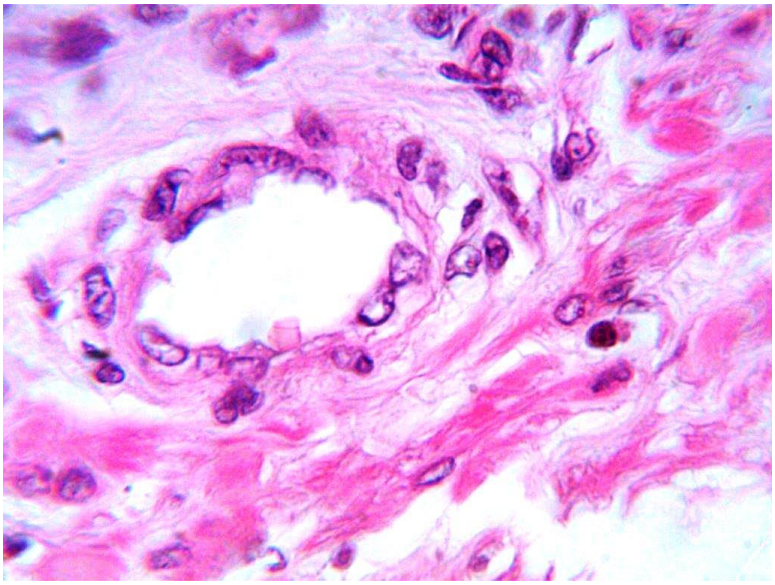

AR-V7: A Driver of Prostate Metastasis?

ABSTRACT

The discovery of AR-V7 a splicing variant of the androgen receptor provided an interesting window on the effects of splicing variants in cancer. This Note discusses the structure and functioning of AR-V7 and focuses on the splice variant characteristic. Many authors have proposed using it as a prognostic metric but it may also be a possible therapeutic target.

Terrence McGarty

TGL 178, August 2020

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Contents

1 INTRODUCTION

Prostate Cancer (PCa) is the most prevalent cancer in men. Many patients can survive with the indolent form but the more aggressive version can rapidly lead to death. A great deal has been learned about this malignancy but it still presents a complex and challenging disorder. There has been a multiplicity of reasons for the development and progression of the cancer and the questions are far from answered[1].

The action of the androgen receptor, AR, is a significant element in controlling this cancer in early stages. Namely ADT, or androgen depletion therapy can be effective. However, this may be effective for limited periods. This Note examines the recent use of an AR variant, AR-V7, expressed as mRNA to determine the state of the cancer and possibly use as a therapeutic target.

Before commencing on this Note it is useful to recognize that whenever we examine a "new" target we are really seeing the complexity of cancers. There is no single silver bullet. In this case we are looking at what is called a splicing variant. Simply the classic paradigm regarding DNA is that a gene is transcribed into RNA, then the RNA is translated into a protein. However, we know that DNA has introns and exons, thus much of the DNA is non-functional. Thus 3 billion base pairs code into 25,000 genes, and most of the base pairs are not recognized. Then the resultant RNA initially starts as a read of the total gene segment, including the introns and it is necessary to splice out these useless segments before we get an operable RNA. This splicing act is another place where errors can occur. Then the spliced mRNA is translated to a protein. Our focus with AR-V7 is what happens when the spliced mRNA and its product protein are produced and not suppressed. This is the case for the androgen receptor in PCa.

As Nimir et al have recently noted:

Detection of androgen receptor (AR) variant 7 (AR-V7) is emerging as a clinically important biomarker in castrate resistant prostate cancer (CRPC). Detection is possible from tumor tissue, which is often inaccessible in the advanced disease setting. With recent progress in detecting AR-V7 in circulating tumor cells (CTCs), circulating tumor RNA (ctRNA) and exosomes from prostate cancer patients, liquid biopsies have emerged as an alternative to tumor biopsy. Therefore, it is important to clarify whether these approaches differ in sensitivity in order to achieve the best possible biomarker characterization for the patient. In this study, blood samples from 44 prostate cancer patients were processed for CTCs and ctRNA with subsequent AR-V7 testing, while exosomal RNA was isolated from 16 samples and tested. Detection of AR and AR-V7 was performed using a highly sensitive droplet digital PCR-based assay.

AR and AR-V7 RNA were detectable in CTCs, ctRNA and exosome samples. AR-V7 detection from CTCs showed higher sensitivity and has proven specificity compared to detection from ctRNA and exosomes. Considering that CTCs are almost always present in the advanced prostate cancer setting, CTC samples should be considered the liquid biopsy of choice for the detection of this clinically important biomarker.

[1] See https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach for a discussion of PCa circa 2010.

Thus the use is as a biomarker. Yet the very presence of AR-V7 means that the PCa has taken on an androgen independent existence and any attempt to use ADT would be futile. Many therapeutics target AR deprivation and thus they would become ineffective.

As Feng and He have noted:

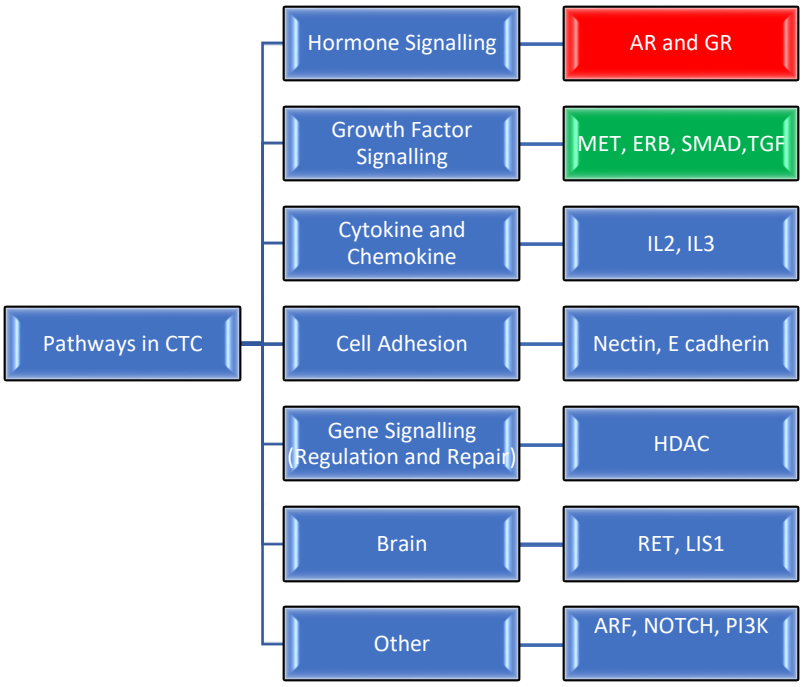
Most prostate cancers are androgen-sensitive malignancies whose growths depend on the transcriptional activity of the androgen receptor (AR).

In the 1940s, Charles Huggins demonstrated that the surgical removal of testes in men can result in a dramatic improvement in symptoms and can induce prostate cancer regression. Since then, androgen deprivation therapies have been the standard first-line treatment for advanced prostate cancer, including: surgical castration, medical castration, antiandrogens, and androgen biosynthesis inhibitors.

These therapies relieve symptoms, reduce tumor burden, and prolong patient survival, while having relatively modest side effects. Unfortunately, hormone deprivation therapy rarely cures the cancer itself. Prostate cancer almost always recurs, resulting in deadly castration-resistant prostate cancer. The underlying escape mechanisms include androgen receptor gene/enhancer amplification, androgen receptor mutations, androgen receptor variants, coactivator overexpression, intratumoral de novo androgen synthesis, etc. Whereas, the majority of the castration-resistant prostate cancers continuously rely on the androgen axis, a subset of recurrent cancers have completely lost androgen receptor expression, undergone divergent clonal evolution or de-differentiation, and become truly androgen receptor-independent small-cell prostate cancers.

There is an urgent need for the development of novel targeted and immune therapies for this subtype of prostate cancer, when more deadly small-cell prostate cancers are induced by thorough androgen deprivation and androgen receptor ablation.

There is a growing interest in circulating tumor cells and related markers for both diagnostic and prognostic purposes. It is essential to understand what these markers are and what they reflect in the state of the cells and the specific cancers. As we have noted above, AR and AR-V7 are markers for use in PCa. AR in a sense drives PSA, a standard marker for PCa. Now from Miyamoto et al in their discussion of circulating tumor cells (CTC) in regards to AR inhibitors they present data summarized below:



The above is noted by the authors as follows:

Signaling pathways enriched in prostate CTCs. Molecular pathways from the PID up-regulated in CTCs versus primary tumors (excluding those enriched in metastases compared with primary tumors), organized by PID categorization. Abbreviations (other than proteins, clockwise from top): HDAC, histone deacetylase; AJ, adherens junction; IL2 and IL3, interleukins; ERBB1, epidermal growth factor receptor B1; TGFBR, transforming growth factor-b receptor

They note as follows:

Prostate cancer is initially responsive to androgen deprivation, but the effectiveness of androgen receptor (AR) inhibitors in recurrent disease is variable. Biopsy of bone metastases is challenging; hence, sampling circulating tumor cells (CTCs) may reveal drug-resistance mechanisms. We established single-cell RNA-sequencing (RNA-Seq) profiles of 77 intact CTCs isolated from 13 patients (mean six CTCs per patient), by using microfluidic enrichment. Single CTCs from each individual display considerable heterogeneity, including expression of AR gene mutations and splicing variants. Retrospective analysis of CTCs from patients progressing under treatment with an AR inhibitor, compared with untreated cases, indicates activation of noncanonical Wnt signaling (P = 0.0064). Ectopic expression of Wnt5a in prostate cancer cells attenuates the antiproliferative effect of AR inhibition, whereas its suppression in drug-resistant cells restores partial sensitivity, a correlation also evident in an established mouse model. Thus, single-cell analysis of prostate CTCs reveals heterogeneity in signaling pathways that could contribute to treatment failure

Their focus was on Wnt. Herein we focus on AR and more specifically on AR-V7. The focus is done to bring out the import of splicing variants. Splicing variants are simply mixed transcriptions of genes which result in mRNAs whose resulting proteins are reflected in the

enhancement, suppression, or others changes in the overall expression of genes that allow growth and proliferation. The new mRNA results in a loss of homeostasis in the cell.

We discuss AR-V7 in this context. The issue we are concerned about is not just its presence but two other issues. First, what caused this splice variant? Was it a result of a single progression in the cell or were the tumor microenvironment a significant player here as well? Second, if we believe that AR-V7 is a primary mover for metastatic behavior, can we find a therapeutic target to suppress it?

2 DNA, RNA AND SPLICING

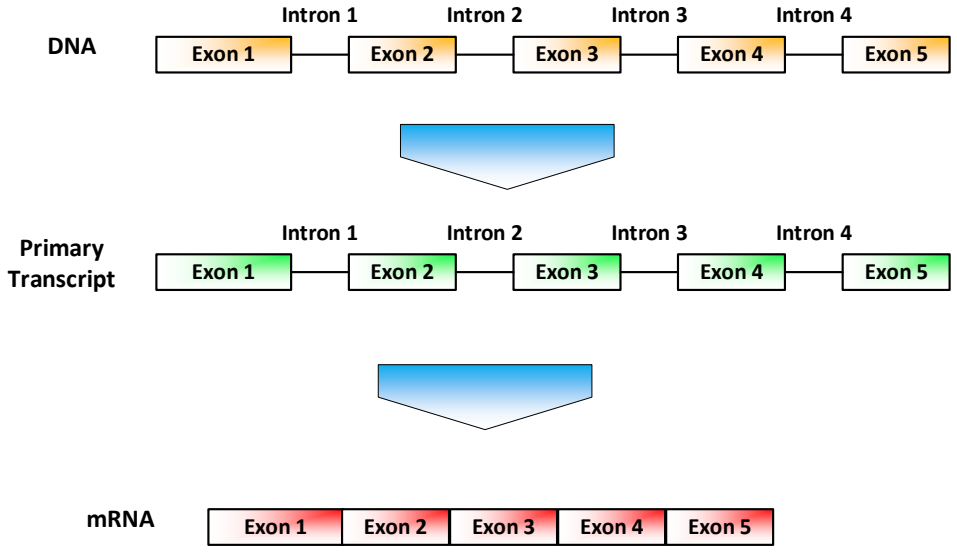
The Paradigm for DNA, mRNA and proteins was a result of the classic paper by Watson and Crick. But as most things there are details that make it much more complex. Also, when examining various cancers we often focus on genes and aberrant pathways. The challenge is to consider how they become aberrant. We have also examined the tumor micro environment, the fibroblasts, macrophages and other external influences which can lead to and support cancerous growth. However, there is also the micro level of such things as miRNAs which can interfere with the normal regulatory DNA expression.

2.1 SPLICING

In 1993 Sharp and Roberts were awarded the Nobel Prize for their work in splicing. Simply, splicing is the process wherein a gene sequence, including introns and exons generate an RNA molecule which is then cut and pasted to yield mRNA. A gene sequence may include orders of magnitude more bases than what would encode the mRNA, the useless ones, namely those not to be included, get spliced out to form the mRNA used to generate the proteins which in turn control cellular functions.

We now review the issue of splicing and then will consider the issue of variants.

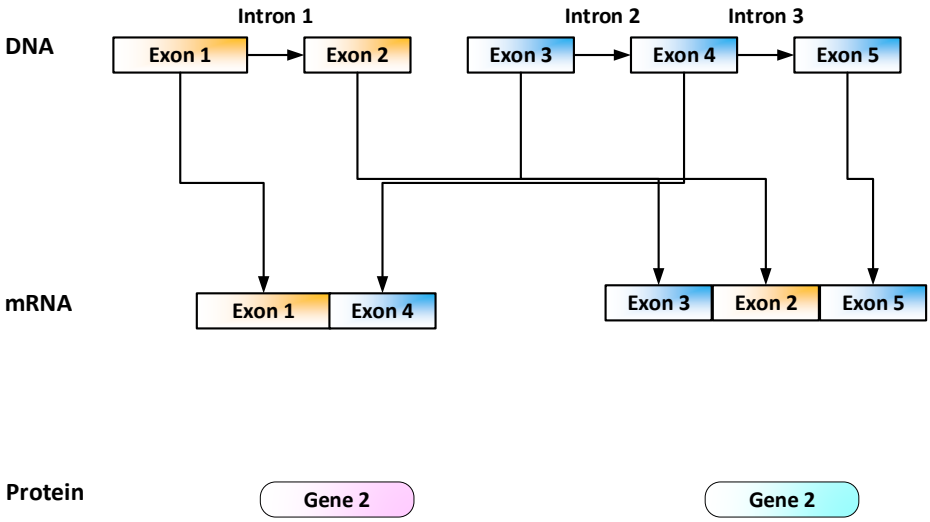
We begin with a basic principle. Namely we have a DNA segment with exons and introns. Pol II transcribes the entire piece while spliceosomes remove the Intro transcribes and produce an MRNA[2].



