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14. ABSTRACT A systematic comparison of the short RNA universe of two prostate cancer cell lines was performed by cloning and deep sequencing to identify microRNAs whose expression may correlate with androgen dependence/independence or microRNAs that are regulated by androgens. The results were confirmed by microarrays and by quantitative reverse transcription and polymerase chain reaction. We have identified five microRNAs that are repressed and three that are induced during progression of prostate cancer from androgen dependent, early stage to androgen independent advanced stage. We have also identified four microRNAs that are repressed by androgens in the androgen dependent cells. The "progression-repressed" microRNAs render the advanced cancer cells unable to grow in charcoal-stripped androgen depleted serum. We have also identified several non-micro-short RNAs that are expressed abundantly in prostate cancer cells and provide evidence that one of them, tRF-1001, is necessary for cell proliferation.					
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## **INTRODUCTION:**

When we wrote our application we proposed to identify the microRNAs that are expressed in three different prostate cancer cell lines, PC3, DU145 and LNCaP. Comparison of the microRNA expression patterns was expected to identify microRNAs that are specific to certain cancer cell lines. As the project progressed, we realized that we would learn more about microRNAs in prostate cancer progression if we compared the microRNA profiles of an early stage and an advanced stage cell line derived from the former. We therefore focused on the androgen-dependent, non metastatic LNCaP cells and its derivative C4-2 that is androgen-independent and metastatic.

We also wanted to characterize microRNAs that are induced or repressed when an androgen responsive prostate cancer cell line, LNCaP is grown in the presence and absence of androgen. Comparison of the microRNA expression patterns was expected to identify microRNAs that may have a role in the cell's response to androgens.

## **BODY:**

### **The Statement of Work and the status of each task:**

Task 1.1: Grow DU145, HeLa and HCT116 cancer cell lines, isolate size-selected microRNAs from each of them. *(These cells were substituted by C4-2 and LNCaP growing in serum.)* Grow LNCaP cells in the absence and presence of androgens and isolate size-selected microRNAs. COMPLETED

Task 1.2: Ligate adapters, make cDNAs of the microRNAs, clone the cDNAs to make libraries representing microRNA complements, and sequence the clones in the libraries. COMPLETED

Task 1.3: De-convolute the sequences and do bioinformatics on public sequence databases to identify predicted and novel microRNAs. COMPLETED

*NEW TASK 1.3b: Assay the microRNAs also on Locked Nucleic Acid based microarrays of microRNAs to profile which microRNAs are altered between LNCaP and C4-2 and in LNCaP plus and minus androgen.* COMPLETED

Task 1.4: Determine which microRNAs should be pursued in task 2. The two independent methods for evaluating the microRNAs (sequencing and microarray) helped us to prioritize which microRNAs gave reproducible results and should be pursued. COMPLETED

Task 2.1: Develop primer extension assays, Rnase protection assays and Northern blot assays for 20-30 microRNAs prioritized in Task 1.4 Quantitative RT-PCR, Rnase Protection assays and Northern blots were used. COMPLETED

Task 2.2: Assay levels of the microRNAs to confirm differences in their levels between cell lines and in response to androgen. COMPLETED

Task 2.3: Prioritize 5-8 microRNAs for follow up in task 3 COMPLETED

Task 3.1: Downregulate microRNAs in PC3 (*changed to LNCaP and C4-2*) cells and assay effects on cell proliferation. ONGOING

Task 3.2: Upregulate microRNAs in PC3 (*changed to LNCaP and C4-2*) cells and assay effects on cell proliferation. COMPLETED

Task 3.3: Alter microRNA levels in LNCaP cells, treat cells with androgens and harvest total RNA at specified time-points. ELIMINATED. *The microRNA alteration was not done. Instead we went straight to Task 3.4 because a short-cut became apparent.*

Task 3.4: Microarray hybridization of the RNA from task 3.3. *Instead, the genes changed by androgens were obtained from microarray experiments in public databases where LNCaP cells were grown in the presence and absence of androgen.* COMPLETED

Task 3.5: Analysis of results from Task 3.4 to determine whether the tested microRNAs modulate the expression of genes affected by androgens. COMPLETED

#### Deliverables

At 12 months: Annual report for year 1 DELIVERED

At 24 months: Annual report for year 2 DELIVERED

At 36 months: Final report of project. CURRENT

2-3 manuscripts for peer review reporting on the microRNAome of prostate cancer cells with an emphasis on microRNAs that are critical for cell proliferation and/or androgen-driven gene expression program. COMPLETED

#### **Summary of our results.**

**Papers:** Two manuscripts have been prepared (attached as appendix):

***1) “MicroRNAs in the androgen-independence and androgen regulation of prostate epithelial cell lines”. Under revision after review. Copy included in the Appendix as LEE1. Figures and Tables in the following section refer to Figures and Tables in the text of LEE1 and in the Supplemental material for LEE1.***

A systematic comparison of the short RNA universe of two prostate cancer cell lines was performed by cloning and deep sequencing to identify microRNAs whose expression may correlate with androgen dependence/independence or microRNAs that are regulated by androgens (**Tables 1 and 2 in attached paper, Lee1**). The results were

confirmed by two independent methods of quantitating microRNAs: locked nucleic acid microarrays, (**Supplemental Tables S1, S2 and S3**) and microRNA-specific qPCR/RNase protection/Northern assays (**Figure 1 and Supplemental Figure S5**). Changes that were consistently detected by all three methods are the decrease of five microRNAs and increase of three microRNAs in the androgen-independent C4-2 cell line relative to its androgen-dependent predecessor, LNCaP (**Fig. 1A**). miR-125b, 100, -99a, -99b and 19b were downregulated in C4-2 and miR-196b, -9 and -557 were upregulated in C4-2. Student's t-test showed that all the changes measured by Q-RT-PCR except that of miR-19b had a  $p < 0.05$ . For the Q-RT-PCR measurement of the decrease of miR-19b in C4-2, the  $p = 0.18$ . Nevertheless, we have decided to keep this microRNA in the list of microRNAs decreased in C4-2 relative to LNCaP because the difference was noted independently in the microarrays and in the cloning/sequencing approach.

Ectopic expression of the microRNAs that were downregulated in C4-2 decreased the growth of C4-2 in the absence of androgen but not in its presence (**Fig. 2A and B**), suggesting that microRNA changes contribute to the androgen-independence. Except for the transfection of single microRNAs miR-99b and -125b in **Fig. 2B**, transfection of all five microRNAs (**Fig. 2A**) or of single microRNAs miR-99a, -100 and -19b (**Fig. 2B**) repressed C4-2 proliferation in androgen-depleted medium ( $p < 0.05$ , Student's t test). All five downregulated microRNAs have been reported to be downregulated in clinically advanced prostate cancer [1], suggesting that they constitute a microRNA signature of prostate cancer progression.

Four microRNAs were consistently shown to be downregulated in multiple assays by androgens: miR-221, -196b, -125b and -99a (**Fig. 1D, Supplemental Table S3, Fig. S1 and S4**). The difference in the level of microRNA in the highest concentration of R1881 relative to no androgen was found to be significant in all four cases ( $p < 0.05$ , Student's t test). We were disappointed by the failure to detect any microRNAs induced by androgens and plan to re-visit the question by additional microRNA microarrays on other androgen-dependent cell lines. This is why we have been unable to ascertain whether transfection of androgen-induced microRNAs change the behavior of the cells (the former Task 3.3). We have, however, examined gene expression changes induced by androgens (new Tasks 3.4 and 3.5) to ascertain whether induction of a gene by androgen could be explained by the repression of the four microRNAs. Intersection of the mRNA changes induced by androgens (available in public microarray datasets) with the computationally predicted targets of the four microRNAs (the TargetScan and MiRanda databases) yielded four mRNAs that could be regulated by the microRNAs. The genes that will be studied are a multi-drug transporter involved in drug resistance (ABCC4), an anti-apoptotic transcriptional factor (Stat3), PTPN18 (a protein tyrosine phosphatase involved in signal transduction) and CPD (carboxypeptidase D, a gene that regulates intracellular NO levels). The first three are predicted to be repressed by miR-125b and the last one by miR-196b. All the genes are induced by androgens and both the microRNAs are repressed by androgens.

Despite the broad concordance in changes in microRNAs detected by cloning/sequencing versus microarrays in nearly four hundred microRNAs studied, there was a significant discrepancy in the changes reported by cloning/sequencing versus microarray hybridization for six microRNAs (**Supplemental Fig. S6**). miR-16, -21, 200c, 106a, 20a and -30b appeared to be 5-50 fold higher in androgen treated LNCaP than in

androgen-depleted cells in the cloning/sequencing experiments. Yet, on the microarrays these microRNAs were equally abundant in the two types of cells. Q-RT-PCR confirmed the equal abundance (**Fig. S6**). This suggests that there is a systematic loss of these microRNAs when they were cloned/sequenced from androgen-depleted cells. Using miR-21 as an example, we followed the yield of this microRNA at various steps in the cloning procedure (**Fig. 3**), and discovered that an as yet unknown 3' end modification of miR-21 in androgen-depleted cells decreases 3' adaptor ligation and thus decreases the cloning frequency.

Finally, cloning and sequencing reveals a large number of non-micro-small RNAs (nmsRNAs) of unknown function that are expressed abundantly in the prostate epithelial cells. Examples of these nmsRNAs are given in **Table 3** of Lee1 and in **Supplemental Fig. S8**. As more of these nmsRNAs are characterized, we expect that novel functions will be attributed to them and that some of them may be as useful for profiling cancers as microRNAs or mRNAs

**2) “A novel class of small RNAs, tRNA-derived RNA fragments (tRFs), is highly expressed in proliferating cells and required for cell proliferation.” Reviewed at Genes & Development, currently being revised for re-submission. Copy included in Appendix as LEE2. Figures and Tables in the following section refer to LEE2 and Supplemental Material of LEE2.**

New types of small RNAs distinct from microRNAs (miRNAs) are progressively being discovered in various organisms. In the course of cloning and sequencing 17-26 base long RNAs from prostate cancer cell lines we discovered a novel class of short RNAs, the tRFs. A significant number of the sequences are derived from precise processing at the 5'- or 3'-end of mature or precursor tRNAs, to form three series of tRFs, the -5, -3 and -1 series (**Table 1 and Fig. 1 of LEE2; also Supplemental Material, Table S1, S2 and Fig. S1-S3**). tRF-5 molecules are generated by the 5'-most 21-22 nucleotides being cleaved from mature tRNAs. tRF-3 molecules are generated by the cleavage of the 3'-most 21-22 nucleotides from mature tRNAs. tRF-1 molecules are generated from precursors of tRNAs by the cleavage that releases the 3' end of the mature tRNA. These sequences, collectively named as tRFs (tRNA-derived RNA Fragments), constitute a class of short RNAs that are second-most abundant to miRNAs.

Northern hybridization and qRT-PCR assays independently measured the levels of at least 16 tRFs (**Fig. 2 and S4**). Their ease of detection is comparable to that of some of the best-studied microRNAs.

The best evidence that the tRFs are not random byproducts of the turnover of tRNAs or their precursors would be if knocking down a specific tRF produces a phenotype suggestive of a specific biological function. Towards that end, we focused on tRF-1001, derived from the 3'-end of a Ser-TGA tRNA precursor transcript that is not retained in the mature tRNA. tRF-1001 is expressed highly in a wide range of cancer cell lines but much less in tissues and its expression in cell lines was positively correlated with cell proliferation (**Fig. 2**). siRNA-mediated knockdown of tRF-1001 impaired cell proliferation with the specific accumulation of cells in G2 (**Fig. 3C**), phenotypes that were reversed by co-introducing a synthetic 2'-O-methyl tRF-1001 oligoribonucleotide resistant to the siRNA (**Fig. 3D**). Although tRF-1001 is localized in the cytoplasm and

similar in size to miRNAs and siRNAs, it did not repress a target mRNA with sites that are complementary to the tRF molecule (**Fig. 4**), suggesting that tRF-1001 does not enter into RISC or miRNP complexes. Our data suggest that tRFs are not random by-products of tRNA degradation or biogenesis but an abundant and novel class of short RNAs with precise sequence structure that have specific expression patterns and specific biological roles.

### **3) *MicroRNAs in Cancer, Annual Review of Pathology, 2009 4: 199-227 (A review)***

Within the past few years, studies on microRNA (miRNA) and cancer have burst onto the scene. Profiling of the miRNome (global miRNA expression levels) has become prevalent, and abundant miRNome data are currently available for various cancers. The pattern of miRNA expression can be correlated with cancer type, stage, and other clinical variables, so miRNA profiling can be used as a tool for cancer diagnosis and prognosis. miRNA expression analyses also suggest oncogenic (or tumor-suppressive) roles of miRNAs. miRNAs play roles in almost all aspects of cancer biology, such as proliferation, apoptosis, invasion/metastasis, and angiogenesis. Given that many miRNAs are deregulated in cancers but have not yet been further studied, it is expected that more miRNAs will emerge as players in the etiology and progression of cancer. Here we also discuss miRNAs as a tool for cancer therapy.

#### **Meeting Abstracts:**

##### 1) ImPact, 2007, Atlanta

MicroRNA profile in prostate cancer cells and response to androgen depletion  
Anindya Dutta; Yong Sun Lee; Hak Kyun Kim

MicroRNAs are short single-stranded RNAs of 18-22 bases length that are produced by the processing of specific transcripts by cellular RNases Drosha and Dicer. They regulate the expression of a wide variety of genes by annealing to the 3 prime untranslated regions (UTRs) of the mRNAs and preventing the expression of the genes. In order to understand whether microRNAs are important for prostate cancer biology we have begun examining the profile of microRNAs in prostate cancer. We have cloned short RNAs of 20-28 bases from three different prostate cancer cell lines, PC3, LnCAP and DU145. The clones have been sequenced to identify the microRNAs that are expressed in these cells. Over a hundred microRNAs have been identified to be expressed in prostate cancer cells, and tens of new short RNAs discovered that could be novel microRNAs or some other type of cellular short RNAs. A distinct microRNA expression profile separates the PC3 and DU145 cells from LnCAP cells, consistent with the different lineage of the latter type of cells. A subset of the microRNAs are expressed specifically in an androgen-dependent manner in LnCAP cells, raising the possibility that changes in expression of microRNAs may be induced by androgens and that microRNAs may contribute to the androgen dependent cell proliferation program in prostate epithelial cells. Finally, overexpression of specific microRNAs modulate the proliferation of cancer cells, so that a systematic examination of the substrates of these microRNAs will reveal genes important for cancer cell proliferation.



2) Forbeck Symposium on microRNAs and cancer, Hilton Head, SC, 2007  
MicroRNAs in cell quiescence and cancer  
Anindya Dutta

We are examining two systems to discover microRNAs that regulate cell proliferation. In the first approach, we focused on miRNAs induced during differentiation of C2C12 myoblasts into myotubes *in vitro*. One of the microRNAs induced during differentiation, miR-206, suppresses cell proliferation. By surveying mRNAs that are downregulated by miR-206 for genes predicted to be targets of the microRNA, we discovered the mRNA of DNA polymerase alpha p180 is a direct target that is destabilized upon miR-206 transfection. We now report that additional microRNAs induced during muscle differentiation target other cell-cycle regulators. After noting that targeted mRNAs are often destabilized by microRNAs, we tested whether global downregulation of microRNAs by knockdown of Dicer or Drosha can help identify microRNA-repressed mRNAs. The principle was proved by the discovery that HMGA2 oncogene is repressed by the growth-suppressive let-7 microRNA.

We have sought other examples of microRNAs and short RNAs that regulate cell proliferation by cloning short RNAs from androgen-dependent prostate cancer cells grown in the presence or absence of androgens. The frequency of a number of clones present in the libraries is changed by androgens and we are currently testing whether the changes reflect changes in the abundance of the microRNAs upon androgen depletion. Over 30-40% of the short RNAs cloned, however, do not correspond to known microRNAs and at least some are produced by cleavage of known mRNAs and noncoding RNAs. We are curious whether other groups cloning short RNAs from other sources are also observing this phenomenon.

3) AACR meeting on Molecular Diagnostics, Philadelphia, 2008

MicroRNAs and non-micro-short RNAs (nmsRNAs) in cell quiescence and cancer  
Anindya Dutta

We are using three approaches to discover microRNAs that regulate cell proliferation. In the first approach, we focused on miRNAs induced during differentiation of C2C12 myoblasts into myotubes *in vitro*. One of the microRNAs induced during differentiation, miR-206, suppresses cell proliferation. By surveying mRNAs that are downregulated by miR-206 for genes predicted to be targets of the microRNA, we discovered the mRNA of DNA polymerase alpha p180 is a direct target that is destabilized upon miR-206 transfection. We now report that additional microRNAs induced during muscle differentiation target other cell-cycle regulators. After noting that targeted mRNAs are often destabilized by microRNAs, in the second approach, we tested whether global downregulation of microRNAs by knockdown of Dicer or Drosha can help identify microRNA-repressed mRNAs. The principle was proved by the discovery that HMGA2 oncogene is repressed by the growth-suppressive let-7 microRNA. Indeed, chromosomal translocations in lipomas and leiomyomas de-repress HMGA2 by deleting the 3'UTR that is normally repressed by let-7. Conversely, downregulation of let-7 has been noted in lung cancers and large leiomyomas and is correlated with over-expression of the HMGA2 oncogene.

In the third approach we have cloned short RNAs from androgen-dependent prostate cancer cells grown in the presence or absence of androgens and subjected them to ultra-high-throughput sequencing. The frequency of a number of clones present in the libraries is changed by androgens and we have identified several microRNAs that change upon androgen-depletion. Over 30-40% of the short RNAs cloned, however, do not correspond to known microRNAs and are produced by cleavage of known mRNAs and noncoding RNAs. The diversity and abundance of the non-micro-short RNAs (nmsRNAs) contrast with how little is known of their function and suggest that much remains to be done before we understand the biological functions of many short RNAs present in the cell. In an extension of the project we have identified several microRNAs that are reproducibly altered in expression level between the androgen-dependent LnCAP cells and androgen-independent derivative C4-2 cells. Interestingly, the microRNAs decreased in C4-2 cells were also reported by others to be decreased as prostate cancers advance in Gleason score or metastasize. This raises the possibility that a microRNA signature may be established for following the molecular stage of prostate cancer and for predicting its predilection to recur in an androgen-independent form.

#### 4) Human Genome Organization, Annual meeting, Hyderabad, 2008

MicroRNAs and non-micro-short RNAs (nmsRNAs) in cell quiescence and cancer  
Anindya Dutta

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In the third approach we have cloned short RNAs from androgen-dependent prostate cancer cells grown in the presence or absence of androgens and subjected them to ultra-high-throughput sequencing. The frequency of a number of clones present in the libraries is changed by androgens and we have identified several microRNAs that change upon androgen depletion. Over 30-40% of the short RNAs cloned, however, do not correspond to known microRNAs and are produced by cleavage of known mRNAs and noncoding RNAs. The diversity and abundance of the non-micro-short RNAs (nmsRNAs) contrast

with how little is known of their function and suggest that much remains to be done before we understand the biological functions of many short RNAs present in the cell.

**Personnel receiving pay from this project.**

Anindya Dutta  
Hak Kyun Kim  
Yong Sun Lee  
Ankit Malhotra  
Mirela Matecic  
Dandan Sun

**KEY RESEARCH ACCOMPLISHMENTS:**

- Discovering microRNAs that are altered when prostate cancer cells become androgen independent. Increased in C4-2 (more advanced): miR-196b, -557 and -9. Decreased in C4-2: miR -100. -125b. -19b, -99a and -99b
- Discovering three microRNAs that are repressed when androgen is added to an androgen-dependent prostate cancer cells: miR-221, -196b, -125b.
- Discovery that certain microRNAs (miR-16, -21, -200c, -106a, -20a and miR-30b) have a 3' end modification in androgen-depleted cells that prevent their cloning.
- Discovery of a novel class of short RNAs, tRNA-derived fragments (tRFs) with biological function.
- Comparison of miRNome by two different profiling approaches: cloning and deep sequencing and microarrays

**REPORTABLE OUTCOMES:**

- One published paper:

1) Lee YS and Dutta A. MicroRNAs in Cancer, **Annual Review of Pathology**, 2009 4: 199-227

-Four Meeting Abstracts:

1) ImPact, 2007, Atlanta

MicroRNA profile in prostate cancer cells and response to androgen depletion. Anindya Dutta; Yong Sun Lee; Hak Kyun Kim

2) Forbeck Symposium on microRNAs and cancer, Hilton Head, SC, 2007

MicroRNAs in cell quiescence and cancer . Anindya Dutta

3) AACR meeting on Molecular Diagnostics, Philadelphia, 2008

MicroRNAs and non-micro-short RNAs (nmsRNAs) in cell quiescence and cancer. Anindya Dutta

4) Human Genome Organization, Annual meeting, Hyderabad, 2008

MicroRNAs and non-micro-short RNAs (nmsRNAs) in cell quiescence and cancer. Anindya Dutta

- Data generated used as preliminary data for an R01 application to the National Cancer Institute.

- Dr. Yong Sun Lee was selected after a National Search to be an Assistant Professor of Biochemistry at the University of Texas Medical Branch in Galveston.

-Dr. Hak Kyun Kim obtained his Ph.D. degree from Seoul National University based on his work on this project. He is currently a postdoctoral fellow at Stanford University School of Medicine.

-Mr. Ankit Malhotra will obtain a Ph.D. in Biochemistry from the University of Virginia at least partly on the basis of his work in this project

-Dr. Anindya Dutta gave talks based on this work:

2007: The Forbeck Symposium on microRNAs in Hilton Head, SC,

2008: The Forbeck Scholars Retreat in Lake Geneva, WI

2008: AACR special meeting on microRNAs in cancer in Philadelphia, PA.

## **CONCLUSION:**

The papers that are now going through various phases of peer-review are expected to be published in high impact journals. Discovering that a handful of microRNAs are altered as prostate cancer cells progress has allowed us to now focus in on these to ascertain whether they contribute to the cancer progression. We were very pleased that the microRNAs we identified were also identified as being altered in a similar manner when clinical prostate cancer tissues are examined [1].

The microRNAs repressed by androgens will allow us to ascertain whether this is a new way by which androgens alter the gene expression program in prostate cancer cells.

The discovery of the tRFs was unexpected. These short RNAs are sometimes more abundant than bonafide microRNAs and we have already demonstrated that some of them have biological functions. The biogenesis and mechanism of action of these tRFs are expected to take up a lot of future time in my laboratory and in Dr. Yong Sun Lee's laboratory. Whether they contribute to prostate cancer progression will be an important topic of inquiry.

We are currently embarking on characterizing which genes are targeted by the microRNAs we have identified. We are also beginning phenotypic assays for cell migration and invasion in C4-2 to ascertain whether overexpression or depletion of the altered microRNAs will alter their migration or invasion phenotypes. If changing the microRNAs alter prostate cancer cell behavior, we hope to go to xenograft assays to demonstrate that microRNA alterations can suppress metastasis.

## **REFERENCES:**

1. Mattie, M.D., et al., *Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies*. Mol Cancer, 2006. **5**: p. 24.

**APPENDICES:** Two papers under review and the pdf of a published review

**SUPPORTING DATA:** None

# MicroRNAs in Cancer

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## Key Words

tumorigenesis, oncogene, tumor suppressor, microRNA expression  
profile, diagnosis and prognosis, therapy

## Abstract

Within the past few years, studies on microRNA (miRNA) and cancer have burst onto the scene. Profiling of the miRNome (global miRNA expression levels) has become prevalent, and abundant miRNome data are currently available for various cancers. The pattern of miRNA expression can be correlated with cancer type, stage, and other clinical variables, so miRNA profiling can be used as a tool for cancer diagnosis and prognosis. miRNA expression analyses also suggest oncogenic (or tumor-suppressive) roles of miRNAs. miRNAs play roles in almost all aspects of cancer biology, such as proliferation, apoptosis, invasion/metastasis, and angiogenesis. Given that many miRNAs are deregulated in cancers but have not yet been further studied, it is expected that more miRNAs will emerge as players in the etiology and progression of cancer. Here we also discuss miRNAs as a tool for cancer therapy.

**siRNA:** small interfering RNA

**pre-miRNA:** precursor microRNA

**UTR:** untranslated region of messenger RNA

## SYNOPSIS

Within the past decade, there was a major advance in biology: the discovery of small RNAs, including microRNA (miRNA) and small interfering RNA (siRNA), as highlighted in the December 2002 issue of *Science* (1). Since the discovery of RNA interference (RNAi) in nematodes (2), siRNA has provided a technical breakthrough for short-term genetics in mammalian systems. The big impact of small RNAs was well celebrated by the 2006 Nobel Prize awarded to the two scientists who discovered RNAi.

Moreover, miRNAs shed new light on the posttranscriptional regulation of gene expression. miRNAs were first discovered in worms (3, 4), and they were later observed in a number of animals, plants, and viruses. During the past several years the miRNA field has expanded, with many recent publications implicating miRNAs in diverse cellular processes.

Cancer is a complex genetic disease caused by the accumulation of mutations, which lead to deregulation of gene expression and uncontrolled cell proliferation. Given the wide impact of miRNAs on gene expression, it is not surprising that a number of miRNAs have been implicated in cancer. In this review, we present a comprehensive discussion of the links between miRNA and cancer.

## INTRODUCTION

MicroRNAs (miRNAs) are small noncoding regulatory RNAs ranging in size from 17 to 25 nucleotides (see miRBase, <http://microrna.sanger.ac.uk/>). The definition of miRNAs is based on their generation by the action of Dicer, an RNase that processes hairpin-structured precursors (known as pre-miRNA) into mature miRNAs (5). miRNAs posttranscriptionally repress gene expression by recognizing complementary target sites in the 3' untranslated region (UTR) of target messenger RNAs (mRNAs).

Eight years after the first miRNA was reported by Ambros's and Ruvkun's groups (3, 4) in 1993, the miRNA era began in earnest when

three groups identified tens of small RNAs from *Caenorhabditis elegans*, *Drosophila*, and human (6–8). During the past seven years, the number of miRNAs identified, as well as the number of publications describing them, has expanded enormously.

## Genes, Sequences, and Genomic Organization

Currently, more than 5000 miRNAs from over 50 organisms are registered in the miRBase database (8a). Five hundred thirty-three human miRNAs are now known, but this number may increase, as bioinformatics studies predict that up to 1000 miRNAs exist (9, 10). miRNAs are named as miR- plus numbers (e.g., miR-125), although there are a few exceptions. miRNAs of similar sequence are usually distinguished by an additional letter following the miRNA number (e.g., miR-125b). A miRNA of identical mature sequence may appear at several genomic loci with different precursor sequences. In such a case, the different miRNA genes would be distinguished by the addition of another number at the end of the sequence (e.g., miR-125b-1).

In humans, approximately one-third of miRNAs are organized into clusters. A given cluster is likely to be a single transcriptional unit, suggesting a coordinated regulation of miRNAs in the cluster. In silico analysis has revealed that more than half of the clusters contain two or more miRNAs of similar sequence (11). However, it is very rare for miRNAs of an identical mature sequence to be duplicated in a cluster. This genomic organization confers simultaneous expression of similar miRNAs, possibly leading to combinatorial diversity and synergy in the biological effects. However, all the miRNAs from a single transcriptional cluster are not expressed at equal levels, suggesting that miRNAs are also regulated posttranscriptionally. Also, a significant portion of miRNAs are located in the intronic region of protein-coding or -noncoding transcription units (12), whereas a minor subset of miRNAs are mapped to repetitive sequences such as long interspersed nuclear elements (13).

## Expression

Many miRNAs exhibit characteristic expression patterns. Some miRNAs are differentially expressed in developmental stages, for instance the two founding members *lin-4* and *let-7* in *C. elegans*. For this reason, they were once known as small temporal RNAs because they are expressed in specific temporal phases of development and regulate developmental timing. Many miRNAs are expressed in a tissue-specific manner. In the few cases where a forward genetic approach in worms or flies led to identification of a miRNA, the function of the miRNA was inferred from the phenotype of the mutant. In mammalian cells, however, a miRNA expression profile is usually the first clue to its possible role. Analogous to mRNA expression, miRNA expression is determined by intrinsic cellular factors as well as diverse environmental variables.

Expression of a miRNA can be measured by molecular biology techniques, such as Northern blot, RNase protection assay, and primer extension assay. The small size of miRNAs initially hampered polymerase chain reaction (PCR)-based methods. However, since the development of adaptor-mediated quantitative real-time PCR (qRT-PCR; reviewed in Reference 14), PCR-based techniques have become very popular due to their high sensitivity. Microarray techniques (reviewed in Reference 15) are widely used to comprehensively assay the entire miRNome (the global miRNA expression profile) in tissues or in cell lines (Table 1). In addition to microarray and qRT-PCR, both in situ hybridization (16) and serial analysis of gene expression (SAGE) adapted for small RNAs (17) have been used to obtain miRNomes. Interest in the SAGE approach was stimulated by recent innovations in ultrahigh-throughput sequencing that provide a powerful tool for various genomics studies. Overall, these technical improvements are expected to greatly widen the repertoire of miRNAs in a variety of biological systems.

## Biogenesis

Biogenesis of a miRNA begins with the synthesis of a long transcript known as a pri-miRNA (Figure 1). In general, pri-miRNAs are transcribed by RNA polymerase II and retain mRNA features such as 5' cap structure and 3' poly(A) tail (18, 19). However, other pathways generate in a minor set of miRNAs, especially from genomic repeats. For example, RNA polymerase III is responsible for transcription of miRNAs in Alu repeats (20).

In the nucleus, pri-miRNA is processed to pre-miRNA by RNase III enzyme Drosha and its interacting partner DGCR8 (DiGeorge syndrome critical region gene 8) (21–23). DGCR8 recognizes the stem and the flanking single-stranded RNA (ssRNA) and serves as a ruler for Drosha to cut the stem approximately 11 nucleotides away from the stem-ssRNA junction to release the hairpin-shaped pre-miRNA (24). A subset of miRNAs known as miRtrons bypass the Drosha requirement by taking an alternative pathway, where pre-miRNAs are derived as a byproduct of a splicing event (25–27). The pre-miRNA is exported by exportin-5 to the cytoplasm (28, 29) and is subsequently converted to mature duplex miRNA by another RNase III enzyme, Dicer (30). The two strands of the duplex are separated by a RNA helicase (31) during the specific assembly of a ribonucleoprotein complex containing miRNA (miRNP). As with the strand selection of a siRNA (32, 33), the strand whose 5' end forms the more unstable duplex with its partner seems to preferentially survive as the miRNA in the miRNP (24, 32, 33). However, the detailed molecular events that take place during the miRNP assembly and strand selection are not yet clear.

## Mechanisms of Action

miRNAs posttranscriptionally suppress the target mRNA expression, mostly through interaction with the 3' UTR (Figure 1). However, the exact mechanism for miRNAs on their targets

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### qRT-PCR:

quantitative real-time polymerase chain reaction

**miRNome:** the global microRNA expression profile

**pri-miRNA:** primary transcript of a microRNA

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**Table 1** Global microRNA expression levels (miRNome) analyses in cancers

Tumor type	Profiling method <sup>a</sup>	miRNAs deregulated in tumors		Notes	Reference
		Upregulated	Downregulated		
Breast cancer	Bead			Classification of breast tumors into five subtypes	93
	qRT-PCR, array	miR-21			165
	Array			Classification according to ErbB2/ER status	158
		miR-21	miR-125b, -145	Clinicopathologic features	185
Prostate cancer	Array		<i>let-7c</i> , miR-125b, miR-145		186
					158
Pancreatic cancer	Membrane			Classification according to androgen dependency	66
	Array	miR-21, -221, -222, -181a, -181b, -181d, -155		Chronic pancreatitis and normal tissues	187
		miR-196a	miR-217	Pancreatic ductal adenocarcinoma versus chronic pancreatitis and normal tissues	160
	qRT-PCR of pre-miRNA	<i>let-7i</i> , miR-100, -155, -221, -301, -21, -181a, -125b, -212, -376a	miR-375	Pancreatic adenocarcinoma, paired benign tissue, normal pancreas, chronic pancreatitis	188
Pancreatic tumor	Array	miR-103, -107, -23a, -26b, -342, -192, -204, -211, -21	miR-155	Endocrine tumors versus acinar cell carcinoma	189
Lung cancer	Array	miR-21, -205	miR-126*	Clinicopathological features	190
Ovarian cancer	Array	miR-200a, -141	miR-199a, -140, -145, -125b-1	Clinicopathological features	191
Cervical cancer	Array			Versus Drosha overexpression	95
Uterine leiomyoma	Array	<i>let-7</i> , miR-21, -23b		Clinicopathological features (tumor size, race, etc.)	192
Hepatocellular cancer	Array	miR-21			134
		miR-221	<i>let-7a</i> , miR-122a	Versus liver cirrhosis	193
		miR-224, -18	miR-199a, -199a*, -200a		194
Thyroid cancer	Array		miR-30d, -125b, -26a, and -30a-5p	Anaplastic thyroid carcinomas versus normal tissues	67
		miR-197, -346 in FTC relative to FA		FTC versus FA	195
		miR-221, -222, -181b		Papillary thyroid carcinoma versus normal tissues	159
		miR-221, -222, -146b		Papillary thyroid carcinoma versus normal tissues	100

(Continued)



**Table 1** (Continued)

Tumor type	Profiling method	miRNAs deregulated in tumors		Notes	Reference
		Upregulated	Downregulated		
Colorectal cancer	Array	miR-25, -92 in MSS relative to MSI-H		Microsatellite stability versus high microsatellite instability	196
	qRT-PCR	miR-31, -96, -135b, -183	miR-133b, -145	miR-31 according to cancer stage	197
	Cloning miSAGE				17
Pituitary adenomas	Array	miR-26a, -149	miR-21, -141, -144	Clinicopathological features	198
Neuroblastoma	qRT-PCR			Neuroblastoma subtypes	69
Glioblastoma	Array	miR-221, -10b	miR-128, -181b, -181a		199
Leukemia	Bead	miR-128a, -128b in ALL compared with AML	let-7b, miR-223 in ALL compared with AML	ALL versus AML	200
	Cloning	miR-21, -150, -155	miR-92, -222	Various CLLs	201
	Array		miR-29b, -181b in aggressive CLL with 11q deletion	Three subtypes of CLL	202
			miR-16-1, -15a	CLL according to ZAP-70 and IgVH	203
Leukemia	Array			B cell CLL	65
Various tumors	Array, cloning			Various sarcomas	204
	Array			Solid tumors (lung, breast, stomach, prostate, colon, and pancreatic)	122
	Bead			Comprehensive collection of various cancers and normal tissues	64

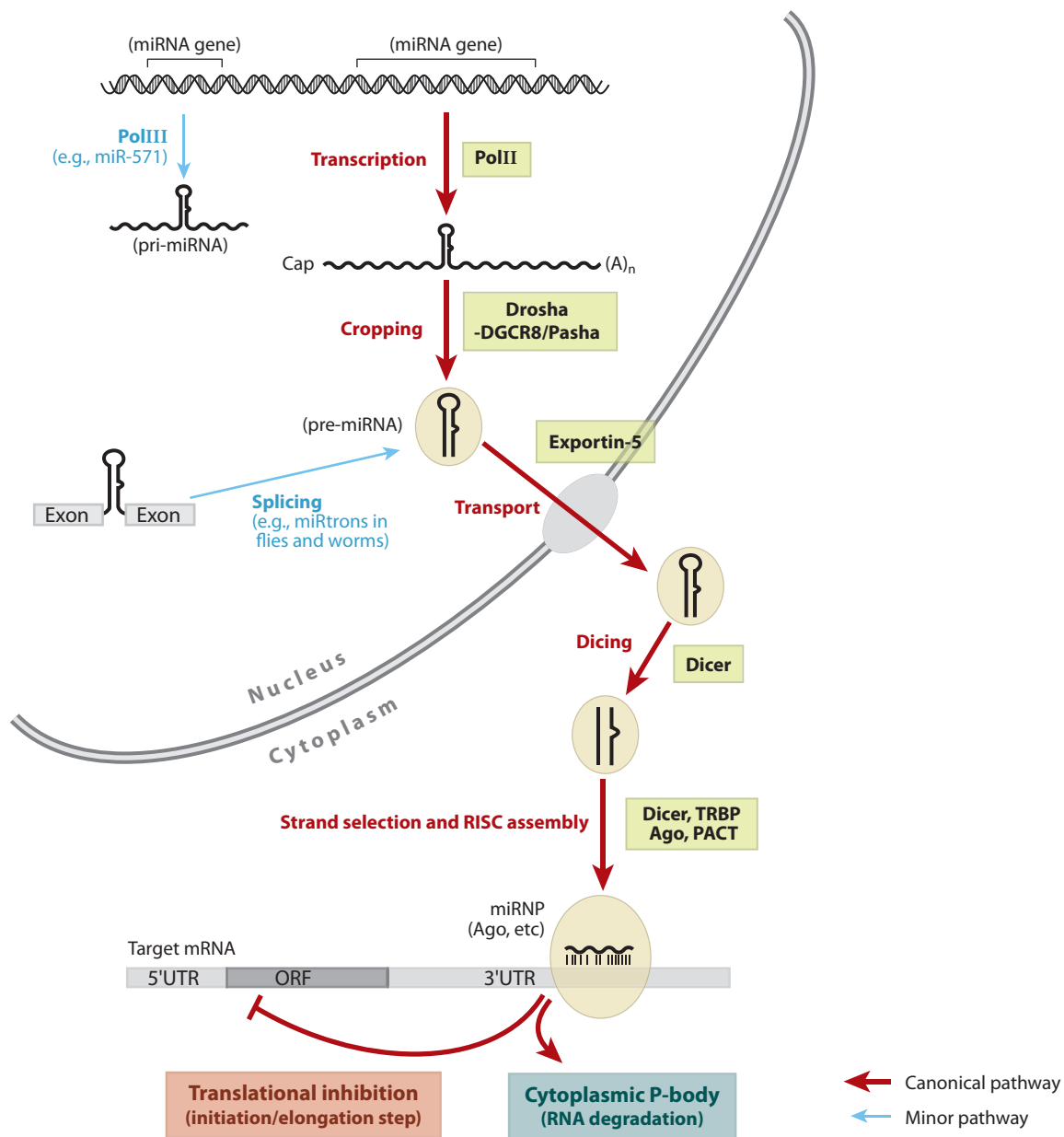
Profiling methods are bead-based flow cytometric profiling (bead), quantitative real-time polymerase chain reaction (qRT-PCR), microarray of miRNA chip (array), membrane spotted array (membrane), cloning, and serial analysis of gene expression of miRNA (miSAGE). miRNAs of notable change are indicated by asterisks.

