Circulating microRNAs: macro-utility as markers of prostate cancer?

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Abstract

The realization that microRNAs (miRNAs) are frequently deregulated in malignancy has had a major impact on cancer research. In particular, the recent finding that highly stable forms of miRNAs can be accurately measured in body fluids, including blood, has generated considerable excitement. Here, we discuss the potential of blood-based circulating miRNAs as diagnostic, prognostic, and predictive biomarkers of prostate cancer. We also describe practical considerations that may influence identification and/or measurement of miRNA biomarkers in the circulation. Finally, evidence is presented for the emerging concept that circulating miRNAs are actively released by their cells of origin and can modulate gene expression at distal sites. These mobile miRNAs, which we term ‘hormomirs’ because of their hormone-like characteristics, could act as local or long-range signals to maintain normal homeostasis or influence the development and progression of diseases such as cancer.

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Micromanagers of gene expression

MicroRNAs (miRNAs) are small nonprotein-coding RNA molecules that act to regulate gene expression. The genes encoding miRNAs are, in general, regulated and transcribed in the same manner as protein-coding genes, generating a primary miRNA transcript of several hundred nucleotides in length. After multiple processing steps in both the nucleus and cytoplasm, an active 21–23nt molecule, termed the mature miRNA, is generated. Mature miRNAs are incorporated into RNA-induced silencing complexes (RISC) and bind with imperfect complementarity to sequences in specific mRNA targets. Formation of these partial miRNA:mRNA duplexes results in decay of the mRNA transcript or, more commonly, inhibition of its translation. The synthesis, transport, and mechanism of action of miRNAs have been comprehensively described in a number of recent reviews (Winter *et al.* 2009, Djuranovic *et al.* 2011, Winter & Diederichs 2011).

Given that miRNAs can bind to multiple mRNA species and that over 1400 human miRNAs have been identified (miRBase release 17; Kozomara & Griffiths-Jones 2010), the miRNA repertoire of a cell

can profoundly impact gene expression programs. Indeed, it has been estimated that ~60% of all protein-coding genes are directly targeted by miRNAs (Friedman *et al.* 2009). It is therefore not surprising that critical functions for miRNAs have been demonstrated in a diverse array of normal physiological processes, including development, cell behavior, stress responses, and survival (Bushati & Cohen 2007). The importance of miRNAs in cellular control of gene expression is highlighted by the observation that loss of *Dicer* (*Dicer1*), an RNase required for miRNA biogenesis, results in embryonic lethality in mice (Bernstein *et al.* 2003) and zebra fish (Wienholds *et al.* 2003).

miRNAs and cancer

In addition to maintaining the normal growth, development, and health of an organism, miRNAs can influence the development and progression of malignancy. Indeed, altered miRNA expression is a common characteristic of tumorigenesis (Croce 2009). Like protein-coding RNAs, miRNAs have the potential to either promote (oncomirs) or inhibit (tumor

suppressor miRNAs) cancer (Calin & Croce 2006, Croce 2009). Oncomirs target tumor suppressor genes and are commonly upregulated in cancer; for example, miR-21 is frequently elevated in many tumor types and causes silencing of *PTEN* and other tumor-suppressing genes (Meng et al. 2007, Pan et al. 2011). Conversely, miRNAs are defined as tumor suppressors if they target oncogenes, such as the *KRAS*-targeting let-7 family members, which are frequently lost or downregulated in malignancy (Peter 2009, Boyerinas et al. 2010).

Aberrant activity of miRNAs in cancer reflects both quantitative and qualitative effects (Schaefer et al. 2010). A quantitative effect refers to a change in miRNA expression or concentration, which can occur by genomic alterations, mutations, or epigenetic changes to miRNA genes, modified regulation of miRNA genes (e.g. by transcription factors), and/or deregulated miRNA biogenesis and transport (for review, see Koturbash et al. (2010)). For example, genes encoding miRNAs are frequently located at the genomic regions associated with loss of heterozygosity, amplification, or break points (Calin et al. 2004). Qualitative changes can occur when an miRNA loses or gains a recognition sequence, either by mutations or by polymorphisms in the miRNA or its target (Schaefer et al. 2010). Emerging evidence suggests that such functional alterations in miRNA activity may occur frequently in cancer (Blitzblau & Weidhaas 2010): one example is a common single nucleotide polymorphism in the *KRAS* 3'-UTR that disrupts a let-7 target site and confers a ~2.3-fold increased risk for developing non-small cell lung cancer in smokers (Chin et al. 2008).

