Bioengineering Novel Chimeric microRNA-34a for Prodrug Cancer Therapy: High-Yield Expression and Purification, and Structural and Functional Characterization[§]

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ABSTRACT

Development of anticancer treatments based on microRNA (miRNA/miR) such as miR-34a replacement therapy is limited to the use of synthetic RNAs with artificial modifications. Herein, we present a new approach to a high-yield and large-scale biosynthesis, in *Escherichia coli* using transfer RNA (tRNA) scaffold, of chimeric miR-34a agent, which may act as a prodrug for anticancer therapy. The recombinant tRNA fusion pre-miR-34a (tRNA/mir-34a) was quickly purified to a high degree of homogeneity (>98%) using anion-exchange fast protein liquid chromatography, whose primary sequence and post-transcriptional modifications were directly characterized by mass spectrometric analyses. Chimeric tRNA/mir-34a showed a favorable cellular stability while it was degradable by several ribonucleases. Deep sequencing and quantitative real-time polymerase chain reaction studies revealed that tRNA-carried pre-miR-34a was precisely processed to mature miR-34a within human carcinoma

cells, and the same tRNA fragments were produced from tRNA/mir-34a and the control tRNA scaffold (tRNA/MSA). Consequently, tRNA/mir-34a inhibited the proliferation of various types of human carcinoma cells in a dose-dependent manner and to a much greater degree than the control tRNA/MSA, which was mechanistically attributable to the reduction of miR-34a target genes. Furthermore, tRNA/mir-34a significantly suppressed the growth of human non-small-cell lung cancer A549 and hepatocarcinoma HepG2 xenograft tumors in mice, compared with the same dose of tRNA/MSA. In addition, recombinant tRNA/mir-34a had no or minimal effect on blood chemistry and interleukin-6 level in mouse models, suggesting that recombinant RNAs were well tolerated. These findings provoke a conversation on producing biologic miRNAs to perform miRNA actions, and point toward a new direction in developing miRNA-based therapies.

Introduction

MicroRNAs are integrated into a large family of genomically encoded noncoding RNAs (ncRNAs) and play a critical role in controlling cancer cell proliferation, apoptosis and invasion, and tumor initiation and progression (Kasinski and

Slack, 2011; Bader, 2012), as well as drug disposition (Yu, 2009; Ingelman-Sundberg et al., 2013) and pathogenesis of other diseases (Yao and Li, 2015). MicroRNA (miRNA or miR) biologic functions contribute to the development of novel anticancer treatments, and several miRNA-based therapies are under or moving toward clinical trials. In particular, oncogenic miRNAs (e.g., miR-10b) are upregulated in cancer cells and may be targeted to achieve the control of cancer cell proliferation and tumor growth (Ma et al., 2007). Furthermore, tumor suppressive miRNAs (e.g., miR-34a) are showing a loss-of-function in cancerous tissues and may be reintroduced into cancer cells to suppress tumor progression (He

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ABBREVIATIONS: CDK6, cyclin-dependent kinase 6; EU, endotoxin units; FPLC, fast protein liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; IL-6, interleukin-6; LAL, Limulus amoebocyte lysate; LPS, lipopolysaccharide; miR or miRNA, microRNA; mir, pre-miRNA; ncRNA, noncoding RNA; pre-miR, pre-microRNA; qRT-PCR, quantitative real-time polymerase chain reaction; SIRT1, sirtuin-1; tRNA, transfer RNA; tRF, tRNA fragment; tRNA/mir, tRNA fusion pre-miRNA; tRNA/mir-34a, tRNA fusion pre-miR-34a; tRNA/MSA, Sephadex aptamer tagged methionyl-tRNA.

et al., 2007; Welch et al., 2007). The later approach, namely “miRNA replacement therapy,” is distinguished from the former miRNA antagonism strategy. The miRNAs or pre-miRNAs used in miRNA replacement therapy have the same sequences as genomically-encoded miRNAs or pre-miRNAs, and therefore are unlikely to produce “off-target” effects. Because miRNAs are normal constituents of healthy cells, reintroduction of therapeutic miRNAs is unlikely to cause major toxicity (Bader, 2012).

Human miR-34a is one of the most promising tumor suppressive miRNAs for cancer treatment. Loss of miR-34a expression has been documented in various tumors, including lung, prostate, breast, pancreas, liver, colon, kidney, bladder, skin, esophagus, brain, cervix, ovary, urothelium, and lymphoid systems (see review in Bader, 2012). The biogenesis of miR-34a is directly controlled by tumor protein p53 at the transcriptional level (Chang et al., 2007; He et al., 2007), and an ectopic expression of miR-34a leads to a dramatic reprogramming of target genes, such as cyclin-dependent kinase 6 (CDK6), hepatocyte growth factor receptor MET, platelet-derived growth factor receptor- α (*PDGFR α*), and GTPase KRAS, and consequently inhibits cancer cell proliferation, induces cell cycle arrest, and enhances apoptosis (Chang et al., 2007; He et al., 2007; Sun et al., 2008; Yamakuchi et al., 2008; Li et al., 2009; Kasinski and Slack, 2012). Meanwhile, miR-34a can stimulate endogenous p53 activity in a positive feedback-loop by targeting the NAD-dependent deacetylase sirtuin-1 (SIRT1) that deactivates p53, and the transcriptional repressor Yin Yang 1 (YY1) that binds to p53 and promotes p53 ubiquitination and degradation (Yamakuchi et al., 2008). Moreover, miR-34a suppresses a clonogenic expansion, tumor regeneration, and metastasis through targeting CD44 and cancer stem cells or tumor-initiating cells (Liu et al., 2011). The anticancer activity of miR-34a has been nicely demonstrated in various human cancer cells in vitro, including lung, liver, pancreas, colon, brain, skin, prostate, bone, ovary, as well as lymphoma and leukemia. Although the performance of miR-34a replacement therapy in animal models depends heavily on the delivery system, a number of successful examples have illustrated the effectiveness of miR-34a in inhibiting progression of many types of xenograft tumors, including non-small-cell lung cancer (Wiggins et al., 2010; Kasinski and Slack, 2012), prostate cancer (Liu et al., 2011), pancreatic cancer (Pramanik et al., 2011), and lymphomas (Craig et al., 2012). As a result, MRX34 (Mirna Therapeutics, Austin, TX), a liposome-formulated miR-34a, has entered phase I clinical trials for the treatment of unresectable primary liver cancer (Kelnar et al., 2014).

Nevertheless, research on miRNA pharmacology and therapeutics rely primarily on the use of synthetic RNAs [e.g., miRNA-mimics and antagomirs, and pre-miRNAs (mir)] and recombinant DNA agents (e. g., viral or nonviral vector-based miRNA or “decoy” antisense RNA expression plasmids). The use of DNA materials may complicate the RNA-based processes, and this approach also relies on the host cells or organisms to transcribe the gene to miRNA precursors before the generation of mature miRNAs. Although organic synthesis of oligonucleotides may be automated, a multimilligram dose of 22-nt double-stranded miRNA-mimics projected for in vivo testing or therapy is very costly. In addition, it is unknown how artificial modifications may alter the folding, biologic activities, and safety profiles, even though synthetic

miRNAs exhibit some favorable pharmacokinetic properties, such as a longer half-life.

