Circulating microRNAs as stable blood-based markers for cancer detection

Patrick S. Mitchell[†‡], Rachael K. Parkin[†‡], Evan M. Kroh[†‡], Brian R. Fritz[†§], Stacia K. Wyman[†], Era L. Pogosova-Agadjanyan[¶], Amelia Peterson[†], Jennifer Noteboom[‖], Kathy C. O'Briant[††], April Allen[††], Daniel W. Lin[‖††‡‡], Nicole Urban[††], Charles W. Drescher[††], Beatrice S. Knudsen[††], Derek L. Stirewalt[¶], Robert Gentleman[††], Robert L. Vessella[‖‡‡], Peter S. Nelson[†¶], Daniel B. Martin[†§§], and Muneesh Tewari[†¶,¶¶]

Divisions of [†]Human Biology, [¶]Clinical Research, and [††]Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98109; [§§]Institute for Systems Biology, Seattle, WA 98103; [‖]Department of Urology, University of Washington, Seattle, WA 98195; and [‡‡]Department of Veterans Affairs, Puget Sound Health Care System, Seattle, WA 98108

Communicated by Leland H. Hartwell, Fred Hutchinson Cancer Research Center, Seattle, WA, May 12, 2008 (received for review March 18, 2008)

Improved approaches for the detection of common epithelial malignancies are urgently needed to reduce the worldwide morbidity and mortality caused by cancer. MicroRNAs (miRNAs) are small (≈22 nt) regulatory RNAs that are frequently dysregulated in cancer and have shown promise as tissue-based markers for cancer classification and prognostication. We show here that miRNAs are present in human plasma in a remarkably stable form that is protected from endogenous RNase activity. miRNAs originating from human prostate cancer xenografts enter the circulation, are readily measured in plasma, and can robustly distinguish xenografted mice from controls. This concept extends to cancer in humans, where serum levels of *miR-141* (a miRNA expressed in prostate cancer) can distinguish patients with prostate cancer from healthy controls. Our results establish the measurement of tumor-derived miRNAs in serum or plasma as an important approach for the blood-based detection of human cancer.

biomarker | miR-141 | plasma | serum | prostate cancer

The development of minimally invasive tests for the detection and monitoring of common epithelial malignancies could greatly reduce the worldwide health burden of cancer (1). Although conventional strategies for blood-based biomarker discovery (e.g., using proteomic technologies) have shown promise, the development of clinically validated cancer detection markers remains an unmet challenge for many common human cancers (2). New approaches that can complement and improve on current strategies for cancer detection are urgently needed.

MicroRNAs (miRNAs) are small (typically ≈22 nt in size) regulatory RNA molecules that function to modulate the activity of specific mRNA targets and play important roles in a wide range of physiologic and pathologic processes (3, 4). We hypothesized that miRNAs could be an ideal class of blood-based biomarkers for cancer detection because: (*i*) miRNA expression is frequently dysregulated in cancer (5, 6), (*ii*) expression patterns of miRNAs in human cancer appear to be tissue-specific (7), and (*iii*) miRNAs have unusually high stability in formalin-fixed tissues (8–10). This third point led us to speculate that miRNAs may have exceptional stability in plasma and serum as well. We show here that miRNAs are in fact present in clinical samples of plasma and serum in a remarkably stable form. Furthermore, we establish proof-of-principle for blood-based miRNA cancer detection by using both a xenograft model system and clinical serum specimens from patients with prostate cancer. Our results lay the foundation for the development of miRNAs as a novel class of blood-based cancer biomarkers and raise provocative questions regarding the mechanism of stability and potential biological function of circulating miRNAs.

Results

Identification and Molecular Cloning of Endogenous miRNAs from Human Plasma. Prior reports have suggested that RNA from human plasma (the noncellular component of blood remaining after removing cells by centrifugation) is largely of low molecular weight (11). We directly confirmed that human plasma contains small RNAs in the size range of miRNAs (18–24 nt) by characterizing the size of total RNA isolated from plasma by using radioactive labeling. PAGE and phosphorimaging of 5′ ^{32}P-labeled plasma RNA demonstrated RNA species ranging from 10 to 70 nt in size, including a discernable species of size ≈22 nt characteristic of most miRNAs [supporting information (SI) Fig. S1]. The detected signal was sensitive to RNase treatment but insensitive to DNase I treatment, confirming that the signal originated from RNA (Fig. S1).

