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microRNAs and prostate cancer

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Abstract

Prostate cancer (CaP) is the most frequently diagnosed malignant tumour and the second leading cause of cancer deaths in American men. One of the most troubling aspects of this disease is that, after androgen ablation therapy, androgen-dependent cancer cells inevitably progress to an androgen-independent status, for which no effective treatment has yet been developed. To date, the mechanisms that underlie the occurrence and progression of CaP remain largely unknown. Recent studies suggest that microRNAs (miRNAs) are involved in human tumorigenesis. Some aberrantly expressed miRNAs have been discovered in CaP cell lines, xenografts and clinical tissues and these CaP-related miRNAs may play critical roles in the pathogenesis of CaP. This review provides an overview of current findings about aberrantly expressed miRNAs in CaP. Although a number of CaP-related miRNAs were discovered, to date, only five are characterized for their functionalities: three as oncogenes and two as tumour suppressors. To understand the mechanisms of miRNA action as oncogenes or tumour suppressors, mRNA targets of miRNAs were characterized. Oncogenic miRNAs down-regulate the expression of apoptosis-related genes, and tumour suppressor miRNAs target the proliferation-related genes. Importantly, there is evidence that CaP-related miRNAs are regulated through androgen signalling and that this regulation may contribute to the development of androgen independence. Due to the oncogenic or tumour-suppressive properties of CaP-related miRNAs, they are highly likely to be of clinical use first as biomarkers but more importantly as therapeutic targets for prostate cancer treatment in the near future.

Keywords: prostate cancer • microRNA • androgen receptor • oncogene • tumour suppressor

Introduction

In the past, most studies of the regulation of gene expression have focused mainly upon transcriptional activity. Over the last few years, microRNA (miRNA)-mediated post-transcriptional regulation of protein-coding genes has emerged as a promising area of research. This regulation provides the cell with a more precise, immediate and energy-efficient mechanism for controlling the expression of proteins. This occurs since it can mediate rapid

changes in protein synthesis without the need for transcriptional activation and subsequent mRNA processing steps [1]. The first miRNA was discovered in 1993 by Lee *et al.* [2] who observed that a 22-base RNA (*lin-4*) could bind to the 3'-untranslated region (3'-UTR) of *lin-14* mRNA and repress its translation in *Caenorhabditis elegans*. This discovery engendered scant attention. It was 7 years later that a second 22-base RNA *let-7* was

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identified in *C. elegans* by another group [3]. Similarly to *lin-4*, *let-7* recognized sequences present in the 3'-UTR of *lin-41* mRNA and repressed Lin-41 protein level. This time scientists immediately realized that these small RNA molecules play an important role in the regulation of gene expression. Identification and functional studies of miRNAs extend rapidly from *C. elegans* to other species including human. To date, nearly 500 human miRNAs have been identified, while up to ~1000 miRNAs have been postulated to exist [4, 5]. In this review, after briefly describing the biogenesis of miRNAs and their role in human cancer, we summarize what is currently known about the involvement of miRNAs in prostate cancer (CaP).

miRNAs and human cancer

miRNAs are a class of naturally occurring, small non-coding RNAs that function in the negative regulation of gene expression. The maturation of miRNAs proceeds in a step-wise enzymatic course [6]. In mammalian cells, miRNAs are initially transcribed in the nucleus as long primary miRNA (pri-miRNA) transcripts that are subsequently processed to produce hairpin-like miRNA precursors (pre-miRNAs) that range in length from 60 to 110 nucleotides (nt). The pre-miRNAs are transported to the cytoplasm where they are further processed into ~22-nt duplexes of mature miRNA strand and an miRNA passenger strand, with 2-nt overhangs at the 3' termini [7]. The mature miRNA strand is incorporated into the ribonucleoprotein complex RISC (RNA-induced silencing complex), while the passenger strand is degraded. The mature miRNA binds to its target mRNA(s) by complete or partial complementarity of the 5'-end nucleotides 2–8 or 2–7 (seed sequences) with a binding site in the 3'-UTR of the target transcripts [8–11]. This subsequently results in either direct cleavage of the targeted mRNAs by nucleases within the RISC or inhibition of translation [12]. The characteristically short seed sequence permits a single miRNA to act on multiple target sites. Bioinformatic analyses have predicted that each miRNA recognizes on average about 100–200 different mRNA targets [5, 13] and thus miRNAs can mediate the regulation of a considerable number of protein-coding genes [14]. Additionally, the 3'-UTR of a given mRNA may contain binding sites for various miRNAs. As a result, miRNAs are involved in almost all cellular processes in mammals, including cell proliferation, differentiation, stress response, apoptosis, immunity and transcriptional regulation [5].

