Histol Histopathol (2014) 29: 1263-1280
DOI: 10.14670/HH-29.1263

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Review

Insight into the heterogeneity of prostate cancer through PSA-PSMA prostate clones: mechanisms and consequences

Awatef Ben Jemaa, Yosra Bouraoui and Ridha Oueslati

Unit of Immunology and Microbiology Environmental and Carcinogenesis (IMEC), Faculty of Sciences of Bizerte, Zarzouna, University of Carthage, Tunisia.

Summary. A major clinical challenge is posed by the current inability to readily distinguish indolent from aggressive tumors in prostate cancer patients. Research efforts are dedicated to overcome this problem by understanding the molecular basis of the transition from normal, benign cells to prostatic intraepithelial neoplasia (PIN), localized carcinoma, and metastatic cancer. Combined with the evidence of the phenotypic heterogeneity of benign prostate hyperplasia, primary tumors and metastases, it is conceivable that several prostate clones emerge progressively during tumor progression. We have identified several PSA-PSMA prostate clones during prostate cancer progression. In this paper we focus on the susceptibilities of these PSA-PSMA prostate clones to factors that promote prostate hyperplastic, neoplastic and metastatic development and their consequences in disease outcome.

Key words: PSA-PSMA, Clones, Heterogeneity, Prostate cancer, Metastasis

Introduction

The prostate gland is composed mainly of stromal, epithelial, and neuroendocrine cells. In the epithelium of secretory acini of the prostate two different cell types can be discriminated on the basis of localization, morphology, and degree of differentiation, the luminal and basal cells. The dynamic balance between cell apoptosis, proliferation and differentiation in general maintains the cellular and tissue homeostasis. This balance is generated by secretion of various types of growth factors, cytokines and chemokines by epithelial and stromal cells (Wong and Wang, 2000; Sung and Chung, 2002). The mitogenic and proliferative effect of growth factors, cytokines and chemokines are mediated in a paracrine manner by activation of several intracellular cascades (Mimeault and Batra, 2006; Sung et al., 2007; Inoue and Ogawa, 2011). Dynamic regulation of these pathways is coordinated in particular by Raf kinase inhibitor protein (RKIP) (Yeung et al., 1999, 2000; Zeng et al., 2008; Keller, 2011). Deregulation in this paracrine communication can result in derangement of the prostate gland, such as benign prostate hyperplasia (BPH) and prostate carcinoma (PC) (Dawson et al., 2004). Despite the difference, androgens have the obligatory role in the etiology of BPH and PC diseases (Chatterjee, 2003; Unni et al., 2004). While not regulating the proliferation/survival of prostate epithelial cell directly, androgen occupation of the androgen receptor (AR) within the nuclei of these cells does regulate expression of prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) (Riegman et al., 1991; Watt et al., 2001). PSA is a 33 kDa glycoprotein produced in the epithelium of the human prostate and has become established as a useful tumor marker to screen for prostate cancer, to detect recurrence following local therapies, and to follow response to systemic therapies for metastatic disease. Nevertheless, as a functional product of normal prostate epithelial tissue, serum PSA levels will reflect changes due to inflammation, trauma or benign proliferation (Roscigno et al., 2004; Williams et al., 2007). The

Offprint requests to: Dr. Oueslati Ridha, Unit Immuno-Microbio Environmental and Carcinogenesis (IMEC), Faculty of Sciences Bizerte, 7021 Zarzouna, Tunisia. e-mail: oueslatiridha12@hotmail.fr

PSMA is a type II transmembrane protein endowed with enzymatic activities and rapid internalization (Carter et al., 1996 ; Veronica et al., 2010). PSMA causes prostate cancer cells to behave more aggressively, making them more likely to move and invade healthy tissue surrounding the tumor (Chang, 2004; Rajasekaran et al., 2005).

Evidence proposed the existence of multiple neoplastic transformation events, many of which may give rise to latent prostate cancer that does not progress to clinically detectable disease. Alternatively, a proportion will give rise to aggressive potential metastatic forms of the disease (Voelkel-Johnson et al., 2000; Sartor et al., 2008; Goldstein et al., 2010). A major clinical challenge is posed by the current inability to readily distinguish indolent from aggressive tumors in prostate cancer patients (True et al., 2006; Sartor et al. 2008; Goldstein et al., 2010). Research efforts are dedicated to overcome this problem by understanding the molecular basis of the transition from normal, benign cells to prostatic intraepithelial neoplasia (PIN), localized carcinoma, and metastatic cancer. Giving the heterogeneity of BPH, primary and metastatic prostate cancer (Kopp et al., 2011; Rodríguez-Berriguete et al., 2012), we have demonstrated the existence of several PSA-PSMA prostate clones during prostate cancer progression (Ben Jemaa et al., 2010). However, susceptibilities of these PSA-PSMA prostate clones to factors that promote prostate hyperplastic, neoplastic and metastatic development and their consequences in disease outcome are largely unknown.

The aim of this paper was to focus on the involvement of PSA-PSMA prostate clones related with several transduction pathways in the transition from normal, benign cells to prostatic intraepithelial neoplasia (PIN), localized carcinoma and metastatic cancer. We also discuss the possible cell-origin of PSA-PSMA prostate clones and their value in pinpointing high- from low-risk patients to develop aggressive PC phenotype.

Heterogeneity of PSA vs. PSMA expression in prostatic hyperplasia

Benign prostatic hyperplasia (BPH) affects aging men and represents the most common urologic disease among elderly men (McVary, 2006). BPH is a growth of both epithelial and stromal cells from both the transition zone and periurethral areas (Roehrborn, 2008). To date, there are multiple theories on the cellular and molecular processes underlying the pathogenesis of BPH. These include embryonic reawakening, aging, estrogens, oxidoreductase, inflammation theories and androgens (Bostwick, 2005; Roehrborn, 2008). It is well known that androgens play a key role in this underlying pathology which indicates that the androgen receptor (AR)/androgen signaling is an early event in BPH disease (Bauman et al., 2006; Ho and Habib, 2011). While not regulating the proliferation/survival of benign prostate epithelial cell directly, androgen occupation of the AR within the nuclei of these cells does regulate expression of prostate-specific marker proteins, such as Prostate-Specific Membrane Antigen (PSMA) and Prostate-Specific Antigen (PSA) (Riegman et al., 1991; Watt et al., 2001). In our previous study, PSMA was shown to be weakly expressed in both normal prostate and BPH, whereas PSA is highly expressed in hyperplastic compared to normal prostate tissues (Ben Jemaa et al., 2010) (Fig. 1). This is in part thought to be due to the biological features of the zonal origin of BPH disease. According to the zonal origin, the transition zone is the exclusive location for the origin of BPH which was found to produce a higher amount of tissue PSA than the other prostatic zones (Balk et al., 2003; Van der Heul-Nieuwenhuijsen et al., 2006, Williams et al., 2007). On the other hand, up-regulation of tissue PSA in BPH is thought to be a consequence of an inflammation area in the benign prostate gland (Bouraoui et al., 2008; Mechergui et al., 2009). Inflammation of the prostate may represent a mechanism for hyperplastic changes to occur in the prostate. There are a variety of growth factors and cytokines that may lead to a proinflammatory process within the BPH. Proinflammatory cytokines contribute in a paracrine and autocrine fashion to hyperplastic cell proliferation and increase survival of such epithelial cells (Nickel, 2008; Robert et al., 2009; Gandaglia et al., 2013). Epithelial cells have the capacity to secrete a wide range of proinflammatory cytokines, such as IL-1, IL-6 and TNFα which can regulate cell growth and PSA production. The capacity of prostate epithelial cells to produce proinflammatory cytokines makes these cells an important component of the immune and inflammatory responses (Gastro et al., 2003; Bouraoui et al., 2008; Nickel, 2008; Mechergui et al., 2009). Penna and associates showed that inflammatory cells can be attracted to the prostate tissue microenvironment and can selectively promote the proliferation of prostate epithelial cells. In *in vitro* assays, they demonstrate that CD4+ T cells activated by BPH stromal cells markedly upregulate secretion of IL-12 and IL-23 (Penna et al., 2009). Further studies demonstrated that healthy prostates do not express IL-17, whereas prostates with inflammation and BPH do express this interleukin. In this latter condition, IL-17 leads to more proinflammatory cytokines, such as IL-6 and IL-8 (Steiner et al., 2003). NO and COX activity may also play an important role in determining the association between inflammation and benign prostate growth (Wang et al., 2004). These cytokines and chemokines may act as ERK, AKT and NF-κB (p50/p65) activators related to inflammation and prostate cell proliferation (McCubrey et al., 2007; Rodríguez-Berriguete et al., 2010). Among signal pathways leading to inflammation and cytokine production, the nuclear factor-κB (NF-κB) family proteins are essential for inflammation, immunity, cell proliferation and apoptosis (Suh and Rabson, 2004; Karin and Greten, 2005; Bouraoui et al., 2012a). In most cell types, NF-κB activity is mediated by the complex

p50/p65 (Rel A), which acts as a transcriptional activator (Hayden and Ghosh, 2004). The sensibility of both NF-κB and PSA to inflammation allowed us to confirm the relationship between these two molecules and its involvement in hyperplastic prostatic disease progression (Bouraoui et al., 2012a).