To directly determine whether miRNAs are present in human plasma, we isolated the 18- to 24-nt RNA fraction from a human plasma sample from a healthy donor (see *SI Text* for details on blood collection and plasma RNA isolation) and used 5′ and 3′ RNA–RNA linker ligations followed by RT-PCR amplification to generate a small RNA cDNA library (Fig. 1*A*). Of the 125 clones sequenced from this library, 27 corresponded to spiked-in size marker oligos or linker–linker dimers. Ninety-one of the other 98 sequences (93%) corresponded to known miRNAs, providing direct confirmation that mature miRNAs are present in human plasma and indicating that the vast majority of 18- to 24-nt plasma RNA species cloned by our protocol are indeed miRNAs (Fig. 1*A*). To quantitate specific miRNAs, we used *Taq*Man quantitative RT-PCR (qRT-PCR) assays (12) to measure three miRNAs (*miR-15b*, *miR-16*, and *miR-24*) in plasma from three healthy individuals. These three miRNAs, chosen to represent moderate- to low-abundance plasma miRNAs (based on the sequencing results described above), were all readily detected in the plasma of each individual at concentrations ranging from 8,910 copies/μl plasma to 133,970 copies/μl plasma, depending on the miRNA examined (Fig. 1*B*).

Stability of Endogenous miRNAs in Human Plasma. We next sought to investigate the stability of miRNAs in plasma, given that this is an important prerequisite for utility as a biomarker. Incubation of plasma at room temperature for up to 24 h (Fig. 2*A Upper*) or subjecting it to up to eight cycles of freeze-thawing (Fig. 2*A Lower*) had minimal effect on levels of *miR-15b*, *miR-16*, or *miR-24* as

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Author contributions: D.W.L., C.W.D., D.L.S., R.L.V., P.S.N., D.B.M., and M.T. designed research; P.S.M., R.K.P., E.M.K., B.R.F., S.K.W., E.L.P.-A., A.P., J.N., K.C.O., and A.A. performed research; N.U., B.S.K., D.L.S., and R.L.V. contributed new reagents/analytic tools; P.S.M., R.K.P., E.M.K., B.R.F., S.K.W., R.G., and M.T. analyzed data; and P.S.M., R.K.P., E.M.K., B.R.F., and M.T. wrote the paper.

The authors declare no conflict of interest.

[‡]P.S.M., R.K.P, and E.M.K contributed equally to this work.

[§]Present address: Illumina, Inc., 9885 Towne Centre Drive, San Diego, CA 92121.

[¶¶]To whom correspondence should be addressed. E-mail: mtewari@fhcrc.org.

This article contains supporting information online at www.pnas.org/cgi/content/full/0804549105/DCSupplemental.