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; FA, follicular adenoma; FTC, follicular thyroid carcinoma; MSS, microsatellite stability; MSI-H, high microsatellite instability; pre-miRNA, precursor miRNA.

(reviewed in Reference 34) is still controversial. In contrast to the perfect sequence complementarity between siRNA and mRNA, there are mismatches and bulges in most miRNA target sites. Comparison between siRNA and miRNA in earlier papers suggested that siRNA destabilizes mRNA, whereas miRNA inhibits mRNA translation without affecting the mRNA level. Therefore, the degree of complementarity be-

tween short RNA and the target was thought to be a major determinant distinguishing the two mechanisms.

Although the translational repression mechanism still holds true for many miRNAs, it has also been demonstrated (35–38) that a miRNA can decrease the level of a target mRNA despite imperfect sequence complementarity between the miRNA and the target. mRNA



**Figure 1**

Pathways of microRNA (miRNA) biogenesis and action. The canonical pathway (red arrows) and minor pathways (light blue arrows) are depicted. Abbreviations: miRNP, ribonucleoprotein complex containing miRNA; ORF, open reading frame; P-body, mRNA-processing body; PACT, interferon-inducible double-stranded RNA-dependent activator; RISC, RNA-induced silencing complex; TRBP, human immunodeficiency virus–transactivating RNA-binding protein; UTR, untranslated region of mRNA.

degradation by a miRNA, which is distinguished from siRNA-mediated mRNA cleavage, can be explained by mRNA-processing bodies (P-bodies), which are sites for RNA decay (39, 40). Plausibly, miRNAs inhibit translation of target mRNAs, which are then sequestered to P-bodies and become subject to degradation. This model fits well for both mechanisms, although miRNAs have also been proposed to lead to the degradation of the target mRNAs without sequestration to P-bodies (41, 42). In some cases where mRNA translation is inhibited by a miRNA but where mRNA levels remain similar, sequestration to P-bodies might be inefficient. Nonetheless, it is possible that different mechanisms apply to individual miRNA–mRNA interactions.

## Target Prediction and Identification

Prediction of miRNA targets (reviewed in Reference 46) is important, given that miRNAs exert their function by regulating target mRNAs. The specificity of miRNA–mRNA interaction is mainly conferred by the first eight nucleotides of a miRNA (known as a seed sequence) (43). The likelihood that a predicted target is a bona fide target is influenced not only by seed pairing but also by other factors such as the number of target sites, the context of surrounding sequence in mRNA (44), and the occlusion of target sites by RNA-binding proteins (45). Currently, several computational algorithms (reviewed in Reference 46) can predict the target mRNA(s), but they are far from perfect. The gold standard is experimental demonstration that (*a*) a luciferase reporter fused to the 3' UTR of the predicted target is repressed by overexpression of the miRNA and (*b*) this repression is abrogated by point mutation in the target sequence(s) in the 3' UTR. Many targets are predicted by *in silico* analyses, but not all of them are confirmed as real targets in this biological assay.

The *in silico* predictions have been complemented by experimental screenings for targets of miRNAs. Because miRNAs destabilize

mRNAs, miRNA depletion by a knockdown of miRNA-processing machinery is expected to result in upregulation of target mRNA, which can be assayed by microarray analysis. This approach identified *HMG2* (high-mobility group AT-hook 2) as a target of *let-7* (38). Microarrays can also identify mRNAs that are decreased upon transfection of a miRNA, and screening these mRNAs for computationally predicted sites targeted by the miRNA yields some bona fide targets (37). A proteomics approach to identify proteins decreased by miRNAs led to the identification of *tropomyosin 1* as a miR-21 target (47). Another screen employed a library of miRNA-expressing vectors and a sensor plasmid containing green fluorescent protein fused with the 3' UTR of the gene of interest. *p27(Kip1)* was found to be targeted by miR-221 and -222 by this approach (48). Another approach is to isolate the miRNA–target mRNA complex by immunoprecipitation of a component of RNA-induced silencing complex (RISC) followed by microarray hybridization of the precipitated mRNAs (49).

Although a growing number of miRNA–target pairs are being identified (Table 2), the fraction of validated pairs is still small, given that 30% of mRNAs have been predicted to be miRNA targets (50, 51). New target identification will be facilitated by the accumulation of validated miRNA–target pairs, which can be utilized to develop a better prediction algorithm. In addition, experimental tools for high-throughput target screening need to be improved.

## Biological Roles

miRNAs play important roles in cell fate determination, proliferation, and cell death. In addition to these vital processes, miRNAs are implicated in diverse cellular activities, such as immune response (reviewed in References 52 and 53), insulin secretion (54), neurotransmitter synthesis (55), circadian rhythm (56), and viral replication (57). This list will undoubtedly expand as experimental data accumulate.

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**HMG2:** high-mobility group AT-hook 2

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**Table 2** Oncogenic or tumor-suppressive microRNAs (miRNAs) and their direct target genes

miRNA	Target gene	Note	Reference
<i>let-7</i>	RAS	Lung cancer	98
<i>let-7</i>	Ccnd2, Cdk6, Cdc25A	Cancer cell lines (A549, HepG2, HeLa)	59
<i>let-7</i>	HMGA2	Ovarian cancer	132
<i>let-7c</i>	c-Myc	Liver tumors	83
<i>let-7</i>	HMGA2	Lung cancer cell lines	38
<i>let-7g</i>	c-Myc, k-RAS		70
<i>let-7</i>	HMGA2		106
<i>let-7</i>	NF2	Cholangiocarcinoma cell lines	206
miR-9, -125a, -125b	trkC	Neuroblastoma	107
miR-10b	HOXD10	Breast cancer	146
miR-16-1, -15a	Bcl2	Chronic lymphocytic leukemia	140
miR-17-5p	AIB1	Breast cancer	162
miR-17-5p, -20	TβRII		74
miR-18	CTGF	Colon cancer model of angiogenesis	147
miR-19	THBS1	Colon cancer model of angiogenesis	147
miR-20a	E2F1, 2, 3		126
miR-21	Pdcd4	Colorectal cancer	145
miR-21	PTEN	HCC	134
miR-21	TPM1		47
miR-21	PTEN	Cholangiocarcinoma	176
miR-27b	CYP1B1	Breast cancer	207
miR-29a, b, c	DNMT-3A, DNMT-3B	Non-small cell lung cancer	82
miR-29s	Mcl-1	Cholangiocarcinoma cell line	84
miR-29b, -181b	TCL1	Chronic lymphocytic leukemia	202
miR-34a	E2F3	Neuroblastoma	76
miR-34a	Ccne2, MET		62
miR-34a, -34b, -34c	Bcl2	Non-small cell lung cancer	60
miR-106a cluster	Myip, Hipk3, Rbp1-like	T cell leukemia	116
miR-122a	Cyclin G1	HCC versus liver cirrhosis	193
miR-124a	Cdk6	Colon cancer, lung cancer	80
miR-125a, -125b	ERBB2 and ERBB3	Breast cancer	144
miR-125b	Bak-1	Prostate cancer	205
miR-127	Bcl6	Bladder (and prostate) cancer	208
miR-206	ERα	Breast cancer	105
miR-221, -222	p27 (Kip)	Glioblastoma	135
miR-221, -222	p27 (Kip)	Glioblastoma	48
miR-221, -222	p27 (Kip)	Prostate cancer	136
miR-372, -373	LATS2	Testicular germ cell tumor	209
miR-378	Sufu, Fus-1	U87 glioblastoma cell line	148

(Continued)

**Table 2 (Continued)**

miRNA	Target gene	Note	Reference
BART 16, 17-5p, 1-5p (EBV-encoded miRNAs)	LMP1 (EBV-encoded oncogene)	Nasopharyngeal carcinoma	111
KSHV miRNAs miR-K12-1, -K12-3-3p, -K12-6-3p, -K12-11	THBS1		112

Listed for each miRNA is its target gene(s). A directly regulatory connection was demonstrated between each miRNA and target a by a luciferase assay using the 3' untranslated region (UTR) of the mRNA.

Abbreviations: AIB1, amplified in breast cancer 1; BART, *Bam*H1 A rightward transcript; Ccnd2, cyclin D2; Ccne2, cyclin E2; CTGF, connective tissue growth factor; DNMT, DNA methyltransferase; EBV, Epstein-Barr virus; ERBB, v-erb-b2 erythroblastic leukemia viral oncogene homolog; E2F, E2F transcription factor; HCC, hepatocellular cancer; Hipk3, homeodomain-interacting protein kinase 3; HMGA, high-mobility group AT-hook 2; KSHV, Kaposi sarcoma-associated herpesvirus; LATS2, large tumor suppressor, homolog 2; MET, hepatocyte growth factor receptor; Mylip, myosin regulatory light chain-interacting protein; PTEN, phosphatase and tensin homolog; Rbp1-like, retinoblastoma-binding protein 1-like; THBS1, thrombospondin 1; TPM1, tropomyosin 1; trkC, tropomyosin-related kinase C; TβRII, transforming growth factor beta-receptor type II.

miRNA transfection results in up- and downregulation of a number of mRNAs. In several cases, a set of genes belonging to a particular cellular pathway are enriched in the genes whose expression was altered (35, 58–62). The length of the miRNA seed sequence is comparable to that of the consensus sequence of transcription factor-binding elements. Thus, although miRNAs are expected to directly regulate a large set of genes simultaneously, random chance is unlikely to explain the enrichment of genes in a particular pathway in the list of putative targets.

# MicroRNA AND CANCER

## Profiling of MicroRNA Expression in Cancers

miRNAs are aberrantly expressed in a variety of cancers. This was first observed in miR-15a and -16-1, which are clustered at chromosome 13q14, a frequently deleted region in B cell chronic lymphocytic leukemia (CLL) and other cancers (63). Concomitantly, reduction of these two miRNAs was observed in the cancer samples relative to the normal tissues.

miRNome analyses have become easier with the introduction of microarray techniques (Table 1). Many miRNAs are found to be up- or downregulated in the cancer samples

relative to the normal tissue counterparts (Supplemental Table 1; follow the Supplemental Material link from the Annual Reviews home page at <http://www.annualreviews.org>). It is beyond the capacity of this review to describe all the miRNAs that are changed in cancers from profiling data. Instead, in Table 1 we summarize the literature reporting miRNomes in tumors. If a change in miRNA expression has been corroborated by a conventional method (e.g., Northern blot, RNase protection assay, or qRT-PCR), it is indicated in Supplemental Table 1.

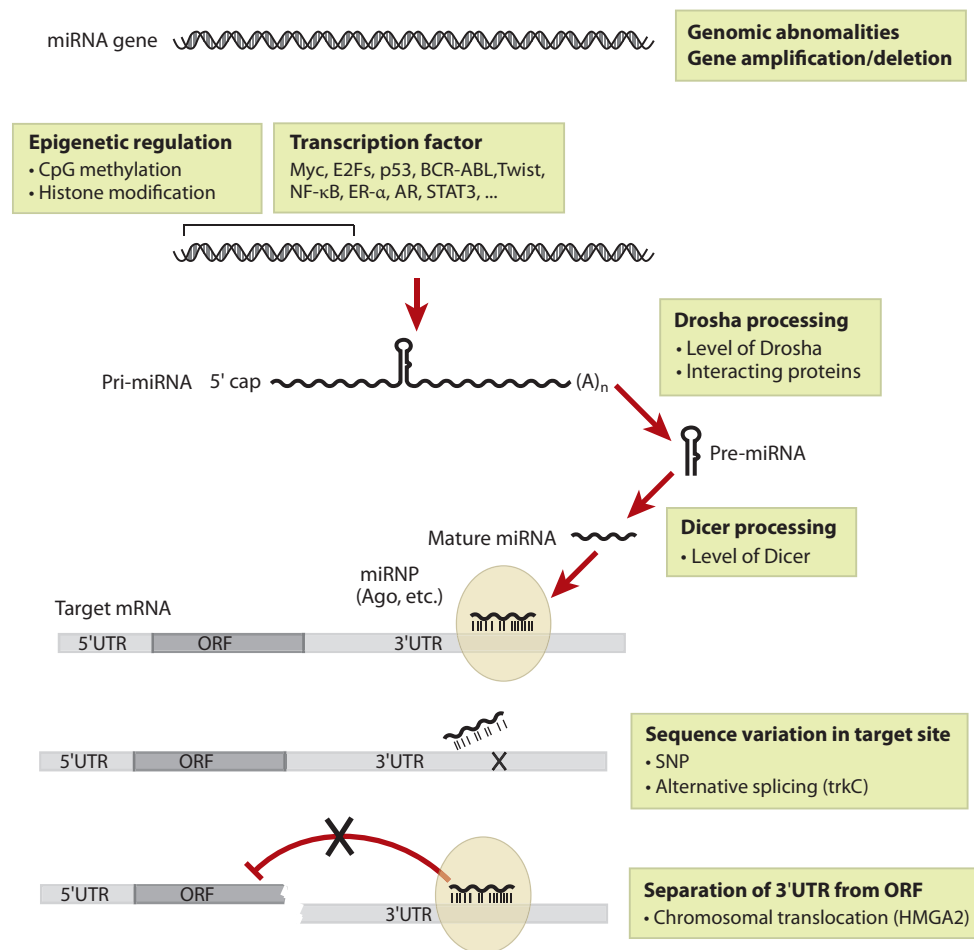
In addition to the distinction of tumors from normal tissue, miRNA expression is characteristic for a cancer type, stage, and other clinical variables. The first systematic analyses of hundreds of cancer samples and normal tissues (64) successfully classified various cancers based on the miRNome. Surprisingly, the miRNome was better at predicting cancer type and stage than the mRNA expression profile; therefore, the miRNome has been proposed as a useful tool for cancer diagnosis and prognosis. For example, clustering of CLL samples according to the miRNome revealed miRNA signatures that correlate with overexpression of *ZAP-70*, a predictor of early disease progression (65). The utility of miRNA in diagnosis is discussed further below.

Although some miRNAs are increased, most are repressed in cancers relative to normal tissue counterparts (64, 66–69). In agreement with these observations, global depletion of miRNAs by knockdown of the miRNA-processing machinery stimulates cell transformation and tumorigenesis in vivo (70). This implies that the miRNA alteration is not simply an end result of tumorigenesis but that it actively contributes to cancer development. Despite the general reduction of miRNAs in cancers, several miRNAs are upregulated, some of which undoubtedly play oncogenic roles.

## Mechanism for MicroRNA Deregulation in Cancers

As described in the previous section, many miRNAs are deregulated in cancers. Aberrant expression of miRNAs can arise through a number of different mechanisms (Figure 2).

**Genomic abnormalities.** As exemplified by miR-15a and -16-1, chromosomal abnormality is one reason for miRNA deregulation in cancers. Tumorigenesis is often accompanied by chromosomal aberrations such as deletion,



**Figure 2**

Various mechanisms for deregulation of microRNA (miRNA) expression. Abbreviations: miRNP, ribonucleoprotein complex containing miRNA; ORF, open reading frame; SNP, single nucleotide polymorphism; UTR, untranslated region.

amplification, and translocation. In silico analysis has revealed that a significant fraction of miRNAs are mapped to these cancer-associated genomic regions or fragile sites in human (71) and in mouse (72). In many cases, miRNA levels are correlated with changes in copy number of the genomic loci, as demonstrated by comparison between array comparative genomic hybridization data and miRNA expression data (66, 73–78).

**Epigenetic factors.** Epigenetic factors can also affect miRNA expression. In many cancers, hypermethylation of CpG islands in promoter regions results in heritable transcriptional silencing of tumor-suppressor genes. Gene silencing by DNA methylation is closely related to histone modification. In silico analyses have indicated CpG islands near dozens of miRNAs (79). In addition, some miRNAs are upregulated (*a*) upon the exposure of cells to the demethylating agent 5-aza-2'-deoxycytidine (79), (*b*) upon mutation of DNA methyltransferases (DNMTs) (80), and (*c*) upon histone deacetylase inhibitor treatment (81). These studies have identified some miRNAs that are repressed by CpG hypermethylation in cancers relative to normal tissue. Representative examples include miR-9-1 in breast cancer

(79) and miR-124a in colorectal tumors (80). In the case of miR-124a, hypermethylation is tumor type specific, as no methylation is seen in neuroblastoma. Also, epigenetic silencing of a miRNA may be a reflection of tissue specificity. For example, miR-124a is normally highly expressed in neuronal tissues, so its epigenetic repression in colorectal tumors is not surprising.

miRNAs may counteract CpG methylation. For example, miR-29 directly targets DNMT-3A and -3B. In agreement with this observation, ectopic expression of miR-29 results in a global reduction of DNA methylation, subsequently leading to a depression of tumor-suppressor genes that had been silenced by promoter methylation in cancer cells (82).

**Transcriptional regulation.** Transcription factors may induce miRNAs by activating the transcription of pri-miRNAs. This mechanism has been well documented in several cases where tissue-specific miRNAs are turned on by transcription factors during differentiation. Given the wide impact of transcription factors on fundamental cellular processes, it is not surprising that many oncogenes or tumor suppressors are transcription factors. Many miRNA–transcription factor relationships have been discovered in cancers (listed in **Table 3**).

**Table 3** MicroRNAs (miRNAs) regulated by transcription factors

Transcription factor	Target miRNA	Note	Reference
AR	miR-125b	Prostate cancer	205
MYCN	miR-17-5p, -92, -320	Neuroblastoma	124
Twist	miR-10b	Breast cancer	146
p53	miR-34a, miR-34b-34c cluster	Cell lines	60
p53	miR-34a	Cell lines	137
p53	miR-34a, miR-34b-34c cluster	Cell lines and in vivo	62
Stat3	miR-21	Myeloma cell lines	91
HIF	miR-26, -210		150
E2F3	miR-17-92 cluster		125
E2F1, 2, 3	miR-17-92 cluster		126
Myc	miR-17-92 cluster	Angiogenesis in colon cancer model	147

Listed are miRNAs whose promoter regions were analyzed by a luciferase reporter assay and were shown to bind to the transcription factor by chromatin immunoprecipitation assay.

Abbreviations: AR, androgen receptor; E2F, E2F transcription factor; HIF, hypoxia-inducing factor; MYCN, Myc myelocytomatosis viral–related oncogene, neuroblastoma derived; Stat3, signal transducer and activator of transcription 3.



We discuss three of these—p53, c-Myc, and E2F—in detail below.

### Regulation at microRNA processing steps.

The steady-state level of a mature miRNA is determined not only by the transcription rate of pri-miRNA but also by the processing efficiency of its precursors and by its stability. miRNAs often exhibit a discrepancy in expression of the mature form relative to that of the precursor (83–88). Although miRNAs in a genomic cluster are usually expressed from a common pri-miRNA, the levels of individual miRNAs in the cluster are not necessarily coordinated (89, 90). A time-course experiment after induction of pri-miR-21 revealed delayed kinetics in accumulation of mature miR-21 (91). Collectively, these observations indicate that miRNA processing and stability are important factors that determine miRNA expression level.

This mechanism has been confirmed in cancers by a comprehensive analysis of expression data (92). As stated above, miRNAs are generally reduced in cancers relative to the normal tissues. When a miRNA resides within a gene, the host gene can be regarded as the pri-miRNA. Comparison of microarray data between mRNA and miRNA revealed that the miRNA reduction in cancers is poorly correlated with a reduction of the host gene expression. Aberrant expression of miRNAs during tumorigenesis is presumably often due to alterations at posttranscriptional steps.

The expression levels of Dicer or Drosha are altered in a number of cancers (70, 93–96). Drosha upregulation is seen in more than half of cervical squamous cell carcinoma (SCC) specimens and is likely due to the copy number gain at chromosome 5p, where the *Drosha* gene is located (95). Hierarchical clustering of miRNA expression data successfully classified cervical SCC samples into two groups according to Drosha overexpression. Notably, some miRNAs were reduced upon Drosha overexpression, indicating that individual miRNAs respond differentially to an elevation of the miRNA processing machinery. Interestingly,

Drosha was reported to interact with an oncogenic fusion protein derived from a chromosomal translocation in some leukemias (97). This interaction affects pri-miRNA selection of Drosha and, as a result, influences miRNA expression patterns.

### Consequence of Aberrant MicroRNA Expression in Cancers

miRNAs regulate the expression of their target mRNAs, so over- or underexpression of miRNAs is expected to result in down- or up-regulation, respectively, of the protein product of the target mRNAs. It is easy to implicate a miRNA in a cancer if a direct target of a miRNA is an oncogene or a tumor suppressor. Since the publication of a study showing that *let-7* miRNA directly regulates *RAS* oncogene (98), a number of other miRNA–target pairs have been studied. However, the number of experimentally validated pairs is still small relative to (a) the number of miRNAs shown to be aberrantly expressed in tumors and (b) the number of in silico predicted pairs, mostly because current target prediction algorithms are not very accurate. A current list of miRNA–target pairs implicated in various cancers is given in **Table 2**, and important examples are discussed below.

If we compare global gene expression profiles in cancers and normal tissues, we find that many miRNAs and mRNAs are deregulated. Therefore, it is plausible that tumorigenesis (or at least the progression of a cancer) results from changes in the entire miRNome, rather than from the change of a single miRNA that regulates an oncogenic (or tumor-suppressive) target gene.

Some miRNAs appear to be deregulated in cancers much more frequently than others (**Supplemental Table 1**). These miRNAs may play key roles during tumorigenesis. For example, the miR-17-92 cluster and miR-155 have been shown experimentally to be bona fide oncogenes, as their ectopic expression accelerates tumor development. These two miRNAs are discussed in detail in separate sections.



## Aberrant Action of MicroRNAs Without Alteration of MicroRNA Expression Level

The function of protein-coding genes is rendered abnormal by point mutations, which either transform proto-oncogenes to oncogenes or abrogate functions of tumor-suppressor genes. In theory, the same mechanism of activation/inactivation may apply to miRNAs. However, mutation in mature miRNA sequence seems to be a rare event (72, 99–101), presumably due to the small size of the mature miRNA. In addition, miRNAs are thought to be more tolerant of point mutations or of single nucleotide insertion/deletion mutations than of protein-coding genes.

In contrast, sequence variation in miRNA target sites may occur and may play a role in cancer. In silico analyses of expressed sequence tag and single nucleotide polymorphism (SNP) databases indicate different allele frequencies of miRNA-binding sites in cancers versus normal tissues (102). Several experiments have shown that sequence polymorphisms in miRNA target sites affect miRNA–mRNA interaction and are implicated in disease phenotypes (100, 103–105) (**Figure 2**).

An interesting illustration of this mechanism can be found in *let-7* and its direct target oncogene, *HMG2* (38, 106). Chromosomal rearrangements at the *HMG2* locus in several tumors separate the open reading frame (ORF) from the 3' UTR that contains *let-7* target sites. As a result, *HMG2* escapes from suppression by *let-7*, is overexpressed, and promotes tumorigenesis (**Figure 2**).

An alternative splicing event may result in a different 3' UTR that displays different miRNA target sites, as exemplified in the targeting of *tropomyosin-related kinase C* by miR-9, -125a, and -125b. One mRNA isoform encodes a truncated ORF that is functionally dominant negative to the intact protein. In this isoform, the 3' UTR contains the target sites of these miRNAs. In contrast, the target sites are absent in another isoform encoding the intact ORF; only the former isoform was repressed by the

miRNAs (107). Although the stop codon is usually located in the last exon, generation of different 3' UTRs by multiple polyadenylation sites or alternative splicing has been known to occur in a small but significant fraction of genes (108). Thus, variation of 3' UTR and of attendant miRNA target sites is expected to be a mechanism for oncogene activation or tumor-suppressor inactivation (**Figure 2**).

Recently, Steitz and colleagues (109) reported that miRNAs activate the translation of the target mRNA in cells arrested at the G<sub>0</sub>/G<sub>1</sub> stage. In addition to aberrant miRNA expression, the switch from repression to activation should be considered in studying the role of miRNAs in differentiation or tumorigenesis, as the same miRNA may exert opposite effects in resting cells and proliferating cancer cells in a given tissue.

## Viruses and MicroRNAs in Tumorigenesis

Several viruses regulate tumorigenesis by expressing viral oncogenes or by activating cellular oncogenes through integration of viral DNA into genomic loci. Both mechanisms are applicable to oncogenic miRNAs. For example, Epstein-Barr virus (EBV)-encoded miRNAs (110) directly target a viral oncogene, *LMP1*, whose overexpression is deleterious to host cells. EBV-encoded miRNAs are thought to enhance EBV-mediated cellular transformation by adjusting LMP1 level to a sublethal dose (111). Another study showed that a viral miRNA repressed a host tumor-suppressor gene. In this study, miRNAs from Kaposi sarcoma-associated herpesvirus (KSHV) directly targeted an antiangiogenic factor, *thrombospondin-1* (*Tsp-1*) (112). Thus viral miRNAs act as viral oncogenes.

Viral integration near miRNA loci may lead to aberrant expression of miRNAs. Indeed, the *BIC* locus harboring miR-155 was originally described as a frequent integration site in virally induced lymphomas (113). Viral integration sites are often mapped to miRNA loci (114) such as the miR-17-92 cluster (115), the

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**KSHV:** Kaposi sarcoma-associated herpesvirus

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miR-106a-363 cluster (116), the miR-29a-29b-1 cluster (117), and the miR-106a cluster (118).

## Various Cancer Types

Comprehensive miRNA profiles have been reported in clinical specimens from various cancers (Table 1). Consistent with the notion that miRNAs are expressed in a tissue-specific manner, miRNAs differ among cancers from various tissue origins. Also, a few miRNAs appear to be frequently deregulated in many cancers (Supplemental Table 1), suggesting that these miRNAs regulate fundamental processes such as cell proliferation and apoptosis.

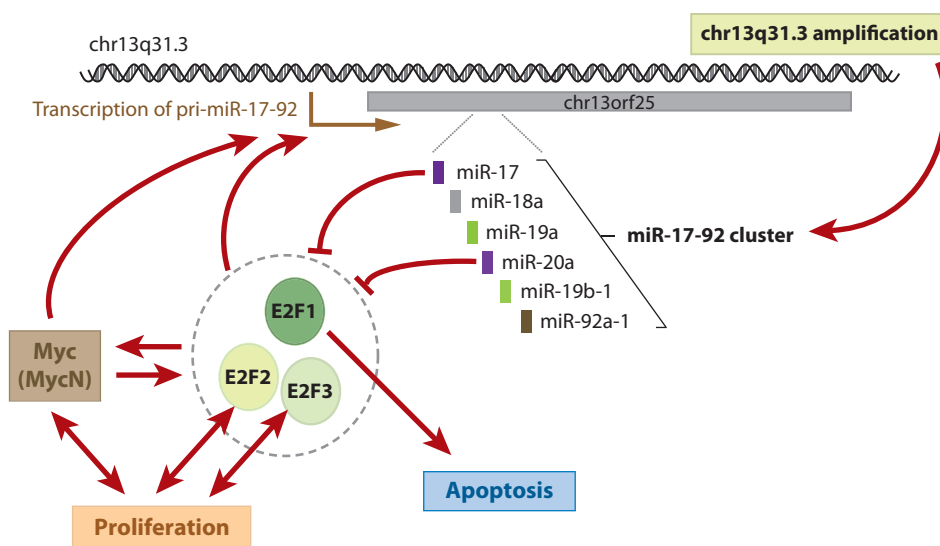
## Oncogenic or Tumor-Suppressive MicroRNAs

**miR-17-92 cluster.** The miR-17-92 cluster, located at chromosome 13q31.3 in humans, is composed of six miRNAs (mir-17, -18a, -19a, -20a, -19b-1, and -92a-1). There exists a similar cluster at chromosome 10 known as miR-106a-363, which also contains six miRNAs (mir-106a, -18b, -20b, -19b-2, -92a-2, and -363). The miR-17-92 cluster contains the

first miRNAs demonstrated to be oncogenic. The development of B cell lymphoma is significantly accelerated by the forced expression of the miR-17-92 cluster in transgenic mice overexpressing the *c-myc* oncogene (119).

Consistent with its oncogenic role, the miR-17-92 cluster is upregulated in a variety of cancers including lymphomas (74, 75, 119, 120), lung cancers (77, 121), and others (122). There appear to be two mechanisms for upregulation of this cluster in cancers (Figure 3). One is the amplification of the chromosome 13q31 locus in several lymphomas and other cancers (74, 77, 78, 120). The other is transcriptional activation of the pri-miRNA, wherein an oncogenic transcription factor c-Myc binds the genomic locus upstream of the miR-17-92 cluster and activates its expression (123). In neuroblastoma cells, MYCN, a protein highly homologous to c-Myc, appears to activate this cluster instead of c-Myc (124). E2Fs also activate this cluster (125, 126) (Table 3).

Given that the E2Fs are direct targets of miR-17 and -20a, the miR-17-92 cluster constitutes a complex regulatory network with c-Myc and E2Fs. E2F1 and c-Myc are known to activate each other to form a positive feedback loop



**Figure 3**

The miR-17-92 pathway.

(**Figure 3**). All of the E2Fs (E2F1, -2, and -3) are capable of activating the miR-17-92 cluster and are subject to repression by miR-17 and -20a from this cluster (**Table 2**). However, degrees of activation and repression vary between individual pairs of miRNA and E2F family members. In general, E2Fs are thought to be proliferative, but E2F1 is also proapoptotic. Although this network is too complicated to describe in a straightforward manner, the miR-17-92 cluster is clearly responsible for fine-tuning the regulatory network. Upon a proliferative cue, c-Myc and E2Fs turn on the miR-17-92 cluster. This cluster represses E2Fs, thereby preventing the uncontrolled amplification of the positive feedback loop between E2Fs and c-Myc. In addition, repression of E2F1 by the miRNAs may help minimize the proapoptotic potential of E2F1. This network is depicted in **Figure 3**.

**miR-21.** miR-21 is upregulated in almost all kinds of cancers (**Supplemental Table 1**). miR-21 is transcriptionally activated by signal transducer and activator of transcription 3 (Stat3) in the interleukin-6 signaling pathway (91) (**Table 3**). miR-21's role in invasion and metastasis is well characterized and is discussed further below.

**miR-155.** As with the miR-17-92 cluster, the oncogenic potential of miR-155 has been demonstrated by transgenic mice expressing miR-155 in B cells (127). The primary transcript for miR-155 is the *BIC* gene, which was originally known as a common viral integration site in lymphomas in chicken. High expression of miR-155 (*BIC*) is reported in various B cell malignancies (128–130) (**Supplemental Table 1**). miR-155 seems to be regulated at multiple levels. miR-155 (*BIC*) appears to be regulated by nuclear factor- $\kappa$ B (NF- $\kappa$ B) through the B cell receptor-mediated signaling pathway (85) or through the Toll-like receptor-activated signaling pathway (131). However, a discrepancy between the levels of miR-155 and *BIC* suggests that miR-155 may also be regulated during processing steps

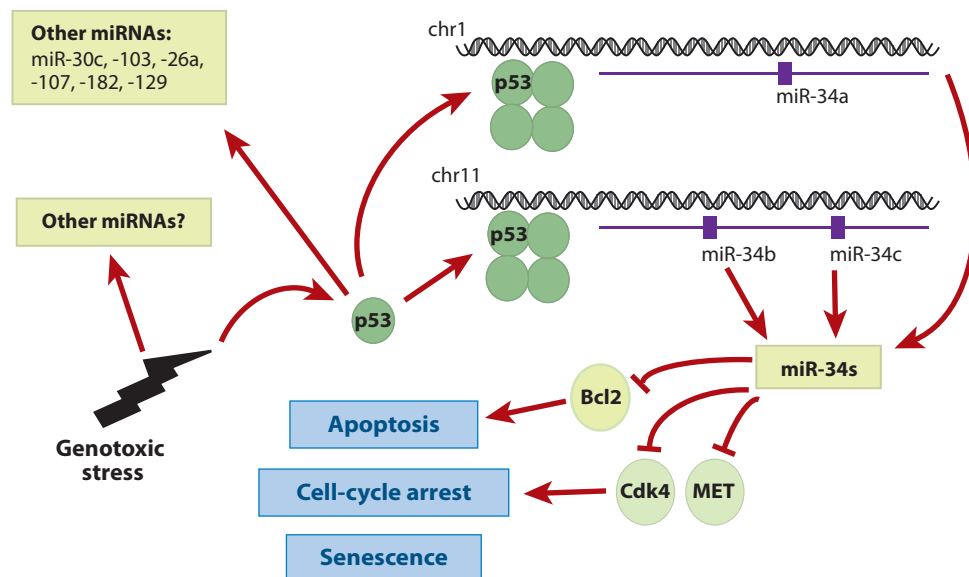
(129). For example, the induction of *BIC* does not lead to upregulation of miR-155 in several Burkitt lymphoma cell lines. In contrast, miR-155 is concordantly increased upon the induction of *BIC* in Raji, an EBV-latency type III-positive Burkitt lymphoma cell line (85).

**let-7.** *let-7* is one of the earliest-discovered miRNAs. In human, there are 12 paralogous *let-7*s. *let-7* is almost absent during embryonic stages or tissues, whereas high expression of *let-7* is seen in most differentiated tissues. The reduction of *let-7* in cancers is reminiscent of *let-7* expression during development in that it is most decreased in less-differentiated, advanced stages of cancer cells with mesenchymal characters (132).

*let-7* is probably more abundant than any other miRNA, so reduction of *let-7* may have a prominent effect on cell physiology compared to other miRNAs. To support this hypothesis, enhanced cellular transformation by global miRNA depletion was largely recapitulated by inhibition of *let-7* alone (70). Well-known oncogenes such as *RAS*, *c-Myc*, and *HMG2* are validated as direct targets of *let-7* (**Table 2**). Hence, *let-7* is an important tumor suppressor.

**miR-34s (miR-34a and miR-34b/-34c clusters).** The miR-34s recently acquired notoriety because they are induced by p53 (**Figure 4**). Consistent with p53's role as a tumor suppressor, miR-34s are downregulated in several tumors such as non-small cell lung cancers (60) and pancreatic cancers (61) (**Supplemental Table 1**). However, reduction of the miR-34s is not always correlated with p53 loss, suggesting a p53-independent mechanism of miR-34 reduction in some cancers. In fact, miR-34a is located in 1p36, a locus that is frequently deleted in a number of cancers (76). We return to our discussion of p53 regulation and miR-34s' roles below.

Although the miR-34s are thought to be tumor suppressors, they have been found to be upregulated in several cancers including renal cell carcinoma (133), colon cancer (58), and



**Figure 4**

The p53 and miR-34 pathway. Abbreviation: MET, hepatocyte growth factor receptor.

hepatocellular carcinoma (134) (**Supplemental Table 1**). The role of high miR-34 and p53 status in these tumors awaits investigation.

## Aspects of Cancer Biology Regulated by MicroRNAs

**Cell cycle.** Cell-cycle regulators often act as oncogenes or as tumor suppressors. The best-characterized example is the cell-cycle inhibitor p27(Kip1). p27(Kip1) is a tumor suppressor, as indicated by its low levels in some cancers. In addition, p27(Kip1) mutation predisposes cells to tumorigenesis upon exposure to carcinogens. p27(Kip1) binds to Cdk2–cyclin E and prevents G<sub>1</sub>-to-S transition. p27(Kip1) is a direct target of miR-221 and -222 in glioblastomas (48, 135) and in prostate cancer cells (136). In these cancer cells, p27(Kip1) is anticorrelated with miR-221 and -222. Targeting p27(Kip1) is responsible for the proproliferative role of these miRNAs, as artificial knockdown of p27(Kip1) mirrors the phenotype of these miRNAs. miR-221 and -222 are overexpressed in other cancers (see **Supplemental Table 1**), suggesting that they play a role in a wide range of cancers.

In addition to regulating p27(Kip1), miRNAs regulate other cell-cycle proteins including Cdk6; Cdc25A; Ccnd2 (cyclin D2) (59); Cdk4 (62), a Rb-family protein (89); and p180 of DNA polymerase  $\alpha$  (37). A number of miRNAs have been shown to perturb normal cell cycle when overexpressed or inhibited (37, 48, 62, 70, 137–139) (**Supplemental Table 2**). However, oscillation of a miRNA during the normal cell cycle has not yet been reported.

**Programmed cell death.** Apoptosis is an active process controlled by a gene expression program that varies depending on the biological context. Because a balance between proliferation and apoptosis is essential for tissue homeostasis and proper differentiation, aberrant apoptosis may give rise to tumors. miRNAs participate in tumorigenesis by directly targeting antiapoptotic genes. Representative examples include the repression of antiapoptotic genes *Mcl-1* and *Bcl-2* by miR-29b (84) and by miR-34s (60), -15a, and -16 (140), respectively (**Table 2**). The loss of these miRNAs due to mutation of p53 or deletion of chromosome 13q14 leads to an increase in

the antiapoptotic gene expression and persistence of tumor cells that would normally have been removed by apoptosis. It is very likely that miRNAs target other genes in the apoptotic pathway, as transfection or expression of a number of miRNAs is associated with apoptosis (69, 76, 121) (**Supplemental Table 2**).

**p53.** p53, a sequence-specific transcription factor, is known as the guardian of the genome owing to its critical role in regulation of the cell cycle and apoptosis in the face of genomic damage. Genotoxic stress and oncogene activation activate p53 to modulate the transcription of several target genes. p53 is the most extensively studied tumor suppressor and its importance is underscored by mutation of p53 in almost 50% of human cancers.

miRNA profiling after p53 induction indicated miR-34a, -34b, and -34c (collectively, the miR-34s) as the most upregulated miRNAs (61, 62, 137, 141) (**Figure 4**). These miRNAs are induced after genotoxic stress in a p53-dependent manner in vitro and in vivo (62, 141). miR-34b and -34c are clustered at chromosome 11, whereas miR-34a is located in a separate genomic locus. Both pri-miRNAs are directly activated by p53. The miR-34s seem to be critical downstream effectors of p53, as ectopic expression of the miR-34s recapitulate the phenotype of p53 activation. The miR-34s promote cell-cycle arrest, apoptosis, and senescence (58, 60–62, 137, 141) (**Supplemental Table 2**). These effects are explained by the repression of several direct targets of the miR-34s such as *Bcl-2* (60), *Cdk4*, and *hepatocyte growth factor receptor* (62) (**Table 2**).

In addition to the miR-34s, other miRNAs may be important in the p53 pathway. miR-30c, -103, -26a, -107, and -182 were induced clearly, albeit less robustly, upon DNA damage in a p53-dependent manner (61). miR-26a expression was also shown to be dependent on p53 (142). In another approach, searching for p53-binding elements in DNA sequence near miRNAs identified miR-129 as a good candidate for regulation by p53 (143).