Regarding tissue PSMA, the significance of its enzymatic activities in the context of benign prostatic cells is not yet identified. Low PSMA expression in BPH tissue may indicate a limited role of this prostate-associated antigen in the context of prostate hyperplastic disease (Ben Jemaa et al., 2010). As tissue PSA, cellular distribution of PSMA was mostly found in the cytoplasm of luminal benign prostate cells (Fig. 1). In our previous study, the primary antibody used against PSMA (3E6) was able to detect both the transmembranous and cytoplamsic variants of PSMA (Ben Jemaa et al., 2010). PSMA is alternatively spliced to produce at least four variants. The most important of which is PSMA', the cDNA of which is identical to PSMA except for a 266-nucleotide region near the 5' end of PSMA cDNA which codes for the transmembrane region of protein. The other three variants of PSMA are PSMA-C, PSMA-D and PSMA-E, which are also thought to exist within the cytosol (Schmittgen et al., 2003; Cao et al., 2007). The implication of such alternative splice variants in prostate cells is not known at present. PSMA has a unique phenomenon, the switch from appearance of soluble and insoluble form of PSMA between benign and prostate cancer disease (Lapidus et al., 2000; Rajasekaran et al., 2005). Therefore, the PSMA detected in luminal hyperplastic prostate cells is more likely to represent the PSMA variants than the form of this protein that appears in plasma membranes, PSMA.

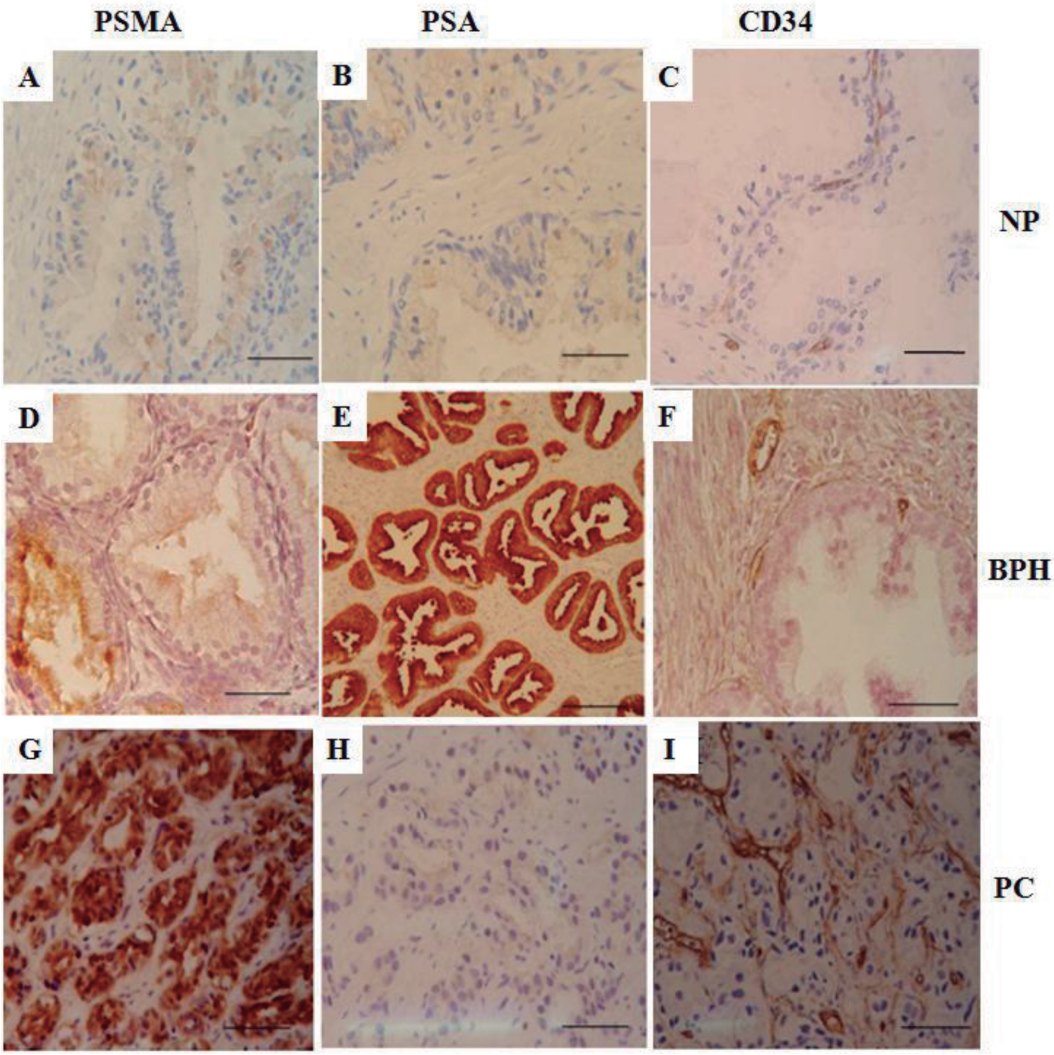

Fig. 1. Immunohistochemical localization of PSMA, PSA and CD34 in normal prostate (NP), benign prostate hyperplasia (BPH) and prostate cancer (PC). NP showing weak cytoplasmic staining for PSMA **(A)** and PSA **(B)** in epithelial cells. CD34 was found at low level in membranous and cytoplasmic endothelial cells in NP **(C)**. BPH showing weak membranous staining for PSMA **(D)** and strong membranous and cytoplasmic staining for PSA **(E)** in prostatic epithelial cells. Capillary vessel network observed, especially near the basal membranes of acinar structures in BPH **(F).** PSMA **(G)** and CD34 **(I)** showed strong immunoreactions in infiltrating prostatic carcinoma. PSA **(H)** showed weak cytoplasmic immunoreactions of epithelial cells in PC. Scale bars: A-D, F-I, 20 µm; E, 30 µm.

Heterogeneity of PSA vs. PSMA expression in prostatic neoplasia

BPH, which reflects non-malignant proliferation of the cells in the transition zone of the human prostate, is histologically distinguishable from the prostate adenocarcinomas (De Nunzio et al., 2011). Prostate cancer occurs when the rate of cell division surpasses cell death, leading to uncontrolled tumor growth (Kyprianou et al., 2000; Dawson et al., 2004). Despite the difference, androgens are involved in the etiology of BPH and prostate cancer diseases (Chatterjee, 2003; Tennakoon et al., 2014). Although serum androgens alone may not promote prostate carcinogenesis, androgen action and the functional status of AR are important mediators of prostate cancer progression (Heinlein and Chang, 2004; Barfeld et al., 2014). In prostate cancer, AR suppresses proliferation of basal cells, supports survival of luminal cells, and promotes metastasis, as shown by analyses of AR conditional deletion in the context of the TRAMP model (Niu et al., 2008). Immunohistochemical studies have shown that AR expression is heterogeneous in prostate cancer (Li et al., 2002; Suzuki et al., 2003). As established as an AR target gene, we have previously shown that tissue PSA evaluated by means of immunohistochemistry presented with high heterogeneity within prostate tumor (Denmeade et al., 2003; Ben Jemaa et al., 2010). Although our prostate cancer cases are mostly poorly differentiated adenocarcinoma, we demonstrated a bi-modal distribution of tissue PSA in carcinomatous prostate samples. In fact, 57% of prostate cancer positive for PSA have a similar PSA expression level distribution to normal prostate, whereas 43% have a similar PSA expression level distribution to benign prostate hyperplasia (Ben Jemaa et al., 2010). These findings may be directly related to the heterogeneous expression of AR within prostate tumour, particularly in poorly differentiated adenocarcinoma (Li et al., 2002; Suzuki et al., 2003). In support of this, Magi-Galluzzi et al. (1997) have reported that the heterogeneity in the expression of the AR increases with increasing Gleason score. Since the AR is responsible for the transcriptional transactivation of the PSA gene (Denmeade et al., 2003), any change in its status is likely to affect PSA expression in some way. A decrease of expression of tissue PSA has been associated with prostate neoplastic compared to hyperplastic transformation (Ben Jemaa et al., 2010). This is in part thought to be due to the differences observed in several biological features between peripheral and transition zone of the prostate gland (Van der Heul-Nieuwenhuijsen et al., 2006). Although the majority of the glandular tissue in prostate is located in the peripheral zone, the PSA tissue is secreted at higher levels by benign prostate epithelium arising exclusively in the transition zone compared to prostate cancer developing mainly in the peripheral zone (Balk et al., 2003; Williams et al., 2007). The normal prostate appears to have a remarkable ability to prevent leakage of seminal fluid components such as active PSA into the blood stream. In the presence of underlying pathology such as BPH the amount of PSA leaking into the serum remains less than 10 ng/mL. In contrast, in conditions of inflammation and malignancy, levels of PSA leaking into the circulation increase significantly, thus forming the basis for the use of serum PSA as a biomarker for early stage prostate cancer (Balk et al., 2003; Williams et al., 2007). Serum PSA testing was initially used for monitoring prostate cancer patients (Catalona et al., 1991). Later, the availability of a highly accessible blood test for PSA has revolutionized the diagnosis of prostate cancer over the past three decades (Hernandez et al., 2004; Roscigno et al., 2004). However, the value of PSA is limited in particular by its poor specificity. The main disadvantage of the test is that PSA can be increased in conditions such as BPH, and conversely can be low in the presence of prostate cancer, thus there is no absolute value at which sensitivity and specificity are maximized (Stamey, 2001; Kehinde et al., 2003). Based on binding kinetics, several serum tests using modifications of PSA have been evaluated to address the weaknesses of serum PSA test and improve specificity in particular (De Angelis et al., 2007). PSA is known to circulate both freely and in complexes with plasma proteins, most commonly α1-antichymotrypsin (Zhu et al., 2013). Our laboratory and multiple other studies have shown that the proportion of PSA that is unbound (ratio of free-total PSA) is lower in men with prostate cancer than in those with benign prostatic hyperplasia and may be particularly helpful in the diagnosis of prostate cancer (Bouraoui et al., 2006; Zhu et al., 2013; Romero Otero et al., 2014). Despite all the modifications and variations of PSA that have been investigated there is still no consensus on what is the best use of PSA to obtain optimum diagnostic and prognostic information (Stangelberger et al., 2008; Agnihotri et al., 2014). Despite the potentially indolent course of prostate cancer, significant numbers of potentially aggressive prostate cancers may go undetected in men with PSA values less than 4.0 ng/mL (Stamey, 2001; Thompson et al., 2005).