There is now abundant evidence that aberrant expression of miRNAs occurs in diverse types of human cancer and in different stages of disease progression [15, 16]. Moreover, several miRNAs have recently been found to promote cell survival and tumour growth [17–19]. Therefore, miRNAs may play a critical role in the pathogenesis of human cancer. Studies have shown that some miRNAs can be overexpressed or down-regulated in cancer cells [20]. Overexpression of miRNAs can be due to amplification, demethylation in the promoter regions of miRNAs or deregulation

of a transcription factor, while down-regulation may be due to deletions, epigenetic silencing or loss of the expression of necessary transcription factors [21]. Overexpressed miRNAs may act as oncogenes since they can repress tumour suppressor or apoptosis-related genes. An example is *miR-155* that is highly expressed in lymphoma. Transgenic overexpression of *miR-155* targeted to B cells caused the development of B-cell malignancies having features of lymphoblastic leukaemia (high-grade lymphoma, thereby indicating that deregulation of a single miRNA can promote malignant transformation [22]. In contrast, down-regulated miRNAs may function as tumour-suppressors since they usually suppress the expression of oncogenes or proliferation-related genes. For instance, deletion or down-regulation of *miR-15* and *miR-16* in chronic lymphocytic leukaemia causes overexpression of the anti-apoptosis molecule Bcl2 [23]. In addition, a given miRNA can be a tumour suppressor if its mRNA targets are oncogenes in one cancer cell type. It can also function as an oncogene if its targets are tumour suppressors in a different tumour cell type. Therefore, if a miRNA functions as a tumour suppressor or oncogene, it depends on its targets in specific cancer tissue [24, 25]. Although many miRNAs have been found to be significantly differentially expressed in different cancer types, to date, only a few have been well characterized for their functional significance. In order to elucidate the contribution of each miRNA to cancer pathogenesis, a large body of work needs to be completed.

Aberrant expression of miRNAs in prostate cancer

CaP is the most frequently diagnosed malignant tumour and the second leading cause of cancer deaths in American men [26]. Retrospective studies suggest that by age 80, more than half of all American men have some cancerous cells in their prostate [27]. Most of these men are asymptomatic and clinical treatment is not needed. In contrast, some cancer cells indeed exhibit aggressive behaviour characterized by possessing a more poorly differentiated phenotype, heightened proliferative index and increased motility and invasiveness. These have the propensity to quickly spread locally and metastasize. Androgen ablation therapy remains the most effective treatment for disseminated disease. However, this treatment is not curative since it results in only a temporary tumour regression. Inevitably, some cancer cells become resistant to hormonal ablation, and relapsed tumour growth emerges usually within 12–18 months. These have been referred to as ablation-resistant, hormone-refractory, or androgen-independent (AI) CaPs [28]. The mechanisms underlying the development and progression of CaP are complex and largely ill-defined. Combined with the heterogeneous nature of the disease, this has impeded the development of effective therapies. Since miRNAs have powerful functions in gene regulation, it is anticipated that identification of CaP-related miRNAs can shed light on understanding the molecular alterations associated with the pathogenesis of CaP.

Aberrant expression of several miRNAs has been found in CaP cells. In an early study, Jiang *et al.* [29] analysed the abundances of selected miRNAs in CaP cell lines using real-time PCR. They found that AI cell lines exhibited 4-fold increased expression of *miR-100* and androgen-dependent (AD) LNCaP cells had 53-fold increased levels of *let-7c*, relative to control cells. In addition, Lee *et al.* [30] observed that PC3 cells highly expressed *miR-125b*. Kore *et al.* [31] employed locked nucleic acids (LNA)-labelled miRNA probes to detect *let-7c* expression in human CaP samples. They found that *let-7c* was clearly present in CaP cells. Recently, Lin *et al.* [32] compared miRNA expression profiles between AI and AD CaP cells. Three miRNAs (*miR-184*, *miR-361* and *miR-424*) were significantly up-regulated, and eight (*miR-19b*, *miR-29b*, *miR-128b*, *miR-146a*, *miR-146b*, *miR-221*, *miR-222* and *miR-663*) were down-regulated in AI cell lines relative to AD cell lines. In order to understand the role of miRNAs in CaP, we recently used Northern blot analysis to examine the abundance of 11 miRNAs in seven CaP cell lines and two immortalized, non-malignant prostate epithelial cell lines. When compared to non-malignant prostate cells, most CaP cell lines had increased expression of *miR-125b*, *miR-92*, *miR-191*, *miR-106a*, *miR-145* and *miR-21*. Furthermore, androgen receptor (AR)-positive cell lines expressed approximately 4-fold increase in *let-7c* and 3- to 5-fold increase in *miR-125b* compared to AR-negative ones. Additionally, when compared to parental cells, AI LNCaP sublines exhibited reduced *miR-92* and *miR-106a* expression and elevated levels of *miR-125b*, *miR-16*, *miR-21*, *miR-30c* and *miR-100*. These findings suggest that: (i) these aberrantly expressed miRNAs may contribute to prostatic tumorigenesis; (ii) the miRNAs differentially expressed in AD and AI CaP cells are associated with androgen independence and (iii) the AR may regulate the expression of a subset of miRNAs in CaP [33].