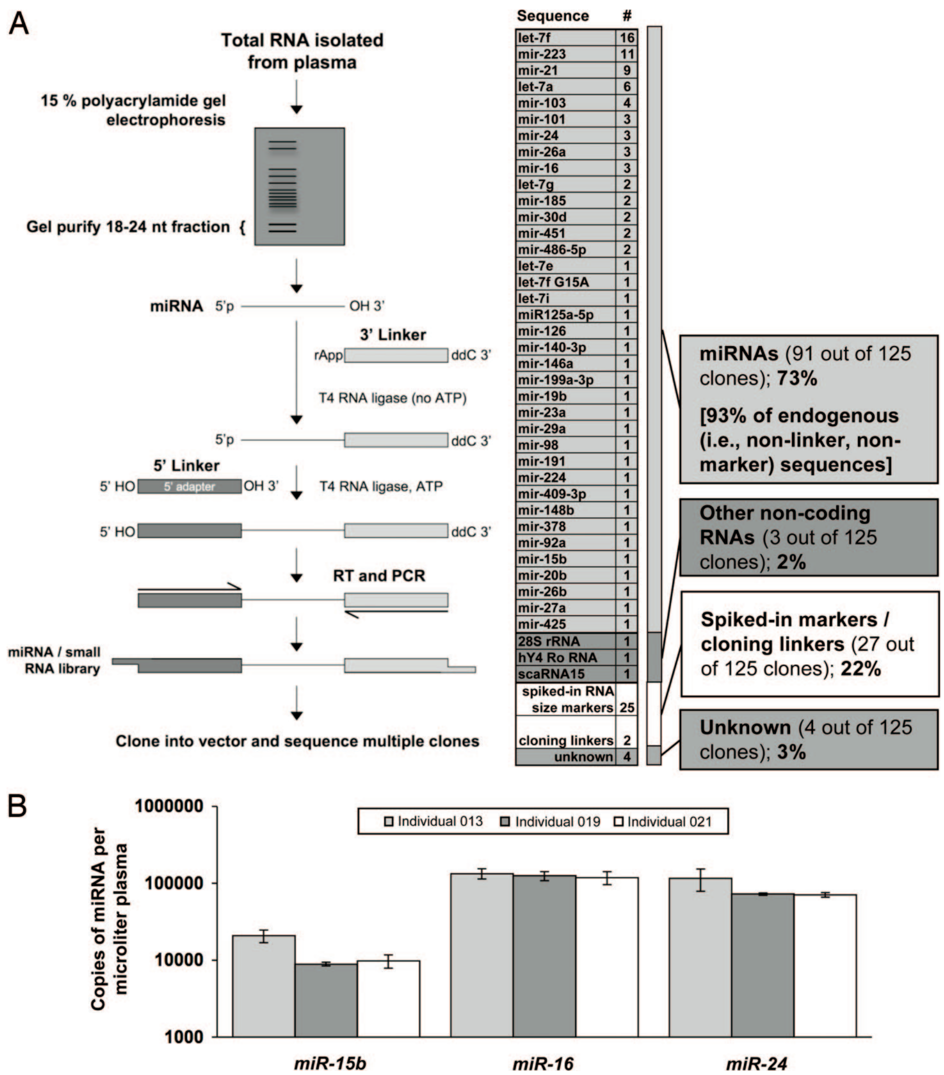

Fig. 1. Identification of miRNAs in human plasma. (*A*) Cloning and sequencing of miRNAs from human plasma. The schematic diagram depicts the preparation of a small RNA library from human plasma. Briefly, the 18- to 24-nt fraction from ≈250 ng of plasma total RNA from a single donor (individual 006; described in Table S6) was isolated by PAGE. Purified miRNAs were then 3′ and 5′ligated to single-stranded oligonucleotides that contained universal primer sequences for reverse transcription and PCR. Reverse transcription and PCR generated a library of small RNA cDNA molecules that were ligated into a plasmid vector (pCR4-TOPO) and transformed into *Escherichia coli*. Inserts from a total of 125 individual colonies yielded high-quality sequence. Sequences were compared to a reference database of known miRNA sequences (miRBase Release v.10.1) (19) and to GenBank. Seventy-three percent of sequences corresponded to known miRNAs as shown. The next most abundant species were matches to the sequence of synthetic RNAs spiked in as radiolabeled 18- and 24-nt molecular size markers during gel isolation steps. When only endogenously derived RNA sequences are considered, miRNAs represent 93% (91 of 98) of the recovered sequences. The miRNA read designated as "let-7f G15A" denotes a sequence matching the known *let-7f* miRNA except for a G-to-A substitution at nucleotide position 15. (*B*) Quantification of representative miRNAs in normal human plasma by *Taq*Man qRT-PCR. The graph indicates the number of copies of each of three representative miRNAs measured in plasma obtained from three healthy individuals. In each case, values represent the average of two replicate reverse transcription reactions followed by real-time PCR. For each miRNA assay, a dilution series of chemically synthesized miRNA was used to generate a standard curve that permitted absolute quantification of molecules of miRNA/μl plasma as shown here (see Fig. S2 for standard curve plots). Values were median-normalized by using measurements of synthetic normalization controls spiked in immediately after addition of denaturing solution during RNA isolation (see *SI Text* for full details). The absence of amplification in reverse transcriptase-negative controls indicated that amplification was originating from an RNA template (real-time PCR plots corresponding to plasma RNA samples and negative controls are provided in Fig. S3).

measured by *Taq*Man qRT-PCR. Given that plasma has been reported to contain high levels of RNase activity (13), we sought to determine whether the stability of miRNAs is intrinsic to their small size or chemical structure, or whether it is caused by additional extrinsic factors. We introduced synthetic miRNAs corresponding to three known *Caenorhabditis elegans* miRNAs (*cel-miR-39*, *cel-miR-54*, and *cel-miR-238*), chosen because of the absence of homologous sequences in humans, into human plasma either before or after the addition of a denaturing solution that inhibits RNase activity. RNA extraction followed by measurement of synthetic miRNAs showed that the synthetic miRNAs rapidly degraded when added directly to plasma (the time between addition of synthetic miRNAs and subsequent addition of denaturing solution was <2 min), as compared with their addition after adding denaturing solution to plasma (Fig. 2*B*). These results confirm the presence of RNase activity in plasma and the sensitivity of naked miRNAs to degradation. The levels of endogenous miRNAs (i.e., *miR-15b*, *miR-16*, and *miR-24*) were not significantly altered in any of the experimental samples (Fig. 2*B*), indicating that endogenous plasma miRNAs exist in a form that is resistant to plasma RNase activity.

Comparison of miRNA Levels Between Plasma and Serum. Given that clinical specimens of serum (the supernatant remaining after whole blood is permitted to clot) are more plentiful than plasma samples in many retrospective clinical sample repositories, we sought to determine whether miRNA measurements are substantially different in serum compared with plasma by measuring *miR-15b*, *miR-16*, *miR-19b*, and *miR-24* in matched samples of serum or plasma collected from a given individual at the same blood draw (Fig. 2*C*). Measurements obtained from plasma or serum were strongly correlated, indicating that both serum and plasma samples will be suitable for investigations of miRNAs as blood-based biomarkers.

