

Chapter 8

Crossroads of Signaling Pathways

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Abstract As studies on PC progression continue to uncover a growing number of crosstalks and co-occurrences of mutations and epigenetic alterations, new drugs are getting approved bringing significant changes in the treatment paradigm of these tumors.

This chapter recapitulates the best known examples of molecular interactions potentially targetable to achieve these therapeutic evolutionary changes, to allow a better control of PC which, in 2012 alone, has still killed more than 28,000 men, in USA (Siegel et al, *CA Cancer J Clin*, 62:10–29, 2012; El-Amm, Aragon-Ching, *Ther Adv Med Oncol*, 5(1):25–40, 2013).

8.1 Background and Aims

Prostate cancer (PC) is a multifocal disease, composed by several independent tumor foci that may show different degrees of molecular alterations. The heterogeneous nature of PC which, at a molecular level, derives from the crosstalk of multiple signal transductions variously acting in promoting growth, survival and therapy-resistance of PC cells (Pittoni et al. 2011).

The development of new therapeutic strategies, particularly focused toward castration-resistant prostate cancer (CRPC), relies on a better understanding of the resistance pathways selectively adopted from prostate cancer cells (El-Amm and Aragon-Ching 2013).

Recently, it has been shown that androgens residual from androgen-deprivation therapy may indirectly favor cancer growth, with a progressive increase of the PSA levels, *via* the over-expression of many HIF-1 dependent, hypoxia-inducible

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genes. The interplay between hypoxia and AR, further cross-talks with several oxidative stress mediators, cytokines, growth factors, DNA-repair pathways, and epigenetic regulators, in a cooperative effort to ensure the survival of neoplastic cells in a highly adverse metabolic (and environmental) background. These interacting signaling mechanisms, indeed, may either potentiate or counteract each other, leading alternatively to cell death or adaptation and radio-chemoresistance. It thus becomes apparent that resistance to therapy can be overcome only through a proper therapeutic manipulation of the right factor(s) that, in turn, will influence the others, triggering PC cells death (Marignol et al. 2008).

As an example, it has been surprisingly shown that cell death can be induced in castration-resistant tumors “still” *via* AR, which can modulate apoptosis and autophagy if targeted in conjunction with PKA pathway members (Attar et al. 2009).

As well, considering that AR is largely expressed in tumor microenvironmental stromal cells, drugs targeting AR signaling in PC cells give rise to a therapeutic favourable effect also on the stromal compartments, as AR is largely expressed in tumor microenvironmental stromal cells (Mantalaris et al. 2001).

Thus, a favourable response to AR targeting will encompass both reductions in serum PSA and bone-specific, osteoblast derived, alkaline phosphatase.

Overall, mounting evidence suggest that the cell fate, in response to therapeutic attack, depends on a plethora of variable factors, ranging from metabolic stress, functional status of cells, the interaction level between the stress-response pathways, paracrine mediators produced by tumor microenvironment, and the epigenetic interactions on DNA damage response and DNA repair (Murr 2010).

During these last years, the efforts of the scientific community have been focused on the correct interpretation of the *complementary pathways* which could kill radio- and chemoresistant cancer cells.

The end-point of such a program will require carefully designed clinical trials, a rigorous patient selection and retrospective analyses of clinical, pathological, and follow-up data.

In the rapidly evolving field of prostate cancer therapy, new drugs are being used; as well, new insights indicating possible different rational approaches to the treatment sequencing with “old” drugs are being proposed. The search for novel biomarkers, useful for the individualized prediction of treatment response and outcome of PC patients, is actively on. This is a particularly complex investigational field (considering that the malignant phenotype of prostate cancer cells results from a highly variable combination of functional, genetic and epigenetic defects in cell cycle metabolism, checkpoint control and DNA-repair pathways, working together to render PC a lethal disease). The real challenge lies not only in detecting all these alterations, but also in defining the multiple layers of their reciprocal intersection (Sarwar and Persson 2011).

We are thus requested to critically review our knowledge about the role of the plethora of molecular guests acting on the scenario of prostate cancer progression, which comprises both epithelial and stromal cells, both contributing to tumor heterogeneity and growth dynamics (Cho-Chung 1989, 1990; Camps et al. 1990; Cunha et al. 1996).

That prostate cancer development and growth is dependent on androgens and can be suppressed by androgen ablation monotherapy is an old concept (Zhu and Kyprianou 2008).