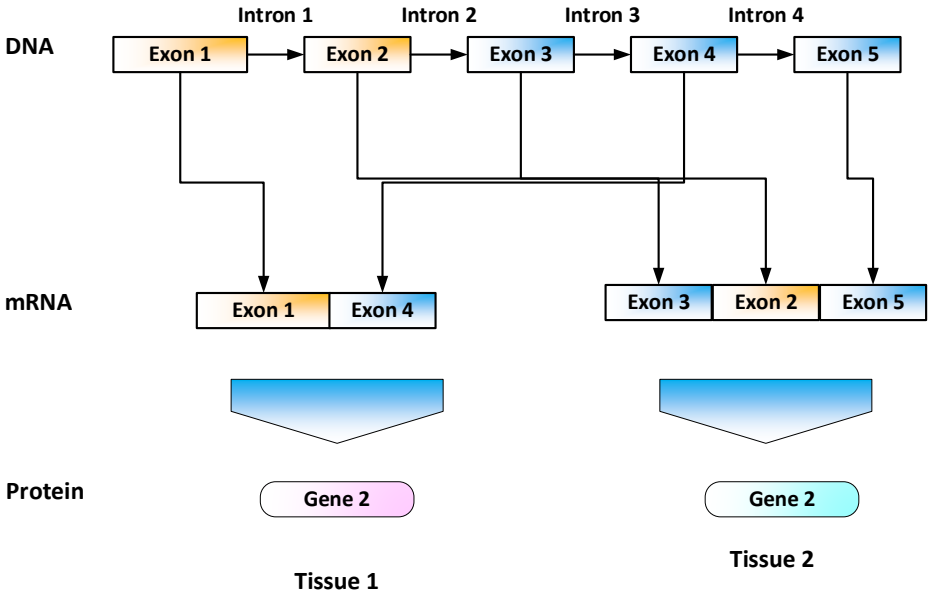
[2] We rely on Latchman for much of this discussion. Also it is worth reviewing the Sharp material as well.

Now there are multiple variants of this scheme. Ones where we can from the same gene produce two different mRNA and in turn proteins. Consider the example below:

We could envision two genes and the creation of two proteins by crossing over of Exons from one segment to the other. We depict this below:



Likewise we can consider the same gene, a segment of Exons and Introns, depending on the possible transcription factors or other similar effects creating two separate proteins from the same gene as shown below.



We can consider a wide variety of other underlying configurations from similar gene pools.

2.2 SPLICING VARIANTS

We noted the variants above. There are a large number of possible sources of splicing variants. We shall focus on one herein, the AR-V7 variant, but with AR alone there are multiple possible variants.

As Sharp (2005) had noted:

It has been estimated that >50% of all human genes are, at some stage, expressed by alternative splicing. Interestingly, a subset of 2000 of these alternatively spliced exons can be computationally identified on the basis of a high evolutionary conservation of flanking sequences. This suggests that, for these genes, the different isoforms have crucial functions. Although there are many documented examples of regulation of alternative splicing, few have been analyzed in detail. For example, the cell-surface protein CD44 is alternatively spliced by inclusion of different combinations of ten variable exons.

Activation of the Ras pathway promotes inclusion of the variable exons that produces CD44 isoforms that mediate cellular invasion of surrounding tissue and recognition of certain growth or scattered factors. This theme of alternative splicing of a set of exons with the various isoforms having different function probably explains the paradox of the obvious difference in the complexity of humans and worms despite the fact that both organisms contain approximately the same total number of genes. It has been estimated that three times more alternatively spliced forms are expressed on average per human gene than per nematode or Drosophila gene

It also explains how so few genes can create so many differing effects. The complexity of variant splicing can be affected by internal and external cellular modulators from transcription factor, miRNA, epigenetic factors like methylation and the like.

Recently Takayama has discussed AR splicing. He notes:

Protein-coding exons are disrupted by non-coding introns. Through the development of genome sequencing analysis, it has become clear that precursor messenger RNA (pre-mRNA) splicing can occur at a large scale and with biological complexity.

Biochemical studies have demonstrated that the RNA cleavage and ligation reactions necessary for intron removal in protein-coding mRNAs occur in a large ribonucleoprotein (RNP) machine called the spliceosome. Alternative splicing pathways result in the expansion of the human proteome and many physiological mechanisms. One of the underlying rationales for the purification of discrete splicing complexes is to determine the structure of these defined intermediates along the splicing pathway. Because of the dynamic nature of the spliceosome, structural biologists have predominantly used electron microscopy to assess the structures of a variety of spliceosomal complexes, as well as isolated snRNPs.

Mechanistically, both the regulatory RNA sequences and their correlated RNA splicing proteins are important for determining splicing site. Some regulatory sequences are called exonic splicing enhancers (ESEs) or intronic splicing enhancers (ISEs) [29]. Moreover, RNA splicing is

closely associated with transcriptional process. Both transcription initiation and elongation rates affect splicing.

The spliceosomal small nuclear ribonucleoproteins (snRNPs) (U1, U2, U4, U5, and U6) interact with the transcribed RNA stepwise to effect the removal of an intron from a precursor messenger RNA (pre-mRNA) including exons [30]. Base pairing of snRNAs to conserved sequences on pre-mRNA, as well as interactions of numerous splicing accessory proteins and RNA–protein interactions, are critical in guiding the huge spliceosomal complex to the sites of pre-mRNA for splicing.

These sites can be either intronic or exonic and can be positive (splicing enhancers) or negative (splicing silencers). In addition to RNA-protein binding, RNA-RNA base pairing can define the splicing site. Connective networks have been formed between chromatin modifications, RNA polymerase II speed, and alternative splicing patterns.

During transcription elongation, these protein complexes move along the gene. The RNA spliceosome complex screens the pre-mRNA to determine and excise the splice sites before the termination of transcription [32]. Thus, the expression of a specific splice variant is regulated by both gene transcription rate and the splicing factor binding to the pre-mRNA during the splicing process.

Recent progress in the field has demonstrated that the catalytic center of the spliceosome is also composed of RNA, so it has been confirmed that the spliceosome is a ribozyme, similar to the ribosome. In addition, investigations have isolated, purified, and characterized the protein composition and biochemical activities to determine the structures of several of these distinct forms of the spliceosome as they proceed along the reaction pathway.

Spliceosome assembly needs to occur repeatedly every time an intron is removed from a pre-mRNA in a eukaryotic nucleus. An intron includes four consensus elements: (i) the 5' splice site (5' SS), which is located at the 5'end of the intron; (ii) the 3' SS, which is located at the 3' end of the intron; (iii) the branch point sequence (BPS), which is located upstream of the 3' SS; and (iv) the polypyrimidine tract, which is located between the BPS and the 3' SS.

These sequences allow the spliceosome to recognize introns and to distinguish introns from exons. The 5' SS contains a GU dinucleotide sequence, whereas the 3' SS contains an AG dinucleotide in majority of introns.

However, these two sequences cannot be used to identify an intron. A variable stretch of pyrimidine nucleotides, which is known as the polypyrimidine tract, is situated between the 3' SS and the BPS. The polypyrimidine tract defines the 3' SS and recruits splicing factors to the 3' SS and BPS. The BPS initiates a nucleophilic attack on the 5' SS, making a branch-like structure. The BPS includes a conserved adenosine nucleotide at which to initiate the splicing process. Five snRNP complexes (U1, U2, U4/U6, and U5) assemble and recognize on each intron to form a catalytically active spliceosome. The spliceosome functions in a dynamic and complex cycle of assembly, reaction, and disassembly.

The earliest steps of spliceosome assembly are the recognition of the 5' SS and BPS by the U1 and U2 snRNPs through base-pairings. The first complex (complex E) is achieved by the binding of: (i) U1 snRNP to the 5' SS; (ii) splicing factor 1 (SF1) to the BPS; (iii) U2 small nuclear RNA auxiliary factor 2 (U2AF2) to the polypyrimidine tract;

2.3 ISSUES

When we examine splice variants, we see the process but seem to lack the mechanism and drivers.

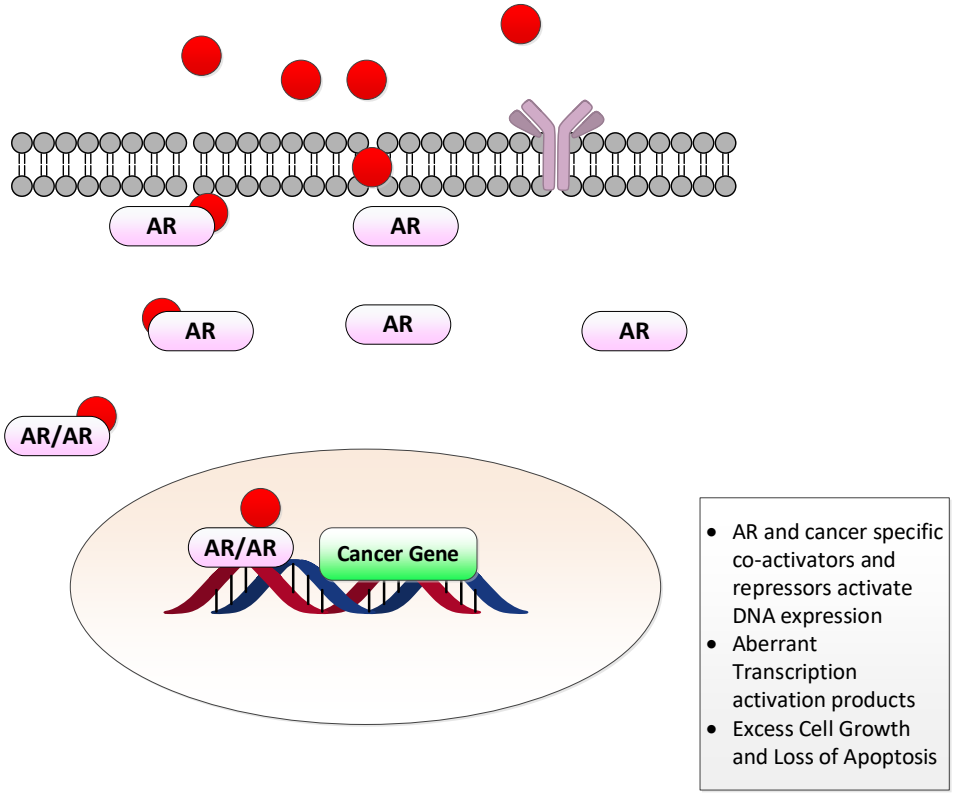
3 AR PATHWAYS

The Androgen Receptor, AR, is located on Xq12. Androgens mediate a wide range of developmental and physiological responses and are especially important in male sexual differentiation and pubertal sexual maturation, the maintenance of spermatogenesis, and male gonadotropin regulation. The principle steroidal androgens, testosterone and its metabolite DHT (5-Alpha-Dihydrotestosterone), mediate their biological effects predominantly through binding to the AR (Androgen Receptor), an androgen-inducible member of the nuclear receptor super-family of transcription factors.

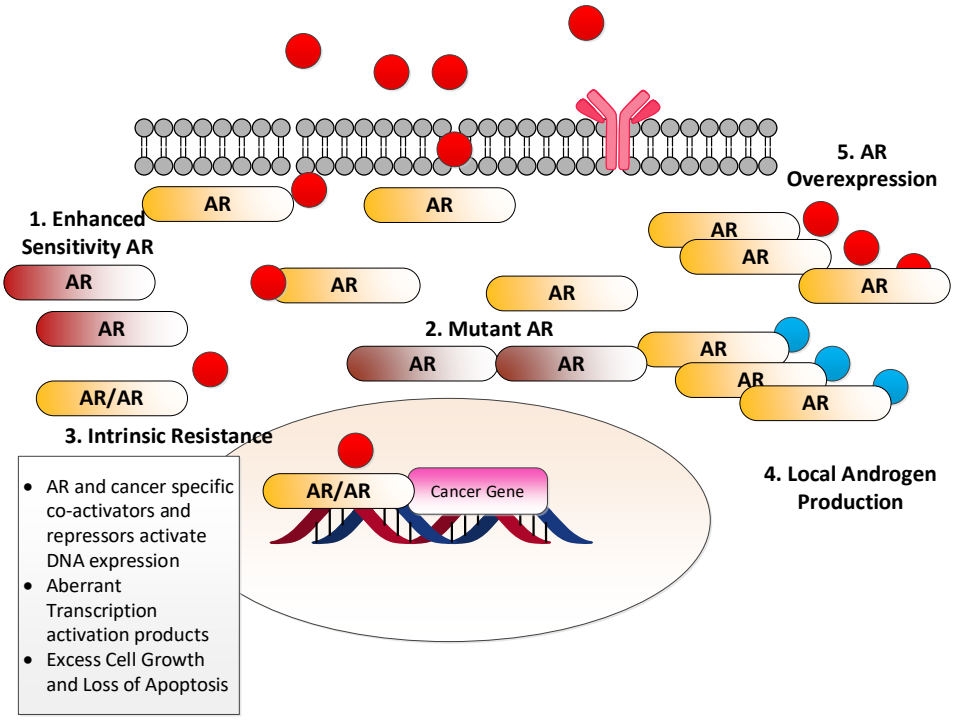
The normal function of the Androgen Receptor is as follows:

1. Testosterone enters the cell
2. If 5-α-Reductase is present the testosterone is converted to dihydrotestosterone, DHT.
3. The DHT then binds with the AR and the entity undergoes a transformation and releases heat shock proteins, HSPS
4. Then there is a phosphorylation
5. The AR translocates to the nucleus where it dimerizes, and there is DNA binding.
6. Target genes are then transcribed.

