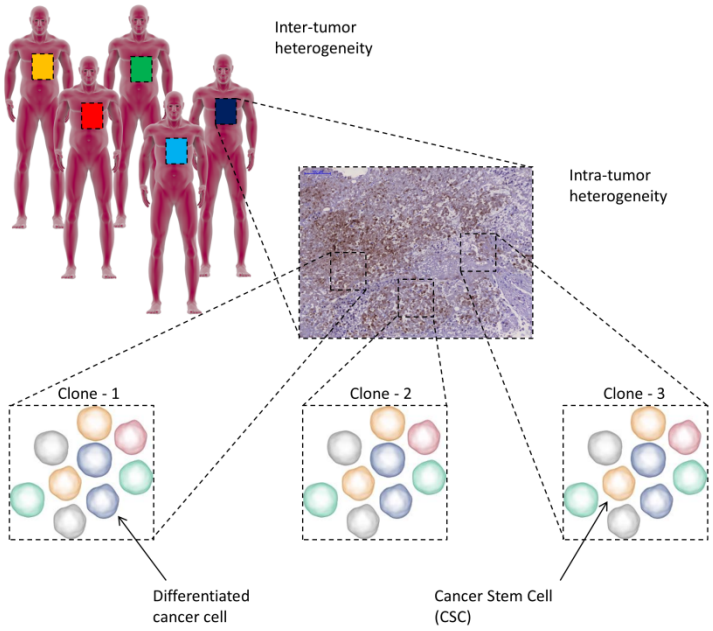

Figure 2. Schematic representation of inter-tumor and intra-tumor heterogeneity. Adapted from [19].

Molecular- and genetic-profiles of cancerous “bulk” tissues indeed cannot discriminate between the aggressive subsets of cancer cells responsible for tumor maintenance, growth & the development of therapy resistance and less aggressive, more differentiated cancer cell subpopulations. This raises the question whether the different clones and subpopulations present in the tumor are properly represented also in a transcriptional analysis between “bulk” tumor and “normal” tissues. One of the aspects of tumor heterogeneity, that has revolutionized the tumor biology in the last years, is indeed the discovery of a subpopulation of cancer cells with tissue stem-like properties, the cancer stem cells (CSCs). The contribution of these cells to the tumor formation and maintenance, metastasis, therapy resistance and their clinical relevance for the identification of new therapeutic strategies will be discussed later.

2. Anatomy of the Prostate

The prostate is a walnut-sized exocrine gland, well encapsulated and positioned in the pelvic cavity inferior of the bladder (it surrounds the first tract of the urethra) and anterior of the rectum (20) (**Fig. 3**). The function of the prostate is to secrete a slightly alkaline milky white fluid, that constitutes about the 30% of the volume of the semen and that contains carbohydrates, phospholipids, and enzymes (e.g. prostate specific acid phosphatase (PAP) and prostate specific antigen (PSA)) (21).

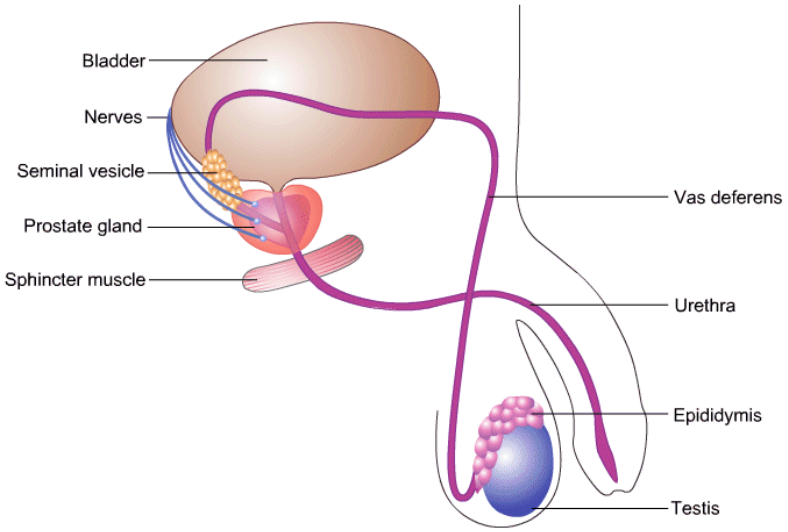

Figure 3. Anatomy of the prostate within the male reproductive system.
Source: http://www.aboutcancer.com/prostate

The prostate can be divided in four "zones" (mainly used in pathology, (22,23)) (**Fig. 4**) or in four lobes (mainly used in anatomy):

1) *Central zone*: it surrounds the ejaculatory ducts and constitutes about 20% of the whole gland and it presents large and irregular ducts. Approximately 1 – 5% of prostate cancer originates from this region and tend to invade the seminal vesicles (24). This part roughly corresponds to the median lobe.
2) *Peripheral zone*: accounts for the majority of the gland and it originates from the mesoderm. Up to 70% of prostate cancer originates from this part. Roughly corresponds to the posterior lobe.
3) *Transition zone*: it surrounds the prostatic urethra and it originates from the endoderm. About 20% of prostate cancer originates from this zone which is also responsible for the formation of benign prostatic hyperplasia (BPH, discussed later (25)). Roughly corresponds to the anterior lobe.
4) *Anterior fibromuscular stroma*: it consists of muscular and fibrous tissue.

The "fourth lobe" is named lateral and spans all zones.

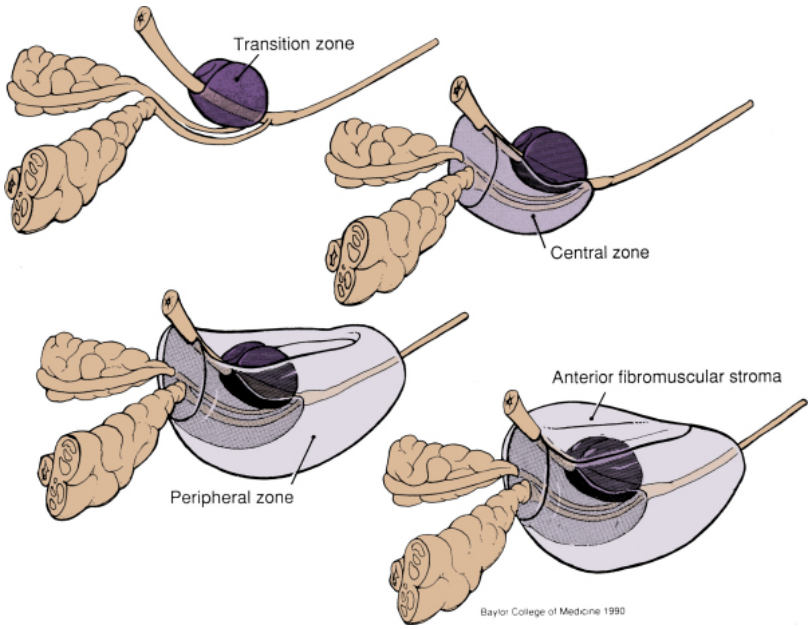

Figure 4. Structure of the prostate. The four zones are indicated: transition zone, central zone, peripheral zone and anterior fibromuscular stroma. From Baylor College of Medicine 1990.

The growth of the prostate is regulated by androgens like testosterone. Testosterone is mainly produced by Leydig cells in the testis and to lower extent in the adrenal cortex

and its synthesis is controlled by luteinizing hormone (LH) and the follicle stimulating hormone (FSH). The secretion of LH from the pituitary gland is regulated by the hypothalamic luteinizing hormone-releasing hormone (LHRH) (26). When testosterone is converted to 5α-dihydrotestosterone (DHT) by 5α-reductase, it can stimulate the growth of the prostate (for example during puberty) (27). DHT in the blood is associated with the sex hormone-binding protein (SHB), responsible for its transportation into the vasculature and to the target cells, where androgens bind and activate the androgen receptor (AR). Activation of the AR by androgens results in the transcription of androgen-responsive genes like PSA or the prostate-specific gene TMPRSS2 (28,29).

2.1. Architecture of the prostate

The prostate has a glandular structure characterized by several ducts constituted by three major cell types: luminal, basal and neuroendocrine cells (30) (**Fig. 5**). These cells are different in morphology, function and significance for tumorigenesis.

Luminal cells: these cells are located along the glandular lumen and have a secretory function. They are terminally differentiated and express specific differentiation markers such as AR and cytokeratin 8/18 (31,32). Additionally, they are androgen regulated and produce PSA and PAP. Cells with self-renewal properties have been identified within the luminal compartment in mice and humans (33,34) and proposed as the cell-of-origin of castration resistant prostate cancer (CRPC, discussed later).

Basal cells: these cells are located between the luminal cells and the basal layer that separates the epithelium from the stroma, which consists of fibroblasts, blood vessels, nerve cells, smooth muscles cells, infiltrating immune cells and connective tissue. The contribution of these cells and components to prostate cancer is crucial, especially during the progression of the disease (35,36). Basal cells are proliferative and characterized by the expression of cytokeratin 5 and 14 (37,38). Experimental evidence have shown the presence of stem-like cells within the basal compartment (39,40) which could maintain basal cells or differentiate into luminal cells and neuroendocrine cells (41,42).

Neuroendocrine cells: these cells are dispersed within the basal layer and are androgen independent; they express different neuropeptides like serotonin and chromogranin A (43). It is hypothesized that their function is to participate in the differentiation of the normal prostate and they also play a role in tumorigenesis (44,45).