The appearance of androgen-independent prostate tumor growth, leading to cancer recurrence and highly metastatic disease, is a well-known phenomenon as well (Wang et al. 2007).

During the entire life-span of prostate cancer, the androgen axis actively cross-talks with a plethora of growth factors, driving the shift of prostate cancer cell toward survival and invasion advantage. Androgenic control of growth and differentiation is tightly regulated in both stromal and epithelial cells (Sar et al. 1990).

This explains why the successful treatment of PC with drugs targeting (AR) signaling (defined as *Epithelial-Stromal Targeting Agents*), leads to reduction in either serum PSA and bone-specific alkaline phosphatase (Mantalaris et al. 2001; Niu et al. 2010).

A poor clinical outcome for prostate cancer patients has been associated, instead, with low-AR levels in the stromal microenvironment (Henshall et al. 2001), and this finding has been proposed as one of the mechanisms involved in the emergence of androgen-independent cancer (Dayyani et al. 2011). The propulsive effect of androgens on prostate epithelial cell proliferation and survival are indirectly regulated by paracrine mediators produced by stromal cells, such as insulin-like growth factor (IGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), (Cunha and Donjacour 1989), vascular endothelial growth factor (VEGF) and transforming growth factor- β (TGF- β) (Byrne et al. 1996).

The epidermal growth factor-1 (EGF) and its receptor (EGFR), (Russell et al. 1998) are frequently up-regulated in advanced stages of PC (Di Lorenzo et al. 2002). Targeting EGFR with monoclonal antibodies or with tyrosine kinase inhibitors, suppresses growth and invasion of androgen-dependent and -independent prostate cancer cells *in vitro*, leading to the conclusion that the multi-crossed signals between EGF/EGFR and androgen signaling is crucial for the acquisition and the maintenance of androgen sensitivity (Bonaccorsi et al. 2004; Festuccia et al. 2005; Leotoing et al. 2007).

Both AR and EGF can activate MAPK and, in a 'functional-symmetry', the EGF-activated MAPK/extracellular signaling-regulated kinase kinase-1 (MEKK1) cascade. This allows EGF to interfere with AR function, modulating the androgen response and blocking androgen-dependent transcription in differentiated cells. MAPK extracellular kinase (MEK) inhibition reverses the EGF-mediated AR down-regulation in differentiated cells (Peterziel et al. 1999).

The alteration of this EGF-AR interplay is an important contributor to prostate tumor progression. The modulation of AR signaling activity by ERBB2 (HER-2/neu), a lead member of the EGFR family of receptor tyrosine kinases, has been found correlated with prostate cancer progression to cell growth of androgen-independent metastatic disease (Heinlein and Chang 2004), *in vitro* and *in vivo* (Craft et al. 1999; Yeh et al. 1999; Mellinghoff et al. 2004; Liu et al. 2005).

Similar positive feedbacks with AR activity in prostate cancer cells have been described also for several other growth factors (Orio et al. 2002). Evidence supports a strict interaction between AR and the IGF signaling. The high IGF1 signaling in prostate cancer cells (HepG2 and LNCaP cells) (Wu et al. 2007) likely depends upon AR up-regulation of IGF1 receptor expression and/or, alternatively, upon the modulation of IGF-binding proteins (IGFBPs), which, in turn, are up-regulated by either androgens and IGF1 in androgen-responsive human fibroblasts (Yoshizawa and Ogikubo 2006). IGF1 enhances AR transactivation under low/absent androgen levels (Culig et al. 1994; Orio et al. 2002) and promotes prostate tumor cell proliferation (Burfeind et al. 1996). According to several reports, high IGF1 levels in the serum can be considered a marker of an increased risk of prostate cancer (Pollak et al. 1998; Wolk et al. 1998).

Even more data concern the cross-talks between AR and Transforming growth factor- β (TGF β). This ubiquitous cytokine, for instance, contributes to the regulation of proliferation, growth and differentiation of prostate stromal and epithelial cells.

