tRNA Fragments: Expression and Function in Ovarian Cancer

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Dedication

I would like to dedicate this dissertation to my parents. They always have full confidence and constant support for me. I would never become what I am today without them.

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Abstract

Deep sequencing studies of noncoding RNA in liquid biopsies are revealing a vast repertoire of potential biomarkers. Ovarian cancer is a difficult-to-diagnose disease, urgently requiring novel and readily accessible biomarkers. We hypothesized that urine, one source of liquid biopsy samples, may contain novel noncoding RNAs (ncRNAs) that could serve as biomarkers for ovarian cancer. We proceeded to deep sequence RNA extracted from urine collected from ovarian cancer patients to better understand the repertoire of small RNAs in this type of liquid biopsy sample. The ncRNAs identified in these urine samples were predominantly microRNAs (miRNAs), ribosomal RNA (rRNA) fragments and tRNA fragments (tRFs). tRFs are a group of ncRNAs, which have been found across the biological kingdom and are increasingly being studied for their role in cancer biology. Several tRFs have been studied in cancer, although not previously in ovarian cancer. We have studied the expression of one specific tRF, 5' fragment of tRNA-Glu-CTC (tRF5-Glu), in five different ovarian cancer cell lines. Several variants of tRF5-Glu were identified and we have now confirmed the expression of tRF5-Glu in ovarian cancer cells by quantitative real-time PCR (qRT-PCR), Northern analysis and ligation PCR. Additionally, we determined that angiogenin (ANG) plays a role in the biogenesis of tRF5-Glu. Furthermore, we have shown that tRF5-Glu targets the mRNA of the Breast Cancer Anti-estrogen Resistance 3 (BCAR3). While BCAR3 is known to regulate cancer cell migration and contributes to anti-estrogen resistance in breast cancer cells, it has not previously been studied in ovarian cancer or shown to be targeted by a tRF. Using synthetic mimics of tRF5-Glu and siRNAs targeting BCAR3, we were able to

show that tRF5-Glu expression and the knock down of BCAR3 expression inhibits proliferation in ovarian cancer cells. These studies demonstrate that tRF5-Glu contributes to the regulation of BCAR3 and provides a novel mechanism of the regulation of proliferation in ovarian cancer cell lines.

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List of Abbreviations

AGO	argonautes
APA	alternative poly A sites
APOER2	apolipoprotein E receptor 2
ARM-seq	AlkB-facilitated RNA methylation sequencing
BCAR3	breast cancer anti-estrogen resistance 3
CA-125	cancer-antigen 125
CLASH	crosslinking, ligation and sequencing of hybrids
CT	computed tomography
DMEM	Dulbecco's modified Eagle's medium
EME	endometrial epithelium
EMS	endometrial stroma
EVs	extracellular vesicles
exRNA	extracellular RNA
GTFB	Gynecologic Tissue and Fluid Bank
HE-4	human epididymis protein 4
HGSC	high-grade serous carcinoma
HITS-CLIP	high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation
LGSC	low-grade serous carcinoma
MRI	magnetic resonance imaging
miRNA	microRNA

MUC16	mucin 16
ncRNA	noncoding RNA
OSE	ovarian surface epithelium
PAR-CLIP	photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation
PBSncRNA	primer-binding small noncoding RNA
pol III	polymerase III
qRT-PCR	quantitative real-time PCR
RIN	RNA integrity number
RNase	ribonuclease
Rny1	RNase T2
rRNA	ribosomal RNA
RSV	respiratory syncytial virus
RT-PCR	reverse transcription PCR
snoRNA	small nucleolar RNA
T4PNK	T4 polynucleotide kinase
tiRNA	stress induced tRNAs
tRF5-Glu	5’ fragment of tRNA-Glu-CTC
tRNA	transfer RNA
tsRNA	tRNA-derived small RNAs
sgRNA	small guide RNA
SHOT-RNAs	sex hormone-dependent tRNA derived RNAs

SRB	sulforhodamine B
TVU	transvaginal ultrasound
UTR	untranslated region
WFDC	WAP four-disulfide core

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Chapter 1: Introduction

Etiology of ovarian cancer

Ovarian cancer is a highly heterogeneous disease which may arise from various cell types, including epithelial cells, germ cells and sex cord-stromal cells. These cells are present in the normal ovary (Figure 1, [1]) and some of them are even located outside the ovary. Despite the complex cellular origins of ovarian cancer, surface epithelial-stromal tumors, also named epithelial ovarian carcinomas, contribute to the majority of ovarian cancers and account for about 90% of all ovarian cancer cases [2, 3]. Epithelial ovarian carcinomas are developed from epithelial cells. Depending on the type of epithelial cell and location of the epithelial cells, they are then classified into five main subtypes including serous (fallopian tube-type), mucinous (endocervix-type), endometrioid (endometrium-type), clear cell (clear cell carcinoma of the endometrium-type) and Brenner carcinoma [4].

Serous carcinomas are the most common subtype of ovarian carcinomas, and they are graded as either low-grade serous carcinoma (LGSC) (still looks similar to healthy tissue and differentiated) or high-grade serous carcinoma (HGSC) (poorly differentiated). The HGSC make up roughly 75% of ovarian carcinomas [5-7]. Another common subtype of ovarian carcinoma is mucinous carcinoma, which represents about 15% of all ovarian neoplasms [8, 9]. The major distinguishing feature of mucinous carcinoma is that the tumors are full of mucus-like fluid, while the serous tumors are filled with watery fluid. Other less common subtypes of epithelial ovarian carcinomas include endometrioid carcinoma, clear cell carcinoma and Brenner carcinoma, which may be benign or malignant, depending on whether the tumor cells have invaded nearby tissues.

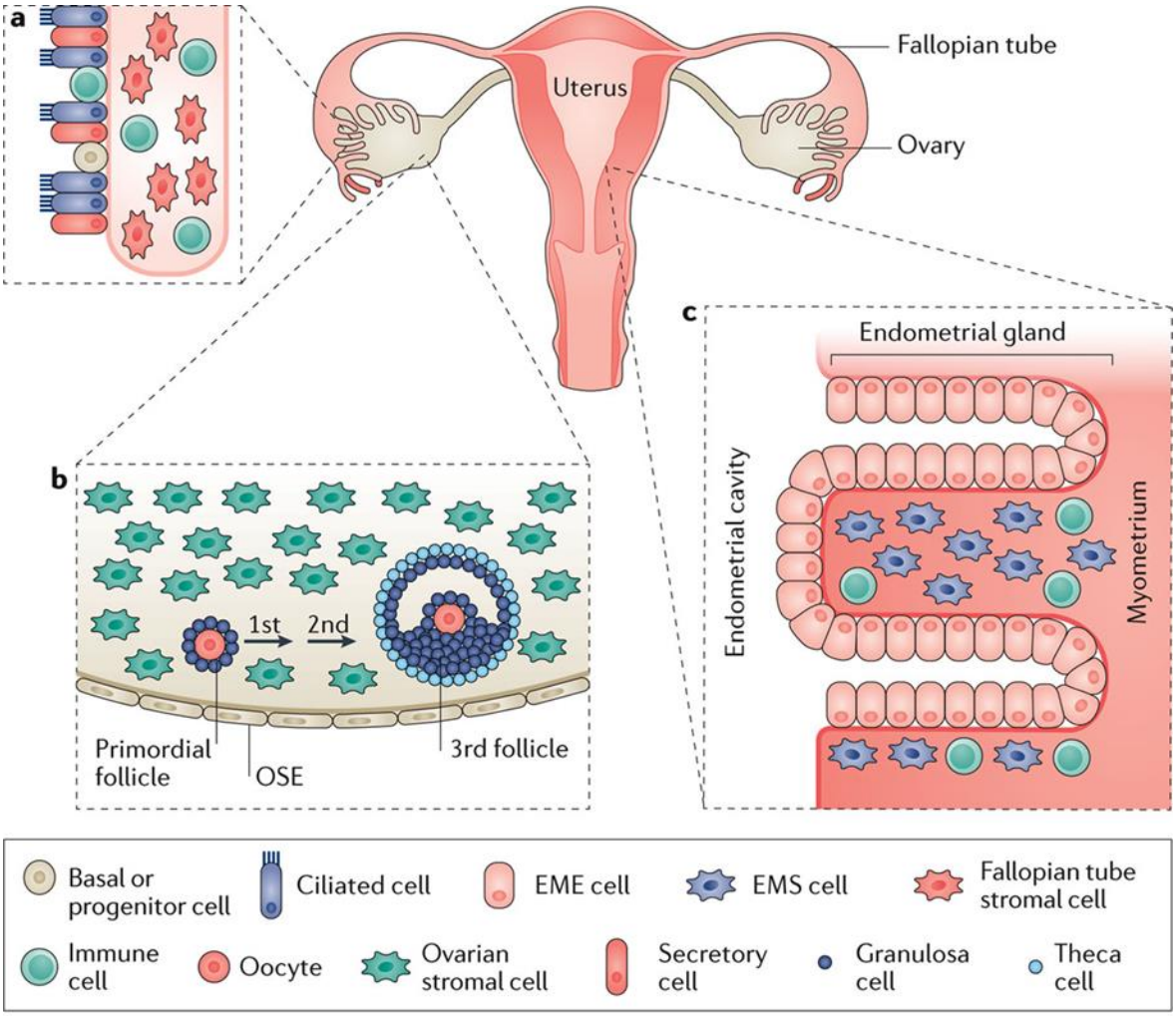


Figure 1. Anatomy and biology of the ovary, fallopian tube and uterus [1]. (**a**) The fallopian tube epithelium contains two principal types of epithelial cell, secretory cells and ciliated cells that are responsible for fluid synthesis and egg transport. Immune cells, which are present in the epithelium and stroma, are thought to play a part in immune surveillance. (**b**) The ovary inset depicts the principal cells of the ovarian cortex — ovarian surface epithelium (OSE), ovarian stromal cells and cells of ovarian follicles at various stages of maturation, such as oocytes, granulosa cells and theca cells. (**c**) The

endometrium is composed of cells of the endometrial epithelium (EME) and endometrial stroma (EMS), which respond to systemic and paracrine signals and undergo cyclical changes during the menstrual cycle.

Although it has been highly controversial and unknown where exactly ovarian tumor cells originate from, the observation of different subtypes of epithelial ovarian carcinomas and recent molecular evidence indicate that ovarian cancer may arise from cells located outside of normal ovarian tissue. For example, serous carcinoma may originate from epithelial cells of the fallopian tube [10]; Both endometrioid carcinoma and clear cell carcinoma are associated with endometriosis (abnormal growth of endometrial tissue), which is the most plausible source of ovarian carcinogenesis [11-13]. The exact derivation of mucinous carcinoma and Brenner carcinoma remains unclear, but recent data suggest that certain subsets of mucinous carcinoma and Brenner carcinoma exhibit a shared clonal relationship and may have similar oncogenesis from the transitional-type epithelium located at the tubal-mesothelial junction [10, 14, 15]. In summary, the evidence provides insight into the development of epithelial ovarian carcinomas, but more effort is need to further characterize the exact origins of each type of ovarian tumors. New understanding of ovarian cancer etiology may help develop more rational approaches of detection, treatment and prevention of ovarian cancer.

Pathogenesis of ovarian cancer

Cancer is a disease characterized by the build-up of mutations and epigenetic changes. Many genetic alterations and other molecular defects have been recorded in various subtypes of ovarian cancer. The majority of the genetic abnormalities include mutations that either activate or inactivate genes controlling the growth and division of ovarian cancer cells. Such genes are known as oncogenes (those that are activated) or tumor suppressors (those that are inactivated), corresponding to their role in cancer biology. The well characterized genetic abnormalities including activation of oncogenes and inactivation of tumor suppressor are summarized for the major subtypes of epithelial ovarian carcinoma (Table 1, [1, 3, 10, 16, 17]).

Carcinoma subtype	Possible cellular origin	Molecular features
HGSC	Ovarian surface epithelium; fallopian tube epithelium	Mutations in *BRCA1*, *BRCA2* and *TP53*
LGSC	Ovarian surface epithelium; fallopian tube epithelium	Mutations in *BRAF* and *KRAS*
Mucinous carcinoma	Unknown; Transitional-type epithelium found at the tubal-peritoneal junction	Mutations in *BRAF*, *ERBB2*, *KRAS* and *TP53*
Endometrioid carcinoma	Endometrial epithelial cell	Mutations in *CTNNB1*, *PTEN*, *PIK3CA* and *BRCA1*
Clear cell carcinoma	Endometrial epithelial cell	Mutations in *KRAS*, *PIK3CA*, *PTEN* and *TP53*
Brenner carcinoma	Unknown; Transitional metaplasia and Walthard cell nests	Mutations in *CDKN2A*, *KRAS* and *PI3KCA*

Table1. Cellular origin and molecular pathogenesis of major ovarian epithelial carcinoma [1, 3, 10, 16, 17]. Although these mutations are associated with the type of ovarian cancer listed, there is great variability between samples and it remains difficult to confirm those tumors that share features of both types, such as low-grade versus high-grade serous carcinoma.

The primary genetic changes leading to ovarian cancer still remain unclear. Families with germline mutations, such as in the tumor suppressor genes *BRCA1* and *BRCA2*, are found to be at higher risk for ovarian cancer. *BRCA1* and *BRCA2* encode tumor suppressor proteins involved in DNA repair pathways. Impairment of DNA repair pathways allows the accumulation of genetic mutations and eventually leads to genomic instability that expedites tumor formation. Together, the germline mutations of *BRCA1* and *BRCA2* account for approximately 10 to 15% of all ovarian cancer cases [18]. In addition, a rare hereditary genetic syndrome named Lynch Syndrome, which is often associated with mutations in DNA mismatch repair genes including *MLH1*, *MSH2*, *MSH6* and *PMS2*, is consider to increase the risk of developing ovarian cancer [19, 20]. Mutations of those genes lead to uncontrolled growth and unchecked division of cells and accounts for about 10 to 15% of hereditary ovarian cancer. Additional germline mutations and the relationship between other rare genetic syndromes and increased risk of ovarian cancer still remains unclear and the identification would provide meaningful clinical implications.

Unlike germline mutations, somatic mutations in ovarian cancer are not inherited

from a parent and are acquired over the lifetime. The mutations are only present in certain cell types and representative mutations of oncogenes and tumor suppressors are listed in Table 1. During the oncogenesis of ovarian cancer, the loss and mutations of *TP53* is one of the most frequent genetic alterations that has been observed in both sporadic and familial cases [21, 22].

Despite the specific DNA mutations of oncogenes and tumor suppressor identified, the comparison of RNA sequencing data from normal and ovarian cancer patient samples also brought about the discovery of tumor-specific RNAs, especially miRNAs [23, 24]. miRNA is known to regulate gene expression and control function of encoded proteins through mRNA turnover and the translation regulation [25]. The altered expression patterns and functions of miRNAs associated with oncogenesis of ovarian cancer may also be categorized into oncogenes or tumor suppressors [26]. Many miRNAs have been revealed to play carcinogenic roles in ovarian cancer development and indicate the prognosis of ovarian cancer [23, 24, 27]. Further study of the roles and basis of miRNA function in ovarian cancer may provide new strategies for tumor detection and treatment.

The risk factors causing the somatic mutations of ovarian cancer are highly diverse. Although chemical carcinogens with mutagenic ability have been recorded to contribute to the development of multiple cancers including ovarian cancer [28, 29], continuous ovulation and disrupted hormone conditions remain to be another important risk factor for ovarian cancer. Following the release of oocytes, quiescent epithelial cells on the surface of the ovary need to proliferate to repair the damage to the ovarian surface, which increases the risk of gaining spontaneous mutations during proliferation. Thus a long

period of ovulation and infertility is thought be one of the fundamental causes of epithelial ovarian carcinoma [30, 31]. Steroid hormones including estrogen and androgen are important for the growth of ovarian epithelial cells. Previous studies suggest that estrogen therapy and hormone therapy for menopausal women increase the risk of developing ovarian cancer due to longer exposure to these hormones [32-35]. Other risk factors including environmental risk factors with psychosocial and psychological stress, aging, obesity and smoking have also been investigated and found to contribute to increased ovarian cancer risks, but more details and the molecular basis for the risk factors need to be studied in depth to confirm their connections to the carcinogenesis of ovarian cancer [36-40].

Early detection and diagnosis of ovarian cancer

Ovarian cancer is a leading cause of cancer mortality among women worldwide, with an expected five year survival of less than 30% [17]. Unfortunately, ovarian cancer is frequently diagnosed at an advanced stage (Stage III & Stage IV), while the five-year survival rate of women that are diagnosed at an early stage can reach up to more than 90% [41, 42]. Although early detection of ovarian cancer is one of the most significant factors that ensure effective treatment of the patients, the difficulties of early detection are attributed to several obstacles including the inaccessible nature of the ovary on physical exam and the absence of specific symptoms until metastasis [43, 44]. Thus, there is a critical need and these would be a significant benefit if effective and reliable early detection strategies for ovarian cancer were to be developed.

Imaging technologies are often used to measure the size and spread of tumors in clinical examination, and they are also being applied in the detection of ovarian cancer. The most widely and commonly used technique is the Transvaginal Ultrasound (TVU) to examine pelvic organs [45]. Even though TVU has been shown to be effective to assess the pain and pressure in the pelvic region, most adnexal masses as well as other gynecologic symptoms such as abnormal bleeding and fibroids, TVU is not a primary early-detection method as it is not able to differentiate malignant and benign tumors efficiently. Other imaging technologies including Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) are also used to evaluate and stage adnexal masses, while they are both limited in the early diagnosis of ovarian cancer due to the inability to

distinguish benign and malignant neoplasms [45, 46].

New advances in genomic and proteomic techniques have led to the discovery of many potential biomarkers. Accessible patient samples including blood and urine are being studied for identification of biomarkers and these samples are now often called liquid biopsies [47, 48]. Candidate biomarkers are not only limited to proteins encoded by susceptible genes, but also include cell-free DNA, various types of RNA, lipids, chromatin alterations and even circulating tumor cells [49-56].

The most comprehensively studied and widely used circulating biomarkers for ovarian cancer are two proteins: cancer-antigen 125 (CA-125) and human epididymis protein 4 (HE-4). CA-125 is a transmembrane protein encoded by the mucin 16 (*MUC16)* gene and elevated serum level of CA-125 has been found in more than 80% of patients with advanced ovarian cancer [57]. In addition, the serum level of CA-125 is also regarded as a standard to determine patient's response to treatment as well as predict the prognosis after treatment. High levels of CA-125 are often associated with poor survival and recurrence of ovarian cancer [58-60]. The detailed biological functions of CA-125 still remains to be defined, but CA-125 is an effective and useful biomarker for ovarian cancer detection following initial treatment. However, the use of CA-125 for early detection of ovarian cancer is restricted due to the lack of sensitivity and specificity. For specificity, although used as a biomarker for ovarian cancer, elevated levels of CA-125 have also been observed in other cancer sites including breast cancer, lung cancer and endometrial cancer as well as other diseases of the ovary and even pregnancy, which often produces false positive early detection for ovarian cancer [58, 61, 62]; For

sensitivity, even though highly expressed in most ovarian cancer patients with high grade disease, more than half of patients at early stages of ovarian cancer fail to exhibit elevated levels of CA-125 in blood. Thus, CA-125 may lead to false negative results for early stage ovarian cancer before the onset of symptoms [62-64]. Another potential proteomic biomarker used for ovarian cancer detection is the HE-4 protein, which is encoded by the WAP four-disulfide core (*WFDC*) gene [50, 65]. With similar sensitivity to detect ovarian cancer patients at advanced stages like CA-125, HE-4 does give a better distinction between benign and malignant ovarian tumors [66]. However, there remains a lack of specificity for the measurements of HE-4 to detect ovarian cancer at early stage and even differentiate ovarian tumors and tumors with similar non-ovary origins [65, 67, 68].

As described earlier, RNAs, especially miRNAs have been revealed to be expressed at different levels in ovarian tumors compared to normal tissues, and different subtypes of ovarian cancer exhibit diverse profiling of miRNA expression
[23, 24, 51, 52, 69]. In addition, recent evidence has shown that several cell-free miRNAs can circulate in body fluids such as urine and blood in normal and ovarian cancer patients [70, 71]. Expression profiles of circulating miRNAs are strongly related to clinical signatures in ovarian cancer patients [52, 72]. Due to their representation of the physiological state and potential function in ovarian cancer, these ncRNA molecules may serve as novel diagnostic biomarkers for ovarian cancer detection, especially early detection.

Various studies have identified many ovarian cancer-associated circulating

miRNAs as possible diagnostic biomarkers of ovarian cancer [73]. The advances in nucleic acid analysis technologies, such as high-throughput small RNA deep sequencing, make it feasible for us to seek novel miRNA as well as other ncRNA biomarkers from the tissue samples or the liquid biopsies of ovarian cancer patients. A recent report characterized a tRF originating from 3'end of Lys tRNA, originally called miR-1274a, as a potential prognostic biomarker in patients with high-grade serous ovarian cancer [74, 75]. It raises the possibility that, besides circulating miRNA, other ncRNA molecules including tRFs could also serve as potential diagnostic and prognostic biomarker for ovarian cancer detection. Toward this effort, we have identified extracellular RNAs (exRNAs) in urine from ovarian cancer patients. Surprisingly, we found some of the most highly expressed ncRNAs in these samples are tRFs.

The introduction of tRFs is adapted from published work:

Diebel et al. Beyond the Ribosome: Extra-translational Functions of tRNA Fragments Biomarker Insights, Jan 28th 2011. doi: 10.4137/BMI.S35904.

What are tRFs?

