



Review

Coordinated AR and microRNA regulation in prostate cancer



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Abstract The androgen receptor (AR) remains a key driver of prostate cancer (PCa) progression, even in the advanced castrate-resistant stage, where testicular androgens are absent. It is therefore of critical importance to understand the molecular mechanisms governing its activity and regulation during prostate tumourigenesis. MicroRNAs (miRs) are small ~22 nt non-coding RNAs that regulate target gene, often through association with 3' untranslated regions (3'UTRs) of transcripts. They display dysregulation during cancer progression, can function as oncogenes or tumour suppressors, and are increasingly recognised as targets or regulators of hormonal action. Thus, understanding factors which modulate miRs synthesis is essential. There is increasing evidence for complex and dynamic bi-directional cross-talk between the multi-step miR biogenesis cascade and the AR signalling axis in PCa. This review summarises the wealth of mechanisms by which miRs are regulated by AR, and conversely, how miRs impact AR's transcriptional activity, including that of AR splice variants. In addition, we assess the implications of the convergence of these pathways on the clinical employment of miRs as PCa biomarkers and therapeutic targets.

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1. Introduction

The androgen receptor (AR) is a ligand-activated transcription factor that is vital both for normal prostate

development and tumourigenesis. Upon ligand binding in the cytoplasm, it dimerises and translocates to the nucleus, where it stimulates target gene transcription through association with androgen response elements (AREs) within

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promoter and enhancer sequences, promoting cell survival and proliferation. Its centrality to the pathogenesis of prostatic diseases has driven the rational development of drugs targeting the AR signalling axis, and thus androgen-deprivation therapies (ADT) remain mainstay treatments. ADT encompasses multiple diverse therapeutic strategies: Reduction in circulating androgen levels through inhibition of pituitary gland-secreted hormones that stimulate testicular androgen synthesis (lutensising hormone-releasing hormone [LHRH] and gonadotropin-releasing hormone [GnRH] agonists), repression of intra-tumoural androgen synthesis (CYP17A inhibitors—Abiraterone) and inhibition of AR activity (anti-androgens/AR antagonists such as Enzalutamide that bind to AR to recruit corepressors and prevent AR target gene transcriptional activation). Unfortunately, resistance to ADT almost invariably develops after 2–3 years and the emergent lethal disease is termed “castration resistant prostate cancer” (CRPC), progressing in the near-absence of circulating androgens.

It is well-established that AR signalling remains a critical driver of growth and progression in the vast majority of CRPC patients. Evidence supports the hypothesis that independence from circulating androgens may be mediated by a number of diverse molecular and genetic mechanisms, including AR gene amplification, promiscuous activation of AR by non-androgenic ligands, AR bypass and activation of AR target genes by alternative nuclear receptors, and expression of AR splice variants [1]. Many of these truncated receptor forms, of which approximately twenty have been described [2], lack the ligand binding domain (LBD) resulting in constitutive expression of an AR full length (AR-FL)-distinct transcriptional programme [3]. Since anti-androgens exert their effects through association with the AR LBD, the above mechanisms render tumours resistant to “gold-standard” ADT. Given the key role of AR at all tumour stages, it is unsurprising that a wealth of coordinated and sophisticated genomic, epigenomic and non-genomic modes of regulation have emerged in recent years.

MicroRNAs (miRs), first identified in 1993, are small ~22 nt non-coding RNAs that post-transcriptionally regulate their targets through binding to complementary sequences, often in the 3′ untranslated region (3′UTR). This frequently leads to translational inhibition and/or transcript degradation, although transcript stabilisation has also been described. miR profiles are perturbed in prostate cancer (PCa) compared to non-malignant prostate [4–9], and in metastatic CRPC compared to organ-confined, hormone-responsive disease [10–14]. Like AR, miRs can have potent oncogenic activity, but also tumour suppressive roles [15], potentially regulating expression of hundreds of transcripts and gene networks, and profoundly impacting disease outcome. Whilst miRs (or their inhibitors) are yet to fulfil their promise as effective therapeutics, a number are currently in Phase 2/3 clinical trials [16–18], and have significant clinical potential due to their small size, stability, potential to target multiple components of same pathway (so-called “vertical” targeting), tissue specificity and capacity for tumour-targeting, and potential as personalised miR/inhibitor “cocktails”.

Notably, in contrast to other genes, the AR 3′UTR is 2.6 times longer than its coding region, is the most miR-

targeted transcript in PCa cells [19], and may undergo dynamic 3′UTR shortening in response to external stimuli [20]. Thus miRs represent an additional and important non-genomic weapon in the arsenal of AR regulation. Recently, the importance of AR variants in PCa pathogenesis and drug resistance has become clear: Variants have distinct 3′UTRs from wild-type (WT) [21,22], and work from our own laboratory and others has identified variant-unique and pan-variant targeting miRs (unpublished data [23,24])—pan-variant miR-targeted therapeutics could circumvent continued AR activity in castrate setting mediated by variants, and thus hold considerable therapeutic promise. Therefore, this review also considers miR-targeting of AR variants.

In the canonical pathway, mammalian miRs are transcribed in the nucleus by RNA polymerase II, where they are 5′ capped and adenylated. They are then cleaved into one or more ~70 nt hairpin-structured precursor miRs (pre-miRs) by the RNase Drosha-containing Microprocessor (MP) complex and exported from the nucleus by Exportin-5 [25–28]. In the cytoplasm, the stem-loop is cleaved by a second RNase, Dicer, to yield a miR duplex. This is unwound and one strand incorporated into the AGO2-containing RNA-induced silencing complex (RISC) [25–28]. The mature miR guides RISC to complementary sequences in target RNAs. The intricate multi-step biogenesis of miRs (with additional complexity afforded by non-canonical pathways that bypass Drosha or Dicer [29], and by post-translational modifications to pathway components [30]) provides opportunities for AR-mediated fine-tuning of their synthesis at many stages. Indeed, mounting evidence supports a complex, responsive and dynamic network of bi-directional interactions between AR and miRs at all steps of biogenesis and during target gene regulation [31,32]. For example, Dicer siRNA completely inhibited dihydrotestosterone (DHT) induction of the well-characterised androgen responsive gene, prostate-specific antigen (PSA) in LNCaP cells, and Dicer^{-/-} mice developed androgen-insensitivity syndrome, indicating that androgen-regulated miRs are vital mediators of AR action *in vivo* [33]. Further, AR upregulates expression of corepressor-targeting miRs to maintain its own expression: 75% of predicted NCoR or SMRT-targeting miRs were upregulated in the prostate by DHT, and Dicer siRNA treatment increased transcription of both NCoR and SMRT [33]. Additionally, AR has been demonstrated to associate with miR gene promoters [34], upregulate Dicer and DGCR8 in PCa cells [35], and increase Drosha cleavage of pri-miRs [36]. Given their importance in PCa development, considerable efforts have been made to assess the impact of AR signalling at miRnome-wide level.

Understanding AR regulation of miR biogenesis, and conversely, miR modulation of AR activity, and how these are perturbed in PCa could inform development of effective novel therapeutics. Identified androgen-regulated miRs can also provide a read-out of AR activity, acting as surrogate AR activity biomarkers informative of disease presence, prognosis and response to therapy. This review summarises our current knowledge of miRs that target AR and its variants, mechanisms by which androgens and AR signalling modulates mature miR levels, and the potential of AR-regulating or -regulated miRs as clinically-informative biomarkers and therapeutics.