AR mediates transcription of proteins which are essential for normal development. However as PCa progresses there is at first normal AR operation, then it is enhanced, and then the PCa which was dependent upon the AR function can become independent of it altogether. We depict that process below. In normal AR operations, we show below the Testosterone coming into the cell and then it binds with the AR. It is this normal bonding which gives the AR the ability to manage a significant portion of the normal growth of the prostate cell. We use the graphics from Turner (2010) as modified below:



In the case of PCa we see the AR playing the role of excess growth enhancer.



As is best understood, the progression towards AR resistant PCa follows the path shown below.

Enhanced AR Sensitivity	The reduced androgens interact with increased ARs so that the efficiency is increased
AR Mutations	The ARs are mutated so that they are more efficient
Alternative Signalling Cascades	The signalling channels on the cells surface may be modified
AR Overexpression	The ARs may have an ability to excessively produce excess receptors
Local Androgen Expression	Local internal cytoplasmic androgen production may occur
Intrinsic Resistance	Cancer cells may be those with low intrinsic resistance

When the cell becomes refractory to AR functions, there may at first be AR overexpression and then a set of PCa specific receptors develop which result in metastatic growth.

The work by Niu et al and that of Vis and Schroder describe in detail many of the specifics of the operation of the AR as currently understood. As regards to some details on specific pathway expressions the work of Nantermet et al states:

The androgen receptor (AR), when complexed with 5-dihydrotestosterone (DHT), supports the survival and proliferation of prostate cells, a process critical for normal development, benign prostatic hypertrophy, and tumorigenesis. However, the androgen-responsive genetic pathways that control prostate cell division and differentiation are largely unknown.

To identify such pathways, we examined gene expression in the ventral prostate 6 and 24 h after DHT administration to androgen- depleted rats. 234 transcripts were expressed significantly differently from controls ($p < 0.05$) at both time points and were subjected to extensive data mining. Functional clustering of the data reveals that the majority of these genes can be classified as participating in induction of secretory activity, metabolic activation, and intracellular signaling/signal transduction, indicating that AR rapidly modulates the expression of genes involved in proliferation and differentiation in the prostate.

Notably AR represses the expression of several key cell cycle inhibitors, while modulating members of the ***wnt and notch signaling pathways****, multiple growth factors, and peptide hormone signaling systems, and genes involved in MAP kinase and calcium signaling. Analysis of these data also suggested that* ***p53 activity is negatively*** *regulated by AR activation even though p53 RNA was unchanged. Experiments in LNCaP prostate cancer cells reveal that AR* ***inhibits p53 protein accumulation*** *in the nucleus, providing a post-transcriptional mechanism by which androgens control prostate cell growth and survival. In summary these data provide a*

comprehensive view of the earliest events in AR-mediated prostate cell proliferation in vivo, and suggest that nuclear exclusion of p53 is a critical step in prostate growth.

The authors continue:

AR induces cell proliferation and apoptosis in part because of its effects on cell-cell communication, particularly the stromal-epithelial interaction. As expected, the insulin-like growth factor (IGF-1) signaling system, which plays an essential role in prostate growth, was regulated at the level of ligand (IGF-1 was induced), extracellular binding protein (IGF-BP3 was repressed), and receptor (the IGF-1 receptor-1 exhibited biphasic expression). Also as expected, epidermal growth factor, which is induced by androgens in the prostate epithelium was upregulated. In addition to these well studied factors, several genes with potentially novel roles in the prostate were identified.

These include the transforming growth factor-2 (TGF-2) secretory partner latent TGF-binding protein- 1 (Ltbp1), which was repressed. Although the role of TGF-proteins in growth repression has been documented (e.g. Ref. 44), latent TGF-binding protein 1 function in the prostate has not been extensively studied, although its expression might be frequently reduced in PCa. DHT also repressed granulin/epithelin (Grn), a cysteine-rich growth factor expressed throughout the reproductive tract that regulates growth in multiple epithelial cell types.

Given the role of Grn in certain epithelial neoplasias, it would be interesting to examine its expression in PCa. Finally, DHT led to the down-regulation of ephrin-A1/B61 (Efna1), a ligand for the Eph receptors expressed in various epithelia. EFNA1 regulates cell growth and inhibits tumor angiogenesis; its function in the prostate is unknown.

In short, other than p53 suppression, and a collection of other genes, there is not significant addition to what is already known. The Chen and Sawyers discussion ends with:

Despite the fact that AR occupies such a central role in prostate physiology and pathology, there ***is little insight into the direct AR target genes responsible for disease progression****. One very intriguing possibility, based on the high frequency of TMPRSS2-ETS fusion, is that the primary effect of antiandrogen therapy is to reduce expression of this presumed oncogene. Even though AR is overexpressed in more advanced stages of prostate cancer, recent profiling studies indicate that many AR target genes are actually expressed at lower levels in high-grade and metastatic lesions.*

This finding is consistent with older data that cancers with a high Gleason grade often produce lower levels of PSA and other markers of differentiation. Therefore, the relationship between the AR pathway and castrate resistance may also reflect the differentiation state of late-stage tumors. A small subset of very aggressive prostate cancers (small cell variant) does not express AR.

Clearly the importance of the AR is critical in PCa as it progresses and yet as noted above the full pathway development is still lacking. From DeVita et al:

Androgen Receptor Pathway: Castration represents the oldest form of targeted therapy.14 Because the majority of castrate resistant prostate cancers still depend on AR signaling (discussed previously), novel therapies targeting this pathway are in development (Fig. 40.5.3). Although the current AR antagonists in clinical practice (e.g., flutamide, bicalutamide, nilutamide) are effective in men who fail castration therapy, the duration of response is generally short.

One possible explanation is that the mechanisms that underlie castration resistance, such as increased expression of AR, can also alter the cellular response to these antagonists, leading to paradoxical agonism that manifests clinically as the antiandrogen withdrawal syndrome.20 Novel antiandrogens that inhibit AR function despite overexpression are entering clinical trials.51 An alternative strategy is to block the production of residual androgen by the adrenal gland with ketoconazole, which inhibits p450 enzymes in androgen synthesis, or the experimental agent abiraterone, a 17-alphahydroxylase/C(17,20)-lyase inhibitor with promising activity in early-phase clinical trials.52 Residual androgen production may not be a purely endocrine problem.

Castrate-resistant prostate cancers can also express genes involved in androgen synthesis, suggesting an autocrine mechanism that may also need to be targeted.24 Indirect approaches to target AR function are also worth consideration. In the cytoplasm, AR is stabilized by binding to the molecular chaperone, heat shock protein 90 (HSP90). HSP90 inhibitors were identified in a broad screen for chemical compounds that inhibit AR signaling. Indeed, the HSP90 inhibitor 17-AAG destabilizes AR and causes regression of prostate cancer in preclinical models.53,54

Dynamic control of histone acetylation is critical for control of transcription, raising the possibility that these agents might block expression of AR target genes. The histone deacetylase inhibitor SAHA has been shown to inhibit the transcription of PSA and has antitumor activity in preclinical models of prostate cancer. Both HSP90 and HDAC inhibitors are in early-phase clinical trials. The ability of various kinase pathways to activate AR function in models raises the possibility that kinase inhibitors may be effective in prostate cancer.

Agents targeting EGFR or HER2 (erlotinib, trastuzumab, pertuzumab) have been studied clinically but showed disappointing results as measured by objective response. The reasons for nonresponse are unclear and may reflect the need for combination therapy and/or molecular criteria for selecting patients whose tumors may be kinase-dependent

3.1 AR STRUCTURE

We now examine the structure of the AR. This is important since it sets the stage for understanding how splicing variants operate. As Saranyutanon et al note:

Androgen receptor (AR) is a nuclear transcription factor and a steroid hormone receptor. The gene encoding for the AR is located on the chromosome Xq11.2-q12, consists of 8 exons and spans 186,587 kb

The structure of AR bears high similarities with other steroid receptors such as progesterone receptor (PR), estrogen receptor (ER), glucocorticoid receptor (GR), and thyroid hormone receptor (TR).

It has four domains:

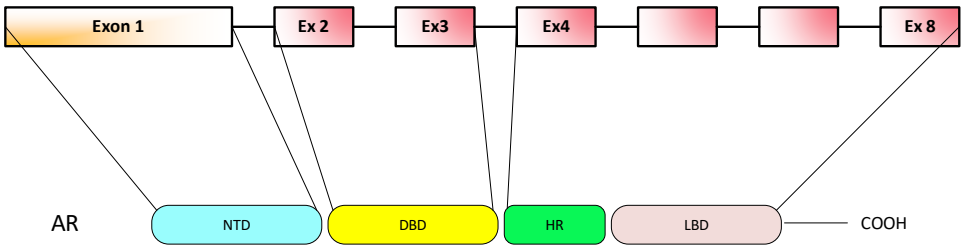
1. a moderately conserved ligand-binding domain (LBD),

2. a highly conserved DNA-binding domain (DBD),

3. a poorly conserved N-terminal domain (NTD), and

4. the hinge region separating the LBD from the DBD.

The structure of each domain is critical to its functioning. It is the binding and activation capacity that drives the ultimate results. The authors depict this as shown as follows:



Now they continue in some detail:

*The **NTD** accounts for more than half of the size of the AR, and is encoded by exon 1.*

Within the NTD, an Activating factor 1(AF1) region is present, which is considered its primary effector region. The AF1 consists of 2 transcription activation units i.e., Tau-1 (aa 100-370) and Tau-5 (aa 360-485) and both of which are indispensable for the complete transcriptional activity of the AR. Tau-1 contains FQNLF motif (aa 23-27) and Tau-5 contains the WHTLF motif (aa 433-437), which are critical in mediating the ligand-dependent, inter-domain interaction between the NTD and the LBD. NTD-LBD interaction is important for the stability of the AR dimer complex.

The NTD also contains a poly-glutamine sequence encoded by a CAG triplet repeat sequence, which begins at codon 58, and CAG repeat length has often been inversely associated with the risk of getting prostate cancer.

***DNA-binding domain (DBD) is encoded by exons 2 and 3, and comprised of two zinc finger motifs.** Exon 2 encodes for the a-helix N-terminal zinc finger, which interacts with the nucleotides located in the hormone response element in the DNA major groove. Exon 3 encodes the second zinc finger containing a conserved D-box motif (ASRND). The selectivity to specific*

DNA response element is known to be achieved by AR dimerization through the Distal box region that allows the AR to bind to direct repeat half-sites in its promoter. The nuclear localization signal present at the junction between the DBD and the hinge region is important for the nuclear import of the AR.

Ligand binding domain (LBD) is encoded by exons 4-8 and required for the binding of AR to its ligands, testosterone and dihydrotestosterone (DHT). *Majority of the AR point mutations in prostate cancer have been identified in the LBD suggesting their importance in pathobiology associated with aberrant AR signaling. Hinge region is a short amino acids sequence that possesses a bipartite ligand-dependent nuclear localization signal (NLS) and helps in the nuclear transport of the AR.*

Furthermore, several AR splice variants (AR-V1, -V7, and -V9) lacking LBD are also commonly reported. *Another splice variant, AR-V567es is also reported in prostate tumor*. The lack of the LBD is a critical change that seems to allow some form of androgen independence and ongoing gene activation. We shall see that this specific change, resulting from a splice variant, is both a challenge and an opportunity.

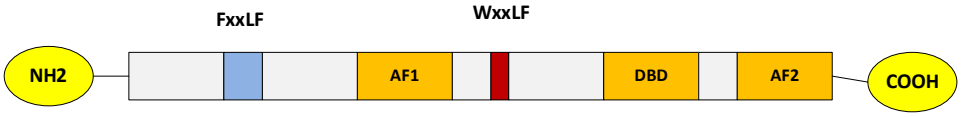
Studies have documented that even though some AR variants lack LBD, they retain their ability to bind to DNA and promote constitutive gene transcription in the absence of androgens. As per the canonical model of AR action, inactive AR resides in the cytoplasm sequestered by multiple chaperones and co-chaperones (such as heat shock proteins, FKBP51, FKBP52, and Cyp40), and cytoskeletal proteins. Ligand binding to the LBD of AR triggers dissociation of chaperone proteins inducing conformational changes that enable AR dimerization and interactions with a cytoskeletal protein, Filamin A to facilitate its nuclear translocation. Nuclear AR binds to the androgen response elements (AREs) in the promoter and enhancer regions of the downstream target genes and forms transcriptional coactivator complexes that remodel the chromatin structure to access the target initiation site.

Then, the complex stabilizes the RNA pol II machinery for repeated rounds of transcription. In parallel, ligand-independent activation of AR signaling via growth factors- and cytokines-mediated mechanisms has also been reported. For example, IL-6 binding to the IL-6 receptor causes MAPK- and STAT3- mediated transcriptional activation of AR.

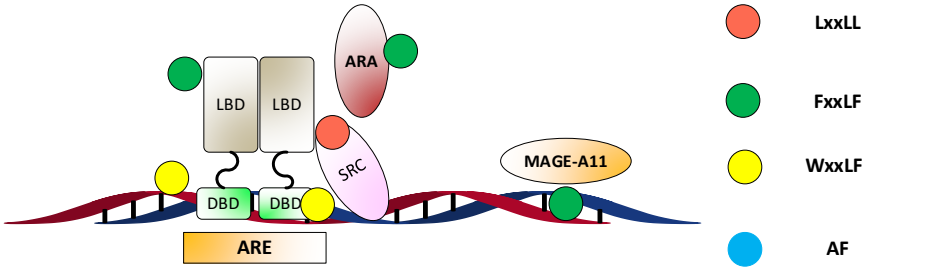
Similarly, EGF in the absence of androgen is also shown to transcriptionally activate AR via RAS/RAF/MAPK and PI3K/Akt pathways*. Besides this genomic mode of action of AR, many of the cellular responses of androgens do not require AR-mediated changes in gene expression. It is established that ligand-bound AR can associate with other molecular effectors in the cytoplasm and inner part of the plasma membrane as well to activate cell signaling cascades. AR-NTD is shown to interact with the SH3 domain of Src and this interaction results in unfolding of Src and subsequent autophosphorylation through.*

The EGF activation is also a critical pathway independent of AR control. We shall discuss this briefly later.