High throughput sequencing studies continue to identify an expanding array of small ncRNAs [76]. These newly identified small ncRNAs are derived from many types of primary RNA transcripts that ultimately give rise to the small, biologically functional molecules of miRNAs, rRNA fragments, small nucleolar RNA (snoRNA) fragments, tRFs, and others [77]. Early high throughput sequencing studies of small ncRNAs generally regarded rRNA and tRFs as nothing more than contaminating degradation products and sequences of little interest for biological study [76]. It was assumed that these fragments were naturally produced, inert RNAs derived from the functional parental rRNA and tRNA molecules. However, with improving bioinformatic methodologies these once dismissed RNA fragments are now being recognized for their importance in many biological pathways including the regulation of translation, stress responses, proliferation and other aspects influencing human disease [76-80]. tRFs are now known to be purposefully processed from both mature and pre-tRNAs. tRFs represent one group of small ncRNAs currently undergoing a new wave of investigation centered primarily on the control mechanisms resulting in their biogenesis and determining their biological function within various cell types and cell growth conditions [76-90].

Biogenesis of tRNAs and tRFs

tRNAs are among the most abundant RNA molecules in a given cell, constituting as much as 4-10% of total cellular RNA [78]. The fundamental role of tRNAs, where a charged tRNA delivers an amino acid to a growing peptide chain within the ribosome, is well studied [91]. The biogenesis of tRNAs begins with their transcription by RNA polymerase III (pol III) from a type 2 RNA pol III promoter. After transcription, several modifications are made to the tRNA transcript in the form of endonucleolytic cleavages, exonucleolytic trimming, non-templated nucleotide additions, and multiple single base modification events. These events occur prior to the addition of a single amino acid to the 3' end of the mature tRNA (**Figure 2**). The highly modified and aminoacylated tRNA may then be used within translation.

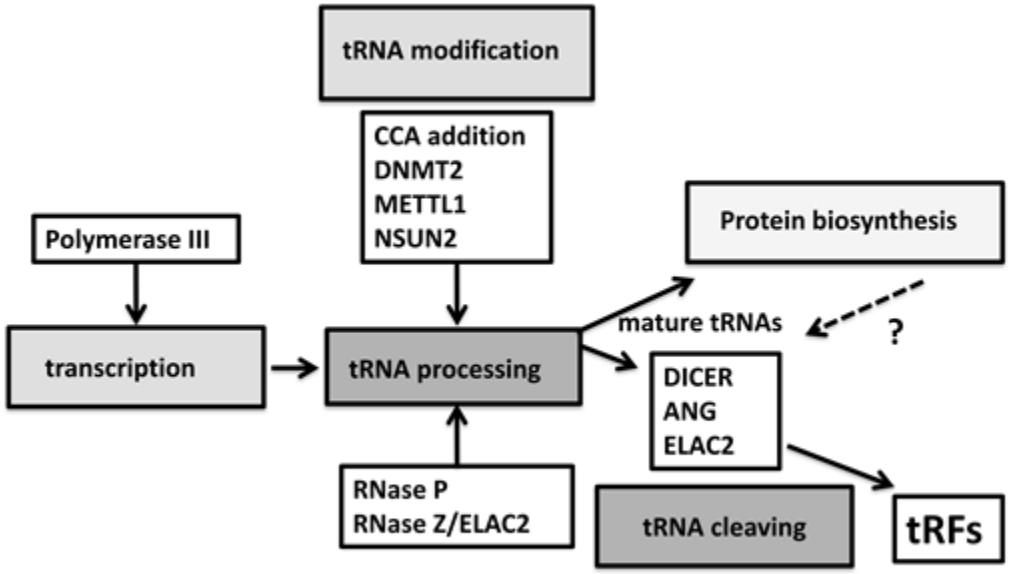


Figure 2. An overview of the process leading to tRNA and tRF biogenesis. tRNAs are initially processed from pre-tRNAs into mature tRNA by removing nucleotides that constitute a 5' leader by ribonuclease P (RNase P) cleavage and then the 3' tail is removed through cleavage of the tRNA primary transcript by RNase Z. A select group of tRNAs harbor introns and must be further processed by a complex known as the tRNA splicing endonuclease, a complex including CLP1 and other proteins (not included in this figure) [90]. In the figure representative enzymes are listed as tRNA modifying or cleaving enzymes and are not meant to be inclusive of all such enzymes. It is highly likely that additional modifying and cleaving enzymes for tRNAs will be identified in the future.

The biogenesis process used by cells to make mature aminoacylated tRNAs consists of many well-regulated steps that result in the interaction of tRNA molecules

with several tRNA modification enzymes [80]. Paramount to understanding tRF biological function is the need to understand tRF biogenesis. Recent research has centered on deducing how a tRF is generated from a molecule essential for protein translation, and which pathways are used to generate mature tRNAs versus those used to generate various tRFs.

Nomenclature of tRFs

Highlighting the emergence of a new field of scientific study, the nomenclature of tRFs has had a varied beginning [92]. In earlier studies of tRFs, the fragments were simply called tRNA halves and included 5'-half-tRNAs as well as 3'-half-tRNAs [83, 88, 92]. In another related study the tRFs were referred to as tRNA-derived small RNAs (tsRNA) [84]. Additional labels have been used for tRFs based on their size, expression or function. In one such study, tRFs expressed in response to stress were called stress induced tRNAs (tiRNA) [88]. In another study, tRFs were named for the location of the cleavage site, thus, those cleaved from the 3' end of the tRNA were called tRF-3 [85]. Meanwhile, tRFs expressed in response to hormone stimulation have been referred to as Sex HOrmone-dependent tRNA derived RNAs (SHOT-RNAs) [89]. **Figure 3** describes frequently identified tRFs and the pathways leading to their biogenesis. As more information becomes available about the function of this group of ncRNAs, a more clear method of naming them will also evolve.

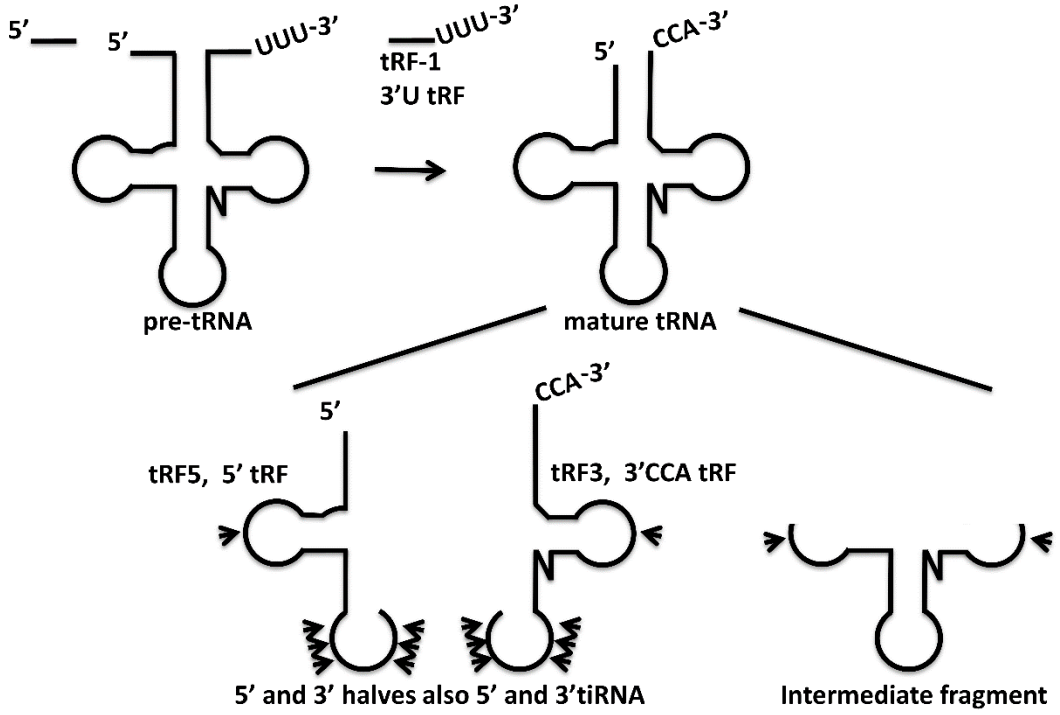


Figure 3. Nomenclature of tRFs. The nomenclature for tRFs is inconsistent due to their recent identification most often as part of deep sequencing data sets. Early terminology included tRF-1 or 3'U tRF for the trailing sequence cleaved by RNase Z during maturation [85, 93]. The tRFs generated following CCA addition and cleaved in the T loop were designated tRF3 or 3'CCA tRF, while the 5' tRFs generated after cleavage by RNase P were designated tRF-5, 5'tRFs or 5'leader-exon tRFs [85, 87, 93]. These fragments are smaller than halve tRNAs and range in size from 13 to 20 base pairs [93]. The tRNA halves are larger and the expected size is 30-40 nucleotides, however, this appears to be quite variable [92-94]. Megel, et al. propose a universal naming where the letter corresponds to the extremity of the tRNA and the number (5 or 3) to the cleavage

site such as tRF3T for a 3' tRF generated by cleavage in the T loop [92]. Utilizing the proposed general naming scheme the newly identified intermediate tRFs would be tRF53DT. Multiple arrow heads are used to designate regions cleaved by ANG that are often variable and may occur at any point in the anti-codon loop [94]. While a single arrow head is used to designate potential Dicer, RNaseP sites or possibly novel RNase cleavage [93].

The biogenesis of tRFs is highly conserved

Initially regarded as degradation products, tRFs were often excluded from published studies and as such were not considered as potential biomarkers in earlier studies [76]. However, the expression of tRFs in response to stress is evolutionarily conserved and includes both prokaryotes and eukaryotes, suggesting a functional role of biological importance [78, 79, 95, 96]. Furthermore, the processing of tRFs from mature tRNAs has been shown to occur by an evolutionarily conserved group of proteins, the RNases. RNases that have been shown to generate specific types of tRFs include PrrC, RNase T2 (Rny1), Dicer, RNase Z, RNase P and ANG, and it is also expected that this list may grow as more fragments are studied [79, 81, 85, 87, 95]. While these studies have provided the groundwork for understanding that the biogenesis of tRFs is a conserved and regulated process, further investigation is needed to determine the ultimate molecular function of specific tRFs. Additionally, improved detection strategies are needed for the identification of tRFs within biological samples. Once characterized,

these tRFs may prove to be robust biomarkers that reveal critical information about a patient's current state or future risk of disease [97, 98].

Interestingly, tRFs observed in many high throughput studies were assumed to be derived from mature tRNAs because the expected cleavage, non-templated nucleotide additions, and base modifications required to generate a mature tRNA had taken place prior to generation of the tRF [76, 80]. tRNAs are initially processed into a mature tRNA by removing nucleotides that constitute a 5' leader or 3' tail through cleavage of the tRNA primary transcript by RNase P and RNase Z respectively. It has recently been shown that the 3' trailer sequence may form a subset of the functional tRFs and this subset has been designated as tRF-1 indicating their biogenesis is neither 3' nor 5' from the mature tRNA [85]. The series of tRF-1 include tRF-1001 which was shown to impair cellular proliferation, when knocked down with siRNA. Thus, biogenesis of pre-tRNAs does result in the formation of a functional class of tRFs [85].

Other subsets of tRFs are generated from the splicing of intron sequences and the cleaving of mature tRNAs [90, 92]. After these cleavage events, the tRNAs gain three additional nucleotides (CCA) at their 3' ends which are required for their subsequent aminoacylation [99]. Mature tRNAs are also modified at as many as 8 or more base pairs during maturation into a biologically functional molecule [80, 99, 100]. These modifications of tRNAs may include but are not limited to methylation events such as m^1A, m^1G, N^2, N^2-dimethylguanosine [80]. Interestingly, some nucleotide modifications by methyltransferases have been reported to block cleavage into tRFs, while other modifications of tRNA, specifically a 2'-O-methylation event in the anticodon loop, have

been shown to enhance cleavage into tRFs in yeast [80, 101-103]. However, many of the known mechanisms of processing tRNAs into tRFs have been defined in organisms other than mammals and these mechanisms will need to be further confirmed for their conservation in higher eukaryotes as the field of tRF biogenesis advances.

RNases involved in the cleavage of tRNAs into functional tRFs

The RNases including Dicer, RNase Z, and ANG have all been studied for their role in cancer cells and as potential biomarkers for predicting cancer risk [104-107]. All three enzymes have also been studied using siRNA knockdowns to confirm that they have a role in the generation of tRFs in cancer cells [81-89]. In contrast to tRF biogenesis, miRNA biogenesis is a well-characterized process and frequently requires initial processing by Drosha and Dicer to produce mature miRNAs. However, the generation of tRFs is thought to be independent of Drosha cleavage, while a subset of tRFs are thought to be generated in a Dicer dependent manner [81, 104, 108].

One example of a tRF generated by Dicer cleavage is the primer-binding small noncoding RNA (PBSncRNA), which was determined to be a tRF derived from tRNA-Lys3 [109]. Knockdown of PBSncRNA allows replication of HIV and PBSncRNA has been identified in argonaute 2 (AGO2) complexes suggesting that the expression of this tRF may target HIV replication. It has been proposed that tRFs may be part of a host response to defend against viral infection, thus, it is hopeful that the function of PBSncRNA will be confirmed to block HIV replication in future studies.

RNase Z also known as ELAC2 has been well studied for its role in the production of mature tRNAs by trimming off excess nucleotides at the 3' end of tRNA primary transcripts. RNase Z has recently been shown to be important in generating a subset of tRFs, particularly tRFs generated from the cleavage of long 3' end tails from primary tRNAs [82, 85]. In addition, to its role in generating tRFs, the nuclease activity of RNase Z can also be guided by tRF to other target RNA molecules inside cells in trans to regulate expression [83]. Thus, RNase Z is currently being used to develop a targeted therapeutic approach for cancer treatment based on its ability to recognize and cleave any pre-tRNA-like complex, a method known as TRUE Gene Silencing [110].

The discovery that RNase Z could be engineered to target RNA molecules led Elbarbary et al., to question if RNase Z bound to endogenous tRFs [82]. Immunoprecipitation of RNase Z and isolation of bound RNA showed that a number of ncRNAs were bound to RNase Z. One of the ncRNAs identified in this study was 5'-half-tRNA^{Glu}. The authors went on to show that 5'-half-tRNA^{Glu} was acting as a small guide RNA (sgRNA) and directly regulated the expression of the PPM1F mRNA a protein that has been shown to induce apoptosis in HeLa cells when over expressed [82].

Of the numerous RNases identified in the human genome, one of the best characterized nucleases shown to be used in the processing of tRNAs to tRFs, is ANG. ANG is a member of the RNase A superfamily and is also known as RNase 5. ANG is well studied for its activity in cleaving mature tRNAs [79]. Several studies have shown that ANG activity increases its cleavage of tRNAs in response to specific stimuli such as nutritional deficiency, hypoxia, heat shock and oxidative stress [79, 111-113]. In

addition, human respiratory syncytial virus (RSV) infection has been shown to stimulate tRF formation through ANG cleavage using lung cancer cells as host for the RSV infection [114]. Within this study the infection of lung cancer cells with RSV was shown to specifically increase the formation of tRF-Glu, tRF-Gly and tRF-Lys. Interestingly infection with a virus from the same family, human metapneumovirus, did not increase the expression of these tRFs showing that there are both substrate specificity in the generation of tRFs and a complex regulatory mechanism governing the activation of ANG to act on tRNAs in order to generate tRFs [114]. The specificity of ANG in producing tRFs in response to RSV infection was determined by knocking down the expression of the RNA cleaving enzymes ANG, Dicer, Drosha, RNase Z, and RNase L. Only the knock down of ANG significantly reduced tRF formation in response to RSV [114].

Mounting evidence from high throughput sequencing studies suggests that many additional cleavage sites in tRNAs may exist and that one part of the molecule is generally found at higher levels suggesting that these ncRNAs are functionally active [94]. Interestingly the total RNA cleavage of a given tRNA is only about a tenth of the total available tRNA suggesting that tRFs are generated from a subpopulation of the total mature tRNA for a given tRNA transcript [113, 115]. Further confirmation that angiogenin and tRNA availability are important in the generation of tRFs came from a comprehensive study by Saikia et al [113]. In this study several conditions including oxidative stress, hypertonic stress and increased angiogenin expression were tested and a micro array based assay was used to detect tRFs [113]. One of the central findings in this

study is that different types and lengths of stress result in differing patterns of tRF generation. As this new area of research moves forward, it will be important to validate each testing condition in various cell types rather than to assume that what was found in one set of conditions will be the same in all other cell types and cell growth conditions.

In a study of the protozoan, Tetrahymena thermophila, half tRNAs were generated during early amino acid starvation [116]. In this study starvation for all amino acids or for any one of the essential amino acids resulted in tRF formation. Tetrahymena thermophila is an excellent model for studies of amino acid starvation induced tRNA fragmentation due to the need of this organism to rapidly respond to changing environmental cues. In contrast, studies of tRNA fragmentation in response to starvation in HeLA cells, was not corrected by the addition of essential amino acids as it was in Tetrahymena [86, 116]. However, tRNA fragmentation was blocked by siRNA shutdown of ANG [86].

The known role of ANG in the generation of tRFs may collide with the role of this same protein in tumor immunology. ANG was known to be elevated in the process of angiogenesis as early as 1987 and continues to be a potential biomarker for tumors in numerous tissue sites and a potential target for therapeutic intervention [117-120]. Furthermore, circulating antibodies against ANG have been found to be a sensitive biomarker for osteosarcoma and are thought to play an important role in tumor immunology for this cancer type [121].

Function of tRFs

Although tRFs have been linked to immune function by their location in exosomes, the specific role of individual tRFs is currently not well characterized [122-124]. Early studies examined the collective extracellular tRF function with one study finding that tRFs in the conditioned media from bladder carcinoma inhibited the growth of endothelial cells [125]. However, at that time it was not possible to characterize the exact tRF responsible for the inhibitory action because methods did not exist to sequence the tRFs in media. Studies confirming the cellular function of tRFs have been summarized in **Table 2**.

Table 2. Representative studies of tRF function.

tRF	Function	Cell type	Study
<u>Alteration of cell phenotype in response to tRF expression</u>			
5'tRFs	Isolated from the media of a urinary bladder carcinoma cell line and used to inhibit endothelial cell growth.	Bovine endothelial cells	[125]
3'tRF^{Ser}	Regulates cell proliferation	HCT116, DU145, LNCaP	[85]
5'tRF^{Val}	Cleavage of tRNAs during stress	HepG2	[87]
5'tRF and 3'tRF^{His}	Ribosomal bound tRFs change with differing growth conditions	Yeast ribosomes	[126]
5'tRF$^{Glu, Gly, Lys}$	Increased tRF expression in	A549 primary small	[114]

			response to infection by RSV.		alveolar epithelial cells		
•	5’ leader	•	Neurodegeneration motor neuron loss, over expression of 5’ leader tRFs.	•	kinase dead CLP1 mice	•	[127]
•	5’tRF$^{Asp, His, Lys}$	•	siRNA to Estrogen and Androgen Receptor reduce expression of 5’ fragments. Androgen receptor dependent cleavage, increases proliferative response.	• • •	MCF7, BT-474, LNCap LNCap-FGC	•	[89]
•	tRFs from all 20 amino acids	•	Predicted in drosophila to bind conserved Seed sequences. Showed significant gene ontology enrichment brain activity and aging.	•	Bioinformatic approach not confirmed in cell lines.	•	[128]
•	5’tRF$^{Glu,Asp, Gly,Tyr}$	•	Displaces YBX1 an RNA binding protein allowing stabilization of 3’ UTRs of oncogenic transcripts.	•	MDA-LM2, four tRFs transfected in mice.	•	[94]
•	<u>Targeting a specific mRNA through Seed binding in the 3’ UTR</u>						
•	3’tRF^{Lys3}	•	Loaded into AGO2 complexes and targets HIV primer binding site.	•	HeLa cells	•	[109]
•	3’tRF^{Gly}	•	A DICER substrate, represses RPA1.	•	HEK293, normal B cells, lost in	•	[129]

					subset of lymphomas		
•	Targeting a specific mRNA by acting as a small guide RNA						
•	5’tRF^{Glu}	•	Down regulates PPM1F transcript	•	HEK293	•	[82]
•	tRFs Regulating Translation						
•	5’-tiRNAs	•	5’-tiRNAs but not 3’-tiRNAs have a distinct inhibitory effect on translation	•	U2OS	•	[88]
•	5’tRF^{Ala}	•	Induces stress granule formation.	•	U2OS	•	[130]
•	5’-tiRNA^{Ala,Cys}	•	YB1 is the only tiRNA binding protein needed for tiRNA induced stress granule formation	•	U2OS	•	[131]
•	5’tRF^{Val}	•	Binds small ribosomal subunit and blocks translation	•	Haloferax volcanii	•	[96]
•	5’tRF^{Gln}	•	Inhibits protein translation.	•	HeLa	•	[132]

A general function of tRFs was shown to be the inhibition of translation by disrupting the cap-binding complex eIF4F [131]. More recently, the function of a subset of tRFs generated by RNase cleavage has been shown to block YB-1 binding of target transcripts [94]. The YB-1 protein (YBX1 gene) is an RNA binding protein that is frequently over expressed in cancer cells and plays a significant role in RNA translation and stability [94, 133]. YB-1 binds to tRF-Ala and specifically inhibits translation [94].

Interestingly, tRF-Ala is one of the tRFs capable of forming a G-quadruplex structure due to the oligo-G nucleotides it contains and this structure is required for translational repression [134]. Earlier studies of G-quadruplex structures showed they cause resistance to nuclease cleavage and have anti-proliferative activity in cancer cells [135].