Due to increased leakage, serum PSA levels increase in the aging male even in the absence of underlying pathology as a consequence of chronic inflammation (De Marzo et al., 2007; McDonald et al., 2014). Chronic inflammation appears to play a role in the development of many types of cancer, including prostate cancer (De Marzo et al., 2007; Nakai and Nonomura, 2013). Inflammation is thought to incite prostate carcinogenesis by causing damage to the cell genome and by creating a tissue environment rich in cytokines that promote cell proliferation and angiogenesis (Palapattu et al., 2005; Sung et al., 2007). The prostate epithelial cells have also been shown to produce proinflammatory cytokine in androgen-dependant and androgen- independent prostate cells and influence the growth and differentiation of prostate cancer cells (Hobisch et al., 2000; Ricote et al., 2004; Bouraoui et al., 2008; Mechergui et al., 2009).

However, the involvement of proinflammatory cytokine in PSA expression within prostate cancer cells is controversial. We have previously shown that NF-κB phosphorylation was notably increased by IL-1β leading to down-regulation of PSA in a dose-dependent manner in LNCaP cells (Bouraoui et al., 2012b). Culig et al. (Culig et al., 1998) have also found that the IL-1 receptor type I was expressed by LNCaP, leading to activation of the IL-1 β signal transduction and inhibition of cell growth, as well as PSA and AR expression in prostate cancer cells. In the same way, Wang et al. (2009) showed that NF-κB activation was increased by TNF-α leading to repression of AR activity and PSA secretion in prostate cancer cell lines. Also, IL-6 might inhibit PSA gene expression through the STAT3 pathway without MAPK involvement by blocking the association between the co-activator p160 and p300 on the PSA gene enhancer and promoter (Jia et al., 2004). Conversely, other authors reported that IL-6 can activate the PSA promoter/enhancer in the presence and absence of androgens (Lin et al., 2001; Lee et al., 2003a). In this context, IL-6 is a multifunctional cytokine that plays an important role in the immune response and inflammation, but also regulates the growth prostate carcinoma and activates the AR dependant gene in prostate cancer cells in the absence of androgen (Lin et al., 2001; Lee et al., 2003a). Signaling pathways activated by cytokines such IL-6 and IL-4 have been shown to activate the AR and stimulate the PSA expression in prostate cancer cell lines (Lee et al., 2003a,b). Clinically, elevated serum IL-6 levels have been also associated with prostate cancer and sera PSA levels (Nakashima et al., 2000; Shariat et al., 2001). These previous reports are consistent with our finding that overexpression of IL-6 in prostate cancer patients was associated with high PSA levels > 20 ng/mL (Bouraoui et al., 2008; Mechergui et al., 2009). Moreover, the cytokine's profiles showed that in (IL-6+, IL-1α+) profile was the most expressed in prostate cancer epithelial cells within the group of PSA levels > 20 ng/mL (Mechergui et al., 2009). In PC proinflammatory cytokines could be implicated indirectly to leakage of PSA in the circulation through their involving in an increased loss of epithelial cell polarity and basal cell number. Therefore, our findings implied that proinflammatory cytokines (IL-6 and IL-1α) play an important role in chronic inflammation of prostate cancer progression and present several profiles of expression among sera PSA levels, suggesting a different profile of PSA produced by prostate malignant cells.

IL-6, which enhances cell proliferation and acts as a survival molecule for many prostate tumor cell lines such as PC3, DU145 and LNCaP (Azevedo et al., 2011), could in turn be regulated by PSMA in androgen-dependent LNCaP cells (Colombatti et al., 2009). Unlike what was previously done by others (Dassie et al., 2009; Zhang et al., 2013), Colombatti et al. decided to stimulate rather than to inhibit or silence PSMA at the surface of LNCaP cells and to overcome the lack of defined PSMA ligand(s) by cross-linking the extracellular domain of the molecule with specific antibodies (Colombatti et al., 2009). By using the androgen-dependent LNCaP cell line, they showed for the first time that the tumor biomarker PSMA, a multifunctional cell surface ectopeptidase endowed with an efficient signaling activity regulated IL-6 production in prostate cancer cells (Colombatti et al., 2009). IL-6 gene induction occurs due to an activation cascade involving RAS, RAC1, p38 and ERK1/2 MAPKs, leading to the phosphorylation of the p65 subunit of NF-κB transcription factor. In addition, they demonstrated that both IL-6 and CCL5 promote the proliferation of LNCaP tumour cells reaching their maximal activity synergistically (Colombatti et al., 2009). In the same way, our previous work suggested that PSMA is also able to induce IL-6 upregulation through bFGF signaling (p38 and ERK1/2 MAPKs) in two different metastatic prostate cancer models, LNCaP and PC3-PSMA cells (Ben Jemaa et al., 2013a). To overcome the problem of the loss of PSMA expression upon prostate cancer progression from an androgen-dependent to an androgen-independent stage like in case of metastatic cell line PC3, PC3 cells were stably transfected with PSMA gene (Ben Jemaa et al., 2013a). IL-6 and bFGF play a fundamental role in the regulation of proliferation, apoptosis, development, progression, and angiogenesis in prostate carcinomas (Lin et al., 2001; Kwabi-Addo et al., 2004; Wu et al., 2013; Hetzl et al, 2013). In an *in vivo* study, we demonstrated the evidence of expression of IL-6 and its receptors in human prostate carcinoma (Bouraoui et al., 2008; Mechergui et al., 2009). Therefore, we consider that this increase in IL-6 expression in prostate tumor tissues could be associated to the paracrine expression of bFGF and its effect on cancer cell proliferation, motility, and angiogenesis. Cytokines, growth factor receptors, adhesion molecules and many other membrane-generated signals all share the ability to efficiently promote IL-6 or CCL5 gene expression and consequently also their downstream effects (Colombatti et al., 2009). In addition, under long-term treatment conditions, IL-6 can activate its own gene expression and, in prostate cancer, autocrine and paracrine loops involving IL-6 and one of its multiple activators, the TGF-β, has been implicated in the regulation of cell proliferation, survival, and neuroendocrine differentiation (Culig et al., 2005). In this context, it could be hypothesized that following stimulation *via* bFGF the prostate tumor cells augment PSMA production, which in turn enhances IL-6 expression. Subsequently, IL-6 will be used as growth factors in both autocrine and paracrine manner, triggering a cell proliferation/survival loop conferring resistance to apoptosis and an overall definite advantage to tumor cell populations. Within this context, the ability of PSMA to activate RAC1 may be highly relevant, inasmuch as RAC1 activation decreases the expression of E-cadherins, thereby loosening intercellular adhesions

and facilitating the cytoskeletal rearrangements required for mitosis (Woods et al., 2007). If so, activated RAC1 could therefore favor the response of LNCaP cells to the mitogenic activity of IL-6 and CCL5, meanwhile participating in the induction of their expression.