Motivated by the idea of deploying biologic RNAs to perform RNA actions for pharmacotherapy, we aimed to bioengineer pre-miRNA agents in common strains of bacteria on a large scale using the transfer RNA (tRNA) scaffold (Ponchon and Dardel, 2007; Ponchon et al., 2009; Nelissen et al., 2012). We hypothesized that the tRNA fusion pre-miRNA (tRNA/mir) could act as a prodrug where pre-miRNA might be selectively processed to mature miRNA in human cells, and tRNA scaffold would be metabolized or degraded to tRNA fragments (tRFs). In contrast to a low-yield production of pre-miR-27b (Li et al., 2014), we present herein an optimal expression and rapid purification of multimilligrams of tRNA fusion pre-miR-34a (tRNA/mir-34a) from 1 liter of bacterial culture in a research laboratory setting. The molecular weight, primary sequence, and post-transcriptional modifications of recombinant tRNA/mir-34a were directly characterized by mass spectrometry studies. Furthermore, unbiased deep-sequencing study and targeted quantitative real-time polymerase chain reaction (qRT-PCR) analyses showed that tRNA-carried pre-miR-34a was indeed processed precisely into mature miR-34a in human carcinoma cells, leading to a 70- to 100-fold increase in cellular miR-34a levels. Consequently, tRNA/mir-34a significantly suppressed the protein levels of miR-34a target genes (e.g., CDK6, SIRT1, and MET) and proliferation of human lung (A549 and H460) and liver (HepG2 and Huh-7) cancer cells in vitro in a dose-dependent manner, compared with the control Sephadex aptamer tagged methionyl-tRNA scaffold (tRNA/MSA). In addition, we demonstrated that recombinant tRNA/mir-34a was well tolerated in animal models, and remarkably repressed A549 and HepG2 xenograft tumor growth in vivo. These findings indicate that biologic RNA agents engineered in bacteria may serve as a new category of RNA agents for drug discovery as well as basic and translational research.

Materials and Methods

Lipofectamine 2000, Trizol reagent, and BCA Protein Assay Kit were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Radioimmunoprecipitation assay lysis buffer was bought from Rockland Immunochemicals (Limerick, PA), and the complete protease inhibitor cocktail was purchased from Roche Diagnostics (Mannheim, Germany). The antibodies against CDK6 (C-21), SIRT1 (H-300), Met (C-28), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Dallas, TX), and peroxidase goat anti-rabbit IgG was from Jackson ImmunoResearch (West Grove, PA). Enhanced chemiluminescent substrate and polyvinylidene fluoride membranes were bought from Bio-Rad (Hercules, CA). All other chemicals and organic solvents of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

Bacterial Culture. All *Escherichia coli* strains were cultured at 37°C in LB broth supplemented with 100 μ g/ml ampicillin. DH5 α and TOP10 (Life Technologies, Grand Island, NY) were used for cloning as well as screening for recombinant ncRNA expression. BL21 (Sigma-Aldrich) and HST08 (Clontech Laboratories, Mountain View, CA) were also used to screen ncRNA accumulation. HST08 was identified and used for large scale production of recombinant ncRNAs.

Human Cell Culture. The human carcinoma cell lines HepG2, Huh-7, A549, and H460 were purchased from American Type Culture

Collection (Manassas, VA). HepG2 cells were cultured in Eagle's minimal essential medium, A549, and H460 cells in RPMI 1640 medium, and Huh-7 cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco/Life Technologies), at 37°C in a humidified atmosphere containing 5% CO₂. Cells in the logarithmic growth phase were used for experiments.

Prediction of RNA Secondary Structure. The secondary structures of various sizes of human pre-miR-34a, tRNA scaffold, and the chimeric ncRNAs were predicted using CentroidFold (<http://www.ncrna.org/centroidfold>), CentroidHomfold (<http://www.ncrna.org/centroidhomfold>), and RNAstructure (<http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html>).

Construction of tRNA/mir-34a Expression Plasmids. The DNA fragments encoding 112-nt and 129-nt human pre-miR-34a (miRBase ID: MI0000268) were amplified from human genomic DNA by PCR using the primers 5'-AGT AAT TTA CGT CGA CGG CCA GCT GTG AGT GTT TCT TTT G-3' and 5'-CGG CCG CAA CCA TCG ACG TCT GGG CCC CAC AAC GTG CAG CAC TT-3', and 5'-AGT AAT TTA CGT CGA CGT GGA CCG GCC AGC TGT GAG TGT T-3' and 5'-CGG CCG CAA CCA TCG ACG TCA TCT TCT CTC TTT GGC CCC ACA ACG-3' (IDT, Coralville, IA), respectively. The amplicons were inserted into the pBSMrnaSeph vector (kindly provided by Dr. Luc Ponchon, Université Paris Descartes; Ponchon et al., 2009) linearized with SalI-HF and AatII (New England Biolabs, Ipswich, MA). Target tRNA/mir-34a expression plasmids were confirmed by sequencing analyses at UC Davis Genome Center.

Expression of Recombinant ncRNAs in *E. coli*. Recombinant ncRNAs were expressed in HST08 as described (Ponchon and Dardel, 2007; Ponchon et al., 2009; Li et al., 2014). Total RNAs were isolated using the Tris-HCl-saturated phenol extraction method, quantitated with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and analyzed by denaturing urea (8 M) PAGE (8%) to assess the expression of recombinant ncRNAs. We usually loaded 0.2–1.0 µg RNAs per lane for the urea-PAGE analysis. The single-stranded RNA ladder and small interfering RNA marker were purchased from New England Biolabs. Images were acquired with a ChemiDo MP Imaging System (Bio-Rad), and intensities of bands were used to provide a rough estimation of relative levels of recombinant ncRNAs present in the total RNAs.

Affinity Purification of Recombinant ncRNAs. Purification of Sephadex aptamer-tagged ncRNAs using Sephadex G-100 beads (Sigma-Aldrich) was conducted as reported (Ponchon et al., 2009; Li et al., 2015), and RNA fractions were analyzed by urea-PAGE.