**Invasion and metastasis.** Features of malignant tumors, distinct from benign tumors, include invasion and metastasis. Malignant tumors are fatal, mostly due to their capacity to invade neighboring tissues and metastasize through the bloodstream to distant organs. An effect of miRNAs on invasion and migration has been reported (**Supplemental Table 2**). Ectopic expression of miR-125 impairs cell motility and invasion in a breast cancer cell line (144), and reduction of global miRNA expression enhances migration of cells (70).

These sporadic in vitro observations were followed by more detailed studies on miR-10b and miR-21. miR-21 is one of the most frequently upregulated miRNAs in cancers (**Supplemental Table 1**). It promotes cell motility and invasion by directly targeting *PTEN* (*phosphatase and tensin homolog*), a tumor suppressor known to inhibit cell invasion by blocking the expression of several matrix metalloproteases (134). Another pathway was recently reported in colorectal cancers, where miR-21 promotes invasion, intravasation, and metastasis by downregulating *Pdcd4* (145).

miR-10b is the other miRNA implicated in metastasis. In metastatic breast cancer cells, miR-10b is upregulated, presumably as a result of transcriptional activation by a transcription factor, Twist. Ectopic expression of miR-10b promotes invasion, intravasation, and metastasis in otherwise noninvasive or nonmetastatic breast cancer cell lines. miR-10b directly targets *HOXD10*, whose reduction induces the expression of a well-characterized prometastatic gene, *RhoC* (146).

**Angiogenesis.** Recruiting blood vasculature is crucial to the survival of neoplastic cells. So far, the miR-17-92 cluster has been characterized in this process. The stimulation of neovascularization by c-Myc involves a downregulation of antiangiogenic factor *Tsp-1*. c-Myc represses *Tsp-1* and a related protein, *connective tissue growth factor* (*CTGF*) by activating the miR-17-92 cluster. *Tsp-1* and *CTGF* appear to be direct targets of miR-19 and -18, respectively. Ectopic expression of the miR-17-92 cluster is sufficient



for promoting angiogenesis (147). A recent observation indicates that other miRNAs—miR-378 and -27a—may play a role in angiogenesis (139, 148) (**Supplemental Table 2**). Viral miRNAs may also play a role in angiogenesis, as *Tsp-1* has been shown to be a direct target of KSHV miRNAs (112).

**Others.** The microenvironment inside a solid tumor is usually hypoxic. Tumor cells under hypoxia tend to be resistant to therapies and to have a poor prognosis. Upregulation of certain miRNAs under hypoxia (149, 150) (**Table 3**) suggests that these miRNAs may influence the phenotype of hypoxic tumor cells. Cancers are predisposed by other external factors, such as genotoxic stress, a folate-deficient diet, and exposure to arsenic. There are a few reports of miRNA profiles under these conditions (151, 152), but whether miRNA changes are responsible for the cancer predisposition remains to be investigated.

## Clinical Applications

**Diagnostic tools.** miRNAs may be used as diagnostic or prognostic tools, as miRNA expression profiles reflect tumor origin, stage, and other pathological variables. miRNAs can function as accurate molecular markers because they are relatively stable and resistant to RNase degradation—probably because of their small size (35, 153, 154). Researchers have shown that miRNAs can be isolated and quantitated from formalin-fixed paraffin-embedded (FFPE) specimens. qRT-PCR and microarray data were reliably and reproducibly obtained from FFPE samples that had been routinely processed and stored for 10 years. The data from FFPE samples are consistent with those from frozen samples (155, 156).

The development of qRT-PCR methods has improved the sensitivity of miRNA detection down to a few nanograms of total RNA (68, 157, 158). This amount can easily be obtained by fine-needle aspiration biopsies (FNABs); in fact, there has been a report of successful

miRNA measurement by qRT-PCR on FNAB samples (159).

miRNA markers that could be used for cancer diagnosis are becoming available. For example, miR-196a is high in pancreatic ductal adenocarcinoma (PDAC) but low in normal tissues and chronic pancreatitis. miR-217 exhibits the opposite expression pattern (**Supplemental Table 1**). Thus, the ratio of miR-196a to miR-217, calculated by qRT-PCR measurement of the two miRNAs from a tiny amount of total RNA, indicates whether the sample contains PDAC (160). Once reliable indicator miRNAs are chosen, they will likely yield easy and accurate tools for cancer diagnosis.

**Cancer therapeutic tool.** A number of miRNAs affect the growth of cancer cells in vitro and in vivo when overexpressed or inhibited. Therefore, cancer cell growth can be controlled by manipulating miRNAs. Overexpression or inhibition of miRNAs can be achieved in several ways. Synthetic miRNA mimics include siRNA-like oligoribonucleotide duplex (161) and chemically modified oligoribonucleotide (162). Conversely, miRNAs can be inhibited by variously modified antisense oligonucleotides such as 2'-O-methyl antisense oligonucleotide, antagomirs, and so on (reviewed in Reference 163). As the first successful tool for knock-down of a miRNA in vivo, antagomirs are of special interest (164). Antagomirs appear to be delivered to all tissues (except brain) after tail-vein injections into mice. The therapeutic value of an antagomir would be greatly enhanced by technical improvements for selective tumor-specific or tissue-specific delivery.

Synthetic oligonucleotides are effective in vivo for at most a couple of weeks, as has been demonstrated by experiments involving cancer cells engrafted in mice (165) and tail-vein injection to mice (166). To circumvent this limitation, miRNAs can be stably expressed through transcription of hairpin RNA from plasmid vector (reviewed in Reference 167). Recently, artificial overexpression of a miRNA

target sequence was shown to inhibit the miRNA function, presumably by titrating the miRNA away from endogenous targets (168, 169). Thus it should be possible to apply such competitive inhibitors for long-term sequestration of a miRNA.

Nonspecific side effects are as important as effectiveness and duration of miRNA expression (or inhibition). Some investigators have argued that miRNA mimics or inhibitors are specific enough to distinguish between similar miRNAs (170, 171). However, cross-reactivity between miRNAs of similar sequence is likely to be unavoidable at high doses of antagonists or agonists. Another possible side effect is that high expression of miRNA mimics may interfere with endogenous miRNA action by saturating the cellular machinery for miRNA processing or action. This may result in a change in expression of other miRNAs, leading to a deleterious effect in the cells. Indeed, a fatal side effect as a result of saturation of miRNA pathway has been reported (172). To minimize undesirable side effects, the expression or knock-down of a miRNA should be improved so that it is more accurate and controllable. An alternate approach to improving specificity is to target the pre-miRNAs with antisense or siRNA strategies (173, 174).

Some chemical compounds alter expression of a group of miRNAs (81); therefore, it may be possible to screen for drugs that could shift the miRNome in a cancer cell toward that of the normal tissue. NCI-60 cancer cell lines may serve as a platform for this screening, as their miRNA expression profiles have been published (68, 175). By modulating multiple miRNAs simultaneously, such a miRNome-modifying approach may be much more effective for therapy than strategies that aim to regulate a single miRNA.

miRNAs affect the drug sensitivity of a cell (165, 176) (**Supplemental Table 2**). Expression or inhibition of a miRNA can therefore be combined with treatment of a drug or other cytotoxic therapy. One example is miR-21 inhi-

bition together with a secreted form of tumor necrosis factor–related apoptosis–inducing ligand, which results in a complete eradication of glioblastoma cells (177).

Collectively, preliminary results suggest that miRNAs could be useful for cancer therapy. However, there is still a significant gap between basic research on miRNAs and clinical application. Extensive preclinical and translational research is necessary to increase the efficacy and decrease the side effects of miRNAs *in vivo*. In parallel, we need to expand our knowledge of the interactions between miRNAs and gene expression programs and how these interactions are altered in tumorigenesis.

## CONCLUDING REMARKS

The discovery of miRNAs has added another dimension to the study of the regulation of gene expression. In addition to transcriptional regulation, posttranscriptional repression by miRNAs may act like rheostats for fine-tuning of gene expression. miRNAs at end stages of differentiation may contribute to keeping cells differentiated by suppressing a large number of genes simultaneously.

In recent years, there has been an explosion of publications on miRNAs. Many of these have emphasized the role of miRNAs in cancer biology. miRNAs play a role in almost all aspects of cancer biology, including proliferation, apoptosis, invasion/metastasis, and angiogenesis. New technical developments are enabling investigators to describe a variety of cancer miRNomes. Therefore, more miRNAs are expected to be identified as oncogenes or as tumor suppressors. Characterization of individual miRNA pathways will be greatly facilitated by an improvement of target prediction algorithms.

miRNA expression is associated with clinical variables of cancers, so miRNAs can be readily used as tools for cancer diagnosis and prognosis. For therapeutic benefit, the methods to manipulate miRNAs *in vivo* should become more robust.

## SUMMARY POINTS

1. miRNAs exhibit an expression pattern characteristic of tumor type, stage, or other clinical variables. Thus miRNAs can be used for cancer diagnosis and prognosis.
2. miRNAs exert an active role in the etiology or progression of cancers by regulating their direct-target oncogenes or tumor-suppressor genes.
3. miRNAs play roles in diverse aspects of cancer such as proliferation, apoptosis, invasion/metastasis, and angiogenesis.
4. Artificial expression/inhibition of a miRNA can be therapeutic.

## APPENDIX

### Other Small RNAs?

In addition to siRNAs and miRNAs, other types of small RNA have been reported in yeast, *Drosophila*, *C. elegans*, and plants. These small RNAs include tiny noncoding RNAs, repeat associated small interfering RNAs, scan RNAs, and others (reviewed in Reference 178). These RNAs are thought to be related to miRNA and siRNA, but are distinct from them. Recently, Piwi-interacting RNA, another small RNA that is slightly larger than miRNA, was discovered from mammalian testes. Other classes of small RNA may exist but have yet to be discovered, given that a significant portion of transcripts are noncoding RNA. Whereas protein-coding genes comprise only 1–2% of the human genome, a significant fraction (~15%) of the human genome is transcribed; these transcripts remain the “dark matter” of the genome (179, 180). The discovery of noncoding RNAs is expected to be greatly accelerated by the widespread adaptation of high-throughput deep-sequencing techniques.

### Other Diseases

miRNAs (or miRNA machinery) have been implicated in diseases other than cancer (182), for example Tourette’s syndrome, fragile X syndrome (181), DiGeorge syndrome (23), myotonic dystrophy, spinocerebellar ataxia type 3 (182), and schizophrenia and schizoaffective disorder (183). Of particular interest is myotonic dystrophy type 1, which is characterized by expansion of CTG repeat in the 3’ UTR of the *dmpk* gene. Tandem CAG sequences are found in some miRNA seed sequences and thus are able to interact with the CTG repeats, raising the possibility that these miRNAs may be involved in DM1 pathogenesis (184).

## DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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62. miR-34s are regulated by the tumor suppressor p53.

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63. The first paper on the correlation between miRNAs and cancer.

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64. The first comprehensive miRNome analysis of hundreds of tissue and cancer samples; it revealed that the miRNA expression profile is better at classifying cancers than the mRNA expression profile.

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71. First genome-wide analysis of miRNA loci; it revealed the correlation between microRNA and cancer more comprehensively.

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## Errata

An online log of corrections to *Annual Review of Pathology, Mechanisms of Disease* articles may be found at <http://pathol.annualreviews.org>

**MicroRNAs in the androgen-independence and androgen regulation of prostate epithelial cell lines.**

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## **Abstract**

A systematic comparison of the short RNA universe of two prostate cancer cell lines was performed by cloning and deep sequencing to identify microRNAs whose expression may correlate with androgen dependence/independence or microRNAs that are regulated by androgens. The results were confirmed by two independent methods of quantitating microRNAs: locked nucleic acid microarrays, and microRNA-specific qPCR/RNase protection/Northern assays. Changes that were consistently detected by all three methods are the decrease of five microRNAs and increase of three microRNAs in the androgen-independent C4-2 cell line relative to its androgen-dependent predecessor, LNCaP. Ectopic expression of the down-regulated microRNAs decreased the growth of C4-2 in the absence of androgen but not in its presence, suggesting that microRNA changes contribute to the androgen-independence. All five downregulated microRNAs have been reported to be downregulated in clinically advanced prostate cancer, suggesting that they constitute a microRNA signature of prostate cancer progression. Discrepancy in the profile obtained by cloning and sequencing versus microarray hybridization suggest that nine microRNAs suffer an as yet unknown 3' end modification in androgen-depleted cells that decreases 3' adaptor ligation. Finally, cloning and sequencing reveals a large number of non-micro-small RNAs of unknown function that are expressed

abundantly in the prostate epithelial cells and may be as useful for profiling cancers as microRNAs or mRNAs.

## **Introduction**

Small RNAs provide a new paradigm in understanding regulation of gene expression. microRNA (miRNA) and small interfering RNA (siRNA) are two representative members of small non-coding regulatory RNAs. Other types of small RNA molecules have also been reported, including tiny noncoding RNAs (tncRNAs), repeat associated small interfering RNAs (rasiRNAs), scan RNAs (scnRNAs) and Piwi-interacting RNA (piRNA) (reviewed in (Tolia and Joshua-Tor 2007)). These RNAs are thought to have functions distinct from miRNAs and siRNAs.

Among these small RNAs, miRNAs have been studied most extensively. Each miRNA exhibits a characteristic expression profile, with levels of expression of a few specific miRNA being dramatically altered by tissue type and developmental stage. miRNAs bind to the 3'untranslated region (3'UTR) of a target mRNA and down-regulate its expression. Many studies demonstrated that miRNAs play critical roles in cancer (reviewed in (Lee and Dutta 2006)).

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths in the male population of the United States. Initially, prostate cancers depend upon androgens for their growth. Therefore, the primary treatment for metastatic prostate cancer is androgen deprivation therapy (ADT), achieved by orchiectomy or anti-androgens. However, prostate cancer often progresses into an androgen-independent stage and becomes

resistant to ADT (reviewed in (Suzuki et al. 2003) and (Agoulnik and Weigel 2006)). Thus, it is of clinical importance to understand the mechanism of the transition from androgen-dependence to androgen-independence.

Several models have been proposed to explain the molecular basis of how prostate cancer cells bypass the requirement of androgen for their growth (reviewed in (Pienta and Bradley 2006)). The androgen receptor (AR), a member of the superfamily of nuclear hormone receptors, is a key molecule in the androgen pathway. Upon binding to androgen, AR undergoes a conformational change, translocates to the nucleus, and modulates the transcription of many target genes by binding to androgen response elements (ARE) in their promoter regions. The coordinated gene expression by androgen through the AR pathway is vital for the survival and proliferation of prostate cells. Therefore, an activating mutation in AR, overexpression of AR or aberrant expression of AR coregulators might make the AR active under low androgen conditions, thereby allowing prostate cancer cells to proliferate even after androgen depletion (reviewed in (Chmelar et al. 2007)). In another model, prostate cancer cells might activate a bypass signal transduction pathway so as to survive independent of androgen and AR.

As described above, reprogramming of gene expression is the basis of many of the proposed mechanisms by which prostate cancer cells become androgen-independent and by



which androgens regulate prostate epithelial proliferation. miRNAs are potent regulators that can inhibit many genes simultaneously due to their small size and relaxed specificity in target recognition. Introduction of a single miRNA into cells can lead to a reprogramming of global gene expression (Lim et al. 2005). Since miRNAs have been implicated in a variety of tumors, we reasoned that miRNAs might play a role during prostate cancer progression into androgen-refractory stage and in the androgen regulation of androgen-dependent cells. Although there are two widely accepted methods for global profiling miRNAs (miRNomes), a head-to-head comparison of the methods has not been published. We, therefore, decided to profile the miRNAs in prostate cancer cells by cloning and deep sequencing (Margulies et al. 2005) and follow up by hybridization to microarrays made of locked nucleic acid probes (LNA-microarray) (Castoldi et al. 2006) and to confirm a subset of the changes by individual miRNA-specific biochemical assays. The results identify miRNAs whose downregulation contributes to androgen-independence and serves as a signature of advanced prostate cancer, suggest that constitutive activity of AR is not the explanation of androgen-independence of C4-2, identify a potential modification at the 3' ends of specific miRNAs in androgen-depleted cells and uncover a large assortment of novel and abundant small RNAs that are not miRNAs (nmsRNAs).

## Results

***Small RNA-ome in prostate cancer cells.*** Small RNAs of 17-26 nts were purified from two prostate cancer cell lines, LNCaP and C4-2. The growth of LNCaP is dependent on androgen while C4-2 is a cell line derived from LNCaP that is independent of androgen (Wu et al. 1994) and is more invasive and metastatic (Thalmann et al. 1994; Wu et al. 1994; Thalmann et al. 2000). In addition, to identify androgen-regulated miRNAs, short RNAs were purified from LNCaP cells grown in charcoal stripped serum or the same supplemented with DHT (4,5-dihydrotestosterone), an active ligand of AR (Anderson and Liao 1968; Bruchovsky and Wilson 1968). Charcoal stripped serum (termed “–DHT”, hereafter) effectively depleted the cells of androgen, as indicated by RT-PCR detection of PSA (prostate specific antigen) mRNA, a representative target gene of the AR (Fig S1). The level of PSA is restored to that of whole serum conditions when DHT is added to –DHT condition, showing AR activity is rescued by DHT (termed “+DHT”).

From the four samples (C4-2, LNCaP, –DHT and +DHT), small RNAs of 17-26 nucleotides were isolated and ligated sequentially to the 3' and the 5' adaptor (see Fig 3 for a brief workflow). cDNA from the ligated RNA was concatamerized before 454 deep sequencing, in order to increase the yield of the amount of sequence reads per run. A flowchart for the analysis of 454 deep sequence data (“454 data” hereafter) is outlined in Fig S2. More than half

million (658,068) sequences were obtained from the four samples (Table 1). The inserts are compared with one another, to yield unique sequences and their cloning frequencies. More than a thousand unique sequences from each sample were cloned five times or more, with a few hundred being cloned more than 50 times. The number of unique sequences relative to total sequences in –DHT (13.81%) is significantly higher than the other samples, suggesting the existence of more diverse small RNAs in cells growing in charcoal stripped serum. Global degradation of the RNA from the androgen-depleted cells was ruled out by ethidium bromide staining of the RNA on a denaturing agarose or acrylamide gel (Fig S3) and so cannot be the explanation for this increase in diversity.

miRNAs were extracted from the unique sequences by searching against miRNA database (miRbase release 10.0; <http://microrna.sanger.ac.uk/sequences/>). 37% of total small RNAs match miRNAs (Table 1). Out of 533 human miRNAs in the database, roughly half (293 miRNAs as a whole in the union of the four sets, with 221 to 240 in each individual sample) were cloned from the prostate cancer cells. The cloning frequency of each miRNA was postulated to be proportional to the quantity of the miRNA. Table S1 contains the raw data for all miRNAs. The most frequently cloned miRNAs were *let-7* family members, miR-125b, -99a, -200c, -17, and -21, suggesting that these miRNAs are abundant in these cell lines (Table S1).

To corroborate these profiles, the RNAs from all four samples were independently hybridized to LNA-microarray of miRNA probes. The raw data is tabulated in Table S1. Based on the 454 and microarray data, miRNAs with interesting changes in expression pattern were collected and summarized in Table S2 and S3. These miRNAs were independently measured by individual miRNA-specific assays (Fig 1; see also Fig S4-6) to confirm changes in their expression between samples.

***miRNAs differentially expressed in LNCaP and C4-2*** There were reproducible changes in several miRNAs between these two cell lines in multiple assays (Table 2 and S2; Fig 1A and left two lanes of Fig 1B; see also lanes 9-10 of Fig S4A-B). In general we give greater weight to the changes that are confirmed and accurately quantitated by the miRNA-specific biochemical assays (qPCR, RNase protection or Northern). miR-100, -125b, -19b, and -99a were most down-regulated in C4-2 relative to LNCaP. The miRNAs in the miR-17-92 cluster (miR-17-5p, -18a, -20a and -92a) were also decreased in C4-2 relative to LNCaP. miR-106a, -99b, -21, -16 were decreased, but not to as great an extent as the others.

In contrast, miR-9 and -557 are significantly up-regulated in C4-2 relative to LNCaP in microarray and miRNA-specific qPCR. Also, a moderate increase of miR-196b in microarray was confirmed by miRNA-specific qPCR assays. The up-regulation of miR-196b is also

supported by the 5-fold higher cloning frequency in C4-2 than LNCaP.

***Altering the miRNA profile of C4-2 changes its growth property in charcoal-stripped serum.***

Having observed a significant decrease of miR-100, -125b, -19b, and -99a in C4-2, we tested whether ectopic expression of these miRNAs confers androgen-dependence on C4-2. miR-99b was also included in this experiment, because it is similar to miR-99a in sequence and is decreased in C4-2 (Fig 1A). Upon transfection of a mixture of these five miRNAs, the growth of C4-2 was not affected in untreated serum but was significantly reduced in charcoal stripped serum (Fig 2A). Transfection of an individual miRNA, at the same concentration that it was in the mixture, showed that the selective growth inhibition in charcoal-stripped serum was recreated by transfection of any of four miRNAs in the mix, except for miR-125b (Fig 2B). Thus, the reduction of miR-100, -19b, -99a, and -99b, seen during the progression from LNCaP to C4-2, could contribute to the transition from androgen-dependence to -independence.

***miRNAs differentially expressed upon addition of androgen.*** The addition of DHT to LNCaP cells growing in charcoal-stripped-serum produced only a few miRNA changes that were reproduced in two out of the three assays (Table 2 and S3; Fig 1C and right two lanes of Fig 1B; see also lanes 7-8 in Fig S4A-B and Fig S6). Again, greater weight was given if a change was reproduced and accurately quantitated by the miRNA-specific assays. These changes were a 2.5-

fold reduction of miR-221 and smaller reductions in miR-196b and -125b. miR-125b was also measured by Northern hybridization (lanes 7-8 in Fig S4A-B) and shown to be reduced similarly to qPCR assays. Therefore, the reduction of miR-125b in the presence of DHT is moderate but reliably reproduced by multiple assays. miR-99a is reduced in RNase protection assays (lane 3-4 in Fig 1B), although this reduction is not marked in qPCR assays (Fig 1C). The repression of miR-221, -196b, and -125b was reproduced by R1881, an androgen analog (Fig. 1D; lanes 1-6 in Fig S4A-B; see Fig S4C for PSA level upon R1881 treatment). The slight suppression of miR-99a by DHT (Fig 1C) was amplified by R1881 (Fig 1D). Upon finding that two of the androgen-depleted miRNAs (miR-125b and -99a) were also depleted in the androgen-independent cell line C4-2 (Fig 1 and Fig S5), we wondered whether the miRNome changes in C4-2 could be explained simply by the constitutive activation of AR. However, the decrease of miR-99b and -19b or the increase of miR-557 and -9 in C4-2 was not recapitulated upon addition of androgen to LNCaP cells (Fig S5). Conversely, the repression of miR-221 and -196b by androgen in LNCaP cells was not paralleled by a decrease of these miRNAs in C4-2. Thus we believe that the miRNA changes in C4-2 are not solely the result of constitutive activation of AR.

In addition, the slight induction of *let-7* in –DHT compared to LNCaP (growing in serum) was more or less abrogated in +DHT (Fig 1B; Table 2), suggesting that these miRNAs are

modestly repressed by androgen.

In summary, miR-221, -196b, -125b and *let-7* were repressed by androgens in LNCaP cells. We were unable to find any miRNAs significantly induced by androgen.

***Corroboration between methods.*** Although the 454 data, the LNA-microarray data, and the results from other detection methods are largely consistent among one another for over several hundred miRNAs, a couple of intriguing discrepancies were noticed (Table S2). miR-100 and -557 were not cloned (Table S1) but were detected by the array hybridization and qPCR assay. In general, all the hybridization-based techniques (microarray, qPCR, RNase protection assay, Northern hybridization) are very consistent among themselves. One exception is miR-130b, which gave comparable signals between LNCaP and C4-2 on the arrays, was 5-fold higher in LNCaP in the qPCR assays but was less frequently cloned in LNCaP than in C4-2.

A very interesting pattern of under-reporting of specific miRNAs by the cloning method was seen in androgen-depleted cells (Table S3 and Fig S6). Comparison between “-DHT” and “+DHT” suggests that some miRNAs are oddly underrepresented in miRNA clones obtained from androgen-depleted LNCaP, even though they are easily detected by the hybridization based methods (bottom nine rows in Table S3 and Fig S6). This observation may be explained by several hypotheses, one of which is that modification of 5’ or 3’ end of a specific miRNA



decreases its efficiency of cloning in androgen-depleted cells without affecting its detection by hybridization based methods.

Using miR-21 as a test case, we first measured the miRNA by other assays to ascertain which method gave the accurate estimate of miR-21 in “–DHT” cells. miR-21 was cloned 2053 times in “+DHT” but only 490 times in “–DHT” (Table S1), giving the relative cloning ratio 5.86 (Table S1 and S3; Fig S6) after normalization to each corresponding total number of sequence. In contrast, comparable levels of miR-21 were measured in “–DHT” and “+DHT” by Northern hybridization (Fig 1B and S7C) and qPCR of total RNA and small RNA tailed by poly-A (Fig 3; i and ii). In a functional assay, where we measured the repression of a luciferase reporter containing a perfect match to miR-21 in its 3’UTR, the repressive activity of endogenous miR-21 was also comparable between “–DHT” and “+DHT” (Fig S7A).

We next checked the yield of intermediate products after each of the cloning steps. Although the amounts of starting miR-21 were equal in the two samples, 3’ adaptor ligation to miR-21 from “–DHT” cells is less efficient than that from “+DHT”. This is revealed by qPCR amplification of cDNA from the 3’ ligated RNA using the 3’ adaptor primer and miR-21-specific primer (Fig 3; iii). This discrepancy persisted (and is slightly amplified) when the ligation products were input into the 5’ adaptor ligation reaction and concatamerized (Fig 3; iv and v).

This extra decrease is not due to an inefficiency of 5' adaptor ligation, as demonstrated by direct 5' adaptor ligation to the small RNA (Fig 3; vii). Our results suggest that the lower efficiency of 3' adaptor ligation to miR-21 from “-DHT” cells was the primary reason why miR-21 clones were under-represented in this sample.

***Novel small RNAs other than miRNAs.*** Approximately 63% of the total sequence yield and 90% of unique clones (Tables 1 and S4) of the small RNAs do not match with miRNA sequence (non-micro-short RNA, nmsRNA). A significant portion of these were cloned multiple times and so could not be attributed to mistakes in sequencing. Tens of these sequences were cloned > 100 times in at least one of the four samples, indicating that these novel small RNAs are present in abundance. In Table 3 are summarized a representative list of nmsRNAs that mapped to genomic sites and were cloned at a high frequency. 20-22 % of the nmsRNAs are mapped within known RNAs such as rRNA, tRNA, snoRNA, piRNA, or Refseq genes, suggesting that the nmsRNAs are derived from these RNAs (Table S4). Northern hybridization confirmed the existence of two nmsRNAs derived from snoRNA U3 and a nmsRNA from a tRNA (Table 3 and Fig S8). 20% of the nmsRNAs map to the human genome sequence if we allow two mismatches at the end due to sequencing errors, suggesting that they are derived from as yet unidentified genes.

Some nmsRNAs were perfectly aligned to a part of human piRNAs or miRNAs from

chicken (see “note” column in Table 3). However, it is very unlikely that they are novel miRNAs in human, as no secondary structure characteristic of hairpin-shaped miRNA precursors was observed from the sequence as embedded in its flanking genomic or EST sequence (data not shown). Some nmsRNAs were encoded within a repeat element (Table 3), suggesting that rasiRNA-like RNA may exist in the prostate epithelial cells. In a few cases, a nmsRNA did not match against the human genome, but matched to human ESTs (for example, ID000653\_3313 in Table 3), suggesting the possibility of base modification at the RNA level.

The nmsRNAs that do not map back to the genome may be explained by errors during library construction (i.e. during PCR amplification) or 454 deep sequencing but is most likely due to sequence variations introduced by post-transcriptional modifications. Recent genome-wide transcript mapping studies have suggested that nearly 67% of the genome is transcribed at low levels with ample evidence of splicing and trans-splicing of transcripts ((Kapranov et al. 2007) and T. Gingeras, personal communication). Thus, 21 base junctional fragments from such splices will be difficult to map back to the genome by BLAST. RNA editing may also alter the small RNA sequence relative to the genomic sequence.

## Discussion

Recent publications have reported miRNomes from prostate cancer cell lines and clinical samples obtained by microarrays (Mattie et al. 2006; Volinia et al. 2006; Porkka et al. 2007; Lin et al. 2008; Ozen et al. 2008). Observing that there were many discrepancies between these reports as to which miRNAs were altered and by how much, we decided to first use cloning and deep sequencing to profile the universe of short RNAs (microRNAs and non-micro-short RNAs) of two well defined cell lines under controlled conditions. The results were then validated by multiple approaches to ascertain the reliability of the miRNA changes noted. We thus arrived at a validated list of miRNAs altered in expression level between the androgen-dependent cell line, LNCaP, and its androgen-independent derivative, C4-2, and upon addition of androgen to the androgen-dependent LNCaP cells.

One reason for the discrepancies between various profiling approaches is the small magnitude of changes reported. Consistent with this, most of the miRNA changes reported in this study are less than three-fold and there are significant differences in the ratios determined by the different methods. However, because of our controls, we could identify five miRNAs (miR-99a, -99b, -100, -125b and -19b) that are decreased and three miRNAs (miR-9, -196b and -557) that are increased in C4-2 relative to LNCaP. C4-2, cells that are normally not affected by serum

deprivation, suffered a decrease in proliferation in charcoal-stripped serum upon transfection of the miRNAs that are decreased in these cells. That proliferation was not decreased in complete medium suggests that this approach will be useful to find miRNAs that contribute to the androgen-independence of C4-2.

These miRNAs will be tested in the future as molecular markers for prostate cancer progression. The utility of miRNA in cancer diagnosis was discussed in several reports. An expression profile of miRNAs was shown to be better at predicting and classifying cancer types and stages than the mRNA expression profile (Lu et al. 2005). Among the down-regulated miRNAs in C4-2, ectopic expression of miR-19b, -100, -99a and -99b resulted in a growth inhibitory effect on C4-2 selectively in the absence of androgen. This opens a possibility that delivery of these miRNAs combined with anti-androgen treatment could be considered for a therapy for an advanced, androgen-independent prostate cancer.

In agreement with our observation that miR-100, -125b, -19b, -99a and -99b are down-regulated in C4-2 than LNCaP, all of them were shown to be markedly decreased in more aggressive (Gleason score 8) and metastatic prostate tumor (Lu et al. 2005; Mattie et al. 2006). Given that C4-2 is readily metastatic and more invasive than LNCaP (Thalmann et al. 1994; Wu et al. 1994; Thalmann et al. 2000), these miRNAs may also play a role in metastasis and invasion.

Our results are also supported by another profiling study where miR-99a, -125b, -19b and -100 were shown to be reduced in prostate carcinomas, with miR-19b and -100 being reduced especially in hormone-refractory carcinomas (Porkka et al. 2007). The corroboration of these independent reports of miRNA changes in clinical prostate cancer by our results emphasize that decrease of these five miRNAs is a reliable signature for progression of prostate cancer. In addition, profiling of miRNAs in clinical samples suffers from the contamination of the cancer cells by stromal and non-malignant epithelial cells, so that any changes noted could be due to changes in the non-cancer portion of the specimen. Our results, however, clearly indicate that the decrease of these miRNAs in the prostate epithelial cells themselves contributes to the cancer progression.

A recent report also attempted to identify miRNAs implicated in androgen dependence/independence in comparison of cell lines (Lin et al. 2008). They observed several deregulated miRNAs between the pairs of cell lines, but comparison between the published data and our data revealed that only miR-19b was consistently down-regulated in androgen-independent cell lines in the two studies. This inconsistency may be due to the diversity of mechanisms by which prostate cancer cells become androgen-independent (Pienta and Bradley 2006). Nonetheless, the discrepancy was still seen when we compared our data on the

LNCaP/C4-2 pair with the published data on the LNCaP/C4-2B pair. A possible explanation could be in the difference between C4-2 and its bone metastatic derivative C4-2B (Thalmann et al. 1994), or experimental variables such as passage numbers of LNCaP cells (Lin et al. 2003). It is worth noting that in (Lin et al. 2008), after the initial array (not on LNA microarrays), the induction of miR-184 and repression of miR-146a during prostate cancer progression was confirmed not by miRNA-specific biochemical assays on the cell lines but by in-situ-hybridization to cancer tissues.

miR-221, -196b, -125b and *let-7* were moderately repressed by androgens in LNCaP cells, though we do not know whether this repression is a primary effect of androgen or due to secondary changes after the activation of the androgen-driven gene expression program. Contrary to our expectation, we did not find any miRNA that was robustly induced by androgens. A recent report indicated that miR-125b is induced by androgens and that miR-125b is up-regulated in androgen independent CDS cell lines relative to LNCaP cells (Shi et al. 2007). Our data from multiple independent assays unequivocally showed that miR-125b is repressed by androgens (Fig 1C-D and Fig S4) and significantly down-regulated in the androgen-independent cell lines C4-2 (Fig 1A and S4), PC3, and DU145 (data not shown). Of note, all these cell lines express very low level of AR relative to CDS cells (Wu et al. 1994; Mitchell et al. 2000; Alimirah et al. 2006), so



that miR-125b induction noted in CDS cells could be related to level of expression of AR.

The differences in levels of specific miRNAs in response to environmental changes (e.g. addition of androgen) or during cancer progression appear to be smaller in magnitude than the >100 fold differences seen when comparing different tissues. However, our careful comparison of miRNA profiles by all the currently available techniques reveals that the smaller differences seen in response to environmental changes or during cancer progression can be identified and must be checked by multiple methods before acceptance.

The significant under-representation of specific miRNAs from androgen-depleted cells in the sequencing approach compared to the hybridization based methods was cautionary. In case of miR-21, the explanation lies in an inefficiency of 3' adaptor ligation in the androgen-depleted cells, suggesting a modification at or near the 3' end of miR-21. In plants, it has been reported that methylation on the ribose of the 3' nucleotide contributes to stabilization of miRNA (Yu et al. 2005). However, such a modification in animal miRNAs has not been reported yet.

One advantage of the deep sequencing technique is that it allowed us to discover novel non-micro-small RNAs (nmsRNAs). The sheer diversity and abundance of the nmsRNAs suggests that they may become very important if one can implicate them in specific biological functions. The two-fold increase in the abundance of nmsRNAs upon androgen-depletion of

LNCaP is interesting and unexplained. Some of the nmsRNAs were mapped to genomic repeats, suggesting that these are human counterpart of rasiRNA in *Drosophila*. However, these novel small RNAs are 18-20 nucleotides long and are smaller than *Drosophila* rasiRNAs that range from 23-26 nucleotides (Aravin et al. 2003). A subset of the novel small RNAs are likely to be processed from abundant noncoding RNAs, such as snoRNA or tRNA. These may not simply be a byproduct of the degradation process because they were of a definite size and derived from a specific small portion of the parental RNA. Taken together with the signal intensity in Northern hybridization, their cloning frequency indicates that some of these novel nmsRNAs are as abundant as miRNAs.

In summary, we obtained a list of miRNAs that differ in expression level between an androgen-independent and an androgen-dependent prostate cancer cell line and upon addition of androgens to an androgen-dependent cell line. The decrease of five miRNAs in the cancer cells appears to be a definitive signature of prostate cancer progression that can be used for prognosis. The roles of these miRNAs in prostate cancer biology and the mode of their regulation remain to be determined. Since relatively little is known about miRNAs in prostate cancer biology, we believe that our list of miRNAs with well verified changes in expression level will be a good starting point to investigate the roles of individual miRNAs in prostate epithelial biology. We also

report for the first time many novel small RNAs, some of which are more abundant than most miRNAs. The biogenesis, regulation of expression, and biological function of this huge universe of non-micro-short RNAs will be an interesting area of inquiry. Finally, the study underlines the extensive variability that could arise from methods of miRNome profiling and emphasizes the need to corroborate the small changes in miRNA expression often reported in the Literature by independent methods of miRNA quantitation.

## **Materials and Methods**

### ***Cell culture***

LNCaP and C4-2 cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. For androgen depletion, LNCaP cells were cultured for four days in a phenol red free RPMI-1640 with 10% charcoal/dextran treated FBS (Hyclone). 4,5 - dihydrotestosterone (DHT) was purchased from Sigma-Aldrich and was added at 10 nM. R1881 was from Perkin-Elmer.

### ***RNA isolation and miRNA measurements***

Total RNA isolation and miRNA analyses (RNase protection assay and Northern hybridization) were performed as previously described (Kim et al. 2006; Lee and Dutta 2007). Microarray profiling of miRNAs was performed with miRCURY™ LNA array (v.9.2; Exiqon).

qPCR measurement of miRNAs was performed with Ncode SYBR GreenER miRNA qRT-PCR kit (Invitrogen) according to the manufacturer's instructions and with ABI 7300 real time PCR system. Each value is an average of triplicate samples. miRNA signals were normalized to those of U6 small nuclear RNA (snU6) as a control. snU6 primer sequence is 5'-ctgcgcaaggatgacacgca-3'.

### ***Cloning of small RNA and 454 deep sequencing***

Isolation of small RNA, ligation of adaptors, cDNA synthesis, PCR amplification, and concatamerization were performed as described in (Lau et al. 2001) with minor modifications. Concatamerized DNAs were subject to 454 deep sequencing (VBI Core Lab at Virginia Bioinformatics Institute in Virginia Tech.).

### ***Transfection of miRNA duplex and cell proliferation assay***

siRNA-like duplexes containing miRNA sequence at one strand (Hutvagner and Zamore 2002) were synthesized Invitrogen Life Technologies and used as a miRNA mimic, with GL2 duplex as a negative control (Elbashir et al. 2001). Transfection into C4-2 cells was performed with Lipofectamine 2000 reagent (Invitrogen Life Technologies) at a final concentration of 20 nM of each siRNA duplex. After 72 hrs, BrdU incorporation was measured as previously described (Machida et al. 2006) and was normalized to cell density measured by MTT assay (CellTiter 96 non-radioactive cell proliferation assay kit from Promega).

## **Supplemental Data**

Supplemental Tables and Figures are included as a single pdf file (Lee et al\_RNA\_supple.pdf).

## **Acknowledgements**

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## Figure legends

### Figure 1. Expression of miRNAs measured by Northern, RNase protection assay, and qPCR.

**A and C.** qPCR was performed as indicated in Experimental Procedures. A miRNA value was normalized to that of snU6. The average and standard deviation from triplicate samples are indicated. miRNAs are arranged from top to bottom according to the fold change. The qPCR values of miRNAs, together with their other results, are tabulated in Tables S2 (panel A; C4-2 versus LNCaP) and Table S3 (panel C; +DHT versus –DHT). In C4-2/LNCaP comparison, the higher value was set as 1 (panel A); while –DHT value was set as 1 in +DHT/–DHT comparison (panel C). miRNAs with an asterisk are also measured in panel B.