Feng and He have detailed AR as follows:



The primary sequence of the androgen receptor contains several functional domains: NH2-terminal Activation Function 1 (AF1), the central DBD, the carboxyl-terminal LBD, and two AR-specific FxxLF and WxxLF motifs



Schematic diagram of homodimeric androgen receptor bound to a palindromic **androgen response element** (ARE). D**imerization of the androgen receptor is mediated by both DBD and LB**D. Shown in the diagram are FxxLF motif-mediated N/C interaction, recruitment of the SRC/p160 by AF1 and AF2, recruitment of FxxLF motif-containing ARA proteins by AF2, and recruitment of MAGE-A11 through the AR NH2-terminal extended FxxLF motif. Competition likely exists among different FxxLF, WxxLF, and LxxLF motifs for binding to the same AF2 site on AR LBD (1). SRC, steroid receptor coactivator; ARA, AR-associated protein; AF1, activation function 1; AF2, activation function 2, a hydrophobic cleft in the LBD; ARE, androgen response element; DBD, DNA binding domain; LBD, ligand binding domain

The above demonstrates the complex structure of the AR dimer and its related elements as part of the DNA promoter process.

3.2 AR Actions

Now we examine the actions that AR assists in. From Feldman and Feldman, we have the classic description as of 2001:

Why do prostate cancer cells normally need androgens to grow and survive? Prostate cancer growth depends on the ratio of cells proliferating to those dying.

Androgens are the main regulator of this ratio by both stimulating proliferation and inhibiting apoptosis. So, prostate cancer depends on a crucial level of androgenic stimulation for growth and survival. Androgen ablation causes cancer regression because without androgen, the rate of cell proliferation is lower and the rate of cell death is increased, leading to extinction of these cells.

Testosterone — the main circulating androgen — is secreted primarily by the testes, but is also formed by peripheral conversion of adrenal steroids. It circulates in the blood, where it is bound to albumin and sex-hormone-binding globulin (SHBG), with a small fraction dissolved freely in the serum. When free testosterone enters prostate cells (BOX 2), 90% is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase (SRD5A2). DHT is the more active hormone, having fivefold higher affinity for the androgen receptor (AR) than does testosterone.

The AR is a member of the steroid–thyroid–retinoid nuclear receptor superfamily. It is composed of an amino terminal ACTIVATING DOMAIN, a carboxy-terminal ligand binding domain and a DNA-binding domain in the mid-region that contains two ZINC FINGERS. Like other nuclear receptors, in the basal state, the AR is bound to HEAT-SHOCK PROTEINS and other proteins in a conformation that prevents DNA binding. Binding to androgens induces a conformational change in the AR that leads to dissociation from the heat-shock proteins and receptor phosphorylation6, in part mediated by protein kinase A7.

The ligand-induced conformational change facilitates the formation of AR homodimer complexes that can then bind to ***ANDROGEN RESPONSE ELEMENTS (AREs)*** *in the promoter regions of target genes. The activated DNA-bound AR homodimer complex recruits co-regulatory proteins, co-activators or corepressors, to the AR complex. As in other nuclear receptors, the ligand-induced, activated conformation involves a shift in the position of helix 12 of the receptor to form a surface to which co-activators can bind. The co-activators allow interaction of the AR complex with the GENERAL TRANSCRIPTION APPARATUS to stimulate or inhibit target gene transcription. Many AR target genes have been identified3, and additional ones are being discovered using cDNA microarray technology.*

They then list several ways in which AR can act in what they call pathways to cause the continuation of the malignancy:

Type	Pathway	Ligand dependence	AR dependence	Mechanism
1	Hypersensitive AR	Androgen dependent	AR dependent	Amplified AR Sensitive AR Increased DHT Androgen antagonists in LNCaP cells and ARccr cells Illicit stimulation by Corticosteroids non-androgens Coregulator mutations 'Flutamide withdrawal' (antagonists acting as agonists)
3	Outlaw AR	Androgen independent	AR dependent	Mutant PTEN Ligand independent Amplified HER-2/neu Activated PI3K Activated MAPK Mutant coregulators
2	Promiscuous AR	Pseudo-androgens	Dependent on a mutant AR	Widened AR specificity
4	Bypass AR	Androgen independent	AR independent	Parallel or alternative survival pathways • Overexpression of BCL2 • Activation of other oncogenes • Inactivation of tumour suppressor genes
5	Lurker cells	Androgen independent	AR independent	Malignant epithelial stem cells

Over the past two decades much of the above has been modified and clarified. Impacts of the tumor micro environment, miRNAs, epigenetic factors, and gene products not then fully understood have added to the above. However, the above does present a useful paradigm for consideration.

3.3 BINDING SITES

The next issue beyond structure is how AR functions starting with the binding and binding sites. As Jin et al note:

For decades, understanding of AR-mediated transcriptional regulation was largely built upon the analysis of a handful of androgen-induced genes, one prototype of which is PSA. AR has been shown to form homodimers which preferentially bind DNA that contains ***androgen-responsive elements (AREs)****.*

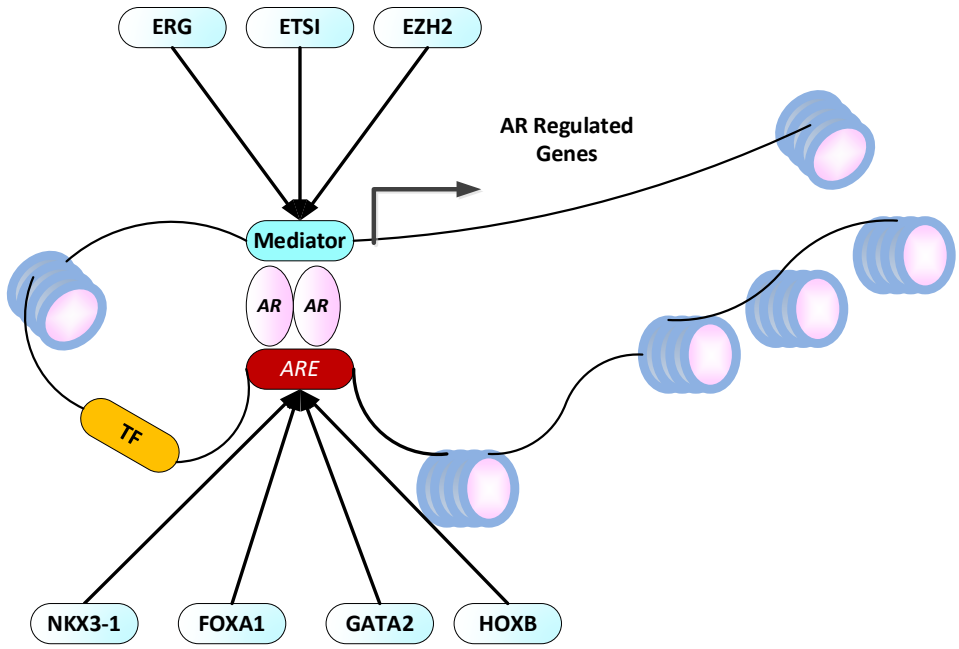
This ***binding activity and thus AR-mediated transcriptional regulation are tightly controlled by a large cohort of AR co-factors****. Despite of these successes, very few AR target genes have been identified and characterized until recent advances in high-throughput genomic technologies. The advent of DNA microarrays at the beginning of this century and the emergence of massively parallel next-generation sequencing have rapidly transformed this field. …*

The identification of AR sites and functions is still in an early stage. Thus when we examine AR-V7 and other variants we must understand the limits of assuming these to be sine qua non. The authors continue:

In addition to ARE, the selective binding of AR across the genome is tightly regulated by a collection of transcription co-factors and/or pioneering factors. Accumulating evidence suggests that AR primarily binds distal enhancers that can be several kb to over 100 kb away from the promoter regions of coding genes. These AR bound enhancers have been shown to interact with the promoters through chromatin looping.

AR recruits the translation initiation complex and regulates transcription through interaction with as many as over 150 co-regulators, some of which are co-activators while others are co-repressors. In order to fully understand AR-mediated transcriptional regulation, in the past decade researchers have put forth significant efforts to determine the DNA binding patterns and the genomic landscapes of AR and its cofactors, taking advantage of modern technologies

Jin et al note the following set of putative processes. The AR are dimerized and bind across the DNA to produce the genes so regulated. The figure below depicts some of this activity. It must be noted that the histone activity is another complex dynamic which in turn can dramatically modulate these functions.



Regarding the above Jin et al note:

Androgen regulates downstream genes by acting as a ligand of the hormonal transcription factor, the AR.

Once liganded, AR translocates into the nucleus, where it homodimerizes and binds directly to DNA through the ARE. The consensus AR-binding motif (i.e., canonical AREs, AGAACAnnnTGTTCT) consists of two hexameric half-sites (5'-AGAACA-3') often arranged as inverted repeats with 3bp of separating nucleotide. AR recognizes and interacts with AREs through its DNA-binding domain (DBD). In addition to ARE, the selective binding of AR across the genome is tightly regulated by a collection of transcription co-factors and/or pioneering factors. Accumulating evidence suggests that AR primarily binds distal enhancers that can be several kb to over 100 kb away from the promoter regions of coding genes. These ARbound enhancers have been shown to interact with the promoters through chromatin looping.

AR recruits the translation initiation complex and regulates transcription through interaction with as many as over 150 co-regulators, some of which are co-activators while others are co-repressors. In order to fully understand AR-mediated transcriptional regulation, in the past decade researchers have put forth significant efforts to determine the DNA binding patterns and the genomic landscapes of AR and its cofactors, taking advantage of modern technologies.

As Cato et al have noted regarding the histones:

ARv7 Negatively Regulates H3K27ac *To further characterize the mechanism whereby ARv7 negatively affects transcription, we utilized ChIP-seq to assess levels of histone H3K27 acetylation (H3K27ac), a mark of active enhancers and transcriptional activity H3K27ac cistromes were determined in the AR KD cell lines, and signals centered at ‘‘high-confidence’’*

AR-binding sites (union of ARN, ARv7, and ARfl peaks) were analyzed. To correlate H3K27ac levels with AR function, we stratified the averaged H3K27ac signals at the AR sites using Pearson correlation.

This allowed us to distinguish two clusters with distinct H3K27ac signals. Cluster I showed decreased H3K27ac levels after ARv7 and ARfl KD, indicative of ARfl and ARv7 positively regulating H3K27ac at these sites. In contrast, H3K27ac levels in cluster II (n = 4,268) were significantly increased upon ARv7 KD, yet decreased upon ARfl KD. This suggests that genes in cluster II sites are mostly differentially regulated by the two AR isoA B C.

***ARv7 Binds to Transcriptional Corepressors NCOR1, NCOR2, and NRIP1 (A) MARCoNI assay** (using a pan-AR antibody) of select corepressor peptides and cell lysates from indicated LNCaP95 cells. …*

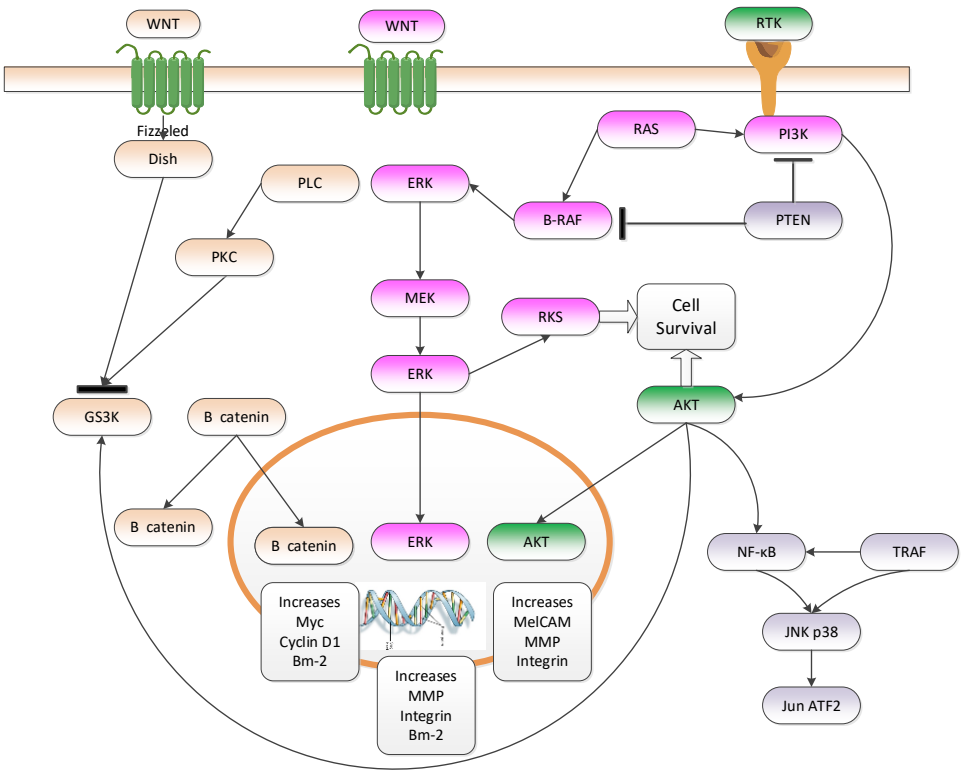
To test this hypothesis, we examined the ARv7- and ARfl-binding sites (Figures 5B and 5C). Although no substantial difference in ARv7 or ARfl binding was apparent between the two clusters, we observed diminished signal intensities in response to KD of either AR isoform. This indicates an interdependent binding of ARv7 and ARfl, in agreement with our previous finding (Figure 3E). We next correlated the cluster-specific AR cistromes with the previously determined AR transcriptomes.

As this analysis depends on a stringent peak-to-gene association, we only considered targets significantly dysregulated (DEseq; $p < 0.05$) upon AR KD, localized within 10 kb of an AR-binding site (Table S4). For cluster I targets, the fold changes for the majority of genes was <0, consistent with these genes being activated by either AR isoform. In contrast, cluster II was biased toward shARv7 upregulated (fold change >0) and shARfl downregulated genes (fold change <0) (Figure 5D), which suggests that cluster II is predominantly associated with ARv7-repressed genes. These findings indicate that ARv7-dependent gene repression is a consequence of ARv7- mediated inhibition of H3K27ac.

