

# Human tRNA-Derived Small RNAs Modulate Host–Oral Microbial Interactions

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## Abstract

Coevolution of the human host and its associated microbiota has led to sophisticated interactions to maintain a delicate homeostasis. Emerging evidence suggests that in addition to small molecules, peptides, and proteins, small regulatory noncoding RNAs (sRNAs) might play an important role in cross-domain interactions. In this study, we revealed the presence of diverse host transfer RNA–derived small RNAs (tsRNAs) among human salivary sRNAs. We selected 2 tsRNAs (tsRNA-000794 and tsRNA-020498) for further study based on their high sequence similarity to specific tRNAs from a group of Gram-negative oral bacteria, including *Fusobacterium nucleatum*, a key oral commensal and opportunistic pathogen. We showed that the presence of *F. nucleatum* triggers exosome-mediated release of tsRNA-000794 and tsRNA-020498 by human normal oral keratinocyte cells. Furthermore, both tsRNA candidates exerted a growth inhibition effect on *F. nucleatum*, likely through interference with bacterial protein biosynthesis, but did not affect the growth of *Streptococcus mitis*, a health-associated oral Gram-positive bacterium whose genome does not carry sequences bearing high similarity to either tsRNA. Our data provide the first line of evidence for the modulatory role of host-derived tsRNAs in the microbial-host interaction.

**Keywords:** tsRNAs, cross-domain interactions, oral microbiome, antimicrobials, microbial-host interaction, sRNAs

## Introduction

The human microbiota exists in a delicate balance with its host, and this equilibrium is maintained via multiple mechanisms of cross-domain communication and modulation (Artis 2008; Peterson and Artis 2014). A variety of host-derived mobile molecules, including antimicrobial peptides, immunoglobulins, and the complex complement network, can serve as inter-domain modulators. These molecules often operate with other mechanisms, such as microbe-associated molecular pattern recognition systems, to maintain microbial-host homeostasis (Ganz 2003; Markiewski and Lambris 2007; Gallo and Hooper 2012; Chu and Mazmanian 2013).

Small regulatory noncoding RNAs (sRNAs) are a class of regulatory elements that have been identified in prokaryotes and eukaryotes and implicated in gene regulation (Shimoni et al. 2007; Waters and Storz 2009; Duran-Pinedo et al. 2015). In eukaryotic cells, sRNAs are RNA molecules that are 19 to 31 nucleotides in length and mainly classified per their biogenesis as microRNA, small interfering RNA, or PIWI-interacting RNA (piRNA; Carthew and Sontheimer 2009). These sRNAs were shown to be involved in the regulation of gene expression, and some of them were implicated in inflammation and cancer (Volinia et al. 2006; Tili et al. 2013), thus potentially serving as biomarkers for cancer screening and diagnosis. For example, while miR-371 acts as a tumor suppressor via regulating SLC7A11 in oral squamous cell carcinoma (Wu et al. 2017), oncogenic miR-155 promotes oral squamous cell carcinoma cell proliferation (Rather et al. 2013). Recent studies further revealed the potential role of sRNAs in interspecies and

cross-domain interactions (Knip et al. 2014; Weiberg et al. 2015). These “social RNAs” could serve as gene silencing and modulating molecules in recipient cells across domains, a phenomenon called *cross-domain RNAi* (Sarkies and Miska 2013).

A novel class of sRNA was recently described: transfer RNA (tRNA)–derived small sRNA (tsRNA; Lee et al. 2009). Depending on the size and mechanism of biogenesis, tsRNAs can be classified into 2 types: the 5′ and 3′ tRNA halves and the shorter, tRNA-derived fragments (tRFs; Dhahbi 2015). tsRNAs are present in most organisms: some are constitutively produced; others are exclusively generated in response to

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A supplemental appendix to this article is available online.

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certain stress conditions (Ivanov et al. 2011; Peng et al. 2012; Loss-Morais et al. 2013; Maute et al. 2013). Increasing evidence indicates that endogenous tsRNAs can function as modulators of gene expression in prokaryotic and eukaryotic organisms (Gebetsberger et al. 2012; Goodarzi et al. 2015).

Host-derived sRNAs, including tsRNAs, can also be found in human body fluids, including blood, tears, and saliva, either freely or contained within exosomes (Lasser et al. 2011; Williams et al. 2013). These secreted sRNAs have been extensively studied for diagnostic applications (Majem et al. 2015); however, their biological functions remain elusive. As a key component of host defense against oral infection, saliva contains innate antimicrobial proteins and adaptive immune mediators. Considering the complexity of commensal microbiota residing within oral cavity and the abundant host-derived sRNAs detected in saliva, it is intriguing to hypothesize that these sRNAs are potentially involved in interdomain interaction. In this study, we provide evidence that host-derived salivary tsRNAs may mediate microbial-host interaction through targeted modulation of oral bacterial growth.