**B.** RNase protection assays (top panels) and Northern hybridization (bottom panels) of selected miRNAs. In case of Northern hybridization of *let-7*, we used a mixture of probes against *let-7b* and *-7e*, the two most divergent *let-7* variants, to cover as many kinds of paralogous *let-7*s as possible (Lee and Dutta 2007). miRNAs with an asterisk are also measured in panel A or C.

**D.** qPCR assays of selected miRNAs from panel C after treating androgen analog R1881 at indicated concentrations. The value of no R1881 (–DHT condition; plain bar) is set as 1.

**Figure 2. The proliferation of C4-2 after transfection of miR-19b, -99a, -99b, -100, and -125b in the presence or absence of androgen.**

**A.** Upon transfection of indicated miRNA(s) or GL2 (Experimental Procedures), C4-2 cells were cultured in untreated serum or charcoal stripped serum. After 72 hrs, BrdU incorporation was measured and normalized to cell density from MTT assay (y-axis). The average and standard deviation from triplicate samples are shown. “miRs” indicates equimolar mixture of the five miRNAs in panel B. The value of GL2 in each condition is set as 1 (panel A).

**B.** Transfection of individual miRNA mimics. Each value in charcoal-stripped serum is again normalized to that of untreated serum and the relative value is shown. GL2 = 1.

**Figure 3. 3' adaptor ligation to miR-21 from –DHT is impaired**

Measurement of various cloning intermediates. Steps i and ii; qPCR with total RNA (step i) or gel-isolated 17-26 mer small RNA (sRNA; step ii). Steps iii and iv are qPCR of cloning intermediates. qPCR assays after control ligation of 5' adaptor directly to sRNA are shown in a separate step vii. The PCR primer pairs are; a miRNA sense primer and the 3' adaptor antisense primer (step iii), or a miRNA antisense primer and the 5' adaptor primer (step iv and vii). miR-21 values are normalized to that of miR-200b (y-axis). All values are an average of triplicate



samples. For step v, 40 nanogram of each concatamer (the last intermediate in the cloning procedure) was subjected to Southern hybridization with a probe specific to miR-21 (left panel). A mixture of probes against *let-7b* and *-7e* was used as a control (right panel). In step vi, the cloning frequency of miR-21 relative to *let-7* is shown.

#### **Table legends**

##### **Table 1. Summary of 454 deep sequencing data**

Numbers of total and unique sequences from each sample (C4-2, LNCaP, +DHT and –DHT; see text for details) are shown. The unique sequences are classified according to cloning frequency. miRNA sequences are extracted from the total sequences, yielding numbers of total and unique miRNA sequences.

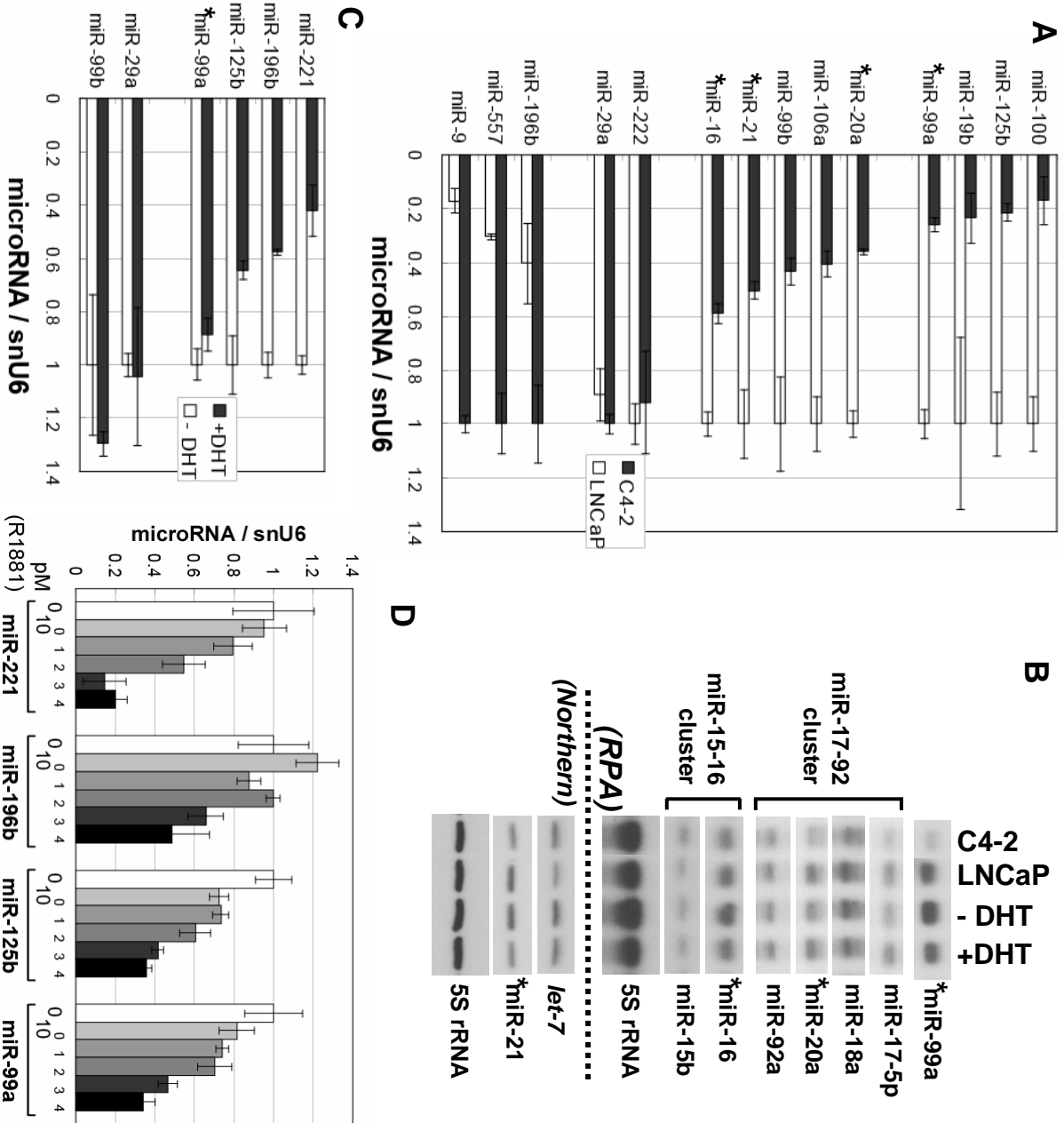
##### **Table 2. List of selected miRNAs in prostate cancer cells**

miRNAs of interesting expression pattern were selected according to the relative expression levels estimated from qPCR, RNase protection assays, and Northern hybridization (Fig 1 and S4).

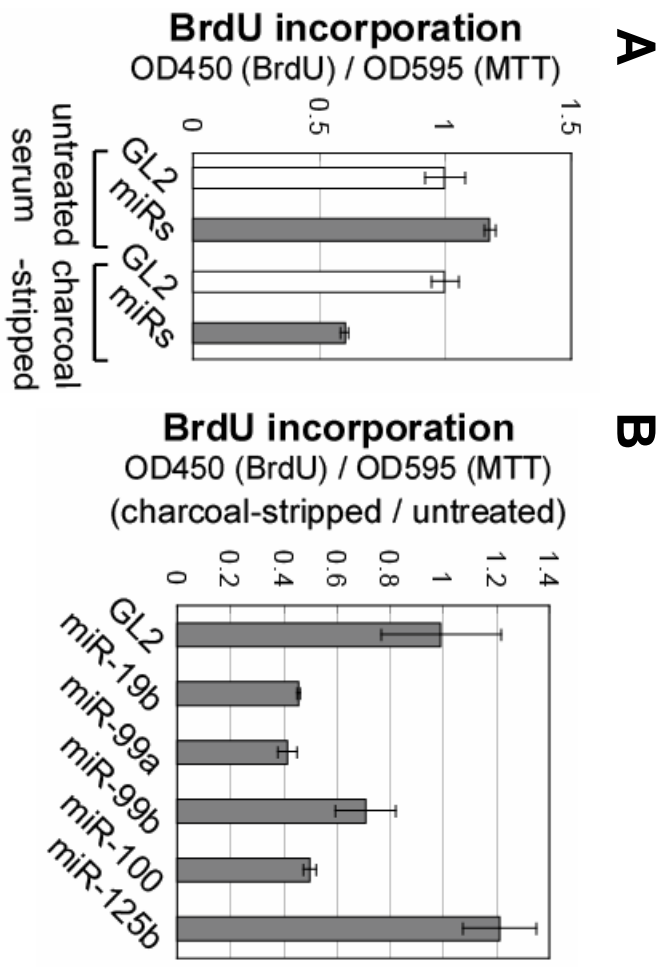
The approximate relative quantities are indicated by the number of “+”, from -/+ (the lowest expression) to five +’s (the highest expression)

**Table 3. Novel small RNAs**

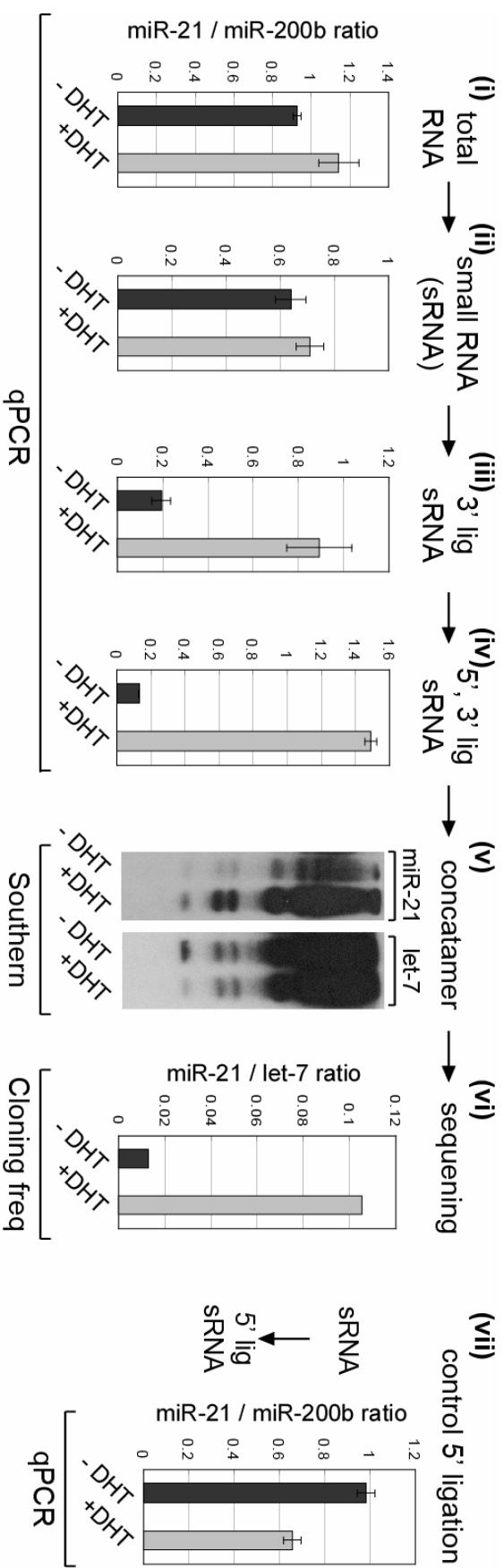
A list of non-micro-short RNA (nmsRNA) that were cloned abundantly (>100 times from at least one sample). Some sequences map to a known non-coding RNA or genomic repeat. If BLAST maps a sequence to more than three loci in the human genome or ESTs, one representative coordinate is shown. Another BLAST search was done against non-coding RNA database (<http://www.noncode.org/>) and the results are shown in the “note” column.



Lee et al\_Figure 1



Lee et al\_Figure 2



Lee et al\_Figure 3

		C4-2	LNCaP	+DHT	- DHT	sum
total number of sequence		191,970	192,025	114,280	159,793	658,068
unique sequences						
	number	15,776	11,756	10,517	22,069	
	ratio (%)	8.22	6.12	9.20	13.81	
cloning frequencies of unique sequences						
<5	number	14,396	10,563	9,485	20,901	
	% of total unique	91.25	89.85	90.19	94.71	
5< <50	number	1,126	978	872	994	
	% of total unique	7.14	8.32	8.29	4.50	
>50	number	254	215	160	174	
	% of total unique	1.61	1.83	1.52	0.79	
microRNA						
	total number	59,448	71,451	43,827	68,825	243,551
	ratio to total (%)	30.97	37.21	38.35	43.07	37.01
	unique microRNAs	240	221	221	221	293

Lee et al\_Table 1

	C4-2	LNCaP	+DHT	- DHT
hsa-let-7	++++	+++	++++	+++++
hsa-miR-100	-/+	+++	+++	++++
hsa-miR-125b	-/+	+++	+++++	+++++
hsa-miR-196b	+++++	++	++	+++
hsa-miR-19b	+	++++	++++	+++
hsa-miR-21	++	+++	++	+++
hsa-miR-221	++	++	++	+++++
hsa-miR-29a	++	++	++	++
hsa-miR-557	++++	+	+	+
hsa-miR-9	+++++	+	+	++
hsa-miR-99a	-/+	+++	+++	+++++
hsa-miR-99b	++	+++++	+++++	++++
miR-17 cluster	++	+++	+++	++

Lee et al\_Table 2

		cloning frequency					
ID	sequence	C4-2	LNcap	+DHT	- DHT	genomic, refseq, or EST coordinate	note
000134_1499	CCACGAGGAAGAGAGGTAGCG	146	102	104	314	613-633 of X14945.1(U3 snoRNA)	
004265_1575	GGAGAGAACGGGTTCTGAGTGGT	93	99	99	483	732-754 of X14945.1(U3 snoRNA)	
001078_1344	ACCCCACTCCTGGTACCA	354	401	281	358	MLT-int (LTR class repeat element)	AM691203_miRNA_chicken
000644_1380	GCGAAGGTGGCGGAATTGG	342	131	80	408	G-rich (low complexity repeat)	
000889_3198	GTCCCTTCGTGGTCGCCA	213	56	71	98	HERV9 (LTR class repeat element) Simple Tandem Repeat	DQ581012_piRNA_human
000407_2746	ATCCGACCGCTGCCACCA	861	604	668	295	chr16:22114547-22114530 (representative coordinate)	tRNA-Leu-CTA AM691362_miRNA_chicken
004221_1576	ACCCCTGCTCGCTGCGCCA	265	101	97	194	chr10:69194334-69194348 chr15:82540146-82540160	tRNA-Ser-TCA AM691336_miRNA_chicken
002521_1423	GCTAAACCTAGCCCAAAAC	236	373	216	256	chr7:145325417-145325436 chr5:79982622-79982603	DQ597483_piRNA_human
000653_3313	ATCGATGTGGTGACGTCGTGCTC	203	117	101	96	61-83 of EST BQ706851.1 (representative EST)	
001257_0208	GCGTTGGTGATATAGTGT	110	243	80	57	chr19:4675082-4675100 (representative coordinate)	tRNA-Gly-GGA DQ571029_piRNA_human

Lee et al\_ Table 3





## Supplemental Figure legends

### **Table S1. Sequencing frequency and LNA -microarray data of all miRNAs.**

Comprehensive list of 454 deep sequencing data and LNA-microarray data. Cloning frequencies (raw value) for each miRNA are indicated. Each cloning frequency of a miRNA was divided by the total number of sequence of the corresponding sample (see Table 1; for example, 192,025 for LNCaP and 191,970 for C4-2) and then the normalized values were used to calculate ratio of C4-2/LNCaP or +DHT/–DHT (for nomenclature, see the text). Each value in microarray data is ratio of median values from LNCaP/C4-2 or +DHT/–DHT pair. “NA” indicates insignificant or poor detection of a miRNA in microarray, probably due to low expression of the miRNA. In cases of some miRNAs that are cloned in our experiment but not present in miRCURY™ LNA array (v.9.2; Exiqon), they are indicated as “NP”.

An asterisk was given to miRNAs of interesting expression profile which include; 1) miRNAs with a two-fold or higher change in LNA-microarray, or 2) miRNAs with a change by more than 33% in cloning ratio among the abundantly (>30 in either one sample) cloned ones. A fraction of these miRNAs were measured by miRNA-specific assays and the results were summarized in Table S2 and S3.

**Table S2. miRNAs that change with androgen independence.**

miRNAs were selected from LNCaP and C4-2 based on changes in expression in microarray data and 454 deep sequencing data (Table S1). The expression of each miRNA was measured by miRNA-specific assays (Fig 1)

**Table S3. miRNAs that change with androgen addition**

miRNAs were selected from +DHT and –DHT. All other descriptions are same as Table S2.

**Table S4. Analyses of unique sequences that are not miRNAs.**

Out of unique sequences, those that do not match miRNA sequences were further analyzed as depicted in Fig S2. The unique sequences are subject to BLAST search against collection of rRNA, tRNA, snoRNA, piRNA, or mRNA (refseq) sequences.

**Figure S1. RT-PCR of PSA (prostate specific antigen) and  $\beta$ -actin**

**A.** RT (reverse transcription)-PCR was performed with the total RNAs from LNCaP, –DHT, and +DHT to measure PSA and  $\beta$ -actin control. Each amplified fragment with its expected size in bp (base pairs) is indicated. The primers for PSA amplification are 5’-

attcccaaccctggcaggtg-3' and 5'-tcgtagagcgggtgtgggaa-3'.  $\beta$ -actin primers were previously described in (Lee, Y. S. & Dutta, A. (2007) *Genes Dev* **21**, 1025-1030).

**B.** PSA/ $\beta$ -actin values from real time-qPCR are shown.

### **Figure S2. Pipeline of analyses of 454 deep sequencing data**

A schematic of the 454 sequencing data analyses is depicted. See the text for details.

### **Figure S3. Quality control of total RNA by ethidium bromide staining**

Total RNAs are subject to gel electrophoresis and visualized by ethidium bromide staining. *Left panel*; 1% denaturing agarose gel with 28S and 18S ribosomal RNA bands indicated. *Right panel*; 15% denaturing polyacrylamide gel with the positions of two dyes (bromophenol blue and xylene cyanol FF) marked.

### **Figure S4. Measurement of miR-125b in the four samples and after R1881 treatment.**

**A.** Northern hybridization was performed to measure miR-125b after treatment of indicated concentrations of androgen analog R1881 (lanes 1-6) and in the four samples (lanes 7-10). 5S rRNA is a loading control. The blot was exposed to phosphorimager Storm 860 (Molecular Dynamics) and each band was quantified with ImageQuant 5.2

software. The intensity of miR-125b was normalized to that of 5S rRNA. “–DHT” samples in each set (lane 1 and 7) were set as 1.

**B.** qPCR assays to detect miR-125b. Results from Fig 1D (lane 1-6) and Fig 1A and C (lanes 7-10) were re-drawn for direct comparison with Northern data in panel A.

**C.** PSA/ $\beta$ -actin values from real time-qPCR after R1881 treatment.

**D.** miR-98/snU6 measured by qPCR, to rule out global miRNA reduction after R1881 treatment.

**Figure S5. qPCR assays to compare C4-2/LNCaP with +DHT/–DHT**

Selected miRNAs in Fig 1 were measured by qPCR assays for side-by-side comparison between C4-2/LNCaP (left panel) and +DHT/–DHT (right panel). Some of the data was reclaimed from Fig 1A and C for ease of comparison.

**Figure S6. qPCR assays to measure miRNAs underrepresented in –DHT in cloning deep sequencing**

Selected miRNAs in Table S3 were measured by qPCR assays. Microarray and deep sequencing values of each miRNA are shown in the left. All other descriptions are same as Fig 1C.

### **Figure S7. Measurement of miR-21 in +DHT and –DHT**

**A.** Luciferase assays were performed as described (Lee, Y. S. & Dutta, A. (2007) *Genes Dev* **21**, 1025-1030). pRLCMV-miR21as is a plasmid containing a sequence perfectly complementary to miR-21 downstream from the Renilla luciferase ORF in the parental vector, pRL-CMV. pRLCMV-miR21as (or pRL-CMV vector control), together with pGL3-Control vector (Promega Corp.), were transfected into –DHT and +DHT using Lipofectamine 2000 reagent (Invitrogen). Renilla luciferase activity was normalized to the firefly (*Photinus pyralis*) luciferase activity from the co-transfected pGL3-Control vector. y-axis indicates ratio of pRLCMV-miR21as to pRL-CMV. The average and standard deviation of triplicate samples are indicated.

**B.** miR-21 sequences were cloned with variable ends, probably due to a flexibility of Dicer or Drosha processing. Among these sequences (490 times in –DHT and 2,053 times in +DHT as a whole) are shown the two most abundantly cloned miR-21 sequences (italicized) and their cloning frequencies in the 454 data. At the bottom, mature miR-21 sequence (capital letters) and its partial flanking sequence from the precursor (lower case letters) are shown from the database (miRBase; <http://microrna.sanger.ac.uk/>).

C. In an attempt to detect 3' end modification in the miR-21 that may interfere with the 3' adaptor ligation, another Northern hybridization was performed on an acrylamide gel of higher resolution (18% denaturing acrylamide sequencing gel). For comparison, a 26-mer oligodeoxyribonucleotide containing miR-21 sequence was phosphorylated at the 5' end by polynucleotide kinase (New England Biolabs), was run in parallel with the unphosphorylated form, and was subject to hybridization to a probe against miR-21. As seen in right panel, a single phosphate group was resolved in this gel. In the miR-21 lanes (left two lanes), we observed two bands that are presumably 22- and 23-mer which represent the two most abundantly cloned forms of miR-21 (panel B). We failed to detect any aberrant migration of miR-21 in –DHT compared to +DHT, suggesting that any modification at the 3' end is too small to be resolved by this technique.

### **Figure S8. Northern hybridization detection of novel small RNAs**

Northern hybridization was performed to detect selected non-micro-small RNAs (nmsRNAs). Two fragments from U3 snoRNA (ID 000134\_1499 and ID 004265\_1575) and a fragment from tRNA (ID 001257\_0208) were chosen from Table 3. A discrete band of expected size (marked by arrowheads) confirmed the existence of the small RNA at a measurable steady-state level. The very strong bands on top of the gel are

most likely due to full length snoRNA U3 or tRNA molecules. We detected additional bands of intermediate size whose intensity is roughly proportional to that of the small RNA, raising the possibility that the novel smallRNAs were derived from snoRNA U3 or tRNA through a stepwise processing, with the intermediate sized bands being processing intermediates.

Lee et al\_Table S1

	Cloning Frequency				LNA array	miRNAs of interesting ratio	Cloning Frequency				LNA array	miRNAs of interesting ratio	measured by other assays in this study?
miRNA_ID	frequency		ratio	ratio	frequency		ratio	ratio					
	C4-2	LNCaP	C4-2 /LNCaP	C4-2 /LNCaP	+DHT		-DHT	+ DHT - DHT	+ DHT - DHT				
hsa-let-7a	5,584	4,733	1.18	0.97		2,911	4,628	0.88	0.87		yes (let-7s but not 7's)		
hsa-let-7a*	4	8	0.50	1.13		9	10	1.26	1.04				
hsa-let-7b	19,212	12,697	1.51	1.02		6,963	12,730	0.76	0.88				
hsa-let-7b*	7	7	1.00	NP		4	4	1.40	NP				
hsa-let-7c	4,238	12,518	0.34	1.04	*	7,865	18,094	0.61	1.04	*			
hsa-let-7c*	1	1	1.00	NP		3	1	4.19	NP				
hsa-let-7d	391	374	1.05	1.10		196	333	0.82	0.94				
hsa-let-7d*	39	26	1.50	0.97		82	9	12.74	1.03	*			
hsa-let-7e	125	110	1.14	0.90		127	182	0.98	0.95				
hsa-let-7e*	0	0		0.99		0	2		0.95				
hsa-let-7f	854	983	0.87	1.13		691	678	1.43	1.02	*			
hsa-let-7f-1*	1	0		NP		0	0		NP				
hsa-let-7f-2*	0	0		0.93		0	0		1.05				
hsa-let-7g	517	770	0.67	0.94	*	491	848	0.81	1.01				
hsa-let-7g*	0	0		0.99		0	0		1.01				
hsa-let-7i	695	433	1.61	1.42	*	266	495	0.75	0.98				
hsa-let-7i*	77	25	3.08	1.00	*	17	46	0.52	1.05	*			
hsa-miR-1	0	0		0.95		0	0		1.05				
hsa-miR-100	0	0		0.38	*	0	2		0.70				
hsa-miR-100*	0	0		1.00		0	0		1.04				
hsa-miR-101	47	63	0.75	0.69		75	46	2.28	1.08	*			
hsa-miR-101*	0	0		0.95		0	0		1.03				
hsa-miR-103	856	705	1.21	0.89		606	742	1.14	1.01				
hsa-miR-105	1	0		1.03		1	0		0.92				
hsa-miR-106a	66	68	0.97	0.49		83	11	10.55	1.31	*			
hsa-miR-106b	323	370	0.87	0.79		260	108	3.37	1.04	*			
hsa-miR-106b*	51	49	1.04	0.78		52	30	2.42	1.04	*			
hsa-miR-107	70	54	1.30	0.87		44	37	1.66	1.05	*			
hsa-miR-10a	1	1	1.00	0.88		1	0		0.84				
hsa-miR-10a*	0	0		1.05		0	0		1.01				
hsa-miR-10b	0	0		1.03		0	0		1.07				
hsa-miR-122	2	0		0.93		0	0		1.15				
hsa-miR-122*	1	0		NP		0	0		NP				
hsa-miR-124	1	0		1.00		0	0		1.03				
hsa-miR-125a-3p	10	5	2.00	0.86		3	8	0.52	1.09				
hsa-miR-125a-5p	165	189	0.87	0.92		108	161	0.94	0.82				
hsa-miR-125b	2,801	9,048	0.31	0.34	*	5,352	12,870	0.58	0.73	*			
hsa-miR-125b-1*	0	0		0.88		0	0		0.95				
hsa-miR-125b-2*	12	26	0.46	NP		29	68	0.60	NP	*			
hsa-miR-126	4	4	1.00	0.68		6	3	2.80	0.89				
hsa-miR-126*	0	0		0.90		1	1	1.40	1.01				
hsa-miR-127-3p	0	0		0.92		0	0		1.14				
hsa-miR-128	24	25	0.96	1.08		16	31	0.72	0.92				
hsa-miR-129*	0	0		0.96		1	0		1.06				
hsa-miR-129-5p	0	0		1.06		0	0		0.95				
hsa-miR-130a	0	0		1.21		0	0		0.98				
hsa-miR-130b	156	42	3.72	1.18	*	57	48	1.66	1.08	*			
hsa-miR-130b*	2	0		0.97		2	2	1.40	1.05				
hsa-miR-132	24	20	1.20	0.96		24	12	2.80	1.05				
hsa-miR-132*	3	0		0.92		2	1	2.80	1.09				
hsa-miR-133a	0	0		NA		0	1		NA				
hsa-miR-134	0	0		1.00		0	0		1.05				
hsa-miR-135a	1	0		0.93		0	0		1.04				
hsa-miR-135b	0	0		0.94		0	0		1.07				
hsa-miR-136	0	0		0.86		0	0		1.17				
hsa-miR-137	0	0		0.97		0	0		0.67				
hsa-miR-138	2	3	0.67	0.88		1	21	0.07	0.99				
hsa-miR-139-5p	1	3	0.33	1.03		0	0		NA				
hsa-miR-140-3p	48	22	2.18	0.93	*	31	24	1.81	1.00	*			
hsa-miR-140-5p	27	17	1.59	0.99		9	43	0.29	0.98	*			
hsa-miR-141	230	225	1.02	0.88		129	52	3.47	1.18	*			
hsa-miR-141*	1	4	0.25	0.84		0	1		1.20				
hsa-miR-142-3p	0	1	0.00	1.06		0	0		0.95				
hsa-miR-142-5p	0	0		1.00		0	0		1.03				
hsa-miR-143	0	0		1.00		0	0		NA				
hsa-miR-143*	0	0		NA		0	0		0.90				
hsa-miR-144	0	0		0.96		0	0		1.06				
hsa-miR-145	0	0		0.98		0	0		1.03				
hsa-miR-145*	0	0		1.00		0	0		1.02				
hsa-miR-146a	0	0		0.72		0	0		1.17				
hsa-miR-146b-5p	0	0		0.93		3	1	4.19	1.15				
hsa-miR-147b	2	1	2.00	0.98		0	0		1.13				
hsa-miR-148a	36	10	3.60	1.56	*	19	2	13.28	1.14				
hsa-miR-148a*	0	0		1.20		0	0		1.13				
hsa-miR-148b	66	58	1.14	1.08		41	38	1.51	1.01	*			
hsa-miR-149	1	5	0.20	0.96		3	7	0.60	0.99				
hsa-miR-149*	3	0		NP		0	0		NP				



hsa-miR-151-3p	10	5	2.00	0.84		24	5	6.71	1.02		
hsa-miR-151-5p	220	189	1.16	0.80		215	242	1.24	1.09		
hsa-miR-152	2	8	0.25	0.87		3	8	0.52	1.03		
hsa-miR-153	2	2	1.00	0.88		6	0		1.41		
hsa-miR-154	0	0		0.96		0	0		1.09		
hsa-miR-154*	0	0		0.98		0	0		1.07		
hsa-miR-155	0	0		0.90		0	0		1.12		
hsa-miR-15a	282	670	0.42	0.58	*	466	219	2.98	1.00	*	
hsa-miR-15a*	6	9	0.67	0.95		5	1	6.99	1.03		
hsa-miR-15b	786	936	0.84	1.07		634	411	2.16	1.06	*	yes
hsa-miR-15b*	8	8	1.00	0.93		8	1	11.19	1.17		
hsa-miR-16	546	901	0.61	0.86	*	531	148	5.02	1.03	*	yes
hsa-miR-16-1*	0	1	0.00	0.75		4	0		1.00		
hsa-miR-16-2*	7	10	0.70	NP		1	0		NP		
hsa-miR-17	2,021	3,324	0.61	0.52	*	2,287	527	6.07	1.21	*	yes
hsa-miR-17*	12	28	0.43	0.53		28	40	0.98	1.31		
hsa-miR-181a	0	1	0.00	0.96		1	4	0.35	0.99		
hsa-miR-181a*	0	0		0.94		0	0		1.03		
hsa-miR-181a-2*	0	1	0.00	0.99		0	1		0.97		
hsa-miR-181b	1	1	1.00	0.87		0	0		1.02		
hsa-miR-181c	0	0		0.91		0	0		1.06		
hsa-miR-181d	0	0		0.98		0	0		1.00		
hsa-miR-182	27	28	0.96	0.82		18	25	1.01	1.00		
hsa-miR-182*	0	0		0.77		0	0		0.99		
hsa-miR-183	31	20	1.55	0.85	*	15	28	0.75	0.86		
hsa-miR-183*	0	1	0.00	0.95		3	0		0.92		
hsa-miR-184	0	0		1.03		0	0		1.06		
hsa-miR-185	101	48	2.10	1.18	*	40	117	0.48	0.87	*	
hsa-miR-185*	2	1	2.00	1.09		1	1	1.40	1.03		
hsa-miR-186	34	23	1.48	0.94		14	24	0.82	1.07		
hsa-miR-186*	3	1	3.00	NP		2	2	1.40	NP		
hsa-miR-187	0	0		0.97		0	0		1.09		
hsa-miR-188-3p	2	1	2.00	1.01		1	2	0.70	0.97		
hsa-miR-188-5p	15	3	5.00	0.91		7	1	9.79	1.10		
hsa-miR-18a	140	178	0.79	0.62		129	26	6.94	1.21	*	yes
hsa-miR-18a*	2	5	0.40	0.77		0	5		1.16		
hsa-miR-18b	0	0		0.64		0	0		1.25		
hsa-miR-18b*	0	0		1.01		0	0		0.96		
hsa-miR-190	1	2	0.50	1.07		3	1	4.19	1.05		
hsa-miR-190b	14	3	4.67	1.13		6	1	8.39	1.34		
hsa-miR-191	424	398	1.07	1.01		302	362	1.17	1.04		
hsa-miR-191*	2	2	1.00	0.91		1	1	1.40	1.15		
hsa-miR-192	10	6	1.67	0.87		4	5	1.12	1.08		
hsa-miR-192	10	6	1.67	0.90		4	5	1.12	1.07		
hsa-miR-192*	0	0		1.00		0	0		1.01		
hsa-miR-193a-3p	184	86	2.14	0.91	*	107	169	0.89	1.35		
hsa-miR-193a-5p	9	4	2.25	1.22		2	18	0.16	1.10		
hsa-miR-193b	70	16	4.38	1.11	*	27	40	0.94	1.07		
hsa-miR-193b*	3	1	3.00	NP		1	2	0.70	NP		
hsa-miR-194	2	8	0.25	0.87		8	8	1.40	1.03		
hsa-miR-194*	0	1	0.00	NP		0	0		NP		
hsa-miR-195	30	60	0.50	0.82	*	43	33	1.82	1.01	*	
hsa-miR-195*	5	10	0.50	NP		6	1	8.39	NP		
hsa-miR-196a	2	3	0.67	1.03		1	2	0.70	1.08		
hsa-miR-196a*	0	0		NP		0	1		NP		
hsa-miR-196b	330	62	5.32	1.39	*	70	172	0.57	0.92	*	yes
hsa-miR-197	12	13	0.92	1.31		11	9	1.71	0.92		
hsa-miR-198	0	0		1.03		0	0		0.76		
hsa-miR-199a-3p	0	1	0.00	0.94		1	0		1.09		
hsa-miR-199a-5p	0	0		0.92		1	0		1.07		
hsa-miR-19a	96	108	0.89	0.56		73	69	1.48	1.17	*	
hsa-miR-19a*	0	0		0.96		0	0		1.06		
hsa-miR-19b	105	247	0.43	0.48	*	161	274	0.82	1.12		yes
hsa-miR-19b-1*	3	0		0.86		1	1	1.40	1.06		
hsa-miR-200a	353	437	0.81	1.01		255	308	1.16	0.89		
hsa-miR-200a*	3	7	0.43	0.94		5	3	2.33	1.05		
hsa-miR-200b	871	817	1.07	1.09		625	247	3.54	0.90	*	yes
hsa-miR-200b*	17	6	2.83	0.93		16	7	3.20	0.98		
hsa-miR-200c	4,759	3,026	1.57	1.13	*	1,820	360	7.07	0.90	*	
hsa-miR-200c*	1	1	1.00	0.97		0	0		1.04		
hsa-miR-203	21	41	0.51	0.89	*	35	36	1.36	0.94	*	
hsa-miR-204	0	0		0.93		0	0		1.12		
hsa-miR-205	0	0		0.97		0	0		1.07		
hsa-miR-206	0	0		0.95		0	0		1.12		
hsa-miR-208	0	0		1.04		0	0		0.93		
hsa-miR-20a	183	472	0.39	0.50	*	256	28	12.78	1.27	*	yes
hsa-miR-20a*	1	1	1.00	NP		2	3	0.93	NP		
hsa-miR-20b	0	0		0.54		0	0		1.22		
hsa-miR-20b*	0	0		0.84		0	0		1.08		
hsa-miR-21	2,760	3,964	0.70	0.71	*	2,053	490	5.86	1.12	*	yes
hsa-miR-21*	21	28	0.75	NP		7	13	0.75	NP		
hsa-miR-210	79	74	1.07	0.85		47	159	0.41	1.08	*	
hsa-miR-211	0	0		0.91		0	0		1.10		
hsa-miR-212	0	0		0.75		1	0		1.17		
hsa-miR-214	0	0		1.10		0	0		0.96		
hsa-miR-214*	0	0		0.91		0	0		1.13		