3.4 Other Regulators

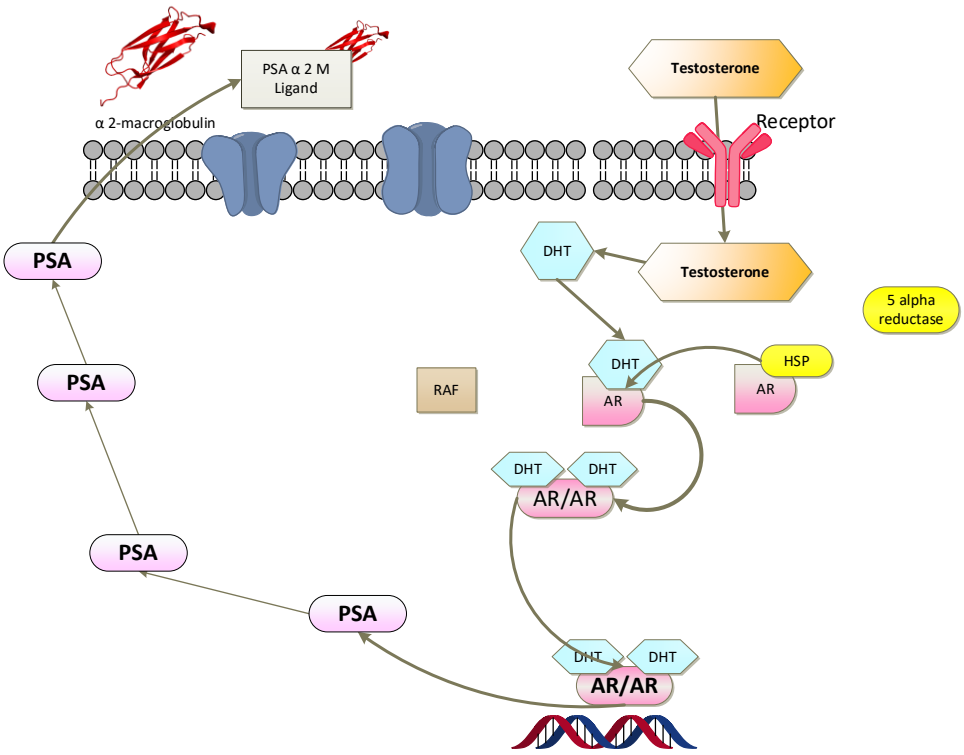
We will summarize many of the other regulators that impact PCa and the AR specifically. This is but a brief overview and not meant to be a tutorial. There are a multiplicity of other pathways, environmental and epigenetic factors as well. Thus AR-V7 is but one more target[3]. The following Figure is a generic of many of the critical operating pathways and their impacts.

[3] See some of the following:
https://www.researchgate.net/publication/341788660_Fibroblasts_and_Cancer_The_Wound_That_Would_Not_Heal , https://www.researchgate.net/publication/340539918_ERG_A_Master_Transcription_Factor ,
https://www.researchgate.net/publication/338412510_mTOR_Target_of_Opportunity ,
https://www.researchgate.net/publication/333704252_EMT_lncRNA_TGF_SMAD_and_Cancers ,
https://www.researchgate.net/publication/329702571_Growth_Factors_Pathways_and_Cancers ,
https://www.researchgate.net/publication/325497685_Neuroendocrine_PCa_Galen_Logic_and_Rationalism?_sg%5B0%5D=3aVZU4qggrzWku4W1Se2x1ET3MWT2DDOvc6QBniPtFgcq6Q3zNtSFUXU6byXEvrzWIeAP1gzi_T9EX8Hctfy5jLEbWTqA87XEBCS0HcK.CRmcrUP8Yn4FrB_0qJy0zfK-NbK_Dg-z1goiVAQWCjoREZv3IpsbmlTdXiQLtvSwy0j5FJT8PQq4UYHqvUrNYA ,
https://www.researchgate.net/publication/304673724_NKX31_and_PCa?_sg%5B0%5D=W2U0ZNFuZi6U6CgoKF

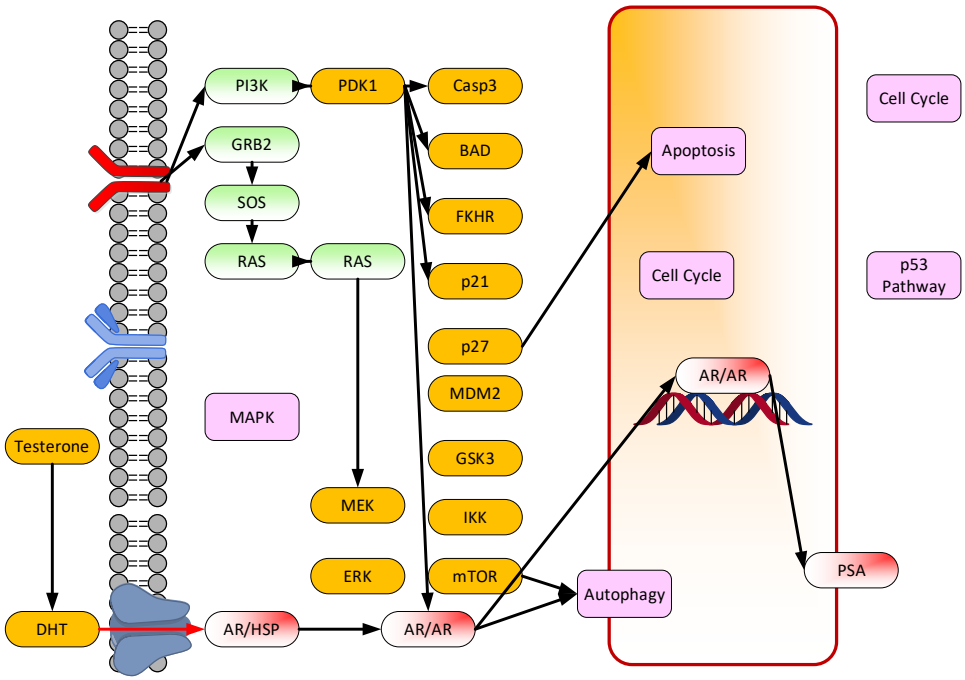


Having examined the broad pathway dynamics of the cell we can show below the dynamics of AR and the result, PSA. PSA is the classic measure of cell activity. AR is essential, when activated, to generate PSA. PSA is a measure of prostate cell activity which is why it is a useful albeit limited metric for cancer observation. If one can abate the androgens that drive the dimerization of the AR molecules then we can downward modulate the malignant proliferation. If however, an aberrant AR, such as AR-7, which can act independent of the androgens, then we must look for an alternative approach. The following Figure depicts the pathway generating PSA. The AR dimer then assists in the promotion of the gene generating the PSA. If we were to block the AR via an ADT then we theoretically should see the PSA go to zero. However as we have noted, changes in the AR allowing autonomous activation can then activate the promoter elements and generate PSA. The proliferation of aberrant cells with autonomous AR generation thus allow for an excess of PSA.

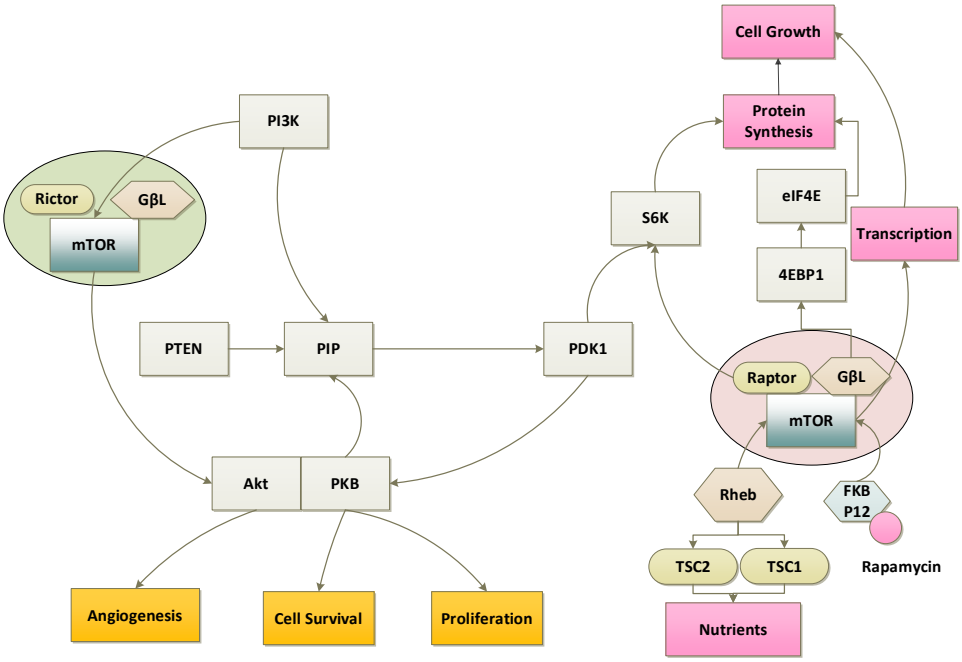
Qmkb6nYLGwY-y9GhI3DsAbcX7j6foA0uHNfLLm9ixowWoJM64GDXAfyXcA1q1Ay3VLX_W_U5KChoSmU7cdtBNK.2DpnxeY3Kdiq6SuPT-632vdX5SxukYnK5ZfOp_dFVZBp0yplCRdKe1l_4apjXiIJEN5z43Qtk3g3yQ-coCgcZw , and others at www.researchgate.net



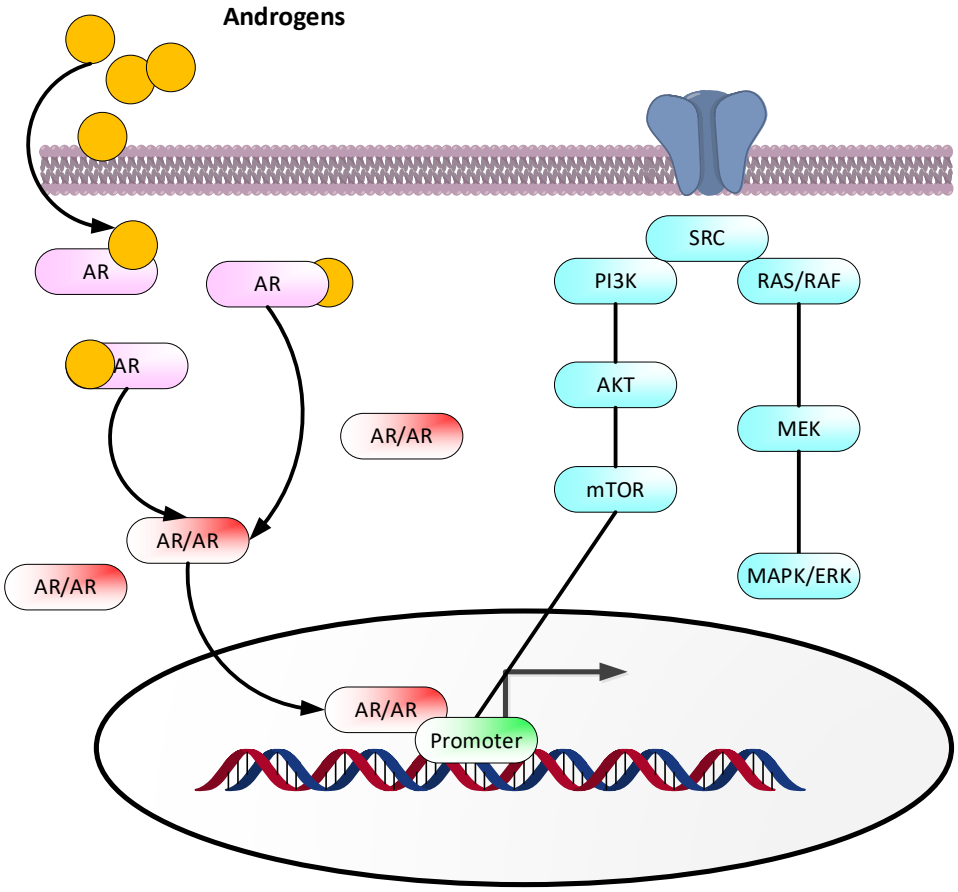
Another view of the PSA path and the AR interface is given in Staibano. We show it modified below:



In summary, some useful therapeutic pathways are demonstrated below. Here we include the function displayed in many of the pathway elements.



Finally a recent paper by Kokal et al has discussed specific targets and putative therapeutics. They use the figure below which is a reconfiguration of the multiplicity above.



Here Kokal et al note:

Androgen at supraphysiological level (SAL) induces cellular senescence through the PI3K/Src/Akt/mTOR pathway. In the cytoplasm, androgen receptor (AR) forms a complex with heat shock proteins (HSPs). Activation of the AR is triggered by binding of androgens (AR agonist) to the receptor. Androgen-activated AR dissociates from HSPs, and subsequently mediates AR signaling.

