AD_____

Award Number: W81XWH-10-1-0452

TITLE: tRNA and its activation targets as biomarkers and regulators of breast cancer.

PRINCIPAL INVESTIGATOR: Tao Pan, Ph.D.
Marsha Rosner, Ph.D.

CONTRACTING ORGANIZATION: The University of Chicago
Chicago, IL 60637

REPORT DATE: September 2013

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2013	Final	1 September 2010 - 31 August 2013

4. TITLE AND SUBTITLE
tRNA and its activation targets as biomarkers and regulators of breast cancer.

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0452

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Tao Pan, Marsha Rosener
Mariana Pavon Eternod, Donghui Zhou,Guanquan Zheng, Thomas Jones, Susanna Gomes, Jiyong Lee.

E-Mail: taopan@uchicago.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Chicago
Chicago, Illinois 60637

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Breast cancer refers to a variety of types that each derive from distinct causes and merit different treatments. It is crucial to develop new classes of molecules that target heretofore unexplored cellular mechanisms, so that tumors not responsive to current treatments might be responsive to new routes of therapy. We are exploring a previously underappreciated avenue that can lead to precise identification of breast cancer sub-types to improve treatment options and identify new therapeutic targets. The genetic code is decoded by transfer RNA (tRNA). We discovered that breast tumors have high levels of tRNA and tRNA over-expression has an aberrant pattern suggesting that tumors use tRNA over-expression to mis-regulate the synthesis of some crucial proteins. Further, we found that a specific tRNA is an oncogene in breast cells suggesting that tRNA over-expression could even originate the development of breast cancer. We have been identifying the mis-regulation targets of tRNA over-expression in breast cancer to serve as biomarkers and establishing the potential of targeting tRNA as a new route for breast cancer treatment.

15. SUBJECT TERMS tRNA, target, biomarker, breast cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	16	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Final Report

Proposal : W81XWH-10-0452,-0453, tRNA and its activation targets as biomarkers and regulators of breast cancer.

Period: 09/01/10-08/31/13.

PIs: Tao Pan, Marsha Rosner.

INTRODUCTION

Transfer RNAs (tRNAs) are small non-coding RNAs that read the genetic code in protein synthesis. They are essential for the proliferation, fitness, and adaptation of the cell. Previously, we showed that elevated tRNA levels are characteristic of breast cancer cells (Pavon-Eternod, M., Gomes, S., Geslain, R., Dai, Q., Rosner, M.R. and Pan, T. (2009) tRNA over-expression in breast cancer and functional consequences. *Nucleic Acids Res*, **37**, 7268-7280). Furthermore, over-expression of one specific tRNA - the initiator methionine tRNA - leads to increased cell proliferation and altered tRNA expression in a non-cancer breast epithelial cell line. Based on these results, we hypothesized that tRNA over-expression alters the translational regulation of key genes involved in cancer development and progression. We aim to identify the protein or RNA targets that are mis-regulated upon tRNA over-expression, and to determine the effect of tRNA over-expression on tumor initiation and progression.

BODY

This collaborative project involves equal efforts between the labs of both PIs. In general, the molecular work was performed in the Pan lab, and the cellular work was performed in the Rosner lab.

Task 1 – Identify gene targets whose translation is mis-regulated upon tRNA over-expression.
a) Establish the method: ribosome profiling. → **Completed successfully.**

We adapted ribosome profiling to identify gene targets whose translation is mis-regulated upon tRNA over-expression. This method, first developed and published in 2009, has been highly successful in the simultaneous identification and quantitation of the translational activity for all mRNAs in the cell.

Our ribosome profiling protocol in human cells includes the following steps: 1) Prepare cell lysate from human cells at ~90% confluency. 2) Digest the cell lysate with a ribonuclease that degrades only the mRNA segments not bound by the ribosome. mRNA segments bound by the ribosome, typically 30-40 nucleotides long, are protected from degradation. 3) Load the digested cell lysate on a sucrose gradient to separate the ribosome protected mRNA segments from other RNAs. The ribosome protected mRNA segments are found in the

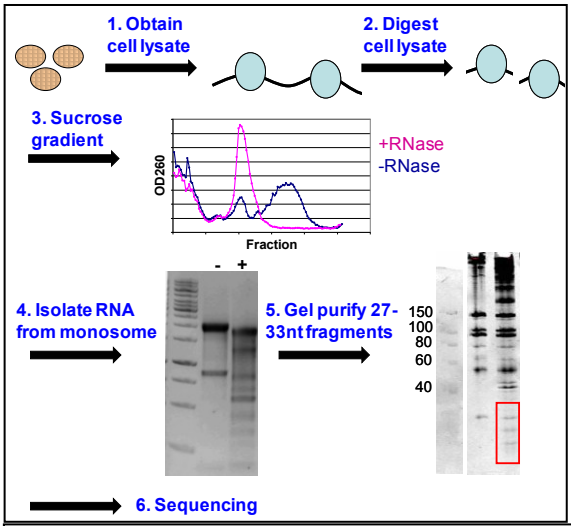

Fig. 1. *The experimental workflow of ribosome profiling.* Sucrose density gradient traces at step 3 and the gel images at steps 4 and 5 are our actual experimental data for a breast epithelial cell line.

monosome peak. 4) Isolate total RNA from the monosome peak. 5) Purify RNA fragments between 30 and 40 nucleotides long, the expected size for ribosome protected mRNA segments. 6) Deep-sequence these RNA fragments to identify sites of active translation (Figure 1).

We first applied this protocol to a breast epithelial cell line, 184 B5. The sequencing statistics are very similar to those obtained in a previous application, confirming the method has been successfully adapted in our laboratory (Figure 2A). We obtained approximately 13 million reads, with 150,000 unique sequences. As expected, a large number of reads map to rRNA and tRNA sequences. About 6% of the reads mapped to mRNA sequences, similar to those in the literature (Figure 2B) – these reads will be the focus of our analysis.

A

	HeLa (ref. 3)	184B5 (Our result)
Total reads	18,029,685	13,136,060
Mapping to genome	9,291,779	10,813,775
% Mapping to genome	52	82
mRNA matches	5,439,248	5,365,365
% mRNA matches	30	40

B

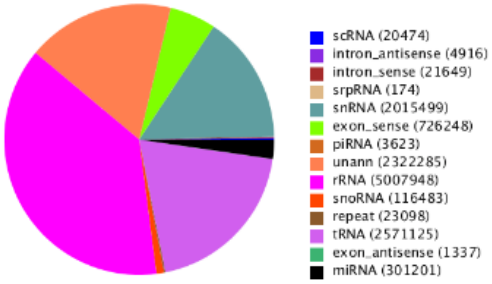

Fig. 2. *Ribosome profiling in breast cells: statistics and mapping to genome.* (A) Statistics. (B) Pie charts showing all categories.

b) Identify gene targets whose translation is mis-regulated upon tRNA over-expression. → **Completed successfully.**