## Materials and Methods

### Identification of Host-Derived Salivary tsRNAs and Determination of Homology to Bacterial tRNA

Salivary RNA sequences were initially trimmed for adapters and low-quality sequences. To identify host-derived salivary tsRNAs, salivary sRNA sequences were first mapped to the human genome (hg19) with Bowtie (version 1.1.2) and the commands “-v 2 -k 100 -best strata” (Langmead et al. 2009). Since tsRNAs are known to have 3' modifications, unmapped reads were subjected to trimming one 3' nucleotide and remapped with bowtie command “-v 1 -k 100 -best strata.” Trimming and remapping of the unmapped reads was performed 3 times to account for 3' CCA extensions found in the tRF 3 series. Mapped reads were overlapped with annotated human (hg19) tRF coordinates (<http://genome.bioch.virginia.edu/trfdb>). Valid overlaps were defined by requiring the mapped read alignment to start either 3 bases before or 2 bases after the tsRNA-annotated start site and ending 2 bases before or 3 bases after the tsRNA-annotated end site. Last, salivary tsRNAs were mapped to bacterial tRNA (<http://gtrnadb.ucsc.edu/>) to determine homology. Homology mapping was performed with a primary round of Bowtie with the previously described parameters and then a secondary round of Bowtie2 (version 2.2.9) on the unmapped reads with the local command to allow soft clipping (Langmead and Salzberg 2012).

### Growth Inhibition Assay

*Fusobacterium nucleatum* subsp. *nucleatum* 23726 and *Streptococcus mitis* ATCC 6249 were used in this study (henceforth, *F. nucleatum* and *S. mitis*). *F. nucleatum* and *S. mitis* were cultured in Columbia and brain-heart infusion broth,

respectively, and incubated at 37 °C under anaerobic condition (10% H<sub>2</sub>, 10% CO<sub>2</sub>, 80% N<sub>2</sub>) until exponential phase. Cells were collected by centrifugation and resuspended in fresh medium to obtain 10<sup>6</sup> CFU (colony-forming units) / mL. Meanwhile, 6 commercially synthesized (IDT, Inc.) sRNAs were serially diluted in medium with concentrations between 0 and 100 μM; 100 μL of bacterial suspension and sRNA solutions were mixed into individual wells of a 96-well plate. As control, sRNAs were pretreated with RNase A for 30 min before being added to the bacterial cultures. Plates were incubated at 37 °C under anaerobic conditions overnight. Bacterial growth was determined by measuring the optical density at 600 nm with a microplate reader.

To test the impact of tsRNAs on bacterial growth dynamics, *F. nucleatum* and *S. mitis* cells were cultured and collected as described earlier. Bacterial cells were seeded into their respective growth medium in 96-well plates containing 100 μM tsRNA-000794, tsRNA-020498, or control RNAs, including piRNA-0016792 and scrambled RNA (sequence provided in the Appendix Table), with a final 10<sup>6</sup> CFU/mL. Plates were incubated under anaerobic condition at 37 °C, and OD<sub>600</sub> was measured with a microplate reader at time intervals indicated in the results.

### Measurement of Protein Synthesis

The Click-iT AHA Alexa Fluor 488 Protein Synthesis HCS Assay (Invitrogen) was used to measure the protein synthesis in bacteria. This assay labels newly synthesized proteins with an analog of methionine that reacts to Alexa Fluor 488 alkyne by click chemistry.

*F. nucleatum* and *S. mitis* cells from the mid-exponential growth phase were used to inoculate fresh media containing RNase inhibitor (1 U/μL) and different concentrations of tsRNA-000794 or tsRNA-020498, with a final bacterial OD<sub>600</sub> of 0.05. Cultures were incubated for 24 h at 37 °C under anaerobic condition and transferred to chemically defined media containing 10 μM glucose with different concentrations of tsRNA and diluted (1:1,000) Click-iT AHA reagent. Bacteria were incubated in the anaerobic chamber at 37 °C for 30 min before being fixed with 75% ethanol for 25 min at room temperature. Fixed bacteria were labeled with Alexa Fluor 488 for 30 min according to the manufacturer's guidelines. Labeled cells were visualized with an inverted confocal microscope (Leica SPE I) equipped with a Leica ACS APO 100X Oil CS objective (NA:1.15).

All images were acquired with the same settings on the microscope and processed with standard FIJI analysis (Schindelin et al. 2012) to quantify the fluorescence intensity per unit area covered by bacterial cells. The regions of interest were selected arbitrarily. However, to ensure that the selected cells were representative of the overall population, we chose 3 to 5 representative images from each experiment (3 independent experiments in total). For each image, we picked >10 cells representing the overall cell population for further quantification.