(1) ***The majority of activated AR forms homodimer and translocates into the nucleus, where it binds to androgen response element (ARE) at the promoter of the target genes and mediates transcription.***

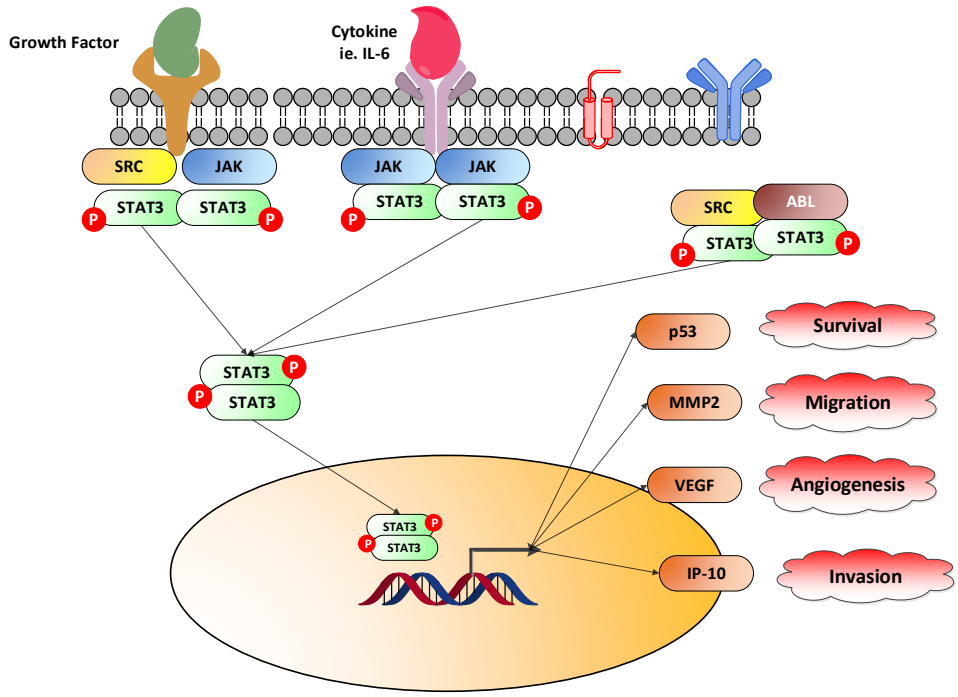
(2) ***A minor fraction of activated AR remains in the cytoplasm, interacts with and functions through several signaling molecules, e.g., Src, PI3K/Akt, S6, Ras/Raf1, MAPK/ERK. Interestingly, co-treatment of SAL with inhibitors of PI3K (3-MA), Src family members (PP2), Akt (Akti), or mTOR (Rapamycin) inhibits senescence induction. This indicates that Src/PI3K/Akt/mTOR pathway is involved in SAL mediated cellular senescence in PCa cells.***

Thus it is essential to view AR and its actions holistically in the context of all the other genes it manages to influence, or influence it. They conclude:

In conclusion, both androgens at supraphysiological levels (Figure 1) and AR antagonists (Figure 2) induce cellular senescence in PCa. This important AR pathway is mediated by membrane and cytosolic transduction factors including PI3K, Src family, Akt and mTOR. AR was shown previously to interact in a non-genomic and rapid signaling with Src and Akt. Analyzing AR ligand-induced cell senescence, the activation of these factors was however also observed after many days of AR ligand treatment.

Therefore, it is suggested that the AR interacts with these factors at the non-genomic level, also in a long-term manner, which eventually changes the transcriptome landscape. Interestingly, despite both ligands inducing cancer cell senescence, AR agonist and antagonist seem to induce a distinct pro-survival pathway. Therefore, targeting senescent PCa cells, the specific pro-survival pathway should be known in order to use a particular senolytic compound.

Now another view we show below depicts cytokine influences such as IL-6. Also growth factors end up playing a significant role as well.



Finally, Chen et al have discussed results for other regulators for AR. They have concluded:

1. PRCAT38 is an Androgen-Regulated lncRNA that Modulates Cell Growth and Migration in Prostate Cancer

2. AR binds to Enhancers between TMPRSS2 and PRCAT38, to Recruit RNA Pol II and H3K27ac to Initiate Transcription

3. Enhancer E1 is Responsive to Androgen Induction and Regulates both TMPRSS2 and PRCAT38 via Interacting with E2 through Chromatin Looping

4. FOXA1 is Recruited by the AR to the Enhancers and Regulates the Expression of TMPRSS2 and PRCAT38

5. FOXA1 is Required for Enhancer Activity and Chromatin Looping between Enhancers and Promoters of TMPRSS2 and PRCAT38

4 AR-V7

We now consider the specifics regarding AR-V7. First we consider how it is generated via a splicing variant. Then how that variant functions in the prostate to evade the loss of androgen control. As Cattrini et al have noted about the history of AR-V7:

In 2014, Antonarakis and colleagues investigated the role of AR-V7 in two small cohorts of patients treated with ARSi. The AdnaTest platform for CTC isolation was adapted for detection and quantification of AR-FL and AR-V7 by quantitative real-time PCR using custom primers.

None of the 18 men who had detectable AR-V7 in circulating tumor cells (CTC) showed response to treatment with enzalutamide or abiraterone acetate. Conversely, detection of AR-V7 was not associated with primary resistance to taxane chemotherapy in the study on 37 mCRPC patients treated with docetaxel or cabazitaxel.

Based on these results, the Johns Hopkins team collected a validation set of 202 patients with mCRPC starting abiraterone or enzalutamide and investigated the prognostic value of CTC and AR-V7 detection. Patients' outcomes were best for CTC− patients, intermediate for CTC+/AR-V7− patients, and worse for CTC+/AR-V7+ patients.

These data led the authors to suggest that the modified-AdnaTest CTC-based AR-V7 mRNA assay should be interpreted using these three separate prognostic categories. Scher and colleagues reproduced the results of Antonarakis using the Epic Sciences CTC platform that detected the nuclear-localized AR-V7 and analyzed the blood sample of 161 patients with mCRPC.

Patients who received ARSi and had basal AR-V7-positive CTC showed worse outcomes in all time-to-event measures, whereas AR-V7 status did not affect the outcome of patients treated with taxanes. In the validation study, Scher and colleagues enrolled 142 patients with mCRPC and tested if AR-V7 status could predict survival according to different treatment received.

In the high-risk group including 70 men, patients with AR-V7+ high-risk disease treated with taxanes showed superior overall survival (OS) relative to those treated with ARSi, whereas patients with AR-V7− high-risk disease treated with ARSi had superior OS compared to those treated with taxanes.

As Nimir et al have noted:

Advanced prostate cancer (PC) tends to be initially hormone sensitive and is treated with androgen deprivation therapy (ADT).

However, resistance to first line therapy usually develops in approximately 20–40% of patients, referred to as castrate resistant prostate cancer (CRPC). Most commonly, PC cells become resistant through molecular changes of the androgen receptor (AR), such as mutations, gene

amplification, and, more recently reported, the expression of transcript AR variants. In particular, the expression of AR variant 7 (AR-V7), the most abundant and clinically relevant of all variants, has been implicated as a cause of CRPC. The translated AR-V7 protein is truncated and lacks the ligand binding domain as well as sequences important for stability and maybe cellular localization.

Importantly, intracellular AR-V7 predominantly localizes to the nucleus and displays ligand independent transcriptional activity, which is thought to be fundamental in its ability to promote ADT resistance. Due to its role in ADT resistance several therapies, such as Galeterone and EPI-506 have been developed to effectively target and reduce AR-V7 levels as shown in cell line studies, with clinical trials underway. Consequently, expression of AR-V7 together with that of full-length AR (AR-FL) have emerged as clinically relevant molecular biomarkers for CRPC. AR-V7 is detectable in tissue at the RNA and protein levels, and can be evaluated using RNA hybridization techniques and immunohistology.

AR-V7 expression is rare in hormone-sensitive PC but correlates with CRPC.

However, in the advanced PC setting, tissue biopsies are generally unavailable for biomarker testing and diagnostic decision making. ***Liquid biopsies have in recent years emerged as an alternative tumor source for biomarker testing****. To date, AR-V7 has been detectable in circulating tumor cells (CTCs) isolated from blood samples, whole blood mRNA, from ctRNA, tumor arisen cellular vesicles, so-called exosomes found in plasma or serum and even urine. Significantly, AR-V7 detection in CTCs has been associated with non-response to novel anti-androgens such as abiraterone and enzalutamide.*

In contrast, the response rates to taxane-based chemotherapy showed no significant difference between AR-V7-positive versus-negative CRPC patients. As such, this has positioned AR-V7 as a potential predictive biomarker that can discriminate between the use of an anti-androgen therapy and taxane chemotherapy.

4.1 GENERATION OF AR-V7

Now we shall examine how AR-V7 is generated. This is an unfinished story and we can but outline some of the know steps. From Ma et al:

In PCa, genetic changes in the androgen receptor (AR), such as point mutation and gene amplification, render the receptor independent of upstream testosterone levels and cause resistance against androgen deprivation therapy (ADT), which is the predominant first line therapy for advanced disease.

Thus far, AR amplification screening using fluorescent in situ hybridization (FISH) and point mutation screening with PCR-based methods from CTC enriched samples have been reported. AR transcriptional variants, for example, AR-V7 and AR-V567es, which encode constitutively active, truncated receptor proteins, cause ligand independent AR activation and are clinically relevant.

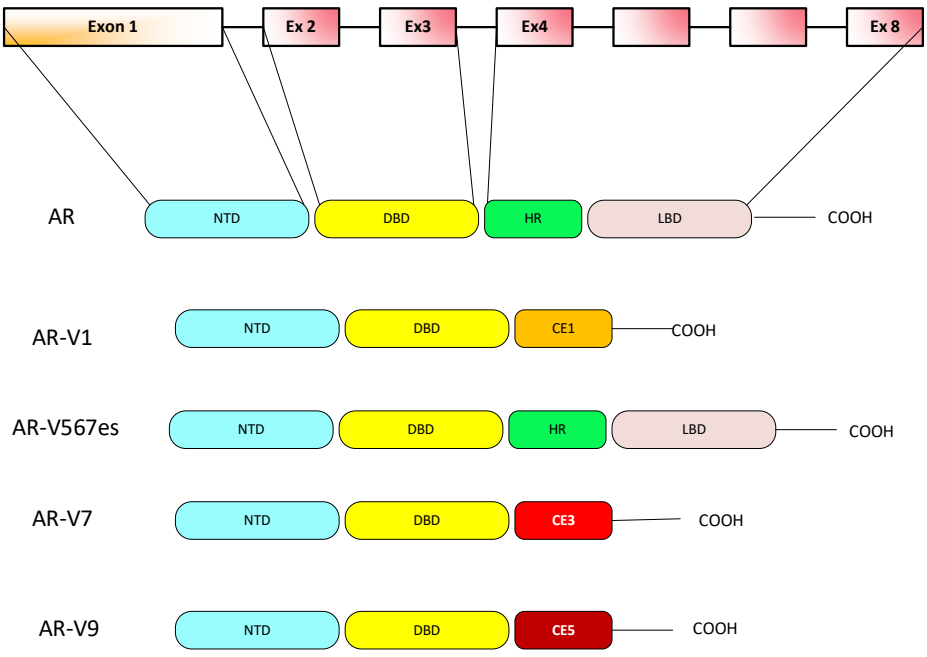
ADT-drug exposure rapidly induces AR-V7 expression in in vivo models and patient PCa cells, likely to compensate loss of regular AR signaling [9,15]. More importantly, the detection of AR-V7 in PCa CTCs has been correlated with metastatic castrate resistant prostate cancer (CRPC) and resistance against enzalutamide and abiraterone, and potentially superior clinical outcomes for patients on taxane therapy, though response to cabazitaxel has been shown to be independent of AR-V7 status.

Taken together, this suggests that AR-V7 may be a useful biomarker on which to base therapy initiation or therapy changes

From Saranyutanon et al:

Genomic organization of the AR gene and frequently reported androgen receptor (AR) splice variants. The gene encoding for androgen receptor is localized on the long arm of chromosome X in the region Xq11.2-q12. It is comprised of 8 exons that code for the mature androgen receptor protein having three functional domains: N-terminal binding domain (NTD), DNA binding domain (DBD), and a carboxy-terminal ligand binding domain (LBD) that is separated from DBD by a short hinge region (HR). Exon1 encodes for the NTD, exon 2–3 encode for the DBD, exon 4–8 encode for the hinge region and LBD.

Several AR splice variants have been reported that show truncation at DBD or LBD. AR-V1 is truncated at the end of exon 3 and contains 19 amino acids from cryptic exon 1 (CE1). AR-V567 is created by skipping of exons 5–7 in the AR mRNA, while AR-V7 has splicing of cryptic exon 3 (CE3) after exons 1–3, and AR-V9 is generated by splicing of cryptic exon 5 (CE5) after exon 3. AR-V1, AR-V7, and AR-V9 all lack LBD



4.2 FUNCTIONS OF AR-V7

As Cato et al have stated (ARfl is full length AR or just AR):

1. LNCaP95 and 22Rv1 Cell Growth is Dependent on ARfl and ARv7 A proposed mechanism underlying the reactivation of AR in CRPC is the increased expression of ARv7 (together with ARfl). Utilizing the CRPC cell line LNCaP95, which endogenously expresses ARfl and ARv7 and is cultured under hormone-starvation conditions, we assessed the dependency of this line for both AR isoforms.

2. ***ARv7 Functions as a Transcriptional Repressor in CRPC Cells***

3. ARfl and ARv7 Bind to the Same Sites in Chromatin and Heterodimerize

*4. A****Rv7 Preferentially Interacts with Transcriptional Corepressors*** *Given the codependent binding of ARfl and ARv7 to chromatin, but divergent transcriptional output, we speculated that other factors might contribute to the divergent genomic function of* the two receptors.

5. ***ARv7 Preferentially Interacts with Transcriptional Corepressors***

6. ***ARv7 Negatively Regulates H3K27ac***

7. ***ARv7 Represses Negative Regulators of Tumor Cell Proliferation***

They conclude (here ARfl is the full length AR and not the variant AR-V7. Specifically it is the classic AR):

In PCa patients, expression of the four genes is negatively correlated with ARv7 protein levels and time to recurrence. This is consistent with the concept that ARv7 acts by repressing tumor-suppressive genes during PCa progression, and that expression of ARv7-repressed genes may serve as a biomarker to assess ARv7 inhibition …

In this work we observed that ARv7 preferentially associates with the NCOR transcriptional corepressors, whereas ARfl associates with both coactivators and corepressors, in agreement with the idea that ARfl and ARv7 display differential coregulatory binding repertoires.

Moreover, increases in ARv7 and NCOR1,2 binding upon ARfl KD suggest that ARfl partially inhibits the ARv7/NCOR interaction, thereby limiting a repressive transcriptional response. We find that the difference in ARfl and ARv7 transcriptional activity is correlated with AR isoform-specific differences in H3K27 acetylation. Therefore, ARv7 likely functions by recruiting corepressors, such as NCOR1 and NCOR2, which in turn control the genomic recruitment of histone deacetylases, such as HDAC3, which negatively regulate H3K27 acetylation.

Reprogramming of the FOXA1 cistrome following ARv7 depletion is probably an important consequence of AR inhibition, but may not be directly linked to ARv7-dependent repression, since it is also observed at ARv7-activated sites. This observation is further supported by the

finding that ARv7 and FOXA1 are unable to interact by coIP. Moreover, additional ARv7 cooperating factors may also exist; HOXB13 was recently indicated as an important mediator of ARv7 function… In PCa patients, expression of the four genes is negatively correlated with ARv7 protein levels and time to recurrence.