hsa-miR-215	0	0		0.93		0	1		1.11		
hsa-miR-216a	0	0		0.92		0	0		1.06		
hsa-miR-216b	0	0		0.98		0	0		1.04		
hsa-miR-217	0	0		1.11		0	0		1.04		
hsa-miR-218	0	0		0.90		0	0		1.08		
hsa-miR-219-1-3p	1	0		NP		1	0		NP		
hsa-miR-219-5p	0	0		0.99		6	3	2.80	1.02		
hsa-miR-22	245	113	2.17	1.12	*	100	310	0.45	1.12	*	yes
hsa-miR-22*	1	1	1.00	1.00		0	0		1.05		
hsa-miR-220	0	0		0.98		0	0		1.05		
hsa-miR-221	780	419	1.86	1.03	*	280	485	0.81	0.52	*	yes
hsa-miR-222	1,142	522	2.19	1.01	*	340	157	3.03	0.51	*	yes
hsa-miR-222*	0	0		0.98		0	0		0.84		
hsa-miR-223	0	0		0.92		0	0		1.01		
hsa-miR-224	18	5	3.60	NP		4	1	5.59	NP		
hsa-miR-23a	65	37	1.76	0.74	*	71	39	2.55	1.00	*	
hsa-miR-23a*	1	0		NP		0	1		NP		
hsa-miR-23b	70	56	1.25	0.73		34	28	1.70	0.99	*	
hsa-miR-23b*	0	0		NP		0	1		NP		
hsa-miR-24	27	32	0.84	0.74		19	32	0.83	0.96		
hsa-miR-24-1 */24-2*	0	0		0.95		0	0		1.05		
hsa-miR-25	254	297	0.86	0.88		204	190	1.50	0.93	*	
hsa-miR-25*	6	1	6.00	NP		16	7	3.20	NP		
hsa-miR-26a	41	51	0.80	0.92		53	44	1.68	0.99	*	
hsa-miR-26a-1*	0	0		1.03		3	1	4.19	1.10		
hsa-miR-26b	26	14	1.86	NP		6	7	1.20	NP		
hsa-miR-26b*	0	1	0.00	NP		0	0		NP		
hsa-miR-27a	21	14	1.50	0.83		12	2	8.39	1.00		
hsa-miR-27a*	2	2	1.00	0.94		0	11		1.04		
hsa-miR-27b	41	51	0.80	0.76		36	8	6.29	1.04	*	
hsa-miR-27b*	2	0		NP		1	5	0.28	NP		
hsa-miR-28-3p	89	46	1.94	0.87	*	78	71	1.54	1.01	*	
hsa-miR-28-5p	23	32	0.72	0.87	*	46	8	8.04	1.11	*	
hsa-miR-296-3p	1	0		NP		0	0		NP		
hsa-miR-296-5p	0	0		1.16		1	0		1.03		
hsa-miR-297	0	0		1.09		0	0		0.95		
hsa-miR-299-3p	0	0		0.95		0	0		1.04		
hsa-miR-299-5p	0	0		1.05		0	0		1.04		
hsa-miR-29a	76	33	2.30	1.35	*	21	29	1.01	0.99		yes
hsa-miR-29a*	1	0		1.00		0	3		1.07		
hsa-miR-29b	68	60	1.13	1.36		48	58	1.16	1.03		
hsa-miR-29b-1*	0	2	0.00	1.01		0	0		1.07		
hsa-miR-29b-2*	4	1	4.00	NP		1	1	1.40	NP		
hsa-miR-29c	90	99	0.91	0.95		111	105	1.48	0.99	*	
hsa-miR-29c*	9	9	1.00	0.92		6	15	0.56	1.08		
hsa-miR-301a	12	11	1.09	0.75		14	3	6.53	1.13		
hsa-miR-301b	5	2	2.50	1.13		10	2	6.99	1.10		
hsa-miR-302a	0	0		0.91		0	0		1.11		
hsa-miR-302b	0	0		1.07		0	0		0.92		
hsa-miR-302c	0	0		NA		0	0		NA		
hsa-miR-302c*	0	0		0.82		0	0		1.03		
hsa-miR-302d	0	0		NA		0	0		NA		
hsa-miR-30a	2	11	0.18	0.88		3	3	1.40	0.92		
hsa-miR-30a*	0	0		0.90		0	1		0.97		
hsa-miR-30b	200	184	1.09	0.87		218	6	50.80	0.93	*	yes
hsa-miR-30b*	0	0		1.33		0	5		0.60		
hsa-miR-30c	141	127	1.11	0.86		94	12	10.95	0.95	*	
hsa-miR-30c-1*	0	0		NP		0	1		NP		
hsa-miR-30c-2*	0	1	0.00	NP		0	0		NP		
hsa-miR-30d	25	15	1.67	0.94		19	12	2.21	0.91		
hsa-miR-30d*	0	1	0.00	0.94		0	1		1.01		
hsa-miR-30e	8	8	1.00	0.77		2	1	2.80	0.99		
hsa-miR-30e*	4	1	4.00	0.76		1	1	1.40	1.03		
hsa-miR-31	0	0		0.95		0	0		1.10		
hsa-miR-31*	0	0		0.94		0	0		1.06		
hsa-miR-32	8	6	1.33	0.68		8	0		1.12		
hsa-miR-32*	3	1	3.00	1.03		2	2	1.40	0.89		
hsa-miR-320	28	21	1.33	0.89		15	62	0.34	0.97	*	
hsa-miR-324-3p	99	84	1.18	NP		71	150	0.66	NP		
hsa-miR-324-5p	113	71	1.59	0.78	*	83	64	1.81	1.09	*	
hsa-miR-325	0	0		1.00		0	0		1.05		
hsa-miR-326	24	10	2.40	1.01		10	9	1.55	0.96		
hsa-miR-328	0	2	0.00	0.96		0	6		1.03		
hsa-miR-329	0	0		1.00		0	0		1.04		
hsa-miR-330-3p	9	5	1.80	1.02		1	7	0.20	0.98		
hsa-miR-330-5p	6	1	6.00	0.92		0	0		1.07		
hsa-miR-331-3p	155	92	1.69	0.89	*	90	30	4.19	1.03	*	
hsa-miR-331-5p	2	0		0.96		0	0		1.02		
hsa-miR-335	8	1	8.00	1.81		4	1	5.59	1.15		
hsa-miR-335*	1	0		1.01		0	0		1.03		
hsa-miR-337-5p	0	0		NA		0	0		1.07		
hsa-miR-338-3p	0	0		0.82		0	0		1.07		
hsa-miR-338-5p	0	0		0.91		0	0		1.12		
hsa-miR-339-3p	17	9	1.89	0.89		17	26	0.91	1.06		
hsa-miR-339-5p	36	50	0.72	0.76	*	47	36	1.83	1.04	*	
hsa-miR-33a	4	2	2.00	0.84		3	0		1.35		

hsa-miR-33a*	4	4	1.00	0.97		3	2	2.10	1.15		
hsa-miR-33b	31	25	1.24	0.94		49	67	1.02	1.10		
hsa-miR-33b*	0	0		0.97		0	0		1.01		
hsa-miR-340	0	1	0.00	0.78		2	1	2.80	1.10		
hsa-miR-340*	2	1	2.00	NP		7	0		NP		
hsa-miR-342-3p	65	47	1.38	0.84		56	59	1.33	1.02		
hsa-miR-342-5p	0	1	0.00	NP		3	13	0.32	NP		
hsa-miR-345	14	14	1.00	0.84		14	24	0.82	0.97		
hsa-miR-346	0	0		1.01		0	0		1.02		
hsa-miR-34a	299	320	0.93	0.81		200	128	2.18	0.87	*	
hsa-miR-34a*	3	1	3.00	0.92		1	0		1.03		
hsa-miR-34b*	0	0		0.97		0	0		1.05		
hsa-miR-34c-5p	0	0		0.93		0	0		1.12		
hsa-miR-361-3p	8	9	0.89	0.92		7	2	4.89	1.09		
hsa-miR-361-5p	0	0		0.76		1	1	1.40	1.04		
hsa-miR-362-3p	1	2	0.50	0.99		3	0		0.98		
hsa-miR-362-5p	1	0		1.07		1	5	0.28	0.96		
hsa-miR-363	0	0		0.98		0	0		1.04		
hsa-miR-363*	0	0		0.99		0	0		1.03		
hsa-miR-365	33	24	1.38	1.35		16	5	4.47	1.02		
hsa-miR-367	0	0		0.93		0	0		1.06		
hsa-miR-369-3p	0	0		0.99		0	0		0.86		
hsa-miR-369-5p	0	0		0.97		0	0		1.00		
hsa-miR-370	0	0		0.89		0	0		1.10		
hsa-miR-371-3p	0	0		0.91		0	0		1.11		
hsa-miR-371-5p	0	0		1.30		0	0		0.91		
hsa-miR-372	0	0		1.01		0	0		1.03		
hsa-miR-373	2	0		0.94		0	1		1.15		
hsa-miR-373*	0	0		1.18		0	0		1.02		
hsa-miR-374a	3	4	0.75	1.03		1	1	1.40	1.03		
hsa-miR-374a*	0	2	0.00	1.03		0	0		1.04		
hsa-miR-374b	6	3	2.00	1.11		6	1	8.39	0.98		
hsa-miR-374b*	1	0		NP		0	0		NP		
hsa-miR-375	2	0		1.14		1	0		1.20		
hsa-miR-376a	0	0		0.94		0	0		1.02		
hsa-miR-376b	0	0		0.95		0	0		1.03		
hsa-miR-376c	0	0		0.96		0	0		1.04		
hsa-miR-377	0	0		0.98		0	0		1.05		
hsa-miR-378	25	8	3.13	0.94		33	38	1.21	1.29		
hsa-miR-378	0	0		0.96		0	0		1.38		
hsa-miR-378*	8	4	2.00	0.95		1	3	0.47	1.06		
hsa-miR-379	0	0		1.02		0	0		1.03		
hsa-miR-380	0	0		NA		0	0		NA		
hsa-miR-380*	0	0		1.03		0	0		0.94		
hsa-miR-381	0	0		1.52		0	0		1.19		
hsa-miR-382	0	0		0.94		0	0		1.10		
hsa-miR-383	0	0		0.93		0	0		1.13		
hsa-miR-384	0	0		NA		0	0		NA		
hsa-miR-409-3p	0	0		1.00		0	0		1.02		
hsa-miR-409-5p	0	0		1.05		0	0		1.00		
hsa-miR-410	0	0		0.99		0	0		0.99		
hsa-miR-411	0	0		0.98		0	0		1.06		
hsa-miR-412	0	0		0.98		0	0		1.06		
hsa-miR-421	5	5	1.00	0.97		1	0		1.06		
hsa-miR-422a	0	0		1.00		0	0		1.18		
hsa-miR-423-3p	44	42	1.05	0.93		38	72	0.74	0.99		
hsa-miR-423-5p	106	74	1.43	0.87		60	301	0.28	0.89	*	
hsa-miR-424	24	14	1.71	1.02		10	14	1.00	1.06		
hsa-miR-424*	1	1	1.00	NP		0	3	0.00	NP		
hsa-miR-425	72	44	1.64	1.07	*	40	116	0.48	0.95	*	
hsa-miR-425*	11	7	1.57	0.94		1	12	0.12	1.05		
hsa-miR-429	8	12	0.67	0.88		7	6	1.63	0.94		
hsa-miR-431	0	0		0.96		0	0		1.03		
hsa-miR-432	0	0		0.98		0	0		1.07		
hsa-miR-432*	0	0		0.97		0	0		1.02		
hsa-miR-433	0	0		0.98		0	0		0.71		
hsa-miR-448	0	0		NA		0	2		NA		
hsa-miR-449a	0	0		0.93		0	0		1.04		
hsa-miR-449b	0	0		0.95		0	0		1.07		
hsa-miR-450a	1	0		0.90		0	0		1.06		
hsa-miR-450b-5p	0	0		0.90		0	2		1.03		
hsa-miR-451	0	0		0.96		0	0		0.97		
hsa-miR-452	0	0		1.14		0	0		0.97		
hsa-miR-453	0	0		0.86		0	0		NA		
hsa-miR-453	0	0		1.02		0	0		1.17		
hsa-miR-454	5	6	0.83	0.97		8	1	11.19	1.03		
hsa-miR-454*	1	4	0.25	1.01		1	0		0.97		
hsa-miR-455-3p	0	0		NA		0	0		0.89		
hsa-miR-455-5p	0	0		0.95		0	0		1.01		
hsa-miR-483-3p	0	0		1.13		0	0		0.95		
hsa-miR-483-5p	0	0		1.43		0	0		0.54		
hsa-miR-484	4	12	0.33	0.80		12	15	1.12	0.91		
hsa-miR-485-3p	0	0		1.01		0	0		0.98		
hsa-miR-485-5p	0	0		0.95		0	0		1.08		
hsa-miR-486-3p	0	0		0.98		0	0		0.99		
hsa-miR-486-5p	4	2	2.00	1.05		0	0		1.00		

hsa-miR-487a	0	0		1.01	0	0		0.96	
hsa-miR-487b	0	0		1.21	0	0		0.74	
hsa-miR-488*	0	0		0.96	0	0		1.10	
hsa-miR-489	0	0		0.94	0	0		1.08	
hsa-miR-490-3p	0	0		1.05	0	0		0.95	
hsa-miR-490-5p	0	0		1.01	0	0		1.04	
hsa-miR-491-3p	0	0		0.72	0	0		1.10	
hsa-miR-491-5p	2	1	2.00	0.93	1	3	0.47	1.16	
hsa-miR-492	0	0		1.87	0	0		1.05	
hsa-miR-493	0	0		0.95	0	0		1.04	
hsa-miR-493*	0	1	0.00	0.93	0	1		1.07	
hsa-miR-494	0	0		1.06	0	0		0.94	
hsa-miR-495	0	0		NA	0	0		1.08	
hsa-miR-496	0	0		1.08	0	0		0.89	
hsa-miR-497	140	134	1.05	0.95	130	206	0.88	0.98	
hsa-miR-497*	0	0		NP	1	0		NP	
hsa-miR-498	0	0		1.23	0	0		0.96	
hsa-miR-499-3p	0	0		1.00	0	0		0.96	
hsa-miR-499-5p	0	0		0.95	0	0		1.04	
hsa-miR-500	0	0		0.94	0	0		1.04	
hsa-miR-500*	0	0		0.98	1	0		0.99	
hsa-miR-501-3p	0	1	0.00	1.06	0	0		0.93	
hsa-miR-501-5p	0	0		0.95	0	1		1.10	
hsa-miR-502-3p	1	0		1.00	0	0		1.00	
hsa-miR-502-5p	0	0		0.97	0	0		1.08	
hsa-miR-503	6	6	1.00	1.14	4	0		0.99	
hsa-miR-504	1	0		0.96	1	1	1.40	1.04	
hsa-miR-505	9	6	1.50	0.95	10	8	1.75	1.11	
hsa-miR-505*	4	3	1.33	0.97	0	1		1.09	
hsa-miR-506	0	0		0.98	0	0		0.95	
hsa-miR-507	0	0		0.90	0	0		1.10	
hsa-miR-508-3p	0	0		0.91	0	0		1.09	
hsa-miR-509-3p	0	0		0.89	0	0		1.12	
hsa-miR-510	0	0		1.01	0	0		1.02	
hsa-miR-511	0	0		NA	0	0		NA	
hsa-miR-512-3p	3	0		0.97	0	0		1.08	
hsa-miR-512-5p	0	0		1.19	0	0		1.01	
hsa-miR-514	0	0		0.92	0	0		1.10	
hsa-miR-515-3p	0	0		NA	0	0		1.03	
hsa-miR-515-5p	0	0		1.02	0	0		1.01	
hsa-miR-516a-3p/516b*	0	0		0.93	0	0		1.11	
hsa-miR-516b	0	0		1.03	0	0		0.97	
hsa-miR-517*	0	0		0.97	0	0		0.99	
hsa-miR-517a	0	0		1.00	1	0		1.04	
hsa-miR-517c	0	0		0.95	0	0		1.05	
hsa-miR-518a-3p	0	0		0.96	0	0		1.07	
hsa-miR-518a-5p	2	0		1.10	0	0		0.99	
hsa-miR-518b	0	0		1.44	0	0		1.00	
hsa-miR-518c	0	0		0.95	0	0		1.05	
hsa-miR-518c*	0	0		1.02	0	0		0.89	
hsa-miR-518d-3p	0	0		1.01	0	0		1.02	
hsa-miR-518d-5p/518f*/518g*	0	0		0.99	0	0		0.92	
hsa-miR-518e	0	0		0.93	0	0		1.06	
hsa-miR-518e*/519a*/519b*	0	0		1.09	0	0		0.94	
hsa-miR-518f*	0	0		1.05	0	0		0.92	
hsa-miR-519b-3p	2	0		1.01	0	0		1.05	
hsa-miR-519c-3p	1	0		0.94	0	0		1.10	
hsa-miR-519d	0	0		1.31	0	0		1.15	
hsa-miR-519e	0	0		0.94	0	0		0.95	
hsa-miR-519e*	1	0		1.05	1	0		0.95	
hsa-miR-520a-3p	2	0		NA	0	0		1.02	
hsa-miR-520a-5p	0	0		0.93	0	0		1.04	
hsa-miR-520b/520c-3p	0	0		1.05	0	0		1.05	
hsa-miR-520c-3p/520f	0	0		1.02	0	0		1.12	
hsa-miR-520d-3p	0	0		0.92	0	0		1.11	
hsa-miR-520e	0	0		0.93	0	0		1.10	
hsa-miR-520g	0	0		1.07	0	0		0.95	
hsa-miR-520g/520h	0	0		0.98	0	0		1.04	
hsa-miR-521	0	0		0.98	0	0		1.04	
hsa-miR-523	0	0		0.93	0	0		1.05	
hsa-miR-524-3p	0	0		1.01	0	0		0.96	
hsa-miR-524-5p	0	0		0.97	0	0		1.06	
hsa-miR-525-3p	0	0		1.03	0	0		0.94	
hsa-miR-525-5p	0	0		1.06	0	0		0.95	
hsa-miR-526b	0	0		1.03	0	0		0.98	
hsa-miR-526b*	1	0		0.98	0	0		1.04	
hsa-miR-532-3p	5	11	0.45	NP	5	10	0.70	NP	
hsa-miR-532-5p	15	10	1.50	0.89	6	3	2.80	1.03	
hsa-miR-539	0	0		0.89	0	0		1.06	
hsa-miR-541*	0	0		NA	0	0		0.94	
hsa-miR-542-3p	3	1	3.00	0.88	0	1		1.05	
hsa-miR-542-5p	0	2	0.00	0.92	0	1		1.03	
hsa-miR-543	0	0		1.07	0	0		0.98	
hsa-miR-544	0	0		NA	0	0		NA	
hsa-miR-545	0	0		0.91	1	0		1.14	
hsa-miR-548a-3p	0	0		0.92	0	0		1.14	

hsa-miR-548a-5p	0	0		1.02		0	0		NA	
hsa-miR-548b-3p	1	2	0.50	0.82		0	0		1.16	
hsa-miR-548c-3p	0	0		1.03		0	0		0.98	
hsa-miR-548d-3p	0	0		1.02		0	0		1.03	
hsa-miR-549	0	0		0.97		0	0		1.02	
hsa-miR-550*	0	0		1.05		0	0		1.00	
hsa-miR-551a	1	0		0.97		4	1	5.59	1.02	
hsa-miR-551b	0	0		0.95		0	0		1.09	
hsa-miR-552	0	0		1.00		0	0		1.00	
hsa-miR-553	0	0		0.99		0	0		1.00	
hsa-miR-554	0	0		0.99		0	0		1.00	
hsa-miR-555	0	0		NA		0	0		1.07	
hsa-miR-556-5p	0	0		NA		0	0		NA	
hsa-miR-557	0	0		1.97	*	0	0		0.93	yes
hsa-miR-558	0	0		0.96		0	0		1.08	
hsa-miR-559	0	0		1.10		0	0		0.89	
hsa-miR-560	0	0		0.95		0	0		1.09	
hsa-miR-561	0	0		1.03		0	0		0.99	
hsa-miR-562	0	0		0.92		0	0		NA	
hsa-miR-563	0	0		0.96		0	0		1.01	
hsa-miR-564	0	0		0.97		0	0		1.03	
hsa-miR-565	0	0		0.91		0	0		0.97	
hsa-miR-566	0	0		0.98		0	0		1.01	
hsa-miR-567	0	0		0.95		0	0		NA	
hsa-miR-568	0	0		1.01		0	0		NA	
hsa-miR-569	0	0		0.95		0	0		1.03	
hsa-miR-570	0	0		0.91		0	0		1.09	
hsa-miR-571	0	0		0.92		0	0		1.09	
hsa-miR-572	0	0		1.03		0	0		0.99	
hsa-miR-573	0	0		0.92		0	0		1.11	
hsa-miR-574-3p	4	7	0.57	1.01		9	3	4.19	0.91	
hsa-miR-574-5p	18	10	1.80	1.03		2	34	0.08	0.93	*
hsa-miR-575	0	0		0.95		0	0		1.08	
hsa-miR-576-3p	0	0		1.01		0	0		1.01	
hsa-miR-577	0	0		NA		0	0		0.98	
hsa-miR-578	0	0		0.87		0	0		1.15	
hsa-miR-580	0	0		0.93		0	0		1.11	
hsa-miR-581	0	0		0.91		0	0		1.08	
hsa-miR-582-3p	0	0		1.05		0	0		0.95	
hsa-miR-582-5p	0	0		1.04		1	0		0.95	
hsa-miR-583	0	0		1.15		0	0		0.68	
hsa-miR-584	0	0		1.38		0	0		1.00	
hsa-miR-585	0	0		0.94		0	0		1.08	
hsa-miR-586	0	0		0.99		0	0		1.03	
hsa-miR-587	0	0		0.97		0	0		1.05	
hsa-miR-588	0	0		0.95		0	0		1.06	
hsa-miR-589	0	0		NP		1	0		NP	
hsa-miR-589*	0	0		1.01		0	0		1.01	
hsa-miR-590-3p	0	0		1.07		2	0		1.06	
hsa-miR-590-5p	8	5	1.60	1.09		6	0		1.22	
hsa-miR-591	0	0		0.87		0	0		1.15	
hsa-miR-592	0	0		0.89		0	0		1.19	
hsa-miR-593*	0	0		0.95		0	0		1.03	
hsa-miR-595	0	0		0.93		0	0		1.10	
hsa-miR-596	0	0		1.01		0	0		0.61	
hsa-miR-597	0	1	0.00	1.02		1	1	1.40	0.83	
hsa-miR-598	5	0		1.34		0	1		1.13	
hsa-miR-599	0	0		NA		0	0		NA	
hsa-miR-600	0	0		0.82		0	0		1.12	
hsa-miR-601	0	0		0.91		0	0		1.07	
hsa-miR-602	0	0		1.67		0	0		1.03	
hsa-miR-603	0	0		0.90		0	0		1.13	
hsa-miR-604	0	0		0.99		0	0		1.01	
hsa-miR-605	0	0		1.00		0	0		0.99	
hsa-miR-606	0	0		NA		0	0		0.98	
hsa-miR-607	0	0		0.99		0	0		NA	
hsa-miR-608	0	0		0.93		0	0		1.16	
hsa-miR-609	0	0		0.93		0	0		1.06	
hsa-miR-610	0	0		1.00		0	0		1.04	
hsa-miR-611	0	0		1.21		0	0		0.83	
hsa-miR-612	0	0		1.03		0	0		1.03	
hsa-miR-613	0	0		0.98		0	0		1.00	
hsa-miR-614	0	0		1.02		0	0		1.00	
hsa-miR-615-3p	47	32	1.47	1.04		14	52	0.38	0.90	*
hsa-miR-615-5p	1	0		NP		0	3		NP	
hsa-miR-616*	0	0		0.86		0	0		1.15	
hsa-miR-617	0	0		0.99		0	0		0.99	
hsa-miR-618	1	0		1.07		1	1	1.40	1.08	
hsa-miR-619	0	0		0.97		0	0		1.07	
hsa-miR-620	0	0		0.97		0	0		1.01	
hsa-miR-621	0	0		0.99		0	0		0.97	
hsa-miR-622	0	0		0.90		0	0		1.13	
hsa-miR-623	0	0		1.33		0	0		1.02	
hsa-miR-624	0	0		1.01		0	0		0.99	
hsa-miR-624*	0	0		0.93		0	0		1.10	
hsa-miR-625	0	0		0.95		1	1	1.40	0.86	

hsa-miR-625*	0	0		1.06		0	0		0.99		
hsa-miR-626	0	0		NA		0	0		1.06		
hsa-miR-627	0	0		1.03		0	0		1.02		
hsa-miR-628-3p	4	1	4.00	0.99		0	0		1.02		
hsa-miR-628-5p	0	0		0.97		0	0		1.04		
hsa-miR-629	15	3	5.00	1.08		5	21	0.33	0.91		
hsa-miR-629*	0	0		0.98		0	1		0.92		
hsa-miR-630	0	0		1.04		0	0		0.95		
hsa-miR-631	0	0		1.01		0	0		0.97		
hsa-miR-632	0	0		0.95		0	0		1.09		
hsa-miR-633	0	0		1.01		0	0		0.97		
hsa-miR-634	0	0		1.00		0	0		1.02		
hsa-miR-635	0	0		1.01		0	0		1.04		
hsa-miR-636	0	0		0.95		0	0		NA		
hsa-miR-637	0	0		1.27		0	0		1.01		
hsa-miR-638	0	0		1.14		0	0		1.00		
hsa-miR-639	0	0		NA		0	0		NA		
hsa-miR-640	0	0		0.98		0	0		0.97		
hsa-miR-641	0	0		1.01		0	0		1.01		
hsa-miR-642	0	0		1.24		0	0		1.01		
hsa-miR-643	0	0		0.84		0	0		1.09		
hsa-miR-644	0	0		0.93		0	0		1.10		
hsa-miR-645	0	0		1.01		0	0		1.02		
hsa-miR-646	0	0		1.00		0	0		1.04		
hsa-miR-647	0	0		1.04		0	0		0.99		
hsa-miR-648	0	0		1.02		0	0		1.00		
hsa-miR-649	0	0		0.96		0	0		1.00		
hsa-miR-650	0	0		0.96		0	0		1.05		
hsa-miR-651	0	0		0.89		0	0		1.03		
hsa-miR-652	9	4	2.25	1.19		4	0		1.20		
hsa-miR-654-3p	0	0		0.99		0	0		0.94		
hsa-miR-654-5p	0	0		1.20		0	0		1.06		
hsa-miR-655	0	0		0.98		0	0		1.05		
hsa-miR-656	0	0		0.96		0	0		1.01		
hsa-miR-657	0	0		1.01		0	0		0.95		
hsa-miR-658	0	0		1.04		0	0		0.82		
hsa-miR-659	0	0		0.99		0	0		0.88		
hsa-miR-660	26	11	2.36	0.95		20	11	2.54	1.07		
hsa-miR-661	0	0		1.01		0	0		1.06		
hsa-miR-662	0	0		1.06		0	0		1.01		
hsa-miR-663	0	0		1.64		0	0		0.99		
hsa-miR-665	0	0		1.22		0	0		0.82		
hsa-miR-668	0	0		1.22		0	0		0.84		
hsa-miR-671-3p	1	1	1.00	NP		0	2		NP		
hsa-miR-671-5p	0	0		1.04		0	0		0.82		
hsa-miR-671-5p	0	0		1.31		0	0		0.91		
hsa-miR-675	0	0		1.08		0	0		0.90		
hsa-miR-7	23	19	1.21	1.01		17	46	0.52	1.04	*	
hsa-miR-708	0	3	0.00	NP		3	6	0.70	NP		
hsa-miR-7-1*	59	22	2.68	1.06	*	26	3	12.12	1.06		
hsa-miR-744	56	61	0.92	0.67		58	86	0.94	1.10		
hsa-miR-744*	3	3	1.00	NP		0	0		NP		
hsa-miR-758	0	0		1.03		0	0		0.99		
hsa-miR-760	18	3	6.00	1.00		4	32	0.17	1.02	*	
hsa-miR-765	0	0		1.06		0	0		0.76		
hsa-miR-766	4	2	2.00	1.28		1	3	0.47	1.00		
hsa-miR-767-3p	1	0		1.02		1	0		0.95		
hsa-miR-767-5p	25	17	1.47	1.05		13	31	0.59	0.91	*	
hsa-miR-768-3p	0	0		0.88		0	0		1.03		
hsa-miR-768-5p	0	0		0.93		0	0		1.07		
hsa-miR-769-3p	6	3	2.00	0.88		1	4	0.35	1.09		
hsa-miR-769-5p	2	5	0.40	0.91		1	15	0.09	1.07		
hsa-miR-770-5p	0	0		1.01		0	0		0.95		
hsa-miR-801	0	0		1.13		0	0		1.00		
hsa-miR-802	0	0		NA		0	0		NA		
hsa-miR-874	1	0		NP		0	0		NP		
hsa-miR-875-3p	0	0		0.91		0	0		1.05		
hsa-miR-876-3p	0	0		1.03		0	0		0.99		
hsa-miR-876-5p	0	0		1.03		0	0		NA		
hsa-miR-877	10	9	1.11	NP		3	9	0.47	NP		
hsa-miR-885-5p	0	0		1.08		0	0		0.95		
hsa-miR-886-3p	0	0		1.05		0	0		1.04		
hsa-miR-886-5p	0	0		1.06		0	0		1.05		
hsa-miR-887	0	1	0.00	1.07		0	0		0.99		
hsa-miR-888	0	0		NA		0	0		1.01		
hsa-miR-890	0	0		0.94		0	0		1.01		
hsa-miR-891a	0	0		1.15		0	0		0.84		
hsa-miR-891b	0	0		NA		0	0		NA		
hsa-miR-9	15	0		2.76	*	1	1	1.40	1.14		yes
hsa-miR-9*	1	0		2.05	*	0	0		1.03		
hsa-miR-92a	73	165	0.44	0.51	*	85	23	5.17	1.11	*	yes
hsa-miR-92a-1*	2	3	0.67	NP		0	4		NP		
hsa-miR-92b	12	7	1.71	0.60		13	3	6.06	1.03		
hsa-miR-92b*	1	1	1.00	NP		4	2	2.80	NP		
hsa-miR-93	966	955	1.01	0.76		500	1,036	0.67	1.07		
hsa-miR-93*	17	8	2.13	0.85		9	33	0.38	1.12	*	

hsa-miR-934	0	0		1.02		0	0		0.86		
hsa-miR-939	0	0		NP		0	1		NP		
hsa-miR-940	3	0		1.76		0	3		0.99		
hsa-miR-941	13	9	1.44	NP		7	3	3.26	NP		
hsa-miR-95	0	0		0.97		1	0		0.98		
hsa-miR-96	2	6	0.33	0.89		0	0		0.87		
hsa-miR-96*	0	0		0.99		0	0		0.82		
hsa-miR-98	99	66	1.50	0.98		58	68	1.19	0.86		
hsa-miR-99a	1,300	6,237	0.21	0.39	*	2,850	6,634	0.60	0.72	*	yes
hsa-miR-99a*	14	24	0.58	0.43		28	25	1.57	0.89		
hsa-miR-99b	151	245	0.62	0.66	*	127	329	0.54	0.88	*	yes
hsa-miR-99b*	10	6	1.67	1.01		12	6	2.80	0.74		

	miRNA	confirmation methods			
		microarray	cloning	qPCR	RPA Northern
		C4-2/LNCaP ratio			
miRNAs significantly decreased in C4-2 relative to LNCaP	hsa-miR-100	0.38	NA	0.17	
	hsa-miR-125b	0.34	0.31	0.21	
	hsa-miR-19b	0.48	0.43	0.23	
	hsa-miR-99a	0.39	0.21	0.26	C4-2 << LNCaP
miR-17-92 cluster	hsa-miR-17-5p	0.52	0.61		C4-2 < LNCaP
	hsa-miR-18a	0.62	0.79		C4-2 < LNCaP
	hsa-miR-20a	0.50	0.39	0.36	C4-2 < LNCaP
	hsa-miR-92a	0.51	0.44		C4-2 = < LNCaP
miRNAs mildly decreased in C4-2 relative to LNCaP	hsa-miR-106a	0.49	0.97	0.41	
	hsa-miR-99b	0.66	0.62	0.43	
	hsa-miR-21	0.71	0.70	0.50	C4-2 = < LNCaP
	hsa-miR-16	0.86	0.61	0.59	C4-2 = < LNCaP
miRNAs with no significant change or inconsistent change among the techniques. let-7s were included because they are the most abundantly cloned.	hsa-miR-222	1.01	2.19	0.92	
	hsa-miR-29a	1.35	2.30	1.12	
	hsa-miR-15b	1.07	0.84		C4-2 >= LNCaP
	hsa-let-7c	1.04	0.34		C4-2 >= LNCaP
	hsa-let-7b	1.02	1.51		C4-2 >= LNCaP
	hsa-miR-200b	1.09	1.07	0.79	
	hsa-miR-22	1.12	2.17	0.81	
	hsa-miR-130b	1.18	3.72	0.20	
	hsa-miR-196b	1.39	5.32	2.48	
	hsa-miR-557	1.97	NA	3.28	
miRNAs significantly increased in C4-2 relative to LNCaP	hsa-miR-9	2.76	NA	5.86	

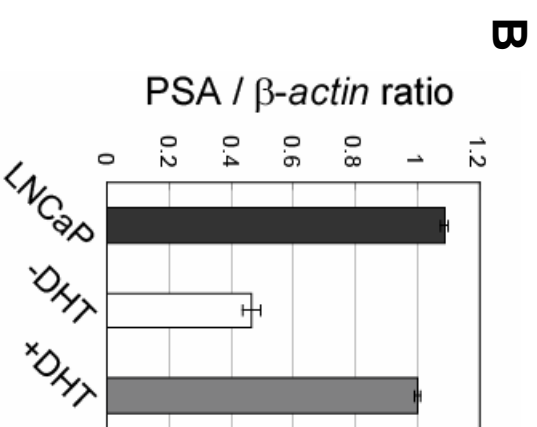
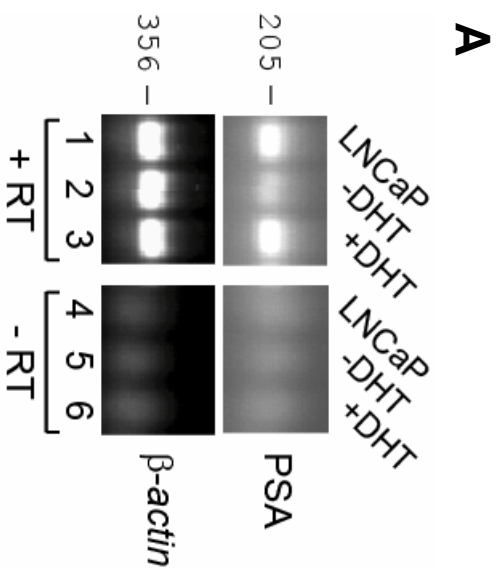


	miRNA	confirmation methods				
		microarray	cloning	qPCR	RPA	Northern
		+DHT/-DHT ratio				
miRNAs significantly decreased in the presence of synthetic androgen	hsa-miR-221	0.52	0.81	0.42		
	hsa-miR-196b	0.92	0.57	0.58		
	hsa-miR-125b	0.73	0.58	0.64		
	hsa-miR-99a	0.72	0.60	0.89	+DHT < -DHT	
	hsa-miR-29a	0.99	1.01	1.05		
miRNAs with no significant change or inconsistent change among the techniques. let-7s were included because they are the most abundantly cloned.	hsa-miR-99b	0.88	0.54	1.30		
	hsa-let-7c	1.04	0.61			+DHT =< -DHT
	hsa-let-7b	0.88	0.76			+DHT =< -DHT
	hsa-miR-15b	1.06	2.16		+DHT = -DHT	
	hsa-miR-16	1.04	5.02	1.03	+DHT < -DHT	
miRNAs with a fold change >5 (+DHT relative to LNA-) in cloning frequency	hsa-miR-92a	1.11	5.17		+DHT = -DHT	
	hsa-miR-21	1.12	5.86	0.98		+DHT =< -DHT
	hsa-miR-17-5p	1.21	6.07		+DHT > -DHT	
	hsa-miR-18a	1.21	6.94		+DHT = -DHT	
	hsa-miR-200c	0.90	7.07	0.60		
	hsa-miR-106a	1.31	10.55	1.26		
	hsa-miR-20a	1.27	12.78	1.18	+DHT >= -DHT	
	hsa-miR-30b	0.93	50.80	1.16		

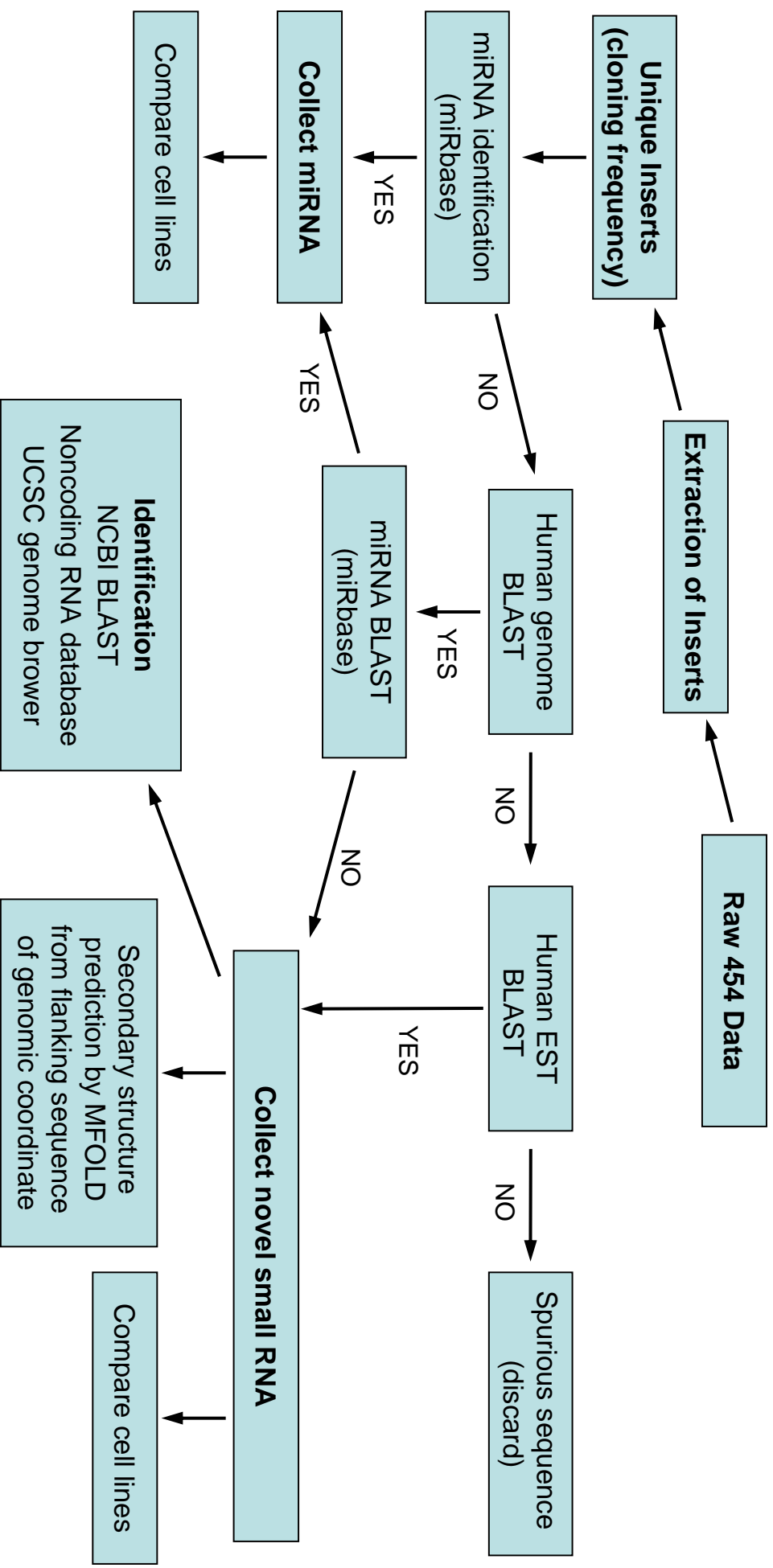
Lee et al\_Table S3

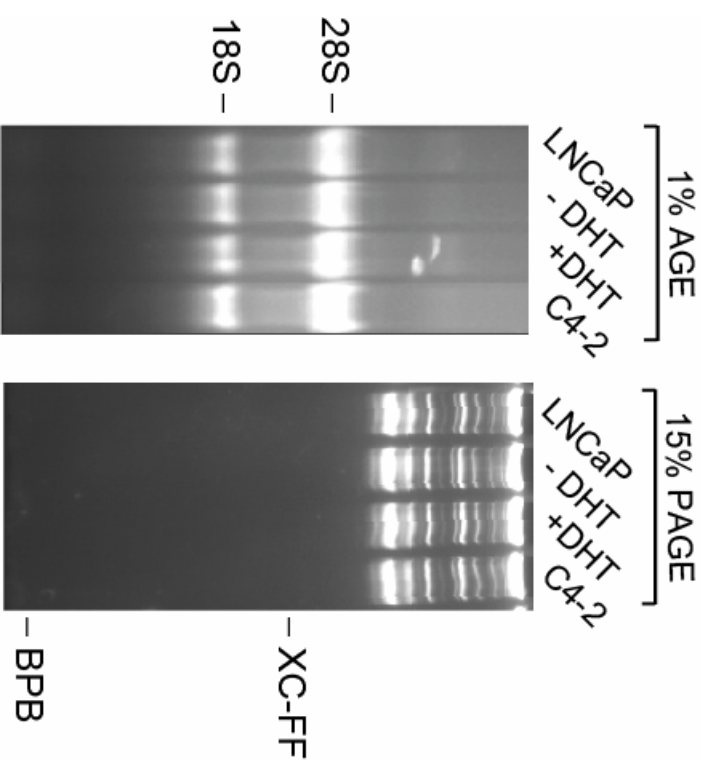
		<b>C4-2</b>	<b>LNCaP</b>	<b>+DHT</b>	<b>- DHT</b>
<b>total unique sequences</b>		<b>15776</b>	<b>11756</b>	<b>10517</b>	<b>22069</b>
<b>unique sequences of non-micro-short RNA</b>		<b>14319</b>	<b>10420</b>	<b>9351</b>	<b>20828</b>
	<b>fraction (%)</b>	90.76	88.64	88.91	94.38
<b>distribution of unique sequences of non-micro-short RNA</b>					
<b>rRNA</b>	<b>number of unique seq</b>	<b>1137</b>	<b>399</b>	<b>394</b>	<b>822</b>
	<b>fraction (%)</b>	7.94	3.83	4.21	3.95
<b>tRNA</b>	<b>number of unique seq</b>	<b>952</b>	<b>821</b>	<b>512</b>	<b>822</b>
	<b>fraction (%)</b>	6.65	7.88	5.48	3.95
<b>snoRNA</b>	<b>number of unique seq</b>	<b>147</b>	<b>139</b>	<b>125</b>	<b>244</b>
	<b>fraction (%)</b>	1.03	1.33	1.34	1.17
<b>piRNA</b>	<b>number of unique seq</b>	<b>358</b>	<b>325</b>	<b>275</b>	<b>423</b>
	<b>fraction (%)</b>	2.50	3.12	2.94	2.03
<b>refseq</b>	<b>number of unique seq</b>	<b>627</b>	<b>323</b>	<b>520</b>	<b>1831</b>
	<b>fraction (%)</b>	4.38	3.10	5.56	8.79
<b>others</b>	<b>number of unique seq</b>	<b>11098</b>	<b>8413</b>	<b>7525</b>	<b>16686</b>
	<b>fraction (%)</b>	77.51	80.74	80.47	80.11