To measure changes in translation upon tRNA over-expression, we initially planned to use stable cell lines over-expressing the initiator methionine tRNA ($tRNA_i^{Met}$) or the elongator methionine tRNA ($tRNA_e^{Met}$). Due to unexpected technical difficulties in maintaining these cell lines, we amended our experimental strategy to compare translation between non-tumorigenic breast cell lines and breast cancer cell lines. From our previous studies, we know that tRNA levels are highly elevated in the breast cancer relative to the non-cancer breast cell lines. We applied ribosome profiling to two non-cancer breast epithelial cell lines – 184 B5 and 184 A1 – and three breast cancer cell lines –BT-474, MDA-MB-231 and 1833 (Figure 3). Furthermore, we focused our analysis on differences in the ribosome density of 5'UTR regions since tRNA levels could significantly affect translational

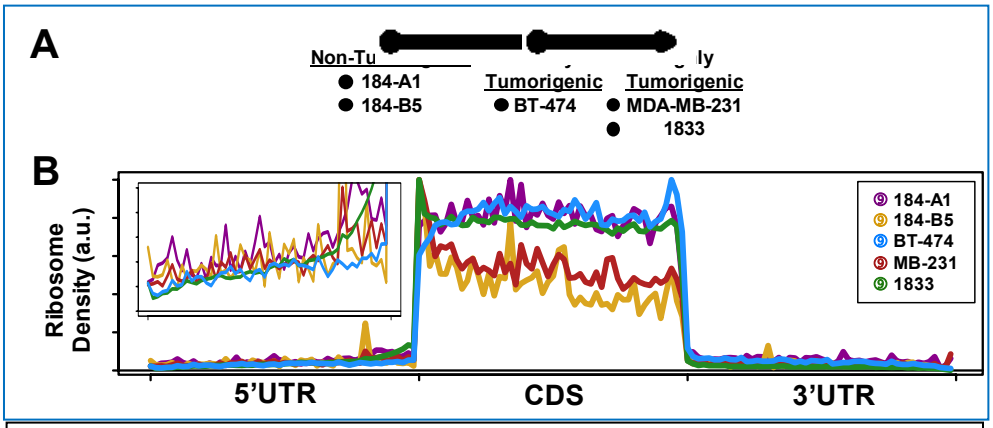

Fig. 3. *Five cell lines used and ribosome profiling results.* (A) Characteristics of the five cell lines, ranging from non-tumorigenic to metastatic. (B) Plots of mRNA matches showing the relative abundance of CDS versus UTR reads.

regulation for genes that contain upstream open reading frames (uORFs) in their 5' UTR.

We found that the ribosome density in the 5' UTR seems to vary significantly among the group of cell lines with similar tumorigenic properties (Figure 4). The five lines we studied can be classified into three groups: 184A1 and 184B5 are non-tumorigenic (NT), BT474 is from a primary, non-metastatic tumor (TP), MDA-MB-231 and 1833 are derived from metastatic tumors (TM). Comparing these three groups shows a surprising, unexpected result: the NT cells have similar ribosome density in 5'UTR among themselves, the TP cell has significantly lower ribosome density in 5'UTR than NT, but the TM cells have significantly higher ribosome density in 5'UTR than TP. Differential ribosome density in 5'UTR was also observed in the differentiation of mouse embryonic stem cells: compared to stem cells, differentiated cells have a significantly lower ribosome density, suggesting a lower degree of translational regulation through 5' UTR after differentiation. Therefore, our result suggests that a primary tumor may also have decreased global regulation of translation when compared to non-tumorigenic or to metastatic breast cancer cells.

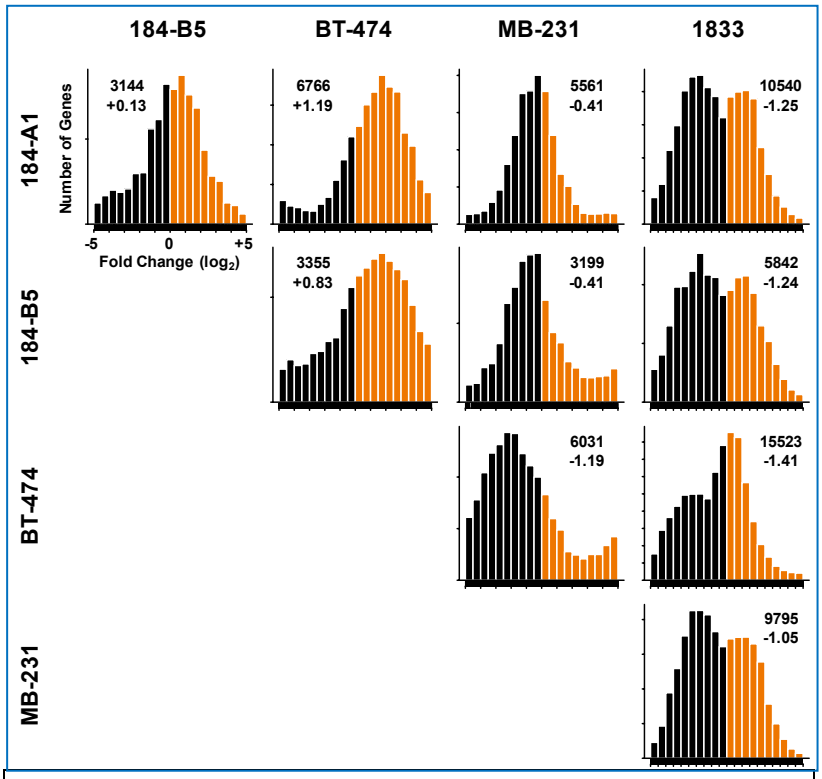

Fig. 4. *Comparing ribosome density in 5'UTR among three groups of breast and breast cancer cells.* TP cells have significantly lower 5'UTR density than NT or TM cells, suggesting a marked loss of translational regulation.

We next performed ribosome profiling of two cell lines derived from the same parent cell to directly address the idea of differential translation regulation as a function of tumorigenic status (Figure 5). This test was necessary because the five lines we used above for ribosome profiling are derived from different people and therefore may have significant genetic differences. The line shCTGL-5 is highly metastatic, whereas shHMAG-6 is similar to a primary tumorigenic cell. We observed the same global difference as our previous result predicted, indicating that translational regulation has decreased significantly in primary breast tumors.

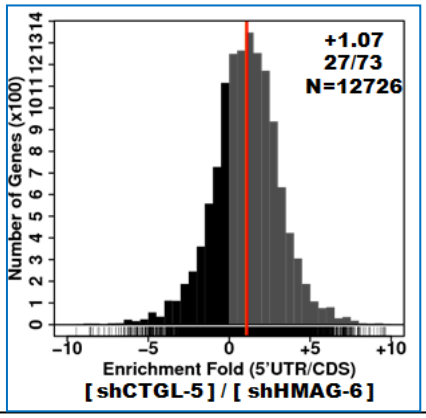

Fig. 5. *Comparing ribosome density in 5'UTR among two lines derived from the same parent cells and therefore with identical genetic background.* Significant difference was observed in 5'UTR density, consistent with our result obtained from five other lines with different genetic background.