Fast Protein Liquid Chromatography Purification of Recombinant ncRNAs. Recombinant tRNA/mir-34a was purified from total RNAs on a UNO Q6 anion-exchange column (Bio-Rad) using a NGC Quest 10 Plus Chromatography fast protein liquid chromatography (FPLC) system (Bio-Rad). After the samples were loaded, the column was first equilibrated with buffer A (10 mM sodium phosphate, pH 7.0) at a flow rate 6.0 ml/min for 0.5 minutes, followed by a gradient elution at the same flow rate, 0–56% buffer B (buffer A + 1 M sodium chloride) in 0.5 minute, 56% buffer B for 2 minutes, 56–65% buffer B in 10 minutes, and then 100% buffer B for 2 minutes, 100–0% buffer B in 0.5 minutes, and 100% buffer A for 5 minutes. The salt gradient elution condition for the control tRNA/MSA was essentially the same as reported (Li et al., 2014). FPLC traces were monitored at 260 nm using a UV/Vis detector. Peak areas were also used to estimate the relative levels of recombinant ncRNAs within the total RNAs, which were consistent with those determined by urea-PAGE analyses. After being analyzed by urea-PAGE, the fractions containing pure ncRNAs were pooled. Recombinant ncRNAs were precipitated with ethanol, reconstituted with nuclease-free water, and then desalted and concentrated with Amicon Ultra Centrifugal Filters (2-ml, 30 kDa; EMD Millipore, Billerica, MA). The quantity of ncRNAs was determined using a NanoDrop 2000 spectrophotometer. The quality was validated by PAGE and high-performance liquid chromatography (HPLC) analysis before other experiments.

HPLC Analysis of Purified ncRNAs. HPLC analysis was conducted using an XBridge OST C₁₈ column (2.1 × 50 mm, 2.5-µm particle size; Waters, Milford, MA) on a Shimadzu LC-20AD HPLC system. The flow rate was 0.2 ml/min, and the column was maintained at 60°C. Mobile phase A consisted of 8.6 mM TEA and 100 mM hexafluoroisopropanol (pH 8.3) in water, and mobile phase B consisted of 8.6 mM TEA and 100 mM hexafluoroisopropanol in methanol. The LC gradient was as follows: 0–1 minute, 16% mobile phase B; 21 minutes, 22% mobile phase B. RNA was monitored at 260 nm using a photodiode array detector.

Removal and Detection of Endotoxin. Endotoxin was further removed from FPLC-purified ncRNAs using the CleanAll DNA/RNA Clean-up Kit (Norgen Biotek, Thorold, ON, Canada) and Endotoxin-free Water (Lonza, Walkersville, MD), as instructed by the manufacturer. Endotoxin activities in total RNAs as well as the FPLC-purified and CleanAll Kit-processed ncRNA samples were determined using the Pyrogen-5000 kinetic Limulus amoebocyte lysate (LAL; Lonza) assay by following the instructions. In particular, a SpectraMax3 plate reader (Molecular Devices, Sunnyvale, CA) was used to measure turbidity at a 340-nm wavelength. Provided endotoxin standards were used to generate a standard curve, and endotoxin levels in RNA samples were expressed in endotoxin units (EU)/µg RNA.

Electrospray Ionization–Mass Spectrometry Analysis of Intact Recombinant ncRNAs. The procedures described by Taucher and Breuker (2010) were followed. The instrumental settings were optimized by automatic tuning with poly-d(T)₈₀. The mass spectra were acquired in negative ion mode using a Thermo LTQ XL ion trap mass spectrometer over an *m/z* range of 600–2000. Electrospray ionization mass spectra of intact ncRNAs were deconvoluted using ProMass software for Xcalibur (version 2.8 rev. 2) (Novatia, Newtown, PA) to determine the average molecular weights of recombinant RNAs.

Nucleoside Analysis by Liquid Chromatography Coupled with UV and Mass Spectrometry Detection. The hydrolysates were prepared with Nuclease P1 (Sigma-Aldrich), snake venom phosphodiesterase (Worthington Biochemicals Lakewood, Lakewood, NJ), and Antarctic Phosphatase (New England Biolabs), resolved on a 5-µm, 2.1 mm × 250 mm Supelcosil LC-18-S column using a Hitachi D-7000 HPLC system, and analyzed by a diode array detector and a Thermo LTQ-XL ion trap mass spectrometer, as described by Russell and Limbach (2013).

RNA Mapping by Liquid Chromatography–Tandem Mass Spectroscopy. RNA mapping and assignment of modifications using a Thermo Surveyor HPLC system coupled to a Thermo LTQ XL ion trap mass spectrometer and a Thermo Micro AS autosampler after the digestion with RNase T1 (Roche, Indianapolis, IN) and bacterial alkaline phosphatase (Worthington Biochemical Corporation) were carried out as described (Krivos et al., 2011). Collision-induced dissociation tandem mass spectrometry was used to obtain sequence information from the RNase digestion products.

Susceptibility to RNases. Recombinant ncRNAs were digested by individual RNases in provided buffer or HEPES (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.4) at 37°C for 1 hour. In particular, 12 µg ncRNAs were incubated with 1 µg/ml human recombinant RNase I (Novoprotein, Summit, NJ), 12 µg ncRNAs with 10 µg/ml Human Recombinant Angiogenin (R&D Systems, Minneapolis, MN), 1 µg ncRNAs with 1 IU of recombinant Dicer (Genlantis, San Diego, CA), 6 µg ncRNAs with 5 IU of bacterial RNase III (Life Technologies), and 5 µg ncRNAs with 5 IU of RNase R (Epicentre, Madison, WI). Likewise, 4 µg RNA was formulated with 0.64 µl in vivo-jetPEI (Polyplus Transfection, New York, NY) delivery agent to a 5% final glucose concentration, and then added to OptiMEM with or without 1 µl human serum (Thermo Fisher Scientific) to a 100 µl volume and incubated at 37°C for 20 minute. The digestion products were analyzed by 8% urea-PAGE.

qRT-PCR. Total RNA was isolated from cells using Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA), and reverse transcribed with NxGen M-MuLV Reverse Transcriptase (Lucigen,

Middleton, WI) and stem-loop primer 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CAA CC-3' for miR-34a, or iScript reverse-transcription Supermix (Bio-Rad) for chimeric ncRNAs, pre-miR-34a, and U6. qRT-PCR was conducted with qRT-PCR Master Mix (New England Biolabs) on a CFX96 Touch real-time PCR system (Bio-Rad), as described (Bi et al., 2014; Li et al., 2014). The primers were as follows: 5'-GGC TAC GTA GCT CAG TTG GT-3' (forward) and 5'-TGG TGG CTA CGA CGG GAT TC-3' (reverse) for chimeric ncRNAs; 5'-GGC CAG CTG TGA GTG TTT CTT TGG-3' (forward) and 5'-GGG CCC CAC AAC GTG CAG-3' (reverse) for pre-miRNA mir-34a; 5'-CGC GCT GGC AGT GTC TTA GCT-3' (forward) and 5'-GTG CAG GGT CCG AGG T-3' (reverse) for mature miR-34a; and 5'-CTC GCT TCG GCA GCA CA-3' (forward), and 5'-AAC GCT TCA CGA ATT TGC GT-3' (reverse) for U6. The relative expression was calculated by using the comparative threshold cycle (Ct) method with the formula $2^{-\Delta\Delta C_t}$.