As miRNA function is dictated by the relative availability of its target mRNAs, tissue-specific expression patterns of miRNAs and mRNAs enable a specific miRNA to possess disparate roles in cells of different lineages (Landgraf et al. 2007, Friedman et al. 2009). Analogously, miRNAs can function divergently in cancers of different origins. An extreme example is miR-125b, which is an oncogene in prostate cancer (PCa), thyroid cancer, neuroblastoma, and glioblastoma but acts as a tumor suppressor in ovarian and breast cancer (for review, see Cortez et al. (2011)). Such dichotomy in respect to miRNA function in cancer is not uncommon and is likely to become more widely recognized as reports of the functional roles of novel miRNAs in different cancers continue to emerge. This phenomenon highlights the importance of identifying miRNA target genes and integrating miRNA/mRNA tissue expression profiles.

miRNAs as biomarkers of malignancy

An increasingly large body of work suggests that miRNA profiles are a rich source of pathognomonic information. This paradigm was first hinted by Lu et al. (2005), who demonstrated that miRNA expression signatures accurately distinguished between tumors of different developmental (i.e. epithelial vs hematopoietic) and tissue origin and could even partition tumors within a single lineage (acute lymphoblastic leukemia). Moreover, such signatures were remarkably useful tools for the diagnosis of tumors of histologically uncertain cellular origin (Lu et al. 2005). Since that time, other studies have highlighted the potential of miRNA expression signatures to define pathological environments (Landgraf et al. 2007, Rosenfeld et al. 2008, Tam 2008, Rosenwald et al. 2010). miRNAs have other requisite traits of a disease biomarker. First, miRNAs are exceptionally stable in various types of clinical samples, including formalin-fixed paraffin-embedded tissues (Xi et al. 2007). Secondly, miRNAs can be readily detected using specific and sensitive quantitative RT-PCR (qRT-PCR)-based assays, providing a significant advantage for analyzing samples that are scant or poorly preserved (Tam 2008). Moreover, these assays are readily amenable to multiplexing, an important consideration given the emerging appreciation that signatures comprised of multiple biomarkers are likely to possess greater diagnostic or prognostic accuracy compared with single analytes. Finally, most miRNAs are highly conserved between species, allowing the use of animal models of disease for preclinical discovery studies.

In 2008, a number of groups isolated cell-free circulating miRNAs from blood fluids (i.e. serum and plasma) and demonstrated that the levels of certain miRNAs were altered in response to various malignancies (Chim et al. 2008, Lawrie et al. 2008, Mitchell et al. 2008), raising the exciting possibility of using blood-based miRNA testing to assist in disease detection and management. Since those seminal papers, the utility of cell-free miRNAs as biomarkers of cancer has become increasingly apparent in preclinical studies: serum or plasma miRNAs have been used for cancer diagnosis and staging (Huang et al. 2010, Liu et al. 2010, Pu et al. 2010, Roth et al. 2010), prognosis (survival or relapse; Pu et al. 2010, Li et al. 2011, Liu et al. 2011), and predicting response to therapy (Zhang et al. 2010a). More recently, miRNAs have been isolated in many other body fluids, including urine, saliva, semen, tears, breast milk, and amniotic fluids, suggesting that their release or export occurs from most tissue and cell types (Hanke et al. 2009,

Park *et al.* 2009, Weber *et al.* 2010). Interestingly, miRNA profiles can define the molecular taxonomy of body fluids in forensic samples, further highlighting their diagnostic potential (Hanson *et al.* 2009).

Other recent reviews have eloquently described the potential of miRNAs derived from body fluids as noninvasive biomarkers for different tumor entities (Brase *et al.* 2010, Cortez *et al.* 2011). Here, we will specifically discuss the application of circulating (serum and plasma) miRNAs to detect and manage PCa.

Circulating miRNAs as novel biomarkers of PCa

Better tools to diagnose and prognosticate PCa are urgently required

PCa is the most common noncutaneous cancer of men in the world and is a major cause of cancer-related mortality (Coleman *et al.* 2008). Currently, testing for this disease heavily relies on the detection of the androgen-regulated serine protease, prostate-specific antigen (PSA), in serum. The expression of PSA in men is highly specific to prostate tissue, and deregulation of androgen signaling during PCa development and progression can result in increased levels of PSA in the blood. Unfortunately, there are a number of problems with PSA as a diagnostic tool. First, elevated serum PSA levels are not specific to PCa, being also associated with benign prostatic hyperplasia (BPH) and prostatitis. Indeed, <50% of men who have undergone a biopsy in response to a PSA reading of >4.0 ng/ml are diagnosed with PCa (Grubb *et al.* 2008, Schroder *et al.* 2009). Secondly, ~15% of men with a ‘negative’ PSA reading (0–4 ng/ml) have PCa (Thompson *et al.* 2004, Lucia *et al.* 2008). Finally, and most importantly, large randomized trials in the USA and Europe have demonstrated that PSA screening contributes heavily to the overdiagnosis and overtreatment of PCa (Andriole *et al.* 2009, Schroder *et al.* 2009). These trials validated earlier work, suggesting that 23–44% of tumors detected by screening would have never caused symptoms in the patient’s lifetime (Draisma *et al.* 2009).

While most men with organ-confined disease are cured by definitive treatment (surgery and/or radiation therapy), up to a quarter experience relapse within 5 years (Greene *et al.* 2004). The outcome for these men is poor. Risk of posttreatment recurrence is assessed using preoperative PSA levels in combination with histological grade (Gleason score) and clinical staging of the tumor. However, it is becoming increasingly

evident that the performance of these parameters is unsatisfactory in terms of accurately differentiating patients with aggressive cancer possessing high metastatic potential from patients with indolent disease. This not only places patients at risk of dying from PCa but also compounds the problem of overdiagnosis and overtreatment outlined above.

