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TITLE: tRNA and its Activation Targets as Biomarkers and Regulators of Breast Cancer

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  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 under-appreciated 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 are 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.					
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## 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 (1). 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 hypothesize that tRNA over-expression alters the translational regulation of key genes involved in cancer development and progression. We aim to (i) identify the protein or RNA targets that are mis-regulated upon tRNA over-expression, and (ii) determine the effect of tRNA over-expression on tumor initiation and progression.

## BODY

Task 1 – Identify gene targets whose translation is mis-regulated upon tRNA over-expression.

a) Establish the method: ribosome profiling. → **Completed.**

We adapted a novel approach, 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. In its initial application, over two million actively translated mRNA segments were sequenced in yeast and in mammalian cells (2,3).

Our ribosome profiling protocol in human cells includes the following steps: 1) Prepare cell lysate from human cells at 90-100% 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 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 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

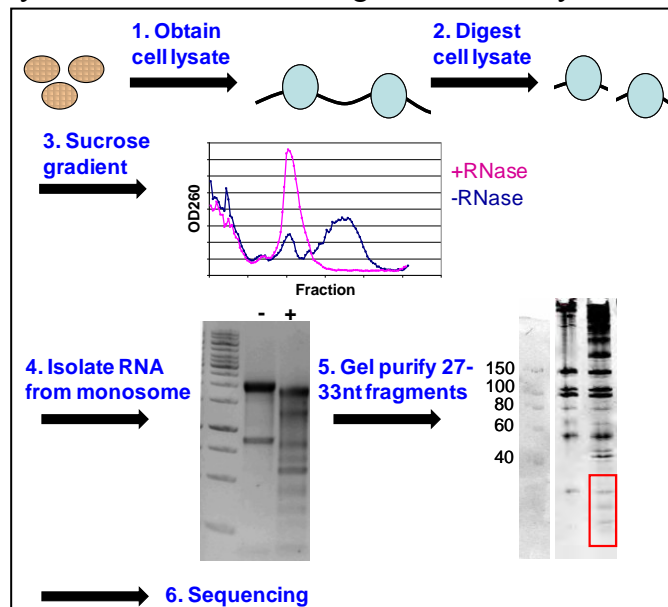
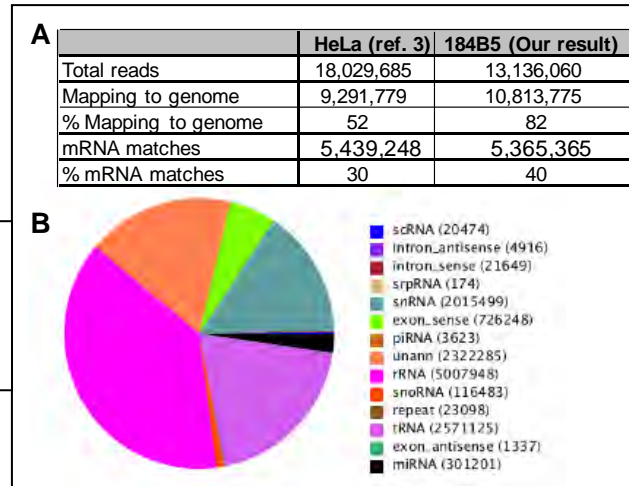


Fig. 1. *The experimental workflow of ribosome profiling in human cells.* Sucrose density gradient traces at step 3 and the gel images at steps 4 and 5 are our actual experimental data for a breast epithelial cell line.

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.

Fig. 2. Ribosome profiling in human 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. → **In progress.**

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 changed 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 have applied ribosome profiling to two non-cancer breast epithelial cell lines – 184 B5 and 184 A1 – and three breast cancer cell lines – MCF7, BT-474, and MDA-MB-231. We are currently analyzing the sequencing data obtained from these samples.

Task 2 – Validate results for selected genes identified in task 1. → **Future direction.**

A comparative analysis of translational activity across non-tumorigenic and breast cancer cell lines will allow us to identify genes whose translation is altered under elevated tRNA levels. In particular, our focus will be these genes that are known to be involved in breast cancer development and proliferation. We will validate changes in translational mis-regulation of such breast cancer relevant genes using standard pulse-labeling and other techniques as described in the original application.

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.**

To determine the effect of tRNA over-expression on tumor initiation and progression, we over-expressed  $tRNA_i^{Met}$  in the non-cancer breast cell line 184 A1.  $tRNA_i^{Met}$  levels were increased by approximately 50% in 184A1+ $tRNA_i^{Met}$  (over-expressing  $tRNA_i^{Met}$ ) as compared to 184A1+v (vector-only control cell line). Remarkably, over-expression of  $tRNA_i^{Met}$  led to over-expression of other tRNAs, in a pattern similar to that observed in breast cancer cell lines (Figure 3).