Recent studies have shown that cells treated with mimics of 5'-tRFs form stress granules. The synthetic mimics of 5'tRFs tRF-Ala, Gly and Val were all tested on U2OS cells and stress granules were visualized [130]. Stress granules are formed in response to cellular stress and they play a role in reprogramming the cell to stop transcription and translation in response to cellular stressors [131]. The specific set of 5'tRFs have been called tiRNAs for stress induced tRFs. Translational inhibition has been linked to 5'tRFs (5'tiRNAs) by a mechanism, which is independent of phospho-eIF2alpha translational repression [136]. Synthetic mimics of 3'tRFs (3'tiRNAs) did not cause stress granule formation. Taken together these studies confirm that one role of a subset of 5'-tRFs is a rapid response to down regulate RNA translation during stress [136].

Modifications of tRNAs are ubiquitous, with as many as 100 or more different modifications and 8 or more modifications per tRNA [100, 137]. Therefore, it has been hypothesized that modifications may alter the cleavage into tRFs. This is an area that will require a great deal of study in the future and will be enhanced by new techniques such as AlkB-facilitated RNA methylation sequencing (ARM-seq) described by Cozen et al. 2015 [80]. ARM-seq allows the removal of modifications so that high throughput sequencing is not blocked by hard stop RNA modifications.

The loss of one such modifying enzyme, NSUN2, resulted in widespread neurological abnormalities in mice and specifically altered the expression of 5'tRFs [102]. NSUN2 is a tRNA methyltransferase known to be mutated in neurological disorders as well as cancer. This is one of the first confirmations of an enzyme functioning in the regulation of tRNA fragmentation resulting in human disease. With over 100 potential modifications, it is expected that tRF expression and function will be altered in many ways as yet undefined. This is an area of research awaiting much study in tumor biology and elsewhere.

tRFs as potential biomarkers

The variable expression and function of tRFs are just now being elucidated. It will take some time to identify clinical applicability and to determine if these small RNAs have potential as biomarkers of disease. Bioinformatic methods to identify tRFs in deep sequencing studies are rapidly evolving and in some cases will allow the mining of historic data sets, where tRFs were initially discarded from analysis [76, 126, 138]. Revisiting data sets where expression of tRFs may be correlated with clinical characteristics will allow the identification of potential tRF biomarkers in well characterized samples. Retrospective studies must be conducted with care and understanding of the complication of tRNA and tRNA-like abundance in the human genome [138]. Telonis et al., outline difficulties in interpreting tRF data in high throughput sequencing studies [138]. Such difficulties include but are not limited to the repetitive nature of tRNA sequences in genomes, the complexity of multiple isoacceptors

for each amino acid, and the existence of base changes thought to be a result of modifications. Bioinformatic analysis must be accompanied by a clear description of the criteria used for data set analysis and then be followed by experimental confirmation.

tRFs have been well documented to exist in patient serum and other convenient biological samples at levels similar to miRNAs [123, 125, 139, 140]. In prospective studies, consistent methods for patient sample collection and analysis of exRNAs will be critical as biomarker studies of tRFs move forward [141]. The methods utilized in each study must be carefully controlled and reported if data is to be comparable across samples and between studies [142]. For example the sample preparation, sample storage and RNA collection methods must be fully described and consistent for all patients. The method of RNA isolation and amplification if different between studies may impact the resulting repertoire of fragments obtained and characterized. The development of biomarkers is a complicated process and as tRFs make their way into the biomarker pipeline, it will be necessary to apply past experience to develop robust biomarkers with clinical value [143, 144].

tRF expression has been detected in cancer patient samples from multiple tissue sites and accessible samples [123, 125, 129]. A study of B cell lymphoma found that tRFs were down-regulated in lymphoma cell lines and primary biopsies as compared to control B cells [129]. While a study of tRF expression in prostate cancer patients, found that tRFs were increased in metastatic samples [140]. Studies of tRF expression in cancer cell lines have revealed a diverse array of tRF expression and have been useful as model systems to study the function of tRFs. Mechanistic studies of tRF expression in the response to

hormones in prostate and breast cancer cells suggest that tRFs enhance cell proliferation and that their expression is tissue dependent [85, 89, 123, 145].

tRFs may act like miRNAs and if confirmed this could mean that one tRF may regulate multiple mRNA targets [109, 129]. Defining the biological expression and function of tRFs is reminiscent of the early studies of miRNAs, where much controversy occurred due to the need for novel method development. Given that their level of expression is similar to miRNAs, and as their functions become known, tRFs are expected to provide a new frontier in cancer biomarker development.

tRFs have been characterized in RSV, HIV and HTLV-1 infection [109, 114, 146]. A pyrosequencing study showed that a tRF derived from tRNA-Lys is increased in cells infected with HIV and that loss of this expression allows HIV replication [109]. A different tRF derived from tRNA-Pro was shown to be incorporated into HTLV-1 viral particles and shown to act as a primer for reverse transcriptase during replication [146]. The specificity of particular tRFs to viral regulation provide hope that these tRFs may eventually provide novel therapeutic targets to block viral infection.

Oxidative stress results in tissue damage and is associated with many disease states. A recent study has shown that even before DNA damage is detectable tRFs are detectable. A specific RNA signature, tRNA-specific modified nucleoside 1-methyladenosine (m1A), is detected in patients who have kidney damage and correlates with mortality [147]. Early detection of tissue damage would be helpful in the detection of many diseases, thus, the emerging field of tRFs provides potential for the discovery of

novel biomarkers aimed at earlier detection. More study is needed to determine if tRFs will provide promising new candidates for future biomarkers of health and disease.

Chapter 2: A Method for Extracting and Characterizing RNA from Urine: for downstream PCR and RNAseq Analysis

This chapter is adapted from published work:

Zhou et al. A Method for Extracting and Characterizing RNA from Urine: for downstream PCR and RNAseq Analysis. Analytical Biochemistry, August 8th 2017. doi:10.1016/j.ab.2017.08.003.

Chapter Summary

Readily accessible samples such as urine or blood are seemingly ideal for differentiating and stratifying patients; however, it has proven a daunting task to identify reliable biomarkers in such samples. ncRNA holds great promise as a source of biomarkers distinguishing physiologic wellbeing or illness. Current methods to isolate and characterize RNA molecules in urine are limited. In this proof of concept study, we present a method to extract and identify small ncRNAs in urine. Initially, qRT-PCR was applied to confirm the presence of miRNAs in total RNA extracted from urine. Once the presence of miRNA in urine was confirmed, we developed a method to scale up RNA extraction to provide adequate amounts of RNA for next generation sequence analysis. The method described in this study is applicable to detecting a broad range of small ncRNAs in urine; thus, they have wide applicability for health and disease analyses.

Introduction

Physiologically representative and accessible samples such as saliva, blood or urine have long been expected to provide a source of biomarkers with high potential for characterizing conditions of health and disease. These types of samples are referred to as liquid biopsies and may harbor circulating cells, protein, DNA, and RNA biomarkers [148]. RNA is one component within these samples that was initially ignored due to its propensity for rapid degradation by RNases. However, with the identification of miRNAs and their notable stability in physiologic samples, RNA has come to the forefront of readily accessible molecules for the discovery of novel biomarkers [47, 149, 150].

ncRNAs found in liquid biopsies include, but are not limited to, rRNAs, tRNAs, tRFs and miRNAs. The wide variety of functions of extracellular ncRNAs are currently under investigation. For example, the tRFs, although having the unfortunate name of tRFs, are actively processed from mature tRNAs and their function is beginning to be elucidated [151]. While the miRNAs are already known to function in most if not all biological processes. Circulating miRNAs as serum biomarkers of health and disease have been robustly explored [152-154]; however, the miRNA repertoire in urine is less studied. The majority of the early studies of RNA in urine were focused on prostate, bladder and kidney disease because these tissues would directly contribute extracellular vesicles (EVs) to urine [155, 156]. In many of the studies of EVs in urine, they are referred to as exosomes due to their size of approximately 40-100 nanometers. The term EV refers to any EV including but not limited to the microvesicles, ectosomes and

exosomes [141]. The misconception that exRNAs must be associated with EVs persists despite recent studies that show exRNAs may be associated with protein and lipid complexes independent of vesicles [157-159]. These findings suggest that exRNA in urine may provide useful biomarkers of physiological relevance to many diseases not limited to those involving urologic disease[160].

Urine based biomarkers would be ideal for many studies because of the accessible nature of urine. Urine is readily collected in many animal models, as well as in the veterinary setting and for human health assessment. A urine based RNA biomarker for ovarian cancer would be particularly useful due to the inaccessible nature of the ovaries and the ability of this cancer to metastasize with few symptoms [17, 43, 44]. Studies of miRNAs in ovarian cancer have delineated a set of differentially expressed miRNAs that are expected to regulate key tumor suppressors such as BRCA1 [161]. Such oncomirs would be expected to be over expressed in biological fluids from cancer patients and have been studied in tissue, serum, ascites fluid and urine from ovarian cancer patients [71, 73, 162-164]. miRNAs associated with EVs are also found in urine from healthy volunteers [165, 166]. It is possible to isolate EVs from large amounts of urine by a combined method of filtration and ultracentrifugation [165, 166]; however, a rapid method to isolate total RNA from small amounts of urine is needed. In this article, we present a proof of concept study of the isolation of total RNA from urine allowing either qRT-PCR or RNA deep sequencing.

Results

In order to identify the repertoire of ncRNAs in urine samples, we developed methods to obtain sufficient RNA for RNA deep sequencing. The amount of total RNA obtained from a one hundred microliter aliquot of urine provided amplifiable RNA for qRT-PCR (**Table 3**), however, not enough RNA for RNA deep sequencing. In order to collect adequate amounts of RNA for deep sequencing, one milliliter samples of urine were obtained from the Gynecologic Tissue and Fluid Bank (GTFB) at the University of Colorado and divided into 100 μl aliquots. Each aliquot was then stored at -80 ^{o}C with 700 μl of the phenol reagent, Qiazol, for at least one hour (**Figure 4**). A final concentration step using low pH phenol was used to combine six samples into one, thus allowing for an increase in total RNA extracted from the same urine sample (**Figure 4**).

Table 3. Total RNA was extracted from eight urine samples and analyzed by qRT-PCR, bioanalysis and RNA deep sequencing. The age, stage and histology for each patient sample is included.

Sample #	Age, Stage	Histology	RNA Quality (Detectable RNA)	RNA Quality (RIN)	Total reads in millions
1	72, IIIC	mucinous adenocarcinoma	RNU6B, miR-29a	2.5	2.6
2	63, IIIC	high grade serous	RNU6B, miR-29a, miR-146a	2.2	49.6
3	48, IIIC	high grade serous	RNU6B, miR-29a, miR-146a	2.6	6.2
4	67, IV	high grade serous	RNU6B	2.5	2.7
5	72, IV	high grade serous	RNU6B	1	1.6
6	57, IIIC	high grade serous	RNU6B, miR-29a, miR-146a	2.5	5.8
7	48, IIC	high grade serous	RNU6B	Less than 1.0	4.8
8	72, IIIC	high grade serous	RNU6B	1.1	2.5

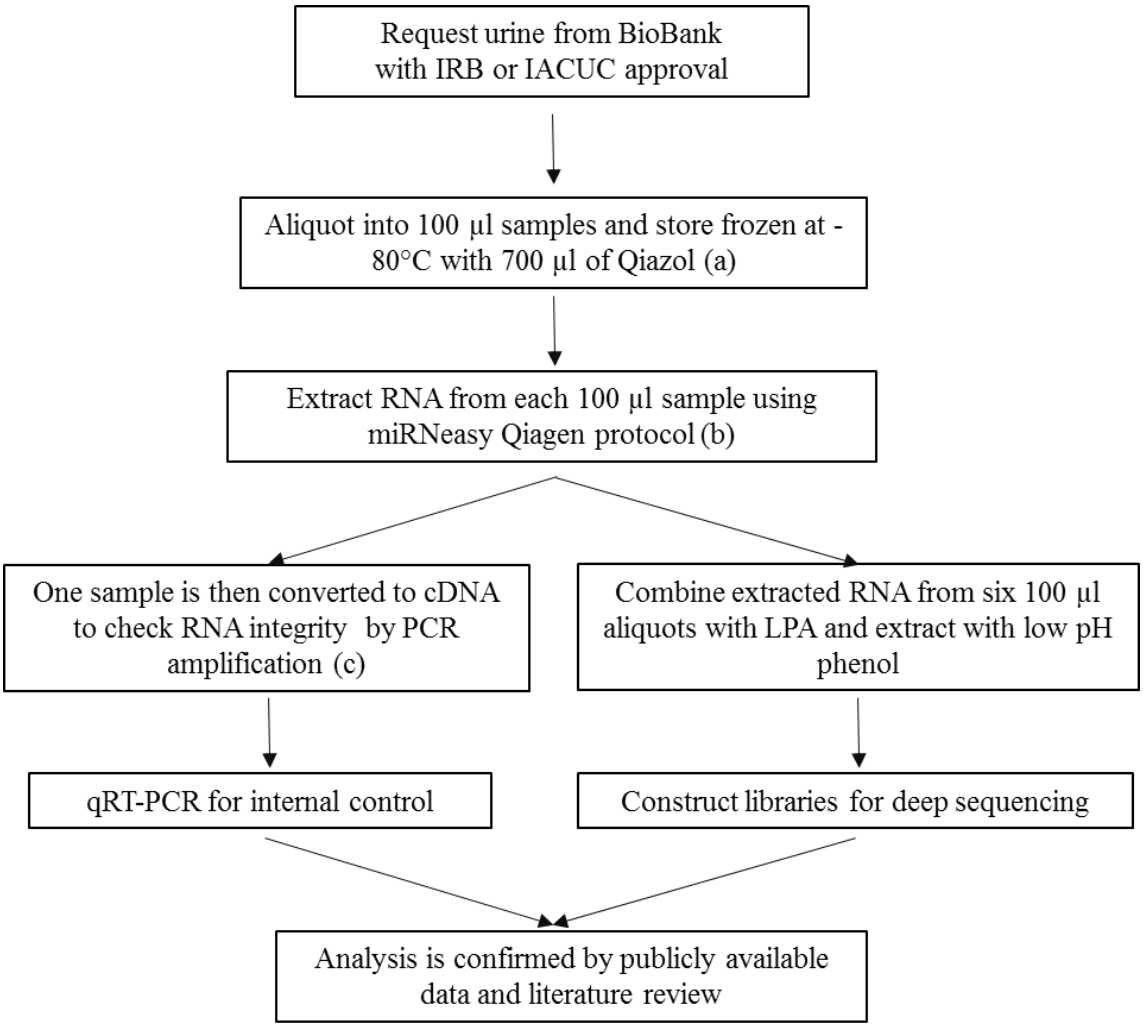


Figure 4. Suggested workflow for ncRNA extraction and analysis from urine obtained from patients or animal models. Notes of specific clarification (**a-c**) of the method are included in **Table 4**.

Table 4. Specific clarification of the methods in Figure 4.

(a). Urine collection
Urine that is collected during surgery from a catheterized patient may be stored unfiltered since it is considered to be sterile at the time of collection. It should be stored at -80 °C for long term storage. Urine that is from the method known as a clean capture in the human clinic and animal samples collected in a nonsterile manner will need to be filter sterilized using a 0.22 or 0.45 micron filter prior to RNA extraction. Filtration before storage is required for samples that will be used for deep sequencing studies where many of the reads could come from bacterial contaminants depending on the method of collection and time before freezing of samples. A product known as a steriflip (MilliporeSigma, US) is especially useful for larger volumes of urine [167].
(b). Volume of urine required for RNA extraction

The miRNeasy kit from Qiagen requires the addition of 100 µl of urine to 700 µl of Qiazol. Once the Qiazol product is added to the urine it may be stored indefinitely at -80 °C. We recommend storing it at -80 °C for at least one hour prior to processing. The theory is that the freeze thaw helps with the release of RNA from proteins or EVs in the urine. Specifically from Tamm-Horsfell protein networks a major confounder in isolation of either protein or nucleic acids from urine [168].

The minimum volume that we have tested is 12 µls of urine and we have been able to generate amplifiable cDNA from RNA extracted with volumes ranging from 12 to 100 µls of urine. It is recommended that the same volume of urine and the same method of collection be used for all samples in the same study.

The Qiagen kit called miRNeasy (Cat No. /ID: 217004, Qiagen) is supplied with Qiazol, which is a phenol based reagent (use caution to avoid contact with skin).

Use the protocol supplied by Qiagen with the following exceptions and reasoning:

There is no need to do the optional DNAse digestion step for urine samples because there is very little contaminating DNA in this type of sample. The miRNeasy kit is optimized for small RNAs less than 200 base pairs.

An optional step of putting the spin column in a new 2ml tube is recommended in the

protocol. However, this is a required step in this urine protocol to prevent carry over of the final RPE buffer. An additional change is that the final spin to dry the column is recommended as 1 minute in the Qiagen protocol and we suggest a three minute spin to be sure that the column is completely dry thereby preventing carry over of ethanol containing buffer that might interfere with downstream use.

The use of the lowest recommended volume of RNase-free water to elute the RNA from the column (30 µls) is used in order to keep the extracted RNA as concentrated as possible.

(c) cDNA preparation and amplification to confirm RNA integrity

The concentration of RNA obtained from 100 µls of urine is much lower than the expected accuracy range of the NanoDrop 1000. Although, the NanoDrop reading does confirm that there is RNA present in the sample. Thus, we use the NanoDrop to confirm RNA extraction while not accepting the accuracy of concentration reading. Amplifiable RNA extraction and cDNA preparation are confirmed by a positive result for qRT-PCR of a small RNA compared to the cDNA water control. The cDNA water control contains everything except RNA, which is required because it is known that the reagents present in cDNA kits and enzymes used for reverse transcription are routinely contaminated with exogenous RNA [169].

The Miscript II kit (Qiagen) for reverse transcription is used with the maximum

volume, allowed by the kit, of 12µls of total RNA per sample. qRT-PCR was conducted using the miScript SYBR Green reagent (Qiagen) and custom primers were designed for the forward primer and specific to the RNA molecule to be amplified.

The primer for miR-29a has an additional three C residues added to increase melting temperature in later steps. The actual melting temperature of miR-29 for qRT-PCR when it is not adapted is too low for the needed specificity (miR-29a-3p 5'-cccTAGCACCATCTGAAATCGGTTA) and for miR-146a-5p (5'-ggT GAG AAC TGA ATT CCA TGG GTT) the melt temperature is increased by the addition of two bases.

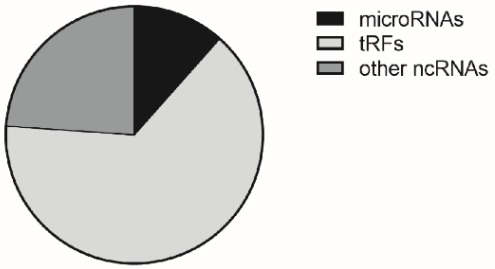
The RNU6B_13 primer from Qiagen is necessary because other variations of RNU6B primers do not routinely amplify extracellular U6. Other internal controls may be more suitable and should be tested for their consistency in the samples of interest [170].

RNA extracted from eight samples was first assessed for the presence of small RNAs by qRT-PCR for either U6, miR-29a or miR-146a. If the sample were positive by qRT-PCR, the sample was then submitted for library preparation regardless of its RNA integrity number (RIN). Later, the total number of reads were compared across the eight samples and it was found that the total number of reads varied widely (**Table 3**).

The libraries of ncRNAs in the urine from eight samples consisted of a wide range of RNA molecules including, but not limited to, rRNA, miRNAs and tRFs. The

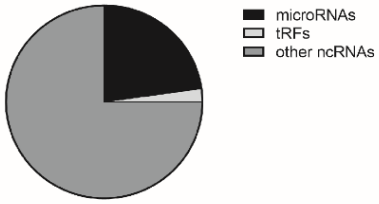
combined deep sequencing results from all eight samples are shown in **Figure 5A**, which is derived from the total annotated reads of ncRNA and shown as the percent of miRNAs, tRFs and other ncRNAs for all libraries. In **Figure 5B** the three categories are shown as percentage of total annotated reads in each individual library.

A

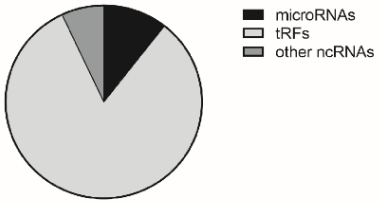


Average from 8 samples

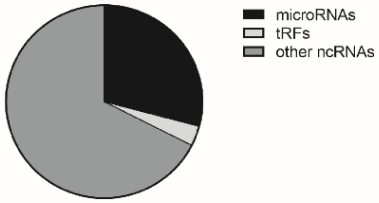
B



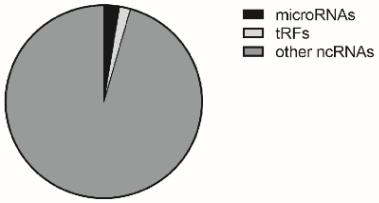
Sample 1



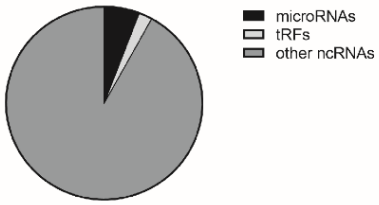
Sample 2



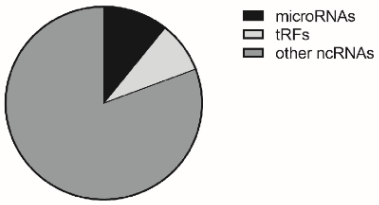
Sample 3



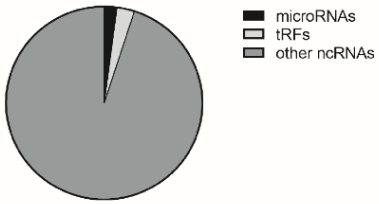
Sample 4



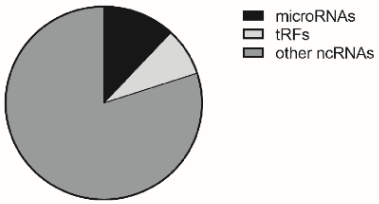
Sample 5



Sample 6



Sample 7



Sample 8

Figure 5. Percentage of ncRNAs. Reads from urine are classified as miRNA, tRFs or other ncRNAs. **(A)** Total reads from 8 urine samples summarized by percent of reads from each sample, microRNAs (black), tRFs (light grey) and other noncoding RNAs (dark grey). **(B)** The percentage of reads for each individual sample again with microRNAs (black), tRFs (light grey) and other noncoding RNAs (dark grey).