Cofilin and prohibitin, two novel signaling effectors of TGF β 1, that serve as potential intracellular effectors of its apoptotic action, were identified in human prostate cancer cells (Zhu et al. 2006). Androgens can inhibit TGF β 1-induced apoptosis in prostate cancer cells (Chipuk et al. 2002) via the AR-associated protein 55 (ARA55/Hic-5; LIM protein superfamily). Overexpression of ARA55 inhibits TGF β -mediated up-regulation of SMAD transcriptional activity in rat prostate epithelial cells, as well as human prostate cells, via an interaction between ARA55 and SMAD3 (Wang et al. 2005). Cancer cells become refractory to the growth inhibitory activity of TGF β due to the loss (or mutation) of transmembrane receptors or intracellular TGF β signaling effectors during tumor initiation (Akhurst and Derynck 2001). In advanced prostate cancer and in PC bone metastasis, TGF β is over-expressed and TGF β 1 ligand overexpression results in pro-oncogenic rather than growth suppressive effect (Coffey et al. 1986; Roberts et al. 1986; Derynck and Zhang 2003; Zhu and Kyprianou 2005).

The androgenic-mediated TGF β enhancement seems to play a role on the epithelial-mesenchymal transition (EMT) in metastasizing cancers (Zavadil and Bottlinger 2005), with a further interplay with E-cadherin. The effects of TGF β 1 expression on stromal cell proliferation and differentiation, depend on the specific stromal cell type, microenvironment, and interactions with other growth factor (Sporn and Roberts 1992). AR and TGF β 1 levels significantly correlate in the stromal component of prostatic intraepithelial neoplasia (Cardillo et al. 2000). Very interestingly, TGF β 1 triggers the AR translocation from nucleus to cytoplasm in prostate stromal cells underlying to myodifferentiation (Gerdes et al. 1998, 2004), while androgens enhance TGF β 1-mediated proliferation of prostatic smooth muscle cells (Salm et al. 2000).

Prostate cancer progression toward androgen-independent disease has been linked also to changes in the expression of several members of the FGF family, characterized by a broad spectrum of functions on cell differentiation, migration, and angiogenesis (Ornitz and Itoh 2001). FGF2 can stimulate also the proliferation of prostate stromal cells, in a synergistic fashion with DHT (Niu et al. 2001).

The synthesis of FGF2 and FGF7 in prostate epithelial cells seems to be mainly regulated by estrogen receptors (ER), whereas ER act in coordination with AR to mediate the synthesis of these growth factors in stromal cells. Androgen depletion rapidly reduces stromal IGF1 expression, after castration, favoring PC cells apoptosis (Ohlson et al. 2007). AR can otherwise directly influence the expression of FGF1, FGF2, FGF8, and FGF10 in either prostate tumor epithelial cells and stromal cells (Saric and Shain 1998; Nakano et al. 1999; Rosini et al. 2002), while a paracrine secretion of FGF10 exert a positive feedback on AR, up-regulating its expression (Memarzadeh et al. 2007). In response to FGFs, AR potentiates FGF-induced survival of prostate cancer cells, possibly through BCL-2 induction, allowing the escape of selected clones from androgenic control (Rosini et al. 2002; Gonzalez-Herrera et al. 2006).

Among the cross-actions between AR and the *multifunctional growth factor signaling pathways*, the interplay between the cellular responses to androgen and hypoxia is emerging as a further key phenomenon in the developing of androgen-independent, metastasizing prostate cancer cell clones (Marignol et al. 2008). In prostate cells, androgens and hypoxia share several regulatory molecular mechanisms: both androgens and hypoxia, in fact, due to the presence of a hypoxia-responsive region in the human PSA promoter, can induce in fact PSA expression (Horii et al. 2007).

It has been then hypothesized that hypoxia, mainly through the hypoxia-inducible factor (HIF1A), may facilitate PC progression through the cross-talk with AR. To further support this idea, it has been recently reported that residual androgens following androgen deprivation induce the expression of hypoxia-inducible genes and stimulate cancer re-growth (Marignol et al. 2008).

This is of particular interest if we consider that, as for most solid cancers, hypoxia is a common feature of prostate tumors. Then, targeting hypoxia looks as a very appealing complementary strategy for the management of aggressive prostate cancers.

The ‘hypoxia-response’ signaling system up-regulates the expression of a wide spectrum of effectors that increase the ability of tumor cells to turn poor oxygenation into survival advantage (Anastasiadis et al. 2003) and radio- and chemoresistance (Zhou et al. 2006).

In response to the decrease in the micro-environmental oxygen (Ellis et al. 2009) HIF-1 α regulates gene expression of several genes involved in multiple physiological responses, such as erythropoiesis and glycolysis (short term solutions) and angiogenesis (long term solution) (Semenza 1998), via the expression of VEGF (DeLongchamps et al. 2006).