We categorized genes that show significant density in the 5' UTR. Figure 6

Category	Term	Count
SP_PIR_KEYWORDS	phosphoprotein	489
SP_PIR_KEYWORDS	alternative splicing	425
UP_SEQ_FEATURE	splice variant	423
SP_PIR_KEYWORDS	nucleus	290
GOTERM_MF_FAT	GO:0046872~metal ion binding	237
SP_PIR_KEYWORDS	cytoplasm	219
SP_PIR_KEYWORDS	acetylation	200

Fig. 6. *Gene categories that show significant ribosome density in 5' UTR.* Hits in 5'UTRs of >1,400 different gene transcripts are present in this non-tumorigenic line.

shows such a list from a non-tumorigenic line. It is clear that these genes are distributed among a wide range of proteins performing distinct functions in the cell.

Task 2 – Validate results for selected genes identified in task 1. → **Still in progress.**

As described under task 1b, we are in the process of completing the analysis of 5'UTR-derived translational regulation across non-tumorignic and tumorigenic cells. Our focus will be on these genes that are known to be involved in breast cancer development and proliferation.

We are validating changes in translational mis-regulation of several breast cancer relevant genes using luciferase reporter constructs. These results should enable validation of several potential gene regulation targets related to tumorigenic states.

Task 3 – Examine tumor initiating cell properties upon tRNA over-expression.

a) Test whether tRNA over-expression promotes cell proliferation and self-renewal in breast cells. → **Completed successfully (publication attached).**

Pavon-Eternod et al.: Overexpression of Initiator Methionine tRNA Leads to Global Reprogramming of tRNA Expression and Increased Proliferation in Human Epithelial Cells, *RNA* 19, 461-6 (2013).

Abstract: Transfer RNAs (tRNAs) are typically considered housekeeping products with little regulatory function. However, several studies over the past 10 years have linked tRNA misregulation to cancer. We have previously reported that tRNA levels are significantly elevated in breast cancer and multiple myeloma cells. To further investigate the cellular and physiological effects of tRNA overexpression, we overexpressed $tRNA_i^{Met}$ in two human breast epithelial cell lines. We then determined tRNA abundance changes and performed phenotypic characterization. Overexpression of $tRNA_i^{Met}$ significantly altered the global tRNA expression profile and resulted in increased cell metabolic activity and cell proliferation. Our results extend the relevance of tRNA overexpression in human cells and underscore the complexity of cellular regulation of tRNA expression.

b) Test whether tRNA over-expression is associated with BT-IC differentiation. → **Unable to complete due to unexpected difficulties maintaining tRNA overexpression.**

Unexpectedly, we run into significant technical difficulties to maintain tRNA overexpression in our studies. We tried three approaches to over-express initiator-tRNA: transient transfection with tRNA transcripts (time required: one week), transient transfection with tRNA vector (time required: one week), and stable transfection with tRNA vector (time required: two months). Only stable transfection led to appreciable overexpression which enabled the characterization described under Task 3a. This result makes sense: tRNAs are derived from multiple

gene copies and are already expressed at very high levels, so that increasing the existing amount in a cell even by ~1.5-fold requires a very high and sustained level of expression. For example, the initiator-tRNA has ten copies in a human genome and a breast cancer cell has at least one million initiator-tRNA molecules to begin with. The 1.5-2 fold overexpression we were able to accomplish requires an additional expression of over 500,000 molecules per cell, several orders of magnitude higher than what is typically needed for endogenous mRNA expression.

To our surprise and dismay, these cells lost tRNA overexpression over a period of several weeks. We repeatedly attempted to re-establish cell lines that overexpress initiator-tRNA (we tried a total of four times, requiring ~ two months each time), and the same thing happened: initiator-tRNA overexpression was lost rapidly after several weeks. At this time, we do not understand the reason for this inability of maintaining overexpression of initiator-tRNA. Using our initial tRNA-transfected cells, we did a number of assays including soft agar growth and mammosphere assays which were consistent with the idea that overexpression of tRNA promotes anchorage-independent cell growth and tumor initiation. Therefore we did not attempt to proceed further with this task which required a much more elaborate treatment course of mammosphere cultured cells.

Task 4 – Examine the effect of tRNA over-expression on tumor formation and metastasis in mice.
→ Unable to complete due to unexpected difficulties maintaining tRNA overexpression.
a) Test whether tRNA over-expression promotes invasion.
b) Determine the effect of tRNA over-expression on human BT-IC tumor formation and lung metastasis in mice.

Please see the description under Task 3b.

KEY RESEARCH ACCOMPLISHMENTS

→ Established a ribosome profiling method to look at active translation in human breast cancer cells.
→ Demonstrated that over-expression of initiator-tRNA leads to increased cell proliferation in a breast epithelial cell line.
→ Applied the ribosome profiling method to a panel of cancer and non-cancer breast cell lines, in order to identify genes whose translation is mis-regulated upon tRNA over-expression.
→ Determined that cells at distinct tumorgenic states have significantly different translational regulation through 5' UTR.

REPORTABLE OUTCOMES

→ A manuscript describing the results under Task 3a has been published in *RNA* 19, 461-466 (2013). Overexpression of Initiator Methionine tRNA Leads to Global Reprogramming of tRNA Expression and Increased Proliferation in Human Epithelial Cells. Authors: Pavon-Eternod, M., S. Gomes, M.R. Rosner & Tao Pan.
→ Poster presentation at the Era of Hope meeting in Orlando, FL, August 2011.
→ Ribosome profiling protocol for human breast cancer cells.

→ Constructs for the over-expression of $tRNA_i^{Met}$ and $tRNA_e^{Met}$ in human cells.

CONCLUSION

Our results show that tRNA over-expression results in increased cell proliferation and greater self-renewal potential in human breast cells. We have adapted a ribosome profiling method and have applied it to a panel of breast cancer and non-cancer breast cell lines. We have already determined that translational regulation through 5' UTR is significantly different depending on the tumorigenic state of breast cells. We are working to validate genes whose translation is altered in breast cancer cells.

Chemotherapy, surgery, and radiotherapy are the current methods of choice to treat breast cancer. The effectiveness of these approaches is unquestionable, but they severely impact the physical and emotional health of the patient. Tremendous efforts are underway to precisely diagnose breast cancer subtypes and predict survival outcomes. This would allow the application of the most effective treatments possible while avoiding unnecessary therapies. We are working on identifying protein or RNA targets that are mis-regulated due to the high levels of tRNA found in breast cancer cells. These targets could serve as unique biomarkers in breast cancer. We also assess the physiological effects of tRNA over-expression on the development and progression of breast cancer. Our effort could establish the potential of tRNA and its regulatory targets as a new class of therapeutic targets.

Bioliography of all publications and meeting abstracts:

Publication:

Pavon-Eternod, M., S. Gomes, M.R. Rosner* & Tao Pan*: Overexpression of Initiator Methionine tRNA Leads to Global Reprogramming of tRNA Expression and Increased Proliferation in Human Epithelial Cells, *RNA* 19, 461-466 (2013).

Meeting abstracts:

Era of Hope 2011: tRNA and its activation targets as biomarkers and regulators of breast cancer. Mariana Pavon Eternod, Suzana Gomes, Tao Pan, Marsha Rosner

List of personnel receiving pay from the research effort:

1. Tao Pan (PI).
2. Marsha Rosner (PI).
3. Mariana Pavon Eternod (Graduate student, worked on Tasks 1, 2, and 3).
4. Dong-hui Zhou (Research Associate, worked on Task 2).
5. Guanquan Zheng (Graduate student, worked on Task 1).
6. Thomas Jones (Research Associate, worked on Task 2).
7. Susanna Gomes (Senior research tech, worked on Tasks 3 and 4)
8. Jiyoung Lee (Research Associate, worked on Tasks 3 and 4).