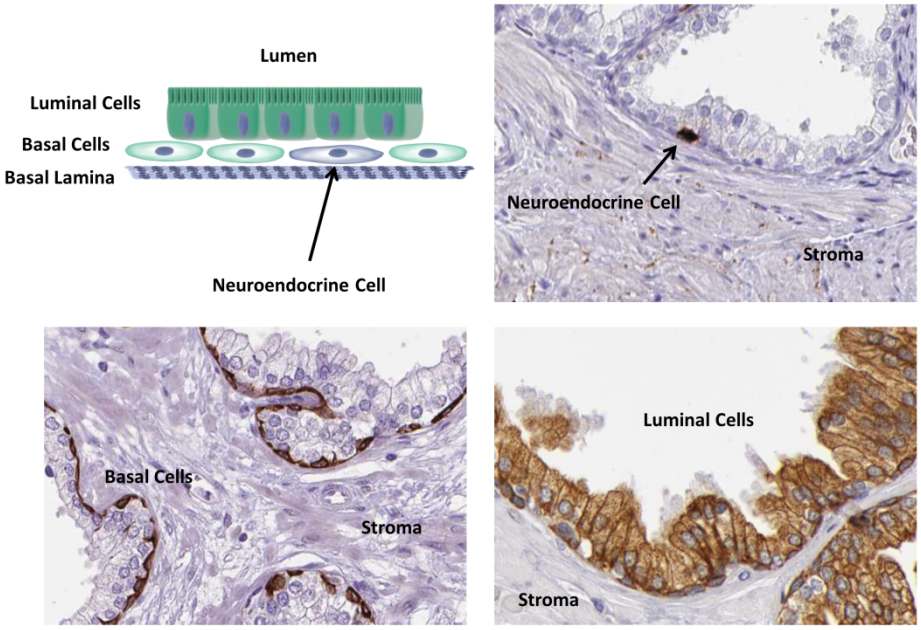

Figure 5. Histology of the prostate. The epithelial layer that characterizes the prostate consists of basal cells (Cytokeratin 5 positive, bottom left), separated from the stroma by the basal lamina; neuroendocrine cells, dispersed within the basal cells (Chromogranin A positive cells, top right) and luminal cells (Cytokeratin 18 positive cells, bottom right). Source: www.proteinatlas.org.

3. Diseases of the Prostate

Due to its high blood perfusion and connection with the urethra, the prostate gland is susceptible to acute and chronic bacterial infection (i.e. prostatitis) typically treated with antibiotics (46). Moreover, during aging, the prostate increases physiologically in size and this can result in benign prostatic hyperplasia (BPH), classified in two types: histologic BPH, characterized by microscopic evidence of epithelial and stromal hyperplasia, and macroscopic BPH, characterized by an enlargement of the prostate (25). Three main theories have been proposed to explain the etiology of BPH (47): 1) the enlargement of the prostate could be caused by a shift in the prostatic androgen metabolism occurring with age, which lead to abnormal accumulation of dihydrotestosterone; 2) changes in epithelial-stromal interaction induce prostatic growth; 3) an expansion in epithelial stem cells. Clinical manifestations of an enlargement of the prostate include lower urinary tracts symptoms such as bladder outlet obstruction and chronic urinary retention which results in additional complications (e.g. infections). Treatment options include pharmacologic agents employed to relax the prostatic smooth muscle (alpha-blockers, 5-alpha reductase

inhibitors) or transurethral resection (48,49). Additionally, there are some similarities between BPH and prostate cancer as both require androgens for growth and development and therefore might respond to antiandrogen treatments (50).

4. Prostate Cancer

4.1. Epidemiology

Prostate cancer is the second leading cause of death from cancer in males in western countries with 220,800 new cases estimated for 2015 (51). The incidence of prostate cancer increases with age (prostate cancer is a rare event in men under the age of 50), and it is higher in the western world compared to less developed countries, due to differences in life-style, eating habits, environmental agents and ethnicity (52). However, there is homogeneity in the age-dependent prevalence of prostate cancer in different countries.

4.2. Prostate Cancer Initiation

Prostate cancer is considered a multifocal disease. The primary tumor often presents multiple histologically independent foci that can be genetically identified for their properties and are relevant for understanding the distinction between latent and clinical disease (30). Although prostate cancer is commonly considered a disease of older men, analysis of specimen collected from younger healthy individuals revealed the presence of histologic foci of prostate cancer also in men in their 20s to 40s, suggesting an early onset of cancer (53). In the majority of the cases, these multifocal lesions will result in a latent disease that will not progress to clinically detectable and relevant prostate cancer. This can be explained by two hypothesis: there is a critical difference between the initiation of the pathogenic program of latent and clinical prostate cancer, or the critical events that are needed to generate a clinical disease do not occur in the latent foci. In the initiation phase, the normal prostate gland present a ductal-acinar histology, characterized by an organized epithelium with luminal secretory cells, basal and neuroendocrine cells and a basal lamina (**Fig. 5**). This organized structure is altered during the “initiation stage”, where histological changes of the luminal epithelium occur and lead to luminal epithelial hyperplasia defined as prostatic intra-epithelial neoplasia (PIN) (54). PIN lesions are classified between low-grade and high-grade, they are multifocal and, at this stage, the cancer is contained by the intact basal layer which prevents the invasion of the surrounding tissues. The morphological integrity of the glandular structures prevents also an increased release of PSA into the blood stream. For this reason PIN lesions are usually detected by biopsy and not by blood test as they don’t produce increased PSA levels (55). High-grade PIN

lesions are characterized by high expression of proliferation markers (56) and by the histological presence of basal cells (30).

A number of genomic alterations such as copy number variations and chromosomal rearrangements (insertions, deletions) associated with prostate cancer and hereditary prostate cancer have been identified with multiple genome analysis studies (30).

Among the most common copy number alterations, those occurring at 8p21 (Nkx3.1), at 10q23 (PTEN) and at 8q24 (MYC) involve key regulatory genes (30,57-59). In addition, genome-wide association studies have shown the involvement of HPC1 and HPC2 (mapped in 1q24-25 and 17p11 respectively) in hereditary prostate cancer (60,61). The down regulation of Nkx3.1 is one of the critical events in prostate cancer initiation and is detected in up to 80% of the prostate tumors (also in PIN lesions and early invasive carcinoma). Nkx3.1 has a critical role in prostate morphogenesis and differentiation and mutant mice for Nkx3.1 develop PIN lesions that resemble closely those detected in human.

Another frequent chromosomal loss detected in a high percentage of prostate cancer cases is represented by PTEN (tumor suppressor gene). Loss of PTEN leads to hyperactive PI3K-AKT-mTOR signaling, which results in aberrant cell proliferation and metabolism (62). Recent studies have shown that the allelic loss of PTEN represents an early event in prostate carcinogenesis and correlates with progression of the disease (59). As for Nkx3.1, also loss of PTEN in mice results in PIN lesions and/or adenocarcinoma (63).

Besides the aforementioned chromosomal losses, genetic studies have identified also gene amplifications in prostate tumors. The oncogene MYC is amplified during initiation of prostate tumors and upregulation of MYC has been registered in PIN lesions (64). Similarly, transgenic mice overexpressing human MYC undergo rapid formation of PIN lesions, followed by progression to invasive adenocarcinoma (65). Another set of genetic alterations is represented by chromosomal rearrangements. Among these, the most common alteration regards the family of transcription factors (ERG, ETV1 and ETV4) and the prostate specific gene TMPRSS2 (66). The most frequent rearrangement produces the fusion gene TMPRSS2-ERG, where the N-terminally truncated ERG protein is expressed under the control of the promoter of the androgen-regulated TMPRSS2 gene (66). This alteration usually occurs in cancer initiation and is also detected as early event during cancer progression.

4.3. Prostate Cancer Detection

The oldest known case of prostate cancer diagnosed reliably by morphological and biochemical techniques dates back to 2,700 years ago (67). Schultz and co-workers

described that a well preserved skeleton of a 40 to 50-year-old Schythian king who lived during the Iron Age in the Southern Siberia (Arzhan) suffered from macroscopically visible osteoblastic and osteolytic lesions throughout the entire skeleton (67). This diagnosis is based on microscopic imaging of the lesions and detection of prostate-specific-antigen (PSA) complexed with α1-antichymotrypsin (PSA/ACT) in the extracellular matrix (ECM) proteins extracted from the compact cortical bone of the skeleton from Arzhan (68,69).

The blood test for PSA, nowadays routinely used in the clinic, has revolutionized the clinical practice over the past four decades and has represented the standard for prostate cancer detection and monitoring. PSA is a glandular kallikrenin-related peptidase produced by the gene KLK3 and its transcription is regulated by androgens which make its expression a main characteristic of the prostate epithelium (70). PSA is continuously produced by the healthy prostate. In the normal prostate, the morphological structure of the glands contains PSA tightly confined and only a reduced amount is released into the blood (0.6 ng/ml in a healthy adult male) where it exits in multiple forms: as pro-protein or mature protein and free or associated with different protease inhibitors (70). In BPH or prostate cancer, the disruption of the normal prostate architecture often results in a massive release of PSA into the blood (>100 ng/ml) that is measured almost exclusively in males with advanced prostate cancer (70). Altered PSA levels in blood are also commonly detected during the occurrence of other alterations of the prostate such as inflammation (prostatitis) and its' levels are also influenced by age. For this reason, PSA is considered as a prostate-specific marker but not a cancer-specific marker. In this perspective, two more specific and clinically-promising markers for prostate cancer detection are represented by the non-coding messenger RNA for the so called Prostate Cancer Antigen 3 (PCA3), identified in 1999 (71) and the fusion gene TMPRSS2-ERG described in 2005 (66). However, although PCA3 and TMPRSS2-ERG (also as combined biomarkers) displayed higher specificity and diagnostic accuracy for prostate cancer outcome, PSA is still the most widely used biomarker in prostate cancer diagnosis (72). Importantly, the expression of KLK3 at molecular level in the prostate epithelium and the increase of PSA level in the blood of men affected by prostate cancer are not directly correlated (73). The detection of augmented PSA level into the blood is indeed determined by an increased release of PSA from the prostatic gland as a consequence of disruption of normal prostate architecture and not by an increase of its transcription. This leads to the documented paradox that during development and progression of prostate cancer, KLK3 expression might slightly decrease (74).