Lee et al\_Table S4

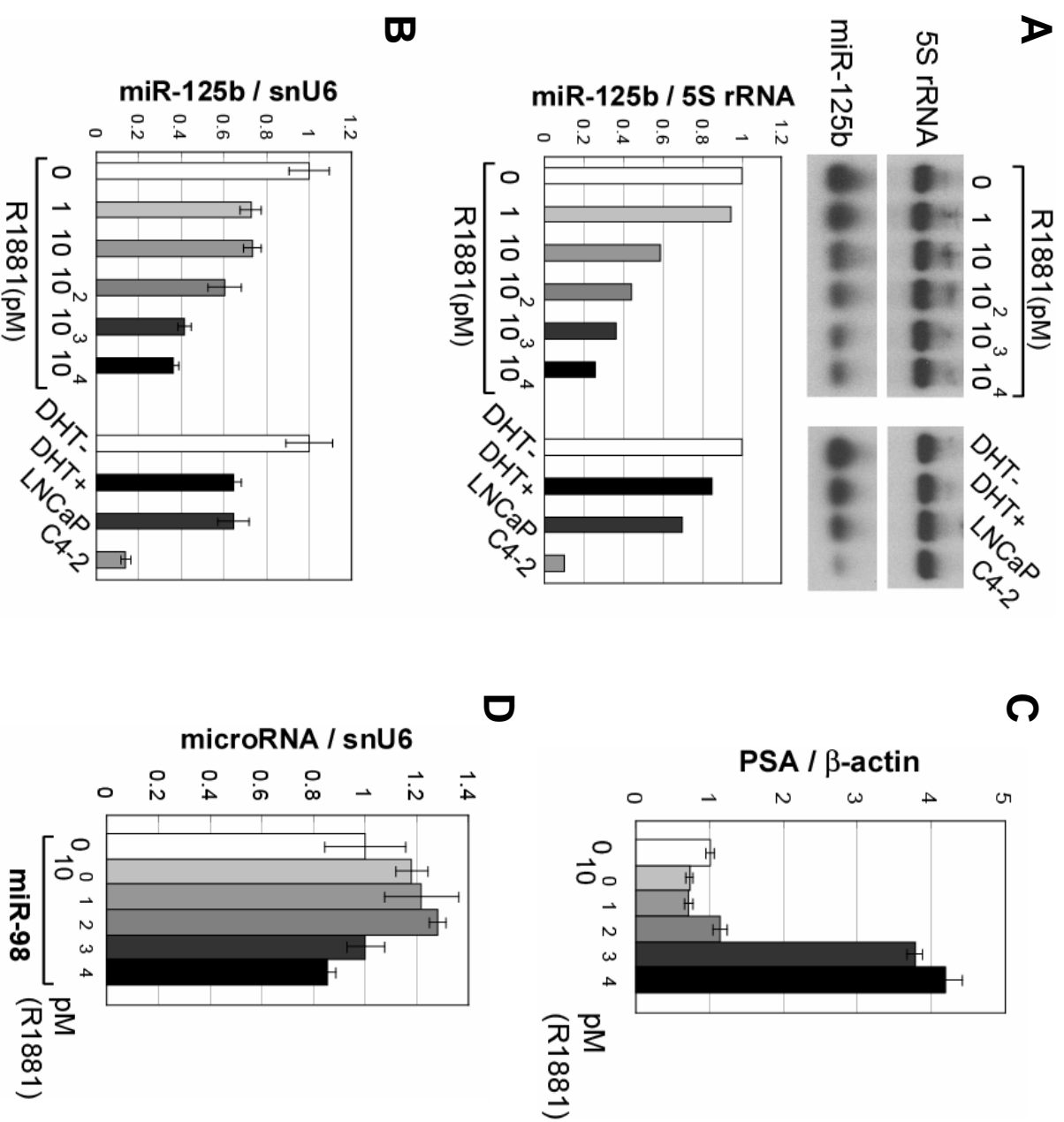


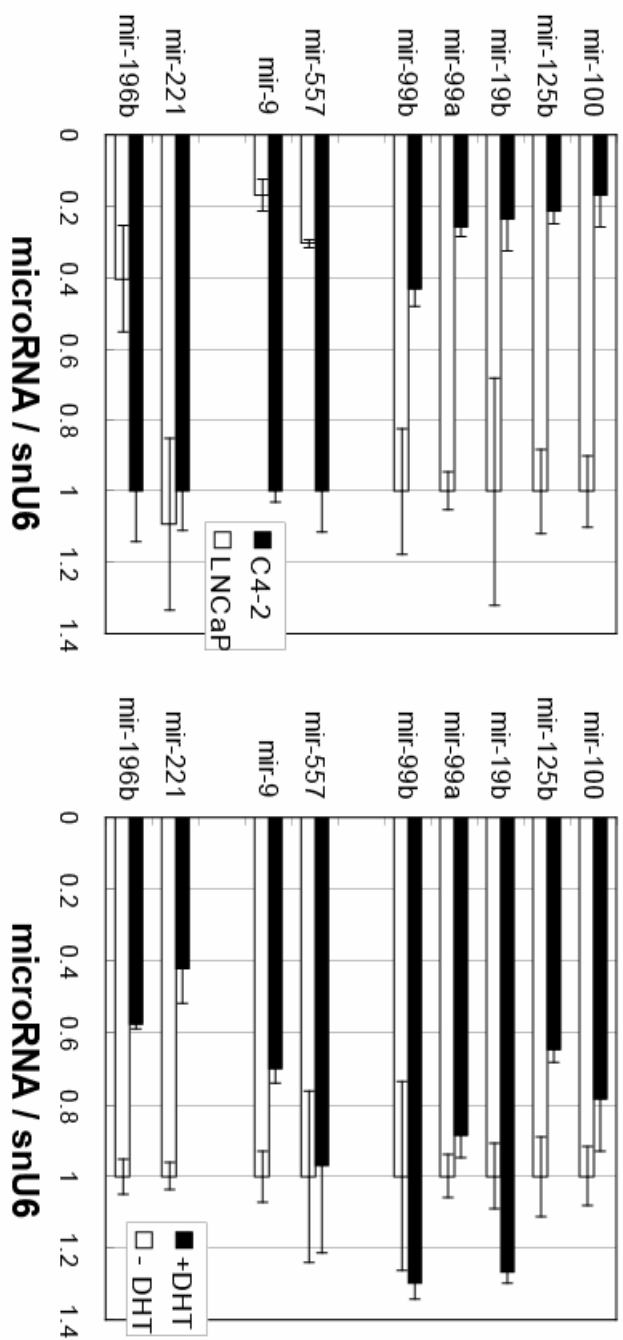
Lee et al\_Figure S1



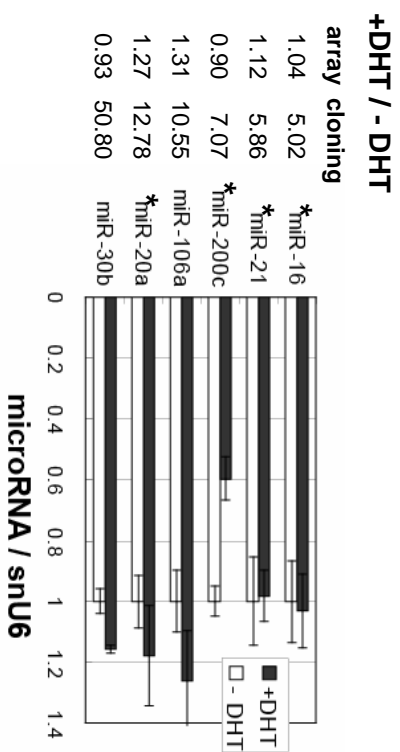


Lee et al\_Figure S3





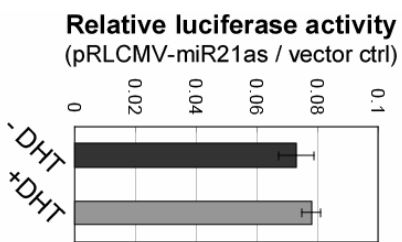
Lee et al\_Figure S5



Lee et al\_Figure S6



A



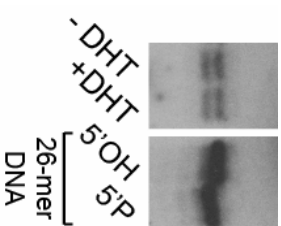
B

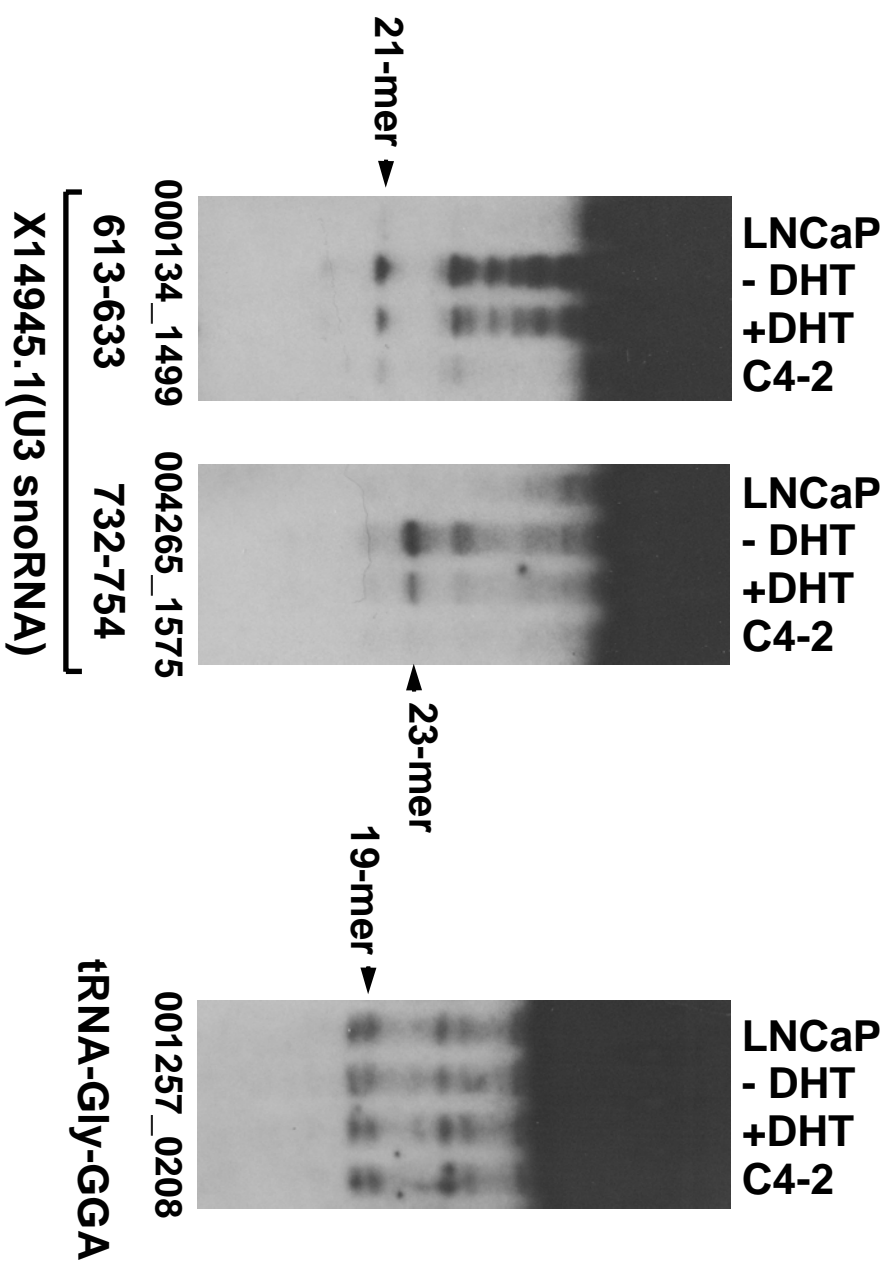
cloned sequence	cloning frequency	
	- DHT	+DHT
TAGCTTATCAGACTGATGTTGAC	187	603
TAGCTTATCAGACTGATGTTGA	201	910
⋮	⋮	⋮
all miR-21s	490	2053

...gggTUAGCUUAUCAGACUGAUGUUGAcugu...

mature miR-21

C





Lee et al\_Figure S8

**A novel class of small RNAs, tRNA-derived RNA fragments (tRFs), is highly expressed in proliferating cells and required for cell proliferation.**

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running title; tRNA-derived small RNAs

key words; small RNA, tRNA, deep sequencing, cancer cell proliferation

## Abstract

New types of small RNAs distinct from microRNAs (miRNAs) are progressively being discovered in various organisms. In order to discover such novel small RNAs, a library of 17-26 base long RNAs was created from prostate cancer cell lines and sequenced by ultra-highthroughput sequencing. A significant number of the sequences are derived from precise processing at the 5'- or 3'-end of mature or precursor tRNAs, to form three series of tRFs, the -5, -3 and -1 series. These sequences, collectively named as tRFs (tRNA-derived RNA Fragments), constitute a class of short RNAs that are second-most abundant to miRNAs. Northern hybridization and qRT-PCR assays independently measured the levels of at least 16 tRFs. tRF-1001, derived from the 3'-end of a Ser-TGA tRNA precursor transcript that is not retained in the mature tRNA, was further investigated. tRF-1001 is expressed highly in a wide range of cancer cell lines but much less in tissues and its expression in cell lines was tightly correlated with cell proliferation. siRNA-mediated knockdown of tRF-1001 impaired cell proliferation with the specific accumulation of cells in G2, phenotypes that were reversed by co-introducing a synthetic 2'-O-methyl tRF-1001 oligoribonucleotide resistant to the siRNA. Although tRF-1001 is localized in the cytoplasm and similar in size to miRNAs and siRNAs, it did not repress a target mRNA, suggesting that it does

not enter into RISC or miRNP complexes. Our data suggest that tRFs are not random by-products of tRNA degradation or biogenesis but an abundant and novel class of short RNAs with precise sequence structure that have specific expression patterns and specific biological roles.

## **Introduction**

Small non-coding regulatory RNAs have emerged as important players in diverse aspects of biology. The most extensively studied among them are microRNAs (miRNAs) that regulate target mRNA post-transcriptionally in animals and plants (reviewed in (Bartel 2004; Du and Zamore 2005; Lee and Dutta 2008)). Another class of small RNA closely related to miRNAs is small interfering RNA (siRNA), a key intermediate in the RNA interference pathway (Elbashir et al. 2001b). Although siRNAs are a popular tool to knockdown genes in mammalian cells, the existence of endogenous siRNAs is still questionable in mammals. This is in contrast to plants where naturally occurring siRNAs, tasiRNA (trans-acting siRNA) and natsiRNA (natural antisense transcripts siRNA), have been discovered (reviewed in (Chapman and Carrington 2007)).

A number of other small RNAs have also been identified in various organisms (reviewed in (Farazi et al. 2008)), including tncRNA (tiny noncoding RNAs), 21U-RNA, rasiRNA (repeat associated siRNA), hcRNA (heterochromatic small RNA), scnRNA (scan RNA), and piRNA (piwi-interacting RNA). Although some of these have been shown to play biological roles in processes such as heterochromatin formation and transposon silencing, most of them are recently discovered and not fully understood.

We believe that additional small RNAs await discovery especially through the application of high-throughput sequencing technology (reviewed in (Morozova and Marra 2008)).

In this paper, we analyzed the global expression profile of small RNA (small RNome) in human prostate cancer cell lines by 454 deep sequencing. Next to miRNAs, the most abundant group of small RNA was fragments derived from tRNA (termed here as “tRNA-derived RNA Fragments; tRFs”). Besides their well-known role in translation, tRNAs have been shown to function in other cellular processes such as reverse transcription, porphyrin biosynthesis, *etc* (reviewed in (Giege 2008)). Here, we identify a new aspect of tRNA biology. The characteristic and abundant expression of individual tRFs reported here as well as their precise sequence composition, indicates that tRFs are not random degradation intermediates during tRNA biogenesis and turnover, but constitute a novel class of small RNA that deserve further scrutiny. One member of tRFs is shown to be required for cell viability.

## Results

**Cloning and identification of tRF-5, -3 and -1 series.** Small RNAs of 17-26 nucleotides were cloned from prostate cancer cells and sequenced by 454 deep sequencing technology (Lee et al, in preparation). More than half of the sequence reads did not match miRNAs. Among these non-micro-small RNAs (nmsRNAs), we focused on 30 sequences that were abundantly cloned (>300 times; Table 1). In comparison, a well-known miRNA with a demonstrated biological function, miR-18a, was cloned 473 times while a highly abundant miRNA, *let-7a*, was cloned 17,856 times. Of 695 miRNAs in the database (miRBase; <http://microrna.sanger.ac.uk/>), a majority of them (635 miRNAs) were cloned <200 times.

Each of these abundant nmsRNA sequences were subjected to BLAST search of the human genome sequence and was further identified by examining its genomic locus on the UCSC genome browser. 28 sequences were mappable to the human genome, in many cases at multiple loci (Fig 1A). Six of these are located within known transcripts and snoRNA (small nucleolar RNA), from which the small RNAs are likely to be derived. Of note, more than half (17 out of 30) of the nmsRNA sequences mapped to tRNA loci (Fig 1A and Table 1). Given that a cloning frequency approximately reflects the intracellular level of a small RNA, these tRNA-related small RNAs are



expected to be more abundant than most miRNAs.

Primary transcripts (pre-tRNA) for mature tRNA are transcribed by RNA polymerase III and contain a 5' leader sequence and a 3' trailer sequence that are trimmed during tRNA maturation (reviewed in (Morl and Marchfelder 2001; Hopper and Phizicky 2003)). For most eukaryotic tRNAs, a CCA sequence is enzymatically added to the 3'-end of the trimmed tRNA intermediate. Among the 17 tRNA-related small RNAs, five and eight sequences are precisely aligned to the 5'- and 3'-ends of mature tRNA, respectively (Fig 1 and S1, tRF-5 and tRF-3 series). The 3'-end sequence of all the eight tRF-3 RNAs includes the CCA that is post-transcriptionally added to the 3'-end of tRNAs during tRNA maturation. We postulate that the tRF-5 and tRF-3 series of small RNAs are derived from cleavage of mature tRNAs after they have undergone 5'-/3'-end trimming and CCA addition. The precise sequence conservation of the ends of all the clones strongly suggests that a precise cleavage event is necessary for the generation of these tRFs.

Four small RNAs of the tRF-1 series are located within pre-tRNA, 3' to the mature tRNA sequence. In all cases, their 5'-ends start precisely after the 3'-ends of mature tRNA (Fig 1 and S1). The 3'-ends fall within DNA sequences of 5-6 consecutive Ts (Fig S1), a canonical termination site of RNA polymerase III transcription

(Hagenbuchle et al. 1979; Koski and Clarkson 1982; Cozzarelli et al. 1983). Most likely, the tRF-1 series of small RNAs have been released from pre-tRNA by an endonucleolytic cleavage during the 3'-end maturation of tRNA.

To obtain a more comprehensive list of tRFs, we expanded our analysis to all small RNA sequences cloned more than 5 times in the library (see Table S1 and S2). Out of 1541 nmsRNA sequences that mapped to the human genome, approximately 40% mapped to genomic loci containing a tRNA and flanking 25 nucleotides (Table S1 and Fig S2). 77% of these were identified as tRFs by their precise start and end sites, while the remaining ~23% match tRNA at random locations and were not further investigated. The fraction of tRF-1 series (14.2%) is lower than that of tRF-3 (44.8%) or -5 series (41.0%).

The lower frequency of tRF-1 clones is explained by the hypothesis that tRF-1 are released by 3' cleavage of the tRNA from the pre-tRNA. Of the 622 tRNA loci in the human genome (<http://lowelab.ucsc.edu/GtRNAdb/>) the distance between the 3'-end of the mature tRNA and the termination signal of the RNA polymerase III is variable (Fig S3). Only 14.6% of the tRNA loci could give rise to tRF-1 of the 16-27 base size range that was included in our cloning, accounting for the lower yield of tRF-1 series.

Thus, tRF-5, -3 and -1 (Table S2) constitute the most abundant class of short RNAs in human prostate epithelial cells, second only to miRNAs. In addition, their precise start and end sites at or near the tRNA ends strongly suggest that the tRFs are derived from tRNA cleavage in a specific manner.

**Expression of tRFs validated by other techniques.** Northern hybridization detected six tRFs tested (Fig S4A). Not only did we detect a band of the correct size, but other bands in the range of 80-100 nucleotides were apparent and could be precursors to the final tRFs. For tRF-1001, -1002, and -1003, the larger bands are likely to be the pre-tRNA from which the tRF is derived. For tRF-5 and -3 series, the probe also detects the corresponding mature tRNA, which is present at a much higher abundance than the tRF. Below the mature tRNA bands, there are additional bands of intermediate size (35-60 nucleotides long) that could correspond to the tRNA cleavage products that were recently reported (Li et al. 2008; Thompson et al. 2008).

The expression level of some tRFs were too low to be detected by Northern hybridization, particularly for tRF-3 and tRF-5 series, where a strong signal from the mature tRNA and intermediate bands interferes with the tRF signal. To circumvent this problem, we employed qRT-PCR detection in a method similar to the detection of miRNAs (reviewed in (Schmittgen et al. 2008)). We gel-purified the RNA of 17-26

nucleotides prior to cDNA synthesis to exclude contaminating signals from larger mature tRNAs or other RNAs. qRT-PCR for 12 tRFs successfully detected all of them (Fig S4B). Interestingly, each tRF exhibited a characteristic expression pattern across the four RNA samples (see Fig S4 and legend), arguing against the possibility that the tRFs are random by-products from nonspecific degradation of tRNA. Such non-specific degradation would have resulted in a similar pattern of expression of all tRFs amongst the four RNA preparations.

**Expression of tRF-1001.** tRF-1001 was chosen for further study, as it is the most abundantly cloned tRF and was clearly detectable by Northern hybridization. tRF-1001 is expressed more abundantly in cell lines than in tissues (Fig 2A; upper panel). Despite the greater degradation of RNA from tissues, as indicated by the ethidium bromide (EtBr) staining (Fig 2A; bottom panel), tRF-1001 levels are not increased in the tissues, reinforcing the notion that tRF-1001 is not a byproduct of non-specific RNA degradation. The higher expression of tRF-1001 in cancer cell lines of many different lineages suggests that tRF-1001 expression may be related to cell proliferation.

Consistent with this, tRF-1001 is decreased upon serum starvation of DU145 prostate cancer, LNCaP prostate cancer and HCT116 colon cancer cell lines and is restored upon serum re-addition (Fig 2B, S5A-B). tRF-1001 levels are also reduced

when cell-density is very high (Fig S5C). Therefore, conditions of poor cell proliferation, as with serum depletion or increased cell density, are associated with a down-regulation of tRF-1001. In contrast, the corresponding mature tRNA level stayed unchanged.

**tRF-1001 is required for cell proliferation.** The best argument against the tRFs being a non-specific byproduct of tRNA metabolism is to demonstrate a functional role for at least one of the tRFs. To investigate this, we knocked down tRF-1001 through an siRNA strategy. We designed an siRNA duplex that targets the pre-tRNA such that it anneals to part of the tRF-1001 and the mature tRNA (without the CCA). The siRNA is centered at +2/+3 position of tRF-1001 (Fig 3A). Thus, siRNA-mediated cleavage of the pre-tRNA at the center of siRNA target sequence (Elbashir et al. 2001b), is expected to cut the pre-tRNA at a site (arrowheads in Fig 3A) incompatible with tRF-1001 generation. Alternatively, the siRNA could sterically interfere with the cleavage event necessary for tRF-1001 generation.

In this experiment, Northern hybridization was not suitable for measuring tRF-1001 knock-down, because the passenger strand of si-tRF1001 shares 12 nucleotides with tRF-1001 and obscured the latter (data not shown). Instead, we took advantage of the fact that qRT-PCR assay of tRF-1001 will fail to detect the DNA passenger strand of

si-tRF1001 for the following reasons. First, the first-step in qRT-PCR is polyadenylation of the small RNA by poly(A) polymerase that requires 3' ribonucleotides for efficient poly(A) synthesis (Zhelkovsky et al. 1998). Second, the 3'-end of the specific primer used in the second step of qRT-PCR anneals to the tRF but not to the passenger strand of si-tRF1001. The reliability of qRT-PCR in measuring tRF-1001 was demonstrated by side-by-side comparison with Northern hybridization (Fig S6).

qRT-PCR assay confirmed the reduction of tRF-1001 by the siRNA (Fig 3B). In addition to standard qRT-PCR procedure for tRFs using gel-isolated small RNA, qRT-PCR with total RNA yielded a similar result (Fig 3B; right panel).

Upon tRF-1001 depletion, cell proliferation was impaired as indicated by a decrease in the number of viable cells (MTT assay in Fig 3C), which was accompanied by a reduction of DNA synthesis (BrdU incorporation assay in Fig 3C) and an accumulation of cells in the G2 phase of the cell cycle (FACS profile in Fig 3D). A closer observation of individual cells revealed an increase in the population of large cells with big nuclei (Fig S8). Although the si-tRF1001 is expected to decrease the maturation of the tRNA associated with tRF-1001, the same tRNA (Ser-TGA) is expressed from several alternate genomic loci different from the tRF-1001 locus. Indeed, Ser-TGA tRNA level is unchanged after si-tRF1001 treatment (Fig S7). Thus, the cell-

proliferation defects induced by si-tRF1001 cannot be attributed to a decrease in the corresponding mature tRNA .

Most importantly, the decrease in cell proliferation and G2 accumulation induced by si-tRF1001 were rescued by co-transfection of a synthetic tRF-1001 in the form of a 2'-O-methyl oligoribonucleotide (Me-tRF-1001, Fig 3C and D), but not by a similar sequence from an irrelevant gene, luciferase (Me-GL2). We used 2'-O-methyl derivatives because this modification is known to make the oligoribonucleotide chemically stable and resistant to cellular nucleases (reviewed in (Lamond and Sproat 1993)). Because the sequence of Me-tRF-1001 overlaps si-tRF1001 by 12 nucleotides (Fig 3A and S9A), we wanted to eliminate the possibility that the rescue was because the Me- tRF-1001 anneals to the siRNA and titrates it away from a non-specific cellular target. The Me-control oligoribonucleotide (Fig S9A) overlaps with another 12 nucleotides of si-tRF1001, but is different from the tRF-1001 sequence. Me-control did not rescue the growth impairment induced by si-tRF1001 (Fig S9B), strongly suggesting that the rescue of cell proliferation by Me-tRF-1001 was because it acted as a tRF-1001 mimic.

**tRF-1001 does not act as an si/miRNA.** tRF-1001 is exclusively localized in the cytoplasm (Fig 4A). Its size (19-mer) and intracellular localization raise the

possibility that tRF-1001 may act like an siRNA or miRNA. To address this question we designed a reporter plasmid containing a perfect antisense to tRF-1001 downstream of the luciferase ORF (pRL-anti-tRF1001 in Fig 4B). If endogenous tRF-1001 is incorporated into the siRNA/miRNA pathway, it is expected to suppress the expression of luciferase that has anti-tRF-1001 in its 3'UTR.

Reduction of endogenous tRF-1001, either by si-tRF1001 or by serum starvation, did not increase the luciferase activity from pRL-anti-tRF1001 (Fig 4C and D). Transfection of single-stranded Me-tRF-1001, a functional form of tRF-1001, also did not inhibit the luciferase expression (Fig 4E). As a technical control, tRF-1001 synthesized and transfected as an siRNA duplex significantly inhibited the luciferase expression of pRL-anti-tRF1001 (Fig 4E). Thus, tRF-1001 will act as an siRNA or miRNA if it is artificially delivered into a RISC complex. Endogenous tRF-1001, however, appears to not be in a RISC, consistent with the lack of any evidence that it is ever part of an RNA duplex : 1) tRF-1001 did not form a stem-loop secondary structure with its flanking genomic sequence and 2) a sequence complementary to tRF-1001 was not cloned in the 454 high-throughput sequencing. Collectively, these results suggest that tRF-1001 is a functionally distinct small RNA from siRNA/miRNA.



## Discussion

We report that tRFs are a novel class of small RNAs that are only second in abundance next to miRNAs. tRF-5 and -3 are derived from mature tRNAs. Although it is conceivable that as many of these tRFs will exist as there are mature tRNAs, we did not clone all the possible tRFs-5 and -3. Whether this is due to our limited cloning and sequencing effort or due to biological differences in the processing of tRNAs will be determined as we accumulate more reports of cloning and sequencing of short RNAs. Of the 622 tRNA loci, an analysis of the distance between the 3'-end of the mature tRNA and 3'-termination site of the pre-tRNA (Fig S3) indicates that fewer tRNA loci can produce tRF-1 series of 17-26 nucleotides. However, it is entirely possible that future cloning of longer or shorter RNAs will yield more tRF-1 clones. Like miRNAs, we expect different sets of tRFs to be expressed in different cell lines and tissues under different growth conditions and future efforts will be directed at expanding the repertoire of tRFs.

The mechanism of biogenesis of the tRFs is not clear yet, even though the juxtaposition of the start and end points of tRFs with that of mature tRNAs suggests that they are generated from cleavage of tRNA or pre-tRNA by specific nucleases. Two endonucleases for 3'-trimming of pre-tRNA, human ELAC1 and ELAC2 (Takaku et al.

2003), are the best candidates for generation of tRF-1 series. It is intriguing that ELAC2 was originally identified as a candidate prostate cancer susceptibility gene (Tavtigian et al. 2001), given that tRF-1001 is highly expressed in cancers and required for cancer cell growth.

Ranpirnase or Onconase is an endonuclease for tRNAs that is also known for its cytotoxic and antitumor activity (Ardelt et al. 1991; Saxena et al. 2002). Ranpirnase belongs to the pancreatic RNase family that has several members in humans (Rybak and Newton 1999). Ranpirnase, however, cleaves preferentially between the two G residues of UGG sequence *in vitro*, in the variable arm or the D-arm regions (Suhasini and Sirdeshmukh 2006). UGG does not define any of the cleavage sites utilized to generate tRF-3 or -5, so that either Ranpirnases are not relevant for the generation of these tRFs or one of the Ranpirnases has a different cleavage site in cells than what has been defined *in vitro*.

The correlation of tRF-1001 expression with cell proliferation raises the possibility that tRF-1001 can be used as a molecular marker for proliferating cancer cells. The utility of miRNAs as a cancer diagnostic tool has been well described (reviewed in (Lee and Dutta 2008)). miRNAs exhibit characteristic expression profile according to a cancer type, stage, and other clinical variables (Lu et al. 2005). The

miRNAs in serum or plasma are in a special form that renders them more resistant to RNase degradation, making them reliable bio-markers in tissues and body-fluids (Mitchell et al. 2008). The sensitive qRT-PCR assays for miRNA allow their measurement in even a few nanograms of total RNA from formalin-fixed paraffin-embedded (FFPE) specimens (Xi et al. 2007). All the advantages of miRNAs as a molecular marker are applicable to tRF-1001, especially since it can be reliably measured from total RNA by qRT-PCR assays (Fig 3B). Since the expression level of tRF-1001 seems to respond quickly to cell proliferation (Fig 2B and Fig S5), tRF-1001 can be used as a proliferation marker similar to the Ki-67 protein (Scholzen and Gerdes 2000).

The tRF-1 series are of particular interest, not only because we show that one of them is required for cell proliferation, but also because the sequence downstream of the tRNA that is encoded in the pre-tRNA is mostly unique among different tRNA genes (Table S2). Mature tRNAs of identical or highly similar sequences are repeated at multiple loci in the genome as expected from the fact that 622 tRNA loci code for tRNAs containing at most 64 anticodons. We speculate that some tRNA genes have additional gene-specific functions by generating unique tRFs from the 3'-flanking sequences, perhaps explaining the duplication and survival of so many tRNA genes

during evolution.

Much more work needs to be done to investigate how many tRFs are expressed, what regulates their levels, how many have discernible biological roles and whether the tRFs exert their biological functions as part of ribonucleoprotein complexes. Despite this, however, it is exciting to consider the obvious parallels between the current status of tRFs with what was known about miRNAs soon after it became evident that hundreds of different miRNAs are present in mammalian cells (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001).

## **Materials and Methods**

### ***Isolation of RNA, 454 deep sequencing, and measurement of tRFs***

Isolation of total, nuclear, and cytoplasmic RNA from cell lines was performed as described in (Lee and Dutta 2007). Small RNAs of 17-26 nucleotides were cloned and subjected to 454 deep sequencing as described in (Lee et al, in preparation). qRT-PCR measurement of tRFs and miRNAs was performed with Ncode SYBR GreenER miRNA qRT-PCR kit (Invitrogen Corp.) and ABI 7300 real time PCR system (Applied Biosystems). Unless described otherwise, total RNA was subjected to 15% denaturing acrylamide gel electrophoresis for isolation of small RNA (17-26 nucleotides long) prior to qRT-PCR steps. The qRT-PCR primer for measuring small nuclear RNA U6 in Fig. 3B is 5'-ctgcgcaaggatgacacgca-3'.

### ***Cell culture and transfection of RNA oligonucleotides***

Cell lines and culture conditions are described in Supplemental Materials and Methods. siRNA duplexes were synthesized by Invitrogen Corp. si-tRF1001 sequence is shown in Fig 3A and siGL2 sequence is described in (Elbashir et al. 2001a). 2'-O-methyl modified GL2 (5'-cguacgcgggaaauacuucga-3') and tRF-1001 (5'-gaagcgggugcucuauuu-3') were synthesized by Dharmacon RNA Technologies. Transfection of short RNA was performed with Lipofectamine<sup>TM</sup> RNAiMAX reagent (Invitrogen Corp.) using 33 nM

siRNA duplex and 67 nM 2'-O-methyl oligonucleotide, according to the reverse transfection procedure in the manufacturer's instructions. The first transfection (day 0) into HCT116 cells was followed by a second transfection at day 2. Total RNA was isolated and various assays were performed at day 4, unless otherwise indicated. During the course of the transfection experiment, HCT116 cells were kept below a density of  $2 \times 10^7$  cells per 10 cm culture dish.

The probe sequences for Northern blots in Fig 2B are 5'-aaataagagcacccgcttc-3' (tRF-1001), 5'-tggcgcagcgagcaggggttcgaa-3' (mature tRNA-Ser-TGA), and 5'-gatcgggcgcgttcaggggtgttat-3' (5S rRNA). The probe for snoU38b, a small nucleolar RNA, for the Northern blot in Fig 4A is 5'-agaactggacaaagtttcatcac-3'.

### ***Measurement of cell proliferation and DNA synthesis***

Cell proliferation was measured with CellTiter 96 non-radioactive cell proliferation assay kit (MTT assay; Promega Corp.). DNA synthesis was assayed by bromodeoxyuridine (BrdU) ELISA as described in (Machida et al. 2006). All experiments were performed in triplicates, from which average and standard deviation are calculated and plotted.

### ***Luciferase assays***

Luciferase assays were performed as described in (Kim et al. 2006; Lee and Dutta 2007) and Supplemental Materials and Methods.

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## Table Legends

### Table 1. List of abundantly cloned tRFs

17 tRFs are displayed with their sequence, length (in nucleotides), cloning frequency (number of sequence reads in 454 deep sequencing data), and relative location in the corresponding tRNA (see also text and Fig 1 and S1). For comparison, we included sequence reads of two miRNAs, miR-18a and *let-7a*, and the number of miRNAs cloned more than or less than 200 times.

## Figure Legends

### **Figure 1. Classification and nomenclature of tRFs revealed by analysis of highly abundant nmsRNAs (non-micro-small RNAs).**

**A.** Identification and classification of 30 nmsRNA sequences that were cloned abundantly. The nmsRNAs are grouped by their identity in the pie chart. “snoRNA”, small nucleolar RNA; “unmappable”: nmsRNAs unidentifiable in the human genome sequence or the human EST database, probably due to as yet unreported splicing or sequence editing events. tRNA-related small RNAs (tRFs; tRNA-derived RNA Fragment) are further classified by their relative location (see panel B) in the tRNA primary transcript (pre-tRNA).

**B.** Diagram of tRFs aligned at a tRNA locus (see Fig S1 for actual sequence alignment).

Wavy line: the genomic sequence for pre-tRNA ending with an oligo-dT stretch, the 3'-termination signal of RNA polymerase III. Grey bar: A mature tRNA after 3'-trimming of the pre-tRNA and addition of the CCA that is not present in the genomic sequence. Black bars: Three groups of tRFs (tRF-5, -3 and -1 series) are aligned with the pre-tRNA or mature tRNA. tRFs are numbered with the first digit indicating the group and the other digits forming a serial number in that group.