Global miRNA microarray profiling is a powerful technique to investigate alterations in miRNAs in human cancer. This approach enables the simultaneous analysis of the entire miRNA expression repertoire of a particular sample in a single assay. Furthermore, the uniform small size of miRNAs and their probes contributes to reducing hybridization-caused variations that can be encountered in mRNA microarray studies [34]. However, the lack of comparability and low accuracy of the derived data have been a major limitation of miRNA microarray studies [35]. Thus, the aberrantly expressed miRNAs detected by microarray have to be validated by using other techniques, such as Northern blot analysis, real-time PCR or *in situ* hybridization (ISH). Recently, three groups investigated the expression of miRNAs in clinical CaP samples using miRNA microarray analysis. Volinia *et al.* [20] examined the differential expression of 228 miRNAs in 56 CaP and 7 benign prostatic hyperplasia (BPH) tissues and identified 42 differentially expressed miRNAs with 36 (84%) being up-regulated and six (14%) down-regulated in CaP samples. Ozen *et al.* [36] analysed expression of 480 human miRNAs in 10 BPHs and 16 clinical CaP tissues. They found that 76 (89%) of 85 miRNAs were significantly down-regulated while only

9 (11%) miRNAs were up-regulated in CaP samples. To identify the miRNA signature specific for CaP, Porkka *et al.* [37] analysed the miRNA expression profiling of 319 miRNAs in 4 BPHs and 9 CaP samples, and detected 50 differentially expressed miRNAs. Of these, 36 (72%) showed down-regulation and 14 (28%) up-regulation. Although these differentially expressed miRNAs need to be validated, these investigators are in agreement that aberrantly expressed miRNAs are associated with the occurrence of CaP. However, these previous profiling studies presented conflicting results. For example, a comparison of two miRNA profiles in which the differentially expressed miRNAs are available [20, 37] revealed that, of 12 differentially expressed miRNAs reported in the two studies, only three (*let-7a*, *miR-184* and *miR-198*) display identical, while nine miRNAs (*let-7d*, *miR-16*, *miR-26a*, *miR-27a*, *miR-29a*, *miR-29b*, *miR-30c*, *miR-92* and *miR-195*) exhibit a reverse differential expression (Table 1). Although many factors can cause this disagreement, including the lack of comparability and low accuracy as described above, one of the major reasons may be contamination of CaP cells with adjacent benign tissue and/or stromal cells, including fibroblasts, smooth muscle cells and endothelial cells. In two profiling studies [36, 37], for example, CaP samples contain up to 40% benign cells. Since benign prostate cells also express miRNAs to different extents, the results obtained from such samples would not reflect the true levels of CaP-related miRNAs. Therefore, accurate microarray analysis of miRNA expression profiles in clinical CaP samples is not trivial and remains a significant challenge. The use of laser-capture microdissection (LCM) to enrich for the CaP cells from clinical CaP samples should be helpful in overcoming this problem.

Biological function of prostate cancer-related miRNAs

The initiation and progression of CaP is a complex biological process involving multiple biochemical and genetic factors. Like other human cancer types, the inactivation of tumour suppressor genes and the activation of oncogenes may be major contributors to this disease. Since miRNAs function as oncogenes (onco-miRNAs) or tumour suppressors (ts-miRNAs) [9, 21, 25], altered expression of miRNAs may contribute to prostatic malignant transformation and subsequent progression to androgen independence. As described above, some CaP-related miRNAs have been identified, but the biological significance of a majority of these miRNAs remains unknown. Therefore, functional characterization of these miRNAs with respect to their abilities to mediate a gain of oncogenic activity or loss of tumour suppressor function is urgently needed. Recently, several miRNAs were reported to be either oncogenic or tumour suppressive, and aberrant expression of these miRNAs promoted the proliferation of CaP cells.