2. miR regulation of the androgen signalling axis

2.1. miRs directly targeting AR

The importance of miRs in regulating AR activity is supported by the observation that AR is the most miR-targeted oncogene in PCa, and in the top 5% of regulated genes overall: Hamilton et al. [19] used AGO-PAR-CLIP-seq to immunoprecipitate crosslinked miR- and AGO2-containing RISC complexes and sequence associated miR RNA targets. Further, Dicer is required for androgen-induction of AR target genes, with Dicer-null mice developing androgen-insensitivity syndrome [33]. miRs are able to directly bind to the 3'UTR of AR, suppressing it post-transcriptionally through modulated transcript stability, translational efficiency and regulatory networks. Initially, miR target prediction algorithms used a 436 nt AR 3'UTR sequence (from NM_000044), but AR 3'UTR length was later shown to be 6777 bp (NM_000044.4). At 2.6-time longer than the coding region, this strongly supports a vital regulatory role for the AR 3'UTR and its associated miRs and RNA-binding proteins (RBPs) in determining AR function. Despite this, the AR 3'UTR is remarkably understudied. It has been reported that LNCaP cells express AR isoforms with two distinct 3'UTR lengths: The canonical 6.8 kb form and a distinct alternatively-spliced isoform that lacks a 3 kb region of the wild-type 3'UTR [37]. Direct and indirect mechanisms of miR regulation of AR are illustrated in Fig. 1.

Several studies have aimed to systematically identify direct AR-targeting miRs in an unbiased manner: Östling et al. [38] coupled protein lysate microarrays with miR expression analysis to quantify miR-mediated changes to AR protein content in PCa cell lines. Seventy-one miRs significantly altered AR protein levels (19 positively and 52 negatively), of which 13 were validated using luciferase-based 3'UTR reporter assays: miR-34a, miR-34c, miR-135b, miR-185, miR-297, miR-299-3p, miR-371-3p, miR-421, miR-449a, miR-449b, miR-634, miR-654-5p and miR-9 [38]. A second study screened a library of 810 miR mimics to identify those altering AR activity through AR and PSA protein lysate microarrays, AR transcriptional reporter assays, and cell viability assays. Forty-three candidate mimics inhibited AR transcriptional activity and AR and PSA protein level, in a minimum of two PCa cell lines. In particular, miR-30 family members were found to be direct AR inhibitors [39], whose inhibition enhanced AR expression and androgen-independent cell growth. Notably, miR-30c-5p and miR-30d-5p were significantly reduced in mCRPC versus healthy control tissue. In a third study, our laboratory transfected hormone-responsive and hormone-resistant PCa cells expressing a luciferase-based AR reporter with a miR inhibitor library, identifying 78 that significantly alter AR activity. Further molecular characterisation showed that miR-346, miR-361-3p and miR-197 inhibitors significantly reduced AR transcriptional activity, mRNA and protein levels, increased apoptosis, reduced proliferation, repressed epithelial-to-mesenchymal transition (EMT), inhibited PCa migration and invasion, and showed additive effects with AR inhibition. These miRs were shown to increase AR activity through direct transcript stabilisation. Interestingly, miR-346 and miR-361-3p were additionally shown to alter levels of constitutively-active

AR variants, and promote variant-driven PCa cell proliferation, and may thus contribute to persistent AR signalling observed in CRPC in the absence of circulating androgens [40].

Importantly, several screen-identified miRs have been identified for their AR-modulatory effects in further candidate-driven studies. For example, miR-185-targeting of AR is consistent with the observations of both Qu et al. [41], who showed that miR-185 reduces AR protein levels to decrease LNCaP cell proliferation *in vitro* and *in vivo*, and Liu et al. [42], who demonstrated that the same interaction yields anti-apoptotic effects. Additionally, another member of the miR-34 family, miR-34b, was demonstrated to directly interact with AR 3'UTR in a study using African-American PCa cell line and patient samples [43]. The authors observed lower expression of miR-34b in African-American compared to Caucasian patient tumours, and proposed this AR-targeting miRs as a determinant of increased PCa predisposition in this population [43]. In agreement with the study of Kumar et al. [24], miR-30 was found to inhibit *in vivo* and *in vitro* PCa cell growth, although the authors did not examine effects on AR [44].

In general, however, there is little agreement in candidate AR-targeting miRs identified by the above screening approaches. There are many likely reasons for this lack of consensus, including differences in miR library types (mimic or inhibitor, with different oligonucleotide stabilisation chemistries), use of different inhibitor/mimic concentrations, use of PCa cell lines with different transfection efficiencies and endogenous miR levels, differing end-point functional assays, and application of contrasting statistical analyses and thresholds. It is also important to note that none of the above methods distinguish direct from indirect miR regulation of AR. In an elegant and more direct elucidation of functional miR: Target interactions as outlined above, Hamilton et al. [19] identified 147 seed complementary sites for 71 miR families within the AR 3'UTR. The validity of the approach was supported by the observation that four of these interactions (miR-9, miR-34c, miR-185 and miR-488) had been previously identified [4,24,38,40].

When assessing candidate-driven AR-regulating miR identification, Fernandes et al. [31] suggest that the most disease-relevant miRs are those that: i) Have been identified in multiple studies through use of several cell/animal models, ii) have been verified for AR-modulatory effects using both miR mimics and inhibitors (since mimics can show non-physiological activity), iii) are downregulated in PCa as compared to benign prostate tissue (as most tumours are "addicted" to oncogenic AR activity, thus theoretically, oncogenic selection pressure would dictate repression of AR-inhibitory miRs), and iv) are inversely-correlated with AR protein levels in clinical samples. These criteria are satisfied by miR-205 [45], miR-145 [46] and miR-34a [31,38]. In addition, miR-205 was shown to enhance chemo- and radiotherapy cytotoxicity of PCa cells [47,48], and to inhibit their migration and invasion [49], providing further support for its tumour suppressive capabilities. Similarly, miR-145 and miR-34a have both been demonstrated to inhibit PCa proliferation in cell line models of PCa [50,51], show utility as PCa diagnostic biomarkers in urine [52,53] and modulate sensitivity to radio-/chemotherapy [54,55].

Evidence for oncogenic selection pressure for down-regulation of AR-repressive miRs was provided by Lin et al.