The typical clinical practice for men with high PSA levels schedules biopsy to assess the possible presence of prostate cancer. The prostate tissue collected is then graded according to the Gleason scoring system introduced for the first time in the clinic

in the 1960s and recently updated in 2005 (75-77). The Gleason scale describes the primary and secondary architectural pattern of the tissue obtained from prostate biopsies and classifies tumors according to their differentiation, from 1 to 5, based on the morphological architecture of the prostate (76,78). Briefly, Gleason 1 corresponds to a transformed prostate epithelium that resembles a normal prostatic epithelial tissue; from Gleason 2 to Gleason 4, the infiltration of cells at the margin of the gland is progressively increasing; Gleason 5 corresponds to a cancerous prostate which has completely lost its epithelial structure and is filled with invading mesenchyme-like cancer cells. The final Gleason score is obtained upon mathematical addition of primary and secondary score and can range from 2 to 10 (79). In addition, the status of the primary tumor is also graded, from organ-confined to fully invasive (T1-4), with or without involvement of lymph nodes (N0 or N1) and with or without presence of distant metastasis (M0 or M1 a-c) (80). These together constitute the so called Tumor-Node-Metastasis (TNM) system of grading.

4.4. Treatment of Localized Disease

There are several options for treating prostate cancer patients with localized disease, depending on the stage and the patient condition.

As previously mentioned, due to improved screening methods, prostate cancer can be detected already at the very initial stage. Active surveillance is considered a logical approach for those men with localized prostate cancer and associated low-risk to prevent overtreatment (81-83). The clinical criteria to define an active surveillance strategy are: confined disease with T1-T2 stage, maximum PSA level of 10 ng/mL and Gleason score <7 (84). Additionally, watchful waiting is considered an alternative for old men with less aggressive disease (85).

Surgical approaches like the removal of the entire prostate are applied to men with high life expectancy and localized disease with the aim to completely eradicate the tumor (86). In these patients, given the low risk of lymph node involvement, the removal of pelvic lymph nodes remains controversial (86,87).

Another therapeutic approach for the localized disease is radiotherapy which employs x- and gamma-rays or alpha emitting radio isotopes (88) to kill tumor cells by causing DNA damage. Two applications are possible: external beam radiotherapy and internal radiotherapy (also called brachytherapy, which consists of implantation near the cancerous region of radioactive plugs which will release slowly the radiation). Recently, also image guided intensity-modulated radiotherapy has been developed to deliver high dose particles to specific regions reducing the impact on the surrounding tissues (89). It is important to consider that although surgical removal and radiotherapy produce a

similar outcome, they have a different impact on the quality of life of the patient (e.g. urinary and sexual function) (90).

4.5. Prostate Cancer Progression and Bone metastasis

When the cancer enters into the “progression phase”, the loss of the basal lamina occurs and results in the switch from high-grade PIN to adenocarcinoma with an invasive phenotype, macroscopically characterized by the lack of basal cells as shown by p63 and cytokeratin 5/14 staining (91). However, whether prostate cancer is originated from luminal or basal prostate cancer stem cells is still under debate (33,92). The majority of prostate adenocarcinomas present with an acinar morphology while ductal and mucinous adenocarcinomas are more rare. In less than 2% of the cases the adenocarcinomas are classified as neuroendocrine variants and mainly occur during recurrence after androgen deprivation therapy (93). This can partially be explained by the fact that neuroendocrine cells lacking of AR expression survive ADT and prevail producing relapse (94).

The terminal phase of prostate cancer progression encompasses systemic metastasis, which coincides with the development of therapy resistance, e.g castration and chemotherapy resistance (95). The majority of aggressive prostate cancers is characterized by their osteotropism leading to the development of predominantly osteoblastic/osteosclerotic lesions and, thus, represent one of the major clinical challenges in uro-oncology. The first explanation for the bone tropism of prostate cancer metastasis was provided in 1940 when Oscar Batson suggested that the venous network that drains the prostate and connect the pelvic veins to the paravertebral venous plexus could explain the dissemination (96). However, another study demonstrated that the venous network does not represent the major driver in the dissemination of prostate cancer cells to the bones (97). Alternatively the interactions between cancer cells and the endothelium was also suggested to underlie organ-specific dissemination (98). Furthermore, the interaction between the chemokine (C-X-C motif) receptor (CXCR) 4 (CXCR4) and its ligand stromal derived factor 1 (SFD1, also known as CXCL12) may be critically important (99). Prostate cancer cells express CXCR4 and experimental evidence has shown that neutralization of CXCR4 reduces prostate cancer bone metastasis in preclinical models (100). Moreover, prostate cancer cells also express various integrins, e.g. integrin $\alpha v\beta 3$, which correlates with prostate cancer bone metastasis and is responsible for the interaction with fibronectin, vitronectin and osteopontin (101-104). The notion that molecular factors might be involved in the specific bone tropism of certain cancer cells was for the first time postulated by Sir Stephen Paget who introduced the “Seed and Soil” hypothesis in which he compared the bone metastatic breast cancer cells to the seed of plants, capable of growing only in

a fertile soil, the bone marrow (105). Today we know that the formation of distant metastasis is a complex process, characterized by multiple bi-directional interactions between the tumor cells and the supportive stroma (106). This process starts at the level of the confined primary tumor where factors systemically released contribute to the conditioning of the metastatic "soil" and provide the establishment of the so called "pre-metastatic niche" (107). The "pre-metastatic niche" is defined as a fertile microenvironment induced in the metastatic target organs that facilitates the future invasion, colonization and the proliferation of metastatic tumor cells (107). During the establishment of the "pre-metastatic niche", bone marrow-derived hematopoietic progenitor cells expressing VEGF receptor 1 (VEGFR1) are recruited to metastatic target organs by specific factors released by the primary tumor (108). Among these factors, LOXL enzymes, VEGFA, VEGFC, TNFα and TGF-β produced by the primary tumor stimulate inflammation, attachment and recruitment of, for example, myeloid cells and the expansion of lymphatic vessels in the proximity of the sentinel lymph nodes (109-112). Interestingly it has been proposed that extracellular vesicles and exosomes released from the primary tumor represent the mechanism of communication between the primary cancer cells and the metastatic sites during the induction of the "pre-metastatic niche" (113) also in prostate cancer (114).

Additionally, in primary and metastatic cancers, tumor cells interact with different cell types that constitute the stroma. Such cells include tumor-associated macrophages (TAMs), cancer associated fibroblasts (CAFs), endothelial cells, pericytes and mesenchymal stem cells (MSCs) reviewed in (6,115). Tumor cells produce several factors that "activate" the surrounding stromal cells and induce remodelling of the EMC. These factors include fibroblast growth factor 2 (FGF2), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), interleukins colony-stimulating factors and TGF-β (116) and proteolytic enzymes (117) that remodel ECM, enabling cell migration. In the progression and castration resistant phase of prostate cancer, cancerous polarized-epithelial cells localized at the site of the primary tumor undergo biochemical changes and acquire an invasive and often mesenchymal phenotype (118,119) which confers them enhanced migratory ability, invasiveness, resistance to apoptosis and resistance to therapy, which are all properties resulting in a clinically-relevant phenotype (120,121). Together these events result in the invasion of the surrounding stroma and in the intravasation and circulation of cancerous cells in the blood stream. Tumor cells which possess stem cell-like characteristics, that survive in the circulation can extravasate at those distant sites where the "pre-metastatic niche" has previously prepared a fertile "soil" for future colonization. Recent research has highlighted the clinically relevant properties of the so called circulating tumor cells (CTCs) capable of surviving into the blood stream and in

distant metastatic sites (122). Once that these CTCs have colonized the metastatic site (e.g. the bone), they may activate a reverse program of mesenchymal-to-epithelial transition (MET) and remain dormant for years (123). Therefore it appears that these disseminating tumor cells (DTCs) can perpetuate in the bone the malignant progression and establish a “metastatic niche”.

Typically, the “metastatic niche” is located at perivascular locations (124). CTCs and DTCs may, potentially, establish a metastatic niche through competition with hematopoietic stem cells (HSCs) for their niche at a perivascular location (124-127). Moreover it has been hypothesized that tumor cells can also create their own niche (125,128). PCa cells amplify the existing hematopoietic niche and induce de novo an ectopic epithelial tissue-of-origin niche which together with the amplified hematopoietic niche generates a hybrid niche, supportive for cancer cell growth (106) and reviewed in (129). DTCs can survive in the bone microenvironment as non-proliferating (dormant) cells that originate microscopic lesions (classified as micrometastasis) (130,131). The mechanisms that induce exit from dormancy are still largely unknown (131). However, it has been shown that a collagen-I enriched fibrotic environment plays a crucial role in the cytoskeletal reorganization in dormant cells and in their awakening from dormancy (132). Once that these cells escape from dormancy, they induce local inflammation, followed by vascular and bone remodelling and establishment of a distant secondary tumor (bone metastasis) (120,133). Recently, it was revealed that the molecular signature of the stroma response in prostate cancer-induced osteoblastic bone metastasis highlights the amplification of hematopoietic and prostate epithelial stem cell niche (106). This observation supports the notion that angiogenesis and osteogenesis are crucial processes involved in the formation and growth of osteoblastic bone metastasis. Moreover, a recent report described the presence of two different type of microvessels: type “H” ($CD31^{high}$ and $endomucin^{high}$) and type “L” ($CD31^{low}$ and $endomucin^{low}$) (134). Interestingly, angiogenesis and osteogenesis have been coupled to the type “H” vessels, that provide also signals for HSCs and where osteoblasts also reside (135). Moreover, the kinetic of type “H” vessels in mice shows a peak at week 4 and loss of type “H” endothelium during ageing has been documented (134). Together this support the involvement of angiogenesis in the homing of metastatic cells in the bones in preclinical mouse models.

The bone remodelling induced by metastatic cancer cells results in either bone formation (osteoblastic bone metastasis) or bone resorption (osteolytic bone metastasis) and interferes with hematopoiesis (133). In prostate cancer, the bone lesions are typically osteoblastic (133,136), however the co-existence of osteobastic and osteolytic response have been documented (137).