**Figure 2. tRF-1001 is highly expressed in proliferating cancer cells.**

**A.** Northern hybridization of tRF-1001 (top panel) and ethidium bromide staining of the gel (bottom panel). Source of tissue RNAs is described in (Kim et al. 2006). tRF-1001 (19-mer) is indicated by an arrowhead on the left. The positions of 18- and 24-mer synthetic oligoribonucleotides (arrowheads on the right), along with 10-bp DNA ladder, are shown as molecular size markers.

**B.** Measurement of tRF-1001 upon serum depletion in DU145 cell line. At 72 hrs after serum starvation (see Supplemental Materials and Methods), DU145 cells were replenished with 10% FBS medium (“re-addition”). Northern hybridization of tRF-1001 (bottom panel), its mature tRNA (middle panel), and 5S rRNA as a loading control (top panel). Different panels have different levels of exposure.

**Figure 3. The impaired growth of HCT116 cells upon siRNA-mediated knock-down of tRF-1001 is rescued by Me-tRF-1001.**

**A.** siRNA duplex was designed against the underlined region of pre-tRNA encompassing tRF-1001 and the mature tRNA. Arrowheads indicate expected sites in the pre-tRNA that will be cleaved by the siRNA (Elbashir et al. 2001b).

**B.** qRT-PCR measurement of tRF-1001 upon siRNA transfection. Left panel: qRT-PCR

was performed with gel-isolated small RNA and the relative expression of tRF-1001 (normalized by tRF-5003) is indicated. Right panel: total RNA was used for qRT-PCR, with snU6 for normalization. Each value is an average of triplicate samples and the error bars indicate standard deviation. The normalized level of tRF-1001 after siGL2 transfection is set as 1.

**C.** MTT and BrdU incorporation assays performed after transfection of siGL2 (plain bar), si-tRF1001 (black bar), and si-tRF1001 with Me-GL2 (light grey bar) or Me-tRF-1001 (dark grey bar). Left panel: A scan of wells after MTT assay at day 4. Right panel: BrdU incorporation normalized to MTT value at days 3 and 4. Average and standard deviation from triplicates are shown. The BrdU/MTT for cells transfected with siGL2 is set as 1.

**D.** FACS of HCT116 cells transfected with indicated oligonucleotides and stained with propidium iodide for DNA content (4 days after first transfection). Cells in G1, S, or G2 were quantitated by ModFit LT for Mac (version 3.2.1). Right panels: change in % of cells in each phase of the cell-cycle between the indicated two samples.

**Figure 4. tRF-1001 is localized in the cytoplasm but is not incorporated into miRNA/siRNA pathway**

**A.** Total, nuclear, and cytoplasmic RNAs were subjected to Northern hybridization of tRF-1001, snoU38 as a nuclear marker, and 5S rRNA as a loading control.

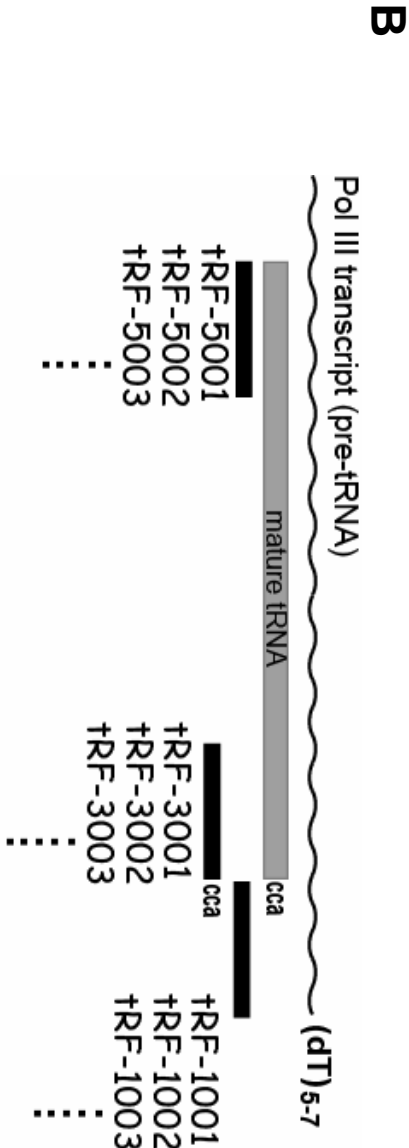
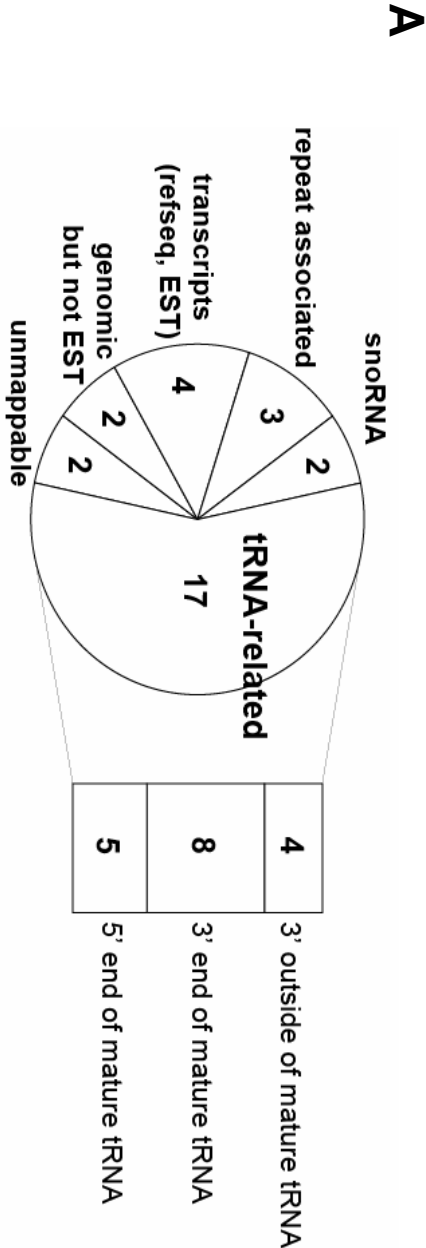
**B.** Diagram of pRL-anti-tRF1001 which contains an antisense to tRF-1001 downstream of the Renilla luciferase ORF (open reading frame) in pRLCMV vector (Promega Corp. and (Kim et al. 2006)).

**C-E.** Luciferase assays were performed in HCT116 cells. Luciferase values were measured at 24 hrs post-transfection of the plasmids. Each Renilla luciferase value (*Rr*) was normalized to that of firefly (*Pp*) from the co-transfected pGL3-Control vector (Promega Corp.). In panel C, the luciferase plasmids were transfected at day 3 after two rounds of siRNA transfection (at day 0 and 2) and assays were performed at day 4 when knock-down of tRF-1001 was confirmed (see Fig 3B). In panel D, serum was depleted at the time of transfection so that assays were done at 24 hrs after serum depletion when tRF-1001 was significantly reduced (Fig S5B). In panel E, transfection of indicated oligoribonucleotides was followed by transfection of the luciferase plasmids at 24 hrs. GL2 value in each pair was set as 1.

Lee\_Table 1

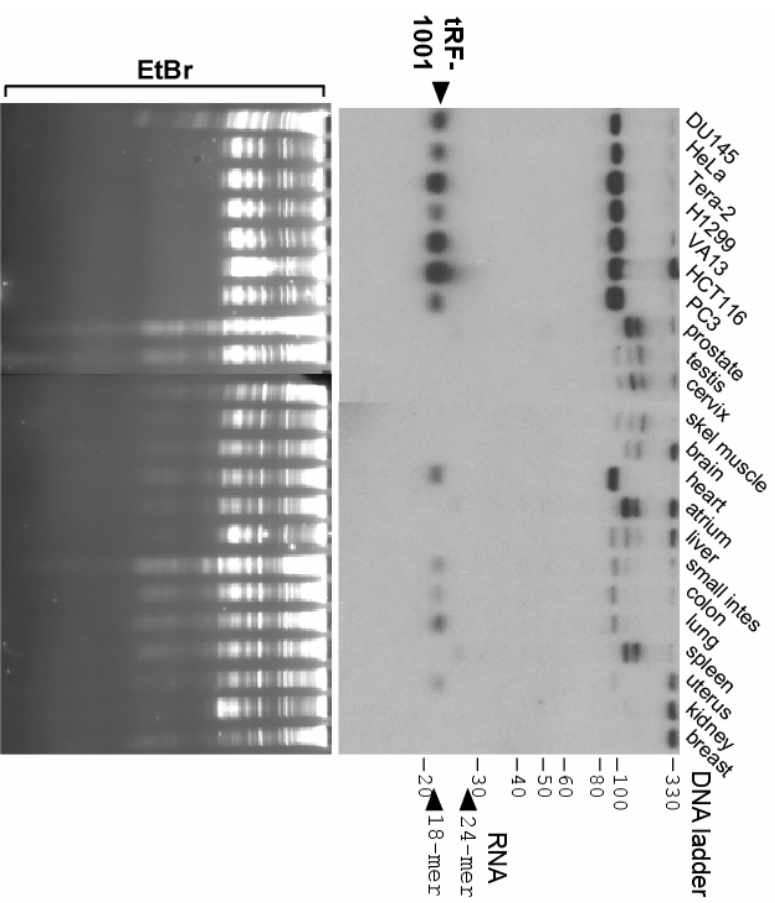
name	sequence (length)		cloning freq (seq reads)	tRNA mapped	
tRF-1001	GAAGCGGGTGCTCTTATTT	19	3995	tRNA-Ser-TGA	3'-out
tRF-1002	GCCGGGTACTTTTCGTATTTT	20	1090	tRNA-Asp-GTC	3'-out
tRF-1003	GCTAAGGAAGTCCTGTGCTCAGTTT	25	378	tRNA-Ser-GCT	3'-out
tRF-1004	GTGTGTAGCTGCACTTTT	18	362	tRNA-Asp-GTC	3'-out
tRF-3001	ATCCCACCGCTGCCACCA	18	2852	tRNA-Leu-TAG	3'
tRF-3002	ACCCTGCTCGCTGCGCCA	18	672	tRNA-Ser-TGA	3'
tRF-3003	TCCCCGGCACCTCCACCA	18	657	tRNA-Ala-AGC	3'
tRF-3004	TCCCCGGCATCTCCACCA	18	775	tRNA-Ala-TGC	3'
tRF-3005	ATCCTGCCGACTACGCCA	18	1219	tRNA-Ser-AGA	3'
tRF-3006	TCGATTCCCGGCCCATGCACCA	22	358	tRNA-Gly-GCC	3'
tRF-3007	TCGATTCCCGGCCAACGCACCA	22	749	tRNA-Gly-TCC	3'
tRF-3008	GTCCCACCAGAGTCGCCA	18	308	tRNA-Arg-TCT	3'
tRF-5001	GACGAGGTGGCCGAGTGG	18	1797	tRNA-Ser-GCT	5'
tRF-5002	TCCCTGGTGGTCTAGTGGTTA	21	906	tRNA-Glu-CTC	5'
tRF-5003	GGTAGCGTGGCCGAGCGGTC	20	710	tRNA-Leu-TAG	5'
tRF-5004	GCGTTGGTGGTATAGTGGT	19	1271	tRNA-Gly-TCC	5'
tRF-5005	GCATGGGTGGTTCAGTGGTA	20	1221	tRNA-Gly-GCC	5'
microRNA			seq reads		
	miR-18a		473		
	<i>let-7a</i>		17,856		
	635 miRNAs		< 200		
	60 miRNAs		> 200		



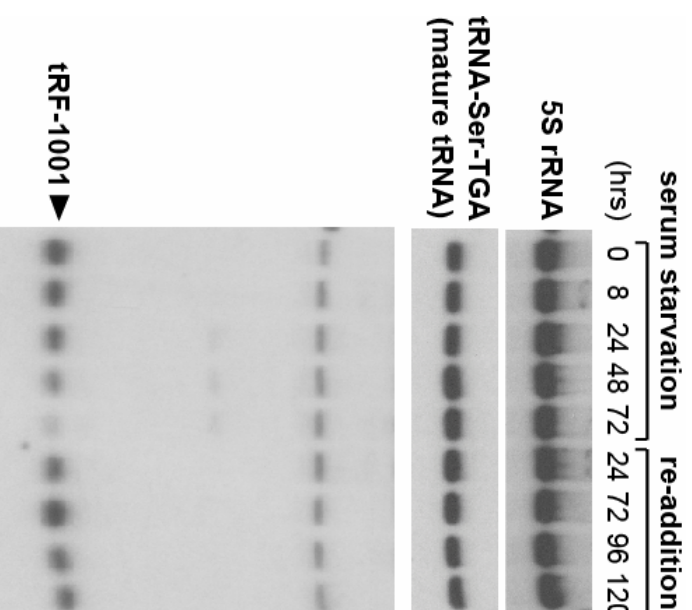


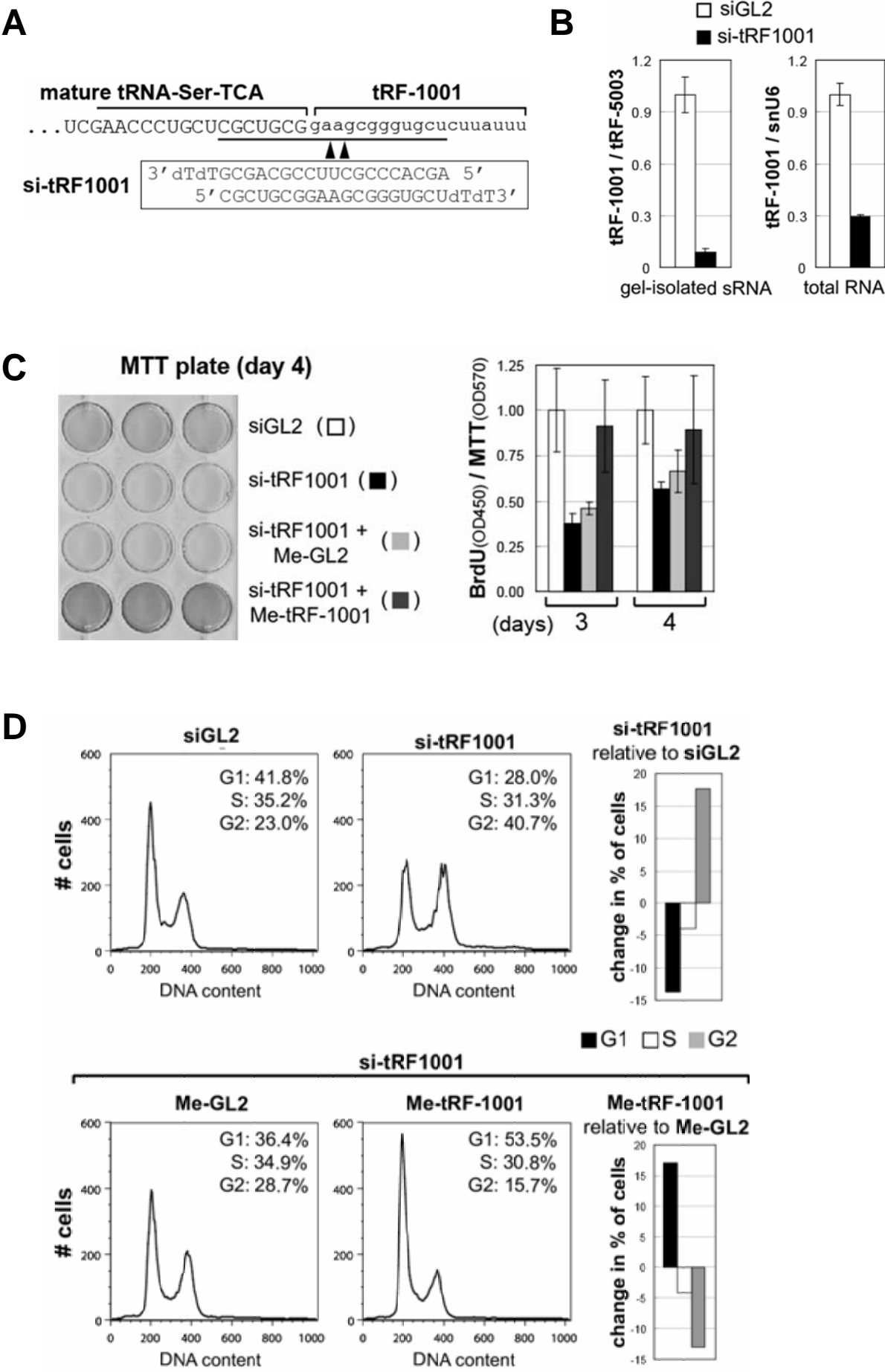
Lee\_Fig 2

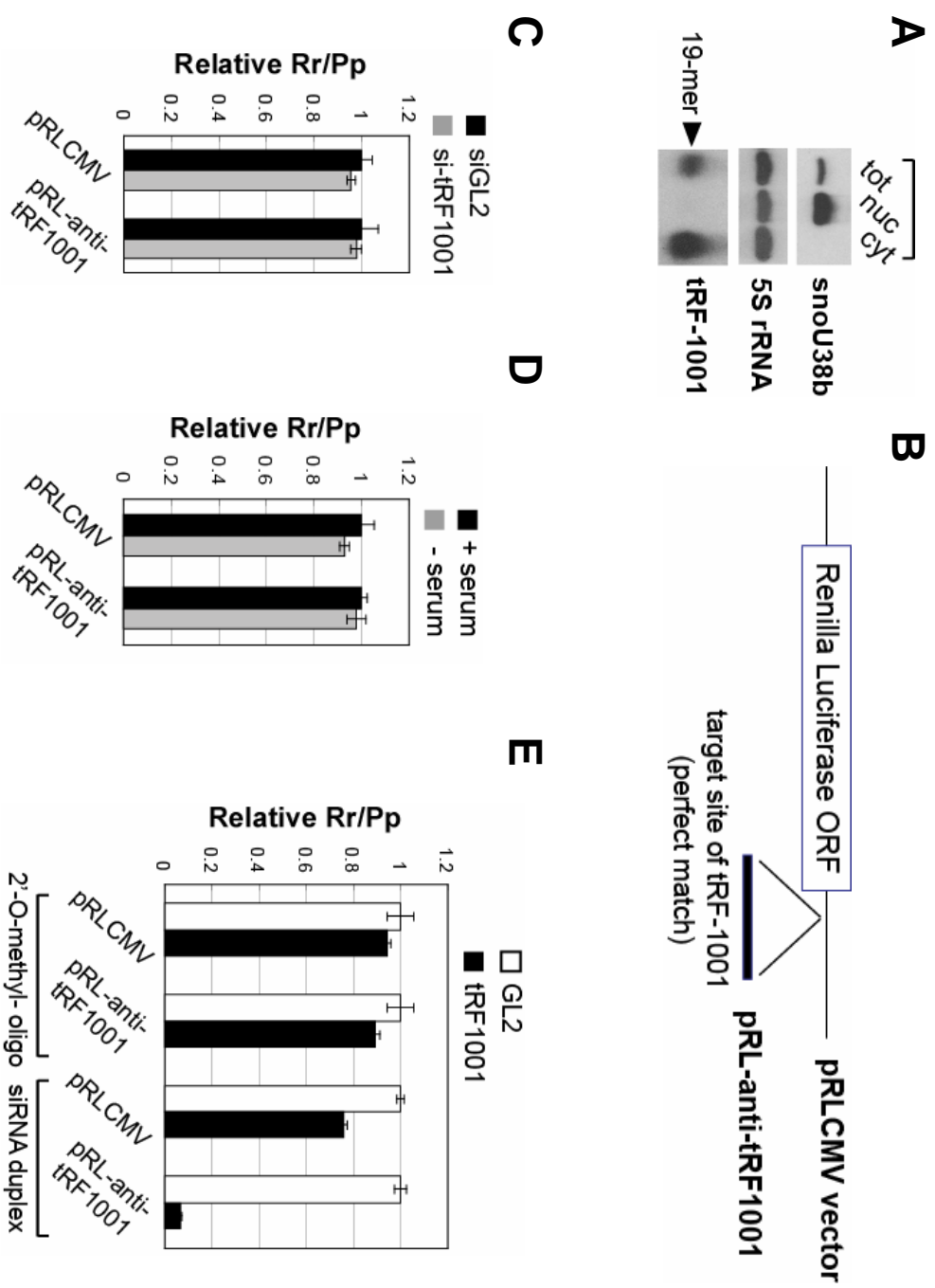
A



B







**Supplemental Information for “A novel class of small RNAs, tRNA-derived RNA fragments (tRFs), is highly expressed in proliferating cells and required for cell proliferation”**

## **Supplemental Materials and Methods**

### ***Cell culture***

Prostate cancer cell lines LNCaP and C4-2 (Wu et al. 1994) were maintained in RPMI-1640 media supplemented with 10% FBS (fetal bovine serum). LNCaP, an androgen dependent cell line, was deprived of androgen (“LNA-” condition) by culturing for four days in a phenol red free RPMI-1640 medium with 10% charcoal/dextran treated FBS (Hyclone). For “LNDHT” condition, 10 nM 4,5- $\alpha$ -dihydrotestosterone (DHT; from Sigma-Aldrich Co.) was added to LNA- condition.

HCT116 colon cancer cell line and DU145 prostate cancer cell line were maintained in McCoy’s 5A medium (Iwakata & Grace modification) and D-MEM (Dulbecco’s Modification of Eagle’s Medium; 4.5 g/L glucose), supplemented with 10% FBS respectively. For serum starvation, the medium was replaced with D-MEM (1.0 g/L glucose) containing 0.1% BSA (bovine serum albumin; fraction V from Sigma-Aldrich Co.).

### ***Staining of cells with DAPI and SP-DiOC<sub>18</sub>(3)***

SP-DiOC<sub>18</sub>(3) (a sulfonated derivative of DiO) was purchased from Molecular Probes<sup>TM</sup>.

Cells were washed with PBS (phosphate buffered saline) twice between each treatment.

HCT116 cells on sterile glass coverslip were treated with SP-DiOC<sub>18</sub>(3) for 5 min at

4°C to stain cellular membranes. Cells were fixed with 3.7% (w/v) HCHO in PBS at

37°C for 10 min and were permeabilized with chilled acetone at -20°C for 10 min.

Nuclei were stained with DAPI (H-1200) (Vector Laboratories, Inc.) for 1 min before

mounting. Images were observed under Nikon Microphot-SA microscope, captured by

Nikon UFX-DX camera, and processed by SPOT (version 3.5.4 for MacOS<sup>TM</sup>) software.

### ***Luciferase assays***

An artificial target site, which is perfectly complementary to tRF-1001, was cloned

downstream of the Renilla luciferase ORF (open reading frame) in pRL-CMV(MCS)

(Kim et al. 2006), to construct pRL-anti-tRF1001 (Fig 4B). pRL-anti-tRF1001 or pRL-

CMV(MCS) vector control was co-transfected with pGL3-Control vector (Promega

Corp.) expressing firefly (*Photinus pyralis*) luciferase. Plasmid transfection was

performed with Lipofectamine 2000 reagent (Invitrogen Corp.) according to the

manufacturer's instructions. Luciferase assays were performed at 24 hours post-

transfection of the plasmids, using Dual-Luciferase Reporter Assay System (Promega

Corp.) and Pharmingen Monolight™ 3020 luminometer. Each value of Renilla luciferase (*Rr*) was normalized to the firefly luciferase value (*Pp*). Each value is an average of three transfections with the standard deviation indicated.

## **Supplemental Table and Figure Legends**

### **Table S1. Number of tRNA-related small RNAs**

Among non-micro-small RNAs (nmsRNAs) that were cloned more than 5 times and mappable in the human genome, tRNA-related sequences (defined as sequences matching anywhere in mature tRNA sequence plus the flanking 25 nucleotides at both ends) were counted and tabulated. tRNA-related sequences were further sorted into the three tRFs (tRNA-derived RNA Fragments; defined and described in the text and Fig 1 and S1) or the rest (“non-specific”). The numbers are plotted in pie charts in Fig S2.

### **Table S2. tRFs captured in the 454 high-throughput sequencing**

All tRFs (cloned >5 times) are tabulated with their name, sequence, cloning frequency (seq reads), and corresponding genomic tRNA loci (chr – tRNA #). The tRNA number on a chromosome is based on the tRNA # in the Genomic tRNA Database

(<http://lowelab.ucsc.edu/GtRNAdb>)

**Figure S1. Sequence alignment of abundantly cloned tRFs (tRNA-derived RNA Eragment) to the corresponding tRNA**

Each tRF sequence (bold letters; see also Table 1) and its relative position (see also Fig 1) in the mature tRNA (shaded capital letters) or the flanking genomic regions (lowercase letters).

**Figure S2. Fraction of tRNA-related sequences in nmsRNAs**

The numbers in Table S1 are depicted in pie charts. See Table S1 legend for details.

**Figure S3. Length distribution of tRF-1 series.**

For 622 tRNA loci in the tRNA database, 3'-end of pre-tRNAs is estimated *in silico* from RNA polymerase III termination signal. Pre-tRNAs are known to terminate in oligo-U stretches (Hagenbuchle et al. 1979; Koski and Clarkson 1982) or in other non-canonical signals such as UUCUU, GUCUU or AUCUU (Thomann et al. 1989). 3'-end of pre-tRNA is defined as the third T in four or more T stretch or the fourth nucleotide in TTCTT, GTCTT or ATCTT. Distribution of the distances between 3'-end of mature



tRNA and pre-tRNA (= estimated lengths of tRF-1 series) are represented in a pie chart. The portion representing 91 tRNA loci, which can potentially generate tRF-1 series of 16-27 nucleotides long that can be capture by our cloning, is highlighted by grey color.

**Figure S4. Detection of tRFs by Northern hybridization or qRT-PCR.**

**A.** Northern hybridization of tRFs. The four samples are; “LNCaP” (cultured in a medium with untreated serum), “LNA-” (LNCaP cells cultured in charcoal stripped serum), “LNDHT” (LNA- supplemented with a synthetic androgen dihydrotestosterone (DHT)), and “C4-2” (an androgen independent cell line derived from LNCaP) (see also Supplemental Materials and Methods). Ethidium bromide (EtBr) staining of total RNA is shown for equal loading (leftmost panel). 18- and 24-mer oligoribonucleotides (arrow heads) and 10-bp DNA ladder are shown as molecular size markers.

**B.** tRFs were measured by qRT-PCR as described in Materials and Methods. qRT-PCR value of each tRF was normalized by that of miR-200b, a miRNA similarly expressed across the four samples (data not shown). Each value is an average of triplicate samples, with the standard deviation indicated.

**Figure S5. Decrease of tRF-1001 upon serum depletion.**

**A.** Northern hybridization of tRF-1001 upon serum starvation in LNCaP cells. EtBr staining of total RNA is shown for equal loading.

**B and C.** tRF-1001 measurement in HCT116 cell line upon serum starvation (panel B) and different cell density (panel C). In panel B, the number of cells at the time of serum starvation was  $\sim 1 \times 10^7$  (in 10-cm culture dish). In panel C, number of cells in 10-cm culture dish was counted at the time of RNA isolation. All other descriptions are same as Fig 2B.

**Figure S6. Comparison of qRT-PCR and Northern hybridization in the measurement of tRF-1001.**

**A.** Northern hybridization of miR-21 and tRF-1001, as well as EtBr staining of total RNA as a loading control. tRF-1001 result was reclaimed from Fig S5B (rightmost lane) and S5C (left lane) for side-by-side comparison.

**B.** qRT-PCR measurement of tRF-1001. The data was normalized by miR-21 (left panel) or tRF-5003 (right panel). Plain bar is set as 1. All other descriptions are same as Fig S4B.

**Figure S7. Level of the mature tRNA-Ser-TGA was unaffected after transfection of**

**si-tRF1001.**

Northern hybridization of tRNA-Ser-TGA (top panel) and EtBr staining of total RNA (bottom panel).

**Figure S8. Cell morphology after tRF-1001 knock-down**

Cellular contour (cellular membranes stained green by SP-DiOC<sub>18</sub>(3)) and nuclei (stained blue by DAPI) are visualized as described in Supplemental Materials and Methods.

**Figure S9. Reduced growth upon si-tRF1001 treatment was not rescued by another control 2'-O-methyl oligonucleotide whose sequence overlaps si-tRF1001 by 12 nucleotides**

**A.** Sequence of Me-tRF-1001 and Me-control. The portions overlapping si-tRF1001 (shown in the top) are underlined.

**B.** MTT assay is performed as described in Materials and Methods. The value of siGL2 is set as 1.

## Supplemental References

- Hagenbuchle, O., Larson, D., Hall, G.I., and Sprague, K.U. 1979. The primary transcription product of a silkworm alanine tRNA gene: identification of in vitro sites of initiation, termination and processing. *Cell* **18**(4): 1217-1229.
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- Koski, R.A. and Clarkson, S.G. 1982. Synthesis and maturation of *Xenopus laevis* methionine tRNA gene transcripts in homologous cell-free extracts. *J Biol Chem* **257**(8): 4514-4521.
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- Wu, H.C., Hsieh, J.T., Gleave, M.E., Brown, N.M., Pathak, S., and Chung, L.W. 1994. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. *Int J Cancer* **57**(3): 406-412.

**Lee\_Table S1**

<b>mappable nmsRNAs (cloned &gt;5)</b>	number of sequences	<b>1541</b>
<b>tRNA-related sequences</b>		
anywhere in tRNA +/- 25 nucleotides	number of sequences	<b>621</b>
	% in mappable nmsRNAs	40 . 3
non-specific	number of sequences	<b>143</b>
	% in tRNA-related seq	23 . 0
all tRFs (tRF-1, -3, or -5 series)	number of sequences	<b>478</b>
	% in tRNA-related seq	77 . 0
tRF-1 series	number of sequences	<b>68</b>
	% in tRFs	14 . 2
tRF-3 series	number of sequences	<b>214</b>
	% in tRFs	44 . 8
tRF-5 series	number of sequences	<b>196</b>
	% in tRFs	41 . 0

Lee\_Table S2

name	sequence	seq reads	tRNA gene	encoded in "chromosome - tRNA # (according to the Genomic tRNA Database; <a href="http://lowelab.ucsc.edu/GtRNAdb/">http://lowelab.ucsc.edu/GtRNAdb/</a> )"
tRF-1001	GAAGCGGTGCTTTAATTT	3995	tRNA <sup>Ser</sup> -TGA	chr10-#2
tRF-1002	GCCGGGTACTTTTCTAATTTT	1090	tRNA <sup>Asp</sup> -GTC	chr12-#10
tRF-1003	GCTAAGGAAGTCCGTGCTCAGTTT	378	tRNA <sup>Ser</sup> -GCT	chr17-#7
tRF-1004	GTGTGTAGCTGCACCTTTT	362	tRNA <sup>Asp</sup> -GTC	chr12-#5
tRF-1005	ATGTGTGGCTTACTTT	289	tRNA <sup>Ser</sup> -GCT	chr15-#10
tRF-1006	GTGTAAGCAGGTCGTTT	231	tRNA <sup>Arg</sup> -ACG	chr6-#8
tRF-1007	TTCAAGGTGAACGTTTT	166	tRNA <sup>Gln</sup> -TTG	chr6-#64
tRF-1008	TAGGGTGTGCGTGTTTTT	120	tRNA <sup>Thr</sup> -CGT	chr6-#121
tRF-1009	TGAGATGTTACCTAGCGTTT	86	tRNA <sup>Val</sup> -TAC	chr11-#17
tRF-1010	TGGTGTGGTCTGTGTTTT	51	tRNA <sup>Val</sup> -TAC	chr10-#6
tRF-1011	GGTGTTAATCTTGCGTTT	41	tRNA <sup>Arg</sup> -ACG	chr6-#36
tRF-1012	GCACGCCCTCCCATTT	36	tRNA <sup>Gly</sup> -GCC	chr1-#43, chr1-#41, chr1-#39, chr1-#37, chr1-#35
tRF-1013	AGGCGATCAGTAGATTT	35	tRNA <sup>Ala</sup> -CGC	chr6-#19
tRF-1014	TGACTGGA CTTTCTTT	27	tRNA <sup>Glu</sup> -TTC	chr15-#11
tRF-1015	CTGTGCTCCGAGTTA CCTCGTTT	22	tRNA <sup>Cys</sup> -GCA	chr17-#26
tRF-1016	GCTGCATAGCAAGCCTTT	22	tRNA <sup>Ser</sup> -GCT	chr6-#62
tRF-1017	TTGCCATGTTAACGTTT	13	tRNA <sup>Arg</sup> -CCG	chr16-#1
tRF-1018	CCGTGTTTCCCCCAGCCTTT	13	tRNA <sup>Thr</sup> -AGT	chr17-#8
tRF-1019	TCGTGGCTACTGTTTT	9	tRNA <sup>Pro</sup> -TGG	chr14-#6
tRF-1020	TGAGAGCGCTCGGTTTT	6	tRNA <sup>Phe</sup> -GAA	chr6-#96
tRF-1021	AAGAGATCGCTTTTT	6	tRNA <sup>Val</sup> -AAC	chr6-#132
tRF-1022	GGAGGGGAAGA GTTTT	6	tRNA <sup>His</sup> -GTG	chr9-#7
name	sequence	seq reads	tRNA gene	encoded in "chromosome - tRNA # (according to the Genomic tRNA Database; <a href="http://lowelab.ucsc.edu/GtRNAdb/">http://lowelab.ucsc.edu/GtRNAdb/</a> )"
tRF-3001	ATCCCA CCGCTGCCACCA	2852	tRNA <sup>Leu</sup> -TAG	chr16-#27
tRF-3002	ACCCGTGCTCGTGGCCA	672	tRNA <sup>Ser</sup> -TGA	chr10-#2
tRF-3003	TCCCGGGACCTCCACCA	657	tRNA <sup>Ala</sup> -AGC	chr6-#65
tRF-3004	TCCCGGGCATCTCCACCA	775	tRNA <sup>Ala</sup> -TGC	chr12-#8, chr12-#13, chr6-#19, chr6-#66, chr6-#10, chr6-#8, chr6-#13
tRF-3005	ATCCTGCGAGCTAGGCCA	1219	tRNA <sup>Ser</sup> -AGA	chr17-#35, chr8-#11, chr6-#145, chr6-#51, chr6-#147, chr6-#50, chr6-#47, chr6-#46, chr6-#44, chr6-#5, chr6-#172
tRF-3006	TCGATTTCCCGGCCCATGCACCA	358	tRNA <sup>Gly</sup> -GCC	chr21-#2, chr1-#41, chr1-#39, chr1-#37, chr1-#35
tRF-3007	TCGATTTCCCGGCCCAACGACCA	749	tRNA <sup>Gly</sup> -TCC	chr19-#2, chr17-#10, chr1-#45, chr1-#70, chr1-#73, chr1-#76, chr1-#79, chr6-#70
tRF-3008	GTCCCA CCAAGATCGCCA	308	tRNA <sup>Arg</sup> -TCT	chr11-#3
tRF-3009	ACCCCA CTCTGTACCA	1440	tRNA <sup>Leu</sup> -TAA	chr6-#83
tRF-3010	GTCCCTTCGTGTCGCA	448	tRNA <sup>Arg</sup> -TCG	chr17-#19, chr16-#1, chr6-#73, chr6-#114