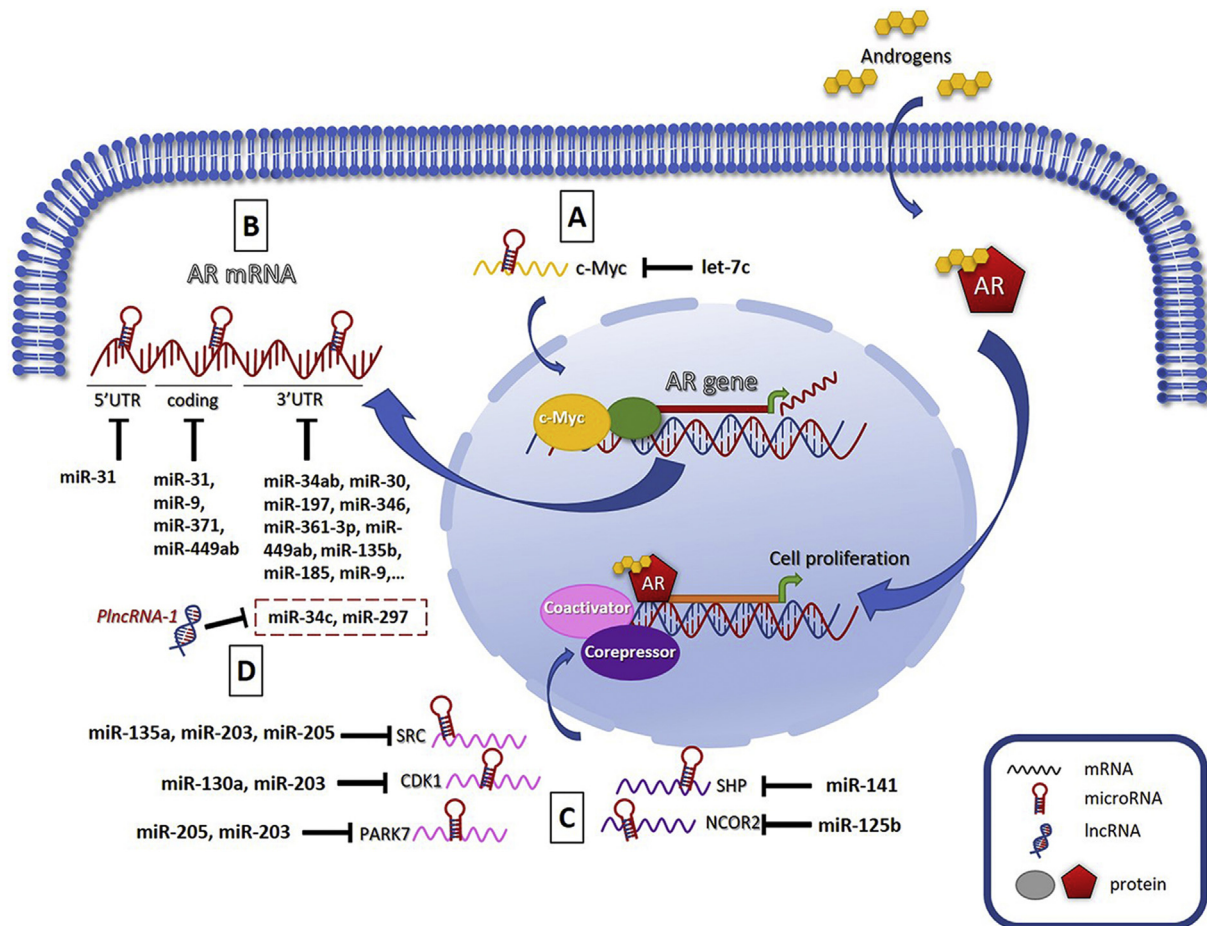


Figure 1 Direct and indirect mechanisms of microRNA (miR)-regulation of androgen receptor (AR) activity. (A) AR transcription—miRNAs modulate expression of AR by targeting AR transcription factors and regulatory proteins. Let-7c indirectly represses AR activity by targeting c-Myc, which acts as a transcription factor for the AR gene [65,66]; (B) AR mRNA—miRNAs target AR directly through binding to microRNA response elements in its transcript. Most microRNAs target the 3'UTR [4,24,31,38–43,45,46], but interactions with the coding region and 5'UTR have also been reported [4,56]; (C) AR-mediated transcription of target genes—miRNAs modulate AR indirectly by targeting cofactors, regulators or effectors of AR activity [19,63,64]; (D) AR competitive endogenous RNAs (ceRNAs) —AR is regulated by ceRNAs containing microRNA response elements (MREs) for AR-targeting miRNAs. For example, PlncRNA-1 protects AR from miR-mediated repression. A direct transcriptional target of AR, this lncRNA contains some of the same miR binding sites as AR, allowing it to sequester two AR-targeting miRNAs: miR-34c and miR-297. These in turn directly suppress PlncRNA-1, creating a feedback loop of AR regulation [87].

[56], who demonstrated that the miR-31 gene promoter is hypermethylated in primary and metastatic PCa as compared to benign tissue. Cell cycle-regulatory miR-31 was shown to repress AR through direct association with the coding region of its transcript, to inversely correlate with AR and disease aggressiveness, and to inhibit *in vivo* prostate tumour growth [56].

2.2. AR 3'UTR modulation alters microRNA binding

Cancer cells are known to evade various regulatory mechanisms to promote continued cell survival and proliferation, and miR-mediated repression in PCa is no exception. Widespread, active and dynamic shortening of oncogene 3'UTRs is a common feature of many cancers, associated with poor clinical outcome [57–59]. 3'UTR shortening enhances oncogene activity through loss of association of

repressive miRNAs; indeed shorter 3'UTR isoforms can yield up to ten-fold increased protein levels than longer 3'UTRs [57]. Together, the observations that i) AR full-length 3'UTR is 2.6-fold longer than its coding region, ii) different lengths on AR 3'UTR have been described in cell lines (in LNCaP, one AR isoform lacks 3 kb of the 3'UTR [37]), iii) that AR is the most miR-targeted oncogene in PCa, and iv) that AR is critical to PCa cell survival and proliferation at all disease stages, all suggest that active 3'UTR shortening in response to environmental cues could have particularly profound effects on AR activity, representing a further mechanism of PCa progression [20], and that at least part of this effect is due to loss of tumour-suppressive miR association.

Dynamic alterations in AR 3'UTR length are likely attributable to alternative polyadenylation (APA) and altered usage of sub-optimal polyadenylation sites (PASs). In addition, intronic APA of AR has recently been described [60], giving rise to constitutively active AR-Vs, and

dysregulation of APA factors such as CPSF proteins has been observed in PCa [60]. miR binding sites within target mRNAs can also be lost through point mutations or chromosomal translocations that show increased prevalence in cancer as opposed to benign tissue. These understudied aspects of AR regulation require thorough mechanistic elucidation and investigation in clinical specimens to permit therapeutic exploitation.

2.3. miRs targeting AR indirectly

AR activity is heavily regulated at different points of its signalling axis by numerous factors, including corepressors, coactivators, pioneer and transcription factors, chaperones, long non-coding RNAs and miRs [61]. In addition to their individual effects on AR, they can modulate each other through feedback circuits and complex regulatory networks to sensitively fine-tune AR activity. AR transcriptional activity is modulated by at least 200 different coregulators as reviewed in Ref. [62], which are under miR control: Oncogenic miR-141, which is frequently overexpressed in PCa, represses the AR corepressor, SHP, resulting in increased AR activity [63]. Another AR corepressor, NCOR2, is targeted by miR-125b to similar effect [64]. In contrast, let-7c indirectly represses AR activity by targeting c-Myc, which acts as a transcription factor for the AR gene [65,66]. Additionally, tumour suppressive miR-135a, miR-203 and miR-204 target multiple oncogenic, positive regulators of AR activity, including nuclear receptor coactivator NCOA2, as well as PARK7, MNAT1, B-catenin, CDK1, KMT2A, FOXA1 and EZH2 (miR-203), and KMT2D, SPOP and p53 (miR-204) [19,64]. Together, these interactions indirectly decrease AR activity through targeting of multiple oncogenic pathways. Their multi-mechanistic functionality may circumvent development of resistance, if employed therapeutically.

Whilst all the aforementioned miRs target the AR, not all effects are equal. Depending on the degree of complementarity between a miR and its target binding site, the target mRNA transcript is either inhibited or degraded [67–69]. The number of binding sites miRs have on their target mRNAs also determines the strength of their effect. For example, miR-204 has four binding sites on AR coactivator SRC2 [19]. The level of indirect miR regulation of AR activity is determined by the function of its direct target: For example, AR corepressor SHP inhibits up to 97% of AR-induced activity through interaction both with AR-LBD and AR-NTD, reversing AR coactivator-mediated activation [70]. Thus miR-141 targeting of SHP is hypothesised to more profoundly derepress AR than other indirect AR-modulatory miRs. However, since miR-141 can also directly target AR, miR-141 likely functions to either positively or negatively modulate AR, as determined by cellular context and the relative levels of miR-141, AR, SHP, and other SHP-modulated AR coregulators, as well as other miR-141 targets acting as competing endogenous RNAs (ceRNAs) to SHP. This highlights the importance of considering miR targeting in the context of complex interaction networks, not as linear exchanges. As a further example of positive and negative feedback loops modulating the AR signalling cascade, androgens that activate AR also induce AR-repressive miRs such as miR-135a [19], and miR-31 and AR

repress each other [56]. miRs can also demonstrate additive and synergistic effects on AR: For example, miR-205 was only observed to modulate AR signalling in combination with miR-130a, greatly enhancing its effect on AR [64]. miRs can also upregulate AR activity: Our group has demonstrated that miR-346, miR-361-3p and miR-197 directly interact with AR 3'UTR to stabilise the transcript, increasing AR protein levels and activity [40].

2.4. Cross-talk between miR biogenesis and androgen receptor signalling

miR biogenesis proteins have been shown to modulate AR signalling at several levels. The effector of miR targeting, the RISC component, AGO2, has been shown to cooperatively repress transcription of AR gene through promoter binding in association with the histone H3-lysine 9 methyltransferase, SETDB1, and a small interfering antineoplastic RNA [71]. This led to recruitment of the repressive SIN3-HDAC complex. Conversely, RISC cofactors PACT and TRBP associate with Steroid Receptor RNA Activator (SRA), an RNA transcript that functions as a nuclear receptor coactivator [72–74]. Both proteins were recruited to AR target gene promoters and increased SRA-enhanced AR reporter gene activity [74]. The identification of a nuclear RISC complex containing PACT, TRBP and SRA supports the potential for coupled miR-mediated repression and AR transcriptional modulation within the same macro-complex, with the balance of activities determined by presence of specific RNA species. Further, the RNA-binding protein, FUS, may couple miR biogenesis to AR transcription activation, since it promotes processing of a subset of pri-miRs within the micro processor [75], acts as an AR cofactor [76], and cytoplasmic Drosha-containing complexes have been described [77,78]. Together these data demonstrate that the static miR-to-mRNA inhibition mechanism is increasingly outdated, with more complex interactions coming to light.