Factors inducing osteoblast recruitment and activity in prostate cancer are: BMP6 (138), and BMP modulators, such as Noggin (NOG) (139); IGF1 (140) VEGFs (141), wnt signaling (142) and modulators of Wnt signaling such as dickkopf (DKK) and Sclerostin (SOST) (139). On the other hand, factors modulating osteoclast recruitment and activity in prostate cancer are: MMP-7, which promotes osteolysis via cleavage of RANKL that stimulates osteoclastogenesis (143); Noggin which antagonizes bone morphogenetic proteins (BMPs) and impairs bone formation (139,144);

It has been hypothesised that osteolytic cancer cells produce PTHrP that stimulates osteoblasts to secrete RANKL. This in turn stimulates ostecolasts progenitor cells and leads to osteoclastogenesis therefore bone resorption. During this process, many factors such as TGF-β, IGF-1 and calcium are released from the mineralised matrix to further feed cancer cell growth, thus perpetuating this "vicious cycle" (133,145). In prostate cancer for example, the expression of the calcium sensing receptor by tumor cells makes them responsive to the release of calcium during bone resorption and leads to increased proliferation and PTHrP release (146,147). However, the inhibition of bone resorption as strategy to impair bone metastasis with agents such as bisphosphonates revealed no effect on cancer cell proliferation in animal studies (148,149) and clinical trials also in prostate cancer (150) suggesting that other mechanisms support tumor cell growth in the bone. In this perspective, the recent identification of the molecular stroma response in osteoblastic prostate cancer (106) supports the coupling of angiogenesis and ostegenesis in bone metastasis (134) and suggest that anti angiogenesis might impact on the growth of osteotropic prostate cancer cells in the bone.

4.6. Treatment of Advanced Disease

As previously described, PSA testing allows an early detection of many cases of the disease when the cancer is still confined and may therefore be successfully resolved by surgery or radiotherapy. However, after local treatment, 20-40% of the cases, biochemical relapse will occur (PSA > 0.2 ng/ml) (**Fig. 6**) (95). Typically these patients will be treated with androgen deprivation therapy (ADT, which consists of chemical or surgical castration and/or treatment with anti-androgens) which will lead to regression of prostate tumors (151).

A strategy consists in the modulation of the testosterone biosynthesis via interference with LH and LHRH. This can be achieved in two ways: employment of LH agonists to produce in the long term a downregulation of the LH receptor thus resulting in a decrease of the testosterone biosynthesis (152); employment of LH antagonists which result in a rapid decrease of testosterone levels (152). Another strategy consists of treatment with anti-androgens such as bicalutamide and enzalutamide (153).

Despite these therapies, 30-70% of the patients treated with androgen deprivation therapy will inevitably display increased PSA levels, acquire resistance to androgen suppression and develop incurable metastatic disease (154). This situation is commonly defined as castration resistant prostate cancer (CRPC) or hormone refractory prostate cancer (HRPC). Although similar, these two terms refer actually to two different clinical situations. Patients who are traditionally identified as HRPC are highly heterogeneous depending on: 1) the clinical status, 2) the level of PSA, 3) the applicability of hormone therapy and 4) the eventual presence of metastasis (95).

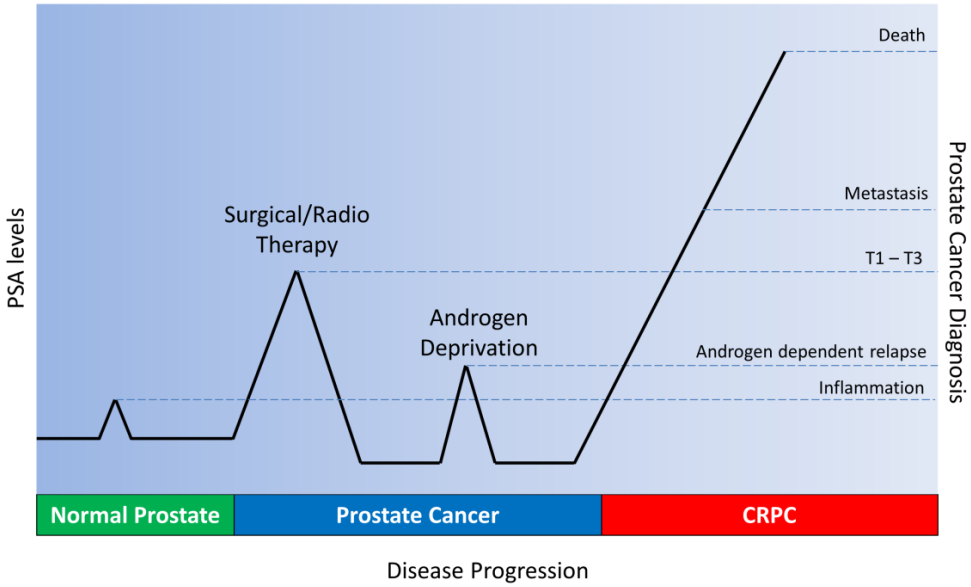

Figure 6. Overview of prostate cancer progression combined with diagnosis and treatment options. Prostate cancer is initially treated with prostatectomy or radiotherapy and in almost 80% of the cases, patients will be cured. In 20-30% of the cases, prostate cancer relapses and these patients will be typically treated with androgen deprivation therapy. However, the development of castration resistance prostate cancer (CRPC) will inevitably occur. Although these patients will be treated with therapies such as docetaxel, cabazitaxel, enzalutamide and abiraterone, the development of incurable metastasis, typically in the bone will occur.

Interestingly, there are documented cases in which the androgen receptor (AR) signaling remains active after androgen deprivation therapy probably through escape mechanisms (30). Such mechanisms include amplification of the AR gene (155-157), gain-of-function mutations of AR (158-162), expression of alternative splice variants (163-165) and endogenous expression of enzymes involved in DHT synthesis by tumor tissue (166-169). For this reason, the term CRPC has been progressively introduced into

the clinic to indicate a condition where response to hormonal therapy is still possible, therefore reveals a different condition from HRPC (95).
Once that tumor acquires resistance to androgen suppression and patients develop metastasis mainly in the bones, treatment options are limited and include symptomatic care with analgesics or radiotherapy to reduce bone pain, treatment with bone-seeking isotopes (e.g. Strontium-89 and the recently FDA-approved Radium-223 chloride) and chemotherapy (170). Typical therapeutic treatments consist of agents targeting the androgen pathway (abiraterone acetate and enzalutamide) and taxanes (docetaxel and cabazitaxel), which target microtubules and result in the arrest of the cell cycle (170-174). Current first-line treatments consist of combination therapy with docetaxel and prednisone, while second line combination treatments are cabazitaxel and prednisone, abiraterone acetate and prednisone and enzalutamide (171-174). Recent studies revealed that simultaneous treatment of ADT and docetaxel significantly increases patient survival (175,176). However, longer follow up of these studies is needed to assess whether this benefit translates also into metastatic-free survival.

4.7. Prostate Cancer Stem Cells

According to what is commonly known as the “cancer stem cell hypothesis”, CSCs appear to be strongly involved in tumor formation, therapy resistance, recurrence and metastasis. As we already mentioned in a previous paragraph, cancer is a disease that originates from a single normal cell after a series of specific genomic and non-genomic alterations. As a result it was hypothesized that cells with self-renewal ability represent good candidates for oncogenic transformation and cancer formation (47). There are two putative sources of cells with self-renewal properties that are believed to generate cancer: adult stem cells (SCs) and non-stem cells that acquire self-renewal properties after de-differentiation and transformation. The majority of prostate cancer have a luminal phenotype and the absence of basal cells is a diagnostic feature of prostate adenocarcinoma (91,177). One could, therefore, speculate that prostate tumors originate from luminal progenitor cells or stem cells within the basal layer that after transformation differentiate into a luminal progeny. However, the histological compartment where the putative cell of origin of prostate cancer resides is still under debate. In hormone-naïve cancer, experimental evidence in rodents and humans support the existence of cells with self-renewal properties and tumorigenic ability in the basal compartment of the prostate ($\alpha_2\beta_1$ integrin^{hi} and CD133^{+} cells (178,179)) (**Fig. 7**). Other common markers include ALDH^{high}, CD44^{+} and CD24^{-} (39,40,180). On the other hand, in CRPC, cells with self-renewal properties exhibit a luminal phenotype. These cells have been identified in castrated mice and are known as castration-resistant Nkx3.1-expressing cells, named CARNS (33) and later the stem-like cells with luminal

phenotype (CARN-like cells) were also identified in humans (34). CARNs are characterized by low AR expression, and display the stem-cell marker ALDH1A1 or NANOG and express the luminal marker NKX3.1 and CK18 (34). Interestingly, the experimental observation in favour of the luminal hypothesis suggest the presence of a residual and dormant subpopulation of cancer cells which are castration-resistant for survival but castration-sensitive for growth (34). Recently the field has been additionally complicated by the experimental evidence that murine luminal (CD49f positive (181)) and basal (CD24 positive (182)) cells and human luminal (CD26 positive (182)) and basal (CD49f positive (182)) cells are capable of generating prostate organoids (183). However, the debate about the localization of the cell of origin of human prostate cancer and its role in the progression to a castration resistant phase is still controversial.

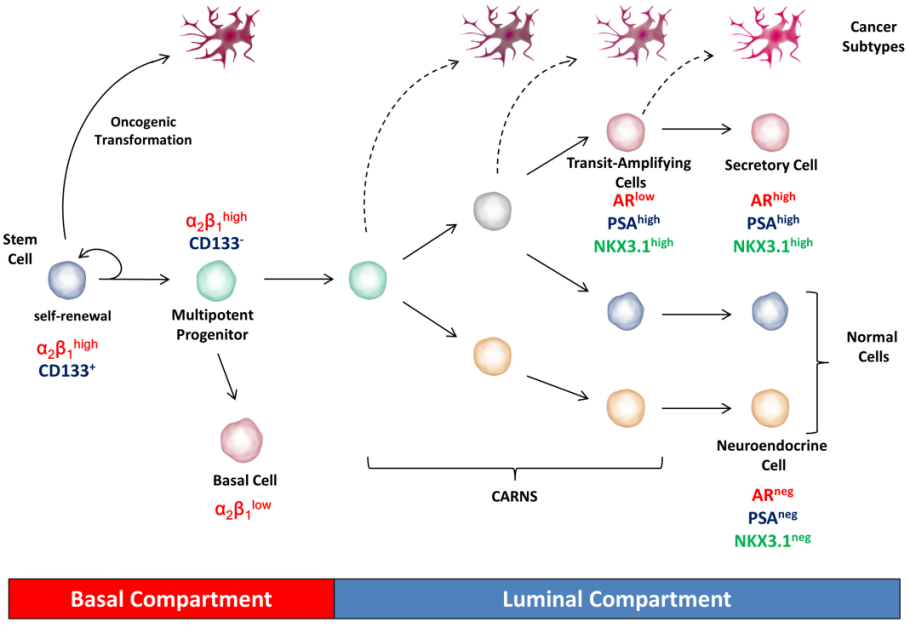

Figure 7. Hierachical model of tumorigenesis: role of normal and transformed tissue stem/progenitor cells. Cells within the different epithelial compartments can be distinguished by their phenotypic characteristics.