Overexpression of initiator methionine tRNA leads to global reprogramming of tRNA expression and increased proliferation in human epithelial cells

MARIANA PAVON-ETERNOD,[1] SUZANA GOMES,[2] MARSHA R. ROSNER,[2,3] and TAO PAN[1,3]

[1]Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637, USA
[2]Ben May Department of Cancer Research, University of Chicago, Chicago, Illinois 60637, USA

ABSTRACT

Transfer RNAs (tRNAs) are typically considered housekeeping products with little regulatory function. However, several studies over the past 10 years have linked tRNA misregulation to cancer. We have previously reported that tRNA levels are significantly elevated in breast cancer and multiple myeloma cells. To further investigate the cellular and physiological effects of tRNA overexpression, we overexpressed $tRNA_i^{Met}$ in two human breast epithelial cell lines. We then determined tRNA abundance changes and performed phenotypic characterization. Overexpression of $tRNA_i^{Met}$ significantly altered the global tRNA expression profile and resulted in increased cell metabolic activity and cell proliferation. Our results extend the relevance of tRNA overexpression in human cells and underscore the complexity of cellular regulation of tRNA expression.

Keywords: initiator methionine tRNA; tRNA; tRNA microarrays

INTRODUCTION

Misregulation of components of the translation machinery is characteristic of many types of tumor cells and can lead to malignant transformation (Bjornsti and Houghton 2004; Pandolfi 2004). Abnormally high levels of RNA polymerase III transcripts, including tRNA and 5S rRNA which are directly involved in translation, are found in a wide variety of transformed cell types (Marshall and White 2008). These cell types include cell lines transformed by DNA tumor viruses (such as hepatitis B virus), RNA tumor viruses (such as human T-cell leukemia virus 1), and chemical carcinogens. These observations have also been confirmed for tumors in situ by RT-PCR, Northern blot, and more recently by microarray analysis (Chen et al. 1997a,b; Winter et al. 2000; Pavon-Eternod et al. 2009). We have previously reported that tRNA levels are elevated in breast cancer and multiple myeloma cell lines (Pavon-Eternod et al. 2009; Zhou et al. 2009). Though abnormal RNA polymerase III activity has long been associated with cancer, it remains unclear whether it contributes to malignant transformation or is simply a byproduct of the cell's cancer state.

Due to its unique function in translation initiation, we are particularly interested in the role of the initiator methionine tRNA ($tRNA_i^{Met}$) in cancer. An appealing possibility is that overexpression of $tRNA_i^{Met}$ could alter the translational regulation of key genes involved in tumorigenesis. The effect may be both quantitative and qualitative: Overall protein synthesis may be increased, and mRNAs encoding cell-cycle or anti-apoptic proteins (such as Myc or cyclin D1) may be preferentially translated. To explore this question in the context of breast cancer, we set out to overexpress $tRNA_i^{Met}$ in human breast epithelial cell lines. Overexpressing tRNAs in human cell lines, however, proved more challenging than we had expected. Here we present our experimental approach to tRNA overexpression and a phenotypic characterization of the resulting cell lines (Fig. 1). We find that tRNA overexpression in human cells requires the generation of stable cell lines, and that only modest increases (1.4- to 2.2-fold) can be achieved. Remarkably, overexpression of $tRNA_i^{Met}$ in both epithelial cell lines changed the levels of other tRNAs, reprogramming the global tRNA expression profile. $tRNA_i^{Met}$ overexpression also resulted in increased metabolic activity and cell proliferation. Our results underscore the need for caution in interpreting the effects of individual tRNA overexpression, as little is known about the regulation of individual tRNA expression in the cell.

Abbreviations: $tRNA_i^{Met}$, initiator methionine tRNA; $tRNA_e^{Met}$, elongator methionine tRNA

[3]**Corresponding authors**
E-mail mrosner@uchicago.edu
E-mail taopan@uchicago.edu

Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.037507.112.

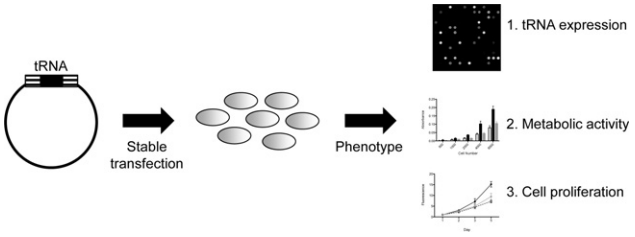

FIGURE 1. Experimental strategy for tRNA overexpression. The tRNA gene of interest with 200-bp flanking regions was cloned into a mammalian expression vector, then stably transfected into the human cell line. The stable cell line was then characterized in terms of tRNA expression profile, metabolic activity, and cell proliferation.

RESULTS AND DISCUSSION

Experimental strategies for tRNA overexpression

Because tRNAs are generally considered non-regulatory housekeeping genes, there are no well-established methods for manipulating the levels of specific tRNAs in mammalian cells. Furthermore, because tRNAs are highly abundant, overexpression even by twofold would require an additional transcription of ~1,000,000 molecules of $tRNA_i^{Met}$ per cell (Pavon-Eternod et al. 2009). In the past, exogenous tRNA has been introduced into cells either as DNA or directly as RNA (Carbon et al. 1983; Buvoli et al. 2000). We tried three different approaches to increase $tRNA_i^{Met}$ levels in two human cell lines: transient transfection with tRNA transcripts, transient transfection with a DNA vector containing the tRNA gene, and stable transfection with a DNA vector containing the tRNA gene. We selected the human breast epithelial cell lines 184A1 and MCF10A for these experiments for the following reasons: (i) They are non-tumorigenic cell lines, (ii) our previous work has shown 184A1 and MCF10A have relatively low levels of tRNA (Pavon-Eternod et al. 2009), and (iii) they are readily transfectable.

Our first approach relied on transient transfection of $tRNA_i^{Met}$ and $tRNA_e^{Met}$ transcripts. In vitro transcribed tRNAs have been reported to be active in translation when transfected into eukaryotic cells. Indeed, they have been used to insert unnatural amino acids into proteins and to induce amino acid substitutions resulting in widespread proteome damage in mammalian cells (Kohrer et al. 2001; Geslain et al. 2010). In vitro transcribed tRNAs are simple to synthesize and allow direct control over the amount of tRNA transfected. However, the question remains whether they are truly fully functional in the cell. In vitro transcribed tRNAs lack the post-transcriptional modifications characteristic of endogenous tRNAs that serve as identity determinants, contribute to tRNA stability, and impact translational accuracy (Alexandrov et al. 2006; Agris et al. 2007; Waas et al. 2007; Phizicky and Hopper 2010). This is particularly relevant for our tRNA of interest: $tRNA_i^{Met}$ transcripts lacking the m^1A_{58} modification are subject to nuclear polyadenylation and rapid degradation (Kadaba et al. 2004, 2006; Vanacova et al. 2005). In any case, we found that in vitro transcribed tRNAs were toxic to MCF10A cells when transfected at high enough concentrations to detectably increase cellular tRNA levels (data not shown).