The correlation between IL-6 and PSMA production in prostate cancer models is confirmed in *in vivo* studies. A number of studies have demonstrated that a prominent role in prostate tumor survival and progression can be attributed to IL-6 present in the tumor micro-environment. IL-6 has a fundamental role in the regulation of development and progression of prostate tumor. In fact, the expression of IL-6 and its receptor is consistently demonstrated in freshly isolated cells from human prostate carcinoma and benign prostate hyperplasia (Hobisch et al., 2000; Bouraoui et al., 2008; Mechergui et al., 2009). Further, elevated levels of IL-6 were found in the serum of patients with prostate cancer metastatic disease, which was associated with poor disease prognosis (Nakashima et al., 2000; Shariat et al., 2001). Regarding PSMA, in an *in vivo* study, we have previously shown that its low expression in normal prostate epithelial cells increases several fold in benign prostate hyperplasia and prostate carcinoma cells. In addition, the highest tissue PSMA levels were found in human prostate carcinoma (Ben Jemaa et al., 2010). Given that its expression increases several fold in high-grade prostate cancers, in metastatic and in androgen-insensitive prostate carcinoma (Silver et al., 1997), PSMA have been proposed as a useful indicator of the severity of the disease in prostate cancer (Chang, 2004; Rajasekaran et al., 2005). Noticeably, we found PSMA expression in a different compartment of luminal prostate epithelial cells. Cell surface positivity for PSMA was observed in the relevant part of the evaluated prostate cancer samples, whereas cytoplasmic staining without evident membrane positivity was observed in a scanty part of the evaluated tumor samples. Nevertheless, both normal and benign prostate epithelial cells showed cytoplasmic localization of PSMA in most prostate tissues (Ben Jemaa et al., 2013b) (Fig. 1). Our data were concordant with the results of Mhawech-Fauceglia et al. (2007). According to their study, the strongest staining pattern of PSMA was apical or with membranous accentuation in luminal cells of the vast majority of prostate cancer (Mhawech-Fauceglia et al., 2007). When we looked to tissue PSA, we found that it is preferentially localized in the cytoplasm of luminal normal, hyperplasia and neoplastic epithelial cells (Ben Jemaa et al., 2013b). Inversely to PSMA, the strongest tissue PSA expression was found in benign prostatic hyperplasia compared to normal and prostate cancer (Ben Jemaa et al., 2010) (Fig. 1). These findings suggested that in addition to their organ specificity, the characteristic stain pattern of PSA and PSMA as revealed by immunohistochemistry in individual hyperplasia and prostate carcinomas can provide valuable information regarding the detection of prostate cancer disease. In fact, high PSA expression is likely reflective of benign prostate hyperplasia, whereas strong PSMA expression with apical pattern is likely indicative of prostate cancer disease. Therefore, simultaneous stains with PSA and PSMA in individual prostate tissue may greatly improve the detection rate and identify a high risk prostate cancer. Consistent with the correlation between PSMA expression and tumor stage, we have demonstrated that increased levels of PSMA are associated with a high Gleason score (8-10), indicating relatively less differentiated late stage prostate carcinomas (Ben Jemaa et al., 2013b). In line with our study, Perner et al. (Perner et al., 2007) found a higher expression of PSMA in high-grade versus low-grade prostate cancers. However, based on our results, tissue PSA does not correlate with Gleason grade in primary prostate cancer (Ben Jemaa et al., 2013b). Unlike expression of PSA, which is down-regulated after androgen ablation, PSMA expression is significantly increased in hormone-naïve metastases as compared with localized prostate cancer cases (Chang, 2004; Rajasekaran et al., 2005). These data implied that with its abundant and restricted expression in prostate tumors, particularly in advanced and metastatic prostate cancer, its membrane location, and rapid internalization, PSMA (Frigerio et al., 2013; Baiz et al., 2013) rather than PSA represents an attractive target for prostate-selective cancer imaging and therapy. Beyond primary prostate cancer, Mannweiler et al. (Mannweiler et al., 2009) showed heterogeneity of PSMA expression in prostate carcinoma with distant metastasis. Differences in the intracellular localization of PSMA immunostaining have been demonstrated in prostate tumors which seem to be related to the tumor differentiation pattern. Moreover, a significant number of the primary tumors (7/51) and metastases (6/51) presented with highly heterogeneous PSMA expression and in a further 2 primary, and 8 metastatic tumors the staining was in the negative range (<10% positive tumor cells) (Mannweiler et al., 2009). As targets for *in vivo* prognostic, imaging and therapeutic approaches (Rajasekaran et al., 2005; Baiz et al., 2013; Frigerio et al., 2013) , we suggest that heterogeneous distribution and stain pattern of PSMA in primary prostate tumors and distant metastasis are of significant interest. The efficacy of these approaches highly depends on a homogenous and tumor cell-selective membrane expression of this molecule. Any variation (negativity, heterogeneity of expression, lack of membrane localization) may significantly limit the access of the therapeutic agent to the target cells, resulting in therapy failure. Previous studies described cytoplasmic PSMA as a splice variant (PSMA') which lost its ability to be integrated in the lipid bilayer as a transmembrane protein. The biological relevance of this variant is not yet known (Lapidus et al., 2000; Schmittgen et al., 2003). We interpret that cytoplasmic PSMA positivity presented in primary prostate tumors and distant metastasis cases represent the overexpression of the PSMA' splice variant in prostate cancer. This kind of overexpression may have a clinical impact as this

PSMA splice variant will not be accessible for antibodies *in vivo* despite the immunohistochemical positivity. Therefore, cytoplasmic PSMA positivity should be considered equal to PSMA negativity in future immunohistochemistry based studies. As prostate cancer is a heterogeneous disease (Boyd et al., 2012), significant attention should be give to PSMA expression as revealed by immunohistochemistry in individual prostate carcinomas which can provide information regarding indication and pitfalls of PSMA based anticancer treatment antibody therapy.

Although most prostate cancers are slow-growing and indolent, a proportion is aggressive, developing metastasis and resistance to androgen deprivation treatment (Sartor et al., 2008; Goldstein et al., 2010). In the case of advanced prostate cancer, serum PSA tests are usually followed or substituted with androgen deprivation therapy, which initially will reduce tumor burden and/or circulating PSA to low or undetectable levels, but ultimately the disease will recur in most cases (Pound et al., 2001). The detection and management of prostate cancer is controversial, especially regarding screening and therapy choice after diagnosis. In fact, a patient can be diagnosed late in life with a prostate cancer and may have a significant ''overtreatment'', which would otherwise require only conservative management. On the other hand, a younger man can have an advanced disease and die within 5 years because of the disease's aggressive progression. Thus, the impact of treatment on prostate cancer survival is small, most likely because overdiagnosis and overtreatment dilutes the benefits of therapy for those who require intervention (Stangelberger et al., 2008; Madu and Lu, 2010). Consequently, a major clinical challenge is posed by the current inability to readily distinguish indolent from aggressive tumors in prostate cancer patients. The Gleason score is widely accepted as the standard for histologic grading of prostate malignancies. It recognizes tumor heterogeneity and incorporates primary and secondary grades into a final score. However, this system does not address the issue of multifocality. Although the Gleason grading system is used to determine an overall score for prostate carcinoma within a specimen, the scores of individual tumors, including the index tumor, often do not agree with this overall score (Arora et al., 2004). Investigators therefore proposed identification of biomarkers that provide greater prognostic significance than Gleason grade determination. This prognostic challenge could be addressed by better understanding of the molecular basis of cancer initiation, which should ultimately lead to the identification of biomarkers that distinguish between indolent and aggressive forms of prostate cancer. At present, however, available panels of molecular biomarkers do not provide greater prognostic significance than Gleason grade determination (True et al., 2006). Evidence clearly illustrates the lack of specificity of PSA and shows that serum PSA test cannot distinguish between the life-threatening and the relatively harmless forms of the disease (Stamey, 2001; Thompson et al., 2005). Additionally, investigation of tissue PSA and PSMA separately do not provide the ability to readily distinguish indolent from aggressive tumors in prostate cancer patients. Based on the heterogeneity of their expression within normal, benign prostatic hyperplasia, primary prostate carcinoma and distant metastasis (Bazinet et al., 1992; Mannweiler et al., 2009; Ben Jemaa et al., 2010), the study of the duality of PSA-PSMA may be potentially relevant for understanding the molecular basis of cancer initiation and distinction between latent, clinical and metastatic prostate cancer.