Table 1 Deregulated miRNAs in clinical CaP samples

Volinia <i>et al.</i> [20]			Porkka <i>et al.</i> [37]		
Up-regulation (36 miRNAs)		Down-regulation (6 miRNA)	Up-regulation (14 miRNAs)		Down-regulation (36 miRNAs)
<i>let-7d*</i>	<i>miR-106a</i>	<i>let-7a**</i>	<i>miR-202</i>	<i>let-7a**</i>	<i>let-7f</i>
<i>let-7i</i>	<i>miR-124a</i>	<i>miR-24</i>	<i>miR-210</i>	<i>let-7b</i>	<i>miR-19b</i>
<i>miR-16*</i>	<i>miR-135</i>	<i>miR-29a</i>	<i>miR-296</i>	<i>let-7c</i>	<i>miR-22</i>
<i>miR-17-5p</i>	<i>miR-146</i>	<i>miR-128a</i>	<i>miR-320</i>	<i>let-7d*</i>	<i>miR-26b</i>
<i>miR-20a</i>	<i>miR-148</i>	<i>miR-149</i>	<i>miR-370</i>	<i>let-7g</i>	<i>miR-27a*</i>
<i>miR-21</i>	<i>miR-181b</i>	<i>miR-218</i>	<i>miR-373</i>	<i>miR-16*</i>	<i>miR-27b</i>
<i>miR-25</i>	<i>miR-184**</i>		<i>miR-498</i>	<i>miR-23a</i>	<i>miR-29a*</i>
<i>miR-26a*</i>	<i>miR-187</i>		<i>miR-503</i>	<i>miR-23b</i>	<i>miR-29b*</i>
<i>miR-27a*</i>	<i>miR-191</i>		<i>miR-184**</i>	<i>miR-26a*</i>	<i>miR-30a-5p</i>
<i>miR-29a*</i>	<i>miR-195*</i>		<i>miR-198**</i>	<i>miR-92*</i>	<i>miR-30b</i>
<i>miR-29b*</i>	<i>miR-196</i>		<i>miR-302c</i>	<i>miR-99</i>	<i>miR-30c*</i>
<i>miR-30c*</i>	<i>miR-197</i>		<i>miR-345</i>	<i>miR-103</i>	<i>miR-100</i>
<i>miR-32</i>	<i>miR-198**</i>		<i>miR-491</i>	<i>miR-125a</i>	<i>miR-141</i>
<i>miR-34a</i>	<i>miR-199</i>		<i>miR-513</i>	<i>miR-125b</i>	<i>miR-148a</i>
<i>miR-92*</i>	<i>miR-203</i>			<i>miR-143</i>	<i>miR-205</i>
<i>miR-93</i>	<i>miR-206</i>			<i>miR-145</i>	
<i>miR-95</i>	<i>miR-214</i>			<i>miR-195*</i>	
<i>miR-101</i>	<i>miR-223</i>			<i>miR-199a</i>	
				<i>miR-221</i>	
				<i>miR-222</i>	
				<i>miR-497</i>	

*Reverse differential expression; **identical expression.

miR-20a

The *miR-17-92* cluster resides on chromosome 13 and comprises seven miRNAs (*miR-17-5p*, *miR-17-3p*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b-1* and *miR-92-1*). A previous study demonstrated that this miRNA cluster could modulate tumour formation and functions as oncogenes [38]. Since *miR-20a* from this cluster is overexpressed in clinical CaP samples [20], Sylvestre *et al.* [39] examined the biological impact of *miR-20a* activity on apoptosis in CaP cells. In that study, PC3 cells were transfected with anti-*miR-20a* and then treated with doxorubicin, a DNA damaging agent that induces apoptosis in PC3 cells. Repression of *miR-20a* activity with anti-miR-20a resulted in a 50% increase in cell death compared to PC3 cells treated with a scramble control. Conversely, overexpression of *miR-20a* mediated a 2-fold reduction in cell death and an approximately 4-fold increase in colony formation in

doxorubicin-treated PC3 cells relative to controls. These results implicated *miR-20a* as being oncogenic *via* its anti-apoptotic function in CaP cells.

miR-125b

Up-regulation of *miR-125b* was observed in PC3 [30], in LNCaP [33, 37], LNCaP AI sublines [33, 37] and in a subset of clinical CaP samples [33, 36]. In a previous study, *miR-125b* was found to be critical for the proliferation of PC3 cells [30]. We recently reported that androgen-independent LNCaP-cds cells markedly overexpressed *miR-125b* compared to parental LNCaP cells, suggesting that *miR-125b* contributes to the progression of CaP. To test the hypothesis, *miR-125b* levels were elevated in three CaP cell lines by transfection with chemically modified, synthetic *miR-125b*.

This manipulation significantly stimulated the growth of these cell lines in the absence of androgens and induced a 3-fold increase in the S-phase fraction in LNCaP cells compared to controls. On the contrary, repression of *miR-125b* activity in LNCaP-cds1 cells by transfection with anti-*miR-125b* significantly inhibited cell growth and induced a 54% reduction of the S-phase fraction and a significant increase of sub-G1 cells, relative to controls. These results demonstrated that *miR-125b* is associated with the AI growth and survival of CaP cells [33].

miR-126*

In human cells, *miR-126** maps to a specific intron of the *Egfl7* gene on chromosome 9, and is consequently co-expressed with the *Egfl7* transcript. Recently, Musiyenko and colleagues [40] observed that due to poor *Egfl7* expression in LNCaP cells, *miR-126** was undetectable. The lack of *miR-126** in CaP cells suggested that this miRNA may act as a tumour suppressor. They thus evaluated the effect of increasing *miR-126** expression on the phenotype of LNCaP cells. It was found that ectopic expression of *miR-126** resulted in a significant reduction in the migration and invasiveness of LNCaP cells, which supported the notion that loss of *miR-126** expression could contribute to the malignant phenotype of CaP cells.