Our second approach relied on transient transfection of a DNA vector containing a $tRNA_i^{Met}$ or $tRNA_e^{Met}$ gene into MCF10A cells. Fragments containing the tRNA gene were PCR-amplified from human genomic DNA and cloned into the pTarget Mammalian Expression Vector. The fragments contained 200 base pairs each upstream of and downstream from the endogenous tRNA gene, which should include all the regulatory elements necessary for tRNA transcription (Geiduschek and Kassavetis 2001; Dieci et al. 2007). This approach is experimentally straightforward and allows the transfection of large amounts of DNA without inducing toxicity. High-copy plasmids have been successfully used to induce tRNA overexpression in yeast and bacteria (Borel et al. 1993; Anderson et al. 1998; Sorensen et al. 2005). Again, transfection with our tRNA vectors did not result in any detectable increase in tRNA levels (data not shown).

Our third approach involved the generation of stable cell lines after transfection with our $tRNA_i^{Met}$, $tRNA_e^{Met}$, and empty control vectors. Using this approach, we successfully generated an 184A1 cell line and an MCF10A cell line overexpressing $tRNA_i^{Met}$ relative to the control cell line (Fig. 2). From this point forward, we designate these cell lines 184A1–$tRNA_i^{Met}$ and MCF10A–$tRNA_i^{Met}$. The levels of $tRNA_i^{Met}$ increased by 1.4- and twofold, respectively, as measured by tRNA microarrays. This level is comparable to that observed in breast cancer cell lines relative to non-tumorigenic breast epithelial cell lines (Pavon-Eternod et al. 2009). We were, however, unable to generate either a 184A1 or MCF10A cell line overexpressing $tRNA_e^{Met}$ relative to the control cell line (Fig. 2A).

To confirm our microarray data, we analyzed $tRNA_i^{Met}$ and $tRNA_e^{Met}$ content in our 184A1 cell lines (control, 184A1–$tRNA_i^{Met}$, and MCF10A–$tRNA_i^{Met}$) by dot blot (Fig. 2B). In all cases, the microarray and dot blot data were in very good agreement. Our selective fluorophore labeling method requires that all tRNAs measured by microarrays contain 3′CCA (Pavon-Eternod et al. 2009) which is characteristic of all mature tRNAs. The agreement between microarray, which measures mature tRNA, and dot blot data, which measure mature and precursor tRNA, indicates that the observed $tRNA_i^{Met}$ overexpression is primarily derived from mature tRNA.

$tRNA_i^{Met}$ overexpression generates unique tRNA expression profiles

Unexpectedly, $tRNA_i^{Met}$ overexpression induced a significant change in the levels of other tRNAs. Compared with the corresponding control line, median nuclear-encoded tRNA abundance increased by 1.4-fold in 184A1–$tRNA_i^{Met}$ and 2.2-fold in MCF10A–$tRNA_i^{Met}$, whereas median mitochondrial-