This is consistent with the concept that ARv7 acts by repressing tumor-suppressive genes during PCa progression, and that expression of ARv7-repressed genes may serve as a biomarker to assess ARv7 inhibition

4.3 CONTROL OF AR-V7

The final question is; how might one control AR-V7? As Nakasoto et al have noted:

Androgen receptors (ARs) are encoded by eight exons (exons 2–3 are DNA binding domains and exons 4–8 are ligand binding domains (LBDs)). The AR splice variant 7 (AR-V7) has exon 3 followed by a cryptic exon 3 and omits exons 4 to 8. It lacks the LBD but retains functional, transcriptive element binding domains that mediate intracellular AR signaling in a ligand-independent manner.

AR-V7 has attracted attention because of its association with castration-resistant prostate cancer (CRPC). Enzalutamide (Enz), an inhibitor of AR signaling that binds to the LBD of the AR, and abiraterone (Abi), an inhibitor of cytochrome P450 17A1 that impairs AR signaling, are the main androgen axis drugs against CRPC. After analyzing AR-V7 in circulating tumor cells *(CTCs), Antonarakis reported that patients with AR-V7-positive CTCs presented high resistance to Enz and Abi.*

Alternatively, docetaxel (DTX) and cabazitaxel (CBZ), which are taxane preparations, showed some promise for patients with AR-V7-positive tumor cells. Thus, AR-V7 has been considered a biomarker to guide treatment choices.

Bernemann found that about 20% of patients expressing AR-V7 in CTCs had a prostate-specific antigen (PSA) reduction of more than 50% after treatment with Enz and Abi, and the PROPHECY study found that the effectiveness of Enz and Abi reached 6–11% of AR-V7 positive patients when the Johns Hopkins Hospital (JHU) method by Antonarakis was used to assess the presence of the biomarker. *These results point to a diagnostic inaccuracy of the biomarker.*

Alternatively, the effectiveness of Enz and Abi was 0% in AR-V7 positive patients when using the Epic Sciences platform to assess the presence of the marker. The positivity of JHU AR-V7 and Epic AR-V7 may vary even in results from a single patient, which indicates that CTC analysis differences may influence the accuracy of AR-V7 as a marker.

5 MIRNA REGULATION

Micro RNAs play a significant and yet to be fully understood role in controlling gene expression. They also are significant in PCa[4]. Thus miRNAs have been found to be control elements in AR as well as possibly AR-V7. We examine some recent work in this area.

5.1 MIRNA AND AR

From Fernandes et al they note:

The androgen receptor (AR) is a ligand-activated transcription factor that drives prostate cancer. Since therapies that target the AR are the mainstay treatment for men with metastatic disease, it is essential to understand the molecular mechanisms underlying oncogenic AR signaling in the prostate. miRNAs are small, non-coding regulators of gene expression that play a key role in prostate cancer and are increasingly recognized as targets or modulators of the AR signaling axis.

In this review, we examine the regulation of AR signaling by miRNAs and vice versa and discuss how this interplay influences prostate cancer growth, metastasis and resistance to therapy. Finally, we explore the potential clinical applications of miRNAs implicated in the regulation of AR signaling in this prevalent hormone-driven disease…

One prominent feature of this interplay is direct targeting of the AR 3'UTR by miRNAs. Indeed, a recent study predicted that the AR 3'UTR is likely to be more heavily regulated by miRNAs than all other PCa driver genes and in the top 5% of regulated genes overall (Hamilton et al. 2016). This elegant work exploited photoactivatable ribonucleoside-enhanced cross-linking immunoprecipitation of the Argonaute protein (Ago-PAR-CLIP) to identify 147 miRNA seed sides corresponding to 71 miRNA families in the AR 3'UTR (Table 1). Importantly, four of the miRNAs identified by Hamilton and colleagues – miR-9, miR-34c, miR-185 and miR-488 – had been previously discovered in two high throughput screens aimed at identifying AR-targeting miRNAs.

In addition to these unbiased approaches, many other studies have characterized specific AR-targeting miRNAs in a more directed manner. Interestingly, in addition to classical targeting of the 3'UTR, miRNA regulation of the AR via 5'UTR (miR-31) and the coding region (miR-421, miR-449a, miR-449b, miR-646, miR-371, miR-193a and miR-9) has also been reported. A list of putative AR-targeting miRNAs and their putative modes of action is provided in Table 1. We propose that the biological relevance of these AR-targeting miRNAs can be prioritized using a set of discrete parameters.

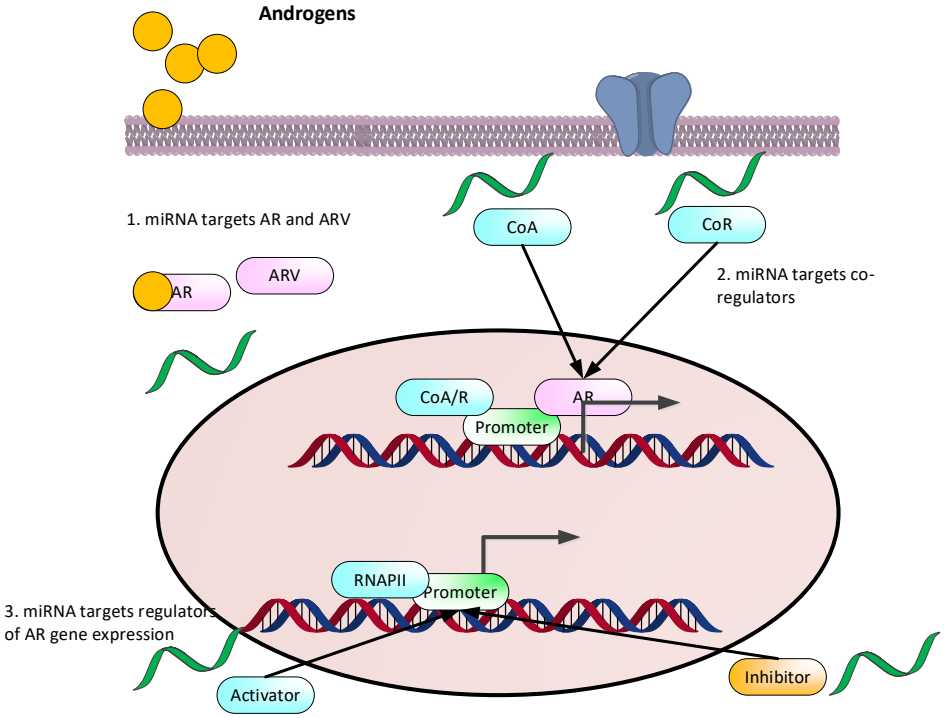
First, miRNAs that have been identified in multiple studies using multiple in vitro models are likely to have greater biological relevance in PCa.

[4] See some of the work regarding this: https://www.researchgate.net/publication/338684968_miRNAS_REDUX , https://www.researchgate.net/publication/277708730_Prostate_Cancer_Metastasis_Some_Simple_Models,

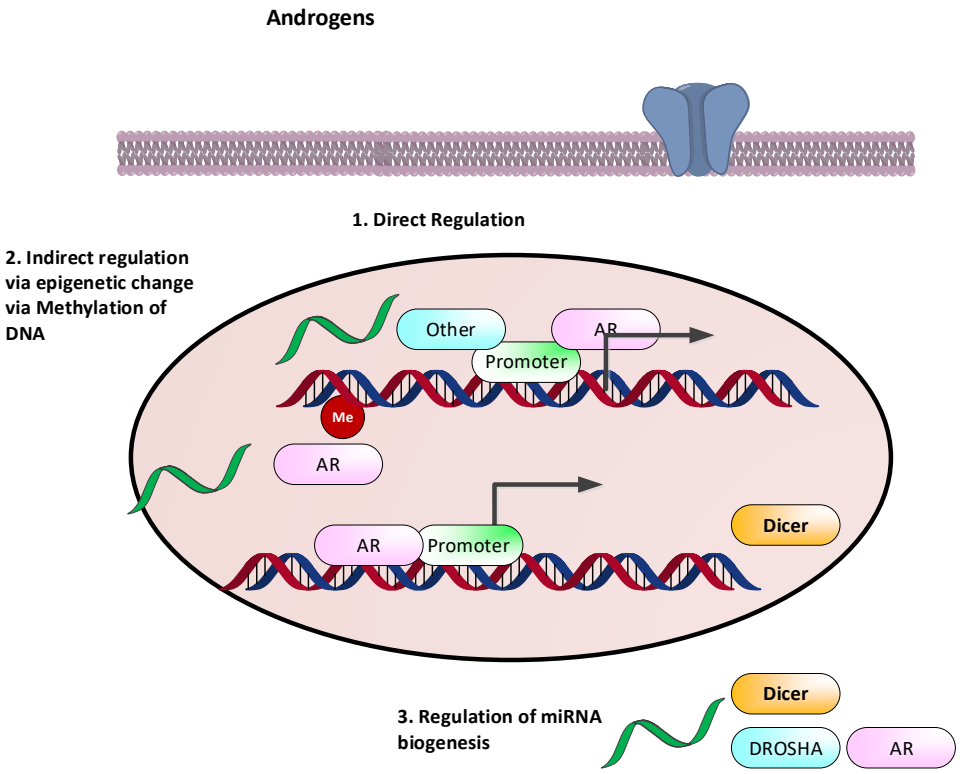
Second, it is known that transfection of cells with miRNA mimics can yield non-physiological miRNA activity; therefore, we prioritize studies that have demonstrated AR targeting using both miRNA mimics and inhibitors.

Third, given that prostate tumors are 'addicted' to AR (Coutinho et al. 2016), one would expect oncogenic selection pressure to downregulate biologically relevant AR-targeting miRNAs.

The following Figures are based upon Fernandes et al and show the ways in which miRNA interact. The first shows the targeting of AR co-regulators and the following figure depicts the targeting that influences AR expression.



The following Figure depicts the potential points of regulation as an extension of what we have shown above.



It is thus clear that miRNAs have significant potential controlling genes, mRNA, proteins, and histones and their ability to enable of disable expression.

5.2 SPECIFIC MIRNAS

We now provide a list of miRNAs and their functions as currently understood (see Fernandes et al).

miRNA	*Target*	*Mechanism*	*Mimic/Inhibit*	*Ago-PAR-CLIP binding site*	*Readouts*
miR-9	AR	3′UTR and coding region	Mimic	Yes	AR protein and mRNA, AR luciferase reporter (wild-type only)
miR-30b-3p	AR	3′UTR	Mimic and Inhibitor		AR protein and mRNA, AR 3′UTR luciferase reporter (wild-type and mutant)
miR-30c-5p	AR	3′UTR	Mimic		AR protein, AR 3′UTR luciferase reporter (wild-type)
miR-30d-5p	AR	3′UTR	Mimic and Inhibitor		AR protein and mRNA, R 3′UTR luciferase reporter (wild-type and mutant)
miR-31	AR	5′UTR and coding region	Mimic		AR protein, AR 3′UTR luciferase reporter (wild-type and mutant)
miR-34a	AR	3′UTR	Mimic	Yes	AR protein and mRNA, AR luciferase reporter (wild-type only)
miR-34c	AR	3′UTR	Mimic	Yes	AR protein and mRNA, AR luciferase reporter (wild-type only)
miR-101-3p	AR and AR-V7	3′UTR	Mimic	Yes	ARlu protein, AR 3′UTR ciferase reporter (wild-type only)
miR-124-3p	AR, AR-V7 AR-V4	3′UTR	Mimic	Yes	AR protein, AR 3′UTR luciferase reporter (wild-type and mutated)
miR-135b	AR and AR-V7	3′UTR	Mimic		AR protein and mRNA, R luciferase reported (wild-type only)
miR-145	AR	3′UTR	Mimic		AR protein and mRNA
miR-149-3p	AR	3′UTR	Mimic		AR protein, AR 3′UTR luciferase reporter (wild-type only)
miR-181c-5p	AR-V7	3′UTR	Mimic	Yes	AR protein and mRNA, AR luciferase reporter (wild-type and mutated)
miR-193a-3p	AR and AR-V7	Coding region	Mimic		AR protein, AR 3′UTR luciferase reporter (wild-type only)
miR-205	AR	3′UTR	Mimic	Yes	AR protein, AR luciferase rep rater (wild-type)

miR-297	AR	3′UTR	Mimic		AR mRNA, AR luciferase porter (wild-type)
miR-298	AR	3′UTR	Mimic		AR protein and mRNA, R luciferase reporter (wild-type only)
miR-299	AR	3′UTR	Mimic	Yes	AR protein and mRNA, AR luciferase reporter (wild-type only)
miR-371	AR and AR-V7	Coding region	Mimic		AR protein, AR luciferase reporter (wild-type only)
miR-421	AR	3′UTR and coding region	Mimic	Yes	AR protein and mRNA, AR luciferase reporter (wild-type only)
miR-425-5p	AR	3′UTR	Mimic		AR protein, AR 3′UTR luciferase reporter (wild-type)
miR-449a	AR	3′UTR and ding re ion	Lentiviral miRNA overexpression		AR protein and mRNA
miR-449b	AR	3 UTR and coding region	Mimic		AR protein, AR 3′UTR luciferase reporter (wild-type)
mir-488	AR and AR-V7	3′UTR	Mimic	Yes	AR protein and mRNA, AR luciferase reporter (wild-type and mutated)
miR-541	AR	3′UTR	Mimic		AR protein and mRNA, R 3′UTR luciferase reporter (wild-type)
miR-634	AR	3′UTR	Mimic		AR protein and mRNA, AR luciferase reporter (wild-type only)
miR-635	AR	3′UTR	Mimic		AR protein, AR 3′UTR luciferase reporter (wild-type)
miR-646	AR and AR-V7	Coding region	Mimic		AR protein, AR 3′UTR luciferase reporter (wild-type)
miR-650	AR	3′UTR	Mimic		AR protein, AR 3′UTR luciferase reporter (wild-type)
miR-654	AR	3′UTR	Mimic		AR protein and mRNA, R luciferase reporter (wild-type only)

5.3 Epigenetics Characteristics

Santos et al summarize many of the epigenetic factors. They note them:

Transformations of proteins associated with DNA or transformations of DNA itself that occur during cell division, different from the habitual sequence of DNA and that will be expressed genetically are termed epigenetics.