Small RNA Library Construction. Total RNAs were isolated with Trizol reagent (Invitrogen) from A549 cells harvested at 48 hours post-treatment with highly purified ncRNAs or Lipofectamine 2000 (Life Technologies) itself, and the small RNA library was generated using the Illumina Truseq Small RNA Preparation Kit (Illumina, San Diego, CA) according to the instructions.

Deep Sequencing and Data Analysis. The purified cDNA library was used for cluster generation on Illumina's Cluster Station and then sequenced on Illumina GAIIx following vendor's instructions. Raw sequencing reads (40 nt) were obtained using Illumina's Sequencing Control Studio software version 2.8 (SCS v2.8) following real-time sequencing image analysis and base-calling by Illumina's Real-Time Analysis version 1.8.70 (RTA v1.8.70). The extracted sequencing reads were used for the standard sequencing data analysis by following a proprietary pipeline script, ACGT101-miR v4.2 (LC Sciences, Houston, TX).

Immunoblot Analysis. The cell lysates were prepared using a radioimmunoprecipitation assay buffer with the cOmplete Protease Inhibitor Cocktail (Roche, Nutley, NJ). Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins were separated on a 10% SDS-PAGE gel and electrotransferred onto polyvinylidene fluoride membranes using Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were incubated with CDK6 (C-21), SIRT1 (H-300), or Met (C-28) rabbit polyclonal antibody (Santa Cruz Biotechnology) and subsequently with a peroxidase goat anti-rabbit IgG (Jackson ImmunoResearch). The membranes were then incubated with Clarity Western enhanced chemiluminescent substrates (Bio-Rad) and visualized with the ChemiDoc MP Imaging System (Bio-Rad).

Cytotoxicity. The effects of tRNA/mir-34a on the proliferation of cancer cells were determined using an MTT assay, as described (Pan et al., 2013; Li et al., 2015). Cells were seeded in 96-well plates at 3000 or 5000 cells per well, and transfected with various concentrations of tRNA/mir-34a using Lipofectamine 2000 for 72 hours. Cells transfected with the same doses of tRNA/MSA or Lipofectamine 2000 were used as controls. The EC_{50} and Hill slope values were estimated by fitting the data to a normalized inhibitory dose-response model (Prism; GraphPad Software, San Diego, CA).

Xenograft Tumor Mouse Models. All animal procedures were approved by the Institutional Animal Care and Use Committee at University of California–Davis. A549 and HepG2 cells were collected, counted, and mixed with Matrigel (BD Biosciences, San Jose, CA) in a 1:1 ratio by volume. Cells (5×10^6) in 100 μ l of medium/Matrigel solution were injected subcutaneously into the lower back region of 5- to 6-week-old male nude mice (The Jackson Laboratory, Bar Harbor, ME). Tumor volumes were measured with a caliper and calculated according to the formula: tumor volume (mm^3) = $0.5 \times [\text{length (mm)} \times \text{width}^2 (\text{mm}^2)]$. Recombinant tRNA/mir-34a and control tRNA/MSA were formulated with in vivo-jetPEI (Polyplus Transfection) and administered intratumorally once tumors reached 150–200 mm^3 . Tumors were collected, fixed in 10% formalin, and cut for histologic verification by pathologist.

Safety Profiles in Mouse Models. Male BALB/c mice at five to six weeks of age (The Jackson Laboratory) were administered intravenously via tail vein with 100 μ g of ncRNAs formulated with in vivo-jetPEI (Polyplus Transfection). Separate groups of animals were treated with in vivo-jetPEI vehicle as a negative control or 20 μ g of lipopolysaccharide (LPS) as a positive control for cytokine induction (Wiggins et al., 2010; Bodeman et al., 2013; Wang et al., 2015). Blood was collected at various time points and serum was isolated using the serum separator (BD Biosciences). Serum cytokine interleukin-6 (IL-6) levels were quantitated using a mouse IL-6 assay kit (Pierce/Thermo Scientific) on a SpectraMax M3 Multi-Mode Spectrophotometer (Molecular Devices), and mouse blood chemistry profiles were determined at the Comparative Pathology Laboratory at UC Davis.

Statistical Analysis. All data were presented as mean $\pm$ S.D. According to the number of groups and variances, data were analyzed with unpaired Student's *t* test, or one-way or two-way analysis of variance (GraphPad Prism). Any difference was considered as significant if the probability was less than 0.05 ($P < 0.05$).

Results

Chimeric tRNA/mir-34a Can Be Efficiently Biosynthesized in a Common Strain of *E. coli* on a Large Scale and Rapidly Purified to a High Degree of Homogeneity. To achieve high-yield production of pre-miR-34a agents in *E. coli*, we chose to use the tRNA scaffold (Ponchon and Dardel, 2007; Ponchon et al., 2009) to assemble a fusion ncRNA namely tRNA/mir-34a (Fig. 1A). The secondary structures predicted by different computational algorithms all indicated that the stem-loop structure of pre-miR-34a consisting of Dicer cleavage sites would be retained within tRNA/mir-34a chimeras. Thus the pre-miR-34a coding sequences were cloned to offer tRNA/mir-34a expression plasmids. Our data showed that slight change in the length of pre-miR-34a did not alter the levels of recombinant tRNA/mir-34a accumulated (Supplemental Fig. 1A). Target tRNA/mir-34a agents were expressed in different *E. coli* strains, and the highest accumulation levels were found in HST08 cells (Fig. 1B) at 9–14 hours post-transformation (Supplemental Fig. 1B). In addition, use of HST08-competent cells prepared within our laboratory offered similar levels of recombinant tRNA/mir-34a (Supplemental Fig. 1C), and the high-level expression (e.g., ~15% of recombinant ncRNAs in total RNAs) was retained when bacterial cultures were scaled up to 0.5 liters, and different batches of cultures were carried out (Supplemental Fig. 1D), demonstrating a consistent and efficient expression of biologic tRNA/mir-34a agents.