2.5. Indirect AR regulation of target genes mediated by miRs

Indirect AR regulation of protein-coding genes may also be attributable to androgen regulation of miRs targeting these transcripts. In evidence of this, Sun et al. [79] showed that the AR-induced gene repertoire is enriched for putative targets of the AR-inhibited miR-99a/let7c/125b-2 cluster. In further *in silico* analysis, Fernandes et al. [31] integrated predicted targets of AR-induced or -repressed miRs with panels of androgen-repressed or -induced transcripts [80], respectively, demonstrating significant enrichment of AR-upregulated genes in predicted targets of AR-repressed miRs and *vice versa* [31]. This therefore constitutes a physiologically-relevant mechanism by which AR indirectly modulates the cellular mRNA pool by exploiting miRs networks.

2.6. ceRNAs in AR and miR regulation

A novel regulatory network of ceRNAs may be involved in AR homeostasis. These RNA species comprise pseudogene

transcripts, lncRNAs and mRNA species containing miR binding sites. They compete with each other for miR binding, reducing the effect of miRs on their target mRNAs by acting as “sponges” ceRNAs [81,82]. Termed ceRNAs, these provide additional layers of complexity to miR regulatory networks and permit “fine-tuning” of gene expression, which is frequently dysregulated in cancer. It has been estimated that transcripts of approximately 7 000 genes can act as miR “sponges” [82], and many studies have applied computational and high-throughput sequencing approaches to the characterisation of these networks. In an elegant demonstration of the contribution of ceRNAs to cancer-associated processes, PTEN tumour suppressor activity is increased by endogenous transcripts harbouring identical miR response elements, functioning as coding-independent tumour suppressors by reducing PTEN:miR association, antagonizing PI3K/AKT pathway activity, and repressing proliferation [83]. Furthermore, modulated expression of ceRNA transcripts during disease progression indicated that ceRNA activity can be adaptive: Karreth et al. [84] reported significant enrichment of putative PTEN ceRNAs amongst a subset of genes whose loss accelerates tumorigenesis in a mouse model of melanoma.

CeRNA networks have also been mapped in PCa, showing lncRNA-miRNA-mRNA and mRNA-miRNA-mRNA interactions, some of which were capable of predicting disease survival [85]. In further support of the importance of ceRNAs in the regulation of PCa-critical tumour suppressor PTEN, and its biological relevance, PTENP1 pseudogene was shown to regulate cellular levels of PTEN and exert growth-suppressive effects. Indeed, the *PTENP1* locus is frequently deleted in human cancers [86]. Long non-coding RNA (lncRNA), PlncRNA-1, was revealed to function as a ceRNA to protect AR from miR-mediated repression. A direct transcriptional target of AR, this lncRNA contained some of the same miRNA binding sites as AR, allowing it to sequester two AR-targeting miRs: miR-34c and miR-297. These two miRs, in turn, directly suppressed PlncRNA-1, creating a feedback loop of AR regulation [87]. In attribution of its physiological importance, PlncRNA-1 was found to be overexpressed in PCa tissue versus healthy controls [88]. It is highly likely that many more ceRNAs play a role in regulation of AR via miR binding, modulating the regulatory network of AR in PCa.

2.7. miRs targeting AR splice variants

AR is expressed not only in its full-length form, but also as alternatively spliced versions, which were originally identified in PCa cell lines [89,90]. These are C-terminally truncated, thus lacking the canonical ligand-binding, and have been termed AR-variants (AR-Vs). More than 20 unique AR variants have been identified to date in human PCa cell lines (22Rv1, CWR-R1 and VCaP), xenograft models (LuCaP 86.2) and primary and metastatic patient tumours, and circulating tumour cells [89–94]. These variants differ in their functional activities and relative abundance in patient samples. The existence of these naturally occurring variants enables androgen-refractory PCa cells to grow in the absence of

androgens and supports the expression of many endogenous AR target genes [89].

To date, only a proportion of these variants have been studied in detail, the most extensively being AR-V7, along with AR-V1, AR-V3, AR-V9 and AR-V567es. As the majority of the variants retain both the N-terminal and DNA binding domains, they remain constitutively active *i.e.* AR-V7 and AR-V567es or are conditionally active (AR-V1 and AR-V9) depending on the cellular context [21,95]. The presence of AR-Vs has been detected in benign tissues, hormone naïve prostate tumours and CRPC samples [21,92,96,97]. Higher expression of AR-V7 was observed in aggressive, but hormone naïve prostate tumors that subsequently progressed to CRPC after surgical treatment, however the presence of AR-V9 and AR-V567es in hormone naïve specimens was not significantly associated with biochemical recurrence [21,97]. Moreover, these variants show significantly elevated levels in CRPC patients as compared to hormone naïve prostate tumours, and studies have shown that patients with elevated AR-V7 or detectable AR-V567es levels have a reduced survival compared to other CRPC patients [21,92,98]. This may be attributable to the association of ARVs with resistance to the androgen-directed therapies, enzalutamide and abiraterone [91,99].

As the current clinically-available androgen-targeted therapies target the LBD and are thus ineffective against ARVs, it is important to understand how other potential mechanisms of regulation, such as miR targeting, function to modulate ARV activity in order to therapeutically-target ADT resistance-associated ARVs. Recent studies have shown that certain miRs are able to bind and regulate both the full length and variant AR by targeting different sites in the 3'UTR region, for example Shi et al. [22,100] have shown that miR-124 is able to target the transcripts of WT/full length AR, AR-V4 and AR-V7 due to the presence of distinct 3'UTR targeting sites. The binding of miR-124 to these sites results in a down-regulation in expression of both WT/full length AR and AR-V7. Our lab has recently reported that miR-346 and miR-361-3p can also alter the levels of both WT/full length AR and the constitutively active variants [40]. Traditionally, studies involving miR regulation of genes focus on target sites within the 3'UTR region of these mRNAs. However, Kumar et al. [24] have reported that miR-646, miR-371-3p and miR-193a-3p can regulate the translation of WT/full length AR and AR-V7 via shared binding sites located within the coding regions, suppressing the protein expression of both AR forms. The ability of miRs to differentially regulate WT/full length AR and ARVs is likely due to the differences in their 3'UTR sequences: Variants lacking the C-terminal exon (*i.e.* AR-v1, v4, v7 & v9) have entirely distinct 3'UTRs as compared to the WT transcript, whereas AR-V567es possesses a 3'UTR with a high degree of sequence similarity to WT-AR, with the addition of exon 9 [2,40]. The aforementioned variants may thus lack miR binding sites found in the WT transcript, or contain additional variant-specific sites: Many of the miRs identified that target WT/full length AR would not affect the expression of the AR variants or vice versa. Whilst the complete repertoire of WT-vs variant-targeting miRs

remains to be determined, miR-181c-5p was shown to effectively suppress AR-V7, but not WT-AR, through association with a variant-specific MRE [23]. In contrast, some miRs have been demonstrated to target both WT and variant AR, but through different miR response elements: miR-124 binds to the WT transcript at a distinct 3'UTR site to that in AR-V3, AR-V4 and AR-V7 [22,100]. Extent of miR-mediated repression is likely to be mediated by the local RNA topology and thermodynamics in addition to the site sequence. Thus these different MREs may have differential impacts on AR activity, and may support co-evolution of miRs and variant forms of the androgen receptor [31]. It is also tempting to speculate that the degree of sequence dissimilarity between WT and variant AR 3'UTRs may lead to the characterisation of additional variant-specific miRs in the coming years. ARV-targeting miRs are summarised in Table 1.