miR-146a

As discussed above, Lin *et al.* [32] found that *miR-146a* was down-regulated in AI CaP cells, suggesting a tumour-inhibitory function for this miRNA. To validate this function of *miR-146a*, *miR-146a* was transfected into PC3 cells, which do not express this miRNA. Ectopic expression of *miR-146a* in PC3 cells markedly reduced their proliferation rate. Furthermore, *miR-146a*-expressing cells displayed neither invasive nor metastatic activity. These data suggest that *miR-146a* is a tumour suppressor that inhibits PC3 cell growth.

miR-221/222

In a recent study, Galardi *et al.* [41] found that aggressive PC3 cells highly expressed *miR-221* and *miR-222*, which are transcribed in tandem from a gene cluster located on chromosome X. However, these two miRNAs were down-regulated in both AR-positive LNCaP and 22Rv1 cell lines that are less aggressive relative to AR-negative PC3 cells. To study the biological significance of *miR-221/222*, LNCaP cells were stably transfected with *miR-221* and *miR-222* alone or in combination and their effects on cell growth and colony formation in soft agar were evaluated. It was observed that ectopic expression of the miRNAs induced a >2-fold increase in growth rate and a 3–4-fold increase in the colony formation in miRNA-transfected LNCaP cells compared to empty

plasmid-transfected cells. Expression of these miRNAs also stimulated a significant increase in the fraction of cells in S phase. Accordingly, antisense-mediated inhibition of *miR-221/222* in PC3 cells strongly reduced (*i.e.* 4.5-fold) the number of the colonies growing in soft agar. These results suggest that *miR-221* and *miR-222* may contribute to the growth and progression of CaP.

In addition to these five CaP-related miRNAs described above, *let-7c* may be involved in the pathogenesis of CaP. Overexpression of *let-7c* has been reported in CaP cells [29, 33]. Although the function of the miRNA in CaP remains to be defined, we recently observed that the miRNA is significantly up-regulated in LNCaP cells exposed to interleukin-6 (IL-6) compared to the LNCaP control. Since IL-6 plays an important role in androgen-independent growth of CaP cells [42], our finding suggests that *let-7c* may be related to IL-6-induced CaP progression.

In summary, only a few CaP-related miRNAs have been characterized for their functional significance. These miRNAs may contribute to the pathogenesis of CaP through oncogenic activities or tumour suppressive functions, respectively. That being said, further investigations are warranted to identify CaP-related miRNAs and to better define the breadth and mechanisms of their biological activities. This will likely provide novel insights into the molecular alterations associated with this disease and potential opportunities for therapeutic interventions of CaP.

Identification of targets of prostate cancer-related miRNAs

miRNAs are powerful repressors of gene expression, and their impact on the growth of CaP cells depends on the functions of downstream targets they suppress. For example, accumulation of an onco-miRNA that targets the mRNAs of tumour suppressors would result in reduction of the tumour suppressors. In contrast, decreased expression of a ts-miRNA that targets the mRNAs of proto-oncogenes could lead to excessive amounts of the oncogenic proteins [9]. The net outcome of both pathways would be an imbalance of the activities of tumour suppressor genes and oncogenes. Therefore, to better understand how miRNAs contribute to CaP's pathogenesis, the mRNA targets of each CaP-related miRNA must be identified. The fact that a single miRNA can inhibit the expression of many mRNA targets and a particular mRNA may be silenced by multiple different miRNAs certainly complicates the validation of true mRNA targets in CaP. Therefore, identification of miRNA targets represents a great challenge.

While various methods have been described for discovering miRNA-targeted mRNAs [43–46], there is no clear agreement as to which procedure should be utilized to determine that a given mRNA is a target of a specific miRNA [43]. Of these methods, bioinformatic prediction followed by experimental validation is the most commonly used strategy for identification of miRNA targets. Many computational algorithms are used to predict miRNA

Table 2 Targets of CaP-related miRNAs and their function

CaP-related miRNA	Function	Targets and function	Methods for validating targets	Ref.
<i>miR-20a</i>	oncomiRNA	E2F1-3: cell cycle regulator, pro-apoptosis	reporter assay, WB	[39]
<i>miR-125b</i>	oncomiRNA	Bak1: pro-apoptotic regulator	microarray, reporter assay, WB	[33]
<i>miR-126*</i>	ts-miRNA	SLC45A3: related to prostate malignancy	transfection, PCR, WB	[40]
<i>miR-146a</i>	ts-miRNA	ROCK1: myosin phosphorylation; PI3K activation	transfection, NB, WB	[32]
<i>miR-221/222</i>	oncomiRNA	p27 ^{Kip1} : inhibition of cell cycle	transfection, reporter assay, WB	[41]

oncomiRNA, oncogenic miRNA, ts-miRNA, tumour-suppressor miRNA; WB, Western blotting, NB, Northern blotting.