Next, we aimed to purify the recombinant tRNA/mir-34a to a high degree of homogeneity (e.g., >98%). Affinity purification was originally carried out for tRNA/mir-34a and the control tRNA/MSA bearing a Sephadex aptamer tag. Although affinity chromatography offered a good purity (>90%), overall yield was not very satisfactory (around 2% of recombinant ncRNA/total RNAs), which may be attributed to an unexpected but obvious inefficient binding (Supplemental Fig. 1E). Thus, we developed an anion-exchange FPLC method for the isolation of tRNA/mir-34a and tRNA/MSA from total RNAs using a salt gradient elution. This FPLC method not only enabled rapid isolation of target ncRNAs (<25 minutes per run; Fig. 1C) to a high degree of homogeneity (e.g., >98% purity, as demonstrated by gel electrophoresis, not shown) and HPLC analysis (Fig. 1D) but

Fig. 1. Production and structural characterization of recombinant tRNA/mir-34a agents. (A) The secondary structure of chimeric tRNA/mir-34a (233 nt) predicted by CentroidFold showed that the stem-loop structure of mir-34a persisted within the chimeric ncRNA. The heat color gradation indicates the base-pairing probability from 0 to 1. (B) Expression of tRNA/mir-34a in various strains of *E. coli*. Among those tested, the highest levels of recombinant ncRNAs were found in HST08. (C) FPLC traces of tRNA/mir-34a and tRNA/MSA during the purification. Total RNAs were separated using anion-exchange FPLC and monitored at 260 nm. (D) HPLC analysis confirmed the high homogeneity (>98%) of purified tRNA/mir-34a and tRNA/MSA. (E) Liquid chromatography–tandem mass spectrometry mapping/sequencing of purified tRNA/mir-34a and tRNA/MSA after the digestion with RNase T1. All post-transcriptional modifications except deoxyadenosine identified by nucleoside analysis (Supplemental Fig. 1) could be mapped to RNase T1 digestion products and assigned to specific sites.

also offered much higher purification yield (e.g., 67% according to the 15% target ncRNA present in total RNAs, or 10% recombinant ncRNA/total RNAs loaded on column). This FPLC method largely facilitated the purification process that allows us to readily obtain milligrams of ncRNAs, i.e., ~1.5 mg of >98% pure tRNA/mir-34a from 15 mg of total RNAs isolated from 0.5 liters of bacterial culture at all times.

Recombinant ncRNAs Carry Post-Transcriptional Modifications. To delineate if the recombinant tRNA/mir-34a comprise any post-transcriptional modifications, which are common for natural RNAs produced in living cells (Novoa et al., 2012), we employed several mass spectrometry–based techniques to analyze the purified ncRNAs after confirming their primary sequences by Sanger sequencing of reversely transcribed cDNAs (data not shown). First, we determined the molecular weights through electrospray ionization mass spectrometry analyses of the intact ncRNAs, which were 73,422.4 Da for tRNA/mir-34a and 34,765.2 Da for tRNA/MSA. The differences between the measured and predicted molecular weights (146.8 Da for tRNA/mir-34a and 149.2 Da for tRNA/MSA; Supplemental Fig. 2A) suggest the presence of modified nucleosides. We then conducted a liquid chromatography–UV and mass spectrometry detection analysis of ncRNA hydrolysates and identified a number of modified nucleosides for the tRNA scaffold (existing in both tRNA/mir-34a and tRNA/MSA), such as dihydrouridine (D), pseudouridine (ψ), 7-methylguanosine (m^7G), 4-thiouridine (s^4U), 2'-O-methylguanosine (Gm), and 3-(3-amino-3-carboxypropyl)uridine (acp³U) (Supplemental Fig. 2B). We thus carried out liquid chromatography–tandem mass spectrometry analyses (Supplemental Fig. 2C) of RNA fragments produced from recombinant ncRNAs by RNase T1, which allowed us to successfully map the ncRNA sequences and localize all modified nucleosides for the tRNA scaffold (Fig. 1E). The deoxyadenosine (da) found in tRNA/mir-34a hydrolysates (Supplemental Fig. 2B) was not mapped to its RNase T1 digestions, which might be attributable to prior carryover or copurified nucleic acid. Together, these data indicate that recombinant ncRNAs obtained from *E. coli* indeed consist of

various post-transcriptional modifications that may be critical for RNA folding and metabolic stability.

tRNA-Carried Pre-miR-34a Is Selectively Processed to Mature miR-34a in Human Carcinoma Cells, Whereas the tRNA Scaffold Is Degraded to tRNA Fragments. To assess whether chimeric tRNA/mir-34a can be selectively processed to mature miR-34a in human cells, we first conducted unbiased deep-sequencing study. The RNAseq data revealed that the tRNA/mir-34a chimera was precisely processed to mature miR-34a in A549 cells, leading to a 70-fold increase in miR-34a levels compared to cells treated with tRNA/MSA or vehicle (Fig. 2, A and B). In contrast, there was no or limited changes in other cellular miRNAs, except a few undefined small RNAs (e.g., hsa-miR-30c-5p_R+1 and hsa-miR-7641-1-p5_1ss6TC, etc.; Fig. 2, A and B; Supplemental Tables 1 and 2), which might be secondary effects that were caused by the changes in miR-34a target gene expression (see the results below). Furthermore, the increase in miR-34a levels was attributed to the 22- and 23-nt isoforms that arose in tRNA/mir-34a-, tRNA/MSA-, and vehicle-treated cells (Supplemental Table 3). In addition, the common tRNA scaffold was degraded in the cells to offer the same tRFs that exhibited similar patterns between tRNA/mir-34a- and tRNA/MSA-treated cells, but at much lower levels than mature miR-34a (Fig. 2B; Supplemental Table 4), supporting the use of tRNA/MSA as a proper control to distinguish the activities of pre-miR-34a.

Recombinant tRNA/mir-34a Exhibits a Favorable Cellular Stability and Is Degradable by Human RNases. Consistent with deep-sequencing data, selective stem-loop reverse transcription qRT-PCR analyses revealed a 70- to 100-fold increase in mature miR-34a levels in human lung (A549 and H460) and liver (HepG2 and Huh-7) carcinoma cells after the transfection with tRNA/mir-34a (Fig. 3A). Moreover, mature miR-34a and pre-miR-34a levels were elevated in tRNA/mir-34a-treated A549 cells in a dose-dependent manner, whereas there was no change of miR-34a levels in cells treated with tRNA/MSA, even though tRNA/MSA levels actually increased (Fig. 3B). Most importantly,

Fig. 2. The tRNA-carried pre-miR-34a was selectively processed to mature miR-34a in human carcinoma cells while tRNA scaffold was degraded to tRFs, as revealed by unbiased deep sequencing studies. Mature miR-34a levels were over 70-fold higher in A549 cells treated with 5 nM tRNA/mir-34a, compared with cells treated with the control tRNA/MSA (A) or vehicle (B). This was associated with a 60- to 65-fold increase in the numbers of reads of 15-nt miR-34a-p3' fragment (5'-CACGUUGUGGGGCCC-3'). In contrast, changes in other miRNAs were small or insignificant (Supplemental Tables 1 and 2), except for several undefined small RNAs (e.g., hsa-mir-7641-1-p5_1ss6TC). Values are mean $\pm$ S.D. of triplicate treatments that were sequenced separately. (C) Mapping major tRFs derived from tRNA/mir-34a and tRNA/MSA in A549 cells. Other low-abundance tRFs are provided in Supplemental Table 4.