VEGF, is “the” angiogenic cytokine, driving endothelial cell proliferation and migration, and vessel assembly (Fong et al. 1995). The expression of HIF1, AR and VEGF expression are tightly correlated (Boddy et al. 2005; Banham et al. 2007). So, AR regulates angiogenesis in androgen-sensitive PC through the HIF1-induced VEGF increase (Boddy et al. 2005). Following androgen-deprivation, the intracellular reactive oxygen species induce, instead, the direct up-regulation of VEGF-C, which favor AR transactivation mediated by the AR co-activator BAG-1L

(Rinaldo et al. 2007). Clonal selection for cells with higher expression of HIF-1 α and/or apoptotic resistance pathways contributes to determine cell specific responses to hypoxia (Zhou et al. 2006).

HIF-1 α over-expression/hyperfunction may be induced by genetic loss of expression/function of pVHL (Ivan and Kaelin 2001, p 53; An et al. 1998) and/or PTEN (Zundel et al. 2000) leading to the activation of PI3K/AKT/mTOR pathway (Zundel et al. 2000; Stiehl et al. 2002) which, in turn, plays a well-known role in proliferation, survival and metastatic ability of hormone independent prostate cancers, as demonstrated by the correlation between elevated phosphorylated AKT and high Gleason grade of PC (Yuan et al. 2007). HIF-1 α may be induced, in addition, by several cytokines and growth factors including insulin (Zelzer et al. 1998), insulin like growth factors (Feldser et al. 1999), P42/44 mitogen activated kinase (MAPK) (Richard et al. 1999).

Furthermore, alternative mechanisms to those mentioned above have been identified which include the tumor microenvironment (Weidemann and Johnson 2008) and mutations within the ODD domain of HIF-1 α (Mabjeesh and Amir 2007). This eloquently explain why targeting only one member of the hypoxia-related angiogenetic pathway is insufficient to permanently inhibit tumor angiogenesis and why tumor cells, treated with a mono-drug therapy, develop resistance by selection of 'hypoxia resistant' cells or by activating alternate angiogenic pathways (Kerbel and Folkman 2002).

To further complicate this scenario, under severe hypoxia, radio-/chemo-resistance and clonal selection may develop as a response of opposing signals delivered by survival and death pathways that allow selection of cells that have a growth advantage either genetically or epigenetically determined (Zhou et al. 2006).

Post-translational epigenetic modifications, including acetylation mediated by histone acetyltransferases (HATS) and deacetylation by histone deacetylases (HDACs), have been shown to be critical to HIF-1 α signaling (Ellis et al. 2009).

HIF-1 α signaling up-regulates the activity of HDACs. Then, HDAC inhibitors are emerging as a new class of anti-angiogenetic cancer therapeutics.

However, the anti-angiogenic properties of HDACI have been associated also with the alteration of numerous pro- and anti-angiogenic genes (Liu et al. 2006) other than HIF-1 α and VEGF. They encompass FGF, *angiopoietin*, tunica intima endothelial kinase 2 (*TIE2*), endothelial nitric oxide synthase (*eNOS*) (Qian et al. 2004; Rossig et al. 2002, p 53), pVHL, thrombospondin 1 (Kim et al. 2001; Kwon et al. 2002; Sasakawa et al. 2003; Kang et al. 2008, p 21)^{WAF1/CIP1}, and survivin (Qian et al. 2004). Even for HDACI, monotherapy has shown limited responses in the clinic, but it seems very promising, as a part of combination therapies. Pre-clinical and clinical studies indicate that HDACI have positive effects on the expression of pro- and anti-angiogenic genes, suggesting their useful role in reinforcing the actions of anti-VEGF therapies.

As it has been largely shown in the past decade, the intricate molecular cross-talks underlying the malignant phenotype and the emergence of androgen-independent prostate tumors encompass the expression and functional defects in HR, single-strand break- (DNA-ssb) repair, MMR and base-excision repair (BER).

Prostate cancer cell lines (Chen et al. 2003; Yeh et al. 2001) have been found to be defective in mismatch–repair (MMR), and up to 23 % of prostate cancers display a high level of microsatellite instability associated with mutations in MMR genes and deficient MMR protein expression (Norris et al. 2007; Prtilo et al. 2005; Sun et al. 2006).

This, in turn, may lead to high mutation rates among microsatellites, ending in a mutator phenotype.