targets, however, TargetScan (<http://www.targetscan.org>), miRanda (<http://microrna.sanger.ac.uk>) and PicTar (<http://pictar.bio.nyu.edu>) are considered to be relatively sensitive target prediction algorithms [47]. They predict miRNA targets based on various parameters, including the degree of base complementarity between the seed sequences of a given miRNA and the 3'-UTRs of its mRNA targets, and the thermodynamic stability of the predicted miRNA:mRNA complex. Presently, all mRNA targets of currently identified miRNAs have been predicted and are available online. For each miRNA, the number of mRNA targets predicted by different algorithms is highly variable and ranges from a handful to more than 800 transcripts [47]. The accurate selection of putative targets for experimental validation is a challenge. Iorio *et al.* [48] and Kuhn *et al.* [43] deemed that if a target is predicted using at least two of the three different algorithms, it should be considered as a good candidate for experimental validation. This rule has been used by several groups for identification of miRNA targets [36, 48, 49]. Compared to other target-prediction approaches like microarray-based analyses, the bioinformatic strategy is very simple, rapid and less expensive. A biochemical validation of computer-predicting miRNA targets is highly desirable. Kuhn *et al.* [43] recommended that four criteria should be met before a computer-predicting miRNA target is considered as a true target. First, a reporter construct containing the 3'-UTR of a predicted miRNA target cloned distal to firefly luciferase should exhibit decreased luciferase activity when transfected into a cell expressing the cognate miRNA. Second, if an miRNA is able to repress the expression of its biological target, the miRNA and its target mRNA must be co-expressed. Third, a given miRNA must have a predictable effect on target protein expression. That is, transient transfection of the miRNA in a cell type known to express the putative target should decrease the expression of this target; conversely, repression of miRNA activity using specific anti-miRNA will increase the target expression. Fourth, if a given target has a known function and is suspected of being specifically regulated by a given miRNA, treatment of the cells with the miRNA should result in a corresponding alteration of the target's function.

Several miRNA targets have been validated in CaP (Table 2). They are E2F1 [39], Bak1 [33], SLC45A3 (solute carrier family 45, member 3) [40], ROCK1 [32] and p27^{Kip1} [41]. E2F1, Bak1 and

p27^{Kip1} are growth-inhibitory and are direct targets of *miR-20a*, *miR-125b* and *miR-221/222*, respectively. Although E2F1 can promote cell cycle progression in many cellular contexts, overexpression of E2F1 promotes apoptosis in PC3 cells [39]. Bak1, a Bcl-2 family member, also functions as a pro-apoptotic regulator by antagonizing the function of anti-apoptotic Bcl-2 family proteins. In previous studies, Bak1 and Bax were reported to modulate apoptotic signalling in CaP synergistically. Knockdown of Bak1 and Bax completely prevented apoptosis induced by a variety of biological molecules and chemical compounds [50, 51]. Loss of Bak1 expression has also been reported to be associated with the progression of CaP. In a clinical study, Bak1 was detected in 77.5% primary and untreated localized CaP, but in only 33% hormone-refractory CaPs [52]. Decreased or absent expression of p27^{Kip1}, a cyclin-dependent kinase inhibitor, was associated with high tumour grade and poor prognosis of CaP [53]. In CaP cells, both SLC45A3 and ROCK1 are proliferation-related genes and their expression is repressed by *miR-126** and *miR-146a*, respectively. SLC45A3, also called prostate cancer-associated protein 6 (PCANAP6) and prostein, is a recently described molecule and is expressed in a prostate-specific manner [54, 55]. This molecule is an important determinant of motility and invasiveness of CaP cells [40]. ROCK1 is a Rho-activated protein kinase that is highly involved in AI growth and metastasis of PC3 cells [56]. Active ROCK1 increases phosphorylation of myosin light chain and subsequent contraction of actin-myosin complex, which might enhance cancer migration and metastasis [57]. Active ROCK1 can also promote the activation of PI3K [56], leading to activation of pro-survival signalling via the Akt/mTOR/eIF4E pathway, demonstrated to promote malignant transformation and drug resistance in hormone-refractory CaP [58].