Tumor-Derived miRNAs Are Present in Plasma. Having demonstrated that circulating miRNAs are detectable and stable in blood collected from healthy individuals, we next sought to determine whether tumor-derived miRNAs enter the circulation at levels sufficient to be measurable as biomarkers for cancer detection. We chose to study a mouse prostate cancer xenograft model system that involves growth of the 22Rv1 human prostate cancer cell line in NOD/SCID immunocompromised mice (14–17). We established a cohort of 12 mice xenografted with 22Rv1 cells injected with Matrigel and 12 control mice inoculated with Matrigel alone (Fig. 3*A*). Plasma was collected 28 days later (once tumors were well established), and RNA was isolated for miRNA quantitation. Because of the lack of an established endogenous miRNA control

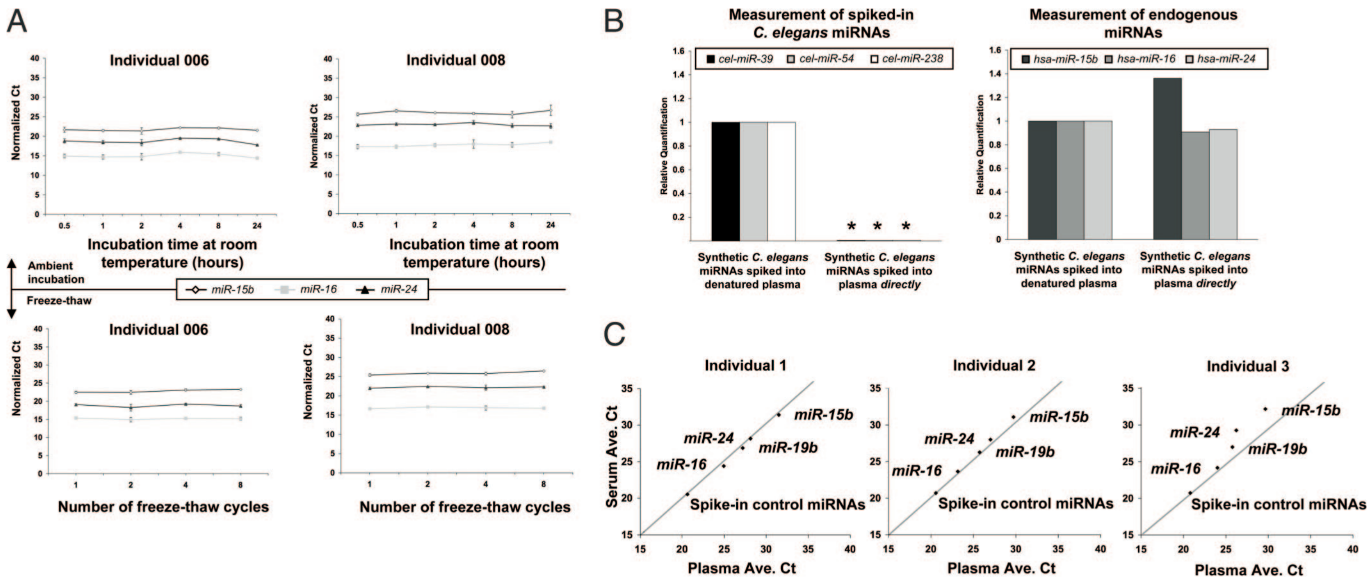

Fig. 2. Characterization of miRNA stability in human plasma. (*A*) miRNA levels remain stable when plasma is subjected to prolonged room temperature incubation or freeze-thawed multiple times. (*Upper*) The graphs show normalized Ct values for the indicated miRNAs measured in parallel aliquots of human plasma samples incubated at room temperature for the indicated times. The experiment was carried out by using plasma from the two different individuals noted. Normalization of raw Ct values across samples is based on the measurement of three nonhuman synthetic miRNAs spiked into each sample at known molar amounts after initial plasma denaturation for RNA isolation (described in detail in *SI Text*). (*Lower*) The graphs show normalized Ct values for the indicated miRNAs measured in parallel aliquots of human plasma samples subjected to the indicated number of cycles of freeze-thawing. Raw Ct values were normalized across samples by using the same approach as described above. (*B*) Exogenously added miRNAs are rapidly degraded in plasma, whereas endogenous miRNAs are stable. Three *C. elegans* miRNAs (chosen for the absence of sequence similarity to human miRNAs) were chemically synthesized and added either directly to human plasma (from individual 003; described in Table S6) or added after the addition of denaturing solution (containing RNase inhibitors) to the plasma (referred to as ''denatured plasma''). RNA was isolated from both plasma samples, and the abundance of each of the three *C. elegans* miRNAs was measured by *Taq*Man qRT-PCR (*Left*), as was that of three endogenous plasma miRNAs (*Right*). Asterisks indicate that the abundance ratios of *cel-miR-39*, *cel-miR-54*, and *cel-miR-238* added to human plasma directly, relative to addition to denatured plasma, were 1.7×10^{-5}, 9.1×10^{-6}, and 1.1×10^{-5}, respectively and therefore too low to accurately display on the plot. (*C*) Abundance of miRNAs in serum and plasma collected from the same individual is highly correlated. Each plot depicts the average Ct values (average of two technical replicates) of the indicated miRNAs measured in serum and plasma samples collected from a given individual at the same blood draw. Results from three different individuals are shown. miRNA measurements were highly correlated in both sample types. Results shown for synthetic *C. elegans* miRNAs spiked into each plasma or serum sample (after addition of denaturing solution) demonstrate that experimental recovery of miRNAs and robustness of subsequent qRT-PCR is not affected by whether it is plasma or serum that is collected.