In addition to haematological malignancies, the presence of a subpopulation of epithelial cells with self-renewal properties is generally recognized in solid tumors including those of the human prostate. Furthermore accumulating experimental and clinical evidence suggest that such cells are highly tumorigenic and may play a key role in distant metastasis in preclinical models (33,178,183) and in clinical reality (184,185).

The assumed role of CSCs in tumor maintenance represents one of the major problems for the identification of new, targeted therapies capable of eradicating the disease. Current therapies are indeed very effective in the treatment of the primary tumor mass (186). However, the relapse that is commonly observed (even after many years) in patients, suggests the presence of subpopulation of cells, resistant to therapy, which probably remain dormant for long time and that are capable of producing a new tumor (186,187).

5. Pathways Involved in Prostate Cancer Progression and Bone Metastasis

The notion that genes involved in developmental process are also likely to be altered in cancer is known and established. Molecular analysis revealed that a wide range of genes, commonly expressed during prostate organogenesis and developmental processes, are also abnormally expressed in prostate cancer.

Wnt pathway: the Wnt signaling pathway is an evolutionarily conserved pathway that regulates crucial aspects of development and cell behaviour, such as differentiation, migration and cell polarity. The Wnt signaling is characterized by two branches: a canonical pathway (Wnt/β-catenin dependent) and a non-canonical pathway (β-catenin independent).

The **canonical Wnt signaling** is activated upon the interaction between a ligand (Wnt) and its receptor (Frizzled, Fz) and co-receptor (low-density-lipoprotein-related protein 5/6, LRP5 and LRP6) (**Fig. 8A**).

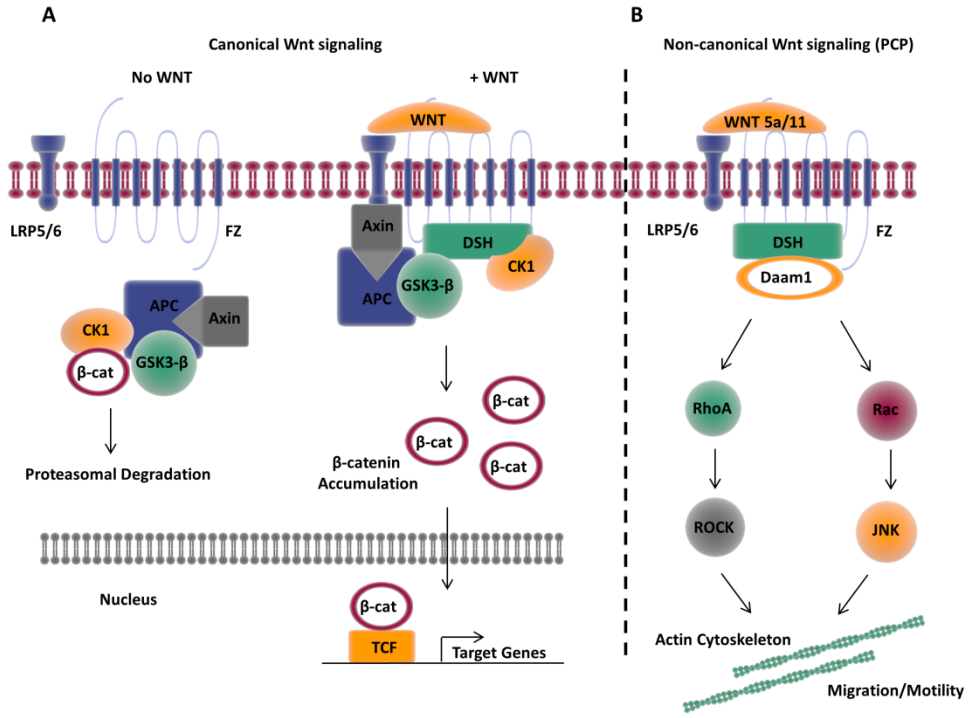

Figure 8. Schematic representation of canonical (A) and non-canonical (B) Wnt signaling.

In the absence of Wnt, a complex of Axin, APC, GSK3-β, CK1 and β-catenin is localized in the cytoplasm. CK1 and GSK3-β phosphorylate β-catenin which is subsequently degraded by the proteasomal machinery. In presence of Wnt, LRP6 is phosphorylated by CK1 and GSK3-β, thus recruiting to the plasma membrane a complex containing Axin and Dishevelled (Dsh), which is sequestered or degraded. This results in stabilization of β-catenin which subsequently translocates into the nucleus and mediates transcription of downstream target genes via interaction with LEF/TCF family members. Canonical Wnt signaling regulates process such as cell fate decision and anteroposterior organization in embryogenesis, as well as important function in organogenesis and stem cell renewal.

Many studies have documented alterations of the Wnt signaling pathway during prostate cancer progression, reviewed in (30,188-191). More specifically, elevated canonical Wnt signaling seems to play a role in the onset of castration resistance in prostate cancer (192). Additionally, alterations or interferences with canonical Wnt signaling, such as modulation of DKK (142) or mutation in sclerostin (SOST), which inhibits LRP5, contribute to disrupt bone formation, a process also regulated by Wnt signaling (193). In addition to the well-established effects of Wnt-signaling on enhanced osteogenesis, Wnt-signaling also induces bone-active factors, such as OPG which prevents the binding of RANKL to RANK thereby inhibiting osteoclast function (194).

The **non-canonical Wnt signaling**, comprise two branches of signaling transduction: the Wnt/Ca^{2+} signaling and the Wnt/planar cell polarity (PCP) pathway (195) (**Fig. 8B**). In Wnt/Ca^{2+} signaling, the interaction between Wnt and Fz activates phospholipase C via G proteins and lead to increase intracellular Ca^{2+}. This can induce, for example, EMT and invasion, therefore promoting cancer progression. In Wnt/PCP, the non-canonical Wnt (Wnt5a and Wnt11) bind their receptor Fz which recruits Dsh at the plasma membrane. This lead to a cascade of interactions which converge on common regulators of cytoskeletal remodelling and actin organization such as RhoA, Rac1 and JNK, which also impact on cell motility.

Wnt/PCP and canonical-Wnt signaling are both part of a negative feedback-loop where Wnt/PCP negatively regulates canonical-Wnt signaling and *vice versa* (196). In cancer, due to aberrant alterations in tumor cells, cancer cells can escape from these control mechanisms and as tumors progress, Wnt/PCP gets activated and promote cell motility, invasion and metastasis (195). Interestingly, β-catenin and GSK3-β have indeed been shown to be decreased in prostate cancer cell lines with high invasive and metastatic potential, such as PC3 (197). Therefore one could speculate that there is a misbalance in these cells between canonical and non-canonical Wnt signaling which results in mesenchymal phenotype and invasive properties.

Notch pathway: the Notch signaling pathways exerts also a crucial role during embryogenesis and organogenesis. In cancer, an aberrant activation of this pathway produces abnormal cell proliferation, increase in self-renewal properties and induction of therapy resistance (198,199). Conversely from Wnt signaling, the Notch signaling pathways requires a direct cell-to-cell contact for its activation. Typically, a signal-sending cell expressing on its plasma membrane the ligand (JAG1/2 or Delta-like 1, 3 and 4, in mammals) stimulates a signal-receiving cell expressing on its membrane the receptor (Notch1/2/3/4). This interaction produces a series of proteolytic cleavages operated by ADAM10 and γ-secretases that convert the full-length transmembrane Notch receptor into a transcriptional activator (Notch intracellular domain, NICD). Subsequently, NICD translocates into the nucleus, where it interacts with RBPjk/CBP transcription factors, resulting in the transcription of downstream target genes (e.g. Hairy and enhancer of split, HES and Hairy/enhancer-of-split related with YRPW motif, HEY) (**Fig. 9**).

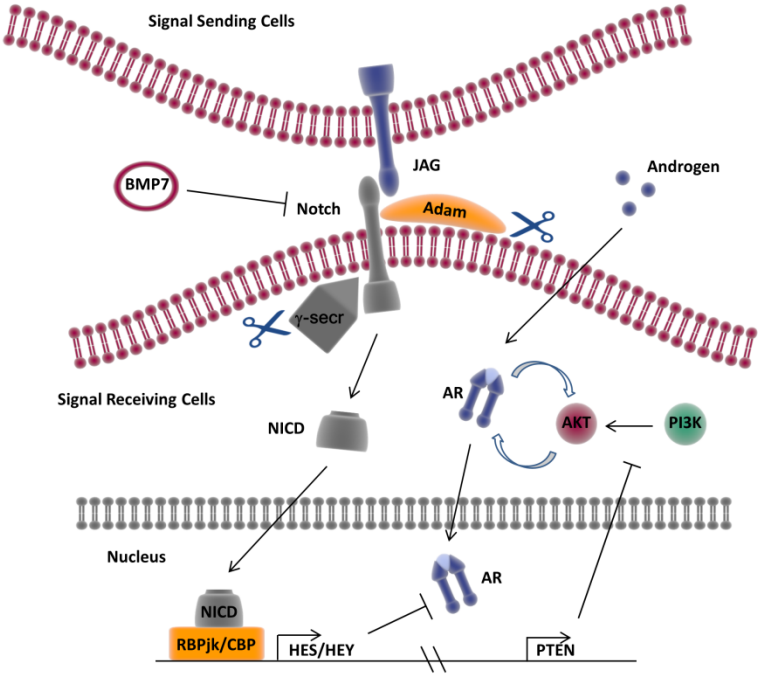

Figure 9. Schematic representation of Notch signaling.