As well, DNA polymorphisms in BER- or DNA-ssb repair associated *Xrcc1*, *Ogg1* and DNA polymerase- β genes have been associated with higher risk for prostate cancer (Chen et al. 2003; Rybicki et al. 2004; Xu et al. 2002).

Most of the molecular therapies targeting the key control pathways involved in prostate cancerogenesis and progression may indirectly influence the DNA-dsb repair activity of neoplastic cells. This has been observed for therapies inhibiting EGFR, IGFR, HDAC and proteasome pathways (Ma et al. 2003; Chinnaiyan et al. 2005; Dittmann et al. 2005a, b) have documented that the radiosensitization mediated by inhibiting EGFR can be related with the altered DNA-PKcs expression, function and cytoplasmic sequestration; as well, an increased DNA-dsbs (Rochester et al. 2005) has been shown to be induced by the inhibition of IGF-1R, via the altered ATM activation, and could be used in combination with radiotherapy (Choudhury et al. 2006) in hypoxic cancers. By converse, the RAS-mediated tumor cell radioresistance could be linked to the augmented DNA-dsb repair (Chang et al. 2005) induced by the use of farnesyl transferase inhibitors (FTIs) via the increase of Ku80 expression.

An additional crosstalk involves DNA-repair genes in human cancers. It concerns the occurrence of silencing DNA repair genes such as *MLH1* and *O*-6-methylguanine-DNA methyltransferase (*MGMT*) leading to microsatellite instability and a failure to repair DNA lesions (Jones and Baylin 2002). This phenomenon is still a matter of investigation in PC.

Another intriguing example of molecular co-sharing in PC is represented by the c-kit receptor (Pittoni et al. 2011). c-Kit receptor is normally expressed by prostate stem cells, that apparently require the c-Kit signaling for prostate regeneration (Leong et al. 2008) in humans, after hormone ablation, c-Kit expression may be observed in a considerable percentage of high-risk prostate cancer cells (Di Lorenzo et al. 2004) and in 10–30 % of this subset of prostate cancers NE differentiation occurs.

In normal prostate, a resident stromal population of mast-cells (MCs), also express the c-kit receptor (Leong et al. 2008).

It was initially thought that MCs can promote tumor growth of WD adenocarcinoma synthesizing MMP-9. MMP-9 has been indicated to correlate with progression of prostate tumor in humans (Castellano et al. 2008). As an ECM-degrading enzyme, it facilitates cell migration and invasion of tumor cells, allowing also the cleavage and activation of growth factors concurrently acting in tumor progression. In addition, peritumoral MCs were shown to stimulate prostate tumor growth in rats by providing proangiogenic factors (Johansson et al. 2010).

For these reasons, it was hypothesized that targeting MCs would be considered useful to counteract the growth of prostate cancers.

However, it was demonstrated, both in mouse and in humans, that poorly-differentiated prostate tumors with features of EMT show an autocrine production of MMP-9 and are devoid of infiltrating MCs.

This implies that MC inactivation would be ineffective when used in therapy for advanced and poorly-differentiated PC, and lead to the intriguing consideration that MC may contribute to the maintenance of prostate stem cell homeostasis and counteract NE tumor formation, serving as “natural decoys” that sequester stimulating growth factors, thus limiting c-Kit signaling in prostate cancer stem cells (Pittoni et al. 2011).

This hypothesis strongly discourages the idea of MC inhibition in PC. Otherwise, the therapeutic use of c-Kit tyrosine kinase inhibitors, such as imatinib, would instead offer the advantage of targeting both adenocarcinoma-promoting MCs (stroma targeting) and NE tumor variants (tumor targeting (Pittoni et al. 2010).

Overall, prostate cancerogenesis emerges as an extremely complex field, involving genetic and epigenetic alterations with multiple layers of merging.

We are still far away to use molecular classifications to unequivocally define different prognostic subcategories of prostate cancer. Key questions remain to be answered before the full range of mutations and genetic alterations will be elucidated.

Nevertheless, it is likely that, as for most of human cancers, also in PC the genes with a high incidence of mutation frequently participate to the evenience of abnormal epigenetic events, and this co-occurrence may be related, for instance, to the abnormal expansion of neoplastic stem cell population (Coussens and Werb 2002; Meng and Riordan 2006) which, in turn, may further select the addiction of oncogenic gene mutations, which drive PC cells to invasion, metastasis, and resistance to therapy.