## Monitoring the Abundance of Extracellular Exosome-Bound sRNAs in Human Normal Oral Keratinocyte Cells

The normal oral keratinocyte (NOKSI) cell line was a gift from Dr. Silvio Gutkind and tested negative for mycoplasma contamination. Cells were cultured with defined keratinocyte serum-free medium (Thermo Fisher Scientific). After cells reached >90% confluence, bacteria resuspended in keratinocyte serum-free medium were added to the NOKSI cell monolayer at a ratio of 100:1. Experiments were stopped at 2 and 4 h. The medium was separated from cells and exosome isolation performed with a differential centrifugation method previously described (Bonifacio et al. 2006) with modifications. Briefly, cells were removed by centrifugation at  $300 \times g$  for 10 min. To remove bacteria and dead NOKSI debris, the supernatant was centrifuged at  $3,000 \times g$  for 20 min, followed by centrifugation at  $17,000 \times g$  for 15 min. The supernatant was filtered through a 0.2- $\mu\text{m}$  filter, followed by ultracentrifugation at  $120,000 \times g$  for 90 min to pellet the exosomes. Isolated exosomes were lysed with QIAzol reagent (Qiagen) and store at  $-80^\circ\text{C}$ . The total exosomal RNA was isolated with the miRNeasy Micro kit (Qiagen).

The abundance of 6 tested sRNAs in the exosomes was measured with droplet digital polymerase chain reaction (ddPCR) with custom TaqMan small RNA assays ordered from Applied Biosystems. Total exosomal RNA was converted to cDNA as described previously. The TaqMan PCR reaction mixtures were assembled with 10  $\mu\text{L}$  of  $2 \times$  ddPCR Supermix (Bio-Rad), 4  $\mu\text{L}$  of cDNA template, 5  $\mu\text{L}$  of water and 1  $\mu\text{L}$  of custom  $20 \times$  TaqMan probes/primers specific for each assay. Droplet formation was carried out with a QX100 droplet generator. The droplets were transferred to a 96-well polymerase chain reaction plate and amplified to the endpoint with the following conditions:  $95^\circ\text{C}$  for 10 min, 40 cycles of  $94^\circ\text{C}$  for 30 s,  $60^\circ\text{C}$  for 1 min, and  $98^\circ\text{C}$  for 10 min at a ramp rate of  $2.5^\circ\text{C/s}$  for all steps. The samples were measured with a BioRad QX100 droplet reader. The fold changes were calculated per comparison with nonchallenged keratinocytes.

## Statistical Analysis

For all analysis, we used 1- or 2-way analysis of variance statistical tests for multiple group comparisons.

## Results

### Detection of Diverse Host-Derived tsRNAs in Cell-Free Saliva

Previously, we used RNA-seq to investigate cell-free human saliva and found that it contains abundant host-derived sRNAs (Bahn et al. 2015). In this study, we performed a more comprehensive bioinformatics analysis of this data set. From the 4 human subjects we previously studied, we identified a total of 68 distinct host-derived tsRNAs, which included 47 fragments derived from 5' ends of tRNA (69%) and 21 derived from 3'

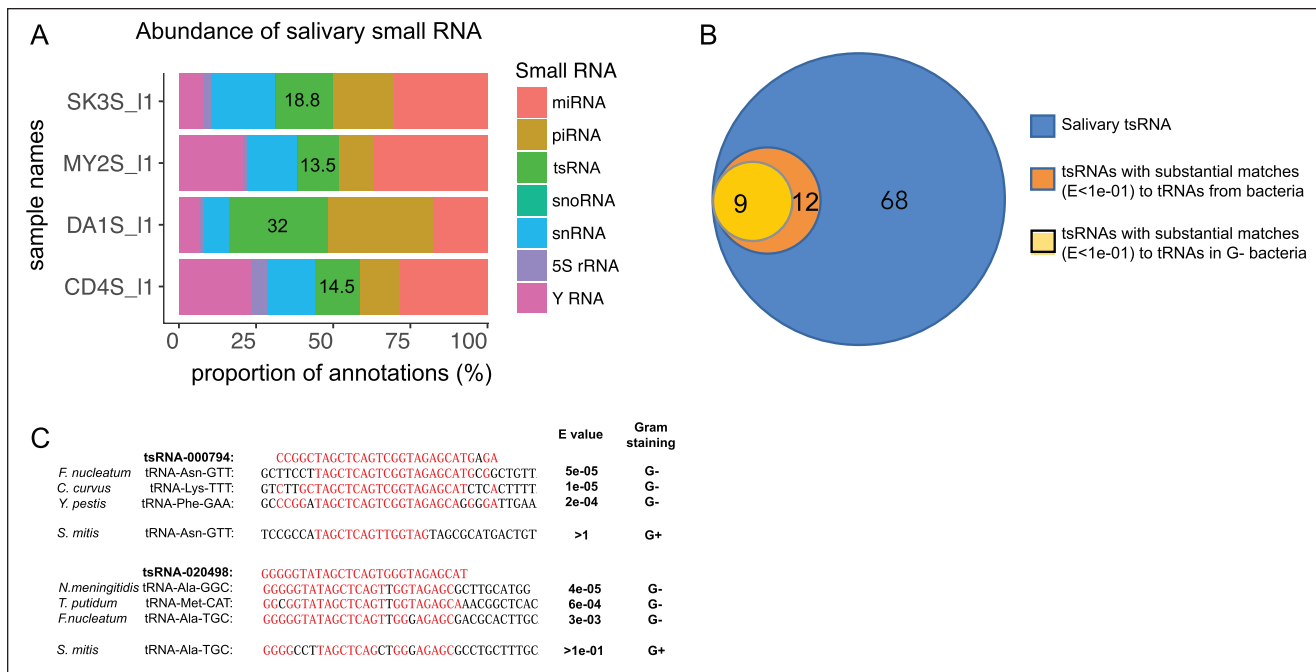
ends (31%), accounting for 13.5% to 32% of the total host-derived salivary sRNA (Appendix Table; Fig. 1A, B). Interestingly, when aligned against the Human Oral Microbiome Database (HOMD; <http://www.homd.org>), 12 of 68 (18%) host-derived tsRNAs substantially matched (E value  $<1e-01$ ) specific microbial tRNA partial sequences (Appendix Table; Fig. 1B). Furthermore, the majority (9 of 12) matched the partial sequence of tRNAs of Gram-negative oral bacteria, including known opportunistic pathogens such as *F. nucleatum*, *Neisseria meningitidis*, and *Treponema putidum* (Fig. 1C).