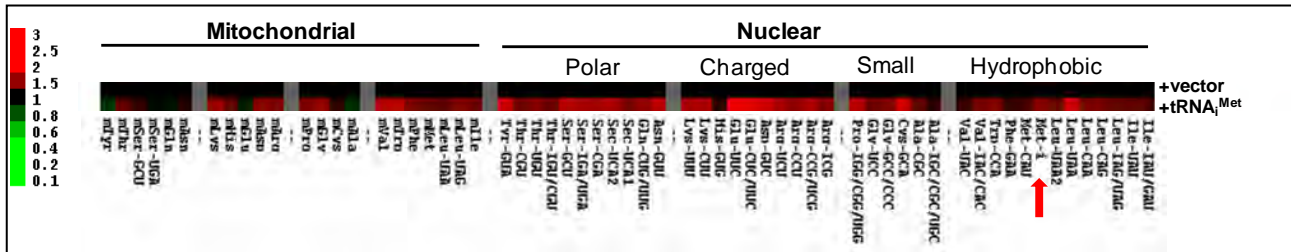


Fig. 3. *tRNA* expression in *184A1+vector* and *184A1+tRNA<sub>i</sub><sup>Met</sup>*. Data is shown as TreeView image. Red indicates an increased expression and green indicates a decreased expression relative to *184A1+vector*. *tRNA<sub>i</sub><sup>Met</sup>* is indicated by a red arrow and is overexpressed by ~50%.

We then measured cell proliferation and metabolic activity in the *184A1+tRNA<sub>i</sub><sup>Met</sup>* and *184A1+vector* cell lines. *184A1+tRNA<sub>i</sub><sup>Met</sup>* showed increased cell proliferation, as measured by Hoechst staining (Figure 4A). Furthermore, *184A1+tRNA<sub>i</sub><sup>Met</sup>* also showed increased metabolic activity, as measured by Calcein AM (Figure 4B) and WST1 assays (similar results, not shown).

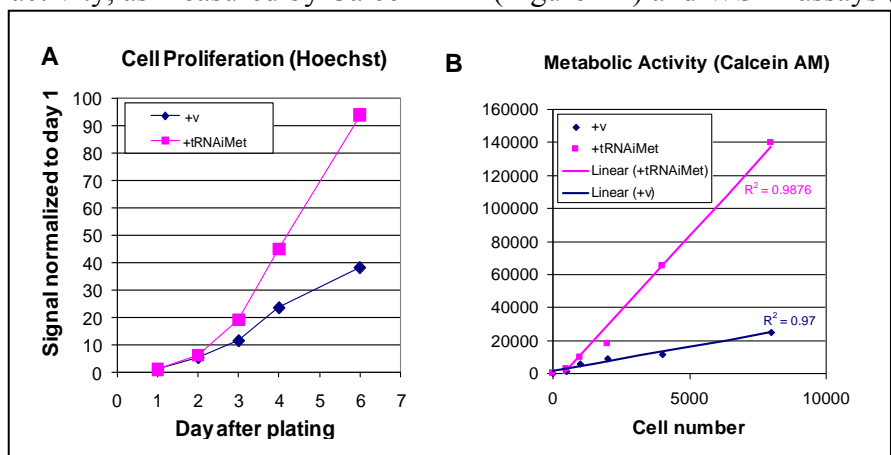


Fig. 4. Cell proliferation and metabolic activity in *184A1+vector* and *184A1+tRNA<sub>i</sub><sup>Met</sup>*. (A) Cell proliferation using Hoechst staining. (B) Metabolic activity using Calcein AM assay.

To assay for self-renewal, we measured colony formation for the *184A1+tRNA<sub>i</sub><sup>Met</sup>* and *184A1+vector* cell lines. Both colony number and colony size are 4- to 5-fold greater for *184A1+tRNA<sub>i</sub><sup>Met</sup>* than for *184A1+vector* (Figure 5). This result suggests cells over-expressing *tRNA<sub>i</sub><sup>Met</sup>* have a greater self-renewal potential than the vector-only control. We are currently working on confirming this result using mammosphere assays. We will also determine the number of cells in

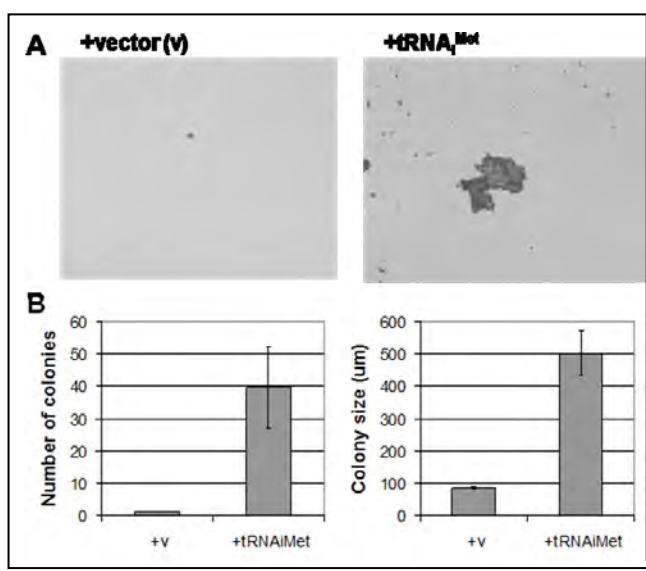


Fig. 5. Soft agar colony assays for *184A1+vector* and *184A1+tRNA<sub>i</sub><sup>Met</sup>*. (A) Microscope images for +vector (left) and +*tRNA<sub>i</sub><sup>Met</sup>* cells (right). (B) Quantitative cell counts (left) and colony size (right).