3. Androgen regulation of miRs

Several authors describe direct and indirect androgen modulation at various stages in miR biogenesis [34,35,50,101–108], from miR transcription to maturation, as summarised in Fig. 2. Further, it has been shown that the miR targetome in AR-positive cell lines is distinct from that

of AR-negative PCa cell lines [19], illustrating the profound impact of AR on miR activity.

3.1. AR modulation of miR gene transcription

As detailed above, AR exerts its function by binding to androgen response elements (AREs), dihexameric motifs located in the enhancers and promoters of its target genes. These regions have been reported in numerous miR-gene promoters as well as in miR host genes promoters. Integration of androgen-regulated miR expression profiling data sets with genome-wide AR cistromes has enabled rapid and specific identification of direct transcriptional AR target miRs. Takayama et al. [34] combined 5' cap analysis of gene expression (CAGE) with CHIP on array (CHIP–chip) to identify miR-222, miR-221, miR-21, miR-125b, miR-218-1, miR-218-2, let7c and miR-100 as AR binding site-adjacent. Pasqualini et al. [102] later identified 32 miRs putatively bound by AR within 25 kb of their transcription start sites, and significantly modulated by androgen treatment, including miR-22, miR-29a (both upregulated), and the miR-17-92 cluster (cell line context-dependent up- or downregulation), which were additionally found to be dysregulated in PCa versus benign tissue. In keeping with these findings, Wang et al. [108] also

Table 1 Reported miRs targeting variant AR in prostate cancer.

Target	miR	Binding location	Identifying experiments	Effects on AR	References
AR-V7	181c-5p	3'UTR	Expression of AR mRNA & protein/AR-V7 3'UTR luciferase reporter assay	miR-181c-5p overexpression represses AR-V7 activity, transcript & protein expression.	[23]
AR-V7/WT AR	101-3p	3'UTR	Expression of AR protein/3'UTR luciferase reporter assay (WT AR only)	miR-101-3p overexpression represses AR-V7 protein expression.	[24]
ARV4,V7/WT AR	124-3p	3'UTR	Expression of AR protein/3'UTR luciferase reporter assay	miR-124-3p overexpression represses AR-V7 activity, transcript & protein expression.	[22,100]
AR-V7/WT AR	135b	3'UTR	Expression of AR protein/3'UTR luciferase reporter assay	miR-135b overexpression represses AR-V7 protein expression.	[38]
AR-V7/WT AR	488	3'UTR	Expression of AR mRNA & protein/3'UTR luciferase reporter assay	miR-488 overexpression represses AR-V7 transcript & protein expression.	[24,38]
AR-V7, V567es/WT AR	346	3'UTR	Expression of AR protein/proliferation assays	miR-346 inhibition reduces AR expression and PCa proliferation.	[40]
AR-V7, V567es/WT AR	361-3p	3'UTR	Expression of AR protein/proliferation assays	miR-361-3p inhibition reduces AR expression and PCa proliferation.	[40]
AR-V7/WT AR	193a-3p	CDS	Expression of AR protein/PSE-PBN luciferase reporter assay	miR-193a-3p overexpression represses AR-V7/WT AR activity & protein expression.	[24]
AR-V7/WT AR	371-3p	CDS	Expression of AR protein/PSE-PBN luciferase reporter assay	miR-371-3p overexpression represses AR-V7/WT AR activity & protein expression.	[24]
AR-V7/WT AR	646	CDS	Expression of AR protein/PSE-PBN luciferase reporter assay	miR-646 overexpression represses AR-V7/WT AR activity & protein expression.	[24]

AR, androgen receptor; AR-V7, AR-variant 7; WT, wild-type; 3'UTR, 3' untranslated region; CDS, coding sequence.

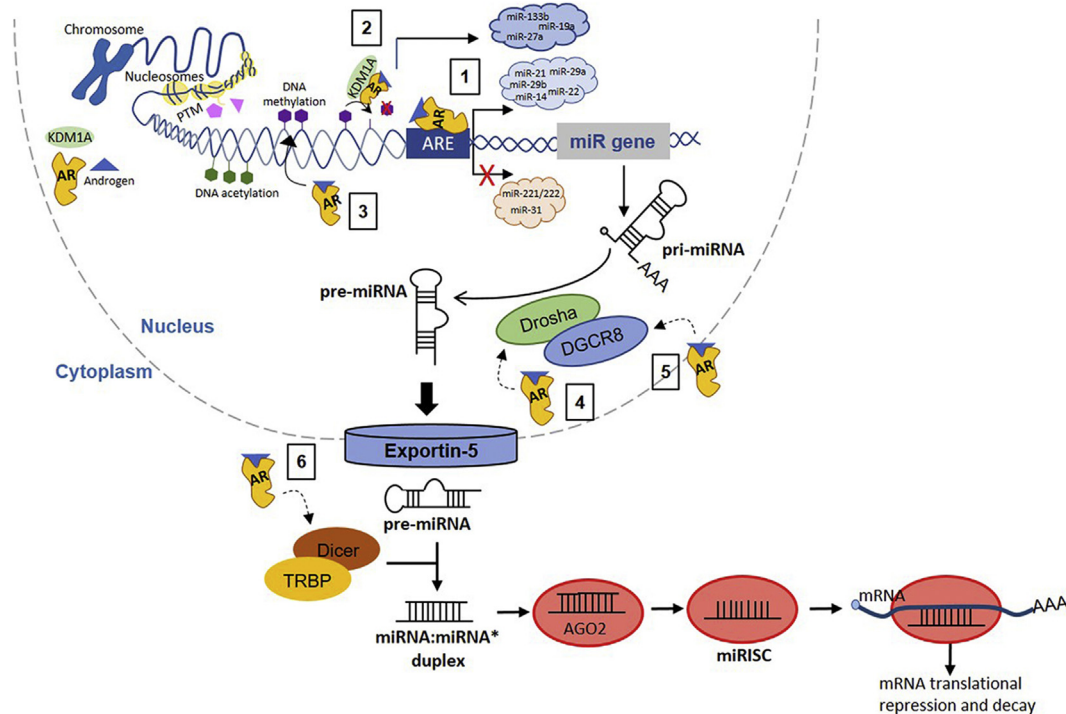


Figure 2 Androgen receptor (AR) regulates microRNA (miR) biogenesis at different stages. 1) AR binds to androgen receptor elements (ARE) at promoter regions of miR genes or miR host genes to increase miR expression (e.g. miR-21, miR-29a, miR-29b, miR-22 or miR-14), or to repress it (e.g. miR-17a, miR-20a, miR-222/-221 and miR-31) [56,103–105]. 2) AR associates with lysine-specific demethylase 1 (KDM1A) to remove repressive methyl marks from AR-target miR genes to enhance transcription of miR-27a, miR-133b and miR-19a through post-translational modifications mediated by KDM1A at miR gene promoters [114]. 3) AR has been linked to altered methylation of miR-22 and miR-29a through an unknown mechanism [102]. 4) AR increases Drosha-mediated pri-miR processing of the miR-23a27a24-2 cluster to promote miR maturation [36]. 5) AR upregulates protein levels of the Drosha accessory factor, DGCR8 [35]. 6) Dicer, the enzyme responsible for generation of miR:miRNA* duplexes from pre-miRs, is transcriptionally-upregulated by androgen signaling [35].

described androgen upregulation of miR-22 and miR-29a amongst 15 miRs found to be coregulated by both vitamin D and testosterone. Notably, they showed androgen-downregulation of miR-17-92 cluster members [108], and their demonstration of androgen stimulation of miR-21 was consistent with the presence of an ARE within the oncogenic miR-21 promoter [104]. In further evidence of the ability of AR to act as a miR transcriptional repressor, AR binds AREs upstream of the miR-221/222 and miR-31 loci, respectively, to repress their gene products [56,109]. Although early reports identified more miRs to be downregulated than upregulated in PCa compared to benign tissues [50,103,105], in general, AR induces expression of oncomiR, pro-proliferative, pro-survival and anti-apoptotic miRs as downstream effectors of AR activity, including miR-21, miR-27a, miR-133b, miR-32, miR-148a, miR-182-5p and miR-29a [34–36,102,104,107,110], and downregulates tumour-suppressive, pro-apoptotic miRs such as miR-99a and miR-421 [34,79,106,111], although exceptions to this model exist (miR-221/222 [34,107,110], miR-1 [101]). AR has also been shown to modulate miRs through transcriptional regulation of intragenic miR host genes [102]. Indeed, of 32 androgen-regulated miRs with AR-bound

promoters, 28 were located within protein-coding genes [102]. It is also important to consider that dynamic, early and late responses to androgen treatment, and differences in response to alternative androgenic compounds in diverse cellular contexts as profiled using different technologies (microarray, small RNA-seq) have resulted in identification of distinct androgen-regulated miRs profiles, with overlapping miRs likely representing the most PCa-relevant AR-target miRs. Further, the number of direct AR transcriptional target miRs is likely greater than thus far reported, as AR is known to modulate transcription through association with enhancer elements far distal to miR gene transcription start sites.