a high level of recombinant tRNA/mir-34a and tRNA/MSA persisted for 6 days in the cells post-transfection with levels gradually decreasing from day 3 (Fig. 3C), indicating a favorable cellular stability. In addition, the change of pre-miR-34a and mature miR-34a levels over time were solely dependent on the tRNA/mir-34a treatment. Further biochemical experiments demonstrated that tRNA/mir-34a

and tRNA/MSA were readily processed by RNase A (or RNase I, the major form of ribonuclease in human serum) and Dicer (RNase III) but to a relatively lower degree by angiogenin (RNase 5) (Fig. 3D), suggesting their involvement in the processing and degradation of recombinant ncRNAs. In contrast, bacterial RNase R was unable to cleave chimeric ncRNAs (data not shown), providing a good explanation

Fig. 3. Cellular stability and RNase susceptibility of chimeric tRNA/mir-34a. (A) Chimeric tRNA/mir-34a was processed to mature miR-34a in various types of human carcinoma cells, as determined by selective stem-loop reverse transcription qRT-PCR analyses. Values are mean $\pm$ S.D. of triplicate treatments. (B) The levels of chimeric ncRNA (tRNA/mir-34a or tRNA/MSA), pre-miRNA mir-34a, and mature miRNA miR-34a were increased in a dose-dependent manner ($P < 0.001$, one-way analysis of variance) in A549 cells at 24 hours after transfection with 1, 5, or 25 nM of FPLC-purified tRNA/mir-34a or tRNA/MSA. Note that mir-34a and miR-34a levels were only elevated in cells treated with tRNA/mir-34a. (C) Chimeric ncRNA (tRNA/mir-34a or tRNA/MSA; 5 nM) exhibited good stability in A549 cells. Note that change in mature miR-34a levels was dependent on tRNA/mir-34a treatment. (D) Chimeric tRNA/mir-34a and the tRNA/MSA were susceptible to a number of human RNases, including RNase A, angiogenin, and Dicer. RNase digestions were analyzed with 8% urea-PAGE.

for why such ncRNAs were accumulated to high levels within bacteria.

tRNA-Carried Pre-miR-34a Is Active in Reducing miR-34a Target Gene Expression and Inhibiting Cancer Cell Proliferation, Equally or More Effectively Than Synthetic miR-34a Agents. We thus assessed the efficacy of tRNA-carried pre-miR-34a in the control of miR-34a target gene expression and cancer cell growth, using tRNA/MSA as a critical control. Recombinant tRNA/mir-34a showed a dose-dependent inhibition against the proliferation of all types of cancer cells tested in our studies, to a much greater degree than the control tRNA/MSA (Fig. 4A; Supplemental Fig. 3). The higher efficacy in suppressing cancer cell growth by tRNA/mir-34a was also indicated by the estimated EC_{50} values (Table 1). Inhibition of A549 and HepG2 carcinoma cell proliferation by tRNA/mir-34a was associated with a remarkable repression of a number of well defined miR-34a target genes such as CDK6, SIRT1, and MET, compared with the tRNA/MSA or vehicle treatments (Fig. 4B). In addition, we compared side-by-side the effectiveness of biologic and synthetic miR-34a agents in human cell line models. Interestingly, our data showed that recombinant tRNA/mir-34a was relatively more effective in suppressing the proliferation of A549 and HepG2 cells and the protein levels of miR-34a target genes (e.g., CDK6, SIRT1, and MET) than the same doses of synthetic pre-miR-34a and miR-34a mimics bearing artificial modifications, compared with corresponding controls (Fig. 5, A and B). These results indicate that tRNA-carried pre-miR-34a is biologically/pharmacologically active in the modulation of miR-34a target gene expression and cancer cell proliferation.

Recombinant Pre-miR-34a Is Effective in Suppressing Xenograft Tumor Progression in Mouse Models. We thus evaluated the therapeutic effects of tRNA/mir-34a in vivo using human lung carcinoma A549 and hepatic carcinoma HepG2 xenograft tumor mouse models. When A549

xenograft tumors reached $\sim 150 \text{ mm}^3$, typically within 3 weeks of inoculation, we treated male nude mice intratumorally with 20 or 100 μg of in vivo-jetPEI-formulated tRNA/mir-34a, which would not be complicated by tissue distribution. Separate groups of animals were administered the same doses of in vivo-jetPEI-formulated tRNA/MSA or only the in vivo-jetPEI vehicle as controls. Our data showed that, compared with the vehicle treatment or the same dose of tRNA/MSA, the higher dose (100 μg) of tRNA/mir-34a led to a complete disappearance of the A549 xenograft tumors (three out of six) and an overall significant repression of the outgrowth of viable tumors (Fig. 6A). The same dose (100 μg) of tRNA/mir-34a also significantly suppressed the growth of HepG2-derived xenografts (Fig. 6B), although to a lesser degree than its effects on A549 xenografts. These findings indicate that recombinant tRNA-carried pre-miR-34a is effective in controlling xenograft tumor progression in vivo.

Chimeric ncRNAs Are Well Tolerated in Mouse Models. We further investigated the safety profiles of recombinant tRNA/mir-34a agents produced in *E. coli*. The LAL assay was first conducted to evaluate whether these biologic ncRNAs contain significant levels of endotoxin that may cause immune response or toxicity in mammalian cells. While total RNAs isolated from *E. coli* showed variable levels of endotoxin (100–1000 EU/ μg of RNA), endotoxin activities were minimal for the ncRNAs purified with FPLC (<10 EU/ μg of RNA) and those further processed with an endotoxin removal kit (<3.0 EU/ μg of RNA). Despite the lack of an endotoxin safety standard for RNA agents and the uncertainty of whether RNAs influence (the mechanism of) the LAL assay, endotoxin activities in our purified ncRNAs measured much lower than 2000 EU/ μg of DNA that is required to significantly inhibit transfection and cell proliferation (Butash et al., 2000).

After verifying that in vivo-jetPEI-loaded tRNA/mir-34a and tRNA/MSA were protected against degradation by serum

Fig. 4. Recombinant mir-34a was biologically/pharmacologically active in suppressing cancer cell proliferation and target gene expression. (A) tRNA/mir-34a inhibited the growth of human carcinoma A549 and HepG2 cells in a dose-dependent manner and to a much greater degree than the control tRNA/MSA ($P < 0.001$, two-way analysis of variance). Antiproliferative activities against H460 and Huh-7 cells are shown in Supplemental Fig. 3, and the estimated ED_{50} and Hill slope values are provided in Table 1. Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay at 72 hour post-transfection. Values are mean $\pm$ S.D. of triplicate cultures. (B) Compared with the control tRNA/MSA, recombinant tRNA/mir-34a sharply reduced the protein levels of a number of miR-34a target genes including CDK6, MET, and SIRT1 in A549 and HepG2 cells. Western blots were conducted using protein-selective antibodies. GAPDH was used as a loading control.