### tsRNA-000794 and tsRNA-020498 Inhibit *F. nucleatum* Growth

Evidence from both eukaryotic and prokaryotic organisms suggests that endogenous tsRNAs can modulate cellular growth (Yamasaki et al. 2009; Zhang et al. 2009). Furthermore, it was shown that Archaea-derived tsRNAs can inhibit bacterial and eukaryotic protein biosynthesis (Gebetsberger et al. 2016). We hypothesize that salivary host-derived tsRNAs, particularly those with high sequence similarity to bacterial tRNAs, may play an important role in mediating bacterial-host interactions.

To test this hypothesis and provide proof-of-concept data, we selected 2 host-derived 5' tRNA halves, tsRNA-000794 and tsRNA-020498, for further analysis based on their high sequence similarity to tRNAs from a group of Gram-negative oral bacteria, which includes *F. nucleatum*, a key oral opportunistic pathogen (Fig. 1C). As controls, we included 2 other tsRNAs that did not have significant sequence matches (E value  $>1$ ) to the microbial tRNA database (<http://gtrnadb.ucsc.edu>) or HOMD: tsRNA-016926 and tsRNA-018570. In addition, 2 host-generated salivary piRNAs were included as controls—piRNA-016792 and piRNA-006465—which displayed no significant sequence similarity to bacterial genome sequences in HOMD. Different concentrations of these 6 commercially synthesized sRNAs (IDT, Inc.) were added to cultures of either *F. nucleatum* or the health-associated Gram-positive oral bacterium *S. mitis*, the tRNAs and genomes of which do not have sequences with high-similarity matches to any of the tested sRNA (E value  $>1e-01$ ). The overnight growth of the differentially treated bacterial cultures was recorded. The results showed that while the 4 control sRNAs had no noticeable effect on the growth of *F. nucleatum*, tsRNA-000794 and tsRNA-020498 clearly inhibited the growth of *F. nucleatum* in a dose-dependent manner (Fig. 2A). At 50  $\mu\text{M}$ , tsRNA-000794 and tsRNA-020498 displayed  $>50\%$  growth inhibition. Furthermore, the inhibition was abolished when tsRNAs were pretreated with RNase A (Fig. 2B). In contrast, *S. mitis* was not sensitive to any of the sRNAs tested (Appendix Fig., Appendix Table). These results support our hypothesis that host-derived tsRNAs may be involved in modulating microbial-host interaction. The data also suggest that a certain level of sequence similarity is required between the tsRNAs and the target species.

We further tested the effect of tsRNA on the growth dynamics of bacteria. As shown in Figure 2C, the growth dynamic of *F. nucleatum* was severely negatively affected by the addition



**Figure 1.** Salivary host-derived tsRNAs. **(A)** Relative abundance of host-derived tsRNAs in total sRNA reads among 4 subjects (SK3S\_I1, MY2S\_I2, DA1S\_I1, and CD4S\_I1). **(B)** A subset of host-derived tsRNAs have substantial matches to bacteria, particularly Gram-negative oral bacterial tRNA sequences. **(C)** Alignment of tsRNA-000794 and tsRNA-020498 with partial sequences of specific tRNAs from oral Gram-negative bacterial species. Letters in red indicate the nucleotides conserved between or among tsRNA and partial tRNA sequences from selected oral bacterial species. sRNA, small regulatory noncoding RNA; tRNA, transfer RNA; tsRNA, tRNA-derived small sRNA.

of 100  $\mu$ M tsRNA-000794 or tsRNA-020498, while addition of piRNA-0016792 or scrambled control RNA not only failed to inhibit the growth of *F. nucleatum* but resulted in higher cell density at a late log phase as compared with the negative control. Meanwhile, the addition of all the tested RNAs did not exert a negative impact on the growth dynamic of *S. mitis* (Fig. 2D). Interestingly, in the presence of scrambled RNA, *S. mitis* culture reached even higher optical density when cells entered the stationary phase as compared with the nonaddition negative control, a phenomenon worth further investigation in the future.