The overall repertoire of ncRNAs varied across samples, with a predominant number of reads falling into the "other ncRNAs" category, although one sample had a higher percentage of tRFs (**Figure 5B**, sample 2). The percent of miRNAs varied widely from a few percent to more than 25% (**Table 5**). To better understand the classification of ncRNA in the urine samples, we queried the eight samples for the five most highly expressed ncRNAs in each library and limited the analysis to those RNAs that were annotated in the GRCH37.57. ncRNA data base (**Table 6**).

Table 5. Annotated Reads for overall ncRNAs.

Patient Sample #	miRNA reads	tRF reads	ncRNA reads	miRNA percentage	tRF percentage	ncRNA percentage	total reads
1	105610	10521	347183	22.79%	2.27%	74.93%	463314
2	2060974	16058115	1363668	10.58%	82.42%	7.00%	19482757
3	444849	51460	1031958	29.11%	3.37%	67.52%	1528267
4	14208	10286	533617	2.55%	1.84%	95.61%	558111
5	7935	3062	125592	5.81%	2.24%	91.95%	136589
6	264617	210105	1989653	10.74%	8.53%	80.74%	2464375
7	11659	16257	532983	2.08%	2.90%	95.02%	560899
8	21564	14468	144149	11.97%	8.03%	80.00%	180181

Table 6. The top 5 ncRNAs in each of the urine samples as determined by percent of total annotated reads. The Ensembl genome number from GRCH37.57. ncRNA is included as well as a sequence name when available.

Top5 ncRNAs / Samples #	1	2	3	4	5
1	ENST00000466130 (rRNA pseudo gene)	miR-10b	ENST00000474885 ENST00000459522 (5.8S ribosomal 1)	ENST00000493956 (rRNA pseudo gene)	ENST00000489202 (rRNA pseudo gene)
2	ENST00000483476 (tRF)	ENST00000486830 (tRF)	miR-10b	miR-30a	miR-10a
3	ENST00000474885 ENST00000459522 (5.8S ribosomal 1)	miR-10b	ENST00000489202 (rRNA pseudo gene)	miR-10a	ENST00000493956 (rRNA pseudo gene)
4	ENST00000466130 (rRNA pseudo gene)	ENST00000474885 ENST00000459522 (5.8S ribosomal 1)	ENST00000496481 (rRNA pseudo gene)	ENST00000493956 (rRNA pseudo gene)	ENST00000492060 (rRNA pseudo gene)
5	ENST00000493956 (rRNA pseudo gene)	ENST00000479524 (rRNA pseudo gene)	ENST00000474870 (rRNA pseudo gene)	ENST00000474075 (rRNA pseudo gene)	ENST00000463737 (rRNA pseudo gene)
6	ENST00000466130 (rRNA pseudo gene)	ENST00000474885 ENST00000459522 (5.8S ribosomal 1)	ENST00000483476 (tRF)	ENST00000486830 (tRF)	miR-10b
7	ENST00000493956 (rRNA pseudo gene)	ENST00000479524 (rRNA pseudo gene)	ENST00000463737 (rRNA pseudo gene)	ENST00000476674 (rRNA pseudo gene)	ENST00000459949 (rRNA pseudo gene)
8	ENST00000493956 (rRNA pseudo gene)	ENST00000479524 (rRNA pseudo gene)	miR-10b	ENST00000466130 (rRNA pseudo gene)	ENST00000474870 (rRNA pseudo gene)

The most highly expressed small RNAs were generally fragments related to rRNAs and pseudo rRNAs. Very little is known about the function of extracellular rRNAs and pseudo rRNAs; however, they have been proposed to be processed in response to stress, including oxidative stress [76, 79].

Among the ncRNAs, miRNAs are currently the most well studied group in urine. Overall analysis of the miRNAs in the eight samples revealed that miR-10b was by far the most highly expressed miRNA in all eight samples followed by miR-10a in five of

the eight samples. Other miRNAs that were second to the highest in at least one patient were miR-22 and miR-30a. Interestingly, a miRNA often associated with oncogenesis, miR-21, was only in the top five in one sample (**Table 7**).

Table 7. The top 5 miRNAs in each of the urine samples as determined by percent of total annotated reads.

Top5 miRNAs / Sample #	1	2	3	4	5
1	miR-10b	miR-22	miR-10a	miR-205	miR-30a
2	miR-10b	miR-30a	miR-10a	miR-21	miR-22
3	miR-10b	miR-10a	miR-22	miR-205	miR-30d
4	miR-10b	miR-10a	miR-30a	miR-22	miR-205
5	miR-10b	miR-10a	miR-92a-1	miR-92a-2	miR-203a
6	miR-10b	miR-10a	miR-22	miR-205	miR-30d
7	miR-10b	miR-10a	miR-30a	miR-204	miR-4454
8	miR-10b	miR-10a	miR-30a	miR-204	miR-22

The most highly expressed miRNA in this study, miR-10b, is not well studied in ovarian cancer, although it has recently been implicated in metastasis in other cancer sites [171]. Based on a literature review we expected to find miR-146a and miR-29a in urine due to their previous association with ovarian cancer and previous findings of their

presence in exRNA samples [73, 163, 172, 173]. However, neither of these miRNAs were highly expressed in any of the eight samples as detected by small RNA deep sequencing.

A large group of reads in the deep sequencing studies derived from tRNAs and belonged to the group of functional ncRNAs known as tRFs [174]. The small RNA reads in this study were mapped against the known human tRNAs using the GtRNAdb data base [175, 176]. The most highly expressed tRFs in the eight samples are reported in **Table 8.**

Table 8. The top 5 tRFs in each of the urine samples as determined by percent of total annotated reads.

Top5 tRFs / Sample #	1	2	3	4	5
1	Glu	Gly	Lys	Val	His
2	Gly	Glu	Val	Lys	His
3	Gly	Glu	Val	Lys	His
4	Glu	Gly	Lys	Val	His
5	Glu	Gly	Lys	Val	His
6	Gly	Glu	Val	Lys	His
7	Gly	Glu	Lys	Val	His
8	Gly	Glu	Lys	Val	His

Highly expressed tRFs in the urine included the tRNA halves as well as a large number of variants differing by only a few bases. One tRF variant of the tRNA halve for tRNA-Glu-CTC (miR-2476) was of particular interest because it had been previously reported as a miRNA in the cow. The miR-2476 had not been previously described in human samples; further, it has been removed from the miRNA data base because of its location near a tRNA, tRF5-Glu-CTC [177]. The tRF5-Glu-CTC variant that was previously called miR-2476 is missing a guanine at the sixth base (**Table 9A**, named tRF5-Glu 1Gv). We examined several deep sequencing studies available at GenBank to determine if this variant of tRF5-Glu-CTC had been observed in other human studies. We queried the publicly available data for the presence of tRF5-Glu 1Gv as well as the most prevalent variant from our urine analysis called tRF5-Glu 2Gv (**Table 9B**). One study of particular interest, where both variants were observed, was a comparison of the RNA cargo in exosomes isolated from the media of ovarian cancer cells, ovarian cancer associated adipocytes, normal adipocytes, cancer associated fibroblasts and normal fibroblasts [178].

Table 9. The expression of tRF5-Glu-CTC and its variants in urine and human samples. (A) The variants detected by small RNA deep sequencing in all eight urine samples. The average in all eight samples is given followed by the range of expression. The length in base pairs refers to the length of the variant. The entire length of tRNA-Glu-CTC-1-1 is included for comparison. **(B)** The two most prevalent variants of tRF5-Glu-CTC are presented here along with the SRA accession number and type of sample.

Table 9A

Sequence of variants	Average in 8 samples (range)	Length in base pairs
TCCCTG-TGGTCTAGTGGTTAGGATTCGGC	2926 (14, 22967)	29
TCCCTGGTGGTCTAGTGGTTAGGATTCGGC	4555 (51, 35617)	30
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCG	4044 (17, 31848)	31
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGC	7064 (37, 49667)	32
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCT	7326 (144, 40615)	33
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCTC	2079 (70, 12402)	34
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCTCT	850 (58, 4303)	35
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCTCTC	452 (10, 2463)	36

tRNA-Glu-CTC-1-1
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCTCTCACCGCCGCGGCCCGGGTTCGATTCCCGGTCAGG
GAA

Table 9B

	Accession number	tRF5-Glu 1Gv	tRF5-Glu 2Gv	tRF5-Glu 1Gv percentage	tRF5-Glu 2Gv percentage	Total reads
Ovarian cancer cell line A2780	SRX1550574	186	1439	0.087%	0.670%	214630
Ovarian cancer cell line HeyA8	SRX1550575	43	105	0.015%	0.037%	281330
Ovarian cancer cell line OVCA433	SRX1550576	32	114	0.009%	0.034%	338204
Ovarian cancer cell line SKOV3	SRX1550577	5	25	0.002%	0.010%	256003
Ovarian cancer adipocytes OMT924007	SRX1550585	8065	10261	1.748%	2.224%	461313
Ovarian cancer adipocytes OMT916645	SRX1550586	1901	1380	0.812%	0.589%	234100
Cancer associated fibroblasts CAF866652	SRX1550580	20	1113	0.007%	0.385%	288787
Cancer associated fibroblasts CAF869881	SRX1550581	116	293	0.027%	0.068%	427929
Cancer associated fibroblasts CAF888242	SRX1550582	111	523	0.023%	0.108%	485258
Normal ovarian fibroblasts NOF151	SRX1550578	60	435	0.020%	0.143%	305013
Normal ovarian fibroblasts NOF81000	SRX1550579	76	254	0.023%	0.078%	324908
Normal omental adipocytes OMN050312	SRX1550583	1473	35	0.434%	0.010%	339618
Normal omental adipocytes OMN923075	SRX1550584	84	97	0.031%	0.036%	269810

tRF5-Glu 1Gv, tRF5-Glu 1G variant
(CCCTG-TGGTCTAGTGGTTAGGATTCGGC)
tRF5-Glu 2Gv, tRF5-Glu most prevalent 2G variant
(CCCTGGTGGTCTAGTGGTTAGGATTCGGCGCT)

The variants of tRF5-Glu (both 1Gv and 2Gv) were found in all 13 samples, with 2Gv being more highly expressed in most samples. The increased expression of the 2Gv is similar to our finding in urine from ovarian cancer patients (**Table 9A**). Both variants were highly expressed in cancer adipocytes as compared to the other samples. The

ovarian carcinoma derived cell line A2780 had increased expression compared to the other three cell lines [179]. The top five tRFs from this study were also analyzed for their expression in data bases available at NCBI in the SRA collection (**Table 10**). In this expanded analysis tRF5-Glu was by far the most highly expressed tRF from urinary exosomes. The use of publicly available data to verify the existence of specific variants of tRFs provides a readily available resource to the research community for follow-up studies of deep sequencing analysis. Confirmation of variant expression by their presence in additional publicly available samples may guide the choice for further study of specific exRNAs.

Table 10. Top five tRFs in publicly available SRA data sets.

	Accession number	tRF-Glu	tRF-Gly	tRF-Lys	tRF-Val	tRF-His	Total reads
Ovarian cancer cell line A2780	SRX1550574	1583	257	380	1	0	214630
Ovarian cancer cell line HeyA8	SRX1550575	184	130	108	4	1	281330
Ovarian cancer cell line OVCA433	SRX1550576	154	55	27	1	0	338204
Ovarian cancer cell line SKOV3	SRX1550577	33	12	15	1	0	256003
Ovarian cancer adipocytes OMT924007	SRX1550585	more than 20,000	954	4851	137	14	461313
Ovarian cancer adipocytes OMT916645	SRX1550586	9821	128	730	62	4	234100
Cancer associated fibroblasts CAF866652	SRX1550580	1164	76	57	2	1	288787
Cancer associated fibroblasts CAF869881	SRX1550581	336	89	47	7	2	427929
Cancer associated fibroblasts CAF888242	SRX1550582	636	313	269	12	2	485258
Normal ovarian fibroblasts NOF151	SRX1550578	162	102	76	5	0	305013
Normal ovarian fibroblasts NOF81000	SRX1550579	308	134	203	5	0	324908
Normal omental adipocytes OMN050312	SRX1550583	1987	26	830	24	1	339618
Normal omental adipocytes OMN923075	SRX1550584	557	59	176	17	3	269810

Sequences used for BLAST analysis:
tRF-Glu (CCCTGGTGGTCTAGTGGTTAGGATTCGGCGC); tRF-Gly (CATTGGTGGTTCAGTGGTAGAATTCTCGCCT); tRF-Lys (CCCGGCTAGCTCAGTCGGTAGAGCATGGGAC);tRF-Val (CTTCTGTAGTGTAGTGGTtATCACGTTCGCCTC); tRF-His (CCGTGATCGTATAGTGGTtAGTACTCTGCGT)

Discussion

Liquid biopsies utilizing novel and robust ncRNA targets hold great potential for the development of new biomarkers [47, 180]. High throughput sequencing studies have fueled the search for novel ncRNA biomarkers from accessible samples including serum, plasma and urine [73, 150]. The unexpected stability of exRNA and new technologies to deep sequence RNA have led to an explosion of new biomarker studies focusing on ncRNAs in accessible samples [47, 148-150]. RNA is a preferred molecule for biomarker discovery in liquid biopsies due to its representation of the physiological state of the organism [180, 181]. RNA as a potential biomarker in urine was recognized as early as the 1970s when tRNAs were detected at increased levels in urine from cancer patients [182]. In these early studies, it was not possible to distinguish the specific ncRNA in urine, while it is now feasible with the advent of RNA deep sequencing technologies and the bioinformatics tools to analyze the vast amount of information.

Methods to extract exRNA from accessible samples are widely ranging and varied in the ability to capture amplifiable RNA [167]. It was initially thought that RNA was only stable in urine if it were protected as cargo in exosomes from bladder and urinary tract cells or from the kidney if there is damage. Thus, studies of exRNA in urine exosomes were initially expected to favor the isolation of ncRNA from the bladder. However, it is now increasingly accepted that exRNA in urine can also come from other tissue sites as well as tumors [183-185].

Many of the recent studies aimed at identifying exRNA in urine through deep sequencing protocols are focused on the ncRNA found in EVs and including the subset of

EVs known as exosomes [167]. The extraction of RNA from urinary EVs requires a large volume of urine to ensure that enough RNA will be extracted for downstream applications. The suggested volume of urine for RNA deep sequencing biomarker discovery protocols ranges anywhere from 5mls to 250mls of urine [165-167, 186]. Furthermore, the collection of exosomes requires either ultracentrifugation or treatment with a proprietary method such as the Exosome RNA Isolation kit from Norgen (Norgen Biotek Corporation, Thorold, ON, Canada) to concentrate the exosomes [186]. Studies of the repertoire of exRNA associated with EVs and specifically exosomal RNA provide a partial understanding of the exRNA present in urine. Methods to extract total RNA from urine are also needed.

Methods designed to conduct RNA deep sequencing on total RNA extracted from urine are less common than exosomal studies. One such study of total RNA extracted from the urine of male goats utilized 500μl samples successfully to accomplish deep sequencing [187]. Other studies based on the extraction of total RNA from a small volume of urine did not attempt deep sequencing, rather these methods were used for miRNA Array studies or qRT-PCR and required as little as 50μls for analysis [188]. The methods we describe in this article require similar volumes of urine and allow the isolation and analysis of total RNA by both qRT-PCR and small RNA deep sequencing. Once biomarkers are discovered in pilot studies such as these, it will be possible to scale up to larger studies and develop methods with direct clinical utility.

In order to determine the full spectrum of exRNA in urine, patient samples were requested from the GTFB at the University of Colorado. These samples had been

obtained with informed consent and had all been collected during surgery and handled in a consistent manner prior to storage at -80 ^{o}C. Consistent collection and storage of samples is required for all studies of exRNA and the importance of collection and storage has been described previously [189, 190]. Ovarian cancer is a very difficult disease to detect and frequently is found at late stage; thus, one goal of the GTFB is to provide a resource for studies to discover new biomarkers for patient care. In this pilot study, we analyzed the miRNAs and tRFs and grouped the rRNA fragments with all other ncRNAs (**Figure 5**). The total tRFs, miRNAs and other ncRNAs were first analyzed for all samples by percent of total annotated reads. Combining the reads from all samples gives an overview of the types of RNAs in ovarian cancer patient urine (**Figure 5A**). However, the grouping of all reads is misleading as individual samples were very different from each other (**Figure 5B**). The variability of classes of RNA in patient samples suggests a potential to discover unique biomarkers in urine. Although this proof of concept project provides methods to identify biomarkers in urine, a larger study with the goal of discovering specific biomarkers for ovarian cancer patients will be required.

The top five most highly expressed exRNAs in most samples were dominated by the group of other ncRNAs; however, miR-10b, miR-10a and miR-30a were also found in this group (**Table 6**). The predominant miRNA was by far miR-10b, which was the top miRNA in all samples (**Table 7**). miR-10a was also one of the top five in all samples and in many samples miR-22 and miR-30a were frequently observed. miR-21, a miRNA reported to be highly expressed in cancer patients, was only observed among the top five molecules in one sample (**Table 7**). Although the goal of this pilot study was to develop

methods to study RNA biomarkers in patient urine, it will be interesting in future studies to determine if there is a role for miR-10a and 10b in ovarian cancer. A literature review revealed that few previous studies of miR-10 in ovarian cancer have been conducted. However, the role of miR-10 has been elucidated in normal granulosa cells of the ovary and is linked to feedback regulation of the TGF-β signaling pathway [191]. We also observed the presence of miR-10a and 10b in publicly available data of normal fibroblasts and cancer associated fibroblasts, where the miR-10 family was equally expressed in both normal and cancer associated fibroblasts [178]. A group of miRNAs expressed in ovarian cancer has been suggested to form a signature predictive of poor outcome in ovarian cancer patients [192]; however, the only miRNA included in that signature that is also detected in the top five miRNAs in this study was miR-30d (**Table 7**). The role of miRNAs in ovarian cancer is still being investigated as is the role of tRFs. These exRNAs are expected to provide new physiologically relevant biomarkers for ovarian cancer.

exRNA contains a complex variety of ncRNAs, including numerous tRFs and their variants whose complexity is just now being realized [79, 85, 111, 145, 193]. The complexity of tRF expression is in part due to the frequent modification of nucleosides in the mature tRNA, which may interfere with tRF detection in high-throughput sequencing studies [80]. In addition to high throughput sequencing, additional methods including qRT-PCR and Northern analysis are routinely used to identify tRFs. However, tRFs are derived from pre- and mature tRNAs making them difficult to distinguish by established assays. Honda et al. have developed methods to specifically analyze tRFs using ligation

PCR [89]. Future studies aimed at applying tRFs as biomarkers in patient samples will require the development of methods specific to tRFs.

In order to confirm the use of deep sequencing of RNA from urine as a potential method of discovering new biomarkers, we chose one tRF, tRF5-Glu-CTC, for further study. We examined publicly available databases of exRNA isolated from exosomes to determine if tRF5-Glu and its variants are detectable in exRNA from EVs (**Table 9B**). Libraries of exRNA isolated from exosomes obtained from ovarian cancer cell lines, cancer associated adipocytes, normal adipocytes, cancer associated fibroblasts and normal fibroblasts were queried for the presence of tRF5-Glu-CTC [178]. The predominant variants from the urine samples were also detected in the RNA from exosomes (**Table 9**). The use of publicly available data to confirm the expression of previously unidentified exRNAs provides an additional resource to study the numerous exRNAs in urine. The methods described here are readily adapted to other species and other disease conditions for the future development of physiologically relevant urine based biomarkers.

Experimental Materials and Methods

Participants and Sample Collection

The GTFB, at the University of Colorado Anschutz Medical Campus, collected urine from women undergoing gynecologic surgery. Urine was obtained from women with ovarian cancer under an IRB approved protocol (COMIRB Protocol 11-0626). Samples were provided as 1 ml aliquots of urine with information on patient age, stage and histology of the ovarian cancer. Urine samples were collected during surgery and centrifuged prior to freezing at -80 ^{o}C for long term storage. All samples were de-identified and data was analyzed under a second IRB approved protocol at the University of Minnesota (study number: 1610E97724).

RNA extraction and qRT-PCR analysis of miRNA

Urine was defrosted once and aliquoted in 100μl aliquots and refrozen following the addition of 700μl of Qiazol Reagent (Qiagen, Valencia, CA). Total RNA was isolated from 100μl of urine using the miRNeasy Mini Kit (Qiagen). The isolation procedure followed the miRNeasy protocol with a few clarifications explained in more detail in **Table 4**. The final elution volume for total RNA from the spin column was the minimum required, 30μl RNase free water, to allow the maximum concentration of RNA per μl for downstream applications. The concentration and quality of extracted RNA were assessed by spectrophotometry on the NanoDrop 1000 (Thermo Scientific, Waltham, MA). However, the concentration of RNA obtained is much lower than the expected accuracy

for the NanoDrop 1000; thus, in the following steps, we ignored concentration estimates and simply used the maximum template volume allowed in the protocol. For example, in the Miscript II kit (Qiagen) for a 20μl reaction it is possible to reverse transcribe a volume of 12μls of total RNA for cDNA preparation. Amplifiable RNA extraction and cDNA preparation were confirmed by a positive result for qRT-PCR of a small RNA compared to the cDNA water control. qRT-PCR was conducted using the miScript SYBR Green reagent (Qiagen) with a custom primer for miR-29a-3p 5'-cccTAGCACCATCTGAAATCGGTTA or miR-146a-5p 5'-ggT GAG AAC TGA ATT CCA TGG GTT. We also used the RNU6B primer available from Qiagen for qRT-PCR assays (RNU6B_13). Although in this study, we did not use an internal control for calibration of qRT-PCR results, it is important to mention that an appropriate internal control for exRNA studies is currently controversial. Several helpful studies have examined this controversy and may be of use to those working in this field [170, 186, 194].