With this in mind, new biomarkers of PCa that improve diagnostic and prognostic accuracy are urgently required. Prognostic biomarkers that enable differentiation of lethal from indolent forms of cancer would allow individuals to make more informed choices, such as deferring treatment of slow-growing tumors or opting for aggressive treatment of life-threatening disease. Diagnostic biomarkers that are highly specific to PCa would facilitate better management of men with elevated PSA and/or a positive biopsy, as was recently proposed for urinary *TMPRSS2:ERG* RNA transcripts (Tomlins *et al.* 2011), or could contradict ‘false negative’ PSA readings. Finally, it is worth noting that novel biomarkers for PCa are likely to be used in other settings: for example, as indicators to monitor response to novel therapeutic strategies for castration-resistant PCa (CRPCa; see Attard & de Bono (2011) for review).

A number of recent studies have highlighted the potential of serum/plasma miRNAs as novel, non-invasive biomarkers for the diagnosis and prognosis of PCa and for predicting response to therapy (Table 1): these are described in more detail below.

Circulating miRNAs as diagnostic markers of PCa

In pioneering work, Mitchell *et al.* (2008) used a mouse PCa xenograft system to elegantly demonstrate that human miRNAs enter the plasma of mice. They measured a panel of PCa-associated miRNAs in the serum of healthy men ($n=25$) and men with advanced disease ($n=25$) and found that miR-141 was highly elevated in the cancer samples. Moreover, miR-141 was correlated with serum PSA levels and could detect individuals with advanced PCa with 60% sensitivity at 100% specificity. A subsequent study used a custom microarray to profile miRNAs in serum from patients with various cancer types and identified 15 miRNAs at elevated levels in the circulation of PCa patients (Lodes *et al.* 2009). However, only six PCa samples from men with significant disparities, including age, disease grades, and modes of treatment, were analyzed. Indeed, the serum miRNA profile described in this study could not discriminate between PCa patients and those suffering from breast, ovarian, lung, or colon cancer.

Table 1 Studies addressing the potential of circulating miRNAs as biomarkers of prostate cancer

Body fluid	Sample size	Methodology	Major findings	Reference
Plasma	25 patients (metastatic PCa), 25 healthy controls	qRT-PCR (6 miRNAs)	miR-141 levels could differentiate PCa patients from healthy subjects	Mitchell et al. (2008)
Serum	6 patients (stages 2–4 PCa), 8 healthy controls	Microarray (custom) (547 miRNAs)	15 miRNAs were elevated in PCa patients. However, serum miRNAs could not distinguish between different cancer types	Lodes et al. (2009)
	56 patients (20 localized PCa, 20 androgen-dependent PCa, 10 CRPC ^a), 6 BPH ^b controls	qRT-PCR (miR-21 only)	miR-21 levels elevated in CRPC patients compared with BPH. miR-21 associated with resistance to docetaxel in CRPC patients	Zhang et al. (2010a,b)
	29 patients (9 low risk, 11 intermediate risk, 9 high risk) ^c , 9 healthy controls	qRT-PCR (677 miRNAs)	10 miRNAs altered in PCa patients compared with healthy controls. 7 miRNAs correlated with different risk groups	Moltzahn et al. (2011)
	Profiling: 7 high-grade, 14 low-grade patients. Validation: 116 patients (various grades)	qRT-PCR (667 miRNAs)	miR-141, miR-200b, and miR-375 were elevated in serum from high-grade patients and correlated with clinicopathological parameters	Brase et al. (2011)
Plasma	21 patients (metastatic PCa)	qRT-PCR (miR-141 only)	miR-141 levels associated with clinical progression. miR-141 positively correlated with prostate-specific antigen	Gonzales et al. (2011)
	51 patients (18 localized PCa, 8 local advanced, 25 metastatic), 20 healthy controls	qRT-PCR (miR-21, miR-141, and miR-221)	miR-21 and miR-221 levels elevated in PCa patients compared with healthy controls. miR-21, miR-141, and miR-221 levels higher in metastatic vs localized disease	Yaman Agaoglu et al. (2011)
Serum	45 patients (37 localized PCa, 8 metastatic), 18 BPH controls, 20 healthy controls	qRT-PCR (5 miRNAs)	miR-26a, miR-195, and let-7i levels elevated in PCa compared with BPH samples	Mahn et al. (2011)
	Profiling: 14 TRAMP mice, 14 healthy controls. Validation: 25 patients (metastatic CRPC), 25 healthy controls	Microarray (Affymetrix), qRT-PCR (609 murine miRNAs, 10 human miRNAs)	miR-141, miR-298, miR-346, and miR-375 levels elevated in patients	Selth et al. (2011)
Plasma and serum	Profiling: 78 patients (various grades, 15 with diagnosed metastases), 28 healthy controls. Validation: 119 patients (47 recurrent after RP ^d , 72 nonrecurrent)	qRT-PCR (742 miRNAs)	12 miRNAs altered in PCa patients compared with healthy controls. 16 miRNAs altered in metastatic vs localized PCa (including miR-141 and miR-375)	Bryant et al. (2012)

^aCastration-resistant prostate cancer.^bBenign prostatic hyperplasia.^c'Cancer of the prostate risk assessment' score.^dRadical prostatectomy.