There are three primary mechanisms of epigenetics:

1. MicroRNA (miRNA) regulation,

2. chromatin remodeling and

3. methylation of DNA.

Methylation of DNA *This mechanism is involved in carcinogenesis at local and global levels because it is essential in the control of innumerable cellular events. The alteration and extent of epigenetics are responsible for these cellular events. Insufficient gene silencing is associated with DNA hypermethylation, while genetic instability and activation of oncogenes is induced by DNA hypomethylation.*

Chromatin remodeling and histone modifications *The nucleosome is the fundamental unit of the nuclear DNA, and chromatin is the higher order of organization. DNA comprising 147 base pairs is involved, forming a frame, with the protein core and its eight histones. Histones are implicated in the control of DNA replication, repair and transcription. They are functional biomolecules that provide sustention to DNA. In healthy cells, modifications of histones are involved in embryonic stem cell growth and specialization, deactivation of chromosome X and genomic imprinting In neoplastic cells, alterations in DNA methylation and modifications of histones happen in a large percentage of the genome.*

Important marks of human cancer are, for instance, hypomethylation of DNA in repetitive sequences, such as decreased lysine 20 trimethylation (H4K20me3) and decreased lysine 16 acetylation (H4K16ac) of H4. Another example is hypermethylation of DNA in silenced genes. Here, we can see association of loss of acetylation of histone 3 (H3ac), methylation of lysines 9 and 27 of histone 3 (H3K9me and H3K27me) and monomethylation of H3K4me. Curiously, identical inhibitory histone signs were found in some specific genes with tumor-suppressor actions that are not stopped by DNA methylation[29]. Therefore, cancer growth and progression have been linked to modifications in the expression of decisive histone regulating enzymes (HDMs, HMTs, HDACs and HATs).

Micro RNAs *MiRNA are a class of small, noncoding RNA, usually an extension of 18 to 25 nucleotides. The synthesis (pri-miR) and the processing steps (pre-miR) occur in the nucleus. After export to the cytoplasm, mature miR ligate to their corresponding mRNA sequences and change their expression via an RNA-induced silencing complex. Every miRNA generally modulates many mRNAs, and mRNAs could be targeted by many miRNAs. MiRNAs control and regulate approximately 30 percent of human genes.*

This process occurs in a specific tissue and under temporal circumstances. The importance of miRNAs in malignance is dependent upon the particular target genes. In terms of programming genes, miRNA expression is changed because of gene mutation, deletion, amplification, abnormalities of chromosomes, alterations in transcription factors, and epigenetic mechanisms. MiRNAs, curiously, can target the posttranscriptional command of crucial enzyme modifiers of chromatin, and therefore are also involved in chromatin structure control. Additionally, miRNAs

can establish a ligation with the principal epigenetic routes, including the usual goals for epigenetic regulation via methylation of DNA or modifications of chromatin of promoters.

For example Richardsen et al note:

We found that high expression of miR-141 to be significantly associated to worse PC outcome. In TE a high expression was associated with BFFS and in TE+TS was associated with a higher risk of BFFS and CFFS. We also found that miR-145 correlated with more aggressive features of prostate cancer. This knowledge may be valuable for further studies, which should provide further mechanistical explanation for the role of miR-145 in PC, in particular regarding target genes of the miR in PC.

6 OBSERVATIONS

The recent work of Takayama presents a good summary of where we have wanted to go. Namely he states:

Although inhibition of the androgen–androgen receptor (AR) axis effectively represses the growth of prostate cancer, most of all cases eventually become castration-resistant prostate cancers (CRPCs).

Enhancement of the expression of AR and its variants along with the downstream signals is important for disease progression.

AR-V7, a constitutive active form of AR, is generated as a result of RNA splicing. RNA splicing creates multiple transcript variants from one pre-messenger RNA (mRNA) by removing introns/exons to allow mRNA translation. The molecular mechanisms leading to marked increases of AR and generation of AR-V7 have been unclear.

However, recent papers highlighted the roles of RNA splicing factors which promote AR expression and production of variants. Notably, a broad range of splicing components were aberrantly regulated in CRPC tissues. Interestingly, expression of various spliceosome genes is enhanced by RNA-binding protein splicing factor proline- and glutamine-rich (PSF/SFPQ), leading to changes in the expression of AR transcript variants.

Moreover, inhibition of several splicing factors repressed tumor growth in vivo. Altered expression of splicing factors is correlated to biochemical recurrence in prostate cancer patients.

*Thus, these findings suggest that splicing factors would be a potential therapeutic targe*t.

6.1 STEM CELLS

The stem cell issue or cancer cell of origin is always an interesting concept. It is not clear how one should view the AR-V7 in this context. Is it an initiator of a metastatic process or the result of some other process? Is it in the putative stem cell of just an artifact of the malignancy?

6.2 DIAGNOSTICS

AR-V7 is not a diagnostic and it may be a prognostic tool. Thus use of this as a blood borne marker may just confirm the androgen resistance stage has been reached. Yet clinically one has many other metrics to see this. In the case of a patient with a prostatectomy, rising PSA is a well-accepted metric. Nuclear scans for bone mets also are standard. Thus one may ask; what does a measurement of AR-V7 provide? It is not clear that anything of much value is added.

6.3 THERAPEUTICS

The targeting of AR-V7 or one its predecessors may be a useful therapeutic target.

The question here may be: knowing AR-V7 drives a malignant process, what therapeutic approach can be taken? The previous analyses by some authors hint at approaches. However perhaps either a direct or an indirect approach may be taken. Namely if one understood the splicing variant drivers then inhibiting them may be productive. Also one wonders if there are "work arounds" with the multiple pathways operating in a PCa environment.

6.4 CAUSES

The underlying question is; what causes this splicing variant? If the cell is already malignant one does not see this variant until late in the process. What prompts this to occur if it is not present at an earlier stage.

6.5 MICROENVIRONMENT

The tumor micro environment, TME, has received significant recent attention. It may pose a protective or disruptive effect on the underlying tumor. In a 2015 paper by Wyatt and Gleave, the authors discuss the putative protective effects as noted:

Cancer cells reside within a complex microenvironment that can either compromise or augment survival and growth. Furthermore, as prostate cancer switches from an endocrine driven disease to a paracrine- or autocrine-driven disease after CRPC development, tumour cells become increasingly reliant on the microenvironment for survival. For example, prostate-cancer-associated stromal cells can facilitate androgen biosynthesis in tumour cells under castrate conditions.

More recently, an elegant study demonstrated that in the aftermath of genotoxic therapy, the innate DNA damage response in benign stromal cells stimulates secretion of cytokines, growth factors and proteases that ultimately promote therapy resistance in tumour cells. Strategies designed to target the tumour microenvironment are attractive, not least since normal cells cannot easily evolve to a resistant state. The most effective approach in many solid malignancies has been to interfere with VEGF-mediated blood vessel recruitment to tumour tissue. Unfortunately, attempts to repurpose anti-angiogenic drugs for CRPC have failed.

For example, the VEGF targeted agents bevacizumab, aflibercept and lenalidomide all failed to improve the overall survival conferred by docetaxel in large phase III trials, collectively implying that VEGF-mediated angiogenesis is not the sole driver of progression in bone-predominant mCRPC. Similarly, endothelin receptor (END1) targeting agents, atrasentan and zibotentan, also failed in phase III studies, despite biologic and preclinical proof-of-principle as well as signals of activity in phase II studies.

Overall, the development of these angiogenesis inhibitors was challenged by lack of single agent activity that compromised detection and/or interpretation of robust activity signals. In the case of

zibotentan, a randomized phase II versus placebo in men with M1 CRPC demonstrated improved markers of bone turnover and initially signalled significantly improved survival but with maturation this benefit disappeared. Based on the initial survival benefit, a phase III trial enrolled 594 patients, but survival was not significantly prolonged, in part due to insufficient sample size. A more successful strategy has been to exploit the remarkable propensity of tumour cells to form metastatic deposits in the bone.

Despite decades of availability, crude radiopharmaceuticals have demonstrated only limited uptake due to incidental bone marrow toxicity from errant beta-particles. However, in 2013, the calcium mimetic radium-223 dichloride (Xofigo) was approved for the treatment of bone metastatic CRPC. Activity is reliant on the potent effect, but short range of alpha radiation emitted from radium-223 decay: reducing peripheral damage to healthy tissue while maintaining powerful anti-tumour efficacy.

Overall, although radiopharmaceuticals do not strictly target the microenvironment, their rational use has demonstrated that it is possible to elicit overall survival gains by selectively targeting the bone niche. Transient cell populations that migrate in and out of the ecosystem can also influence tumour dynamics. Tumours arise in an immunocompetent environment, interacting with innate and adaptive branches of the host immune system.

Although the host immune system is capable of mounting an antitumour response, tumour cells frequently enjoy an excess of regulatory and suppressor T cells, blunting the effector response. In the apoptotic aftermath of initial androgen deprivation therapy, leucocytes are further recruited to tumour tissue, but rather than reacting to the cancer, they may promote progression to CRPC. The field of immunotherapeutics seeks to exploit the potent and intact anti-tumour response and is reviewed in-depth elsewhere. The most advanced clinical strategies for CRPC are therapeutic vaccines that induce a novel antitumour response, and immune checkpoint modulators that prevent suppression of the existing response.

Sipuleucel-T (provenge) is a therapeutic vaccine generated by ex vivo stimulation of antigen presenting cells. It became the first immunotherapy approved for use in prostate cancer after demonstrating a significant overall survival benefit in asymptomatic.

As regards to other elements Bonollo et al have noted:

CAFs and cancer cells interact with each other through the secretion of soluble factors to establish a network of paracrine communication that sustains tumor growth. Among the secreted signaling molecules, TGFβ certainly has a dominant role in the regulation of TME biology, inducing fibroblasts to acquire a CAF phenotype. PCa cells release TGFβ to induce normal fibroblasts-to-MFBs transition and stimulate TNC deposition. This TGFβ-mediated effect on CAFs is further increased after castration, suggesting that stromal remodeling underlies the development of CRPC. TGFβ released from PCa cells also determines NADPH-oxidase 4 (Nox4) expression in CAFs (Figure 1), which induces metabolic changes characterized by high ROS production.

CAF-derived ROS, in turn, enhance tumor cell proliferation and migration. In PCa reactive stroma, loss of TGFβ signaling activity in subgroups of CAFs has been identified. In vitro and in vivo experiments showed that a mixture of fibroblast populations positive and negative for TGFβRII induced malignant transformation of non-tumorigenic prostate cells, similarly to CAFs, whereas this effect was not seen with the single populations. The co-culture of the two fibroblast types enhanced the production of pro-tumorigenic factors TGFβ1, CXCL12, and FGFs.

These observations reveal the complexity of TGFβ-mediated signaling in prostate TME. Further investigations are needed to dissect these signaling interactions and to target TGFβ signaling for therapeutic purposes. IL-6, which is produced by both PCa and stromal cells, is another fundamental player within the PCa microenvironment that influences many aspects of prostate tumorigenesis, including proliferation, angiogenesis, and insensitivity to androgens.

For example, CAF-secreted IL-6 induces VEGF secretion from PCa cells, stimulating tumor angiogenesis. Due to its multiple effects on prostate TME, the clinical use of inhibitors of IL-6 or its related transcription factor STAT3 is under investigation. Other examples of molecular players involved in paracrine communication between cancer cells and CAFs are secreted frizzled-related protein 1 (SFRP1), FGF2, FGF10, heat shock protein 90 (HSP90), YAP signaling and Hedgehog (Hh) signaling components.

Extracellular vesicles have been found to play a relevant role in the paracrine communication between cancer cells and CAFs in prostate TME, acting as transporters of cellular DNA, RNA, and proteins. PCa cells release exosomes that contain TGFβ1, which induces MFB transition. By disrupting the exosome secretion process in PCa cells, the stroma loses its growth-promoting properties.

In addition, CAFs secrete exosomes containing non-coding RNAs, such as microRNA-409, which inhibits the translation of tumor-suppressor genes, promoting EMT and thus tumor invasiveness (Figure 1) [133]. Factors secreted by CAFs might also have a systemic/endocrine effect on the organism. A study suggests that the TGF-β/BMP family member GDF15, secreted by CAFs, exerts not only a paracrine effect on tumor growth, migration, and invasion, but also has tumor-instigating properties on distant PCa cells. Indeed, by using a xenograft model the authors showed that GDF15-expressing fibroblasts induce the outgrowth of PCa cells implanted in a distinct site in a manner that is distinct from control fibroblasts.

Supporting its systemic effect, high serum GDF15 levels are correlated with weight loss and anorexia in advanced PCa patients and in PCa xenograft models. PCa cells and CAFs can establish signaling interactions through direct cell–cell contact. Physical interaction between the two types of cells is relevant for the regulation of cancer cell motility through modulation of Eph-Ephrin signaling. A study using in vitro co-cultures of PC-3 prostate cancer cells and fibroblasts demonstrated that the transmembrane EphrinB2 ligand interacts with its receptor EphB3/4 in PC-3 cells activating Cdc42 signaling, which stimulates cancer cell migration and causes a failure of contact inhibition of locomotion.

This study suggests that direct contact between CAFs and cancer cells promotes PCa invasiveness and this hypothesis is supported by the high expression in advanced human PCa of EphB3/4 and EphrinB2 in epithelial and stromal cells, respectively. Another study suggests that direct cancer–stromal cell contact enhances the tumorigenic properties of the reactive stroma by activating Notch signaling in stromal cells through the interaction between Jagged1 ligand, bound to PCa cell membranes, and its cognate receptors on CAFs. This causes the formation of inflammatory foci and activates TGFβ signaling in stromal cells, thus promoting tumor progression. Interestingly, CAFs can also support PCa cell oxidative metabolism by releasing lactate and forming cellular bridges to transfer their mitochondria to cancer cells, as it has been shown in both in vitro and in vivo PCa models. Mitochondrial metabolism promotes cell proliferation and inhibits apoptosis.

Its activation is related to the reverse Warburg effect, a phenomenon observed in many cancer types including PCa, in which CAFs undergo aerobic glycolysis to release pyruvate and lactate that are used by cancer cells to activate mitochondrial oxidative metabolism.

The fact that CAFs and PCa cells establish cellular bridges to transfer mitochondria indicates that these tumors strongly depend on the stroma to sustain their metabolism and growth.

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