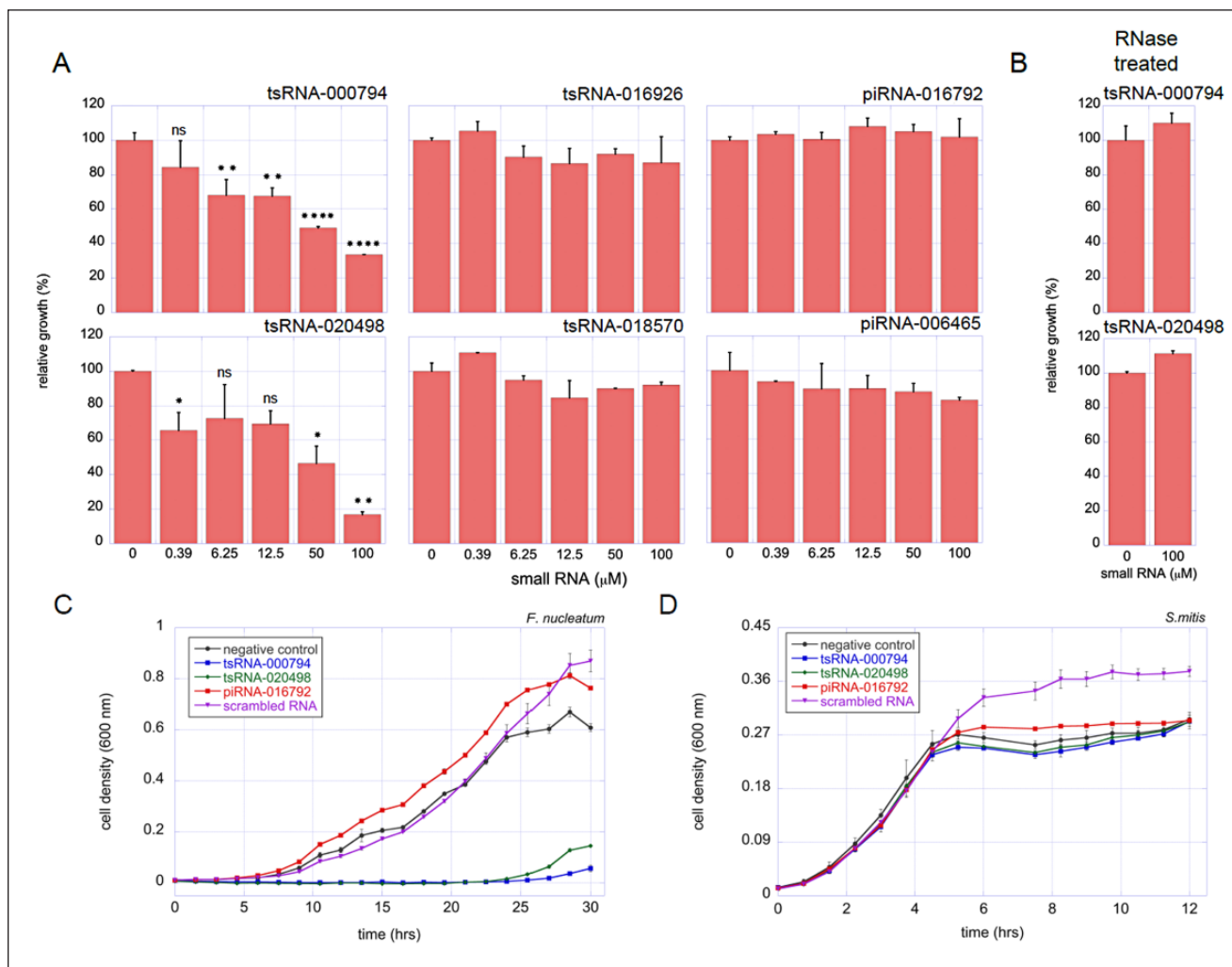
### tsRNA-000794 and tsRNA-020498 Inhibit *F. nucleatum* Protein Biosynthesis

Endogenous tsRNAs are capable of globally downregulating protein synthesis in eukaryotic and prokaryotic organisms (Yamasaki et al. 2009; Zhang et al. 2009; Gebetsberger et al. 2012). To test if the observed tsRNA-induced growth inhibition was a result of reduced protein biosynthesis, we monitored the amount of global protein synthesis in *F. nucleatum* with the modified Click-iT AHA Alexa Fluor 488 assay. The results showed that the addition of tsRNA-000794 and tsRNA-020498 at 100  $\mu$ M resulted in a drastic reduction in the incorporation of the methionine analog, as reflected by significantly reduced fluorescence signal intensity (Fig. 3A, B). At 100  $\mu$ M, treatment with either of the tsRNAs led to a similar level of reduction in fluorescence intensity per area unit covered by bacterial

cells as compared with the samples treated with 25  $\mu$ g/mL of kanamycin (Fig. 3C), while more drastic fluorescence reduction was observed for cultures treated with higher concentrations of kanamycin (50 and 100  $\mu$ g/mL). Conversely, treatment of *S. mitis* with tsRNA-000794 or tsRNA-020498 did not affect the fluorescence intensity as compared with the negative control (Fig. 3A, B), which is consistent with the lack of an observed effect on growth (Appendix Fig.).