for plasma or serum, we introduced three synthetic *C. elegans* miRNAs (described earlier) after the addition of denaturing solution to the plasma samples and used these as normalization controls to correct for technical variations in RNA recovery (detailed in *SI Text*).

We first sought to establish that endogenous (murine) miRNAs exist in mouse plasma and determine whether the presence of cancer may lead to a general increase in plasma miRNAs, whether they be tumor- or host-derived. *miR-15b*, *miR-16*, and *miR-24* (which are perfectly conserved in mature sequence between human and mouse) were all readily detectable in healthy control mice (Fig. 3*B*) and were not expressed at substantially different levels in xenograft-bearing mice, indicating that the presence of tumor does not lead to a generalized increase in plasma miRNAs.

We next sought to identify candidate tumor-derived miRNAs for examination in plasma by first profiling the expression of 365 known miRNAs in 22Rv1 cells by using a microfluidic *Taq*Man low-density miRNA qRT-PCR array (Applied Biosystems). We identified two miRNAs, *miR-629** and *miR-660*, that (*i*) were expressed in these cells as indicated by low cycle threshold (Ct) values and (*ii*) did not have known mouse homologs and therefore would be expected to be tumor-specific markers in this setting (Table S1). We next analyzed plasma samples from control and xenograft mice for the levels of *miR-629** and *miR-660* by *Taq*Man qRT-PCR. Levels of *miR-629** and *miR-660* were generally undetectable in the control mice, whereas they were readily detected (ranging from 10 to 1,780 copies/μl plasma for *miR-629** and 5,189–90,783 copies/μl for *miR-660*) in all of the xenografted mice (Fig. 3*C*). Levels of both *miR-629** and *miR-660* were able to independently differentiate xenografted mice from controls with 100% sensitivity and 100% specificity. These data establish proof of the principle that tumor-derived miRNAs reach the circulation where their measurement in plasma can serve as a means for cancer detection.

To understand the basis for the wide variation in miRNA abundance observed among the different xenografted mice, we compared plasma levels of *miR-629** and *miR-660* with tumor mass in each mouse. Levels of these miRNAs were moderately correlated with tumor mass (Fig. S4 and see Table S7), indicating that variation in miRNA abundance across animals reflects, at least in part, the differences in tumor burden.

Tumor-Derived miRNAs in Plasma Are Not Cell-Associated. We sought to further explore the mechanism of protection of tumor-derived miRNAs from plasma RNase activity by testing the hypothesis that they are present inside circulating tumor cells that might have escaped pelleting during primary centrifugation for plasma isolation. We took two approaches to address this hypothesis. In the first experiment, we filtered pooled plasma generated from the xenograft or control groups through a 0.22-μm filter, followed by RNA extraction from the filtrate and the material retained on the filter (referred to as the retentate). Measurement of *miR-629** and *miR-660* by qRT-PCR in each of the samples demonstrated that virtually all of the tumor-derived miRNAs passed through the 0.22-μm filter (Fig. S5*A*). As expected, tumor-derived miRNAs were essentially undetectable in all of the samples from the control group. In a second independent experiment, we subjected plasma