TABLE 1
Estimated EC₅₀ and Hill slope values for the suppression of human carcinoma cell proliferation by recombinant tRNA/mir-34a and control tRNA/MSA

Cell Lines	EC ₅₀		Hill Slope	
	tRNA/MSA	tRNA/mir-34a	tRNA/MSA	tRNA/mir-34a
<i>nM</i>				
A549	Not fitted	7.80 ± 1.19	Not fitted	-2.18 ± 0.59
H460	158 ± 1	5.72 ± 1.09*	-0.86 ± 0.20	-1.32 ± 0.14*
HepG2	87.9 ± 1.1	4.75 ± 1.21*	-1.74 ± 0.40	-0.96 ± 0.18*
Huh-7	Not fitted	11.1 ± 1.2	Not fitted	-0.66 ± 0.07

Goodness of fit, *R*² > 0.75. Not fitted, goodness of fit is less than 0.50.
**P* < 0.05, significantly different from the control tRNA/MSA in the same cell line.

RNases (Fig. 7A), we directly assessed the effects of in vivo-jetPEI-formulated ncRNAs on the immune response as well as hepatic and renal functions in immunocompetent BALB/c male mouse models. The activation of immune response is often indicated by the increase of blood levels of various cytokines, among which the pro- and anti-inflammatory cytokine IL-6 is the most sensitive in response to nucleic acids (Wiggins et al., 2010). As a positive control, LPS-treated mice showed an immediate sharp surge of serum IL-6 levels 1–6 hour after injection (Fig. 7B), in addition to obvious signs of stress (e.g., hunched posture and labored movement), and then fully recovered within 24 hours. In contrast, serum IL-6 levels were just elevated slightly in mice 6 hours after intravenous administration of 100 μg tRNA/mir-34a or tRNA/MSA. The change was very mild compared with the LPS treatment and it was similar to that reported for synthetic miR-34a mimics (Wiggins et al., 2010), which might not indicate an adverse drug response. Furthermore, mouse blood chemistry profiles, including the levels of alanine

aminotransferase, aspartate aminotransferase, albumin, alkaline phosphatase, total bilirubin, blood urea nitrogen, creatinine, and total protein were not significantly altered by recombinant tRNA/mir-34a (Fig. 7C), suggesting that the biologic ncRNA agents did not induce acute liver or kidney toxicity.

Discussion

In contrast to the great efforts to develop miRNA-based therapies, translational and clinical research is often hampered by a lack of access to large quantities of inexpensive natural miRNA agents. Motivated by the concept of deploying biologic RNA agents to perform RNA actions and the principle of “prodrug,” we established a novel strategy to cost effectively produce multimilligrams of tRNA fusion miR-34a biologic agents in 1-liter cultures of a common strain of *E. coli* in a research laboratory setting. The better expression of recombinant ncRNA in HST08 strain may be related to the lack of gene clusters in HST08 cells for digesting methylated DNA or a lower capacity to polyadenylate ncRNA for degradation. Notably, our strategy is different from a newly reported approach of generating fully-processed small interfering RNAs using p19-expressing bacteria (Huang et al., 2013). A high-yield accumulation of recombinant tRNA/mir-34a in bacteria (~15% of total RNAs) also facilitated the purification by anion-exchange FPLC method to a high degree of homogeneity (>98%). In addition, we were able to characterize the primary structures and modified nucleosides of recombinant ncRNAs through liquid chromatography–UV and mass spectrometry detection analyses of hydrolysates and liquid chromatography–tandem mass spectroscopy analyses of RNase T1-cleaved fragments. Our findings illustrate fundamental post-transcriptional modifications of the tRNA

Fig. 5. Recombinant mir-34a was equally or more effective than synthetic miR-34a mimics in reducing human carcinoma cell proliferation and target gene expression. (A) The effects of 5 nM biologic tRNA/mir-34a, and synthetic miR-34a precursor and mimics on the growth of A549 and HepG2 carcinoma cells. Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay at 72 hours post-transfection. Values are mean ± S.D. of triplicate treatments. **P* < 0.05, compared with corresponding control. (B) The effects of 25 nM recombinant mir-34a and synthetic miR-34a mimics on the protein levels of miR-34a target genes CDK6, MET, and SIRT1 in A549 and HepG2 carcinoma cells, as determined by Western blots at 72 hours post-transfection.

Fig. 6. Recombinant mir-34a was effective in controlling xenograft tumor progression in mouse models. (A) Compared with the same dose of tRNA/MSA or vehicle control, tRNA/mir-34a (100 µg) significantly ($P < 0.001$, unpaired t test) suppressed the growth of A549 xenograft tumors. Note that the tumors in three mice completely disappeared after the treatment with 100 µg of tRNA/mir-34a. (B) Growth of HepG2 xenograft tumors were also significantly ($P < 0.01$, unpaired t test) suppressed by the 100-µg dose tRNA/mir-34a treatment, compared with the same dose of tRNA/MSA or vehicle. FPLC-purified ncRNAs were administered intratumorally every the other day for three times. Values are mean $\pm$ S.D. ($N = 6$ per group for A549 xenografts; $N = 4$ per group for HepG2 xenografts). Separate groups of mice ($N = 4$) were treated with vehicle as additional controls.

scaffold that are critical for its stability (Alexandrov et al., 2006).

Chimeric tRNA/mir-34a showed a rather surprisingly favorable stability within human carcinoma cells, suggesting that the tRNA carrier also offered a “stealth delivery” of target pre-miR-34a into human cells beyond the high-yield production of chimeric tRNA/mir-34a in bacteria. As expected, chimeric tRNA/mir-34a acted as a prodrug in human carcinoma cells, where pre-miR-34a was selectively processed to mature miR-34a by intrinsic miRNA processing machinery,

and the tRNA carrier was degraded to tRFs (Lee et al., 2009; Li et al., 2012). The 70-fold higher levels of mature miR-34a were also accompanied by 60-fold increase in miR-34a-p3 small RNA derived from pre-miR-34a. Therefore, chimeric tRNA/mir-34a may serve as an optimal carrier to assemble small RNAs of interests (Chen et al., 2015) that cannot be produced with tRNA scaffold. In addition, these results offer a good understanding of the fate of recombinant ncRNAs in human cells and the susceptibility to a few commercially-available human RNases, even though the precise contribution

Fig. 7. Biologic ncRNAs were well tolerated in mouse models. (A) In vivo-jetPEI-loaded ncRNAs were protected against degradation by serum RNases. (B) Compared with vehicle control treatment, in vivo-jetPEI formulated recombinant ncRNAs (100 µg i.v.) did not cause significant change in mouse serum IL-6 levels whereas lipopolysaccharide did (two-way analysis of variance with Bonferroni post-tests). (C) Recombinant ncRNAs (100 µg i.v.) had no significant influence on blood chemistry profiles, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), alkaline phosphatase (ALP), creatinine, total bilirubin, albumin, and total protein. The gray-shaded area indicates the guideline ranges reported by the Comparative Pathology Laboratory at UC-Davis. Values are mean $\pm$ S.D. ($N = 3-4$ mice per time point and treatment).

of specific RNases to the metabolism and pharmacokinetics of biologic tRNA/miR-34a warrants further investigation. While the effects of these tRFs are unknown, the same tRFs were produced from tRNA/MSA and tRNA/miR-34a at comparable levels in human cells, supporting the validity of using tRNA/MSA expressed in the same strain of *E. coli* and purified in the same manner as a control for the assessment of bioactivities of tRNA-carried pre-miR-34a.