### *F. nucleatum* Induces Release of Exosome-Associated tsRNA-000794 and tsRNA-020498 by Human NOKSI Cells

We next investigated if tsRNA-000794 and tsRNA-020498 secretion can be induced by *F. nucleatum* and if the tsRNAs are secreted via exosomes. We designed a coculture system in which NOKSI cells were challenged with *F. nucleatum*. NOKSI cells were cocultured with bacteria at a multiplicity of infection of 100 and harvested after 2 and 4 h. The secreted extracellular exosomes were extracted, and the absolute copy number of the sRNAs within the exosomes was measured with ddPCR. Results showed that a significant increase ( $P < 0.05$ ) in tsRNA-000794 and tsRNA-020498 was detected within exosomes when cells were challenged with *F. nucleatum*, especially at the 4-h time point (Fig. 4A). No significant change was observed for control tsRNAs (Fig. 4A). Furthermore, when NOKSI cells were challenged with *S. mitis*, there was no increase detected for any of the tested sRNAs in the secreted



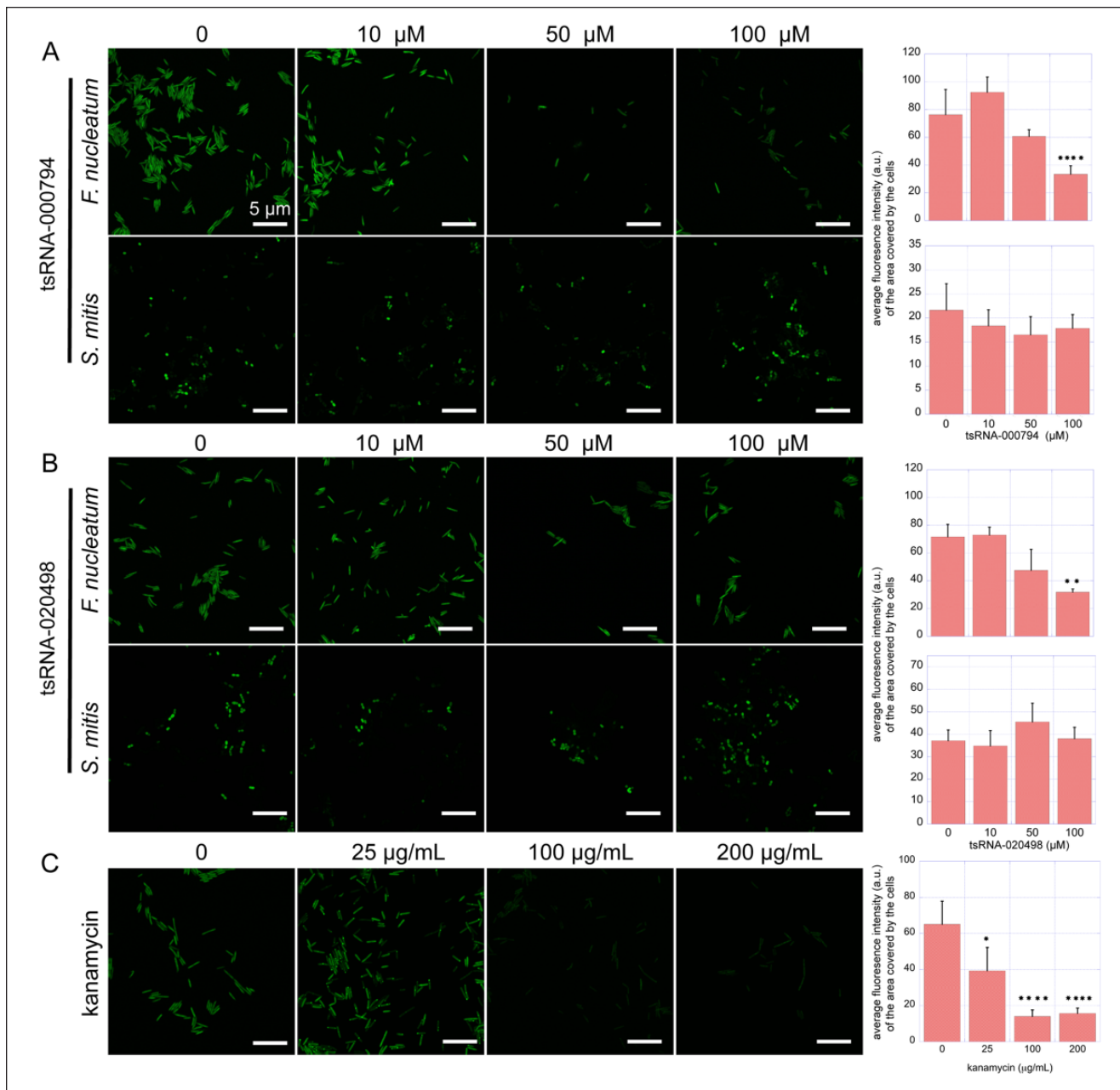
**Figure 2.** tsRNA-000794 and tsRNA-020498 induced growth inhibition in *Fusobacterium nucleatum*. **(A)** Different concentrations of tsRNA-000794, tsRNA-020498, tsRNA-016926, tsRNA-018570, piRNA-016792, and piRNA-006465 were added to *F. nucleatum* cultures. Cultures were incubated overnight at 37 °C under anaerobic condition before OD<sub>600</sub> was measured. The growth inhibition is expressed as percentage of absorbance to that of the negative control (no addition of tRF). **(B)** tsRNA-000794 and tsRNA-020498 were pretreated with RNase A before being added to the *F. nucleatum* culture, and impact on bacterial growth was similarly monitored. tsRNA-000794 (100 μM), tsRNA-020498 (100 μM), piRNA-016792 (100 μM), or scrambled control RNA (100 μM) was added to *F. nucleatum* **(C)** and *Streptococcus mitis* **(D)**, and the growth curves were measured. Assays were performed in triplicates, and mean ± SEM values are shown. ns,  $P > 0.05$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ . All the columns without “ns” or stars are nonsignificant according to statistical tests. piRNA, PIWI-interacting RNA; tsRNA, transfer RNA-derived small sRNA; tRF, tRNA-derived fragment.