Notably, Notch plays a crucial role during prostate organogenesis and is involved in its regeneration (198). Importantly, and relevant for the purpose of this thesis, the Notch

signaling pathway is characterized by multiple cross-talk with other major signaling pathways involved in prostate cancer progression and bone metastasis formation (e.g. TGF-β, AR and PI3K/AKT) (**Fig. 9**) (200-205). Members of TGF- β superfamily can control Notch signaling: for example, Bone morphogenetic protein 7 (BMP7) can inhibit the branching morphogenesis of the prostate during development via down-regulation of Notch signaling (206). Notch can also suppress AR signaling which is crucially involved in prostate growth and disease. Upon binding of androgen to the AR, the receptor undergoes a homodimerization and traslocates into the nucleus where it can recruit coactivators such as p300/CBP and steroid receptor coactivator 1 (SRC1). The downstream target of Notch, HEY1 can directly bind the N-terminal activation domain of AR thus preventing androgen signaling, supporting a role of Notch in the acquisition of a castration resistant phenotype (204). Finally, Notch can also suppress the PI3K/AKT pathway that is fundamental for prostate growth and cell migration (207). The activation of this pathway triggers a cascade of sequential phosphorylation that can be suppressed by PTEN (198). It appears that NICD contributes to induction of PTEN expression, therefore suppressing indirectly PI3K/AKT pathway (208). This has led to the paradox that Notch signaling (particularly when triggered by Notch1) can exert a tumor suppressive role in the prostate. The complexity of the interaction between the Notch signaling and AR and PI3K/AKT pathways is further increased by a reciprocal feedback mechanism between PI3K/AKT and AR signaling: recently it was indeed demonstrated that inactivation of PI3K/AKT induces activity in AR signaling, while suppression of AR pathway induces increase in PI3K/AKT (209). Given the established increased in PI3K/AKT in advanced prostate cancer (59,210), one could speculate that an increase in Notch signaling (as documented during prostate cancer progression), through its downstream target HEY1, produces a decrease in the AR pathway (castration resistant phase) which results in increased PI3K/AKT signaling (increase migration and metastasis). Notch signaling has also been shown to critically be involved in prostate cancer progression and bone metastasis formation and JAG1 has been found to be elevated in metastatic prostate cancer compared to primary tumor (211). Additionally, in the bone microenvironment, tumour-derived JAG1 activates the Notch pathway in osteoblasts and osteoclasts and the activation of the pathway in osteoblasts results in a growth advantage to bone metastatic tumour cells (212). Interestingly, mechanistic studies showed that the proliferative effect was dependent on osteoblast-secreted IL-6, which was transcriptionally regulated by the Notch signaling in breast cancer (213). The Notch signaling pathways represents a promising target for therapy against tumor growth (214) and bone metastasis (213). However, the presence of studies addressing the possible application of γ-secretase inhibitors in breast but not prostate cancer is remarkable.

TGF-β superfamily signaling pathways: during the early phase of prostate tumor growth, TGF-β acts as tumor suppressor by reducing proliferation and inducing apoptosis (215). However, during tumor progression, TGF-β switch gains a tumor promoter role and facilitates EMT and therefore metastasis (9,216). The transforming growth factor (TGF)-β superfamily of ligands includes more than 30 factors such as Bone morphogenetic proteins (BMPs), Growth and differentiation factors (GDFs), activins, inhibins, nodal and Anti-müllerian hormone (AMH). For the purpose of this thesis we will mainly focus on TGF-β members and BMPs. TGF-β is a pleiotropic cytokine that regulates many biological processes such as tissue growth and morphogenesis, cell proliferation and apoptosis, adhesion, differentiation, migration and metastasis (217). The TGF-β cytokine family consists in different members (TGF-β1, β2 and β3) whose bioactive cytokine molecule is a dimer consisting in a polypeptide chain which is cleaved from a latent precursor into the biologically active product (218).

BMPs include approximately twenty members and these are less homologous compared to the TGF-β isoforms (219). They are functionally involved in skeletal and joint morphogenesis, bone remodeling and in different cellular processes including osteogenesis, cell differentiation, anterior/posterior axis specification, growth, and homeostasis (220). In normal tissues, basal release of TGF-β by local sources is enough for the maintenance of homeostasis. In case of tissue injury, TGF-β is abundantly released by blood platelets and stromal components to prevent aberrant regenerative cell proliferation and inflammation. This occurs also in tumor microenvironment, where TGF-β is frequently present initially as factor to prevent premalignant progression, and eventually as factor that cancer cells may use to their advantage (218).

TGF-β superfamily members bind to type I and type II serine/threonine kinase receptors (**Fig. 10**). In human, seven different human type I receptors have been identified (ALK1-7) and five type II receptors, namely, TGF-β receptor II (TβRII), BMP receptor II (BMPRII), activin receptor II (ActRII), ActRIIB and AMH receptor type II. TGF-β binds TβRII and ALK5 and in endothelial cells it signals also via ALK1. BMP signaling occurs via BMPRII, ActRII and ActRIIB in association with ALK1,2,3 or 6 depending on the molecular context (221-231).

Binding of TGF-β and BMPs to their heterodimeric transmembrane receptors induces phosphorylation of type I receptor threonine/serine kinases. The signal is then transduced via Smad intracellular proteins, which later translocate into the nucleus and regulate transcription. The Smad pathway is also named **canonical signaling pathway**. TGF-β type I receptor propagates the signal by phosphorylating receptor-regulated Smad proteins (R-Smads) Smad-2 and -3. On the other hand, BMPs induce phosphorylation of Smad-1, -5 and -8 (218,232) (**Fig. 10**). This phosphorylation operated

by activated type I receptor occurs at the C-terminal SXS domain that is shared by all Smad proteins and that represent a nuclear localization signal (218,233).

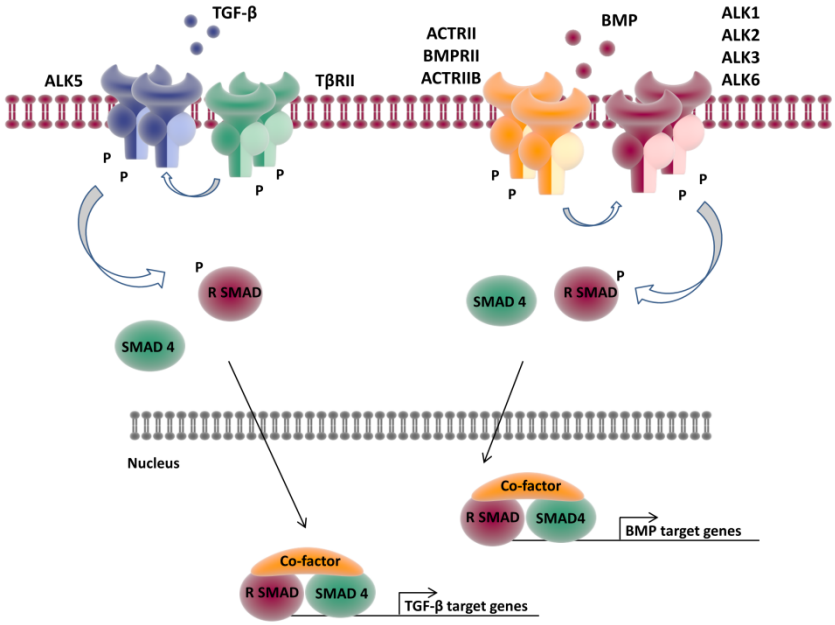

Figure 10. Schematic representation of the TGF-β and BMP signaling.

Depending on their phosphorylation state, Smad-2 and Smad-3 linked to Smad-4 undergo constant nucleo-cytoplasmic shuttling, in a sort of rapid activation-deactivation cycle, determined by repeated cycles of dephosphorylation and rephosphorylation, involving direct interactions with both nuclear pore proteins and importins and exportins, a protein family of transport factors (233).

TGF-β also regulates alternative pathways via Smad-independent signaling (**non-canonical Smad signaling**). These signaling include the extracellular-signal- regulated kinase (ERK1 and ERK2), p38, MAPKs, c-Jun N-terminal Kinase (JNK), PI3K-Akt and small GTPases. The non-canonical Smad signaling pathways have been extensively reviewd in (234-236).

Each step in the TGF-β signaling pathways is controlled by specialized factors. These factors include encapsulation of the extracellular ligand by binding proteins, inhibition of activation of latent TGF-β, receptor-interacting partners (BAMBI, SARA and FKBP12), inhibitory Smads (Smad6 and Smad7) and post-translational modification by E3 ubiquitin ligases, co-repressors and phosphatases, reviewed in (237).

Integrins: integrins belong to a family of heterodimeric transmembrane glycoprotein receptors which consist of an α and a β subunit and which play important roles in tissue development and cancer (238,239). To date 18 α and 8 β subunits have been identified from which 24 different functional heterodimers can be generated (240). Integrins regulate many processes such as cell adhesion, migration, proliferation, neo angiogenesis (241) and have been shown to undergo changes in their expression during the transition to neoplastic phase (242,243). Integrins establish the connection between the cell and the extracellular environment (mostly the extracellular matrix molecules) and the cytoskeleton and transduce signals from the outside and into the cells and vice versa (reviewed in (238,240,244-246)) (**Fig. 11**). In addition, integrins can modulate the signaling cascade of multiple growth factor receptors via RasGTP, such as the epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) receptors, thereby lowering the threshold level in different signaling pathways (247). Moreover, ligation and clustering of integrins can lead to the activation of focal adhesion kinase (FAK) and extracellular signal-related kinase kinase (MEK) (248) which have been implicated in prostate cancer progression and metastasis (249,250). Previous studies have shown that specific integrins (such as αvβ3 (101,102)) correlate with poor survival and are involved in the formation of bone metastasis (251-254). Furthermore, targeting of α_v integrin (knockdown or selective drug targeting) in human prostate cancer cells, abolished the formation of bone metastasis in preclinical mouse model (255,256).