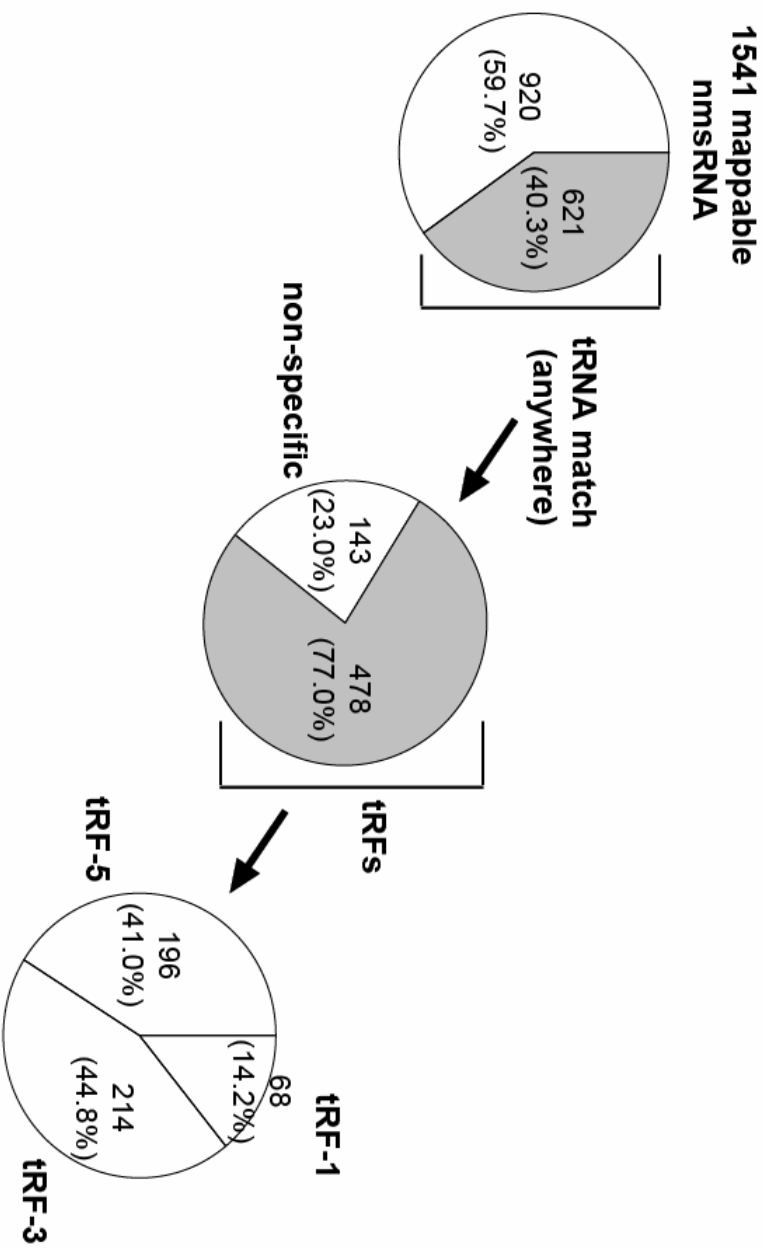
tRF-3011	AACGGGCGGAACACCA	441	tRNAVal-CAC	chr5-#6, chr6-#9, chr5-#10, chr3-#2
tRF-3012	AGTCCGATCTGGGTGCGCA	341	tRNAArg-CCG	chr17-#23
tRF-3013	TCGAGCCGACCGAGGACGCCA	333	tRNAAsn-GTT	chr19-#1, chr17-#31, chr10-#4, chr17-#37, chr17-#7, chr1-#7, chr6-#8
tRF-3014	GTCCTGCCCGCGGTGCGCA	313	tRNAArg-TCG	chr15-#4
tRF-3015	TCAGTCCCTGTTCGGGCGCCA	294	tRNALys-TTT	chr17-#2, chr11-#14, chr11-#5, chr6-#76, chr1-#62, chr1-#54
tRF-3016	TCGAATCCGACTCTCTGACACCA	260	tRNALeu-CAG	chr6-#7, chr1-#67, chr1-#42, chr1-#40, chr1-#38, chr1-#36, chr1-#34
tRF-3017	TCGAATCCGATTCCTCGTCGCCA	251	tRNASer-GCT	chr6-#175
tRF-3018	TCGATTCGCGGCCAATGCACCA	216	tRNAArg-GCC	chr17-#5, chr1-#68
tRF-3019	ATCCCGGACGAGCCCCCA	175	tRNAPro-TGG	chr16-#8, chr16-#28, chr16-#11, chr14-#3, chr11-#12
tRF-3020	ATCCGACGAGATCGCCA	162	tRNAArg-TCI	chr17-#4, chr11-#3, chr9-#4
tRF-3021	ACCCGACTTCTGTACCA	144	tRNALeu-TAA	chr11-#4
tRF-3022	ATCCGACCACTGCCACCA	124	tRNALeu-TAG	chr14-#2
tRF-3023	TGCCGATCTCCACCA	106	tRNAAla-AGC	chr2-#3
tRF-3024	TCGATCCCGGCATCTCCA	101	tRNAAla-TGC	chr12-#13, chr6-#119, chr6-#66, chr6-#10, chr5-#8
tRF-3025	TCGAATCCGAGTAGCCTCCA	80	tRNAThr-TGT	chr6-#127
tRF-3026	TCGAATCCCACTTCTGACACCA	75	tRNALeu-CAA	chr6-#74, chr6-#100, chr6-#140, chr6-#141, chr1-#58
tRF-3027	CCCCGGCTCTCCACCA	70	tRNAAla-CGC	chr6-#70
tRF-3028	ATCCCTTGTGTGCGCA	70	tRNAArg-TCG	chr6-#124
tRF-3029	TCCCGAGTACCTCCACCA	68	tRNAAla-AGC	chr6-#108
tRF-3030	ATCAGTCGGGTCTACCA	67	tRNATrp-CCA	chr17-#39, chr17-#6, chr12-#6, chr7-#1, chr6-#168, chr6-#170, chr17-#12
tRF-3031	TCGACTCCCGGTGTGGAAAC	62	tRNAGlu-TTC	chr15-#11
tRF-3032	ATCCGACCTTCTGCGCA	58	tRNASer-GCT	chr6-#43
tRF-3033	TCGATTCGCGGCGCGCACCA	56	tRNAGly-CCC	chr16-#34
tRF-3034	TCCGGGTTTCGGACCA	51	tRNAPhe-GAA	chr12-#11
tRF-3035	ATCCGGGACGAGCCCCA	46	tRNAPro-CGG	chr17-#37, chr16-#9, chr16-#29, chr16-#6, chr16-#4, chr16-#3, chr14-#6, chr14-#22, chr14-#23, chr11-#9, chr7-#2, chr6-#30, chr6-#12, chr5-#14, chr1-#65, chr1-#52
tRF-3036	TCCCGACACCTCCACCA	46	tRNAAla-TGC	chr6-#104, chr6-#107
tRF-3037	ACCCGTCGCTGCTCCA	45	tRNAThr-CGT	chr17-#14, chr16-#15
tRF-3038	ATCCGACGCTGCACG	45	tRNALeu-TAG	chr17-#42, chr16-#16, chr14-#1, chr6-#78, chr6-#98, chr6-#126, chr5-#7, chr5-#16, chr5-#3, chr5-#19
tRF-3039	TCAAATCCGGGTGCCCTCCA	38	tRNAcys-GCA	chr17-#26, chr17-#27, chr17-#28, chr17-#15, chr17-#29, chr4-#3
tRF-3040	TCGAATCCGAGTCAGGACCA	37	tRNAThr-CGT	chr6-#121, chr6-#135, chr6-#34, chr6-#167
tRF-3041	TCGAATCCGAGTCAGGACCA	32	tRNAHis-GTG	chr15-#1, chr15-#8, chr15-#9, chr9-#7, chr6-#33, chr17-#19, chr14-#1, chr12-#5, chr1-#16, chr6-#64
tRF-3042	TCCCGTACTGGCCACCA	32	tRNAIle-AAT	chr6-#153
tRF-3043	TCCCGAGCACTCCACCA	31	tRNAAla-AGC	chr1-#87
tRF-3044	TCGAATCCTGTTCGTGACGCCA	30	tRNASer-CGA	chr12-#2
tRF-3045	TCGATCCCGGCACCTCCA	29	tRNAAla-TGC	chr6-#110, chr6-#113
tRF-3046	TCGATTCAGCTCGAAGACCA	28	tRNATyr-GTA	chr14-#18
tRF-3047	ACCCGACTCTCGGTACCA	28	tRNALeu-TAA	chr6-#134
tRF-3048	AGTCCGACCTGGGGTACCA	27	tRNAArg-CCT	chr17-#18
tRF-3049	TCGAACCATCCTCTGTACCA	27	tRNAMet-CAT	chr17-#20, chr6-#129, chr6-#142, chr6-#150, chr6-#169, chr6-#171, chr6-#2, chr1-#32, chr9-#1
tRF-3050	ATCCTGCTACAGGCGCA	27	tRNASer-CGA	chr6-#35

tRF-3051	GTACAGTCGGGGTACCA	26	tRNA <sup>Tp</sup> -CCA	chr17-#12, chr17-#39, chr17-#6, chr12-#6, chr7-#1, chr6-#168, chr6-#170
tRF-3052	TCGATCCCGGTACGGGCCACCA	26	tRNA <sup>lle</sup> -AAT	chr6-#80, chr6-#57, chr6-#154, chr6-#158, chr6-#28, chr6-#165
tRF-3053	TCGATTTCCCGACGGGGAGCCA	25	tRNA <sup>asp</sup> -GTC	chr17-#38, chr12-#10, chr12-#12, chr12-#4, chr6-#144, chr6-#48, chr6-#45, chr1-#44, chr1-#69, chr1-#72, chr1-#75, chr1-#78, chr1-#81
tRF-3054	ATCCCACTCTCTGCCA	24	tRNA <sup>Ser</sup> -GCT	chr6-#31
tRF-3055	ACTCCGGTGTGGGAACCA	23	tRNA <sup>Glu</sup> -TTC	chr13-#3
tRF-3056	GAGCTCAGACGGGGACCA	23	tRNA <sup>Met</sup> -CAT	chr16-#22
tRF-3057	TCGATTCGGCTCGAAGGACCA	21	tRNA <sup>Tyr</sup> -GTA	chr2-#2
tRF-3058	TCGATTCGGGCAATGCACC	20	tRNA <sup>Gly</sup> -GCC	chr16-#19, chr16-#24, chr6-#128, chr2-#19
tRF-3059	TCGAATCAGTCGGGTCACCA	18	tRNA <sup>Tp</sup> -CCA	chr12-#6
tRF-3060	TCGAATCCAGCGAGGCTCCA	16	tRNA <sup>Thr</sup> -AGT	chr6-#60
tRF-3061	TCGACTCTGGCTGGCTCGCC	15	tRNA <sup>Arg</sup> -ACG	chr14-#7, chr6-#36, chr6-#156, chr6-#8, chr6-#6
tRF-3062	TCCCGGGCGGAACACCA	15	tRNA <sup>Val</sup> -CAC	chr19-#13, chr6-#132, chr6-#136, chr6-#9, chr5-#10, chr5-#12, chr5-#15, chr5-#6, chr5-#5, chr5-#4, chr5-#2, chr3-#2, chr1-#85, chr1-#90
tRF-3063	ACCGGGCAGAAGCACCA	12	tRNA <sup>Val</sup> -CAC	chr6-#152
tRF-3064	ATCCCTCCGTGTTACCA	11	tRNA <sup>Arg</sup> -TCG	chr6-#4
tRF-3065	GTCCCTGTCCAGGCGCCA	11	tRNA <sup>Lys</sup> -TTT	chr6-#149
tRF-3066	TCTCGTGGGCTCCA	10	tRNA <sup>Thr</sup> -TGT	chr14-#20
tRF-3067	TCGAACCGGGGGAACACC	10	tRNA <sup>Val</sup> -AAC	chr6-#132, chr6-#136, chr5-#12, chr5-#15, chr5-#5, chr5-#4, chr5-#18, chr5-#2, chr1-#85, chr1-#90
tRF-3068	TCCTCAGACGGGGACCA	10	tRNA <sup>Met</sup> -CAT	chr8-#10
tRF-3069	TCGACTCTGGCTGGCTGCCA	9	tRNA <sup>Arg</sup> -ACG	chr6-#138, chr3-#11
tRF-3070	TCGAGCCCCAGTGAACACCA	8	tRNA <sup>Ala</sup> -TAC	chr11-#16
tRF-3071	TCGACTCCGGTATGGAACCA	8	tRNA <sup>Glu</sup> -TTC	chr13-#5, chr2-#20
tRF-3072	TCAAATCTCGGTGAACCTCCA	8	tRNA <sup>Gln</sup> -CTG	chr17-#3, chr15-#7, chr6-#99, chr6-#49, chr6-#42, chr6-#1
tRF-3073	TCGAATCTCTGCACAGGCCA	8	tRNA <sup>Ser</sup> -CGA	chr17-#41
tRF-3074	AGTCCCACTCGGGTACCA	6	tRNA <sup>Arg</sup> -CCT	chr16-#2
tRF-3075	ATCTCGGTGGACCTCCA	6	tRNA <sup>Gln</sup> -TTG	chr17-#16
tRF-3076	GGTCCCTGTCCAGGCGCCA	6	tRNA <sup>Lys</sup> -TTT	chr6-#53, chr6-#149
tRF-3077	TCCCCGGGCACTCCACC	6	tRNA <sup>Ala</sup> -AGC	chr6-#101, chr6-#102, chr6-#110, chr6-#113
name	sequence	seq reads	tRNA gene	encoded in "chromosome - tRNA # (according to the Genomic tRNA Database; <a href="http://lowelab.ucsc.edu/GtRNAdb/">http://lowelab.ucsc.edu/GtRNAdb/</a> )"
tRF-5001	GACGAGTGGCCGAGTGG	1797	tRNA <sup>Ser</sup> -GCT	chr17-#7, chr15-#10, chr11-#8, chr6-#123, chr6-#62, chr6-#43, chr6-#31
tRF-5002	TCCCTGGTGTCTAGTGTTTA	906	tRNA <sup>Glu</sup> -CTC	chr6-#87, chr6-#77, chr1-#59, chr1-#71, chr1-#74, chr1-#77, chr1-#80, chr6-#110
tRF-5003	GGTAGCGTGGCCGACGGTCC	710	tRNA <sup>Leu</sup> -TAG	chr17-#42, chr16-#16, chr14-#1, chr6-#78, chr6-#98, chr5-#7, chr5-#16, chr5-#3, chr5-#19
tRF-5004	GCGTTGGTGTATATAGTGGT	1271	tRNA <sup>Gly</sup> -TCC	chr19-#2, chr17-#10, chr1-#45, chr1-#70, chr1-#73, chr1-#76, chr1-#79, chr1-#82, chr6-#70
tRF-5005	GCATGGGTGGTTCAGTGTA	1221	tRNA <sup>Gly</sup> -GCC	chr21-#2, chr1-#41, chr1-#39, chr1-#37, chr1-#35
tRF-5006	GCATTGGTGGTTCAGTGTA	429	tRNA <sup>Gly</sup> -GCC	chr17-#5, chr16-#19, chr16-#18, chr16-#24, chr16-#25, chr6-#128, chr2-#19, chr1-#68, chr1-#58, chr17-#7
tRF-5007	GGCCGATTAGCTCAATTGG	355	tRNA <sup>lle</sup> -AAT	chr17-#34, chr17-#9, chr14-#10, chrX-#5, chrX-#6, chrX-#7, chr6-#80, chr6-#59, chr6-#41, chr6-#154, chr6-#158, chr6-#28, chr6-#163, chr6-#165, chr6-#11
tRF-5008	GGGGGTGTAGCTCAGTGG	261	tRNA <sup>Ala</sup> -TGC	chr11-#18, chr6-#101, chr6-#102, chr6-#104, chr6-#107, chr6-#110, chr6-#113, chr6-#115, chr6-#70, chr6-#68, chr6-#67, chr6-#65, chr3-#7

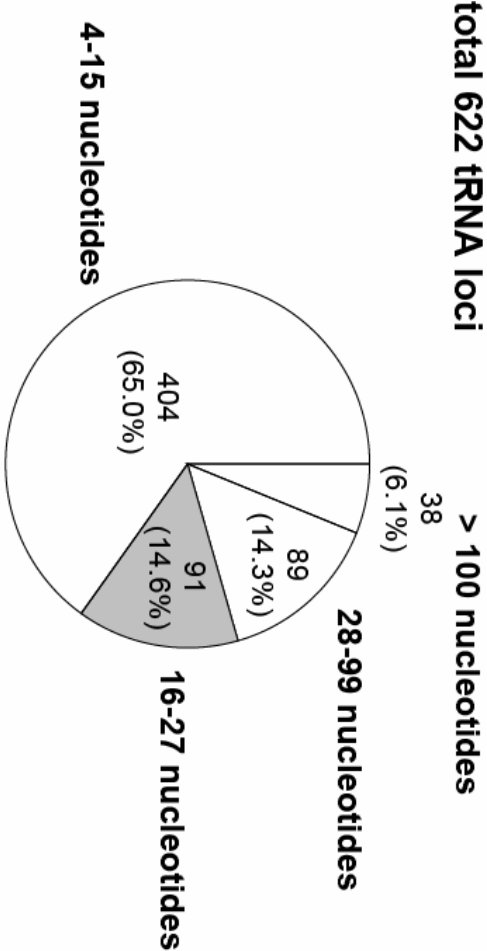


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tRF-5011	GGTAGCGTGGCCGAGTGGTC	141	tRNALeu-TAG	chr16-#27, chr6-#126
tRF-5012	GCCCGGATAGCTCAGTGG	132	tRNALys-TTT	chr17-#2, chr11-#14, chr11-#5, chr6-#76, chr1-#62, chr1-#54
tRF-5013	GTCAGGATGGCCGAGCGGTC	91	tRNALeu-CAG	chr16-#26, chr16-#17, chr6-#7, chr1-#67, chr1-#42, chr1-#40, chr1-#38, chr1-#36, chr1-#34
tRF-5014	GCTGTGATGGCCGAGTGG	84	tRNASer-CGA	chr17-#41, chr6-#137, chr6-#35
tRF-5015	GTAAGTCGTGGCCGAGTGGTTA	73	tRNASer-AGA	chr17-#35, chr8-#11, chr6-#145, chr6-#51, chr6-#147, chr6-#50, chr6-#148, chr6-#47, chr6-#46, chr6-#44, chr6-#5, chr6-#172
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tRF-5017	GTCACGGTGGCCGAGTGG	59	tRNASer-CGA	chr12-#2
tRF-5018	GGGCCAGTGGCCGCAATGG	56	tRNAArg-ACG	chr14-#7, chr6-#138, chr6-#36, chr6-#156, chr6-#8, chr6-#6, chr3-#11
tRF-5019	GCAGCGATGGCCGAGTGG	54	tRNASer-TGA	chr10-#2
tRF-5020	TCCCTGCTGGTCTAGTGGCTA	52	tRNAGlu-TTC	chr1-#84, chr1-#33, chr1-#94, chr2-#6, chr10-#2
tRF-5021	TCCACATGGTCTAGCGGTAGG	38	tRNAGlu-TTC	chr15-#11, chr13-#3
tRF-5022	GTTTCCGTAGTGTAGTGGTTA	27	tRNAVal-AAC	chr6-#132, chr6-#133, chr6-#136, chr6-#139, chr6-#37, chr6-#9, chr5-#10, chr5-#12, chr5-#6, chr5-#5, chr5-#4, chr5-#18, chr5-#2, chr3-#2, chr1-#85, chr1-#90, chr1-#98, chr5-#15
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tRF-5025	GGCGCGGTGGCCAGTGGT	20	tRNAThr-CGT	chr17-#14, chr16-#15
tRF-5026	GCCTGGGTAGCTCAGTCGG	19	tRNA-derived pseudogene	chr6-#118, chr6-#149
tRF-5027	ACCAGAATGGCCGAGTGGTTAA	16	tRNALeu-TAA	chr11-#4
tRF-5028	AGCAGAGTGGCGCAGCGGAA	14	tRNAMet-CAT	chr17-#20, chr6-#129, chr6-#61, chr6-#142, chr6-#150, chr6-#169, chr6-#171, chr6-#2, chr1-#32
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tRF-5032	GACCTCGTGGCGCAATGG	7	tRNATrp-CCA	chr17-#12
tRF-5033	GCCCGGATGATCCTCAGTGG	7	tRNASec(e)-TCA	chr19-#8
tRF-5034	TCCCATATGGTCTAGCGGTTA	6	tRNAGlu-TTC	chr13-#5, chr2-#20
tRF-5035	GGGGTATAGCTCAGTGG	6	tRNACys-GCA	chr17-#26, chr17-#27, chr17-#28, chr17-#15, chr15-#3, chr6-#108, chr4-#3
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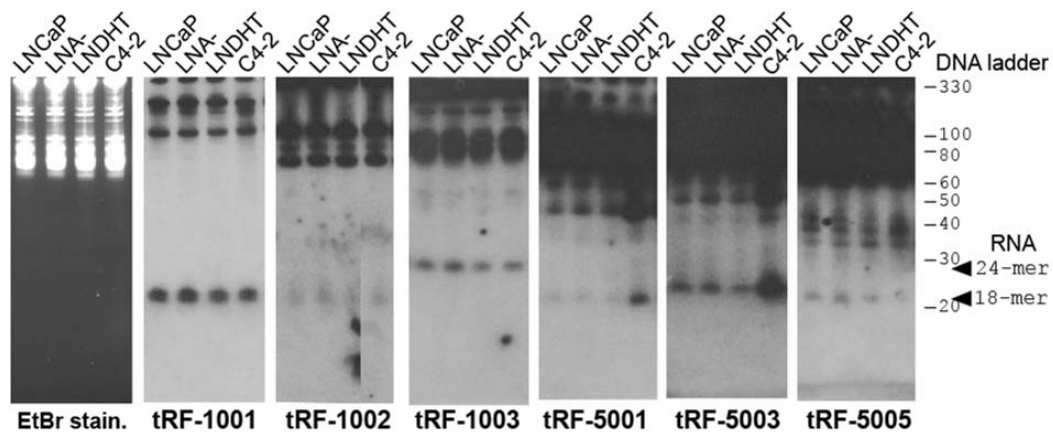
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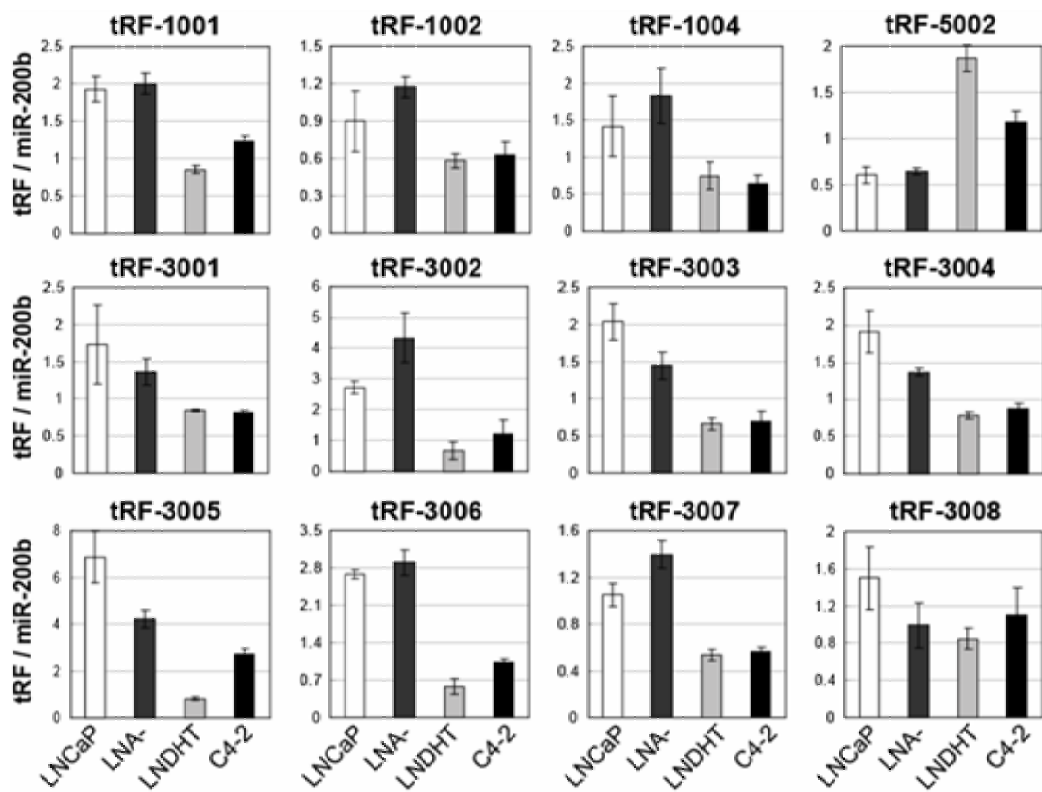
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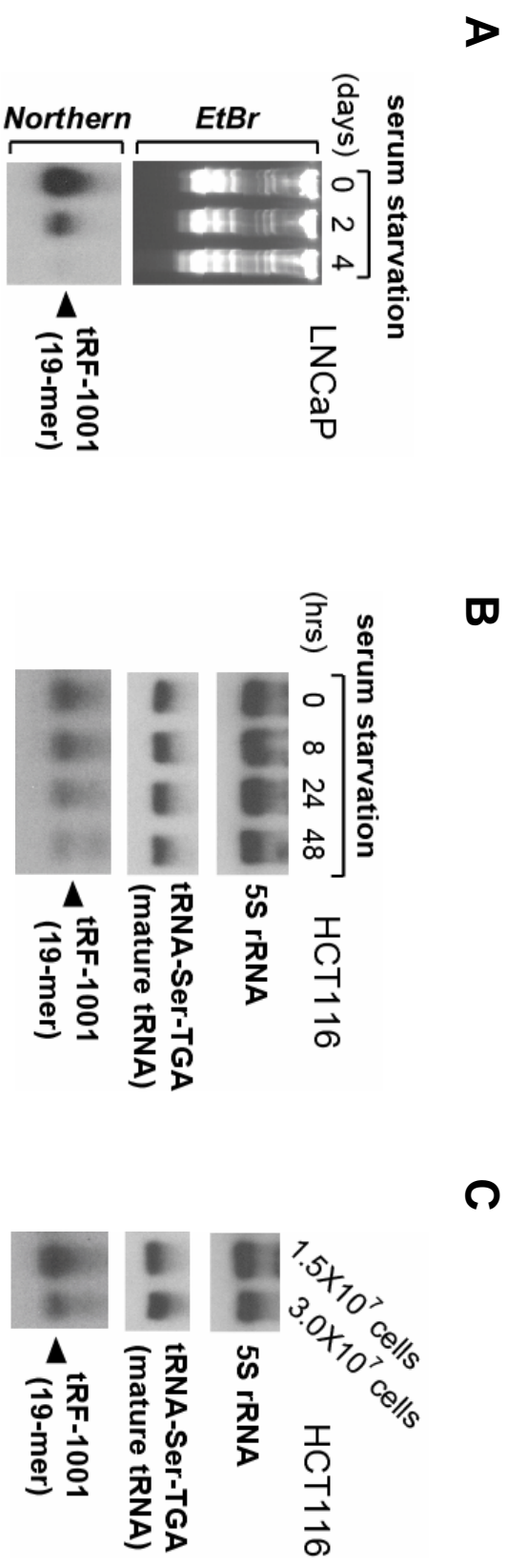
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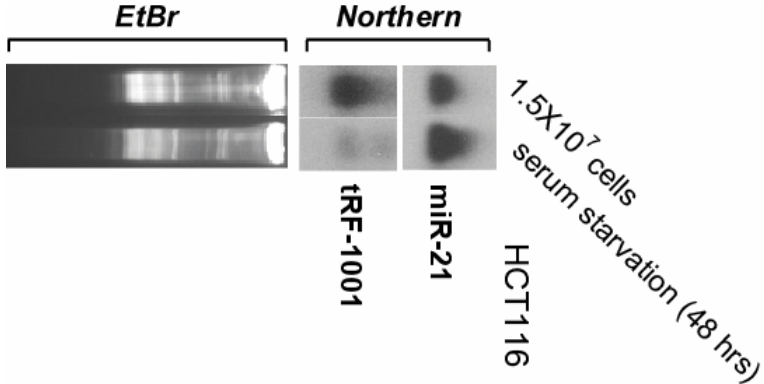
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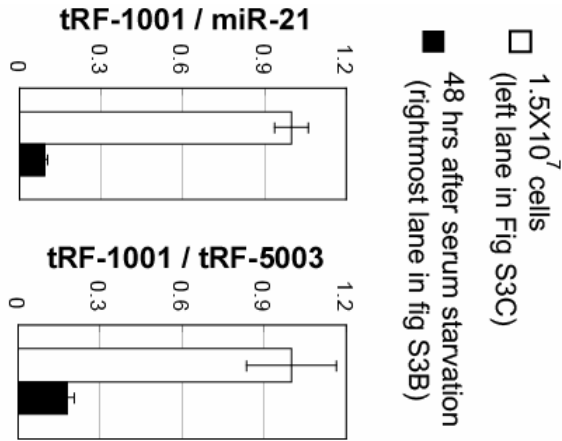
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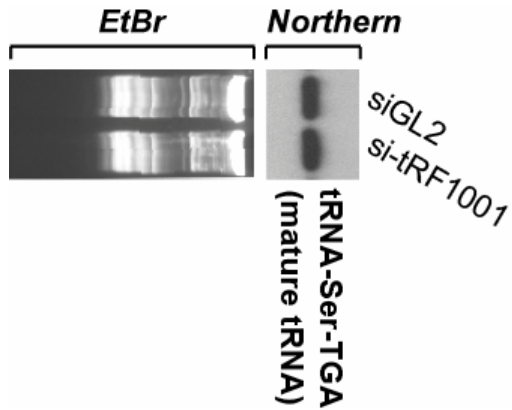
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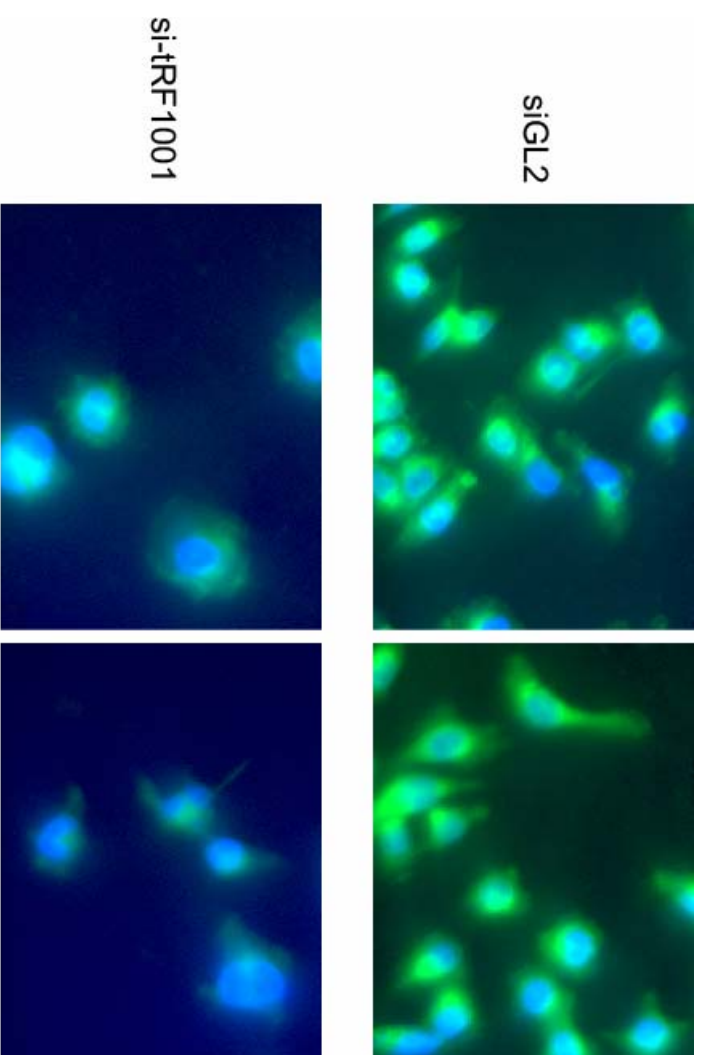


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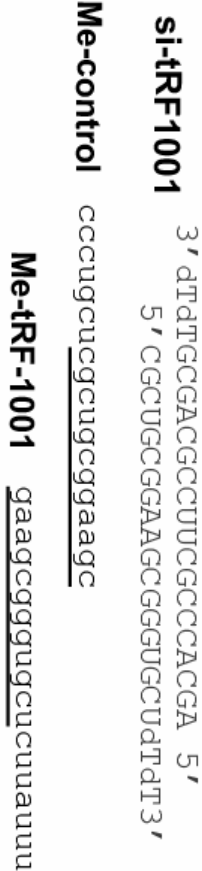




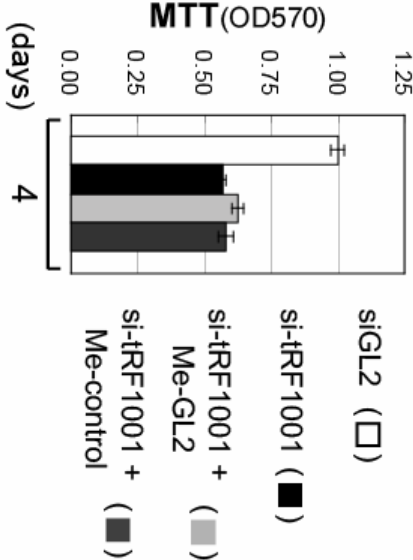
Lee\_Fig S8



A



B



**Supplemental Information for “A novel class of small RNAs, tRNA-derived RNA fragments (tRFs), is highly expressed in proliferating cells and required for cell proliferation”**

## **Supplemental Materials and Methods**

### ***Cell culture***

Prostate cancer cell lines LNCaP and C4-2 (Wu et al. 1994) were maintained in RPMI-1640 media supplemented with 10% FBS (fetal bovine serum). LNCaP, an androgen dependent cell line, was deprived of androgen (“LNA-” condition) by culturing for four days in a phenol red free RPMI-1640 medium with 10% charcoal/dextran treated FBS (Hyclone). For “LNDHT” condition, 10 nM 4,5- $\alpha$ -dihydrotestosterone (DHT; from Sigma-Aldrich Co.) was added to LNA- condition.

HCT116 colon cancer cell line and DU145 prostate cancer cell line were maintained in McCoy’s 5A medium (Iwakata & Grace modification) and D-MEM (Dulbecco’s Modification of Eagle’s Medium; 4.5 g/L glucose), supplemented with 10% FBS respectively. For serum starvation, the medium was replaced with D-MEM (1.0 g/L glucose) containing 0.1% BSA (bovine serum albumin; fraction V from Sigma-Aldrich Co.).

### ***Staining of cells with DAPI and SP-DiOC<sub>18</sub>(3)***

SP-DiOC<sub>18</sub>(3) (a sulfonated derivative of DiO) was purchased from Molecular Probes<sup>TM</sup>.

Cells were washed with PBS (phosphate buffered saline) twice between each treatment.

HCT116 cells on sterile glass coverslip were treated with SP-DiOC<sub>18</sub>(3) for 5 min at

4°C to stain cellular membranes. Cells were fixed with 3.7% (w/v) HCHO in PBS at

37°C for 10 min and were permeabilized with chilled acetone at -20°C for 10 min.

Nuclei were stained with DAPI (H-1200) (Vector Laboratories, Inc.) for 1 min before

mounting. Images were observed under Nikon Microphot-SA microscope, captured by

Nikon UFX-DX camera, and processed by SPOT (version 3.5.4 for MacOS<sup>TM</sup>) software.

### ***Luciferase assays***

An artificial target site, which is perfectly complementary to tRF-1001, was cloned

downstream of the Renilla luciferase ORF (open reading frame) in pRL-CMV(MCS)

(Kim et al. 2006), to construct pRL-anti-tRF1001 (Fig 4B). pRL-anti-tRF1001 or pRL-

CMV(MCS) vector control was co-transfected with pGL3-Control vector (Promega

Corp.) expressing firefly (*Photinus pyralis*) luciferase. Plasmid transfection was

performed with Lipofectamine 2000 reagent (Invitrogen Corp.) according to the

manufacturer's instructions. Luciferase assays were performed at 24 hours post-

transfection of the plasmids, using Dual-Luciferase Reporter Assay System (Promega

Corp.) and Pharmingen Monolight™ 3020 luminometer. Each value of Renilla luciferase (*Rr*) was normalized to the firefly luciferase value (*Pp*). Each value is an average of three transfections with the standard deviation indicated.

## **Supplemental Table and Figure Legends**

### **Table S1. Number of tRNA-related small RNAs**

Among non-micro-small RNAs (nmsRNAs) that were cloned more than 5 times and mappable in the human genome, tRNA-related sequences (defined as sequences matching anywhere in mature tRNA sequence plus the flanking 25 nucleotides at both ends) were counted and tabulated. tRNA-related sequences were further sorted into the three tRFs (tRNA-derived RNA Fragments; defined and described in the text and Fig 1 and S1) or the rest (“non-specific”). The numbers are plotted in pie charts in Fig S2.

### **Table S2. tRFs captured in the 454 high-throughput sequencing**

All tRFs (cloned >5 times) are tabulated with their name, sequence, cloning frequency (seq reads), and corresponding genomic tRNA loci (chr – tRNA #). The tRNA number on a chromosome is based on the tRNA # in the Genomic tRNA Database

(<http://lowelab.ucsc.edu/GtRNAdb>)

**Figure S1. Sequence alignment of abundantly cloned tRFs (tRNA-derived RNA Eragment) to the corresponding tRNA**

Each tRF sequence (bold letters; see also Table 1) and its relative position (see also Fig 1) in the mature tRNA (shaded capital letters) or the flanking genomic regions (lowercase letters).

**Figure S2. Fraction of tRNA-related sequences in nmsRNAs**

The numbers in Table S1 are depicted in pie charts. See Table S1 legend for details.

**Figure S3. Length distribution of tRF-1 series.**

For 622 tRNA loci in the tRNA database, 3'-end of pre-tRNAs is estimated *in silico* from RNA polymerase III termination signal. Pre-tRNAs are known to terminate in oligo-U stretches (Hagenbuchle et al. 1979; Koski and Clarkson 1982) or in other non-canonical signals such as UUCUU, GUCUU or AUCUU (Thomann et al. 1989). 3'-end of pre-tRNA is defined as the third T in four or more T stretch or the fourth nucleotide in TTCTT, GTCTT or ATCTT. Distribution of the distances between 3'-end of mature

tRNA and pre-tRNA (= estimated lengths of tRF-1 series) are represented in a pie chart.

The portion representing 91 tRNA loci, which can potentially generate tRF-1 series of 16-27 nucleotides long that can be captured by our cloning, is highlighted by grey color.

**Figure S4. Detection of tRFs by Northern hybridization or qRT-PCR.**

**A.** Northern hybridization of tRFs. The four samples are; “LNCaP” (cultured in a medium with untreated serum), “LNA-” (LNCaP cells cultured in charcoal stripped serum), “LNDHT” (LNA- supplemented with a synthetic androgen dihydrotestosterone (DHT)), and “C4-2” (an androgen independent cell line derived from LNCaP) (see also Supplemental Materials and Methods). Ethidium bromide (EtBr) staining of total RNA is shown for equal loading (leftmost panel). 18- and 24-mer oligoribonucleotides (arrow heads) and 10-bp DNA ladder are shown as molecular size markers.

**B.** tRFs were measured by qRT-PCR as described in Materials and Methods. qRT-PCR value of each tRF was normalized by that of miR-200b, a miRNA similarly expressed across the four samples (data not shown). Each value is an average of triplicate samples, with the standard deviation indicated.

**Figure S5. Decrease of tRF-1001 upon serum depletion.**

**A.** Northern hybridization of tRF-1001 upon serum starvation in LNCaP cells. EtBr staining of total RNA is shown for equal loading.

**B and C.** tRF-1001 measurement in HCT116 cell line upon serum starvation (panel B) and different cell density (panel C). In panel B, the number of cells at the time of serum starvation was  $\sim 1 \times 10^7$  (in 10-cm culture dish). In panel C, number of cells in 10-cm culture dish was counted at the time of RNA isolation. All other descriptions are same as Fig 2B.

**Figure S6. Comparison of qRT-PCR and Northern hybridization in the measurement of tRF-1001.**

A. Northern hybridization of miR-21 and tRF-1001, as well as EtBr staining of total RNA as a loading control. tRF-1001 result was reclaimed from Fig S5B (rightmost lane) and S5C (left lane) for side-by-side comparison.

B. qRT-PCR measurement of tRF-1001. The data was normalized by miR-21 (left panel) or tRF-5003 (right panel). Plain bar is set as 1. All other descriptions are same as Fig S4B.

**Figure S7. Level of the mature tRNA-Ser-TGA was unaffected after transfection of**



**si-tRF1001.**

Northern hybridization of tRNA-Ser-TGA (top panel) and EtBr staining of total RNA (bottom panel).

**Figure S8. Cell morphology after tRF-1001 knock-down**

Cellular contour (cellular membranes stained green by SP-DiOC<sub>18</sub>(3)) and nuclei (stained blue by DAPI) are visualized as described in Supplemental Materials and Methods.

**Figure S9. Reduced growth upon si-tRF1001 treatment was not rescued by another control 2'-O-methyl oligonucleotide whose sequence overlaps si-tRF1001 by 12 nucleotides**

**A.** Sequence of Me-tRF-1001 and Me-control. The portions overlapping si-tRF1001 (shown in the top) are underlined.

**B.** MTT assay is performed as described in Materials and Methods. The value of siGL2 is set as 1.

## Supplemental References

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