In an alternative approach designed to minimize biological variability in the discovery cohort, we analyzed serum miRNA profiles from a mouse model of PCa, transgenic adenocarcinoma of mouse prostate (TRAMP), using microarray technology (Selth *et al.* 2011). We validated four of the TRAMP-associated miRNAs – miR-141, miR-298, miR-346, and miR-375 – as markers of disease in sera from human patients with advanced disease. This study was the first to demonstrate that certain serum miRNAs are common between human PCa and a mouse model of the disease, highlighting the potential of such models for the discovery of novel miRNA biomarkers. The potential advantages of mouse models for these types of studies are discussed in more detail below.

More recently, Moltzahn *et al.* (2010) collected serum from 29 men with early-stage PCa immediately before radical prostatectomy (RP) and nine healthy men. Profiling using high-throughput (multiplexed) quantitative real-time PCR (qRT-PCR) identified ten miRNAs significantly altered in the malignant samples. Receiver operating characteristic (ROC) curves generated for individual miRNAs revealed that most of these miRNAs possessed significant diagnostic capability: for example, the area under the curve (AUC) for both miR-106a and miR-1274 was 0.928.

In a study aimed at testing the diagnostic utility of three specific PCa-associated miRNAs, miR-21, miR-141, and miR-221, Yaman Agaoglu *et al.* (2011) found that miR-21 and miR-221 were elevated in the plasma of men with localized cancer compared with healthy controls. Moreover, these three miRNAs were increased in samples from men with bone metastases compared with men with localized/locally advanced disease, and miR-141 could accurately distinguish between these groups (AUC=0.755). Mahn *et al.* (2011) conducted a similar study but profiled four different PCa-associated miRNAs: miR-26a, miR-32, miR-195, and let-7i. They found that miR-26a, miR-195, and let-7i were increased in the serum of men with localized PCa compared with men with BPH, and a signature comprising all four miRNAs effectively distinguished between these groups (AUC=0.758). Of note, none of the candidate PCa-associated miRNAs were significantly altered in the serum of men with PCa compared with healthy individuals. This calls into question the utility of these particular miRNAs as markers of PCa, although it must be noted that the healthy control samples were collected from much younger population of men.

Most recently, Bryant *et al.* (2012) assessed the diagnostic capacity of plasma miRNAs by

high-throughput qRT-PCR profiling. Twelve miRNAs were differentially quantitated in the circulation of men with PCa (of diverse grade and stage) compared with healthy men, with miR-107 showing the greatest fold-change. In ROC analyses, miR-107 had an AUC of 0.62 compared with an AUC of 0.79 for PSA. Interestingly, five of the PCa-associated miRNAs found in plasma were detectable in urine, and miR-107 and miR-574-3p were present at significantly higher concentrations in men with PCa compared with healthy controls. Of note, in this cohort, the urinary levels of miR-107 and miR-574-3p were more diagnostically informative than *PCA3* mRNA, a well-characterized urine marker of PCa (Hessels *et al.* 2003).

The studies described earlier suggest that circulating miRNAs may assist in the diagnosis of PCa. Further validation of these putative new markers and comparisons with current diagnostic tools in large, carefully selected cohorts that represent different clinical groups – for example, PSA- or non-PSA-screened, biopsied or not – are now warranted.

Circulating miRNAs as prognostic markers of PCa

Three studies have investigated the prognostic potential of circulating miRNAs in PCa. Brase *et al.* (2011) compared serum from men with primary and metastatic cancer by multiplexed qRT-PCR to discover circulating miRNAs associated with PCa progression. This experiment identified 69 miRNAs elevated in men with systemic disease. A subset of these miRNAs was then measured in serum taken from 42 men with localized disease at the time of RP. Three miRNAs, miR-141, miR-200b, and miR-375, were elevated with increasing tumor stage and Gleason score in this validation set, suggesting that they may be indicators of high-risk cancer. Moltzahn *et al.* (2010) compared serum collected before RP from 29 patients with different ‘cancer of the prostate risk assessment’ (CAPRA) scores. A number of miRNAs, including miR-24, miR-106a, miR-451, and miR-93, were altered in malignant samples and associated with CAPRA score. Finally, a recent study identified 16 miRNAs, including miR-141, miR-200b, and miR-375, at differential levels in the plasma of men with localized ($n=55$) or metastatic ($n=11$) PCa (Bryant *et al.* 2012). The authors went on to validate the association of miR-141 and miR-375 with metastatic disease in an independent set of serum samples, although the prognostic utility of these biomarkers in men with localized disease was not assessed in this study.

These studies suggest that serum/plasma miRNAs could be used to predict high-risk tumors and, as such, be a useful complement to the current prognostic armamentarium. In particular, the robust association of miR-141, miR-200b, and miR-375 with metastatic disease is noteworthy: these markers could potentially be applied at the time of diagnosis to identify patients with aggressive disease/micrometastases and/or to predict recurrence following primary treatment. Future studies must validate candidate prognostic miRNAs in cohorts with long-term clinical follow-up to test whether circulating miRNAs are predictive of disease progression, either following definitive treatment for putative localized disease or for men on active surveillance regimes.