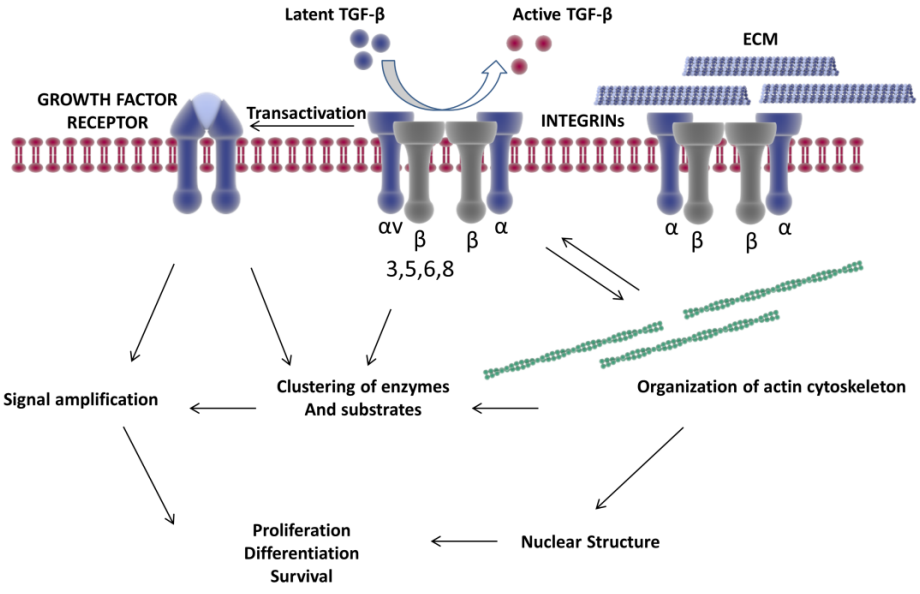

Figure 11. Schematic representation of the multiple roles of integrins.

Additionally, α_v integrins appear to be up-regulated in tumor- and metastasis-initiating prostate cancer cells ($ALDH^{high}$ (39,257)) and these integrins are involved in the activation of latent TGF-β, thereby modulating TGF-β signaling (and *vice versa* in a feedforward loop (244,258-260)) (**Fig. 8**). As previously outlined, high bone turnover provides a significant contribution to the development and the relapse of bone metastasis (261). Interestingly, it has been reporter that increased expression of integrin alpha-v enhanced the TGF-β mediated osteoclastogenesis (262). Therefore it appears that changes in integrin expression play an important role in malignat disease and impact not only on primary tumor growth and invasion but also on the bone microenvironment.

Cripto pathway: Cripto (TDGF1, CRIPTO-1) is a small, GPI-anchored/secreted fetal-oncoprotein that plays important roles in regulating stem cell differentiation, embryogenesis, tissue growth and remodelling (263). An essential mediator for the Cripto signaling is the Glucose-regulated protein 78 (GRP78) (264). As for Wnt and Notch, Cripto represents one of those embryonic signaling pathways that when corrupted can drive tumor initiation and progression. The Cripto pathway modulates the signaling of multiple TGF-β ligand that transduce the signal via Smad2 and 3 such as Nodal, GDF1 and GDF3 (265-267) (**Fig. 12**). Interestingly, Cripto has also been shown to negatively regulate the activation of Smad by Activin-A (268,269), Activin-B (270) and TGFβ-1 (269,271) leading to suppression of the cytostatic effect of these ligands (271,272). These cross-talk with the TGFβ pathway, also crucially involved in prostate cancer bone metastasis, highlight the interest of elucidating the role of Cripto signaling in the contest of bone metastasis. Additionally, even though the soluble vs. secreted effects regulated by Cripto are not yet been entirely elucidated (273), Cripto has also signaling activities that are independent from the TGF-β pathway. Relevant for the purpose of this thesis, soluble Cripto can activate and promote signaling routes of extreme relevance in prostate cancer formation and progression, such as the already discussed PI3K/AKT. In this context, blockade of Cripto binding to cell surface GRP78 inhibits oncogenic Cripto signaling via MAPK/PI3K and Smad2/3 signaling routes (270). Moreover, Cripto is also known to modulate Wnt and Notch signaling pathways (274-277). The interaction between Cripto and PI3K/AKT pathway is mediated via Glypican-1, a GPI-anchored heparan sulphate proteoglycan, that activates a cascade of phosphorylation in which MAP Kinase are involved. This lead to the subsequent activation of PI3K/AKT pathway which promotes proliferation and motility (278). Cripto has also been shown to cross-talk with Wnt signaling; it can bind LRP5/6 facilitating the interaction with Wnt3a, therefore stimulating Wnt pathway through cytoplasmic stabilization of β-catenin (279). Cripto is also involved in the processing of the Notch receptors by enhancing its

cleavage from the plasma membrane, thereby potentiating Notch signaling (277). Finally, Notch signaling can also modulate the expression of Nodal, further complicating the cross-talk between Notch and Cripto /Nodal signaling (280). As we previously mentioned, one of the key processes that characterize the switch from non-invasive to invasive disease in prostate cancer is represented by EMT. Interestingly, Cripto exerts an important role in this process in prostate cancer, where its overexpression produces increase in the mesenchymal marker Vimentine, decrease in the epithelial marker E-Cadherin and augment PI3K/AKT and FGFR1 activity, thus inducing migration (281).

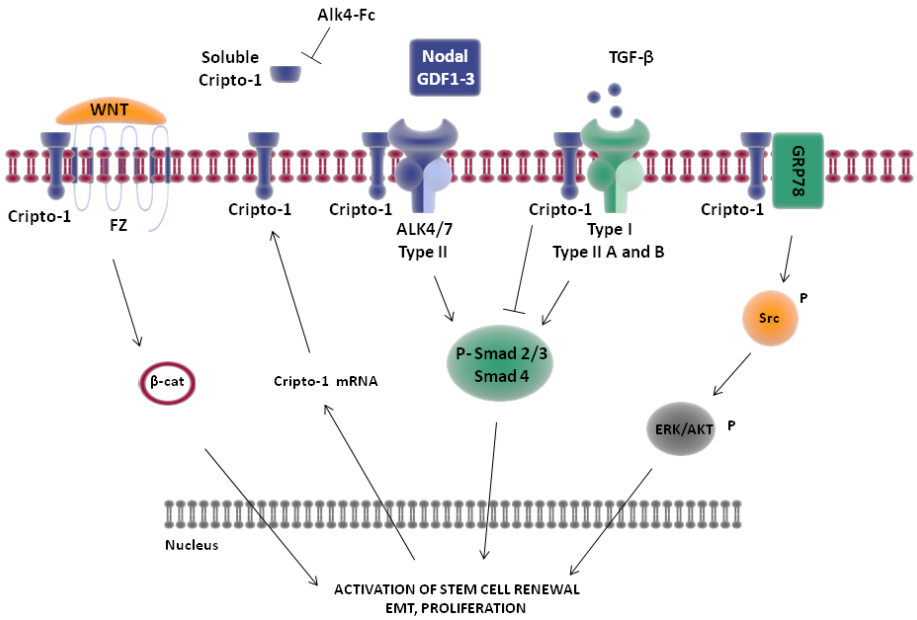

Figure 12. Schematic representation of the multiple interactions of Cripto signaling.

The genetic alterations and the signaling pathways discussed in this paragraph obviously do not cover the entire complexity of aberrant genetic events and abnormalities that characterize multiple pathways and molecules from the onset to the progression of prostate cancer. Among these, we have focused our interest on the alteration and the role of a class of small non coding RNA, namely microRNA, that regulate gene expression. The properties of these molecules, the mechanism of action and their functional relevance in the maintenance of aggressive subpopulation of

cancer cells during prostate cancer progression and metastasis are presented in the next paragraphs.

5.1. The involvement of microRNA in prostate cancer

microRNAs (miRs) are a class of small non-coding RNAs that derive from larger precursor (pri-miRNA) folded into a stem-loop configuration. miRs are transcribed by RNA polymerase II (Pol II) and subsequently processed into the ~70-nucleotide precursors (pre-miR) (282,283). The pre-miR is then cleaved to generate a ~21-25-nucleotide mature miR. miRs localized within Alu-repetitive element, can be transcribed by RNA polymerase III (284). miRs can be positioned at different genomic locations; for example, they can map within introns of both protein-coding or non-coding genes (285). These are transcriptionally regulated through the promoters of these genes (286,287). The transcription of miRs held in the same cluster is regulated by the same promoter and all the miRs from that cluster are transcribed at the same time.

The processing of miR is catalyzed by different multiprotein complexes (**Fig. 13**) (reviewed in (288)). A complex localized in the nucleus and composed by an RNase III enzyme Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha, process the pri-miR (289). This enzymatic reaction produces a 2-nucleotide-long 3' overhangs at the cleavage site. The processing of the pri-miR into ~70-bp pre-miRs by Drosha depends on the terminal loop size and the flanking sequence of the Drosha cleavage site. Shortening of the terminal loop, disruption of complementariety within the sequence, or mutations of flanking sequence at the Drosha cleavage site, can significantly reduce, if not abolish the processing of the pri-miR.

After the pri-miR is cleaved by Drosha, the resuting pre-miR is exported from the nucleus into the cytoplasm by Exportin 5 (Exp5), a nucleo/cytoplasmic cargo transporter Ran-GTP dependent (290-292). In the cytoplasm, another RNase III enzyme (Dicer) cleaves the hairpin into a small imperfect dsRNA duplex that contains both the mature miR strand and its complementary strand (293-295). The ability of Dicer to recognize the pre-miR molecules is due to the presence of a PAZ (Piwi-Argonaute-Zwille) domain that allows a low-affinity interaction with the 3' end of ssRNAs (296-298). For this reason, the pre-miR that presents 2-nucleotide 3' overhangs resulting from Drosha cleavage, can be easily recognized and processed by Dicer.

Dicer cleavage generates mature miRs ~21-25-nucleotide long. After the dsRNA duplex is formed, the target specificity and the functional efficiency of a miR, requires that the mature miR strand is incorporated into the RNA-induced silencing complex (RISC) (reviewed in (288)). In human cells, transactivating response (TAR) RNA-binding protein (TRBP), recruits the Argonaute protein Ago2 and together with Dicer they form a trimeric complex that initiates the assembly of the RISC complex (reviewed in (288)).