The functions of tRNA-carried pre-miR-34a were nicely demonstrated by the selective reduction of protein expression levels of a number of previously verified miR-34a target genes, such as CDK6, MET, and SIRT1, in both A549 and HepG2 cells, compared with tRNA/MSA. These genes are critical for many cellular processes such as cell cycle and apoptosis. Therefore, the suppression of miR-34a target genes by recombinant pre-miR-34a provides a mechanistic explanation for its antiproliferative activities. The broad anticancer activities of recombinant pre-miR-34a against various types of cancer cells are consistent with previous findings on miR-34a functions in targeting multiple oncogenes and oncogenic pathways among different types of cancer cells (Chang et al., 2007; He et al., 2007; Sun et al., 2008; Yamakuchi et al., 2008; Li et al., 2009; Liu et al., 2011; Kasinski and Slack, 2012). Meanwhile, different human carcinoma cell lines did exhibit variable sensitivities to tRNA/miR-34a, which may be attributable to the variability in genome and gene expression profiles as well as the apparent effects of pre-miR-34a on target gene expression in different cell lines. While we posit that the activities of tRNA-carried pre-miR-34a in the modulation of miR-34a target gene expression and cancer cell growth is attributable to the mature miR-34a selectively produced from chimeric tRNA/miR-34a, we cannot exclude the possibility that pre-miR-34a itself is responsible for some of the effects noted.

Non-small-cell lung cancer A549 cells containing Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation, wild-type p53, and wild-type epidermal growth factor receptor, commonly found in human lung carcinomas, are proper models for human lung carcinogenesis and tumor progression (Lehman et al., 1991; Nomoto et al., 2006). By contrast, the hepatocellular carcinoma HepG2 cell line represents a pure human liver carcinoma cell line free of viral infections, comprises an NRAS mutation, and is often used as an hepatocellular carcinoma model (Hsu et al., 1993; Charette et al., 2010; Costantini et al., 2013). Therefore, the A549 and HepG2 cells were used to produce xenograft tumors in mouse models to evaluate the effectiveness of recombinant pre-miR-34a in the control of tumor growth in vivo. Our study revealed a significant suppression of both A549 and HepG2 xenograft tumor growth by the higher dose (100 μ g) of tRNA/miR-34a, compared with the vehicle treatment or the same dose of tRNA/MSA. After monitoring tumor growth for 6 weeks, we demonstrated that tRNA/miR-34a strikingly eradicated A549 tumors (three out of six mice), although it is unknown whether and when recurrence would occur. Although we did not measure the half-life of tRNA/miR-34a in vivo, we observed that expression levels persisted until day 6 in A549 and HepG2 cell lines after single-dose transfection. Meanwhile, we showed a much greater degree of inhibition against A549 xenografts than HepG2 by tRNA/miR-34a, which is in agreement with the efficacy of tRNA/miR-34a defined with cancer cell line models in vitro. While this study is limited to

an intratumoral drug administration, it provides direct evidence to support the effectiveness of biologic miR-34a agents in vivo. Nevertheless, the utility of recombinant miR-34a agents for cancer treatments should be challenged by using targeted drug delivery systems and/or more clinically relevant tumor animal models before clinical investigations.

Our study also illustrated that a relatively higher dose intravenous bolus, FPLC-purified chimeric miR-34a biologic agents, did not cause any stress to the mice (e.g., hunched posture and labored movement) within 48 hours after drug administration or alter the liver and kidney functions as manifested by the unchanged blood chemistry profiles. The levels of serum IL-6, the most sensitive cytokine in response to nucleic acids, were only slightly perturbed by chimeric ncRNAs compared with LPS treatment. The minor change in IL-6 levels caused by recombinant tRNA/miR-34 and tRNA/MSA within a short period (6 hours) was actually comparable to those reported for synthetic miR-34a mimics (Wiggins et al., 2010). Owing to unchanged levels of alanine aminotransferase, aspartate aminotransferase, bilirubin, albumin, blood urea nitrogen, creatinine, and total proteins in tRNA/miR-34a-treated mice 48 hours postinjection, these findings indicate that recombinant ncRNAs are well tolerated in mouse models and do not induce acute toxicity. Nevertheless, further studies are needed to critically define the safety profiles following chronic administration of biologic ncRNAs in different species of animal models before clinical studies.

It is also noteworthy that recombinant tRNA/miR-34a, as first described in the current study, was proven to be equally or more effective than synthetic pre-miR-34a and miR-34a mimics in the regulation of target gene expression and suppression of cancer cell proliferation, compared with corresponding controls. This might be related to the differences in their secondary structures and metabolic stabilities within the cells, and consequently the efficiencies of miRNA processing machinery and RNA-induced silencing complex in utilizing these agents for the regulation of target gene expression and control of cellular processes. Nevertheless, the advantages and disadvantages of using recombinant miRNA agents versus synthetic miRNAs, as well as recombinant DNAs for research and therapy, will be undoubtedly subjected to discussion and examination.

In conclusion, our results demonstrated that chimeric pre-miR-34a agents could be efficiently produced in a common strain of *E. coli* on a large scale. The biologic tRNA/miR-34a bearing natural modifications exhibited a favorable cellular stability and was degradable by RNases. Furthermore, tRNA-carried pre-miR-34a was pharmacologically active in suppressing human carcinoma cell proliferation through the regulation of miR-34a target gene expression after being selectively processed to mature miR-34a. In addition, chimeric miR-34a was effective in controlling xenograft tumor progression and it was well tolerated in mouse models. Our findings indicate that chimeric miRNA agents engineered in bacteria may be useful tools for the discovery and development of novel pharmacotherapies.

Authorship Contributions

Participated in research design: Yu, Wang, Ho, Chen, Addepalli, Limbach, M.-M. Li, Wu, Jilek, Qiu, Zhang, T. Li, Wun, White, Lam.

Conducted experiments: Wang, Ho, Chen, Addepalli, M.-M. Li, Wu, Jilek, Yu, Qiu.

Contributed new reagents or analytic tools: Yu, Limbach, Li, Addepalli, Lam.

Performed data analysis: Wang, Ho, Chen, Addepalli, Limbach, M.-M. Li, Wu, Jilek, Qiu, Zhang, T. Li, Wun, White, Lam, Yu.

Wrote or contributed to the writing of the manuscript: Yu, Wang, Ho, Chen, Addepalli, Limbach, M.-M. Li, Wu, Jilek, Qiu, Zhang, T. Li, Wun, White, Lam.

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