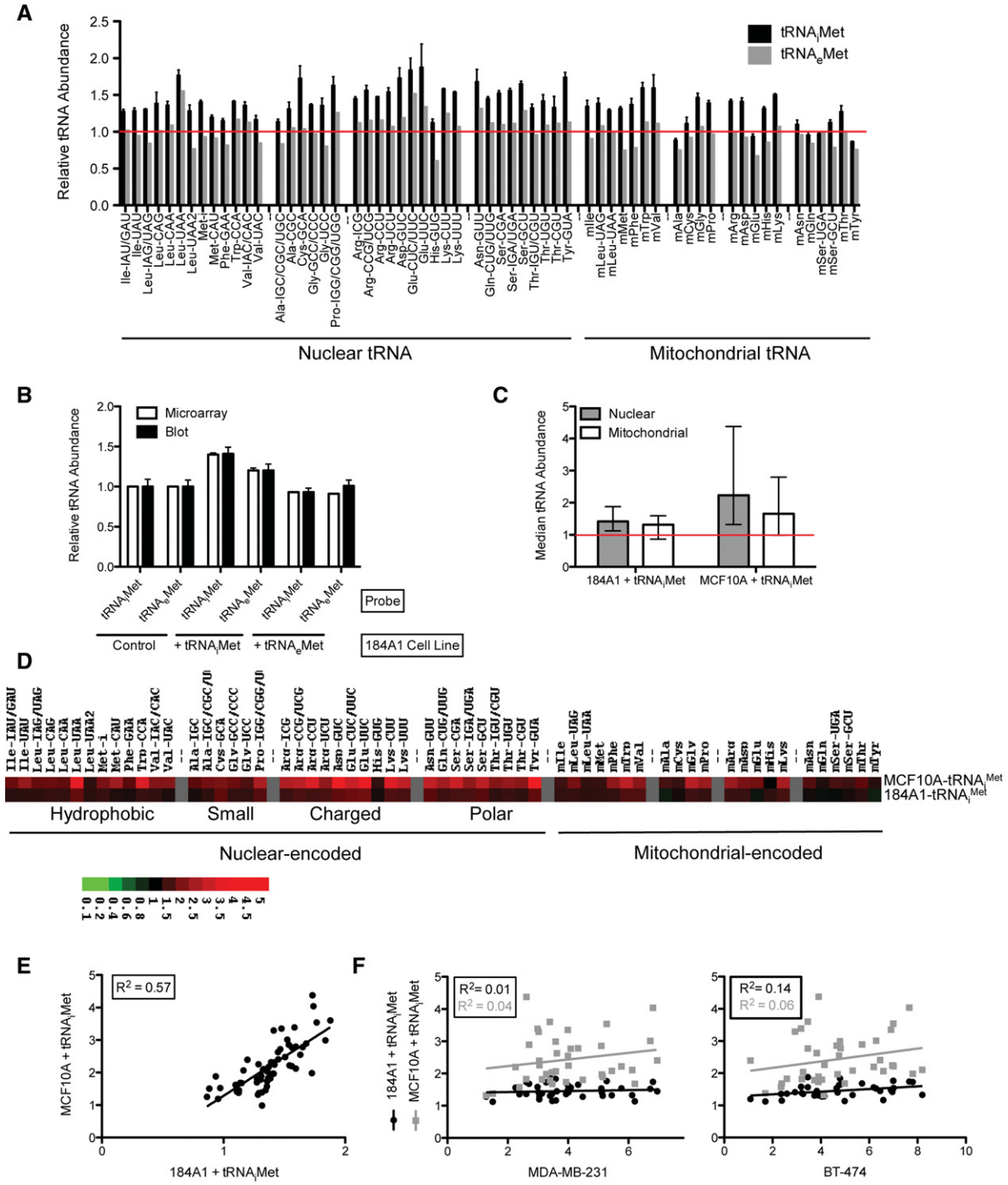

FIGURE 2. tRNA$_i^{Met}$ overexpression generates unique tRNA expression profiles. (*A*) Individual tRNA abundances in 184A1–tRNA$_i^{Met}$ and 184A1–tRNA$_e^{Met}$ cell lines. Individual tRNA abundance values are shown for 184A1–tRNA$_i^{Met}$ (black) and 184A1-tRNA$_e^{Met}$ (gray) relative to an empty vector control cell line (set to 1, red line). A value of 1 indicates no change, a value <1 indicates a decrease, and a value >1 indicates an increase in tRNA levels relative to the control cell line. Data are grouped according to amino acid type. The tRNA$_i^{Met}$ and tRNA$_e^{Met}$ probes are labeled Met-i and Met-CAU, respectively. Where error bars are present, values are averages from dye-swapped experiments and error bars indicate standard deviation. One sample *t*-test was performed to determine the statistical significance of the changes: $^*P$-value <0.05. (*B*) Validation of microarray data by dot blot. As in *A*, relative tRNA abundance is defined as the ratio between the indicated cell line and the control cell line. Relative tRNA abundance values obtained by microarray (white) and dot blot (black, average of three replicates, error bars indicate standard deviation) are plotted for tRNA$_i^{Met}$ and tRNA$_e^{Met}$ in the three 184A1 cell lines generated for this study (control, 184A1–tRNA$_i^{Met}$ and 184A1–tRNA$_e^{Met}$). (*C*) Median tRNA abundance upon tRNA$_i^{Met}$ overexpression. Median values for 184A1–tRNA$_i^{Met}$ and MCF10A–tRNA$_i^{Met}$ relative to control cell lines (set to 1, red line). Median values for nuclear-encoded tRNAs (gray) and mitochondrial-encoded (white) tRNAs are shown. The *upper* and *lower* bars indicate the range of individual tRNA abundances. (*D*) Heat map of tRNA abundances upon tRNA$_i^{Met}$ overexpression. Relative tRNA abundance levels of nuclear and mitochondrial-encoded tRNAs in 184A1–tRNA$_i^{Met}$ and MCF10A–tRNA$_i^{Met}$ are shown as TreeView images. Data are grouped according to amino acid type. Green indicates a decreased level of expression, red indicates an increased level of expression, and black indicates no change in expression level relative to the reference sample. (*E*) Individual tRNA abundances in 184A1–tRNA$_i^{Met}$ compared with MCF10A–tRNA$_i^{Met}$. Individual tRNA abundance values for 184A1–tRNA$_i^{Met}$ and MCF10A–tRNA$_i^{Met}$ are relative to the corresponding control cell lines. (*F*) Individual nuclear-encoded tRNA abundances in two breast cancer lines, MDA-MB-231 (*left*) and BT-474 (*right*) compared with 184A1–tRNA$_i^{Met}$ and MCF10A–tRNA$_i^{Met}$. Individual tRNA levels for the two breast cancer cell lines, MDA-MB-231 and BT-474, are relative to the breast epithelial MCF10A cell line. Individual tRNA levels for the 184A1–tRNA$_i^{Met}$ and MCF10A–tRNA$_i^{Met}$ cell lines are relative to the corresponding control cell lines.

encoded tRNA abundance increased by 1.3-fold in 184A1–$tRNA_i^{Met}$ and 1.7-fold in MCF10A–$tRNA_i^{Met}$ (Fig. 2C). These changes are more striking for individual tRNAs (Fig. 2A,D). Due to the nature of our microarray measurements, we express individual tRNA abundances in 184A1–$tRNA_i^{Met}$ and MCF10A–$tRNA_i^{Met}$ relative to the corresponding control cell line. While some tRNAs are increased up to fourfold upon $tRNA_i^{Met}$ overexpression, others are not affected. Remarkably, $tRNA_i^{Met}$ overexpression generates very similarly altered expression profiles for nuclear-encoded tRNAs in both 184A1 and MCF10A cell lines ($R^2 = 0.57$, Fig. 2E).

We also compared whether the tRNA expression profiles induced by $tRNA_i^{Met}$ overexpression in breast epithelial lines are similar to those measured in breast cancer cell lines. Our previous study of tRNA expression in breast cancer revealed that tRNA overexpression is characteristic of breast cancer cells, and that this overexpression is highly selective based on tRNA identity (Pavon-Eternod et al. 2009). We therefore plotted tRNA levels (relative to the breast epithelial cell line MCF10A) in two breast cancer cell lines, MDA-MB-231 and BT-474, vs. the tRNA levels (relative to the corresponding control cell line) in our 184A1–$tRNA_i^{Met}$ and MCF10A–$tRNA_i^{Met}$ cell lines. We find a very poor correlation between the tRNA levels in bona fide breast cancer cell lines and the tRNA levels induced by $tRNA_i^{Met}$ overexpression ($R^2 < 0.15$ in all cases) (Fig. 2F). While the tRNAs carrying charged and polar amino acids were consistently among the most overexpressed tRNAs in the breast cancer cell lines examined, we observe no such trends in our 184A1–$tRNA_i^{Met}$ and MCF10A–$tRNA_i^{Met}$ lines.

Initiator methionine tRNA overexpression leads to increased cell metabolism and proliferation

Elevated tRNA levels are characteristic of breast cancer cells (Pavon-Eternod et al. 2009) which often exhibit altered metabolic activity and unregulated growth compared with noncancer cells. We therefore measured the metabolic activity and cell proliferation of 184A1–$tRNA_i^{Met}$ relative to the 184A1–control cell line (Fig. 3). We also included the 184A1–$tRNA_e^{Met}$ cell line as an additional control: Even though $tRNA_e^{Met}$ expression was not increased in this line as we had expected, we detected punctual changes in the levels of several other tRNAs (such as $tRNA^{Leu(UAA)}$ and $tRNAGlu^{(CUC/UUC)}$) (Fig. 2A), which may have an effect. We first measured the metabolic activity using two assays: Calcein AM, which relies on the activity of cytoplasmic esterases, and WST1, which relies on the activity of mitochondrial dehydrogenases. Both assays showed increased metabolic activity for 184A1–$tRNA_i^{Met}$ relative the control cell line, but no change in metabolic activity for 184A1–$tRNA_e^{Met}$. We also measured cell proliferation by Hoechst staining. Again, 184A1–$tRNA_i^{Met}$ showed increased cell proliferation relative to the control cell line, but no significant change was seen for 184A1–$tRNA_e^{Met}$.

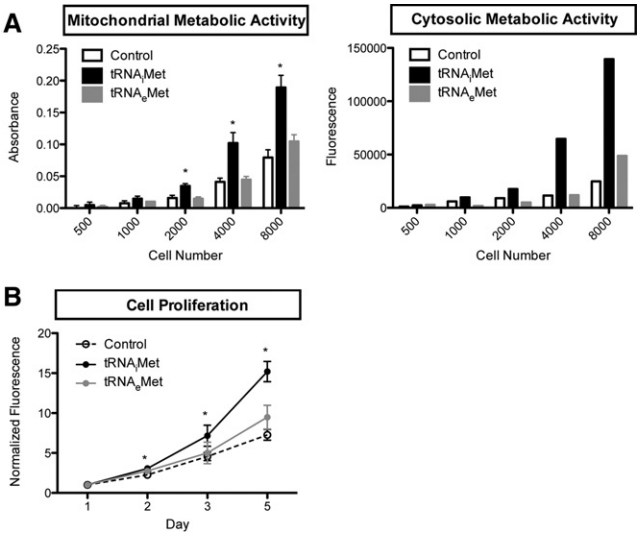

FIGURE 3. $tRNA_i^{Met}$ overexpression leads to increased cell metabolism and proliferation. Data are shown for the 184A1 cell lines 184A1–control, 184A1–$tRNA_i^{Met}$, and 184A1–$tRNA_e^{Met}$. *T*-tests were performed to determine the statistical significance of the differences observed relative to 184A1–control: **P*-value <0.05. (*A*) Metabolic activity. Mitochondrial metabolic activity was measured by WST1, which relies on the activity of mitochondrial dehydrogenases. Cytosolic metabolic activity was measured by Calcein AM, which relies on the activity of cytoplasmic esterases. Where error bars are indicated, assays were performed in triplicate and the error bars indicate standard deviation. (*B*) Cell proliferation. Cell proliferation was measured over 5 d by Hoechst DNA staining. The assays were performed in triplicate; error bars indicate standard deviation.