Using circulating miRNAs to predict treatment response in PCa

Zhang *et al.* used qRT-PCR to measure miR-21 in patients with localized PCa ($n=20$), androgen-dependent PCa (ADPC; $n=20$), CRPCa ($n=10$), or BPH ($n=6$). miR-21 levels were not significantly different between BPH, localized cancer, or ADPC with PSA < 4 ng/ml but were increased in CRPCa and ADPC with PSA > 4 ng/ml. Of particular interest, levels of miR-21 were significantly higher in the four CRPCa patients who were resistant to the chemotherapeutic docetaxel (Zhang *et al.* 2010a). This work provided the first evidence that circulating miRNAs can predict response to a cancer treatment.

A more recent study specifically aimed to assess the utility of plasma miR-141 as a biomarker of treatment response (Gonzales *et al.* 2011). In 21 patients with either castration-resistant or hormone-sensitive metastatic PCa receiving a range of therapies (i.e. chemotherapy, hormone therapy, or new agents as part of clinical trials), miR-141 had a sensitivity of 78.9% and specificity of 68.8% in predicting clinical progression. This prognostic performance was similar to PSA, circulating tumor cells (CTCs), and serum lactate dehydrogenase, and miR-141 was highly correlated with these other markers. While the latter observation suggests that miR-141 may not contribute much additional prognostic information in this cohort of men, the study highlights the potential of circulating miRNAs for evaluating treatment efficacy.

Specificity of circulating miRNA biomarkers

Notwithstanding the deserved excitement surrounding the findings described above, it has been noted that no single analyte is likely to achieve the desired level of diagnostic or prognostic accuracy for PCa (Steuber

et al. 2008). The evidence thus far suggests that this will also be true for circulating miRNAs. For example, many different tumor entities are associated with increased levels of circulating miR-21, including lymphoma (Lawrie *et al.* 2008), pancreatic cancer (Kong *et al.* 2011), and breast cancer (Asaga *et al.* 2011). Similarly, a circulating miRNA that is robustly associated with PCa, miR-141, is also elevated in plasma from patients with colon cancer (Cheng *et al.* 2011). With these results in mind, the application of circulating miRNAs in a clinical setting is likely to involve evaluation of a signature of multiple miRNAs rather than a single miRNA.

Beyond biomarkers: the role of circulating miRNAs in PCa

Certain miRNAs are elevated both in the circulation of patients and in prostate tumors, suggesting that they originate from the malignant tissue and play a role in tumor development and/or progression (Mitchell *et al.* 2008, Brase *et al.* 2011, Selth *et al.* 2011). For example, miR-375 is a serum/plasma marker of PCa and is also consistently upregulated in tumors (Szczyrba *et al.* 2010, Brase *et al.* 2011, Selth *et al.* 2011, Szczyrba *et al.* 2011, Bryant *et al.* 2012). By interrogating data from the Prostate Cancer OncoGenome Project (Taylor *et al.* 2010), we found that the intratumoral expression of miR-375 at the time of RP can predict biochemical relapse, with high expression being associated with an unfavorable outcome (hazard ratio = 3.49; Selth *et al.* 2011). Bioinformatic prediction of miR-375 targets revealed enrichment for genes involved in PCa development and progression (Selth *et al.* 2011). Indeed, another study found that miR-375 targets *SEC23A*, which encodes a putative tumor suppressor in PCa (Szczyrba *et al.* 2011). Further elucidation of the functions and targets of miR-375 will reveal whether targeting this miRNA is a viable therapeutic strategy.

Like miR-375, miR-141 is elevated in both the circulation of men with PCa and prostate tumors (Mitchell *et al.* 2008, Brase *et al.* 2011, Gonzales *et al.* 2011, Selth *et al.* 2011, Yaman Agaoglu *et al.* 2011, Bryant *et al.* 2012). miR-141 is a member of the miR-200 family, which functions to repress the epithelial to mesenchymal transition (EMT) by targeting the ZEB family of transcription factors (Bracken *et al.* 2009). Of note, miR-141 is cotranscribed with another miR-200 family member, miR-200c, that is also elevated in malignant prostate tissue (Szczyrba *et al.* 2010). While EMT is known to play a key role in cancer metastasis (Nauseef & Henry 2011), it is currently unclear why

epithelial specific miRNAs are upregulated in primary prostate tumors. Interestingly, miR-141 was correlated with CTC concentration in one study, suggesting that CTCs may retain some epithelial characteristics (Gonzales *et al.* 2011). Clearly, more work is required to elucidate the function of this factor; however, the available evidence supports the hypothesis that miR-141 plays an important role in cancer development and/or progression to metastasis.

These examples suggest that miRNAs identified as circulating markers of PCa can be derived from the primary tumor and influence its development and progression to lethal disease. Therefore, studies aimed at identifying new noninvasive miRNA biomarkers are not only useful for their potential application in clinical settings but are also likely to provide fundamental and important insight into the biology of PCa and result in the identification of novel drug targets.