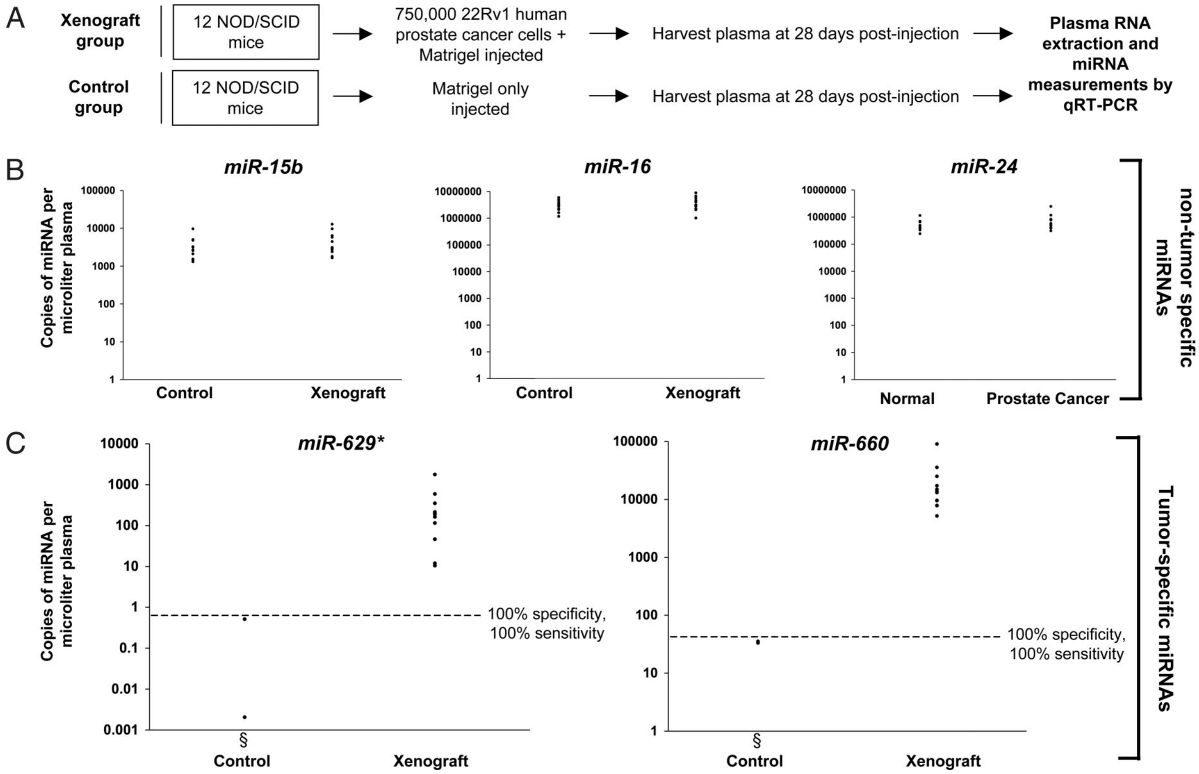

Fig. 3. Tumor-derived miRNAs are detectable in plasma. (*A*) Schema for 22Rv1 human prostate cancer xenograft experiment. (*B*) MiRNAs are present in plasma of healthy control mice and their levels are not nonspecifically altered in cancer-bearing mice. Plasma levels of *miR-15b*, *miR-16*, and *miR-24* were measured in 12 healthy control mice and 12 xenograft-bearing mice. The mature sequence of these miRNAs is perfectly conserved between mice and humans. Ct values were converted to absolute number of copies/μl plasma by using a dilution series of known input quantities of synthetic target miRNA run on the same plate as the experimental samples (dilution curves are provided in Fig. S2). Values shown have been normalized by using measurements of *C. elegans* synthetic miRNA controls spiked into plasma after denaturation for RNA isolation (details of the normalization method are provided in *SI Text*). (*C*) Tumor-derived miRNAs are detected in plasma of xenograft-bearing mice and can distinguish cancer-bearing mice from controls. Plasma levels of *miR-629** and *miR-660* (two human miRNAs that are expressed in 22Rv1 cells and do not have known murine homologs) were measured in all control and xenografted mice. Ct values were converted to absolute number of copies/μl plasma and normalized as described for *B* (see Table 5) threshold. Given that homologous miRNAs are not believed to exist in mice, the low level of signal detected for a few mice in the control group, particularly for the *miR-660* assay, is likely to represent nonspecific background amplification. As expected, in the control (nontumor-bearing) mice group, qRT-PCR for *miR-629** or *miR-660* in plasma from most animals could not detect any appreciable signal. These points are therefore not shown on the graph, even though plasma samples from the entire group of 12 mice in the control group were studied.

pools from the xenograft and control groups to a series of two centrifugations (one at 2,000 $\times$ *g*, which should pellet any intact cells remaining after the initial centrifugation used to collect plasma, followed by another at 12,000 $\times$ *g*, which should pellet any large cell fragments). We assayed for miRNA expression in the starting material, any pelleted material obtained from each centrifugation, and the supernatant remaining after the 12,000 $\times$ *g* centrifugation. As shown in Fig. S5*B*, virtually all of the tumor-derived miRNA was present in the supernatant of the 12,000 $\times$ *g* spin. Taken together, the data indicate that tumor-derived miRNAs are not associated with intact cells or large cell fragments. These results do not exclude the possibility that circulating tumor cells or fragments of the same may have been lysed during the process of blood collection or plasma processing. Even if that is the case, however, our results show that miRNAs that may have been released are ultimately present in a stable, protected form of size much smaller than that of a typical epithelial cell.

