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Chimeric MicroRNA-1291 Biosynthesized Efficiently in *Escherichia coli* Is Effective to Reduce Target Gene Expression in Human Carcinoma Cells and Improve Chemosensitivity

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ABSTRACT

In contrast to the growing interests in studying noncoding RNAs (ncRNAs) such as microRNA (miRNA or miR) pharmacoepigenetics, there is a lack of efficient means to cost effectively produce large quantities of natural miRNA agents. Our recent efforts led to a successful production of chimeric pre-miR-27b in bacteria using a transfer RNA (tRNA)–based recombinant RNA technology, but at very low expression levels. Herein, we present a high-yield expression of chimeric pre-miR-1291 in common *Escherichia coli* strains using the same tRNA scaffold. The tRNA fusion pre-miR-1291 (tRNA/mir-1291) was then purified to high homogeneity using affinity chromatography, whose primary sequence and post-transcriptional modifications were directly characterized by mass spectrometric analyses. Chimeric tRNA/mir-1291 was readily processed to mature miR-1291 in human carcinoma MCF-7 and PANC-1 cells. Consequently, recombinant tRNA/mir-1291 reduced the protein levels of miR-1291 target genes, including *ABCC1*, *FOXA2*, and *MeCP2*, as compared with cells transfected with the same doses of control methionyl-tRNA scaffold with a sephadex aptamer (tRNA/MSA). In addition, tRNA-carried pre-miR-1291 suppressed the growth of MCF-7 and PANC-1 cells in a dose-dependent manner, and significantly enhanced the sensitivity of ABCC1-overexpressing PANC-1 cells to doxorubicin. These results indicate that recombinant miR-1291 agent is effective in the modulation of target gene expression and chemosensitivity, which may provide insights into high-yield bioengineering of new ncRNA agents for pharmacoepigenetics research.

Introduction

The discovery of genomically encoded, functional noncoding RNAs (ncRNAs) such as microRNAs (miRNAs or miRs) and long noncoding RNAs in the control of various cellular processes, including drug disposition and cell proliferation, has expanded our knowledge of "genes" in the cells. Some miRNAs (e.g., miR-519c, -328, -326, -379, -1291, and -124) (To et al., 2008; Pan et al., 2009, 2013; Liang et al., 2010; Haenisch et al., 2011; Markova and Kroetz, 2014) that negatively regulate the expression of ATP-binding cassette efflux transporters (e.g., ABCC1, ABCC2, ABCC4, and ABCG2) may be used to improve the sensitivity of human carcinoma cells to anticancer drugs. Furthermore, a number of oncogenic miRNAs dysregulated in tumor tissues may be directly targeted to manage tumor progression (Trang et al., 2008; Bader et al., 2010). These approaches are indeed under active investigations toward an improved understanding of miRNA pharmacoepigenetics and development of novel miRNA-based therapies.

However, research on miRNA pharmacoepigenetics and therapies is hampered by the lack of an efficient method for producing large quantities of inexpensive and natural miRNA agents. The broadly used viral or nonviral vector-based miRNA expression systems use DNA agents rather than RNAs, and this approach relies on the host cells or organisms to transcribe DNA to miRNA precursors before getting into the cytoplasmic miRNA machinery. The other major group of miRNA agents consists of miRNA mimics, precursors, and antagomirs, which are all produced via chemical synthesis. Although organic synthesis of oligonucleotides may be automated, a projected dose of miRNA mimics or precursors for in vivo studies is very costly. It is also unclear to what extent chemical modifications would alter miRNA structure, folding, biologic activity, and safety profile, despite

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ABBREVIATIONS: acp^{3}U, 3-(3-amino-3-carboxypropyl)uridine; D, dihydrouridine; ESI, electrospray ionization; LB, Lysogeny broth; LC, liquid chromatography; m^{1}G, 1-methylguanosine; m^{7}G, 7-methylguanosine; miR, microRNA; miRNA, microRNA; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MSA, methionyl-tRNA with a sephadex aptamer; MTT, 3-(4,5 -dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; m^{5}U, 5-methyluridine; MW, molecular weight; ncRNA, noncoding RNA; PCR, polymerase chain reaction; rRNA, ribosomal RNA; RT-qPCR, reverse-transcription quantitative real-time polymerase chain reaction; tRNA, transfer RNA; ψ, pseudouridine.

the fact that artificial miRNA mimics show some favorable pharmacokinetic properties such as a longer half-life. In vitro transcription may be used to produce target RNA agents, whereas it normally generates RNA molecules in a test tube on microgram scale, and the production of larger quantities of RNAs requires more and costly RNA polymerases. Recently, transfer RNA (tRNA) (Ponchon and Dardel, 2007; Ponchon et al., 2009; Nelissen et al., 2012) and rRNA (Liu et al., 2010) have been successfully used as scaffolds to biosynthesize recombinant RNAs for structural and biophysical analyses, given the fact that tRNAs and rRNAs are present as stable RNA molecules in the cells. This recombinant RNA technology provides a novel method for cost-effective and rapid production of large quantities of recombinant RNAs (e.g., milligrams of target RNAs from 1 l of bacterial culture).