RNA precipitation, RNA quality assessment and Illumina Mi-seq methods

Urine is a complex liquid containing EVs, protein, nucleic acids and many other metabolites [195]. Tamm-Horsfell is a protein in urine that is known to form networks that trap EVs [168]. In the study presented here we extracted RNA from six 100μl aliquots of urine and then combined these in the following step to reduce the concentration of contaminating substances such as Tamm-Horsfell protein. Total RNA from 600μl of urine (in 100μl aliquots) was extracted using the above protocol. The 30μl

aliquots of purified RNA were combined into 1 tube for a total of 180 μls. This was extracted with addition of GenElute LPA as described by the manufacturer (Sigma-Aldrich, St. Louis, MO) as previously described [196]. The use of LPA as a carrier is required because alternatives such as glycogen or yeast tRNA are isolated from biological sources and maybe contaminated with small ncRNA [196]. After LPA addition, the samples were extracted with low pH phenol (Ambion 9710) and chloroform:isoamyl alcohol (49:1) with a standard ethanol precipitation using 3M NaAcetate pH 5.2 (**Table 11**).

Table 11. Protocols for combining extracted RNAs for small RNA deep sequencing.

Concentrating fractions of small RNA (Protocol)
1. First extract RNA from six 100 µl samples of urine, this is important rather than drying down a 600 µl sample because it reduces some of the protein contamination. By using more dilute samples and then recombining, we avoid some of the protein contamination [168]. 2. Combine six samples (180 µl of total RNA) then add 400 µl of low pH phenol (Ambion 9710) and 130 µl of CHCl3 (chloroform 49:1 with isoamyl alcohol) mix and spin at 13,200 rpm (Centrifuge 5415, Eppendorf) and collect supernatant. 3. Add 0.75 µL LPA (GenElute, Sigma-56575, Sigma-Aldrich) (In many protocols, glycogen is often used as a carrier for nucleic acid percipitation. However, we and

others find LPA as the preferable carrier because Glycogen is a natural product and for that reason it may be contaminated with RNA or DNA [196]).

4. Add 50 µl of 3M sodium acetate, pH 5.2 and 1 ml of 1:1 ethanol:isopropanol, precipitate over night at -80 ºC.

5. Centrifuge samples for 20min at 4°C at 14000 rpm (Centrifuge 5417R, Eppendorf)

6. Remove supernatant with a pipet, being careful to avoid dislodging the RNA pellet

7. Wash pellet with 500 µL 70% ethanol

8. Centrifuge samples for 15min at 4°C at 14000 rpm to reseat the pellet

9. Remove supernatant as quickly as possible as the pellet can become slippery in 70% ethanol

10. Air dry samples and resuspend in nuclease free water. *do not over dry samples

11. Store at -80°C and submit for library construction (next day)

Library preparation and Illumina sequencing analysis

RNA was forwarded to the UCD Genomics and Microarray Core for library construction. In the core facilities, RNA was assessed for quality on the Agilent Bioanalyzer 2100 using the Eukaryote Total RNA Pico Chip (Agilent Technol., Palo Alto, CA). RNA libraries were constructed using a volume of 5µls of total RNA rather than the recommended concentration of RNA need to prepare the Illumina HiSeq

libraries. The TruSeq Small RNA kit uses a 3′ adapter modified to target miRNAs and other small RNAs that have a 3′ hydroxyl group. Enriching for RNA with a 3'hydroxyl allows the detection of RNA that has been enzymatically cleaved by Dicer or other RNA processing enzymes. Small RNA template libraries were sequenced using small RNA deep sequencing technology on the Illumina HiSeq2000 platform at the University of Colorado's Genomics and Sequencing Core Facility.

miR-seq data analysis

The miRNA sequence reads were identified for known and novel miRNA sequences using the program miRDeep. We calculated the expression of the miRNA variants based on normalized read counts and tested for significant differences using ANOVA in R. Galaxy cutadapt was used for each fastq file to remove adapters. Then fastq files were imported into CLC Genomics workbench to identify and count unique small RNAs using two databases as reference (miRBase Release 19 and Homo sapiens GRCh37.57 ncRNA). The individual data files of trimmed reads have been uploaded to the publicly available data base at https://doi.org/10.5281/zenodo.801484.

Additional data sets were searched from publicly available data at NCBI on the SRA website [178].

Chapter 3: A tRNA Fragment, tRF5-Glu, Regulates BCAR3 Expression and Proliferation in Ovarian Cancer Cells

This chapter is adapted from published work:

Zhou et al. A tRNA Fragment, tRF5-Glu, Regulates BCAR3 Expression and Proliferation in Ovarian Cancer Cells. Oncotarget, Sept 8th 2017. doi: 10.18632/oncotarget.20709.

Chapter Summary

Ovarian cancer is a complex disease marked by tumor heterogeneity, which contributes to difficulties in diagnosis and treatment. New molecular targets and better molecular profiles defining subsets of patients are needed. tRFs offer a recently identified group of ncRNAs that are often as abundant as miRNAs in cancer cells. Initially their presence in deep sequencing data sets was attributed to the breakdown of mature tRNAs, however, it is now clear that they are actively generated and function in multiple regulatory events. One such tRF, tRF5-Glu, is processed from the mature tRNA-Glu and is shown in this study to be expressed in ovarian cancer cells. We confirmed that tRF5-Glu binds directly to a site in the 3'UTR of BCAR3 mRNA thereby down regulating its expression. BCAR3 has not previously been studied in ovarian cancer cells and our studies demonstrate that inhibiting BCAR3 expression suppresses ovarian cancer cell proliferation. Furthermore, mimics of tRF5-Glu were found to inhibit proliferation of ovarian cancer cells. In summary, BCAR3 and tRF5-Glu contribute to the complex tumor heterogeneity of ovarian cancer cells and may provide new targets for therapeutic intervention.

Introduction

Worldwide, ovarian cancer is a leading cause of cancer mortality among women, with the five-year survival rate for women with advanced stage disease expected to be less than 30% [17]. The poor survival is attributed to several factors including nonspecific symptoms of early disease, late stage of diagnosis, and the molecular heterogeneity of the disease [17, 43]. A recent summary and review of microarray studies of tissue samples from ovarian cancer patients demonstrates that heterogeneity is associated with the wide diversity of patient outcomes [44]. In addition to the molecular heterogeneity of mRNA expression, it is now being realized that regulation by ncRNAs contributes to the diversity of gene expression in cancer patients [197, 198].

The impact of newly identified regulatory mechanisms of small ncRNAs on the heterogeneity of gene expression in cancer is exemplified by the recent explosion of miRNA studies including their expression in ovarian cancer [52, 162, 164, 199]. Furthermore, due to an increase in studies utilizing high throughput sequencing technologies even more prevalent classes of ncRNA are being identified [77, 200]. One group of recently identified ncRNAs implicated in cancer biology are the tRFs [85, 174, 201]. tRFs are evolutionarily conserved, from prokaryotes to eukaryotes, and include segments of tRNAs from both pre-tRNAs and mature tRNAs [77]. Due to their recent identification, the naming convention for members of the tRFs is not yet consistent [85, 92, 145]. The tRFs as a class include but are not limited to tRNA halves, tiRNAs, tsRNAs, intermediate tRFs, 5'tRFs, 3'tRFs, and SHOT-RNAs [82, 84, 86, 88, 89].

The biogenesis of tRFs is an active process resulting from the cleavage of tRNAs by multiple enzymes including ANG, RNase Z, and Dicer [82, 84, 88]. The potential functions of tRFs are the focus of many current studies. tRFs have been isolated as part of AGO complexes implying that one function of a subset of tRFs may be similar to that of miRNAs [129, 201, 202]. For instance studies initially aimed at identifying miRNA targets , including high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP), photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) and crosslinking, ligation and sequencing of hybrids (CLASH) studies, have been reanalyzed to confirm the association of tRFs with mRNAs from numerous cellular sources, including a subset from cancer cell lines [129, 145, 193, 201]. An association with AGO implies that tRFs may be regulators of mRNA expression. To date, however, only a few direct mRNA targets of tRFs have been confirmed [129, 203].

A subset of tRFs were initially misclassified as miRNAs and were eventually discovered and eliminated from miRBase if their genomic location was near or embedded in a tRNA gene [75, 204]. For example, miR-2476, which is one base pair different from the 5'-tRF derived from tRNA-Glu-CTC, was discovered in the cow and pig and is now called a dead entry because of its location near a known tRNA (miRBase Accession: MI0011537) [177]. Prior to its elimination from miRBase, miR-2476 was included in TargetScan (6.2) for the cow and predicted to bind the 3' untranslated region (UTR) of many potential mRNA targets. In a pilot study, we found that tRF5-Glu is present in the

urine of ovarian cancer patients (**Chapter 2**) and questioned if it might be present in ovarian cancer cells.

In order to determine if tRF5-Glu is expressed and functional in ovarian cancer cells, we examined its expression in five ovarian cancer cell lines. We have shown that tRF5-Glu is present in ovarian cancer cells grown in culture and is capable of directly binding a predicted target BCAR3. BCAR3 has been well studied in breast cancer for its role in anti-estrogen resistance and breast cancer cell proliferation [205]. BCAR3 is also known to be expressed in B cells, colorectal cancer, and more recently has been shown to function during development [206-209]. However, regulatory mechanisms governing the expression of BCAR3 remain poorly understood. The aim of this study was to identify the expression pattern, function and regulation of BCAR3 and tRF5-Glu in ovarian cancer cells. In this study, we show decreasing BCAR3 expression and increasing tRF5-Glu inhibits the proliferation of ovarian cancer cells. These studies begin to define the regulatory mechanisms of tRFs in ovarian cancer, providing a potential new class of molecules to target for therapeutic development.

Results

tRF5-Glu is expressed in ovarian cancer cell lines

In order to confirm that ovarian cancer cells express tRF5-Glu, we interrogated a panel of ovarian cancer cell lines by qRT-PCR and Northern analysis. The five ovarian cancer cell lines included PEO1, PEO4, SKOV3, 2008, OVCAR3 and were chosen for their molecular heterogeneity and previous use in studies of ovarian cancer [210-212]. The cell line HEK293T was used as a positive control because the expression of tRF5-Glu in this cell line had been previously described in RNA sequencing studies [202]. Expression of tRF5-Glu was measured by qRT-PCR in these cell lines (**Figure 6A**). Northern Blot analysis provided further confirmation that tRF5-Glu is expressed in ovarian cancer cells (**Figure 6B**).

The ovarian cancer cell lines and HEK293T expressed tRNA-Glu and tRF5-Glu variants. The full length mature tRNA-Glu is visible as larger than 70 nucleotides as expected. The tRF5-Glu variants range in size from 29-35 nucleotides (**Figure 6B**, box). Models of the mature tRNA and the tRFs are included next to the corresponding bands of the Northern analysis (**Figure 6B**). The Northern analysis revealed that the ovarian cancer cell lines were able to express tRF5-Glu.

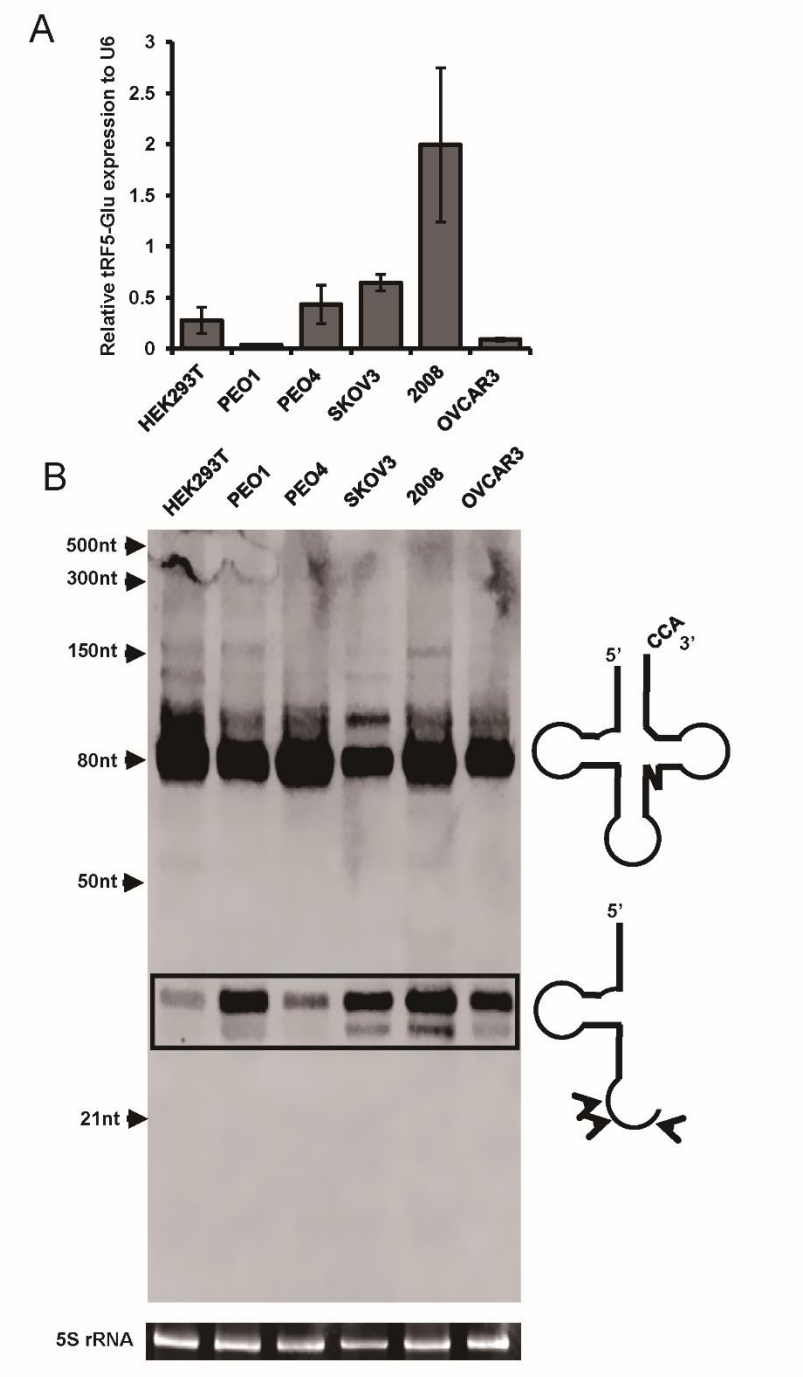


Figure 6. tRF5-Glu is expressed in ovarian cancer cell lines and HEK293T cells. Ovarian cancer cells were grown to confluence and lysed in passive lysis buffer for fifteen minutes prior to RNA extraction. (**A**) tRF5-Glu expression was assessed using

qRT-PCR. tRF5-Glu expression is shown relative to the expression of the internal control, RNU6B (U6). qRT-PCR experiments are replicates of three independent experiments and error bars refer to +/- standard error. (**B**) Validation of tRF5-Glu expression by Northern blot analysis. Northern analysis utilized a 5' biotinylated probe designed to hybridize antisense to the tRF5-Glu region of tRNA-Glu. This probe binds unprocessed tRNA-Glu (greater than 80 base pairs), mature tRNA-Glu (70-80 base pairs) and tRF5-Glu (29-35 base pairs, box). A schematic of the mature tRNA-Glu and the tRF5-Glu is included next to the northern blot showing the mature tRNA-Glu (top) and predominant cleavage sites for tRF5-Glu (bottom). Potential cleavage sites resulting in various size fragments are shown with arrowheads on the schematic of tRF5-Glu. Ethidium bromide stained 5S rRNA below the blot serves as a loading control for the Northern analysis.

ANG modulates the biogenesis of tRF5-Glu in ovarian cancer cells

ANG is an RNase implicated in the biogenesis of the class of tRFs known as the tRNA-halves and the tiRNAs [87, 88]. The tRFs observed by northern blot correspond to the size expected for tRNA-halves. We performed RNAi knockdown of ANG expression in ovarian cancer cell lines to determine if ANG has a role in generating tRF5-Glu in ovarian cancer cells. Two ANG siRNAs were shown to significantly reduce ANG protein expression as compared with a control siRNA in the cell lines (**Figure 7**).

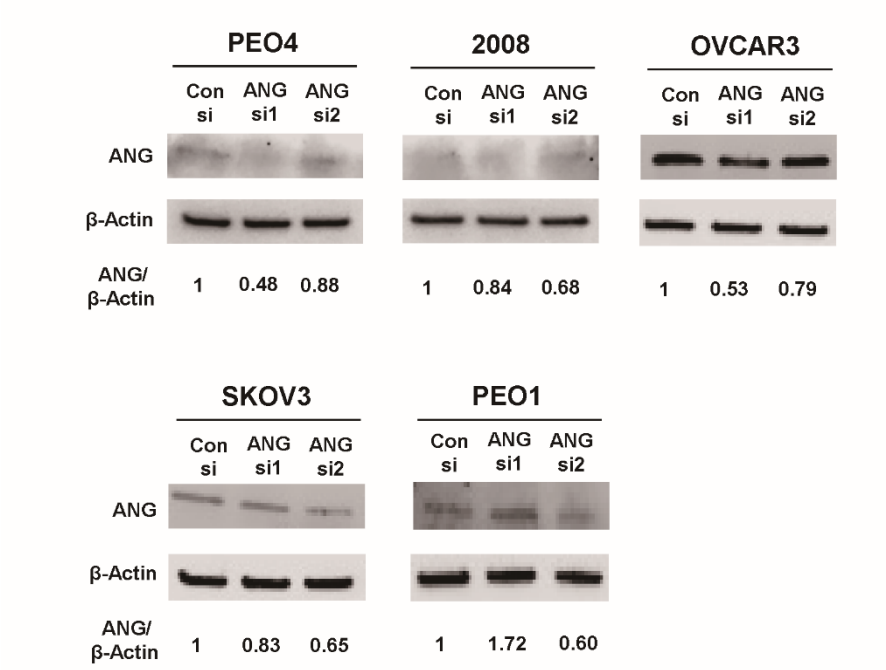


Figure 7. Western analysis of ANG protein expression following 48 hours treatment with control siRNA (con si) and ANG siRNAs (ANG si1, 2) in five ovarian cancer cell lines. Densitometry of each band was measured and the relative ratio of ANG/β-Actin is shown.

tRF5-Glu is part of the mature tRNA-Glu and it is not possible to accurately measure the fragment by routine qRT-PCR without also amplifying the mature tRNA. In order to accurately quantify tRF5-Glu using qRT-PCR methods, we modified an assay previously described by Honda et al., [89] (**Figure 8A**). The modified method is called ligation PCR and includes an internal control of U6 and a specific primer for tRF5-Glu. Ligation PCR confirmed that the tRNA half, tRF5-Glu, is reduced in all five cell lines treated with ANG siRNA (**Figure 8B**). We also confirmed changes in tRF5-Glu by Northern analysis in two cell lines treated with siRNAs to ANG (**Figure 8C**). The

loading control of 5S rRNA was used to show equal loading and we did observe a reduction in the mature tRNA-Glu, as has been shown in a recent study of another tRF [151]. Taken together ligation PCR and Northern analysis confirm that ANG participates in the biogenesis of tRF5-Glu in ovarian cancer cells.