Significance of PSA-PSMA duality in normal, hyperplastic, malignant and prostate metastatic transformation

Prostate cancer generally does not present any symptoms until it becomes locally advanced and metastatic disease (Madu and Lu, 2010). Advanced and metastatic prostate cancer is a highly devastating disease with limited and largely ineffective treatment options (Jadvar, 2013). Despite advances in diagnosis and treatment of prostate cancer, development of metastases remains a major clinical challenge (Larsson et al., 2011). Moreover, the relationship of disseminated tumor cells to the formation of metastases remains unresolved, and the molecular factors that promote metastases of prostate cancer to lymph node, bone or brain are poorly defined (Goldstein et al., 2010; Stoyanova et al., 2013). Consequently, understanding the molecular basis of cancer initiation and the distinction of indolent from aggressive tumors in prostate cancer patients remain a major clinical challenge. Research efforts are dedicated to overcome this problem by understanding the molecular basis of the transition from normal, benign cells to prostatic intraepithelial neoplasia (PIN), localized carcinoma, and metastatic cancer (Khamis et al., 2012). There are several molecular-biological features that have been associated with the development of hyperplastic, malignant and prostate metastatic androgen-independence. The AR, in particular, plays a key role. AR activity is implicated in hyperplastic transformation and all phases of prostate cancer, including the final stages of the disease that ensue following the failure of androgen ablation therapy which is frequently termed androgen-independent (Heinlein and Chang, 2004; Barfeld et al., 2014). While not regulating the proliferation/survival of prostate epithelial cells directly, androgen occupation of the AR within the nuclei of these cells does regulate expression of PSA and PSMA (Riegman et al., 1991; Watt et al., 2001). Profiling analyses of normal, benign hyperplasia and prostate cancer specimens allowed us to identify four PSA-PSMA prostate clones: (PSA+,PSMA+), (PSA+,PSMA-), (PSA-,PSMA-) and (PSA-,PSMA+). The preponderance of each PSA-PSMA prostate clone fluctuates between normal and prostatic pathologies.

Among them (PSA+,PSMA+) and (PSA-,PSMA+) are the two individual prostate clones most expressed in normal prostate, BPH and PC samples (Ben Jemaa et al., 2010). When PSA and PSMA are coexpressed, these biomarkers are more present in BPH compared to normal prostate. However, among the above PSA-PSMA clones, PSA and PSMA are inversely regulated in BPH and PC samples. In fact, the highest tissue PSA was concomitant with low PSMA expression in BPH cells. By contrast, the PSMA was highest in neoplastic cells and is associated with low tissue PSA expression. Among (PSA-,PSMA+) prostate clone, the expression of tissue PSMA increased significantly from normal, BPH to PC specimens (Ben Jemaa et al., 2010). Accordingly, while BPH and PC patients may share the same PSA-PSMA prostate clone, PSA and PSMA expression largely depend on the cellular context. It is hypothesized that susceptibilities of PSA and PSMA expression to factors that promote prostate disease may differ between hyperplastic and neoplastic development. The prostate gland is composed mainly of stromal, epithelial, and neuroendocrine cells. The dynamic balance of cell proliferation, differentiation, and apoptosis in general maintains the cellular and tissue homeostasis. This balance is generated by the continuous cross talk among these cell populations (Sung and Chung, 2002). For this purpose, epithelial and stromal cells secrete various types of cytokines, growth factors, chemokines, and neuropeptides (Wong and Wang, 2000). Deregulation in this paracrine communication can result in derangement of the prostate gland, such as benign prostate hyperplasia and prostate carcinoma (Dawson et al., 2004). The heterogeneity of PSA versus PSMA expression under the same PSA-PSMA prostate clone is, in part, thought to be due to the effect of androgen, cytokines, growth factor receptors, adhesion molecules and many other membrane-generated signals that all share the ability to efficiently regulate PSA and PSMA gene expression. In the prostate, interaction of dihydrotestosterone (DHT) with AR, and the subsequent binding of DHT/AR dimerized complex to androgen response elements in the DNA causes transcription of various genes, resulting in the production of proteins such as PSA and PSMA and regulatory proteins important for cellular growth and function (Denmeade et al., 2003; Williams et al., 2007). Many lines of evidence support that androgens are permissive but insufficient for the induction and maintenance of BPH. BPH prevalence increases with age, while levels of serum androgens decline. Thus, while androgens are clearly important in BPH, other factors are likely involved (Bauman et al., 2006; Ho and Habib, 2011). The histopathology of BPH strongly implicates local paracrine and autocrine growth factors and inflammatory cytokines in its pathogenesis. A complex milieu of growth-regulatory proteins includes members of the fibroblast, insulin-like, and transforming growth factor families. It appears that these proteins and downstream effector molecules, in addition to a variety of interleukins, are overexpressed in BPH and, working together, create a landscape of increased stromal and epithelial growth and mesenchymal transdifferentiation that leads to disease progression (Bouraoui et al., 2008; Nickel, 2008; Mechergui et al., 2009). IL-8 can directly promote autocrine/paracrine proliferation of BPH cells, which express both IL-8 cognate receptors CXCR1 and CXCR2. In addition, IL-8 production by prostate epithelial and stromal cells *in situ*, as documented by immunohistological analysis of BPH specimens, is associated with the presence of CD15+ neutrophils, suggesting the capacity of locally produced IL-8 to recruit lymphomononuclear cells into the prostate. Interestingly, IL-8-mediated BPH cell growth can be induced by a combination of IFN-γ and IL-17, thus establishing a possible relationship between the autoimmune response induced by BPH cells and prostate cell growth (Steiner et al., 2003; Wang et al., 2004; Penna et al., 2009). IL-8 as well as IL-6, another autocrine growth factor for prostate cells (Bouraoui et al., 2008; Penna et al., 2009; Mechergui et al., 2009), are induced at high levels also following triggering of TLRs expressed by BPH cells. TLRs expressed by BPH cells are functional, with the apparent exception of TLR9, and their triggering by viral or bacterial products, such as LPS, induces production of proinflammatory chemokines like IL-8 and CXCL10 and cytokines like IL-6. In addition to the growth-promoting properties of IL-8 and IL-6 on prostate cells, the capacity of IL-8 and CXCL10 to recruit inflammatory cells could play a role in inducing and maintaining chronic inflammatory conditions of the prostate, such as those observed in BPH patients (Penna et al., 2009). These molecular-biological features provide the evidence that BPH represents an immune inflammatory disease. Chronic or recurrent inflammation is responsible for the development of many human cancers, including prostate cancer (De Marzo et al., 2007; Nakai and Nonomura, 2013). The presence of histological inflammation within the BPH and PC tissues was shown to correlate significantly with serum PSA levels. In our previously *in vivo* study, we demonstrated that proinflammatory cytokines, IL-1α, IL-6 and TNFα, were produced by prostate epithelial cells, in normal, hyperplasia and cancer samples. Moreover, we have confirmed the expression of IL-6 receptors (IL-6Rα and Gp130), IL-1 receptors (IL-1R1, IL-RII and IL-1RA) and TNFα receptors (TNFRI) in BPH and PC patients. While expression of TNFα was found to be the same in normal, BPH and PC, IL-6 and IL-1α profiles differ between BPH and PC patients (Bouraoui et al., 2008; Mechergui et al., 2009). Depending on the cut-off of serum PSA, coexpression of IL-6 and IL-1α was mostly found in PC patients with serum PSA levels > 20 ng/mL. It is suggested that IL-6 and IL-1α may contribute synergistically to leakage of PSA in the circulation as well as increased serum PSA levels (Mechergui et al., 2009). To reach the circulation, PSA must cross the prostate epithelial and basal cell layers and the basement membrane. It then enters the prostatic stromal