3.2. AR regulation of epigenetic modifications

Chromatin is organized around nucleosomes, which are composed of histones. Epigenetic editing of these histones through post-translational modifications (PTM) switch “on” or “off” gene transcription, including that of miR genes. Acetylation activates transcription, whilst methylation events constitute repressive modifications [112]. Both processes can be altered by AR activity. For instance, AR associates with lysine-specific demethylase 1 (KDM1A) to

remove repressive methyl marks of AR-target genes such as miR-27a, miR-133b and miR-19a [113]. Additionally, AR modulates methylation of promoter elements of androgen-upregulated miR-22 and miR-29a, although the precise mechanism remains to be elucidated [102].

AR has additionally been shown to indirectly regulate 5-hydroxymethylation signatures through induction of the TET2-targeting miR-29 family [114]. AR-upregulated miR-29 binds directly to the TET2 3'UTR, leading to loss of oxidation of methylated cytosine (5-mC) to 5-hmC. Indeed, miR-29 is increased in hormone-refractory versus -sensitive PCa, and high levels predict poor outcome of PCa patients. Mechanistic investigations revealed that increased global 5-hmC may inhibit PCa progression through repression of FOXA1 activity, whilst 5-hmC loss activates key PCa drivers including AR and mTOR [114]. This study constitutes an important demonstration of the ability of miRs to effect AR transcriptional activation and pro-proliferative activity through targeting of epigenetic modifiers. Of note, a separate study demonstrated TET2 association with AR and AR coactivators in PCa cells, whilst TET2 knockdown increased PSA expression [115]. Further, TET2 binding sites and resultant hydroxymethylcytosine residues were identified in the proximal PSA promoter [115]. When considered in the context of the observation that other AR-regulated miRs also target TET2 (for example, miR-200a and miR-125b [116]), it is likely that AR upregulation of TET2 targeting miRs constitutes an important epigenetic positive feedback loop to maintain AR activity in PCa.

3.3. AR modulation of miR biogenesis

MiRs are known to be deregulated in many cancer types, and to contribute to disease progression. AR is the main driver of PCa and some of its roles in modulating the miR landscape (miRnome) in disease have been stated above. The influence of AR in the PCa miRnome was investigated by Hamilton et al. [19] in AGO-PAR-CLIP-seq analysis, where PCa cells treated with the anti-androgen, enzalutamide, decreased global miR:mRNA 3'UTR interactions by 25%. This provides evidence for a role for AR in modulating activity of the miR biogenesis cascade. Indeed, several studies have determined roles for AR in miR processing and maturation at various stages. Data from our own laboratory showed that not only does AR enhance miR-23a27a24-2 cluster transcription through binding to the gene promoter, but it also promotes miR maturation by enhancing Drosha-mediated pri-miR processing [36]. Dual-mechanism AR upregulation of miR-27a constitutes a positive feedback loop by which AR maintains its own activity through repression of the AR corepressor, Prohibitin (PHB). Similarly, AR reinforces its own activity by increasing levels of oncomiR-21, which in turn enhances AR protein levels and transcriptional output, possibly through repressing PTEN-mediated AR degradation [117–119].

In addition, several genes with key roles in miR biogenesis are androgen-upregulated, including Dicer, the enzyme responsible for generation of miR:miR* duplexes from pre-miRs, and the Drosha RNase cofactor, DGCR8 [35]. This is consistent with the observation that Dicer protein levels

are elevated in PCa compared to benign tissue [120], but contrasts with estrogen receptor alpha (ER α)-mediated inhibition of Drosha in breast cancer tissues [121]. This highlights the potential for nuclear receptor-mediated amplification or repression of distinct miR subsets to orchestrate tissue development through determination of cell fate and identity.

Regulatory factors are also shared between the miR biogenesis cascade and the AR transcriptional complex: Microprocessor component, p68, was identified as an AR-interacting coactivator in PCa cells that is recruited to AREs following ligand treatment. Phosphorylation at Y⁵⁹³ was shown to be required for its coactivation of AR [122], although the impact of this modification on Microprocessor activity has not been investigated.

3.4. Additional mechanisms of AR miR regulation

AR can also modulate miR levels indirectly through transcriptional regulation of their interacting ncRNAs: Association of AR with the host gene promoter of the circular RNA, circHIAT1, reduced its expression and consequently decreased cellular levels of miR-195-5p/29a-3p/29c-3p, since circHIAT1 was demonstrated to upregulate these miRs in clear cell renal cell carcinoma [123].

4. MiRs as modulators of resistance to androgen deprivation therapy (ADT)

ADT, also known as medical or surgical castration, is the main therapeutic strategy in PCa and aims to suppress intratesticular or intra-prostatic androgen synthesis and/or inhibit AR activity [124–126]. Despite initial efficacy, patients commonly relapse within 2 or 3 years of ADT, progressing to CRPC, which nevertheless requires AR activity. The mechanisms driving continued AR activity in CRPC are varied and described above [1].

The ability of miRs to modulate response and resistance to ADT, to promote cell survival and proliferation under androgen-ablated conditions, to enhance metastasis-associated cell invasion, and to drive emergence of metastatic CRPC (mCRPC) have been investigated in several studies [127,128]. Sun et al. [129] identified upregulation of miR-221/222 and downregulation of miR-23b and miR-27b in 90% of mCRPC cases compared to primary tumours, although only miR-221/222 and miR-203 altered cellular response to androgen, indicative of a potential role in progression to mCRPC. Despite their lack of modulation of androgen response, members of the CRPC-downregulated miR-23b/27b cluster were found to repress PCa invasion and migration in an independent study, potentially indicative of pro-metastatic functionality independent of the AR signalling axis [130]. Through integration of miRs dysregulated in CRPC vs. primary disease in the MSKCC cohort [131] with purported CRPC-associated miRs from three miR profiling studies, Fernandes et al. [31] sought to define a robust repertoire of CRPC-dysregulated miRs that includes miR-30a, miR-30e-5p, miR-99a, miR-100, miR-125b, miR-133a, miR-205 and miR-452 (downregulated), and miR-7, miR-18a, miR-25, miR-95, miR-130b, miR-182, miR-183,

miR-185, miR-425 and miR-625 (upregulated) [110,131–133]. Of note, greater numbers of miRs were found to be decreased than increased in mCRPC, and such downregulated miRs frequently inhibit oncogenic processes. For example, miR-145-3p was shown to promote cell cycle progression and survival through targeting of MELK, NCAPG, BUB1 and CDK1, all of which are significantly predictive of CRPC patient survival [133]. In agreement with the above analyses, miR-125b was shown to be increased in androgen-independent LNCaP derivatives versus their androgen-dependent parental lines [134]. In mechanistic studies in cell line models, miR-21 was shown to be sufficient in isolation to drive progression to castration resistance [104]. Whilst levels of miR-21 are elevated in primary PCa versus benign tissue [34,104,107], miR-21 is not dysregulated in mCRPC as compared to hormone-responsive primary disease.