Association between androgen signaling and circulating miRNA markers of PCa

The androgen receptor (AR) signaling axis plays a pivotal role in prostate carcinogenesis and progression to more advanced forms of the disease (Heinlein & Chang 2004, Scher *et al.* 2004). Thus, factors regulated by or impinging on this axis are often dysregulated in PCa, some of which may have potential as biomarkers. The classic example is PSA, which is encoded by a gene (*KLK3*) that is directly regulated by AR. Given that the miRNA transcriptome is profoundly affected by androgen signaling in PCa (for review, see Coppola *et al.* (2009)), it is not surprising that androgenic regulation of many of the circulating miRNAs described earlier has been documented (e.g. miR-141 and other miR-200 family members (Szczyrba *et al.* 2010, Waltering *et al.* 2010), miR-375 (Szczyrba *et al.* 2010, Waltering *et al.* 2010), miR-21 (Shi *et al.* 2007, Ribas *et al.* 2009, Narayanan *et al.* 2010), and miR-221 (Sun *et al.* 2009)). These observations provide important mechanistic information regarding the origins of these potential biomarkers, which may be critical for their effective clinical implementation.

While the evidence implies that circulating miRNA markers of PCa are likely to be influenced by androgen signaling, it is worth noting that the activity of this pathway is not always an informative indicator of cancer behavior or even malignancy, a concept that is perhaps best exemplified by the limitations of PSA (discussed earlier). In our opinion, putative or known links between biomarkers and cancer pathophysiology may be instructive but are unnecessary if the biomarker has robust, validated clinical utility.

Methodologies and strategies for identification of circulating miRNA markers of cancer

Isolation of miRNAs from serum and plasma is relatively straightforward. In general, most protocols use guanidinium–phenol extraction of the sample followed by either precipitation of the miRNA-containing aqueous phase or column-based purification of miRNAs from the aqueous phase (Kroh *et al.* 2010, Weber *et al.* 2010). A number of important considerations for extracting miRNAs from blood-based fluids have been described already (Kroh *et al.* 2010). One issue that we would like to reiterate is the potential of contaminating cellular material, which has a higher concentration of miRNAs than plasma or serum, to confound miRNA quantitation (Kroh *et al.* 2010). This contamination could arise from the presence of intact blood cells in serum/plasma or lysis of these cells (hemolysis) during sampling and processing (Duttagupta *et al.* 2011, Kirschner *et al.* 2011, McDonald *et al.* 2011). Recent work found that an additional centrifuge step after plasma preparation efficiently removes intact blood cells and a significant fraction of contaminating cellular miRNAs (Duttagupta *et al.* 2011). Hemolysis, which frequently occurs at low levels during collection of serum/plasma, is a more difficult problem to circumvent. Two studies have highlighted the profound effect hemolysis can have on miRNA concentrations in blood fluids (Kirschner *et al.* 2011, McDonald *et al.* 2011). The extent of hemolysis can be estimated by the measurement of free hemoglobin and certain miRNAs (e.g. miR-15b, miR-16, and miR-451), which may be necessary to determine whether a serum/plasma sample is suitable for analysis (Kirschner *et al.* 2011, McDonald *et al.* 2011). We believe that standardization of miRNA isolation from serum/plasma and other bodily fluids, taking into account factors such as phlebotomy protocol, sample processing, and miRNA extraction methodology, is one of the most urgent requirements for preclinical experimentation and will maximize the translatability of these studies.

After purification, a general strategy for identifying cancer-associated miRNAs in the circulation is to globally profile and compare the miRNA populations from cancer samples and healthy controls (discovery) and then measure candidate miRNAs by qRT-PCR in a larger, independent cohort (validation). Profiling in the discovery phase is done, in general, using one of the three techniques: qRT-PCR, next-generation sequencing (NGS), or miRNA microarrays. The first stage of qRT-PCR profiling is a reaction in which all miRNAs

of interest (often the entire known miRNAome) are reverse transcribed. This can be achieved by tailing the miRNA molecules and using a common RT primer (i.e. oligo-dT) or by using a pool of RT primers that are specific for each miRNA. Subsequently, miRNAs are amplified by qPCR using 96-well or 384-well miRNA arrays. A consensus protocol for analyzing miRNAs from serum or plasma using qRT-PCR is emerging, and this will likely apply to other body fluids as well (Kroh et al. 2010). NGS and microarray profiling of miRNAs are less frequently used techniques because they require more RNA and hence more starting material. A workflow to circumvent this issue by pooling RNA samples from cancer and healthy groups before Illumina NGS was recently proposed (Zen & Zhang 2010). In this experimental strategy, validation of the NGS data by qRT-PCR is subsequently carried out at an individual patient level using separate training and testing cohorts. NGS has the benefit of being able to identify unknown RNA species that would not be amplified by qRT-PCR. This may be a significant advantage given the emerging notion that miRNA 5'- and 3'-end structural variants, termed isomirs, are commonly expressed and have been linked to cancer (Lee et al. 2010, Ryan et al. 2010). However, pooling samples reduce the statistical power of the profiling experiment. Moreover, NGS and microarray profiling are more technically challenging for most laboratories and require extensive validation, while NGS remains expensive and labor intensive. These issues may explain why qRT-PCR-based profiling strategies, which can quantify the miRNAome from minute quantities of individual patient material, are more frequently used.