Concluding remarks

In our experience, overexpressing a specific tRNA in human cell lines is not trivial. Of three possible approaches attempted by us, only one was successful: the generation of stable cell lines after transfection with a DNA vector containing the tRNA gene. Even so, we were able to generate cell lines stably overexpressing the desired tRNA for only one of two tRNAs. While we successfully generated stable cell lines overexpressing $tRNA_i^{Met}$, we were unable to generate stable cell lines overexpressing $tRNA_e^{Met}$ to any detectable level. This may be due to some intrinsic properties of $tRNA_e^{Met}$, or other random factors such as the site of integration and copy number. Our results also indicate that manipulating the levels of one specific tRNA—in this case $tRNA_i^{Met}$—significantly affects the levels of other tRNAs in the cell, suggesting some kind of feedback regulatory mechanism in the cell. Remarkably, $tRNA_i^{Met}$ overexpression in two different cell lines resulted in similar patterns of tRNA expression. We expect that the tRNA expression profile induced by overexpressing a specific tRNA is dependent on many factors, including but not limited to the identity of the tRNA being introduced, the genetic background of the cell, and the integration sites. Regardless, care must be taken in attributing phenotypic changes to overexpression of an individual tRNA. The increase in metabolic

activity and cell proliferation we measure in our 184A1–tRNA$_i^{Met}$ cell line may indeed be due to tRNA$_i^{Met}$ overexpression, but also to overexpression of a number of other tRNAs or even to globally increased tRNA levels.

Our findings highlight the fact that little is known about the regulation of individual tRNA expression in the cell, and how cells respond to perturbations in tRNA levels. It is generally believed that tRNA transcription via RNA polymerase III is globally regulated in response to nutrient availability and other environmental signals, in coordination with rRNA transcription via RNA polymerase I. Current models hold that transcription at tRNA genes is coordinately regulated by shared transcription factors, acting at highly related promoter sequences (Phizicky and Hopper 2010). This view does not account for the tissue-specific differences in individual tRNA expression or the differential overexpression of individual tRNA species in breast cancer cells (Dittmar et al. 2006; Pavon-Eternod et al. 2009). A systematic study of individual tRNA expression is required to elucidate the functional significance and underlying regulatory mechanisms.

MATERIALS AND METHODS

DNA vectors

Fragments containing tRNA genes were PCR-amplified from human genomic DNA, using the following primer pairs: 5′-TGAG TTGGCAACCTGTGGTA and 5′-TTGGGTGTCCATGAAAATCA for tRNA$_i^{Met}$, 5′-AGCGACCTTCCCACA and 5′-GTCTCCCATT CCTACACG for tRNA$_e^{Met}$. These fragments were cloned into the pTarget Mammalian Expression vector (Promega) following the manufacturer's instructions.

Cell lines

All cell lines were purchased from American Type Culture Collection (ATCC). MCF10A and 184A1 cells were cultured in 1:1 DMEM/F12 with 2.5 mM L-Gln and 15 mM HEPES (Thermo Scientific HyClone) supplemented with 10% FBS, 1% Penicillin/Streptomycin, 5 µg/mL insulin, 10 ng/mL EGF, and 0.5 µg/mL hydrocortisone. MDA-MB-231 and BT-474 were cultured in RPMI 1640 1× medium (Thermo Scientific HyClone) supplemented with 10% FBS and 1% Penicillin/Streptomycin.

To generate stable cell lines, cells were transfected using Amaxa Nucleofector technology (LonzaBio). After 48 h, medium was supplemented with 500 µg/mL G418 (Sigma) for selection. After 2–4 wk, G418 resistant colonies appeared and the G418 concentration was scaled down to 200 µg/mL. Medium was supplemented with 200 µg/mL G418 for routine culture.

Transfer RNA microarrays

Total RNA for each cell line was obtained at 80%–90% confluency using the miRVana miRNA Isolation Kit (Ambion). This procedure isolates RNA species as short as 15 nt and is therefore not biased against tRNA. Total RNA quality was verified by agarose gel electrophoresis.

The tRNA microarray experiment consists of four steps starting from total RNA: (1) deacylation to remove any amino acids still attached to the tRNA, (2) selective fluorophore labeling of tRNA, (3) hybridization, and (4) data analysis. The tRNA microarray method, including reproducibility and result validation by Northern blot, has been extensively described in previously published papers (Dittmar et al. 2004, 2006; Pavon-Eternod et al. 2009, 2010; Zhou et al. 2009).

Dot blots

The following DNA probes, identical to those spotted on the tRNA microarrays, were used to quantify tRNA$_i^{Met}$ and tRNA$_e^{Met}$ in total RNA: 5′-AGCAGAGTGGCGCAGCGGAAGCGTGCTGGGCCCAT AACCCAGAGGTCGATGGATCGAAACCATCCTCTGCTA-3′ for tRNA$_i^{Met}$, and 5′-GCCYYCTTAGCGCAGYDGGCAGCGCGTCA GTCTCATAATCTGAAGGTCCTGAGTTCGAGCCTCAGAGRGGG CA-3′ for tRNA$_e^{Met}$. Probes were 5′-radiolabeled using T4 polynucleotide kinase and γ-^{32}P-ATP (Perkin-Elmer), followed by purification on a denaturing urea polyacrylamide gel. To detect tRNA$_i^{Met}$ and tRNA$_e^{Met}$ in total RNA, 100 ng total RNA was spotted and UV cross-linked on a Hybond XL membrane (GE Healthcare). The membrane was pre-hybridized in hybridization buffer (300 mM NaCl, 1% SDS, 20 mM phosphate buffer pH 7) for 30 min at room temperature, then hybridized overnight at 60°C in hybridization buffer containing 100,000–300,000 cpm of the radiolabeled probe. The membrane was then washed three times for 20 min at room temperature in wash buffer (300 mM NaCl, 0.1% SDS, 20 mM phosphate buffer pH 7, 2 mM EDTA). Phosphorimaging was used to quantify the amount of tRNA$_i^{Met}$ and tRNA$_e^{Met}$ present in each sample.