Detection of Human Prostate Cancer Based on Measurement of a Prostate Cancer-Expressed miRNA in Serum. We next sought to extend this approach to cancer detection in humans. We reasoned that an ideal marker would be (*i*) expressed by the cancer cells at moderate or high levels and (*ii*) present at very low or undetectable levels in plasma from healthy individuals. We established a list of likely blood-based miRNA biomarker candidates for prostate cancer by (*i*) compiling a list of miRNAs expressed in human prostate cancer specimens based on published miRNA expression profiling data (7, 18) and (*ii*) filtering out miRNAs detected in healthy donor-derived plasma in our miRNA cloning experiment (Fig. 1*A*) or detected on a microfluidic *Taq*Man qRT-PCR array analysis of plasma from a normal healthy individual (details provided in *SI Text* and see Table S8). This process generated a list of six leading candidates (*miR-100*, *miR-125b*, *miR-141*, *miR-143*, *miR-205*, *and miR-296*) for further investigation.

We chose to analyze these candidates in a case-control cohort of serum samples collected from 25 individuals with metastatic prostate cancer and 25 healthy age-matched male control individuals. To efficiently screen multiple miRNA biomarker candidates, we first generated two pools of serum aliquots derived from the individuals in the case and control groups, respectively. We isolated RNA from both pools and screened them for differential expression of the six candidate biomarker miRNAs by *Taq*Man qRT-PCR assays. Results of this screen indicated that five of six of these candidate miRNA biomarkers showed increased expression, although to varying degrees, in the prostate cancer serum pool compared with the healthy control group serum pool (Table S2). For one of the candidates (*miR-205*), no conclusion could be reached because miRNA levels in both pools were lower than the limit of detection of the assay (as determined by a standard curve using a dilution series of a synthetic *miR-205* RNA oligonucleotide).

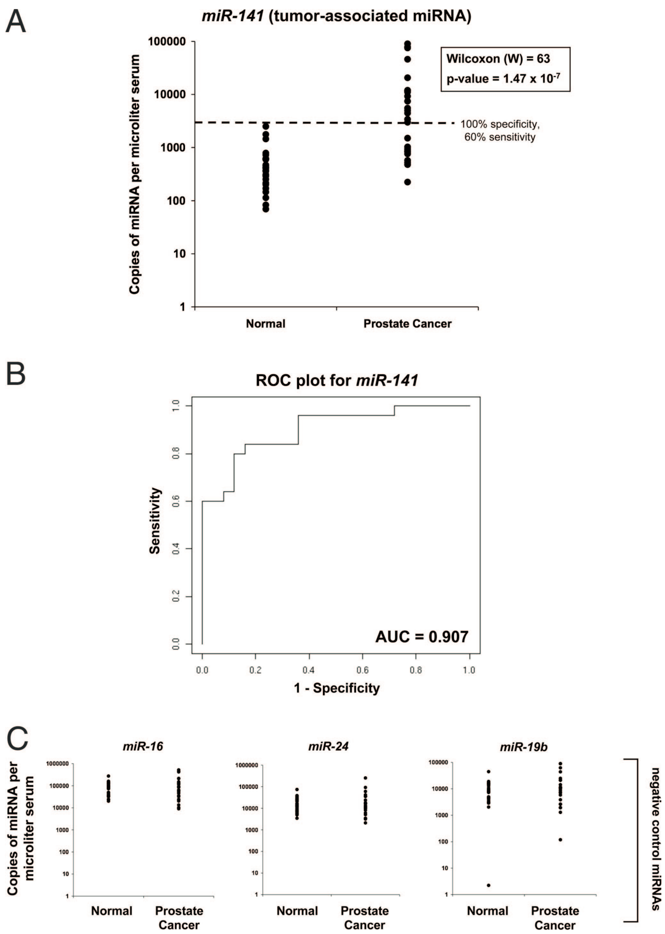

Fig. 4. Detection of human prostate cancer by serum levels of tumor-associated miRNA miR-141. (*A*) Serum levels of *miR-141* discriminate patients with advanced prostate cancer from healthy controls. Serum levels of the prostate cancer-expressed miRNA *miR-141* were measured in 25 healthy control men and 25 patients with metastatic prostate cancer (clinical data on subjects is provided in Table S3). Ct values were converted to absolute number of copies/μl serum by using a dilution series of known input quantities of synthetic target miRNA run simultaneously (on the same plate) as the experimental samples (dilution curves are provided in Fig. S2). Values shown have been normalized by using measurements of *C. elegans* synthetic miRNA controls spiked into plasma after denaturation for RNA isolation (details of normalization method are provided in *SI Text*). The dashed line indicates a 100% specificity threshold. (*B*) Receiver Operating Characteristic (ROC) plot. The data shown in *A* were used to draw the ROC plot shown. (*C*) Serum levels of nontumor-associated miRNAs are not substantially different between patients with prostate cancer and controls. Serum levels of *miR-16*, *miR-24*, and *miR-19b* were measured as negative controls as they are not expected to be cancer-associated in the serum. Absolute quantification of miRNAs and data normalization were carried out as described for *A*.