There are a number of unresolved issues relating to miRNA profiling of serum and plasma. The first is how to best normalize miRNA concentrations to account for biological and technical variability. Quantitating the amount of miRNA in body fluid specimens is often not possible because of the extremely low concentrations found in such samples. One method to overcome this issue would be to use internal reference (housekeeping) miRNAs, as is commonly done for mRNA profiling. However, there is currently no consensus on suitable small RNA reference genes in circulation (Kroh et al. 2010). A number of studies have used miR-16 for normalization purposes (Lawrie et al. 2008, Huang et al. 2010), but more recent work has found that this molecule is highly expressed in erythrocytes and that its levels in the cell-free content of blood can vary significantly due to hemolysis (Kirschner et al. 2011, McDonald et al. 2011). Our current preference is to process all samples from identical input volumes and

then correct for technical variability of the RNA extraction using spiked-in control miRNAs from *Caenorhabditis elegans* that lack human homologs, as originally proposed by the Tewari Laboratory (Mitchell et al. 2008). Another common point of difference in studies that use qRT-PCR to analyze miRNAs in the circulation is whether or not to 'preamplify' the cDNA before real-time PCR. Commercially available preamplification reagents can be used to increase sensitivity of the qRT-PCR protocol, and we have found that this greatly increases the number of detectable miRNAs in serum from patients with PCa (L A Selth, unpublished observations). Again, the application of miRNA biomarkers in a clinical setting will require rigorous standardization of these protocols.

An alternative discovery strategy that deserves mention is to use blood fluid samples from mouse models of disease. Many cancers show significant heterogeneity at the molecular level, which is likely to be reflected in the circulating miRNA fingerprints associated with that pathology. Genetically engineered mouse models of human cancers exhibit greatly reduced biological and nonbiological heterogeneity by providing defined stages of tumor development, genetic uniformity, homogenized breeding and environmental conditions, controllable health status, and standardized sampling (Kuick et al. 2007). In a recent study, we used the TRAMP model of PCa in the discovery (profiling) phase and then validated a subset of circulating miRNAs in human patient samples (Selth et al. 2011). Other recent studies have further highlighted the utility of mouse models of disease for the discovery of blood-derived miRNA markers (Cacchiarelli et al. 2011, LaConti et al. 2011, Mizuno et al. 2011, Starkey Lewis et al. 2011). We believe that animal models will be increasingly used for this purpose in the coming years in order to fast track the development of novel clinical biomarkers and therapeutics.

Can circulating miRNAs act non-cell-autonomously?

When searching for circulating miRNAs that demarcate disease and could have important clinical applications, one could be forgiven for overlooking a simple question: what is the function of these molecules? Are they simply the remnants of dead cells that have accumulated in the blood? Or do miRNAs secreted into bodily fluids mediate inter-cellular gene regulation? This latter hypothesis has precedence in the plant kingdom: the movement

of miRNA-165/166 communicates radial position information between cells in the root via modulation of target gene expression, a signaling mechanism that is required for normal root development (Carlsbecker *et al.* 2010).

If circulating miRNAs have an intrinsic capacity to function non-cell-autonomously, mechanisms should exist to protect them from degradation in body fluids (Cortez *et al.* 2011). Indeed, miRNAs in serum and plasma are resistant to ribonucleases and severe physicochemical conditions such as boiling, extended storage, freeze–thawing, and extreme pH levels (Chen *et al.* 2008, Mitchell *et al.* 2008). Emerging evidence indicates that this stability is related to sequestration of extracellular miRNAs in various types of protective structures. First, miRNAs can be encapsulated in lipid vesicles, including exosomes (Valadi *et al.* 2007, Kosaka *et al.* 2010, Kuwabara *et al.* 2011), microvesicles (Skog *et al.* 2008, Zhang *et al.* 2010b), and apoptotic bodies (Zernecke *et al.* 2009). Secondly, miRNAs can be bound by RNA-binding proteins, including nucleophosmin 1 (NPM1; Wang *et al.* 2010) and argonaute 2 (AGO2 (EIF2C2); Arroyo *et al.* 2011). This latter finding is particularly interesting: AGO2 is part of the RISC complex and therefore a key mediator of target mRNA silencing. Thus, if AGO2:miRNA complexes are efficiently taken up by recipient cells, they could rapidly and effectively transduce a gene-silencing signal. Finally, a unique population of miRNAs in the blood is associated with high-density lipoprotein (HDL; Vickers *et al.* 2011). Collectively,

these data provide evidence for structurally diverse extracellular miRNA-containing entities, which likely underlie the remarkable stability of miRNAs in the circulation. Moreover, they suggest that targeted enrichment of these entities may improve the utility of miRNAs as biomarkers. For example, isolation of organ- or tissue-specific exosomes using protein surface markers could enrich for a cancer-specific miRNA population, as has been described for ovarian tumor-derived exosomes (Taylor & Gercel-Taylor 2008).