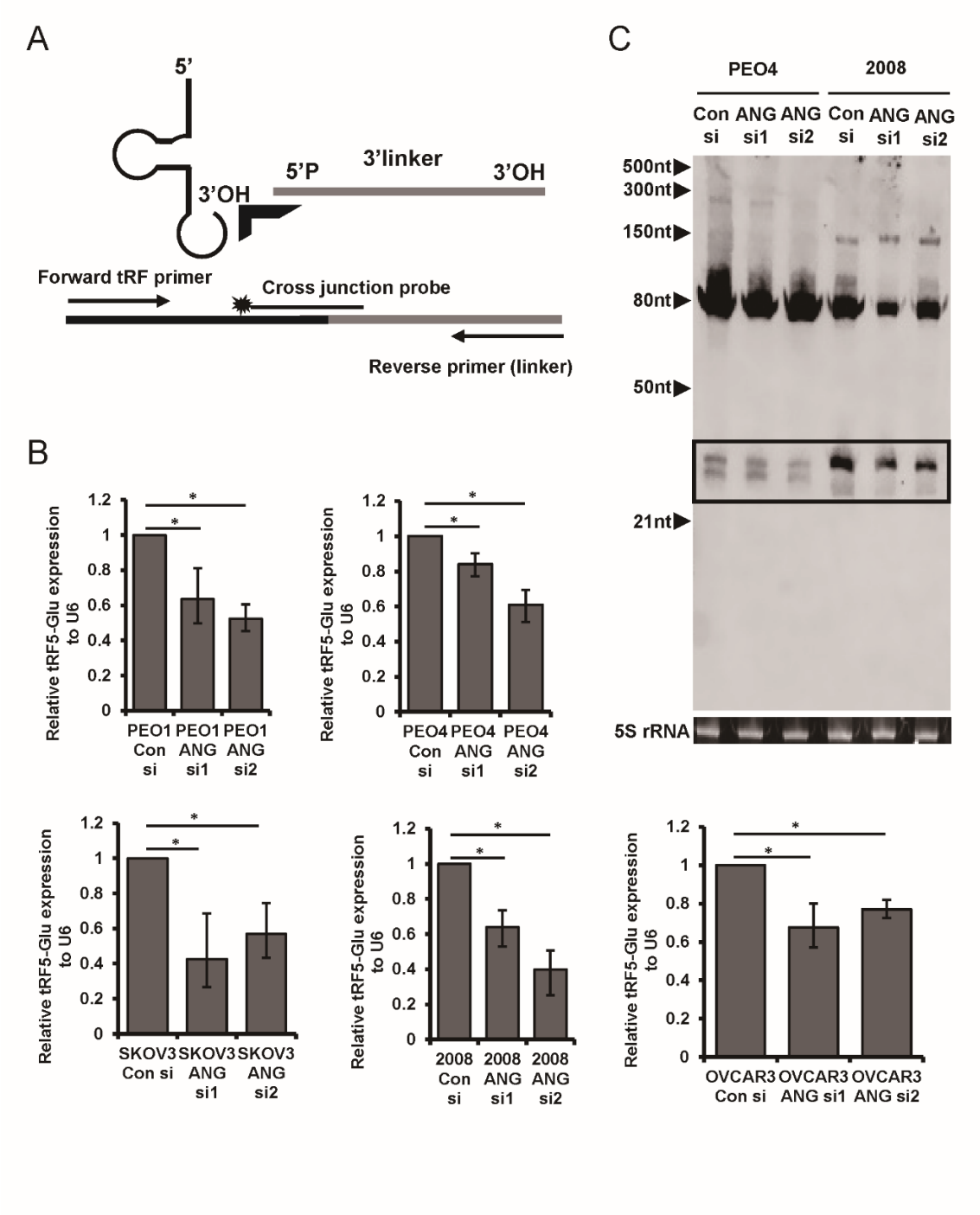


Figure 8. The biogenesis of tRF5-Glu is regulated by ANG in ovarian cancer cells. ANG expression was blocked by transfection of cells with ANG siRNA to determine if tRF5-Glu expression is altered. (**A**) Ligation PCR provides a method to quantify tRF5-

Glu, a diagram of the primer placement for Ligation PCR is included. A labeled probe covering the junction between the end of the 3' linker and target sequence is shown. The linker is designated in grey, the cross junction probe has a star and arrows designate the primers. (**B**) Ligation PCR of cDNA from PEO1, PEO4, SKOV3, 2008, and OVCAR3 cells transfected with control and ANG siRNAs was conducted. tRF5-Glu expression is compared relative to the internal control U6. Ligation PCR experiments are replicates of three independent experiments and error bars refer to +/- standard error (* p-value <.05). (**C**) Northern analysis of RNA collected from cells transfected with a control siRNA and two ANG specific siRNAs (ANG siRNA1 and ANG siRNA2). The tRF5-Glu bands are shown within a box.

tRF5-Glu and its predicted target BCAR3 are simultaneously expressed in ovarian cancer cells

A variant of tRF5-Glu was previously misreported to be miR-2476 (miRBase accession number: MI0011537). miR-2476, now recognized as a variant of tRF5-Glu, was included in TargetScan version 6.2 and thus a list of potential mRNA targets for miR-2476 is available. A somewhat controversial question about tRFs is if they function in a manner similar to miRNAs. We examined the list of predicted targets of miR-2476 in TargetScan 6.2 (available at http://www.targetscan.org/vert_61/) and selected potential targets for further analysis based on their previous expression in cancer (**Table 12**).

Table 12. Potential targets of miR-2476 expressing in cancer cells [213-220]**.**

Targets of miR-2476 with a previous association with cancer. **TargetScan 6.2 (http://www.targetscan.org/vert_61/)**	**Total Context+ score**	**Previous studies in cancer for each of the top genes.**	**Selected for further study**
CBX5 (HP1α)	-0.78	Regulated by an epigenetic methylation	No
PCGF2 (Mel-18)	-0.63	The 3' untranslated region was shown to be associated with tumor suppression	No
CUX1 (CUTL1)	-0.52	Not the gene associated with ovarian cancer at 7q22	No
BCAR3	-0.50	Associated with anti-estrogen resistance in breast cancer	Yes
TCF20 (SPBP)	-0.43	Associated with anti-estrogen resistance in breast cancer	No
CNTN2 (Tag-1)	-0.41	Early studies mostly in neuronal tumors	No
HIF3A	-0.41	Not as much reported about HIF3A as about HIF1 or HIF2 could be interesting in future studies	No
FLT1	-0.41	This is becoming significant in ovarian cancer but at the time these studies were initiated it was not as well studied	No

BCAR3 was selected from the genes predicted for regulation due to its expression in the ovarian cancer intraperitoneal xenograft model previously published and available from GEO Profiles (GDS4066:204032) [210]. The available GEO data indicates that both PEO4 and 2008 cells express the mRNA of BCAR3. In order for BCAR3 mRNA to be targeted by tRF5-Glu, they both must be present in cells grown under the same conditions. To determine if BCAR3 and tRF5-Glu are present in ovarian cancer cells, PEO4 and 2008 cells were grown in the presence or absence of estrogen for 48 hours, similar to methods used in the GEO experiment [210].

PEO4 and 2008 cells were both shown to express BCAR3 as well as tRF5-Glu under both growth conditions. Interestingly, BCAR3 expression decreased in estrogen stimulated PEO4 cells compared to estrogen starved PEO4 cells. However, there was no significant difference in BCAR3 expression between estrogen treated or estrogen deprived 2008 cells (**Figure 9A** and **9B**). In contrast, tRF5-Glu expression was increased in the PEO4 cell line stimulated with estrogen, while again no change was observed in the 2008 cell line (**Figure 9C** and **9D**, box). These data demonstrate a potential correlation between BCAR3 and tRF5-Glu under estrogen stimulated growth conditions in PEO4, while no significant change in BCAR3 or tRF5-Glu was observed in 2008 cells.

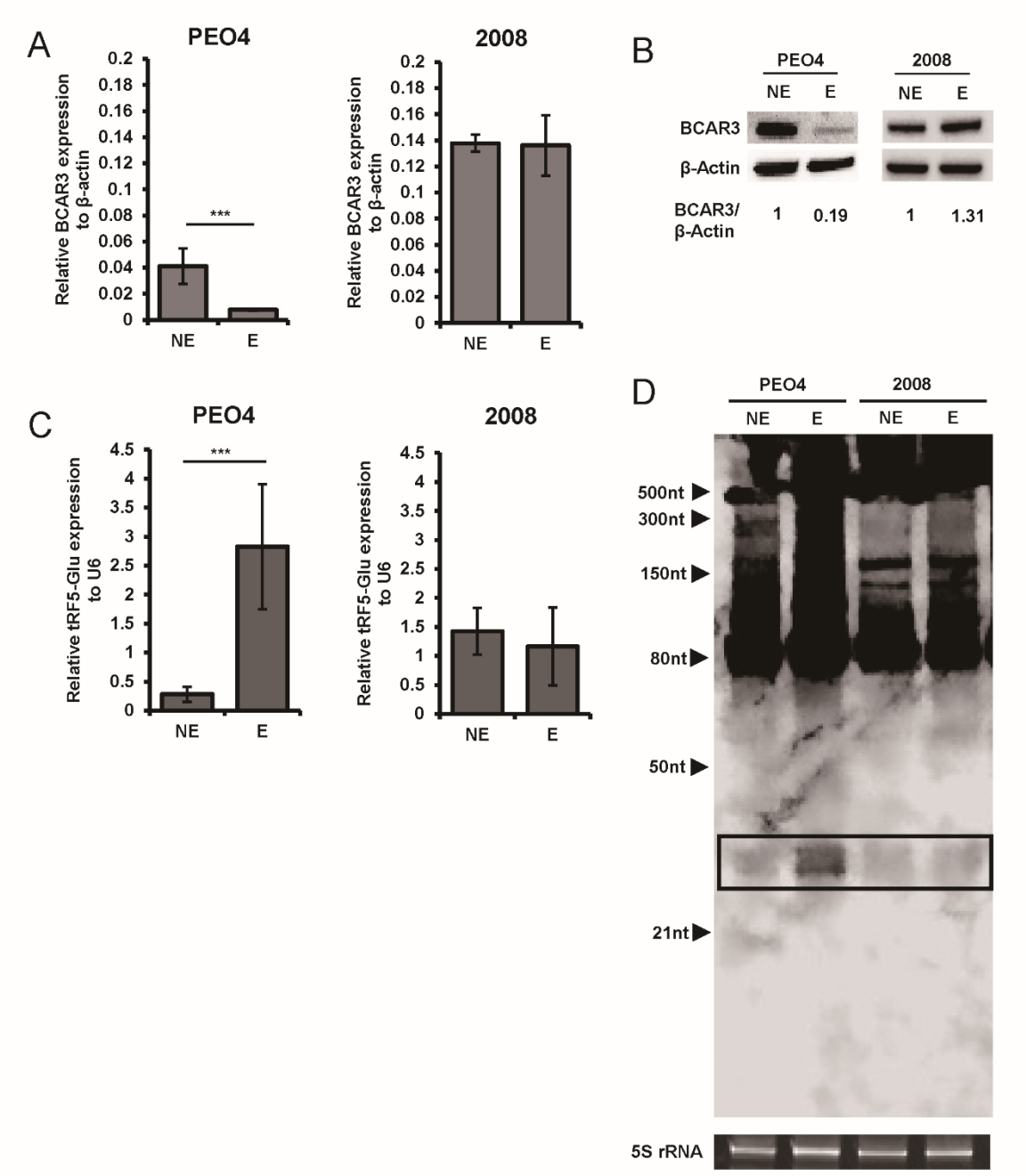


Figure 9. tRF5-Glu and its potential target BCAR3 are expressed in ovarian cancer cell lines. Ovarian cancer cell lines were grown under estrogen starved (NE) or estrogen stimulated (E) conditions to determine the best conditions to study the potential relationship between tRF5-Glu and BCAR3. (**A**) Expression of BCAR3 mRNA in cDNA

from PEO4 and 2008 cells estrogen starved (NE) or estrogen stimulated (E) as assayed by qRT-PCR. qRT-PCR experiments are replicates of three independent experiments and error bars refer to +/- standard error (*** p-value<.005). (**B**) Western analysis of BCAR3 protein expression at 48 hours of estrogen starvation (NE) or estrogen stimulation (E) in the two ovarian cancer cell lines. Densitometry of each band was measured and the relative ratio of BCAR3/β-Actin is shown. (**C**) Expression of tRF5-Glu amplified from cDNA of PEO4 and 2008 cells estrogen starved (NE) or estrogen stimulated (E) as assayed by ligation PCR. (**D**) Northern analysis of tRF5-Glu in estrogen starved (NE) or estrogen stimulated (E) PEO4 and 2008 cells. Cells were grown for 48 hours in estrogen depleted media and then the media was replaced by new estrogen depleted media (NE) or media supplemented with estrogen for an additional 48 hours (E). Cells were then lysed by direct addition of Qiazol for RNA extraction. The tRF5-Glu bands are shown within a box.

tRF5-Glu directly binds BCAR3 mRNA in ovarian cancer cells

In order to confirm direct binding of the 3'UTR of BCAR3 by tRF5-Glu we modified the previously described miR-Catch assay [221]. We used a biotinylated probe targeting a region of BCAR3 predicted to have an open secondary structure upon RNA folding and distal to the predicted binding site for tRF5-Glu (**Figure 10A**). Cell lysates from PEO4 cells treated with estrogen for 48 hours were used because we had previously observed regulation of BCAR3 expression in this cell line with these growth conditions (**Figure 9**). Lysates were collected and hybridized to a BCAR3 specific biotinylated

capture probe or a random probe (**Figure 10B**). BCAR3 mRNA was enriched by pulldown with a BCAR3 probe while it was not enriched by pulldown with a randomized probe. Pulldown with a BCAR3 specific probe showed enrichment for tRF5-Glu.

To investigate if there is direct regulation of BCAR3 by tRF5-Glu, comparable to that expected of a miRNA, a reporter binding assay placing the 3'UTR of BCAR3 downstream of the luciferase reporter was developed. Another reporter construct with two mutations in the predicted binding site was also generated (**Figure 10C**). HEK293T, PEO4 and 2008 cells were transfected with these constructs in the presence of either a control or a specific mimic of tRF5-Glu. Forty-eight hours after transfection there was a significant 40% and 30% reduction in luciferase activity with the wildtype construct as compared to control in HEK293T and PEO4, respectively. A significant reduction in luciferase activity was not observed in 2008 cells (**Figure 10D**). There was no significant reduction in luciferase activity with the luciferase assay containing mutations in the predicted miR-2476/tRF5-Glu binding site in HEK293T, PEO4 or 2008 (**Figure 10D**). These results confirm that BCAR3 is directly regulated by tRF5-Glu. However, the lack of response to tRF5-Glu mimics in 2008 cells suggests an alternative mechanism of BCAR3 regulation in this cell line.

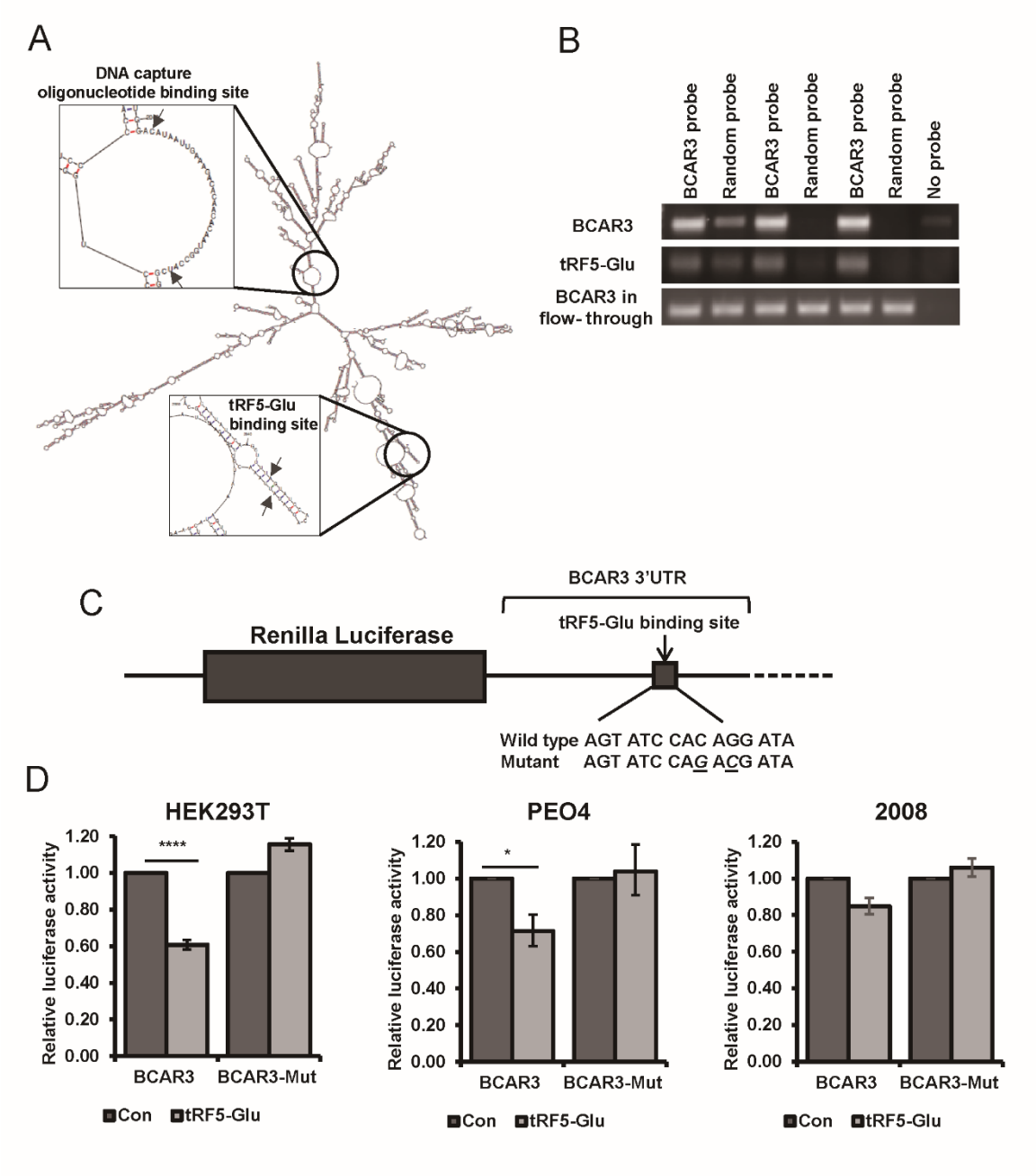


Figure 10. Binding of the BCAR3 mRNA by tRF5-Glu is demonstrated using miR-Catch and luciferase assays. (A) Secondary structure of the BCAR3 mRNA with placement of the DNA capture probe in relationship to the tRF5-Glu binding site is shown with arrows. **(B)** Triplicate samples of PEO4 cell lysates probed with either

BCAR3 or a random probe were amplified by reverse transcription PCR (RT-PCR) for BCAR3 and tRF5-Glu expression. The BCAR3 mRNA in the flow-through was amplified and is shown for each sample. (**C**) A schematic of the BCAR3 3'UTR and predicted binding site for tRF5-Glu is shown with the wild type and mutant seed sequences listed below. Luciferase reporter plasmids were constructed containing the predicted binding site intact or mutated at two base pairs within the predicted seed region (designated by underlined letters). (**D**) Luciferase constructs with the wild type or mutant BCAR3 3'UTR were transfected into HEK293T, PEO4 and 2008 cells with a control mimic or a mimic of tRF5-Glu. Transfections were performed in triplicate. The result was compared to a control mimic and error bars refer to +/- standard error (* p-value <.05 and **** p-value <.001).

tRF5-Glu directly regulates BCAR3 expression in ovarian cancer cells

Further investigation of the regulation of BCAR3 by tRF5-Glu was studied in five cell lines, PEO1, PEO4, SKOV3, 2008, and OVCAR3 under growth conditions where BCAR3 was readily expressed. All five ovarian cancer cell lines were transfected with control mimics and inhibitors or a tRF5-Glu specific mimic or inhibitor. Western analysis for BCAR3 protein expression was conducted on cell lysates following 48 hours of treatment (**Figure 11A**).

In response to treatment with a mimic of tRF5-Glu, BCAR3 expression was observed to decrease in PEO1, PEO4 and OVCAR3 cells as compared to a control mimic; however, in SKOV3 and 2008 cells, BCAR3 protein expression remained

unchanged. PEO1, PEO4 and OVCAR3 cells also increased expression of BCAR3 after receiving an inhibitor of tRF5-Glu compared to a control, whereas BCAR3 expression in SKOV3 and 2008 cells was unaltered.

In order to further explore the regulation of BCAR3 by tRF5-Glu we constructed vectors expressing only a 50 base pair region of the 3' UTR of BCAR3 (**Figure 11B**). The wild type and mutant constructs were transfected into HEK293T, PEO4 and 2008 cells (**Figure 11C**). When the smaller region of the BCAR3 UTR is included in the luciferase construct, regulation through the predicted binding site is possible regardless of the cell line where the luciferase constructs are expressed. These results suggest that other regions of the BCAR3 UTR are required for the lack of regulation of BCAR3 by tRF5-Glu observed in 2008 cells.

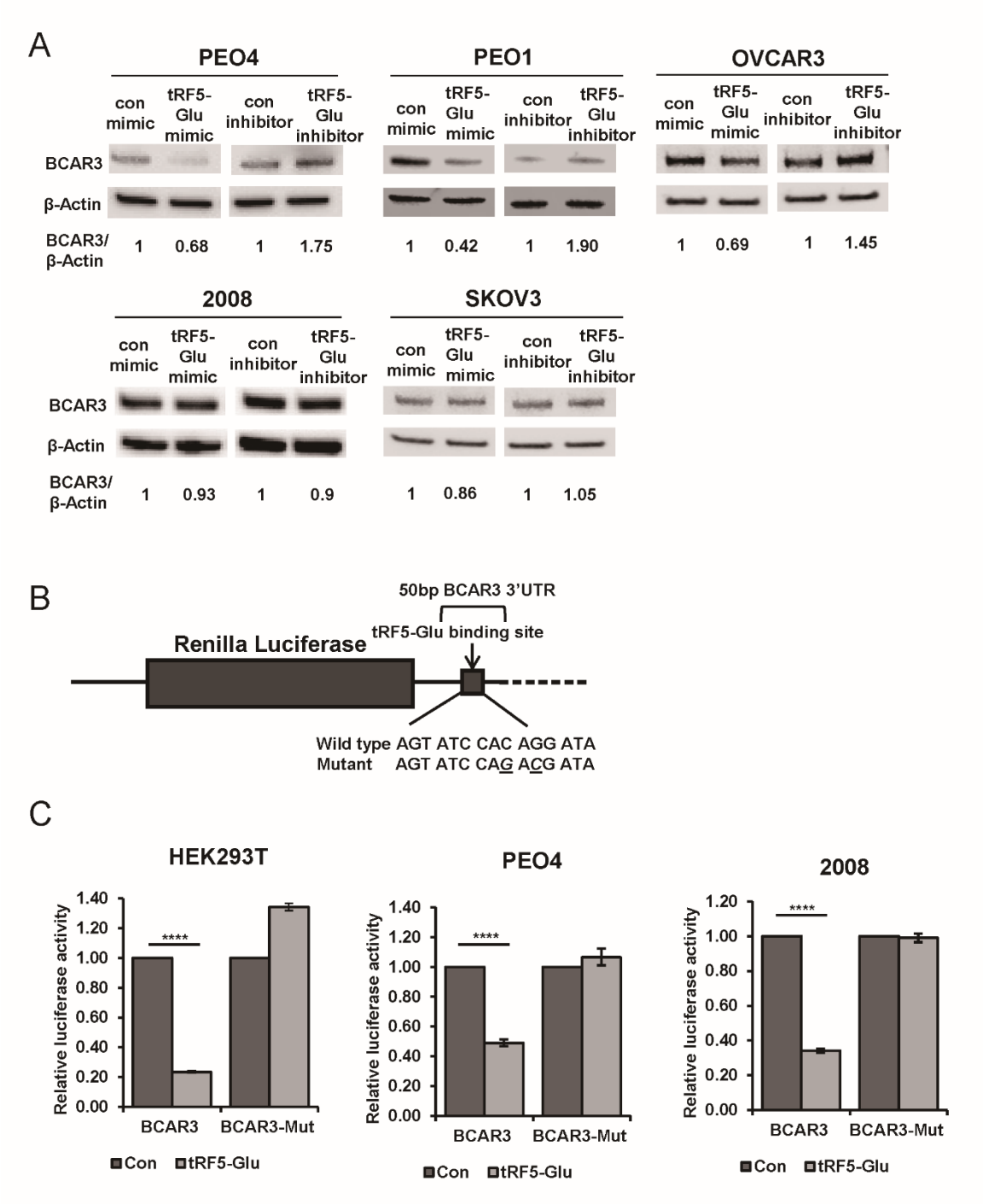


Figure 11. Direct regulation of BCAR3 by tRF5-Glu is predicted in TargetScan 6.2 which shows a binding site for miR-2476/tRF5-Glu in the BCAR3 3'UTR. Mimics and inhibitors of tRF5-Glu were used to determine if BCAR3 is regulated by tRF5-Glu.