exosome (Fig. 4B). These data suggest that oral keratinocytes can respond to the presence of specific oral bacteria by releasing specific tsRNAs. Interestingly, exosome-associated tsRNA-020498 level was decreased at the 4-h time point when cells were challenged with *S. mitis* as compared with nonchallenged control, a phenomenon worth further study.

## Discussion

tsRNAs have been reported as endogenous modulators of gene expression in prokaryotic and eukaryotic organisms (Gebetsberger et al. 2012; Godarzi et al. 2015). A recent study also revealed the potential role of bacteria-generated tsRNAs in modulating the host immune response (Koeppen et al. 2016). Our data strongly suggested that host-derived tsRNAs, particularly

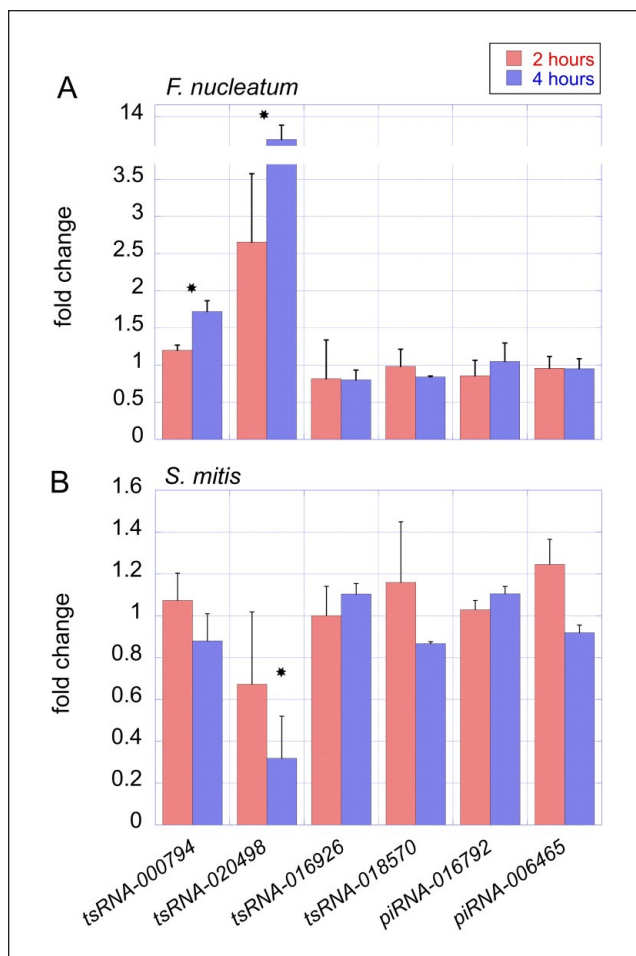
those displaying strong sequence similarity to bacterial tRNAs, might serve as interdomain mediators to regulate the growth of host-associated bacteria. Various mechanistic models have been proposed to explain the role of endogenous tsRNAs in modulating gene expression, such as interacting with Argonaute proteins and silencing expression of target mRNAs (Shigematsu and Kirino 2015) or negatively affecting global protein biosynthesis via initiation (Ivanov et al. 2011; Gebetsberger et al. 2016) and elongation (Gebetsberger et al. 2012). Sobala and Hutvagner (2013) recently demonstrated that selected 5'-tRNA-derived sRNAs inhibit translation of an mRNA reporter in vitro, possibly by affecting translation elongation, which differs from the inhibition of translation initiation caused by 5'-tiRNAs. Similarly, in *Haloflex volcanii*, an archaeal species, a stress-induced 26-nt 5'-tRNA fragment derived from