Metabolic activity assays

Cells were plated in 100 µL medium in 96-well plate at the following cell densities: 500, 1000, 2000, 4000, and 8000 cells/well. For WST1 assays: 10 µL WST1 reagent (Roche 05 015 944 001) was added to each well. Absorbance at 440 nm was read after 1 h incubation at 37°C. For Calcein AM assays: Cells were incubated in 200 µL Calcein AM (BD 354216) working solution (1 µM in HBSS) for 1 h at 37°C. Fluorescence was measured at 490ex/520em.

Cell proliferation assays

Cell proliferation was measured over 5 d using Hoechst DNA staining (Invitrogen H1398). Cells were plated in 100 µL medium at 500 cells/well in 96-well plates. To stain DNA, cells were incubated in 100 µL of 0.1 µg/mL Hoechst solution in HBSS for 1 h at 37°C. After washing with HBSS to remove any unbound dye, fluorescence was measured 355ex/460em. Fluorescence is directly proportional to the number of cells present, regardless of cell type.

Statistical significance

T-tests and one-sample *t*-tests were performed using GraphPad QuickCalcs (http://www.graphpad.com/quickcalcs/contMenu/).

ACKNOWLEDGMENTS

This work was supported by a grant from CDMRP (W81XWH-10-1-0452, 10-1-0453 to T.P. and M.R.R.). M.P.-E. was supported in

part by a Ruth Kirshstein Pre-doctoral Fellowship from the NIH (1F31CA139968).

Received November 26, 2012; accepted January 23, 2013.

REFERENCES

Agris PF, Vendeix FA, Graham WD. 2007. tRNA's wobble decoding of the genome: 40 years of modification. *J Mol Biol* **366:** 1–13.

Alexandrov A, Chernyakov I, Gu W, Hiley SL, Hughes TR, Grayhack EJ, Phizicky EM. 2006. Rapid tRNA decay can result from lack of nonessential modifications. *Mol Cell* **21:** 87–96.

Anderson J, Phan L, Cuesta R, Carlson BA, Pak M, Asano K, Bjork GR, Tamame M, Hinnebusch AG. 1998. The essential Gcd10p-Gcd14p nuclear complex is required for 1-methyladenosine modification and maturation of initiator methionyl-tRNA. *Genes Dev* **12:** 3650–3662.

Bjornsti MA, Houghton PJ. 2004. Lost in translation: Dysregulation of cap-dependent translation and cancer. *Cancer Cell* **5:** 519–523.

Borel F, Hartlein M, Leberman R. 1993. In vivo overexpression and purification of *Escherichia coli* $tRNA^{ser}$. *FEBS Lett* **324:** 162–166.

Buvoli M, Buvoli A, Leinwand LA. 2000. Suppression of nonsense mutations in cell culture and mice by multimerized suppressor tRNA genes. *Mol Cell Biol* **20:** 3116–3124.

Carbon P, Haumont E, Fournier M, de Henau S, Grosjean H. 1983. Site-directed in vitro replacement of nucleosides in the anticodon loop of tRNA: Application to the study of structural requirements for queuine insertase activity. *EMBO J* **2:** 1093–1097.

Chen W, Bocker W, Brosius J, Tiedge H. 1997a. Expression of neural BC200 RNA in human tumours. *J Pathol* **183:** 345–351.

Chen W, Heierhorst J, Brosius J, Tiedge H. 1997b. Expression of neural BC1 RNA: Induction in murine tumours. *Eur J Cancer* **33:** 288–292.

Dieci G, Fiorino G, Castelnuovo M, Teichmann M, Pagano A. 2007. The expanding RNA polymerase III transcriptome. *Trends Genet* **23:** 614–622.

Dittmar KA, Mobley EM, Radek AJ, Pan T. 2004. Exploring the regulation of tRNA distribution on the genomic scale. *J Mol Biol* **337:** 31–47.

Dittmar KA, Goodenbour JM, Pan T. 2006. Tissue-specific differences in human transfer RNA expression. *PLoS Genet* **2:** e221.

Geiduschek EP, Kassavetis GA. 2001. The RNA polymerase III transcription apparatus. *J Mol Biol* **310:** 1–26.

Geslain R, Cubells L, Bori-Sanz T, Alvarez-Medina R, Rossell D, Marti E, Ribas de Pouplana L. 2010. Chimeric tRNAs as tools to induce proteome damage and identify components of stress responses. *Nucleic Acids Res* **38:** e30.

Kadaba S, Krueger A, Trice T, Krecic AM, Hinnebusch AG, Anderson J. 2004. Nuclear surveillance and degradation of hypomodified initiator $tRNA^{Met}$ in *S. cerevisiae*. *Genes Dev* **18:** 1227–1240.

Kadaba S, Wang X, Anderson JT. 2006. Nuclear RNA surveillance in *Saccharomyces cerevisiae*: Trf4p-dependent polyadenylation of nascent hypomethylated tRNA and an aberrant form of 5S rRNA. *RNA* **12:** 508–521.

Kohrer C, Xie L, Kellerer S, Varshney U, RajBhandary UL. 2001. Import of amber and ochre suppressor tRNAs into mammalian cells: A general approach to site-specific insertion of amino acid analogues into proteins. *Proc Natl Acad Sci* **98:** 14310–14315.

Marshall L, White RJ. 2008. Non-coding RNA production by RNA polymerase III is implicated in cancer. *Nat Rev Cancer* **8:** 911–914.

Pandolfi PP. 2004. Aberrant mRNA translation in cancer pathogenesis: An old concept revisited comes finally of age. *Oncogene* **23:** 3134–3137.

Pavon-Eternod M, Gomes S, Geslain R, Dai Q, Rosner MR, Pan T. 2009. tRNA over-expression in breast cancer and functional consequences. *Nucleic Acids Res* **37:** 7268–7280.

Pavon-Eternod M, Wei M, Pan T, Kleiman L. 2010. Profiling non-lysyl tRNAs in HIV-1. *RNA* **16:** 267–273.

Phizicky EM, Hopper AK. 2010. tRNA biology charges to the front. *Genes Dev* **24:** 1832–1860.

Sorensen MA, Elf J, Bouakaz E, Tenson T, Sanyal S, Bjork GR, Ehrenberg M. 2005. Over expression of a $tRNA^{Leu}$ isoacceptor changes charging pattern of leucine tRNAs and reveals new codon reading. *J Mol Biol* **354:** 16–24.

Vanacova S, Wolf J, Martin G, Blank D, Dettwiler S, Friedlein A, Langen H, Keith G, Keller W. 2005. A new yeast poly(A) polymerase complex involved in RNA quality control. *PLoS Biol* **3:** e189.

Waas WF, Druzina Z, Hanan M, Schimmel P. 2007. Role of a tRNA base modification and its precursors in frameshifting in eukaryotes. *J Biol Chem* **282:** 26026–26034.

Winter AG, Sourvinos G, Allison SJ, Tosh K, Scott PH, Spandidos DA, White RJ. 2000. RNA polymerase III transcription factor TFIIIC2 is overexpressed in ovarian tumors. *Proc Natl Acad Sci* **97:** 12619–12624.

Zhou Y, Goodenbour JM, Godley LA, Wickrema A, Pan T. 2009. High levels of tRNA abundance and alteration of tRNA charging by bortezomib in multiple myeloma. *Biochem Biophys Res Commun* **385:** 160–164.