(**A**) Western analysis was conducted on protein extracts from PEO4, PEO1, OVCAR3, 2008 and SKOV3 cells, estrogen starved and transfected with either a control mimic, a control inhibitor, a tRF5-Glu mimic or a tRF5-Glu inhibitor. Protein lysates were collected at 48 hours post transfection. Densitometry of each band was measured and the relative ratio of BCAR3/β-Actin is shown. (**B**) A schematic of the 50 base pair region of the BCAR3 3'UTR and predicted binding site for tRF5-Glu is shown with the wild type and mutant seed sequences listed below. Luciferase reporter plasmids were constructed containing the predicted binding site intact or mutated at two base pairs within the predicted seed region (designated by underlined letters). (**C**) Luciferase constructs with the wild type or mutant 50bp region of BCAR3 3'UTR were transfected into HEK293T, PEO4 and 2008 cells with a control mimic or a mimic of tRF5-Glu. Transfections were performed in triplicate. The result was compared to a control mimic and error bars refer to +/- standard error (**** p-value <.001).

Usage of an alternative poly-A site may modulate tRF5-Glu regulation of BCAR3 expression in 2008 ovarian cancer cells

The lack of response of BCAR3 expression to mimics or inhibitors of tRF5-Glu in SKOV3 and 2008 was reminiscent of miRNA studies where the 3'UTR in rapidly growing cells may be shortened thereby deleting a binding site for a miRNA repressor [222-224]. The 3'UTR of mRNAs contain many regulatory sites including, but not limited to, ncRNA binding, RNA binding protein binding sites and alternative poly A sites (APA) [225]. It has been shown that APA sites may be used in some rapidly

proliferating cells, thereby eliminating a regulatory region and shortening a normally longer 3'UTR [226]. Regulation of mRNA expression through RNA binding sites in the 3'UTR are often affected by the use of tandem APA sites [227].

The discrepancy between the regulation of BCAR3 by mimics of tRF5-Glu in some ovarian cancer cell lines in contrast to that of SKOV3 and 2008 cells raised the possibility that the 3'UTR of BCAR3 might have been truncated due to the use of an alternative tandem APA in the UTR and 5' to the tRF5-Glu binding site. A poly-A site prediction search was conducted to determine if a tandem poly-A site is present in the BCAR3 3'UTR (**Figure 12A**) [225]. A qRT-PCR assay relating the total BCAR3 mRNA expression to the longer 3'UTR was developed and substantiated the hypothesis that a shorter UTR was used in SKOV3 and 2008 cells, while the more distal poly-A site was frequently utilized in PEO1, PEO4 and OVCAR3 cells (**Figure 12B**). Further confirmation was achieved by amplifying and cloning the BCAR3 3'UTR from the poly-A containing fraction of total RNA from PEO4 and 2008 cell lines (**Table 13**).

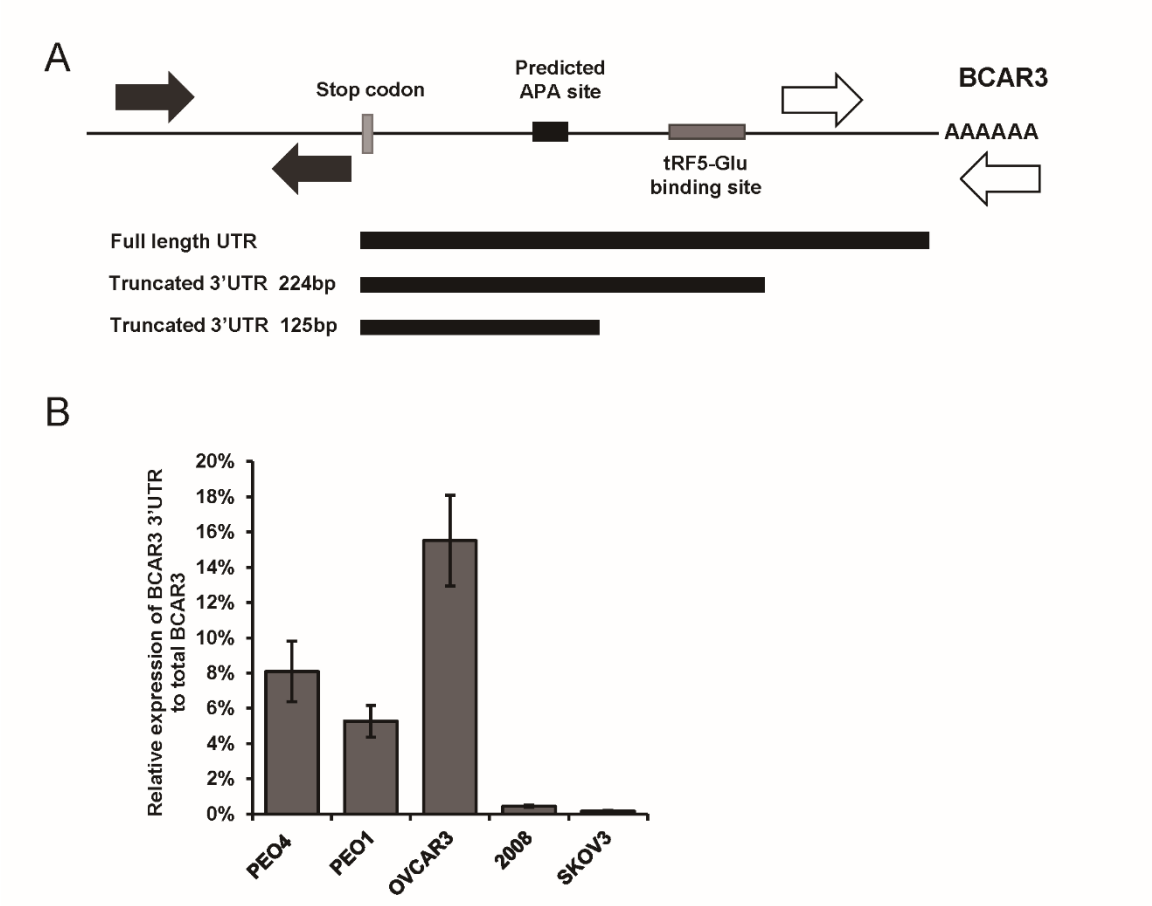


Figure 12. APA sites in the 3'UTR of BCAR3 are utilized and may prevent regulation by tRF5-Glu. (**A**) Schematic diagram of the 3'UTR of BCAR3 designating the APA site (black box) and the predicted site for tRF5-Glu binding (grey box). The length of the cloned 3'UTRs for BCAR3 are depicted starting from the stop codon of BCAR3. The primers used for qRT-PCR are designated by solid arrows for the amplicon in the coding region and hatched arrows are used to designate the 3'UTR specific primers. (**B**) Relative expression of the longest BCAR3 3'UTR to total BCAR3 mRNA assayed by qRT-PCR for PEO4, PEO1, OVCAR3, 2008 and SKOV3 cells. qRT-PCR experiments are replicates of three independent experiments and error bars refer to +/- standard error.

Table 13. Representative sequences of the 3'UTR of BCAR3 in ovarian cancer cells

Representative sequences of the 3' UTR of BCAR3		
Cell line		**Sequence**
PEO4	full length	CCTGTAAAGCAGGCAGAGCTTTGATAACTCTCCAGAGAAC CTTTAGAATATCTTTTCAAGTTTCCCCAGCTTCATCTTTGGG AAAGCTTACTGTTTTTGATAAAGTAATAATGTGCAAATCTG ACAATATACAAGCTTTTAGTATCCACAGGATATTAAACGTG TAAATTGCACAGAGCACACTTATTTATGAATTGTCTAAAGT TACTACTGATTTTAAAATGAATAATTTATTATTAAGGTAAC TACTGCTAATGTTGATCAGCAAATTTAAGAGAAGACCTAG CTATGTTGGCTGGTTGCTTTCTATTATCATGGTATTTGACCA TTTTAGTTTTAATTCCATGTCAGATAAGTGTAAATAGAAGA
2008	full length	CCTGTAAAGCAGGCAGAGCTTTGATAACTCTCCAGAGAAC CTTTAGAATATCTTTCCAAGTTTCCCCAGCTTCATCTTTGGG AAAGCTTACTGTTTTTGATAAAGTAATAATGTGCAAATCTG ACAATATACAAGCTTTTAGTATCCACAGGATATTAAACGTG TAAATTGCACAGAGCACACTTATTTATGAATTGTCTAAAGT TACTACTGATTTTAAAATGAATAATTTATTATTAAGGTAAC TACTGCTAATGTTGATCAGCAAATTTAAGAGAAGACCTAG CTATGTTGGCTGGTTGCTTTCTATTATCATGGTATTTGACCA TTTTAGTTTTAATTCCATGTCAGATAAGTGTAAATAGAAGA GTTTAAAAGCATGAAACATTTCAGAAGGTATCAGTTATAT GATATTCTTTAAACAAATATGAAAAATGTAAATACTCATG AATGAAAATACATCTTTTTGTG
2008	224 bp	CCTGTAAAGCAGGCAGAGCTTTGATAACTCTCCAGAGAAC CTTTAGAATATCTTTTCAAGTTTCCCCAGCTTCATCTTTGGG AAAGCTTACTGTTTTTGATAAAGTAATAATGTGCAAATCTG ACAATATACAAGCTTTTAGTATCCACAGGATATTAAACGTG TAAATTGCACAGAGCACACTTATTTATGAATTGTCTAAAGT TACTACTGATTTTAAAATG
2008	125 bp	CCTGTAAAGCAGGCAGAGCTTTGATAACTCTCCAGAGAAC CTTTAGAATATCTTTTCAAGTTTCCCCAGCTTCATCTTTGGG AAAGCTTACTGTTTTTGATAAAGTAATAATGTGCAAATCTG AC

The qRT-PCR analysis and cloning of the 3'UTR sequences in PEO4 cells confirmed that in this cell line the distal poly-A is more frequently used, while in 2008 proximal sites may be used for poly-A addition. The use of the proximal poly-A site

closest to the stop codon in BCAR3 prevents regulation of BCAR3 by tRF5-Glu because the binding site is not present in the shortened BCAR3 mRNA. This finding implies that the regulation of BCAR3 is more complicated than previously known and may vary between cell lines.

Over expression of tRF5-Glu reduces ovarian cancer cell proliferation

It had previously been shown that BCAR3 expression increased proliferation of breast cancer cells [205]. We hypothesized that knocking down BCAR3 expression in ovarian cancer cells would similarly inhibit the proliferation (that is, overall cell number resulting from the balance of cell death and cell division) of ovarian cancer cells. BCAR3 expression was down regulated with siRNAs targeting BCAR3. In both PEO4 and 2008 cells siRNAs targeting the coding region of BCAR3 were able to block BCAR3 expression (**Figure 13**). The loss of BCAR3 and subsequent reduction of proliferation had not previously been shown in ovarian cancer cells. In **Figure 14** we show that siRNA inhibition of BCAR3 results in a significant decrease of proliferation when compared to control.

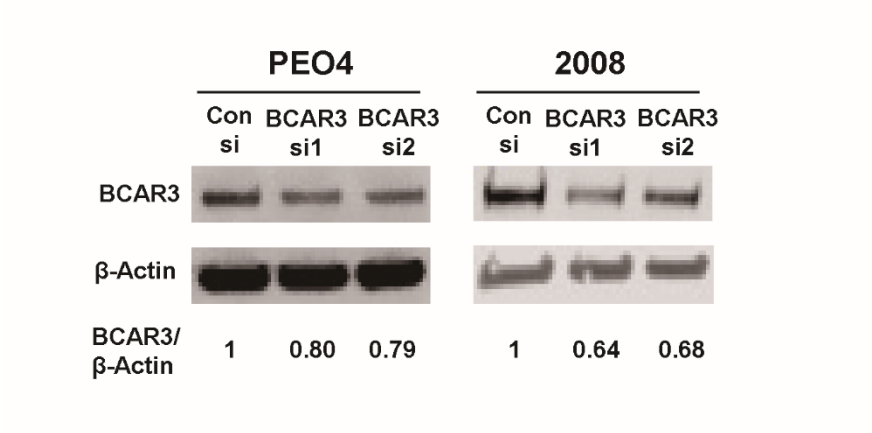


Figure 13. Western analysis of BCAR3 protein expression following 48 hours treatment with control siRNA (con si) and BCAR3 siRNAs (ANG si1, 2) in PEO4 and 2008 ovarian cancer cell lines. Densitometry of each band was measured and the relative ratio of BCAR3/β-Actin is shown.

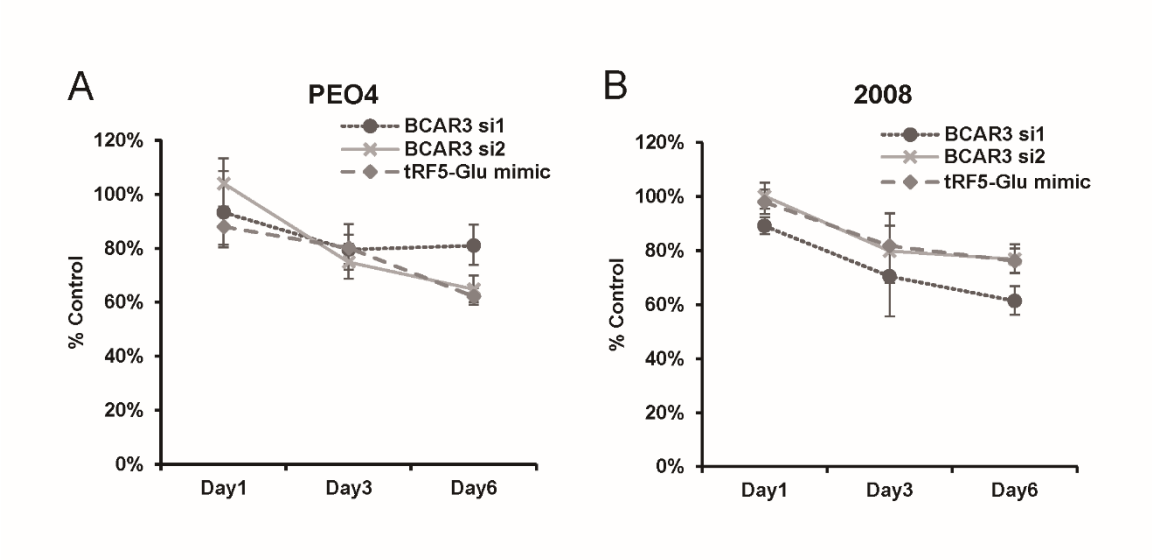


Figure 14. Proliferation of ovarian cancer cells is inhibited by either siRNA to BCAR3 or mimics of tRF5-Glu. (**A**) PEO4 cells transfected with either a control siRNA, siRNAs targeting BCAR3 or mimics of tRF5-Glu were grown in culture for up to

six days and then fixed and assessed by Sulforhodamine B (SRB) assay. Proliferation measures the overall cell number resulting from the balance of cell division and cell death over time. Values are shown as percent of control (control siRNA transfected cells; set to 100%) at day one, day three and day six. (**B**) 2008 cells were transfected and grown as described above for PEO4 cells. Cell proliferation experiments are replicates of three independent experiments and error bars refer to +/- standard error. p-value was less than 0.05 for PEO4 and 2008 cells treated with siRNAs to BCAR3 and tRF5-Glu mimic on day six.

Additionally, studies of tRFs in cancer cells have previously shown that increasing tRF concentration may decrease cancer cell proliferation. For example the over expression of tRF-1001 (tRF3-Ser) has been shown to decrease proliferation of prostate cancer cells [85]. Thus, it was logical to assess the ability of tRF5-Glu to alter the proliferation rate of ovarian cancer cells in culture. We found that mimics of tRF5-Glu significantly reduced the proliferation of both PEO4 and 2008 cells (**Figure 14**). These results indicate that BCAR3 is not the only target of tRF5-Glu because mimics of tRF5-Glu do not alter BCAR3 expression in 2008 cells (**Figure 11A**). Therefore, tRF5-Glu would be expected to bind to additional mRNA target sites in additional genes, similar to the mechanism of canonical miRNAs where multiple targets are common. Future studies will be needed to identify additional targets of tRF5-Glu.

Discussion

Ovarian cancer is known to have the highest mortality rate of the gynecologic malignancies [228]. The high mortality rate is often attributed to the late stage of diagnosis, lack of treatment options and the molecular heterogeneity of the disease [228, 229]. Next-generation sequencing is becoming routine in the clinical setting and adds to our understanding of tumor heterogeneity, however, much work is needed to define useful information from the massive amounts of data now clinically available [230]. The ncRNAs, including the miRNAs are frequently found highly expressed in next-generation sequencing studies and are adding to our understanding of tumor heterogeneity [197]. Even more recently many rRNA and tRNA fragments have been described and the tRFs are now being implicated in cancer biology [74].

Studies of a variety of tRFs have shown that they are actively generated from mature tRNAs and the 5'tRFs are frequently generated by cleavage from tRNAs by ANG. ANG is a well characterized RNase previously associated with the process of angiogenesis, one of the hallmarks of cancer [231]. The biogenesis of tRF5-Glu has previously been shown to involve cleavage by ANG in response to respiratory syncytial virus [114]. We applied similar methods to confirm that ANG is responsible for the biogenesis of tRF5-Glu in ovarian cancer cell lines. siRNA inhibition of ANG followed by northern analysis and ligation PCR confirmed that with reduced expression of ANG, there is reduced expression of tRF5-Glu.

tRFs have been confirmed to function in the stress response to regulate stress granule formation as well as to directly target the RNA binding protein, YBX1, and

prevent binding to its mRNA targets [94, 232]. Two other tRFs are known to directly bind mRNA targets in a manner similar to canonical miRNAs [129, 203]. Further indication that tRFs may function to regulate RNA targets has come from their inclusion in AGO HITS-CLIP data sets [201, 202, 233]. The CLASH data set is particularly informative because of its ability to link RNA-RNA hybrids, and indicates that both tRF5-Glu and a variant of tRF5-Glu, homologous to the former miR-2476, are able to target mRNAs in an AGO1 dependent manner [202]. As tRFs are further characterized it is expected that they will contribute to the complexity of tumor heterogeneity.

BCAR3 was especially interesting as a potential target of tRF5-Glu because it has been extensively studied in breast cancer as a protein associated with anti-estrogen resistance [234]. BCAR3 has not been studied in ovarian cancer and little is known regarding its regulation at the mRNA level. Thus, we chose to examine this protein for its potential as a target of tRF5-Glu with the expectation that it would lead to new understanding of a regulatory mechanism previously undefined in this disease.

Modifying the method of miR-Catch and using two luciferase reporter assays we were able to confirm that tRF5-Glu binds directly to the BCAR3 mRNA. The BCAR3 specific probe allowed the pulldown of the BCAR3 mRNA and the associated tRF5-Glu and was not pulled down with a random probe. Luciferase assays confirmed the predicted site in the 3' region of BCAR3 was capable of binding mimics of tRF5-Glu and allowing down regulation of the target. We were also able to confirm that tRF5-Glu is capable of targeting BCAR3 mRNA expression in multiple cell lines, while in still others it appeared incapable of regulating BCAR3 protein expression. APA sites have been shown to alter

miRNA regulation in rapidly growing cells and provide another layer of regulation through the 3'UTR of mRNA [226]. Our finding that BCAR3 has an APA site upstream from the tRF5-Glu binding site suggests that this may provide an alternative mechanism for the regulation of BCAR3.

The expression of BCAR3 in breast cancer cells has been previously associated with increased proliferation [205]. In contrast, tRF-1001 has been reported to inhibit prostate cancer cell proliferation [85]. Taken together these two findings led to our hypothesis that increased expression of tRF5-Glu would reduce the proliferation of PEO4 cells, due to direct binding of tRF5-Glu in the 3' UTR of BCAR3. However, our studies also indicate that mimics of tRF5-Glu reduced the proliferation of 2008 cells, suggesting that other targets of tRF5-Glu are regulated in the 2008 cell line. These results and the regulation of apolipoprotein E receptor 2 (APOER2) by tRF5-Glu demonstrate that, much like miRNA regulators, tRF5-Glu has multiple targets [203].

Future studies will be required to identify the impact of tRF5-Glu expression and regulation of its targets in ovarian cancer and its impact for patient outcome. The study of tRFs is just beginning and this group of ncRNAs may open the way for the development of new therapeutic targets as more information is gathered. tRFs add another layer of complexity to discerning the heterogeneity of gene expression and regulatory mechanisms of ncRNA.