compartment where it can interact with fibroblasts, macrophages, and other inflammatory cells. In addition, unlike benign prostate epithelial cells, prostate adenocarcinomas lose the basal cell layers (Balk et al., 2003; Williams et al., 2007). Therefore, the high expression of cytokines IL-6 and IL-1α could be involved in an increased loss of epithelial cell polarity and basal cell number, leading to a decrease of tissue PSA in PC compared to BPH samples. However, the cytokines, growth factors and chemokines cited above are more likely implicated in low PSMA expression in prostate hyperplastic compared to neoplastic cells among (PSA+,PSMA+) and (PSA-,PSMA+) prostate clones. Overall, it appears that androgen, cytokines, growth factors and chemokines are likely to play different cell type-specific roles, leading to different expression of PSA and PSMA in BPH and PC disease. Androgen, cytokines, growth factors and chemokines act through several signaling pathways to maintain BPH and PC progression. Among signal pathways leading to inflammation and cytokine production, the nuclear factor-ϰB (NF-ϰB) family proteins are essential for inflammation, immunity, cell proliferation and apoptosis (Suh and Robson, 2004). In most cell types, NF-κB activity is mediated by the complex p50/p65 (Rel A), which acts as a transcriptional activator (Hayden and Ghosh, 2004). The sensibility of both NF-ϰB and PSA to inflammation allowed us to confirm the relationship between these two molecules and their involvement in hyperplastic and neoplastic prostatic disease progression (Bouraoui et al., 2012a). According to serum PSA levels, we demonstrated the presence of different NF-ϰB (p50/p65) profiles between BPH and PC diseases. In PC there was an association between the high expression of NF-ϰB (p50/p65) subunits and elevated serum levels of PSA (Bouraoui et al., 2012a). The NF-ϰB (p50/p65) pathway is affected by AKT. AKT is a major pathway activated constitutively in metastatic human prostate cancer relating to the stimulation of cell migration and invasion (Bellacosa et al., 2005 ; Floc'h and Abate-Shen, 2012). AKT activation involves the phosphorylation of two residues: threonine 308 (T308) in the activation loop by PDK1 (phosphoinositoldependent kinase 1) and serine 473 (S473) in the C-terminal hydrophobic motif by PDK2. A number of kinases have been suggested to function as the so-called PDK2, including mammalian target of rapamycin complex 2 (mTORC2) and integrin-linked kinase (ILK) (Manning and Cantley, 2007 ; Morgan et al., 2009). Interestingly, we have demonstrated that overexpression of NF-κB (p50/p65) subunits was concomitant with an increase in p-AKT (T308/S473) and serum PSA levels in PC compared to BPH patients in both (PSA+,PSMA+) and (PSA-ßPSMA+) prostate clones (data not shown). In addition to these signaling pathways, we showed that Raf-1/MEK/ERK cascade was also significantly increased in PC compared to BPH patients in the two most immunoexpressed PSA-PSMA prostate clones (data not shown). Components of these pathways are aberrantly expressed in human prostate disease and are overactivated in most advanced prostate cancer (McCubrey et al., 2007; Inoue and Ogawa, 2011). In turn, some of these components could be sequestered away from their pathways by means of Raf kinase inhibitory protein (RKIP), thereby abrogating intracellular growth signals. By influencing the Raf kinase and NF-κB pathways, RKIP is considered to play a pivotal role in the pathogenesis of BPH and PC (Yeung et al., 1999, 2000; Zeng et al., 2008; Keller, 2011). Loss of RKIP is considered to be indicative of the emergence of prostate cancer pathology (Al-Mulla et al., 2011, 2013). In support of this concept, we have demonstrated a loss of RKIP expression in PC compared to BPH patients among (PSA+,PSMA+) and (PSA-,PSMA+) prostate clones (data not shown). Our findings implied that through its involvement with signal transduction pathways (Raf, NF-ϰB and AKT), RKIP may drive a different pathogenic program leading to BPH and PC disease, resulting in reciprocally expression of PSA and PSMA in benign and prostate cancer cells. Importantly, we have demonstrated that PSMA and angiogenic activity are higher in (PSA+,PSMA+) compared to (PSA-ßPSMA+) clones in prostate cancer patients (Ben Jemaa et al., 2013c). In human prostate cancer, overexpression of PSMA has been postulated to be a late event in tumor progression, as its increase was detected in advanced carcinoma and metastatic disease compared to normal and benign tissue (Chang, 2004; Rajasekaran et al., 2005). Colombatti et al. (2009) showed for the first time that the tumor biomarker PSMA, a multifunctional cell surface ectopeptidase endowed with an efficient signaling activity regulated IL-6 production in prostate cancer cells. IL-6 gene induction occurs due to an activation cascade involving RAS, RAC1, p38 and ERK1/2 MAPKs, leading to the phosphorylation of the p65 subunit of NF-ϰB transcription factor. In addition, they demonstrated that both IL-6 and CCL5 promote the proliferation of LNCaP tumour cells reaching their maximal activity synergistically (Colombatti et al., 2009). Within this context, the ability of PSMA to activate RAC1 may be highly relevant, inasmuch as RAC1 activation decreases the expression of E-cadherins, thereby loosening intercellular adhesions and facilitating the cytoskeletal rearrangements required for mitosis (Woods et al., 2007). If so, activated RAC1 could therefore favor the response of LNCaP cells to the mitogenic activity of IL-6 and CCL5, while participating in the induction of their expression. In line with this view, Zhang et al. (2013) reported that PSMA stimulate prostate cancer cell proliferation, migration and survival through the p38 MAPK pathway, revealing a novel mechanism for PSMA playing a positive role on LNCaP cells. Recently, we have shown that bFGF stimulates PSMA expression in metastatic prostate cancer cells, LNCaP and PC3-PSMA. This finding implied that PSMA could participate in neovessel growth in developing tumors (Laidler et al., 2005; Ben Jemaa et al., 2013a). PSMA regulates the angiogenesis process

through modulating the adhesion/de-adhesion processes *via* activation of focal adhesion kinase (FAK) in LNCaP cells or *via* activation of p21-activated kinase1 (PAK1) in normal endothelial cells (HUVEC) (Conway et al., 2006). PSMA expression was also shown to be associated with tube formation by primary human umbilical vein endothelial cells (HUVECs) cultured in Matrigel and induced by tumor-conditioned medium (TCM) derived from human breast cancer cells (MDA-MB-231) (Liu et al., 2011). In addition, the study of Tsui et al. (2005) reported that PSMA expression seems to correlate with VEGF, which stimulates the proliferation and migration of endothelial cells towards malignancies through the process of angiogenesis. In many types of carcinomas, a strong PSMA expression was also seen in the newly formed vessels resembling tumor-related angiogenesis (Silver et al., 1997; Chang, 2004). Notably, prostate cancer can manifest itself in several forms, including a percentage of cancers that show reduced levels of PSA and can progress without the need for the normal ligand to active AR (Paliouras and Diamandis, 2008). Low PSA levels in prostate cancer may result in the involvement of proinflammatory cytokine. We have previously shown that NF-κB phosphorylation was notably increased by IL-1β leading to down-regulation of PSA in a dose-dependent manner in LNCaP cells (Bouraoui et al., 2012b). Culig et al. (1998) have also found that the IL-1 receptor type I was expressed by LNCaP, leading to activation of the IL-1 β signal transduction and inhibition of cell growth, as well as PSA and AR expression in prostate cancer cells. In the same way, Wang et al. (Wang et al., 2009) showed that NF-κB activation was increased by TNF-α, leading to repression of AR activity and PSA secretion in prostate cancer cell lines. Also, IL-6 might inhibit PSA gene expression through the STAT3 pathway without MAPK involvement by blocking the association between the co-activator p160 and p300 on the PSA gene enhancer and promoter (Jia et al., 2004). Low levels of PSA are associated with tumor-propagating cells in prostate cancer xenografts (Qin et al., 2012). This form of the prostatic disease is a characteristic of a more aggressive, hormone refractory stage or androgen-independent cancer that is often associated with loss of androgen sensitivity (Paliouras and Diamandis, 2008). As stated, molecular changes occur that enable ligand/AR interactions within the malignant epithelial cells to regulate directly the expression of prostate marker proteins such as PSA and PSMA, in addition to the autocrine production of growth and survival factors without stromal requirement (Denmeade et al., 2003). Numerous studies indicate that in the secretory epithelial cells of prostate gland, both PSA and PSMA transcriptions are androgen-dependent (Serda et al., 2008; Kuroda et al., 2009). The emergence of androgen-insensitive tumor cells may arise as a consequence of an adaptation to androgen withdrawal or from pre-existing androgen-independent clone (Hernes, 2005). Angiogenesis is often coordinately upregulated toward hormone-refractory PC and contributes to their more malignant or aggressive phenotype (Gustavsson et al., 2005). According to the androgen levels, PSA and PSMA are different in several ways. In human PC, a loss of expression of tissue PSA has been associated to advanced prostate cancer and to transition into hormone refractory tumor growth (Berner et al., 1993; Sivridis et al., 2001). However, in a previous report Denmeade et al. (Denmeade et al., 2003) identified PSMA as a gene that was up-regulated in the more aggressive androgen independent prostate cancer cell line C4-2B compared to the androgen-dependent cell line LNCaP. Therefore, our findings implied that (PSA+,PSMA+) is more likely than (PSA-,PSMA+) prostate clone to have the characteristic of aggressive metastatic prostate cancer phenotype (data not shown).