In the above studies, only one target per CaP-related miRNA was identified. Identification of a single target may not reflect the entire biological function of an miRNA, since a single miRNA species can target multiple mRNAs for degradation or translational repression. For example, *miR-125b* down-regulates the pro-apoptotic regulator Bak1. While repression of *miR-125b* with anti-*miR-125b* induced apoptosis in CaP cells [33], siRNA-mediated knockdown of Bak1 expression did not result in significantly increased proliferation, suggesting that silencing of multiple targets

is required to mimic the function of *miR-125b*. Therefore, in order to elucidate the biological significance of *miR-125b*, additional targets of this miRNA must be identified. We have identified another *miR-125b* target, *SGPL1* that is involved in tumour suppressor and cancer-surveillance pathways [59]. Further identification of *miR-125b* targets in CaP cells will lead to an enhanced understanding of the role of *miR-125b* in the development and progression of CaP. Identification and functional validation of CaP-related miRNA targets is providing new insights into the molecular mechanism underlying the oncogenic effects of miRNAs. Although validation of the targets is a daunting task, it is indeed worthwhile and necessitated by the goal to achieve a precise and comprehensive understanding of miRNA function.

Regulation of miRNAs in prostate cancer

Limited information is available regarding the regulation of the expression of miRNAs. Studies have shown that factors that affect the expression of protein-encoding genes can disrupt the regulation of miRNAs in cancer cells, including miRNA gene copy number (*i.e.* gain or loss), mutations of the precursor miRNAs, histone deacetylation and hypermethylation of miRNA promoters, and aberrant miRNA processing. Saito *et al.* [60] demonstrated that treatment of human tumour cells with demethylating agents highly induced the expression of *miR-127*. Their results suggested that epigenetic silencing serves as one mechanism of miRNA regulation in cancer cells. In addition, there is evidence showing that aberrantly expressed miRNAs are regulated indirectly through their processing enzymes, including RNA polymerase II and two nuclear RNases (Drosha and Dicer). For instance, Thomson *et al.* [61] reported that altered Drosha function regulates the abundance of miRNAs and Chiosea *et al.* [62] observed an increased expression of *Dicer* and most of its partners in metastatic prostate tumours. The up-regulation of Dicer may be associated with a global increase of miRNA expression in CaP. Since these enzymes regulate miRNA expression in a generalized, non-specific manner, the participation of additional factors is most likely required for the regulation of the cellular levels of individual miRNAs. Accordingly, studies have shown that transcriptional factors involved in mRNA transcription also contribute to miRNA transcriptional regulation. An example is tumour suppressor p53 that transcriptionally activates the expression of *miR-34* [63, 64]. Additionally, the transcription factors E2F1–3 can directly bind to the promoter of the *miR-17-92* cluster and induce the expression of seven miRNAs including *miR-20a*. The miRNA in turn binds to the 3'-UTRs of *E2F1-3* transcripts and represses their translation, thereby establishing an autoregulatory feedback loop between *E2F1-3* and *miR-20a* [39].

The role(s) of nuclear steroid hormone receptors in the transcriptional regulation of miRNA expression is virtually unknown.

However, several lines of evidence suggest that the AR may be involved in the regulation of the expression of miRNAs. First, chromatin immunoprecipitation (ChIP)-on-Chip analysis identified and mapped the locations of 90 AR-binding sites along chromosomes 21 and 22 in LNCaP cells [65]. Fifty-four (62%) of these sites were found to be very far from transcription start sites of androgen-regulated genes (*e.g.* 500–8000 kb) while only 34 (38%) sites are located within 500 kb [65]. Since not all these distant AR-binding sites act as transcriptional enhancers of neighbouring protein-encoding genes, some of these sites may have other functions. Analysis of these ChIP-on-Chip data revealed that in the chromosome 21 an AR-binding site is located within 83 kb from *miR-802*, while the site is 995 kb far from the transcriptional start site of the neighbouring *MORC3* gene that is regulated by androgen. Similarly, in chromosome 22 an AR-binding is located within 49 kb from *miR-130b* and *miR-301b*, but it is 2241 kb away from the androgen-regulated *GSTT2* gene [65]. Since the oestrogen receptor (ER) can bind to the distal (144 kb) ER binding site to regulate the transcription of target gene [66], it is possible that the AR-binding sites nearby *miR-802*, *miR-130b* and *miR-301b* might drive the expression of these miRNAs. Secondly, global gene expression profiles of androgen-treated CaP cells have identified a large number of androgen-regulated genes. In LNCaP cells, for example, approximately 1.5% to 4.3% of the transcriptome are either directly or indirectly regulated by androgen [67]. However, only a small number of genes have been confirmed to be directly regulated by the AR [68]. A majority of these genes are believed to be indirectly regulated by androgen-AR signalling. How androgen-AR signalling indirectly regulates gene expression has not been clearly delineated. It is possible that there is an androgen-AR-miRNA-target gene-signalling pathway in CaP cells. Confirmation of this pathway may provide a mechanistic explanation for androgen-mediated regulation of gene expression. Thirdly, Porkka *et al.* [37] found that miRNA expression was associated with the AR status in CaP cell lines and xenografts, suggesting that androgens may regulate the expression of a subset of miRNAs. In another report, transfection of the AR into PC3 cells induced the differential expression of 11 miRNAs compared to AR-negative PC3 cells [32].