It is noteworthy that thus far, few studies have specifically investigated miRs dysregulated in cell line, organoid or animal models resistant to specific PCa therapeutics, and thus much remains to be learnt about miRs driving drug resistance, and those dysregulated in response to specific hormonal therapies.

5. AR-regulating/-regulated miRs as PCa biomarkers

PCa suffers a lack of reliable diagnostic or prognostic predictors. Current biopsy and imaging techniques are invasive, costly and associated with considerable morbidity. Whilst introduction of PSA as a circulating biomarker has contributed to a reduction in PCa mortality, this comes at the cost of over-detection and over-treatment of indolent disease and also false-negative results: PSA is frequently elevated in serum of men with benign prostatic hyperplasia and prostatitis, and cases of PSA-negative PCa (0–4 ng/mL) comprise 15% of diagnosed PCas [135]. Indeed, less than 50% of men undergoing biopsy following a raised PSA result are diagnosed with PCa, and biopsy carries a significant risk of infection, incontinence, impotence and missed detection of clinically-significant disease [136].

MiRs represent promising circulating diagnostic, prognostic and predictive biomarkers due to their long half-life, high stability in biofluids, and ease of detection. Further, miR profiles are altered in tumours and biofluids of many cancer types, suggesting that miRs may be informative of carcinogenic processes [137,138]. Their ease of detection also permits repeat sampling for disease monitoring [139]. Furthermore, quantitation can be achieved in low biofluid volumes [140].

Given the key role of AR in PCa, AR-associated miRs are particularly compelling biomarker candidates. Levels of androgen-stimulated miR-141 were elevated in PCa tissue versus healthy controls, as well as between PCa and benign prostate hyperplasia (BPH) [141]. Differential expression was also evident in patient serum [9,10,142]. MiR-27a, also androgen-upregulated [36], is found at higher levels in tumour and serum of PCa patients compared to healthy controls [143]. MiR-21 and MiR-221 have been proposed as

diagnostic biomarkers, demonstrating sensitivity and specificity of 90% and overexpression in PCa patient serum [144]. Interestingly, miR-221 has previously been demonstrated to be androgen-repressed and both up- and down-regulation in PCa have been reported [34,107,110]. MiR-375, which is indirectly androgen-upregulated through AR suppression of its promoter DNA methylation [145], has been consistently reported as a PCa diagnostic biomarker [9,10,146,147]. Further, this miR is well-correlated with AR activity in numerous tumour data sets [148], and is released from PCa cells upon androgen treatment [149], suggestive of its PCa tumour origin and potential use as an indicator of intra-tumoural AR activity.

In addition to androgen-regulated miRs, AR-targeting miRs demonstrate diagnostic biomarker utility: The AR-targeting miR-320 family members are elevated in PCa patients versus BPH and healthy controls and exhibit higher expression in serum of older PCa patients versus younger [150,151]. Further studies have exploited AR-targeting/-targeted miR panels to increase biomarker specificity: A urine-based signature of miR-21-5p (androgen-regulated), miR-141-3p and miR-205-5p (AR-targeting) was able to differentiate malignancy from BPH with enhanced specificity and similar sensitivity to that of PSA [152]. Similarly, a combination of miR-141-3p, miR-21-5p, and miR-375 has additionally been proposed to be diagnostic for PCa in serum [153].

As discussed above, AR continues to play a vital role in the biology of ADT-resistant PCa. Hence AR-associated miRs may represent effective prognostic and/or predictive biomarkers. Proposed diagnostic miRs, miR-141, miR-375, miR-320 and miR-27a also exhibit overexpression in CRPC versus primary PCas [10,11,141,143,151]. In addition, miR-141 and miR-221 had significantly higher levels in the circulation of metastatic versus localised PCa patients [154]. Other AR-associated miRs, miR-125b and miR-222 (androgen-induced and -repressed, respectively), predict early treatment failure post-radical prostatectomy when assayed in patient serum [155]. MiR-21 demonstrated higher expression in serum samples from hormone-refractory PCa [156], whilst another plasma-based study identified miR-20a, miR-21, miR-145, and miR-221 as distinguishing high versus low risk PCa by D'Amico score with area under curve (AUC) of 0.824 [12]. Similarly, a combination of urine-derived miR-125-5p, let-7a-5p, miR-151-5p predicted time to biochemical recurrence (BCR) post-prostatectomy independently of routine clinicopathological parameters [157], and miR-146b-3p and miR-194 were elevated in serum of PCa patients who subsequently experienced BCR [158]. Importantly, the prognostic utility of the Fredsoe- and Selth-identified miR signatures was validated in larger independent patient cohorts [157,158].

5.1. Challenges of AR-targeting/-targeted miRs as PCa biomarkers

An important caveat that should be considered when interpreting data from the above biomarker studies are the broad inter- and intra-tumour heterogeneity observed in PCa, particularly since the disease is frequently multi-focal

[159–161]. Indeed, AR activity was found to vary between different foci from the same PCa patient [160], which would be hypothesised to result in differential modulation of AR-modulated miRs, and differential contribution of distinct tumour clones to plasma and urine AR-informative miR profiles. Further, intra- and inter-tumour heterogeneity of *PTEN* loss in PCa needle core biopsies was found in 68% of cases [162], an important observation since *PTEN* mediates AR degradation, an effect repressed by androgen-upregulation of *PTEN*-targeting miR-21 [117–119]. Thus androgen-regulated miR signatures may demonstrate diversity between different tumour clones. It should also be remembered that inter- and intra-tumoural AR activity heterogeneity may be mediated or modulated by AR-associated miRs.

Whilst monitoring of subclonal populations increasingly informs as to tumour clone phylogenetics [163], it is increasingly apparent that, the “snapshot” of biomarker molecules released into body fluids may provide an incomplete or misleading picture: Tumour origin of miRs is hard to definitively ascertain, and it is currently not known whether high levels of individual miRs present in PCa patient biofluids reflect large tumour volume, or tumour cell apoptosis resulting from successful treatment. Further, miRs and other small RNAs are secreted from almost all cell types, including red and white blood cells. Thus these common plasma contaminants may skew identified miR profiles.

A further key limitation of many of the above studies is the use of small numbers of patient samples. Identified biomarkers cannot be considered for clinical implementation without robust validation of sensitivity and specificity in large, well-defined PCa cohorts. The lack of concordance between biomarker studies may well reflect use of small patient groups, in addition to type of biofluid interrogated, technology used for miR quantification, lack of consensus standard operating procedures (SOPs) for sample collection and absence of standardised normalisation procedures.

As the sensitivity and diversity of small RNA detection technologies increases, the number of clinically-informative non-coding RNA biomarkers is likely to grow to include isomiRs and other recently-identified small RNA species, such as tRNA fragments and snoRNAs. Such factors may increase sensitivity, specificity and robustness of biomarker panels, and provide opportunities for further molecular stratification. Whilst most research currently relies on amplification-based qPCR, technologies are increasingly available for rapid, amplification-free and isothermic miR profiling that is amenable to clinical implementation by non-expert end users [164]. Peptide nucleic acid (PNA)-based fluorogenic biosensors have already been validated for quantitation of miRs in human blood samples [164], and electrochemical detection using magnetic capture sensitively assays miR-21 in human serum samples [165], showing promise for rapid and reproducible miR biomarker profiling.

Identifying biomarkers of response to specific therapies represents a further substantial challenge, particularly in the context of advanced disease, where patients are likely to have diverse treatment histories, with resultant modulated tumour genetics, transcriptomics and non-coding RNA

profiles. These above challenges are inherent to the use of any biomarkers, and not unique to miRs.