Although our PC cases which coexpressed PSA and PSMA are mostly poorly differentiated adenocarcinoma (Gleason score ≥ 8), they reacted differently with RKIP. In fact, some of the PC patients exhibited a positive immuostaining to RKIP, whereas other missing the expression of this protein. Among (PSA+,PSMA+) PC clones, loss of RKIP expression was associated with increased levels of both PSA and PSMA expression (Ben Jemaa et al., 2013d). Moreover, loss of RKIP has been associated with an increase of components of Raf-1/MEK/ERK and NF-κB signaling. However, AKT (T308/S473) seems to be activated independently to RKIP status (Ben Jemaa et al., 2013d). Although each pathway is conceptually linear, Raf-1/MEK/ERK, NF-κB, and AKT pathways are often coordinately deregulated toward hormone-refractory PC and contribute to their more malignant or aggressive phenotype (Bluemn and Nelson, 2012). RKIP has been found to play a major role in the regulation of epithelial mesenchymal transition (EMT) by intervening in Raf-1/MEK/ERK-1/2 and NF-κB mediated signaling (Wu and Bonavida, 2009; Tang et al., 2010; Al-Mulla et al., 2013). NF-κB activation and subsequent activation of its downstream transcriptional target SNAIL induces EMT through downregulation of E-cadherin and negatively regulates RKIP in cancer cells (Wu et al., 2009). Moreover, RKIP loss enhances cellular motility by inducing the expression/stabilization of β-catenin, vimentin, MET and p21-activated kinase 1 (PAK-1) (Al-Mulla et al., 2011). On the basis of our results, it seems that according to RKIP, our PC patients with (PSA+,PSMA+) profile could exhibit the feature of two different PC phenotypes: an androgen-dependent phenotype for PC patients keeping the RKIP and an androgen-independent phenotype for those missing the RKIP (Ben Jemaa et al., 2013d). Consequently, our study supports the heterogeneity and the complex process of tumor clonality during all stages of prostate cancer development. It is possible that this later PC phenotype might reflect a subpopulation of prostate tumor that will eventually escape hormonal control and relapse to an androgen-independent state that is basically lethal. In keeping with this and combined with the

evidence that PC is an heterogeneous disease, RKIP seems to contribute to the complex PSA-PSMA clonal progression of PC with different molecular phenotypes through its involvement with signal transduction pathways (Ben Jemaa et al., 2013d). In this way, through their involvement in expansion of several PSA-PSMA prostate clones, RKIP/Raf-1, RKIP/NF-κB and RKIP/AKT signaling may be driving both human prostate cancer initiation as well as progression to a metastatic phenotype.

Insight into cell origin and evolution of PSA-PSMA prostate cancer clones

Since the existence of several PSA-PSMA prostate cancer clones with distinct molecular-biological features (Ben Jemaa et al., 2010), we suggested that these clones may arise from distinct cells of origin. In the prostate gland, there are three types of epithelial stem cell - luminal cells, basal cells, and rare neuroendocrine cells (Pignon et al., 2013). The cell-of-origin model in cancer biology suggests that some tumors are more aggressive than others because of differences in the cell lineages from which they arise. Luminal cells have long been considered the cellular origin of prostate cancer (Wang et al., 2013). Additional evidence is suggested by detailed histopathological analysis of Myc expression in high-grade PIN samples, which still retain basal cells, which shows that Myc up-regulation is associated exclusively with luminal cells, and is not detected in their basal neighbors (Gurel et al., 2008); similar findings have also been reported with respect to telomere shortening (Meeker et al., 2002). Also in favor of a luminal cell of origin is the recent finding that AR mediates formation of the TMPRSS2-ERG fusion in human prostate cancer cells (Lin et al., 2009; Mani et al., 2009; Haffner et al., 2010), suggesting that initiating events take place in AR expressing luminal cells.

However, recent evidence from a prostate regeneration assay suggests that prostate basal cells can also give rise to prostate cancer. Prostate adenocarcinoma can be serially propagated by cells with

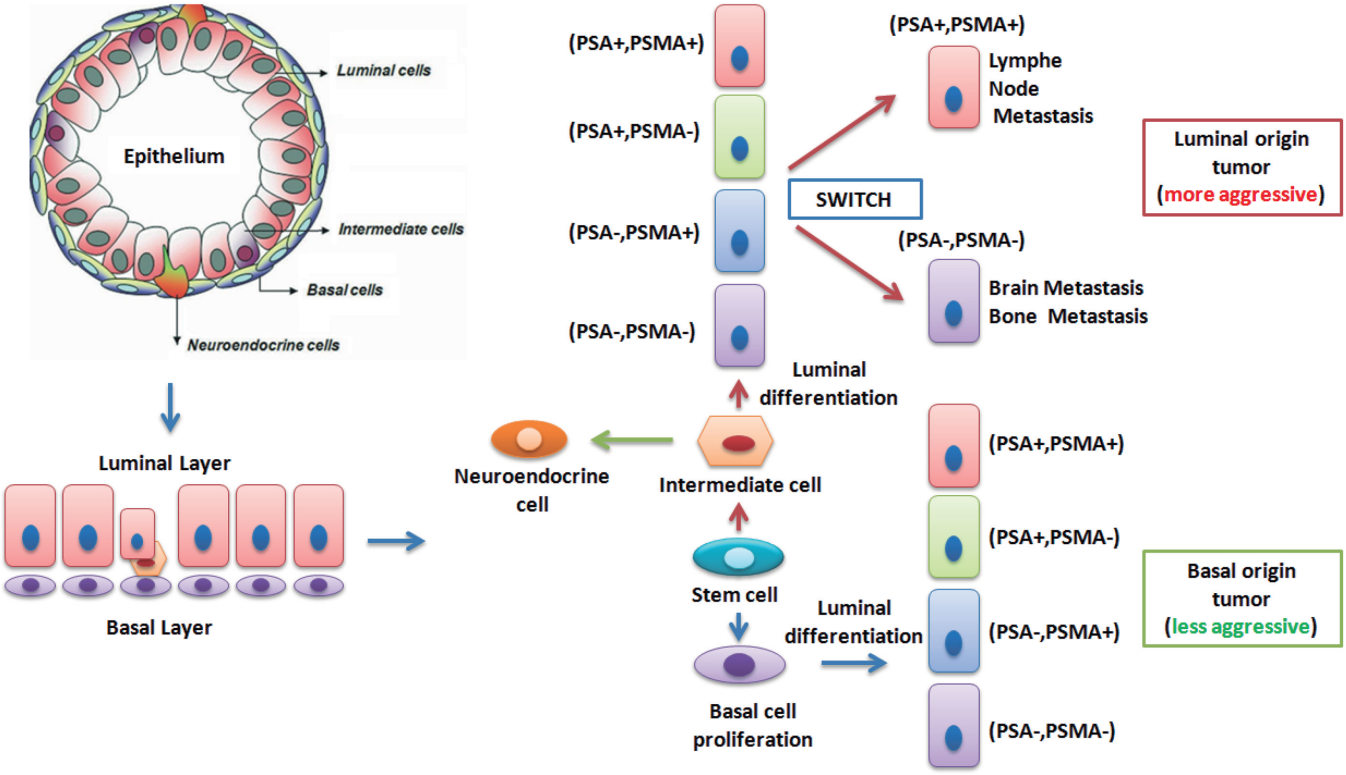

Fig. 2. Model of human prostate cancer initiation and propagation by distinct phenotypic cell populations. Prostate adenocarcinoma can be serially propagated by cells with a luminal phenotype or by a subset of basal cells within the tumor to ensure continuous production of malignant luminal-like cancer cells. Luminal origin tumor may generate several PSA-PSMA prostate clones able to switch from (PSA+/-,PSMA+/-) to a final (PSA+,PSMA+) phenotype when it propagates to lymph node, or to (PSA-,PSMA-) phenotype when it propagates to the brain or the bone. However, prostate cancer originating from a subpopulation of basal cells could generate distinct PSA-PSMA prostate clones with a low probability to spread from primary tumor. In this model, RKIP through its involvement in ERK, AKT and NF-κB pathway seems to initiate and drive heterogeneous PSA-PSMA prostate cancer clones from basal or luminal cells. Therefore, PSA-PSMA prostate cancer clones might recapitulate the histological and molecular features of human prostate cancer.

a luminal phenotype, whereas it has been recently reported that human prostate tumors may set aside a subset of basal cells within the tumor to ensure continuous production of malignant luminal-like cancer cells (Stoyanova et al., 2013) (Fig. 2). A basal cell of origin has been suggested by analyses of Pb-Cre4; Ptenflox/flox mice, which display an expansion of basal cells as well as intermediate cells coexpressing basal and luminal markers in tumors (Wang et al., 2006). More recently, a comparison of basal and luminal epithelial populations isolated by flow cytometry from the mouse prostate has shown that basal populations are readily transformed by lentiviral expression of ERG and AR in tissue reconstitution experiments, whereas luminal cells are not transformed (Lawson et al., 2010). Human prostate cancer cells with a basal phenotype have been reported to produce luminal cancer progeny *in vitro* (Collins et al., 2005). Using cell lines that were originally derived from human prostate tumors, it was shown that basal cell marker CD44 enriched for tumor-propagating cells in the absence of differentiated luminal cell markers (Qin et al., 2012). A recent study demonstrates that advanced chemotherapy-resistant prostate cancer is maintained by cells lacking basal or luminal cytokeratins (Domingo-Domenech et al., 2012). Basal and luminal cell lineages are largely independent in the adult prostate, but rare basal and luminal stem/progenitor cells can potentially compensate for imbalances in cell number during regeneration and tissue homeostasis. One possible model is that rare basal (and luminal) stem/progenitor cells may reside at the top of an epithelial lineage hierarchy, and a larger subpopulation of basal cells, perhaps corresponding to transit-amplifying cells, can exhibit plasticity in sphere and tissue reconstitution assays. Alternatively, basal cells may exhibit stochastic stem/progenitor properties, with a low probability in the adult prostate epithelium and a higher probability when explanted or transformed (Stoyanova et al., 2013; Wang et al., 2013).