The mechanism by which the RISC complex incorporates the mature miR strand of the dsRNA duplex is driven by the different stability of the miR duplex. Potentially the

mature miR strand can reside on either strand of the hairpin, but, because of thermodynamic reasons, it mostly derives from the strand with the less stable 5'.

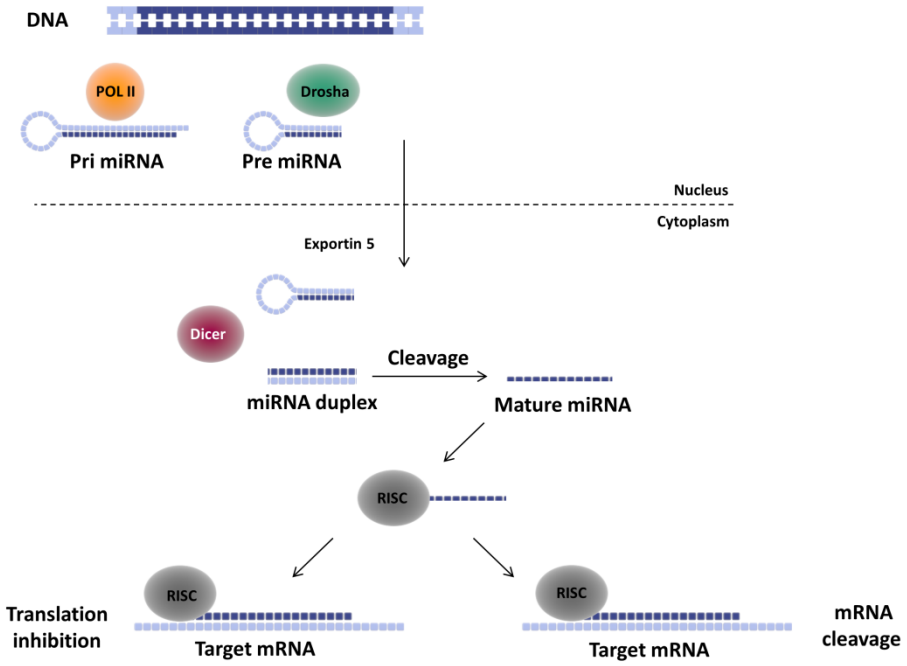

Figure 13. Schematic representation of the microRNA processing pathway.

The detection of microRNA in blood and urines represent an interesting and non-invasive approach to diagnose prostate cancer (299). Independent studies have shown that miR-141 and miR-375 are significantly elevated in the blood of prostate cancer patients with bone metastasis and in the respective exosomes (300,301). Interestingly elevated levels of the two microRNAs are also associated with higher Gleason score, positive lymph nodes and were also detected in the urine of prostate cancer patients (302,303). miR-375 has also been identified as prognostic marker in castration-resistance prostate cancer together with miR-1290 in exosomes (304). Additional microRNA that have been measured in the urine and associated with prostate cancer are miR-107, miR-574-3p and miR-200b (303). Interestingly, the last has also been associated by an independent study with docetaxel resistance (305). In the same study, miR-429, miR-200a, miR-21, miR-200c, miR-375, miR-132 and miR-20 have been associated with lower survival (305). A recent report investigated the expression of microRNAs in patient-derived stem like cells ($CD133^{+}$, $\alpha 2\beta 1$ $integrin^{high}$) enriched from

benign prostatic hyperplasia, Gleason 7 treatment-naive prostate cancer, and CRPC and identified miR-548c-3p as functional biomarker involved in prostate cancer progression (306).

5.2. Mechanisms of microRNA Post-Transcriptional Repression

After incorporation into the RISC complex, the miR interact with its target mRNA by base-pairing interactions. If mRNA/miR complementarity is perfect or near-perfect, the target mRNA can be cleaved and degraded; otherwise the translation is repressed (294).

The target complementarity is determined by base-pairing of nucleotides in the so called “seed sequence” of the miR (307). This sequence is essential for the binding of the miR to the mRNA. The seed sequence is an heptametrical sequence located at positions 2-7 from the miR 5´-end and has to be perfectly complementary to the target mRNA complementary sequence. The miR seed sequence is exploited to develop computational approaches for target prediction.

The microRNA target site is positioned at the 3’UTR region, probably because the movement of ribosomes that occur during translation will contrast RISC binding and interaction (308). Different and “non-canonical” miR-mediated mechanisms of mRNA expression modulation are also emerging. In fact some miRs can bind to the open reading frame (ORF) sequences or to the 5’UTR region of the target genes, determining gene activation rather than repression (309). The RISC action on target mRNA is modulated by the Ago protein that is incorporated in the complex and by the grade of complementarity between the miR strand and its mRNA target. Ago2, for example, is able to cleave RNA, but this event requires extensive base pairing between the miR strand and the mRNA target (310,311).

To date, six models have been proposed for the miR translational repression:

1) the RISC complex induces de-adenylation determining a decrease of translational efficiency by blocking target mRNA circularization (312);
2) RISC complex blocks cap function by interacting with both the cap or eIF4E (313);
3) Argonaute proteins recruit eIF6, which blocks the recruitment of 60s ribosomal subunit (314);
4) RISC complex blocks the translation elongation or promotes premature dissociation of ribosomes (ribosome drop-off) (315);
5) RISC complex induces the proteolysis of nascent peptides during translation (in this model the translation is not inhibited) (316);

6) RISC complex recruits target mRNAs to processing bodies, where the mRNA is degraded or stored in an inactive state for translation (317,318).

5.3. microRNA and Cancer Stem Cells

microRNAs regulate multiple biological process, such development and cell growth and have been proposed as one of the important players during pathogenesis and cancer. In human prostate cancer, several studies in patient samples and xenografts have revealed their characteristic pattern in benign vs. aggressive disease and highlighted their role in castration-resistant prostate cancer and their implication in bone metastasis formation (see microRNAs described in paragraph 3 and reviewed in (299,300,302)).

However, the number of studies addressing the role of specific miRs in the regulation of stem-like properties in bulk prostate cancer cells lines is limited. Remarkably, there is an even lower number of studies, that investigated the expression of miRs directly in selected subpopulation of cells, characterized by stem-like properties and capable of maintaining the tumor and producing metastasis. In these studies new miRs have been identified using expression profiling of subpopulation of cells enriched for cancer stem cells isolated from bulk prostate cancer cell lines. For example, prostaspheres from PC3 cells have been compared to adherent PC3 cells and miR-143 has been identified as promoter of prostate cancer metastasis (319). In other approaches, a fraction of prostate cancer stem cells (PCSCs, described as $CD44^+/CD133^+$) has been isolated by viable cell sorting from culturedLNCaP cells and miR-101 has been found to inhibit cell growth and promote apoptosis in PCSCs (320). With a similar approach, miR-409-3p/5p has been identified in embryonic stem cells and then studied in prostate cancer, where it was found to promote bone metastasis (321). Other studies have also employed cell sorting of various stem/progenitor cell population, including $CD44^+$, $CD133^+$, integrin $\alpha2\beta1^+$ and side population of cells isolate from bulk cell lines and found multiple tumor-suppressive microRNA down-regulated, including miR34a, let-7, miR-106a and miR141 (322). Interestingly, a 'near-patient' approach highlights that a population of $CD44^+$ cells isolated from xenografts led to the identification of miR-34a as master regulator of metastasis (323) and, as a consequence, this was subsequently validated in $CD44^+$ cells isolated from primary prostate tumors. Moreover, in patient-derived stem like cells ($CD133^+$, $\alpha2\beta1^{high}$), it was recently found that miR-548c-3p can be considered as functional biomarker involved in prostate cancer progression (306) as enforced overexpression of this miR in differentiated cells induced stem-like properties and radioresistance. Finally other studies have used Hoechst 33342-based flow cytometry to isolate a CSC-like side population and confirmed the tumor suppressive role of miR-34a and additionally identified miR-200c as mediator of chemoresistance.

Strikingly, there is lack of microRNA expression profiles of cancer-stem like/progenitor cells obtained from clinical prostate cancer specimens. The molecular characterization of this subpopulation of highly tumorigenic cells, could indeed provide novel insights in tumor progression and facilitate the identification of new therapeutic targets and strategies.

6. Outline of the thesis

Cancer is a heterogeneous disease and the presence of multiple genetically distinct foci in the primary prostate cancer supports this notion. The identification of the molecular properties of highly aggressive and metastatic subclones might facilitate the identification of new targets for therapy and putative markers for monitoring the progression of the disease.

In **Chapter 2** of this thesis, we established a microRNA signature common to three key signaling pathways in prostate cancer progression and bone metastasis formation (i.e. TGF-β, Wnt and Notch). With this approach we identified a signature of validated microRNA targeting the process of epithelial-to-mesenchymal transition (EMT) that may be critically involved in the spreading of many aggressive cells from the primary tumor and the formation of distant metastases.

Chapter 3 is focused on a candidate tumor suppressor microRNA that is downregulated in highly metastatic, stem-like $ALDH^{high}$ cells vs. non-metastatic, more differentiated $ALDH^{low}$ prostate cancer cells. We studied the functional role of miR-25 in the maintenance of aggressive behaviour of $ALDH^{high}$ compared to $ALDH^{low}$ subpopulation of cells *in vitro* and *in vivo*. Our analysis revealed that miR-25 represents an important player in the regulation of invasiveness in human prostate cancer through the interaction with at least three signaling pathways.

Chapter 4 describes a follow-up study for the regulatory role of miR-25 in human prostate cancer biology, in particular its role in the cross-talk between the TGF-β and Wnt signalling in prostate carcinogenesis and progression.

In **Chapter 5** we show that the soluble chimeric protein ALK1Fc reduces BMP-9 induced activation of Notch signaling and proliferation in human prostate cancer cells. Alk1Fc is capable of reducing tumor growth in an orthotopic model of human prostate cancer *in vivo*.

Chapter 6 contains a study about the role of Cripto and GRP78 in the maintenance of an aggressive behaviour in human prostate cancer cells *in vitro* and their role in metastatic dissemination in two different preclinical models of prostate cancer invasion and metastasis *in vivo*. The general conclusions are included in **Chapter 7**, the general discussion of this thesis.

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