Non-cell-autonomous/signaling functions of miRNAs would likely be associated with active and selective export of these molecules, rather than passive release in response to, for example, tissue damage. A number of observations provide evidence for selective export of miRNAs into circulating fluids. First, small RNAs in the blood are highly enriched for miRNA species (Chen *et al.* 2008). Secondly, the levels of miRNAs in circulation vary widely according to gender (Dutttagupta *et al.* 2011), age, physiological events (e.g. pregnancy (Gilad *et al.* 2008)), and environmental factors (Cortez *et al.* 2011). As an aside, this observation indicates that control samples will need to be carefully selected in biomarker discovery studies (Cortez *et al.* 2011). Thirdly, there is evidence for selective secretion of miRNAs in exosomes. For example, the bulk of miR-1246 and miR-451 was found to be released by breast cancer MCF7 cells in exosomes, but these miRNAs were retained by a nonmalignant mammary epithelial cell

Figure 1 Potential transport mechanisms underlying noncell-autonomous action of miRNAs. miRNAs are transcribed in the nucleus and processed to mature forms in the cytoplasm. Subsequently, miRNAs could be released into circulating body fluids as nucleoprotein complexes (i.e. bound to AGO2 or NPM1), in microvesicles or in apoptotic bodies. Alternatively, ‘free’ miRNAs could penetrate the cell membrane or be released by necrosis/apoptosis and enter the circulation where they may associate with high-density lipoprotein (HDL), which would protect them from extracellular nuclease attack. The ceramide secretory pathway (CSP), which triggers cellular export of miRNAs in exosomes, may repress export to HDL. Circulating miRNA complexes could be delivered to recipient cells by endocytosis, phagocytosis, specific receptors (e.g. scavenger receptor class B member 1 (SR-BI (SCARB1)) may import HDL:miRNA complexes), or other as-yet-unknown receptors, where the miRNAs could regulate the expression of target genes. See main text for references.

line (Pigati *et al.* 2010). Similarly, a subset of miRNAs is specifically released in exosomes from heart cells in response to myocardial infarction (Kuwabara *et al.* 2011). Finally, recent evidence suggests that exosomal miRNA complexes are actively released in a process involving ceramide-dependent secretory machinery, with an enzyme involved in ceramide synthesis, neutral sphingomyelinase 2 (nSMase2 (SMPD3)), seemingly playing a key role (Kosaka *et al.* 2010). Interestingly, NSMASE2 and the ceramide machinery appear to repress the export of miRNAs to HDL, suggesting that the exosomal and HDL pathways have distinct and opposing roles in the export of miRNAs (Vickers *et al.* 2011).

Thus, the stability of miRNAs in circulation and evidence for their selective release or export support the idea that cells secrete functional miRNAs (Fig. 1). More direct evidence for this idea has recently been provided by *in vitro* studies demonstrating that exosome-, microvesicle-, apoptotic body-, or HDL-associated miRNA can regulate expression of both endogenous target genes and exogenous reporter constructs in recipient cells (Valadi *et al.* 2007, Skog *et al.* 2008, Yuan *et al.* 2009, Zerneck *et al.* 2009, Pegtel *et al.* 2010). The mechanisms of miRNA export and import remain to be fully elucidated, and it must be noted that noncell-autonomous actions of miRNAs in human pathology or normal physiology are yet to be demonstrated *in vivo*. However, the evidence outlined here strongly supports the existence of ‘hormomirs’ that are released by donor cells, enter the circulation, and regulate gene expression in target cells, tissues, and organs (Fig. 1), a paradigm originally hypothesized by Cortez *et al.* (2011). In normal physiology, hormomirs may be key regulators of homeostasis. By contrast, tumor cells could deliver miRNAs to the surrounding microenvironment to facilitate growth and invasion (Skog *et al.* 2008), a hypothesis that warrants further experimental attention given the potential of targeting circulating miRNAs therapeutically.

Concluding remarks: future work and challenges

The identification of miRNAs in body fluids, including the blood, has triggered considerable excitement in the biomarker field. We believe that this excitement is well founded: based on their stability and relative ease of detection, circulating miRNAs may become one of the most important pieces in the personalized medicine armory. However, their utility has so far been limited by conflicting data between studies, probably due to inconsistency in methodology, the lack of suitable

reference genes for normalization, small sample sizes, and differences in fluid and miRNA preparation (Cortez *et al.* 2011). Standardized methodologies and larger sample sets are now vital to more effectively explore the clinical potential of circulating miRNAs. Furthermore, it is vital to thoroughly characterize how circulating miRNAs, which appear to be quite dynamic, change in response to age, normal physiological conditions, and extrinsic factors. We believe that these challenges will be rapidly overcome and that noninvasive detection of circulating miRNAs will soon be an important aspect of detection and management of many diseases, including PCa. Indeed, a miRNA-based diagnostic test for pancreatic ductal adenocarcinoma was recently launched by Asuragen (Lee *et al.* 2007, Szafranska *et al.* 2008, Park *et al.* 2011, Szafranska-Schwarzbach *et al.* 2011).

Finally, it is worth noting that at least a fraction of the circulating miRNA population may be acting as ‘hormomirs’ to transduce gene modulation signals and thereby systemically influence normal homeostasis and disease pathology. Thus, in addition to their potential as biomarkers, circulating miRNAs could represent a new class of molecules that are amenable to manipulation for therapeutic purposes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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