**Figure 3.** tsRNA-000794 and tsRNA-020498 affect protein synthesis in *Fusobacterium nucleatum*. The effect of the addition of tsRNA-000794 and tsRNA-020498 on protein synthesis in *F. nucleatum* and *Streptococcus mitis* (A and B) and the effect of kanamycin on protein synthesis in *F. nucleatum* (C). The left panels show representative fluorescence images of differentially treated cultures. The right panel shows the quantification of fluorescence intensity per area unit covered by bacterial cells. au, arbitrary units. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ . All the columns with no “ns” or stars are nonsignificant according to statistical tests. tsRNA, transfer RNA–derived small sRNA.

tRNA<sup>val</sup> inhibits translation by directly binding to the 30S small ribosomal subunit and inhibiting peptidyl transferase activity (Gebetsberger et al. 2012). Furthermore, these Archaea-derived tsRNAs can also inhibit eukaryal and bacterial protein biosynthesis, suggesting a role in mediating cross-domain interaction, as well as a functionally conserved mode of action (Gebetsberger et al. 2016). Our data are in line with these findings, as we observed tsRNA-induced inhibition of *F. nucleatum* growth and showed that it may directly result from

tsRNA-mediated protein biosynthesis inhibition. However, we could not completely rule out the possibility that the observed protein biosynthesis reduction may be a consequence of tsRNA-induced decreased bacterial growth. Further studies are required to investigate the detailed mechanism. In our in vitro system, the amount of exogenously added tsRNAs is likely higher than physiologic concentrations. However, our data suggest that in the oral cavity these host-derived tsRNAs are likely packaged in exosomes, which would allow them to be



**Figure 4.** *Fusobacterium nucleatum* specifically induces the secretion of tsRNA-000794 and tsRNA-020498 from human oral keratinocytes. Human oral keratinocyte NOKSI cells were cocultured with *F. nucleatum* or *Streptococcus mitis* at a multiplicity of infection of 100. **(A)** The level of exosome-associated sRNAs in NOKSI medium when cells were challenged with *F. nucleatum* was measured by ddPCR. The fold changes were calculated per the comparison with nonchallenged keratinocytes. **(B)** The levels of exosome-associated sRNAs in NOKSI medium when cells were challenged with *S. mitis* were measured by ddPCR. The fold changes were calculated per the comparison with nonchallenged keratinocytes. Assays were performed in triplicates, and mean  $\pm$  SEM values are shown. \* $P < 0.05$ . All the columns with no “ns” or stars are nonsignificant according to statistical tests. ddPCR, droplet digital polymerase chain reaction; NOKSI, normal oral keratinocyte; sRNA, small regulatory noncoding RNA; tsRNA, transfer RNA-derived small sRNA.

delivered to the targeted bacteria in small but concentrated doses. Additional studies with better in vitro systems are needed to better understand the details of the molecular mechanism.

An intriguing finding is that the majority of the bacterial tRNA sequences that show high sequence similarity with the host-derived salivary tsRNAs are from Gram-negative bacteria (Fig. 1B). Within the oral cavity, the gingival sulcus is one of the sites of the most intense microbial-host interactions; a dysbiosis at this site can eventually lead to periodontitis (Teles et al. 2013; Costalonga and Herzberg 2014). Therefore,

homeostatic host-bacterial interactions at the gingival sulcus are crucial for health. One of the hallmarks of periodontitis is a shift in the community from mostly Gram-positive to mostly Gram-negative species, which contain highly toxic and immunogenic lipopolysaccharide and other virulence factors (Darveau 2010). tsRNAs may represent a mechanism evolved in the host to suppress the growth of Gram-negative bacteria, either commensal opportunistic pathogens (e.g., *F. nucleatum*) or possibly exogenous pathogens (e.g., *Yersinia pestis*; Appendix Table) to maintain health, an intriguing hypothesis that warrants further testing.

More investigation is required to fully understand the mechanisms underlying the observed microbial-host interactions mediated by tsRNAs. Unanswered questions include how specific tsRNAs are induced and selected for transport in the presence of bacteria, how tsRNAs are transported outside of the cells, and the mechanism of target cell recognition. Understanding the mechanism by which tsRNAs affect the physiology of bacterial cells, either triggering the event by entering the target cells or remaining extracellularly, and determining if this is unique to the oral cavity or is more universal among microbiome-host interactions are important fundamental questions requiring further exploration. Nevertheless, our study expands the repertoire of known molecules that can act endogenously to modulate gene expression as well as exogenously, in this case as mediators of microbial-host homeostasis.

### Author Contributions

X. He, contributed to design, data analysis, and interpretation, drafted and critically revised the manuscript; F. Li, contributed to design, data acquisition, analysis, and interpretation, critically revised the manuscript; B. Bor, K. Koyano, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; L. Cen, contributed to data acquisition and analysis, critically revised the manuscript; X. Xiao, contributed to design, data analysis, and interpretation, critically revised the manuscript; W. Shi, D.T.W. Wong, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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