6. Opportunities and challenges of AR-associated miRs as therapeutic targets

MiRs possess many desirable properties as therapeutics: They are small, readily synthesisable, relatively inexpensive and modifiable for *in vivo* stability and efficient cellular uptake. So too are miR inhibitors, which bind their sequence-complementary miRs in a highly specific manner and inhibit its activity through sequestration. Several miR mimics and inhibitors are in clinical trials for solid tumours [166–169], aiming to increase levels of tumour suppressor miRs and inhibit oncomiRs. Whilst a recent trial of miR-34 mimic was discontinued due to serious adverse effects, these were likely sequence- or liposomal formulation-specific. Other trials of miRs/inhibitors suggest they are well tolerated, even long-term, with no serious adverse effects, e.g. Santaris phase II trials of anti-miR-122 (NCT01200420, NCT02508090) [169–171], miRagen phase II for anti-miR-155 (NCT02580552) after successful phase I [172], also phase I for miR-29 mimic (NCT02603224). Oligonucleotides are hailed as the 3rd major drug-development platform (after small molecules and biologics) with predicted market worth of USD 4.6 billion by 2022 [173,174].

Previous sections have discussed the reliance of the majority of PCa tumours on AR activity, even in the advanced CRPC setting. Androgen-independent tumours frequently demonstrate only a modest increase in AR expression which could be effectively downregulated by miR-based therapeutics [175]. Studies using siRNAs, antisense AR, and selective AR modulators have successfully shown that targeting AR increases apoptosis and reduces cancer cell proliferation [176,177]. A study comparing patient samples from Caucasians versus African-Americans identified an AR-targeting miR-34b to have lower expression in the latter group, who frequently present with more aggressive PCa [43]. In addition, tumour suppressor miRs, miR-22, miR-29a, miR-99a and miR-135a are repressed in PCa as compared to healthy control or BPH tissue [34,79,102,104,106,107,178,179]. This provides further evidence for the role of AR-targeting miRs in determining tumour aggressiveness, and justification for the pursuance of AR-associated miRs for therapeutic intervention: Therapy would aim to reinstate AR-targeting miRs, and/or inhibit AR activity-amplifying, oncogenic miRs. For example, reconstitution of the established tumour suppressor miR, let-7c, has been proposed as a therapeutic option in PCa, given its regulation of AR through targeting of c-Myc, as well as its function in repressing the Ras oncogene [65]. Androgen-regulated miR-221 and miR-222, which target the cell cycle regulator p27, are two of the most commonly overexpressed miRs in cancer, including CRPC. Their overexpression is sufficient to drive acquisition of androgen-independence in cell line PCa models [109,180,181], and thus their inhibitors may be hypothesised to inhibit PCa proliferation and potentially maintain response to ADT.

Given the number of miRs targeting AR, the ability of AR to modulate miR activity to enhance its own function, and the potential for functional redundancy between miRs of

the same family targeting protein components of the same signalling pathways, use of a single miR or miR inhibitor may provide little therapeutic benefit. Greater efficacy may be achievable with miR/inhibitor “cocktails” to exert increased inhibitory effects on AR and circumvent development of resistance. Indeed, AR-modulatory miRs can display additive or synergistic effects: A combination of miR-203, miR-205 and miR-130a was shown to repress AR and MAPK signalling pathways to effectively induce apoptosis and cell cycle arrest [64]. An additional advantage of miR-based therapeutics is the potential for exploitation of individual “master” tumour suppressive miRs targeting multiple oncogenic pathways, or inhibition of miR that constitute central “nodes” in tumourigenic gene networks. Indeed, many miRs target protein components of the same signalling axis (so-called “vertical targeting”). In illustration of this, miR-34a reduces PCa cell viability through targeting AR in addition to more than 30 other oncogenes (including MET, MEK1, MYC, PDGFR- α , CDK4/6, BCL2, WNT 1/3, NOTCH1, and CD44) and modulators of tumour immune evasion (PD-L1 and DGK ζ) across diverse tumourigenic pathways [168]. Indeed, liposomal delivery of miR-34a mimic showed evidence of anti-tumour activity in refractory advanced solid tumours. Strategic re-expression of master tumour suppressor miRs may prove effective in bypassing resistance development. In addition, DNA demethylating agents and histone deacetylase inhibitors that restore expression of epigenetically-silenced, AR-targeting miR-124 and miR-320a, respectively, resulted in decreased AR protein levels and activity in PCa cells [100,150], thus offering proof-in-principal of the viability of this therapeutic approach.

Whilst multiple-oncogene-targeting miRs may prove advantageous for PCa therapy, conversely, ceRNA networks may act to promote resistance by restoring oncogene activity through sequestration of therapeutic miR mimics: Upregulation of ceRNAs acting as sponges to AR-targeting miRs may act to restore activity [82]. Precedents for this may be found in other cancer types: In liver cancer, HULC lncRNA acts as a ceRNA for miR-372, derepressing activity of other miR-372 targets including PRKACB, leading to enhanced phosphorylation of its target, CREB, and ultimately enhancing chromatin accessibility and cancer progression [182]. In melanoma, ZEB2 transcript acts as a miR decoy for PTEN, maintaining its tumour suppressive activity in a miR-dependent, protein-coding-independent manner [84]. Given the relevance of loss of PTEN activity in PCa tumourigenesis, it is highly likely that this ceRNA network exerts similar functions in this malignancy, and it would be interestingly to assess the correlation between ZEB2 mRNA and PCa outcome. Expression of transcripts harbouring AR-targeting miR seed sequences following chronic exposure of cell lines and near-patient models to AR-targeting miR mimics should be assessed as evidence of resistance development, and the effect of different miR mimic formulations on this process should be evaluated.

A further potential mechanism of resistance to AR-targeting miR therapeutics is shortening of the AR 3'UTR. Indeed, widespread 3'UTR shortening of oncogenes has

been observed in cancers, resulting in loss of tumour suppressive miR binding sites [57]. At 2.6 times longer than the coding region, AR 3'UTR length is supportive of a vital regulatory role for miRs in regulating AR activity, and indeed, AR is the most miR-targeted oncogene in PCa [19]. Although different AR transcript lengths have been reported [37], how these differ during PCa progression, their functional implications and their consequences for efficacy of AR-targeting miR therapeutic strategies have not been investigated.

7. Summary

The complexity of the miR biogenesis cascade provides a myriad of opportunities for androgen regulation of miR synthesis that includes and extends beyond transcriptional effects. Conversely, miRs can alter AR activity through direct targeting of its mRNA, repression of its numerous cofactors, and modulation of its effector molecules. Indeed, cropping enzyme Dicer is required for androgen-induction of target genes. Additional intricacy of AR:miR cross-talk is afforded by non-canonical pathways that bypass Drosha or Dicer, post-translational modifications to pathway components, the poorly-characterised phenomenon of AR 3'UTR shortening, and the modulated abundance of AR splice variants during disease progression. The convergence of the multi-step miR biosynthesis pathway and AR signalling axis permits responsive and dynamic fine-tuning of the miRnome, with implications for extended ceRNA networks. Dysregulation of this delicate equilibrium is evident in PCa and other malignancies, and yields both PCa-specific, AR-related miR signatures for liquid biopsy, and miR targets for therapeutic intervention. Given that AR remains key to continued PCa growth and progression in the absence of circulating androgens, it is unsurprising that AR-targeting tumour suppressive miRs are lost during PCa progression, whilst those that promote AR activity are frequently gained. Moreover, AR-regulated miR signatures are enriched for miRs that target its effectors and cofactors, resulting in feedback loops to amplify or restrict signalling. Further, the ability of miRs to drive resistance to ADT and other PCa therapies is increasingly appreciated but mechanistically under-investigated, being hampered by interrogation of samples from small patient cohorts, widely acknowledged limitations of PCa cell lines, and considerable inter- and intra-tumour heterogeneity.

Despite the wealth of miR research in the PCa context, much remains to be learnt: The discovery of shared cofactors between the AR transcriptional unit, and the Microprocessor and Dicer-containing pre-miR processing complexes within the nucleus suggests direct coupling of AR-driven transcription and miR biogenesis, and warrants further comprehensive investigation. Intriguingly, the presence of AGO2- and AR cofactor-containing RISC bound to DNA within the nucleus may indicate direct miR modulation of AR-mediated transcription. It is of vital importance to comprehend the complex bi-directional cross-talk between miRs and androgen signalling to fulfil the

therapeutic and biomarker promise of miRs, and to improve design of drugs targeting the key PCa oncogene, AR.

Author contributions

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Conflicts of interest

The authors have no conflict of interest.

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