each condition that express T-IC markers, namely CD44<sup>+</sup>CD24<sup>-</sup>, by FACS analysis using FITC-tagged antibodies to CD44 and PE-tagged antibodies to CD24.

b) Test whether tRNA over-expression is associated with BT-IC differentiation. → **Future direction.**

Once we have isolated human BT-ICs from mammosphere cultures as described above, we will determine whether these cells lose the CD44<sup>+</sup>CD24<sup>-</sup> markers upon plating in differentiation conditions on collagen in DMEM with 10% serum. We will also assay the cell lysates by immunoblotting for two stem cell-associated markers: SOX2 and OCT4. We anticipate that these cells will rapidly lose all of these T-IC markers by 10 days of culture. In addition, we will test the cells for changes in tRNA expression with differentiation by tRNA arrays. These studies will indicate whether tRNA expression is suppressed upon differentiation of tumor-initiating cells.

To determine whether the differentiated cells are of mixed lineage, another hallmark of T-ICs, we will immunostain the cells with antibodies to myoepithelial markers (CK14, alpha-SMA), or luminal epithelial cells (CK18, MUC1) as described above. We will also determine whether the differentiated cells have lost their capacity for self-renewal using a mammosphere assay. If the original human tumor cells were enriched for T-ICs, then we would expect them to be multipotent and thus differentiate into different lineages.

Task 4 – Examine the effect of tRNA over-expression on tumor formation and metastasis in mice.

a) Test whether tRNA over-expression promotes invasion. → **In progress.**

As described under Task 3a, we have two cell lines derived from 184A1 (non-cancer breast cell line): 184A1+tRNA<sub>i</sub><sup>Met</sup> which over-expresses tRNA<sub>i</sub><sup>Met</sup> by 50%, and 184A1+vector which is a vector-only control cell line. We have measured cell proliferation in these two cell lines, and found that 184A1+tRNA<sub>i</sub><sup>Met</sup> proliferates at a much greater rate than 184A1+vector. We are currently measuring the invasion potential of these two cell lines using Matrigel invasion assays.

b) Determine the effect of tRNA over-expression on human BT-IC tumor formation and lung metastasis in mice. → **Future direction.**

To test efficacy of tumor formation by human tumor cell lines expressing tRNAs that grow on mammosphere, we will inject 10<sup>3</sup> -10<sup>4</sup> dissociated mammosphere cells orthotopically into the mammary glands of 10 mice and determine the number of mice that express tumors. We will use cells that have been labeled by luciferase, injected into mice and monitored by optical scanning with Xenogen. If the cells are cancer stem cells, we expect at least 8-10 mice to express tumors. By contrast, injection of control cells that overexpress non-transforming tRNAs should not increase the number of mice expressing tumors.

Similarly, some investigators have postulated that T-ICs are the source of metastatic cells. To test this possibility, we will determine the efficiency of lung metastasis by mammosphere cells either expressing control vector or selected tRNAs by tail vein injection. For the growth promoting, tRNA-expressing cells lines, we will use fewer cells than in the parent population to see if the

efficiency is enhanced by mammosphere passage. As in the parent cells, we expect that expression of tRNAs such as the initiator methionine will dramatically enhance BT-IC bone metastasis.

## KEY RESEARCH ACCOMPLISHMENTS

- Established a ribosome profiling method to look at active translation in human cells.
- 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.
- Demonstrated that over-expression of tRNA<sub>i</sub><sup>Met</sup> leads to increased cell proliferation and self-renewal in a breast epithelial cell line.

## REPORTABLE OUTCOMES

- Poster presentation at the Era of Hope meeting in Orlando, FL, August 2011.
- Ribosome profiling protocol for human cells.
- Constructs for the over-expression of tRNA<sub>i</sub><sup>Met</sup> and tRNA<sub>e</sub><sup>Met</sup> 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 are currently investigating whether tRNA over-expression also increases the invasion potential of these cells, and plan to move our studies into mice in the near future. Furthermore, we have adapted a ribosome profiling method and have applied it to a panel of breast cancer and non-cancer breast cell lines. Analysis of the data profiling data should identify genes whose translation is altered under the high tRNA levels found in the breast cancer cell lines.

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 therefore 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 currently 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 should establish the potential of tRNA and its regulatory targets as a new class of therapeutic targets.

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