Our recent results provided direct evidence that androgen-AR signalling regulates the expression of certain miRNAs. We evaluated the effect of androgens on the expression of *miR-125b* in AR-positive CaP cell lines using locked nucleic acid-probe-mediated *in situ* hybridization (LNA-ISH) analysis. In the absence of androgens, reduced levels of *miR-125b* were detected in these cell lines while androgen treatment stimulated a marked increase in *miR-125b*. In addition, transfection of the AR into AR-negative prostate epithelial cells followed by treatment with androgen also increased the abundance of *miR-125b*. Since a typical TATA box and potential androgen responsive elements (AREs) were identified in the 5'-region of *miR-125b-2*, we tested the possibility that this was a direct transcriptional target of the AR. ChIP analysis demonstrated that the AR was recruited to the 5' DNA region of the *miR-125b-2* locus in an androgen-dependent manner. These results

strongly suggest that androgen-AR signalling mediates the regulation of *miR-125b-2* in CaP cells [33].

Potential application of miRNAs in prostate cancer

Although the utilization of miRNAs as cancer biomarkers has not yet been implemented in clinical practice, studies have demonstrated that the expression of several miRNAs is different in benign and malignant prostate tissues and in different stages of disease [20, 36, 37]. As a result, CaP-related miRNAs represent attractive novel biomarkers for clinical CaP as they might be potentially useful for diagnosis, as prognostic indicators, and in monitoring response to treatment. The cellular levels of miRNAs in clinical CaP samples can be measured using various RNA detection approaches. Global miRNA expression profiling is a powerful tool and can be used to identify the aberrant expression of miRNAs. However, employing this approach as a diagnostic tool for clinical cases of CaP would not be practical due to the heterogeneity of clinical CaP samples. LNA-modified miRNA probes markedly increase hybridization affinity to miRNAs compared to traditional RNA or DNA probes. LNA-ISH has been employed for detection of miRNA levels in formalin-fixed archival human cancer samples in which miRNAs remain largely intact and remarkably stable [33, 69]. Therefore, once certain miRNAs are confirmed as useful biomarkers in CaP, LNA-ISH analysis of their expression can be readily adapted to clinical practice.

Since miRNAs act as either oncogenes or tumour suppressor genes, they have significant potential as therapeutic targets for cancer treatment [70]. In recent studies, repression of CaP-related oncogenic miRNAs (*miR-20a*, *miR-125b* and *miR221/222*) using specific anti-miRNAs resulted in a significant increase in cell death [33, 39,41]. These findings hold promise that CaP-related miRNAs may eventually be exploited as therapeutic targets. The miRNA-based therapeutic manipulation can be carried out by the utilization of an antagonistic miRNA that specifically inhibits an oncogenic miRNA and/or the employment of a synthetic miRNA that specifically targets oncogenic mRNAs. An advantage of an miRNA-based therapeutic strategy over the current single gene therapy is the capacity of an miRNA to target multiple downstream effectors. As a result, miRNA-mediated targeting therapy may be more effective than traditional single gene therapy. However, there is a long way to go before miRNAs can be used for CaP treatment. To achieve this goal, several key

obstacles need to be overcome. First, CaP-related miRNAs that silence critical tumour suppressors or activate key oncogenes need to be identified. Only when these important miRNAs are identified and their functions elucidated, can we manipulate them for therapeutic benefit in a rational manner. A second major challenge is presented by the methods required to deliver a specific miRNA into solid CaP tissue and maintain continuous activity; this is common to many nucleic acid-based therapeutic approaches. The recent finding that Pol II promoter-driven expression of an shRNA directed against *miR-30* may provide a potential way to overcome this problem [71].

Conclusion

From the foregoing discussion, it is evident that we have already learned a great deal about the function and role of miRNAs in CaP cells. Knowledge of these small RNAs can significantly improve our understanding regarding the pathogenesis of this disease. It is clear that some miRNAs are implicated in this cancerous disease process, which results in augmented expectation that miRNAs will be useful targets for clinical practice. However, the study of miRNAs is a relatively new area in CaP. Many aspects of these small RNA molecules remain to be clarified, including their abundance in different disease stages, their actual *in vivo* functions, regulation of CaP-related miRNA expression and potential clinical applications. It is expected that the current CaP-related miRNA database will be updated with additional aberrantly expressed miRNAs. Functional characterization of these miRNAs remains a great challenge and a more efficient biochemical strategy is desired to precisely identify the key targets of each CaP-related miRNA. Elucidation of the signal transduction pathways that regulate the expression of CaP-related miRNAs is another significant challenge. If an androgen-AR-miRNA-target signal pathway is verified to exist, this would represent an important contribution to our understanding of AR signalling and would help elucidate the mechanisms regarding AI growth of CaP cells. In this regard, it is fair to have optimism as scientists historically have risen to these challenges.

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