A combination of molecular and bioinformatics analysis showed that tumors that originate from luminal cells are more aggressive than those that develop from other cell types. Bioinformatics studies also identified a cross-species molecular signature within mouse luminal cells that correlates with human patient outcomes (Wang et al., 2013). Importantly, we have demonstrated that (PSA+,PSMA+) is more likely than (PSA-,PSMA+) prostate cancer clone to have the characteristics of aggressive metastatic prostate cancer phenotype (data not shown). Thus, it is hypothesized that (PSA+,PSMA+) prostate cancer clone may originate from luminal cells. Conversely, the (PSA-,PSMA+) prostate cancer clone may originate from those that develop from other cell types, such as a subset of basal cells. However, we have demonstrated that (PSA+,PSMA+) prostate cancer clone, which is a poorly differentiated adenocarcinoma (Gleason score ≥ 8), reacted differently with RKIP signaling. On the basis of our results, it seems that according to RKIP, our PC patients with (PSA+,PSMA+) profile could exhibit the features of two different PC phenotypes: an androgen-dependent phenotype for PC patients keeping the RKIP and an androgen-independent phenotype for those missing the RKIP (Ben Jemaa et al., 2013d). In this way, RKIP signaling may play a role in tumor cell survival and disease recurrence after androgen withdrawal (Keller, 2011; Al-Mulla et al., 2011, 2013). It is possible that the first PC phenotype might reflect a subpopulation of prostate tumor that originates from basal cells. However, the later PC phenotype might reflect a subpopulation of prostate tumor that originates from luminal cells (Fig. 2). In this context, through it is interference with ERK, AKT and NF-κB signaling, RKIP may be involved in the emergence of basal and luminal prostate cancer cells to initiate heterogeneous PSA-PSMA prostate cancer clones. Accordingly, the cooperative effects of RKIP/ERK, RKIP/AKT and RKIP/NF-κB pathway and AR in basal and luminal cells confer the histological and molecular features of human prostate cancer, with loss of basal cells and expansion of luminal cells expressing or not PSA and PSMA.

Coexpression of PSA and PSMA are commonly found in normal, benign prostatic hyperplasia, primary prostate cells and androgen-dependent metastatic prostate cancer cells (like the LNCaP prostate cancer cell line derived from lymph node metastasis) (Laidler et al., 2005; Williams et al., 2007). However, the problem of the loss of their expression upon cancer progression from androgen-dependent to androgen-independent stage, like in the case of two metastatic cell lines PC-3 (derived from bone metastasis) and Du-145 (derived from brain metastasis) remains open (Laidler et al., 2005; Williams et al., 2007). Interestingly, we have previously demonstrated that (PSA-,PSMA-) prostate clone appeared only in BPH patients, whereas it is not detected in either normal prostate or prostate cancer patients (Ben Jemaa et al., 2010). Cells within the basal fraction can regenerate benign prostate tissue in immunodeficient mice. In addition, the introduction of oncogenic alterations in these target basal cells can induce a disease that mimics human prostate cancer (Goldstein et al., 2010; Stoyanova et al., 2013). Isaacs (1999) has postulated that androgen-independent prostate cancer eventually develop, because a subpopulation of androgen-independent tumour cells pre-exist even before therapy was initiated. Accordingly, our findings support this model and suggest that change to androgen-independent growth could result from pre-existing androgen-independent (PSA-,PSMA-) prostate clone.

Despite the phenotypic heterogeneity of metastatic prostate cancer (Shah et al., 2004), molecular and cytogenetic analyses showed that multiple metastases in the same patient are clonally related (Mehra et al., 2008), indicating that advanced prostate cancer is monoclonal (Mehra et al. 2008; Liu et al., 2009). These observations lead us to conclude that, in the same patient, malignant luminal cancer cells switch from (PSA+/-,PSMA+/-) to a

final (PSA-,PSMA-) phenotype during their propagation to the bone or to the brain. Alternatively, malignant luminal cells switch from (PSA+/-,PSMA+/-) to a final (PSA+,PSMA+) phenotype during their propagation to the lymph. This switch seems to implicate several mechanisms in which RKIP/ERK, RKIP/AKT and RKIP/NF-κB pathway and AR play a key role (Fig. 2). The functional consequences of AKT pathway activation are particularly relevant for initiating malignant transformation of the prostate and evolution of PSA-PSMA prostate cancer clones. The consequences of AKT activation are mediated in part by activation of NF-κB signaling *via* stimulation of IKK (Dan et al., 2008). Importantly, Stoyanova et al. (2013) demonstrated that tumors driven by expression of oncogenes Myc and myristoylated/activated AKT (myrAKT) initiating in basal cells exhibit features of adenocarcinoma. Since AKT and AR signaling pathways have recently been shown to regulate each other through complex reciprocal feedback mechanisms, it is suggested that AKT and AR may cooperate in basal cells to exhibit malignant phenotype (Carver et al., 2011). Furthermore, evidence indicated that while mammalian target of rapamycin complex 2 (mTORC2) acts as the PDK2 in LNCaP (PSA+ßPSMA+) cells, integrin-linked kinase (ILK) plays a major role in facilitating S473-AKT phosphorylation in PC-3 (PSA-ßPSMA-) cells (Lee et al., 2013). Surprisingly, the angiogenic factor basic fibroblast growth factor (bFGF) involved in AKT activation (Hart et al., 2001) has been shown to restore the expression of PSMA in markedly dedifferentiated androgen-independent metastatic prostate cancer PC-3 and Du 145 cells (Laidler et al., 2005). The expression levels of pAKT have been proposed as a useful indicator of the severity of the disease in prostate cancer. Its low expression in normal prostate epithelial cells increased several fold in high-grade prostate cancers, in metastatic and in androgen-insensitive prostate carcinoma (Malik et al., 2002; Floc'h and Abate-Shen, 2012). Accordingly, the progressive emergence of (PSA-,PSMA-) clones in response to phosphorylation of AKT at T308 and S473 during prostate tumor progression may represent one mechanism by which prostate cancer cells escape hormonal control.

Although human prostate cancer displays significant phenotypic heterogeneity, >95% of prostate cancers are classified pathologically as adenocarcinoma, which has a strikingly luminal phenotype (Humphrey, 2012). Focal regions of neuroendocrine differentiation are more commonly observed in prostate adenocarcinoma, particularly following recurrence after prostatectomy and androgen deprivation therapy (Yuan et al., 2007; Komiya et al., 2009), and expression of the neuroendocrine marker chromogranin A is associated with the development of castration-resistant tumors and shortened time to disease recurrence (Kokubo et al., 2005; Berruti et al., 2007). This prevalence of neuroendocrine differentiation after recurrence may be due to the lack of AR expression by neuroendocrine cells, which are inherently castration-resistant. In the human prostate cancer cell line LNCaP, STAT3 mediates IL-6-induced growth and neuroendocrine differentiation (Spiotto and Chung, 2000). In the same prostate cancer cell line, PSMA has been shown to regulate the IL-6 production through activation of the cascade involving RAS, RAC1, p38 and ERK1/2 MAPKs, leading to the phosphorylation of the p65 subunit of NF-κB transcription factor (Colombatti et al., 2009). In parallel, we have demonstrated that PSMA may be able to induce IL-6 upregulation through bFGF signaling (p38 and ERK1/2 MAPKs) in two different metastatic prostate cancer models, LNCaP and PC3-PSMA cells. LNCaP cells are androgen-dependent prostate cancer cells known to express both PSA and PSMA (Ben Jemaa et al., 2013a). In line with this view, we suggested that PSMA may be able to induce IL-6 production, leading to neuroendocrine differentiation of LNCaP cells. In this way, in response to PSMA-induced IL-6 expression, LNCaP cells with (PSA+,PSMA+) phenotype may switch to (PSA-,PSMA-) phenotype, which is characterized by neuroendocrine cells that lack AR expression.

Perspectives

Based on the available evidence, prostate cancer can indeed arise from distinct cell types of origin, but it remains unclear whether different cells of origin are involved in human prostate cancer initiation, or whether they might result in differing molecular subtypes. Furthermore, the relationship between the cells that initiate and maintain human prostate adenocarcinoma is not known. Identifying cells of PSA-PSMA prostate clones origin and determining their relationship with cells that maintain human prostate adenocarcinoma holds potential for distinguishing high- from low-risk patients at early stages of the disease. Further research in this area could provide valuable information that could be used to predict prostate neoplastic transformation and treatment response.

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Accepted April 30, 2014