Of all of the candidates, *miR-141* showed the greatest differential expression (46-fold overexpressed) in the prostate cancer pool compared with the control pool (Table S2). We therefore focused our study on *miR-141* by measuring the abundance of this miRNA in all of the individual serum samples comprising the case and control groups. Consistent with results from the analysis of pooled samples, serum levels of *miR-141* were, in general, substantially higher in cancer cases compared with controls (Fig. 4*A*). Comparison of the two groups by a Wilcoxon two-sample test yielded W = 63 with a $P = 1.47 \times 10^{-7}$, confirming a significant difference in *miR-141* levels between the two groups. Furthermore, serum levels of *miR-141* could detect individuals with cancer with 60% sensitivity at 100% specificity (Fig. 4*A*). Representation of the data using a Receiver Operating Characteristic plot (Fig. 4*B*) reflects strong separation between the two groups, with an area under the curve (AUC) of 0.907. Comparison of *miR-141* levels to prostate-specific antigen (PSA) values among the prostate cancer patients demonstrated Pearson and Spearman (rank) correlation coefficients of +0.85 and +0.62, indicating that *miR-141* and PSA levels are moderately correlated (Table S3). Serum levels of nonbiomarker candidate miRNAs *miR-16*, *miR-19b*, and *miR-24* were not significantly different between cases and controls, supporting the notion that *miR-141* is specifically elevated in prostate cancer, as opposed to reflecting a nonspecific, generalized increase in serum miRNA levels in the setting of cancer (Fig. 4*C*). Taken together, the results extend to human cancer the concept that circulating miRNAs can serve as markers for cancer detection.

miR-141 Is an Epithelial-Associated miRNA Expressed by Several Common Human Cancers. miR-141 is a member of an evolutionarily conserved family of miRNAs that includes, in humans, *miR-141*, *miR-200a*, *miR-200b*, *miR-200c*, and *miR-429* (19). The expression of zebrafish homologs of this family, when studied by *in situ* hybridization, was found to localize to various epithelial tissues (20). To gain more insight into the potential biological role of *miR-141*, we explored the large miRNA expression profiling dataset generated by Lu *et al.* (7), who profiled a diverse range of human cancer types. Consistent with findings from the zebrafish studies, the expression of *miR-141* was tightly associated with expression in epithelial samples compared with nonepithelial samples (Fig. S6), and *miR-141* was expressed in a wide range of common epithelial cancers including breast, lung, colon, and prostate.

To determine the relative expression of this miR-141 specifically between the epithelial and stromal compartments of prostate tissue, both comparatively between the two cell types and relative to all other known miRNAs within a cell type, we generated small RNA libraries from primary cultures of human prostate epithelial and stromal cells and subjected them to massively parallel sequencing (detailed in *SI Text*). We found that *miR-141* (and two of its family members, *miR-200b* and *miR-200c*) was readily detected in the prostate epithelial cell dataset but strikingly absent in the prostate stromal cells (Table S4). In fact, of all miRNAs in this analysis, *miR-141* and *miR-200b* were the two most overexpressed in prostate epithelial cells relative to prostate stromal cells (Table S4). Taken together, the data are consistent with the notion that *miR-141* is an epithelial-restricted miRNA that can be detected in the circulation as a prostate cancer biomarker.

Discussion

Our Results Establish That Tumor-Derived miRNAs, Detected in Plasma or Serum, Can Serve as Circulating Biomarkers for Detection of a Common Human Cancer Type. Although there is a long history of investigation of circulating mRNA molecules as potential biomarkers (21), blood-based miRNA studies are in their infancy. Recently, Chim *et al.* (22) reported the detection by qRT-PCR of miRNAs of presumed placental origin in the plasma of pregnant women, and Lawrie *et al.* (23) reported detecting elevations in miRNAs in serum from lymphoma patients. Beyond confirming the early reports, our study yielded (*i*) a more comprehensive view of plasma miRNAs by direct cloning and sequencing from a plasma small RNA library, (*ii*) unique results on miRNA stability that provide a firm grounding for further investigation of this class of molecules as blood-based cancer biomarkers, and (*iii*) evidence that tumor-derived miRNAs can enter the circulation even when originating from an epithelial cancer type (as compared with hematopoietic malignancies like lymphoma). Most importantly, our study of *miR-141* in prostate cancer patients demonstrates that serum levels of a tumor-expressed miRNA can distinguish, with significant specificity and sensitivity, patients with cancer from healthy controls.

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