Experimental Materials and Methods

Cell culture and reagents

Ovarian cancer cell lines PEO1, PEO4, SKOV3, 2008, OVCAR3 were provided by Dr. Monique Spillman [210-212] and HEK293T cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). All the cell lines were grown in RPMI 1640 with 10% fetal bovine serum and Plasmocin (Invivogen, San Diego, CA). Estrogen depletion and addition studies were conducted using cells cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% charcoal stripped fetal bovine serum [235]. Cells were incubated at 37°C with 5% CO2 and have been tested to be mycoplasma free.

RNA extraction, qRT-PCR and cloning of PCR products

Total RNA was extracted from cells using miRNeasy (Qiagen, Valencia, CA) as previously described [236]. Equal amounts of RNA were reverse transcribed into cDNA using the miScript II RT Kit and qRT-PCR was conducted with miScript SYBR Green (Qiagen). mRNA expression was normalized to beta actin, while small RNA expression was normalized to RNU6B (RNU6B_13, Qiagen). The polyA site was identified in BCAR3 from PEO4 and 2008 cells using Thermoscript RT (Invitrogen) and oligo dT as previously described [237]. PCR products were amplified using the 5' forward BCAR3 primer with the Universal reverse primer from Qiagen and amplified using GoTaq Green (Promega, Madison, WI). Primers are listed in **Table 14**. PCR products were cloned

into T-vector (Life Technologies, Carlsbad, CA) and sent for direct sequencing (University of Minnesota Genomic Center, Minneapolis, MN).

Table 14. Sequences of primers and probes.

Primer Type	**Sequence and information about the primer design**
adj-tRF5-Glu	ggCCCTGTGGTCTAGTGGTTAGGATTC adjusted to achieve improved melting temperature
RNU6B forward primer	Commercially available RNU6B_13. It is critical that RNU6B_13 be used because RNU6B-11 does not work for these assays (Qiagen).
BCAR3 forward	TTGAAAGACACAACACAATGGCCATCGG
BCAR3 reverse	GGAACACATGTGGACTCTCTGCCTTC (cross exon)
Beta actin forward	ATCCACGAAACTACCTTCAACTC
Beta actin reverse	GAGGAGCAATGATCTTGATCTTC (cross exon)

3’RNA adaptor [89]	5’/5Phos/GAACACUGCGUUUGCUGGCUUUGAGAGUUCU ACAGUCCGACGAUC/3ddC/-3’
tRF5-Glu probe	5’-/56FAM/CGCTCTCGA/ZEN/ACACTGCGTTTGC/3IABkFQ/-3’
RNU6B probe	5’-/56FAM/TTTGAACAC/ZEN/TGCGTTTGCTGGC/3IABkFQ/-3’
tRF5-Glu forward	CCCTGTGGTCTAGTGGTTAGGATTC
3’ adaptor reverse primer [89]	CGTCGGACTGTAGAACTCTCAAAGC
Luciferase construct BCAR3	The amplified region included 415 of the 458 base pairs reported in GenBank as the 3’UTR of the human BCAR3 (NM_001261408.1).
Xho1 BCAR3	aacctcgagCCTGTAAAGCAGGCAGAGCTTTGA
Not1 BCAR3	aaggatataagcggccgcgAATATCATATAACTGATACCTTCTGA AATGTTTC

BCAR3 mut forward	CAAGCTTTTAGTATCCAGACGATATTAAACGTG
BCAR3 mut reverse	CACGTTTAATATCGTCTGGATACTAAAAGCTTG
BCAR3 3'end forward	AGAGAAGACCTAGCTATGTTGGCTGGTTG
Qiagen Universal reverse	Use Qiagen universal reverse with BCAR3' end forward and with tRF5-Glu adjusted primers.
Biotin BCAR3 capture probe	5'-/5BiotinTEG/ATGGCCATTGTGTTGTGTCTTTCAATTATG-3'
Biotin random capture probe	5'-/5BiotinTEG/GATTAGTGTCACAACTTTTGTTCGTTGTTG-3'
Xho1 50bp BCAR3	TCGAAGTAATAATGTGCAAATCTGACAATATACAAGCTTTTAGTATCCACAGGATAT
Not1 50bp BCAR3	GGCCATATCCTGTGGATACTAAAAGCTTGTATATTGTCAGATTTGCACATTATTACT
50bp BCAR3 mut forward	TCGAAGTAATAATGTGCAAATCTGACAATATACAAGCTTTTAGTATCCAGACGATAT

50bp BCAR3 mut reverse	GGCCATATCGTCTGGATACTAAAAGCTTGTATATTGTC AGATTTGCACATTATTACT

Quantification of tRFs by Ligation qRT-PCR

The sequence of the adapters, probes and primers for tRF quantification by Taqman qRT-PCR are included in **Table 14**. The ligated tRFs were prepared with methods described by Honda et al., [89]. RNA extracted from siRNA treated cells was treated with T4 polynucleotide kinase (T4PNK) to convert the 3' cyclic phosphate group into a hydroxyl group prior to ligation of a 3' linker and then copied into cDNA. A forward primer specific to either tRF5-Glu or U6 and a reverse primer specific to the linker were used to amplify targets from the total cDNA pool. Ligated RNA were subjected to qRT-PCR using the QuantiTect Probe RT-PCR Kit (Qiagen). The quantified tRF5-Glu levels were normalized to RNU6B.

The probe placement was determined by Sanger sequencing of the PCR product which identifies the base pairs at the junction of the tRF5-Glu and the linker. The sequence identified the specific variant of tRF5-Glu to be one base longer than that previously predicted to be cleaved by ANG [87]. Once the junction between the linker and the most 3' base of tRF5-Glu and of U6 were determined we were able to design probes for ligation PCR (**Table 14**).

Northern Blot

Northern blot analysis was modified from methods previously described [238]. Denaturing polyacrylamide gels (15%) containing 7M urea was used to separate small RNA. Equivalent loading and RNA integrity were confirmed by gel imaging of the rRNA using a Licor FC imager. Blots were probed with the 5'-biotinylated RNA probes: tRF5-Glu probe (**Table 14**), and the miRNA marker miR-21 probe was used for size analysis (New England Biolabs, Ipswich, MA).

Western Blot

Protein was extracted from cells using Passive Lysis Buffer (Promega) and samples were loaded onto 4%-20% Mini-PROTEAN TGX gel (Bio-Rad Laboratories, Hercules, CA). The primary antibodies include rabbit anti-BCAR3 #A301-671A (Bethyl Laboratories, Montgomery, TX), ANG 1 (C-20): sc-1408 (Santa Cruz Biotechnology, Dallas, TX), rabbit anti-β-actin #4970 (Cell Signaling Technologies, Danvers, MA). All antibodies has been tested for specificity.

Transfection with siRNA, mimics and inhibitors

Cells were seeded 24h prior to transfection in 35mm petri dishes or 24-well plates and transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The Allstars negative control and the miScript inhibitor negative control were used in the transfection at a concentration of 1nM (Qiagen). Mimics and inhibitors for tRF5-Glu (MSY0012067 and MIN0012067, Qiagen) were also used in the transfection at a concentration of 1nM. The siRNAs were obtained from Qiagen and

consisted of Gene Solution siRNA product number 1027416 as a control. Two siRNAs were selected from each set of four for further use including Hs_BCAR3_3 SI00053102 (BCAR3 siRNA 1), Hs_BCAR3_6 SI0381603 (BCAR3 siRNA 2), Hs_ANG_6 SI02780197 (ANG siRNA 1) and Hs_ANG_7 SI03071866 (ANG siRNA 7).

Luciferase Assay and constructs

Luciferase constructs containing either 471 base pairs of the BCAR3 3'UTR or a 50 base pair region containing the predicted binding site and a second construct, containing site directed mutations in the predicted binding site were prepared using methods as previously described [239]. The BCAR3 3'-UTR including the predicted binding site for tRF5-Glu was cloned into psiCheck-2 dual luciferase vector. Mutant BCAR3 3'UTR luciferase vectors were generated using primers designed to exchange a C for a G and a G for a C in the tRF5-Glu predicted binding region. These plasmids and a tRF5-Glu mimic were co-transfected into HEK293T cells. Finally, luciferase assays were conducted using the dual luciferase reporter kit (Promega). All primer sequences for vector construction are included in **Table 14**.

miR-Catch and BCAR3 mRNA pulldown

Five variants of BCAR3 are listed in GenBank and each variant was subjected to unifold (IDTDNA.com) to identify regions that would remain single stranded and available for probe binding. A biotinylated probe targeting the BCAR3 mRNA was designed such that it would bind to a predicted single stranded region of the BCAR3

coding sequence and conjugated to a streptavidin labeled magnetic bead. A similar biotinylated probe was generated from a randomized sequence and used as a negative control. Cell lysates from PEO4 cells following 48 hours of Estrogen stimulation were collected by resuspending and lysing cells in passive lysis buffer (Promega) and hybridizing for 90 minutes at 37 ^{o}C with either the BCAR3 specific probe or the randomized probe. Magnetic beads were then captured on a Mylteni separator and washed with 1X PBS 3 times. The flow through and magnetic particles were collected for RNA extraction. RNA was converted to cDNA for qRT-PCR amplification and analysis for BCAR3 mRNA and tRF5-Glu were conducted. All primer sequences for miR-Catch probes are included in **Table 14**.

SRB proliferation assay

SRB assays were used to measure cell proliferation, essentially as described by Skehan et al., [240], with a few modifications [241, 242]. The SRB assay measures total cellular protein, which has been shown to be linear with cell number [240]. PEO4 and 2008 cells were seeded in 48 well-plates at a concentration of 1×10^4 cells/ml and grown under the described experimental conditions for up to 6 days. The cells were then fixed with ice-cold methanol containing 1% acetic acid. Subsequently, cells were incubated with 0.5% SRB solution for 1h at 37 °C, then rinsed 3-5 times with 1% acetic acid washed to remove unbound dye, and then air-dried. Bound SRB was eluted with 10mM Tris, pH10, and the absorbance at 540 nm measured in a multi-well plate reader. Results were expressed as a percentage of signal of the controls.

Statistical analysis

All the data were expressed as means with standard error and represent at least three independent experiments. Comparison of gene expression was made by using the Student's t-test. A p-value less than 0.05 was considered to be significant.

Chapter 4: Conclusions and Future Directions

Summary and significance of results

Given the low five-year survival rate of ovarian cancer patients, which is largely driven by non-specific symptoms and a lack of early diagnosis, a simple and effective method of early detection is urgently needed. Previously, I have discussed several widely used detection methods for ovarian cancer in **Chapter 1**, however, none of them are sufficient and specific enough to be applied in the early diagnosis of ovarian cancer.

With the advance of deep sequencing techniques for nucleic acids, ncRNAs have recently been identified in cancer patients' tissue samples as well as in liquid biopsies. Several ncRNAs have been identified to be associated with the diagnosis and prognosis of multiple cancers [73]. Liquid biopsy samples, such as urine, act as a good source for biomarker discovery due to the easy accessibility and the ready collection from patients. In my project the use of urine was especially helpful due to the inaccessible nature of the ovaries. In reports from our physician collaborators (MA Spillman and K Behbakht), the use of urine was preferable in this disease.

My first hypothesis was that exRNAs are present and detectable in the urine obtained from ovarian cancer patients. To test this hypothesis, we have performed small RNA deep sequencing on urine samples of ovarian cancer patients with late stage disease. Our goal was to identify potential ncRNA biomarkers of ovarian cancer. In this study, we have developed a novel method to isolate exRNAs from a small volume of urine instead of urinary exosomes which requires a large volume of urine. The use of small volumes of urine rather than large amount of urine is advantageous for several reasons. Although exosomes from urine are more often used in other published studies, limited analysis of

exosomes misses the totality of the RNA in urine. While it is possible to collect large volumes of urine, it is harder to handle large volumes in the clinic. When the ultimate goal is a clinically applicable method, it is better to start with a stream lined approach to begin with. The first hypothesis that there are detectable ncRNAs in urine proved to be correct and a larger number of potential biomarkers were identified than initially expected.

In the follow-up analysis, we have compared the classes of exRNAs among all eight urine samples. Notably, functional ncRNA were present at significant levels and included certain miRNAs and a novel class of small ncRNAs named tRFs. Analysis of the deep sequencing data was performed to obtain the top 5 miRNAs and tRNA fragments, which helps narrow down potential biomarker candidates or functional molecular candidates for potential biomarkers of ovarian cancer (**Table 7** and **Table 8**).

The discovery of several tRFs in the urine of ovarian cancer patients led to my project described in **Chapter 3**, where I worked to discover the function of a specific tRF, tRF5-Glu. Following extensive bioinformatics analysis of tRF5-Glu expression, tRF5-Glu was the most highly expressed in all eight urine samples. In addition, tRF5-Glu had been misclassified as miR-2476 and thought to be specific to the cow and pig and not found in humans (http://www.targetscan.org/vert_61/). Our first goal was to determine if this ncRNA could originate from the ovarian tumor. The reasoning was that, even though tRF5-Glu was identified in the urine of patients with ovarian cancer, it could be either secreted from the ovarian cancer cells themselves into circulation or from some other sources, such as bladder cells and urinary tract cells into urine directly which have direct

access to urine. Several studies have indicated that miRNAs can be released from cells into circulation through cellular mechanisms including as cargo in exosomes, microvesicles, proteins and lipid complexes [158, 243-245]. Reports of miRNAs from other tissue sites, such as the breast, helped to convince us the possible origin of tRF5-Glu from ovarian cancer cells [73]. We went on to show that tRF5-Glu is expressed in five different ovarian cancer cell lines (**Figure 6**).

To our knowledge, only a few tRFs has been studied in cancer cells and a tRF, tRF-Lys, is identified as a possible prognostic biomarker of high-grade serous ovarian cancer [75]. Thus, the tRFs identified in our study could be functional molecules in ovarian cancer cells and may be associated with tumor growth and progression. At the time of these studies, the function of tRFs had not been studied in ovarian cancer cells. In **Chapter 3**, we further studied the function of tRF5-Glu. We were able to confirm that ANG is involved in the biogenesis of tRF5-Glu from the mature tRNA-Glu and the production of tRF5-Glu was in response to the estrogen treatment in ovarian cancer cell line, PEO4. We also evaluated that tRF5-Glu binds to the 3'UTR of BCAR3 gene and regulates its expression in a manner similar to a miRNA. In addition, the overexpression of tRF5-Glu also inhibits the proliferation of ovarian cancer cells not only through downregulating BCAR3, but may also regulate through targeting other pathways. Unlike previous work showing certain tRFs regulate the translation process in cooperation with particular RNA binding proteins, my work exhibits another pattern of how a tRF is involved in the regulation of tumor growth. This work also provides a model for studying the functions of other tRFs as miRNA-like regulators in cancer cells. The results and

methods used in this study were published recently in two manuscripts (**Chapter 2** and **Chapter 3**).

Reflections and future directions

A novel method to identify potential exRNA biomarkers for ovarian cancer was proposed in **Chapter 2** and a novel tRF, tRF5-Glu, was identified from those studies. Evaluation of tRF5-Glu expression and its function were then conducted in **Chapter 3** to confirm the role of tRF5-Glu in ovarian cancer cells. In this section, possible challenges and future directions will be addressed to better clarify the potential impact of my studies.

In **Chapter 2**, we have performed a pilot small RNA deep sequencing study in the urine samples collected from 8 patients to seek potential RNA biomarkers. While several miRNAs and tRFs were found to be highly enriched in urine, a future study containing a larger number of urine samples needs to be conducted to further test the possibility that certain tRFs or miRNAs are potential biomarkers of ovarian cancer. While we were looking at the pilot small RNA deep sequencing database, rRNA fragments are actually much more prevalent than miRNAs and tRFs discussed in my study. However, there is still less known about the function of rRNA fragments at this time and it is unknown if they are associated with cancer diagnosis or tumor biology.

Among the top 5 miRNAs summarized in **Table 7**, miR-10b was identified as the top one in all samples, which led to a future question whether miR-10b could be a potential biomarker of ovarian cancer or if it plays a role during ovarian cancer growth and progression. Previous studies have already evaluated the oncogenic role of miRNA-

10b in many cancer sites including non-small cell lung cancer and glioblastoma [246, 247]. And we have found it regulates the NCOR2 mRNA and protein expression in ovarian cancer cells, which in turn affects the expression of androgen receptor (AR). Our laboratory previously showed that the downregulation of NCOR2 by miR-125b allows increased expression of the AR [236]. One future goal in the Bemis laboratory will be to further explore the functional mechanisms of miRNA-10b in ovarian cancer cells.

Finally, we analyzed the tRFs identified in patients urine and summarized the top5 tRFs in **Table 8**. Although we only studied the two most abundant variants of tRF5-Glu-CTC in urine samples and in an available database of human samples, the same analysis could be applied to the study of other tRFs, such as tRF-Gly, tRF-Lys, tRF-Val and tRF-His (**Table 10**). Previous studies have shown that the fragment from tRNA-Lys enhances the proliferation of prostate cancer cells, while another study showed that a fragment from tRNA-His could inhibit the translation initiation process in human osteosarcoma epithelial cells [89, 131]. These studies illustrate that the implications of tRF expression may be broader than firstly thought and more extensive than standard miRNAs. There remain many unanswered questions in the study of tRFs including the functional study of less known tRFs as well as the different tRF variants from the same mature tRNA.

The studies in **Chapter 3** use tRF5-Glu as a model to study the function of the tRF in ovarian cancer. We characterized the functions and detailed the expression of this specific tRF in ovarian cancer cells. However, several challenges were encountered in this work and we have developed possible solutions to conquer them. A clear limitation

to the tRF study is the lack of methods to quantify the exact amount of tRFs. Due to the short sequences and origins of tRFs, a regular qRT-PCR is not applicable to measure the exact copies of tRFs. Thus, in our study, we have adapted a method called ligation PCR from Honda et al. to the study of tRF5-Glu expression thereby allowing us to determine the precise levels of tRF5-Glu [89]. In this method, a specific TaqMan probe was designed to bind to the junction of the tRF and the 3' linker. Furthermore, with the binding of two primers, one at the 5' end of tRF5-Glu and the other at the 3' linker correspondingly, only ligated tRF5-Glu will be amplified and the fluorescence can then be measured to tell the exact amount of tRF5-Glu. However, one TaqMan probe can only be matched with one ligated tRF5-Glu. Given the multiple variants of tRF5-Glu, we were able to pick up the most prevalent variant based on our sequencing and cloning results, while it does not represent the total levels of each type of variant of tRF5-Glu. If a more accurate measurement of tRF5-Glu levels in cells is required, it would be the combination of multiple TaqMan probes targeting all possible tRF5-Glu variants or small RNA deep sequencing to identify all the variants, which could be time-consuming and costly. Thus, although ligation PCR has been developed to quantify the levels of tRFs, it requires more improvement to measure the total amount of tRFs.

Another challenge is the collection conditions for RNA extraction to study tRFs. In 2009, Fu et al. described that the cleavage of tRNA into tRFs is a stress-induced response [87]. tRFs may be generated in response to many stresses including starvation, such as PBS starvation as short as 30min, would induce the production of tRFs [87]. This raises a concern, if cells are not collected properly, whether tRFs measured by ligation

PCR and Northern blot represent the real-time amount in cells under different treatments. Unfortunately, there is a lack of details available about cell collection and RNA extraction in most studies of tRFs. In my study, we have used two different methods to collect cells for RNA extraction. One way is a 15min exposure to passive lysis buffer (Promega) incubation first, then add 700μl Qiazol (Qiagen) to save for RNA extraction; the other way is adding 700μl Qiazol to collect cells directly and then saving for RNA extraction. Two Northern blots of tRF5-Glu were shown in **Chapter 3** to represent the first method (**Figure 6B**) and the second method (**Figure 9D**). Even though there is detectable tRF5-Glu bands in both Northern blots, it appears that there is a cleaner background and higher amount of tRF5-Glu in the first Northern Blot where cells undergo 15min of lysis buffer treatment. It is likely that the gentle lysis buffer treatment mimics the starvation process and it somehow increases the amount of tRFs and then improves the sensitivity of the Northern blot. Interestingly, I have obtained significant results from both collection methods but more studies need to be done to identify the optimal way to collect cells for RNA extraction and tRF study.

In studies of the function of tRF5-Glu we used luciferase assay to study if there is direct regulation between tRF5-Glu and BCAR3 and we observed a 30-40% decrease for the relative luciferase activity (**Figure 10D**). Although significant, compared to the activity of miRNAs in luciferase assay where there is usually more than a 50% decrease, it indicates a potential difference in the RNA interference machinery between regulation by tRF and the mechanism of miRNA regulation. tRFs have been shown to be associated with different AGOs (Ago1-Ago4) [85], but it is possible that novel proteins may be

involved in this particular silencing complex, which needs to be further examined in future studies. Many questions remain about the regulatory mechanisms of tRFs and their role in the regulation of gene expression.

In terms of the effects of tRF5-Glu on ovarian cancer cell proliferation, we have shown that overexpression of tRF5-Glu inhibits ovarian cancer cell growth in the PEO4 and 2008 cell lines. However, the mechanism of regulation must be different because tRF5-Glu only regulates BCAR3 in PEO4 cells and BCAR3 is not regulated by tRF5-Glu in 2008 cells. We have confirmed that at least part of this difference is due to the reduction in the expression of the full length 3' UTR of BCAR3 in 2008 cells (**Figure 12**). The observance of reduced cell proliferation of 2008 cells treated by tRF5-Glu mimic indicates the possibility of multiple targets of tRF5-Glu in ovarian cancer cells. Multiple targets for tRFs is a reminiscent of the function of miRNAs and may be a similarity to other ncRNAs. Using the predicative software, TargetScan, and the methods describe in **Chapter 3**, further study of other potential targets regulated by tRF5-Glu is feasible. Future characterization of additional targets of tRF5-Glu will also provide new insight and advance the understanding of the novel functions of tRFs in cancer cells.

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