Our knowledge of the intricated cross-links between genetic and epigenetic events occurring in PC has registered exciting progresses during the last decade.

The role of the three-dimensional texture and regulation of chromatin function in PC has been partially uncovered, and this has led to hypothesize the therapeutic use of drugs and small molecules such as HDAC inhibitors or DNA methylating and demethylating agents, acting as epigenetic modulators, as alternative or complementary tools for fighting aggressive PC (Murr 2010). The emerging data confirm that prostate carcinogenesis bases upon a definite group of interconnecting key signaling pathways.

Large scales of studies and carefully designed clinical trials will be required to validate novel effective therapeutic strategies for the treatment of PC.

The availability of next-generation sequencing will provide us with a broad genotyping platform which contribute to further refine our notions, shortening the time occurring to set-up multi-faceted molecular strategies tailored against the multiple molecular alterations responsible for the killing ability of advanced, androgen-independent, prostate cancers (Dias-Santagata et al. 2010) (Fig. 8.1).

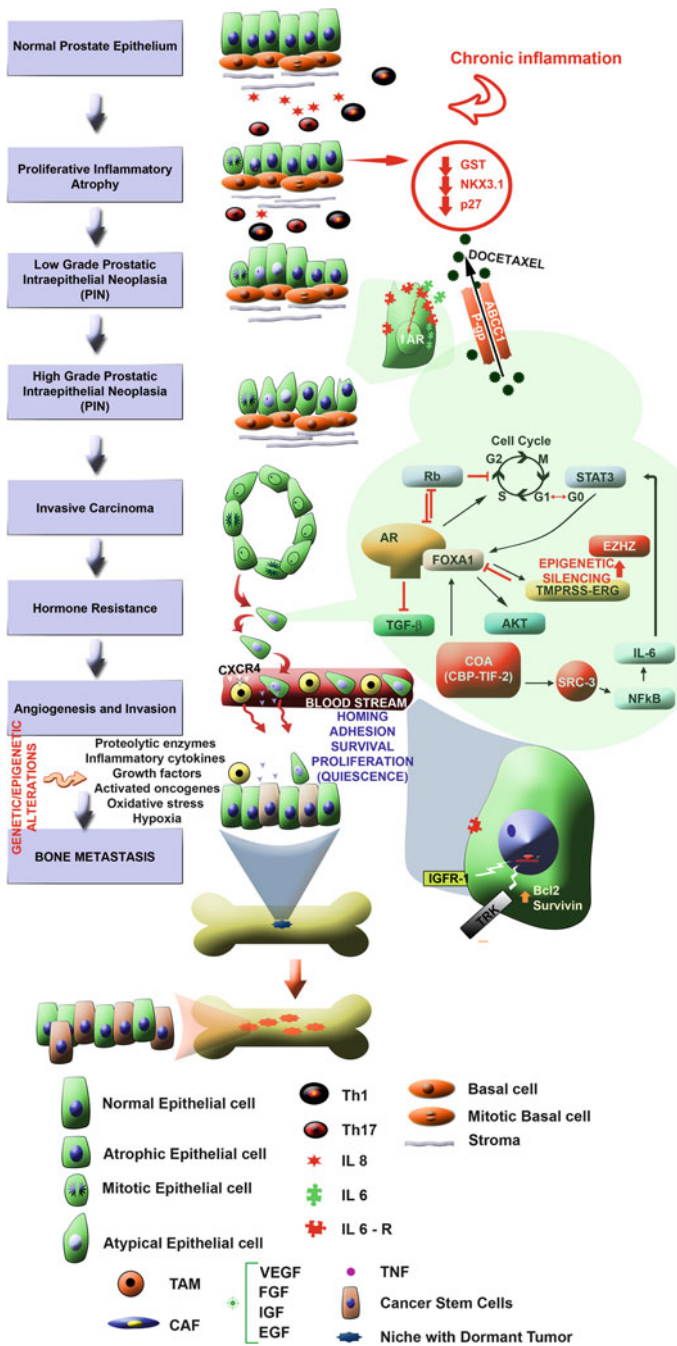


Fig. 8.1 Crossroads of molecular pathways involved in PCa. In this picture the most frequent intersections between the major molecular pathways promoting growth, survival, invasiveness, metastasis and therapy-resistance of PC cells, potentially targetable for therapeutic strategies are summarized

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