In an effort to produce natural miRNA agents to perform miRNA actions, we had demonstrated that the tRNA scaffold could be used to produce chimeric pre-miR-27b (tRNA/mir-27b) agents in *Escherichia coli* to study miR-27b functions in the modulation of drug metabolism in human cells (Li et al., 2014). However, the yield of recombinant tRNA/mir-27b is extremely low (e.g., <2% of chimeric tRNA/mir-27b in total RNAs). Herein, we show that various lengths of human pre-miR-1291 chimera could be assembled using the same tRNA scaffold, and much expected high-level expression (e.g., >10% of fusion tRNA/mir-1291 in total RNAs) was identified for pre-miR-1291 around 120 nucleotide (nt) in length in a common *E. coli* strain HST08, which may be used to investigate the functions of relatively less understood miR-1291 (Pan et al., 2013; Bi et al., 2014). Sephadex aptamer–tagged recombinant tRNA/mir-1291 was purified by affinity chromatography, and mapped/sequenced through mass spectrometry (MS)–based studies, including the characterization of post-transcriptional modifications. Furthermore, chimeric tRNA/mir-1291 was processed to mature miR-1291 in human carcinoma MCF-7 and PANC-1 cells. Compared with the control methionyl-tRNA scaffold with a sephadex aptamer (tRNA/MSA), tRNA/mir-1291 reduced the protein expression levels of miR-1291 target genes (e.g., ABCC1) and sensitized the ABCC1-overexpressing human carcinoma cells to the anticancer drug doxorubicin (a known substrate of transporter ABCC1). These findings shall provide insight into developing recombinant miRNA agents for pharmacoepigenetic and therapeutic studies.

Materials and Methods

Chemicals and Materials. Doxorubicin was purchased from LC Laboratories (Woburn, MA). Lipofectamine 2000, TRIzol reagent, and BCA protein assay kit were bought from Thermo Fisher Scientific Inc. (Waltham, MA). radioimmunoprecipitation assay lysis buffer was purchased from Rockland Immunochemicals (Limerick, PA), and the complete protease inhibitor cocktail was bought from Roche Diagnostics (Mannheim, Germany). The antibody against MeCP2 was purchased from Cell Signaling Technology (Danvers, MA), the antibodies against ABCC1 and FOXA2 were purchased from Abcam (Cambridge, MA), and the antibody against glyceraldehyde-3-phosphate dehydrogenase was bought from Santa Cruz Biotechnologies (Santa Cruz, CA). The horseradish peroxidase goat anti-rabbit or mouse secondary antibodies were bought from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence substrate and polyvinylidene fluoride membrane were obtained 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 Inc.

Prediction of RNA Secondary Structure. The secondary structures of tRNA/MSA, pre-miRNAs, and chimeric RNAs (Fig. 1A) were predicted using CentroidFold (http://www.ncrna.org/centroidfold) and CentroidHomfold (http://www.ncrna.org/centroidhomfold).

Construction of Plasmids. To express pre-miR-1291 using the tRNA scaffold (Fig. 1A), the DNA fragments encoding 123-nt, 164-nt, and 197-nt pre-miR-1291 were amplified by polymerase chain reaction (PCR) with the primers 5′-ACGCGTCGACGAGTTCTGTCCGTGAGCCTTGG-3′ and 5′- CATCGACGTCACAGCCAACAGACCACAGGAAG-3′, 5′-ACGCGTCGACAGCCTTGGGTAGAATTCCAG-3′ and 5′-CATCGACGTCGAGCTGTAGGTTGTTTCTTCC-3′, and 5′-ACGCGTCGACGAGTTCTGTCCGTGAGCCTTG-3′ and 5′-CATCGACGTCCCTCTTCCAATGGGATGGTGAG-3′, respectively, and then cloned into the vector pBSMrnaSeph (provided by Dr. Luc Ponchon, Université Paris Descartes, Paris, France) (Ponchon and Dardel, 2007; Ponchon et al., 2009) (Fig. 1B) that was linearized by restriction endonucleases SalI and *Aat*II (New England Biolabs, Ipswich, MA). All inserts were confirmed by Sanger sequencing analysis.

Expression of Chimeric RNAs in *E. coli*. Expression of tRNA/mir-1291 chimeras and the control tRNA/MSA was performed as described (Ponchon and Dardel, 2007; Ponchon et al., 2009; Li et al., 2014). In brief, freshly transformed HST08 *E. coli* cells (Clontech, Mountain View, CA) were plated on a Lysogeny broth (LB) agar plate containing 100 μg/ml of ampicillin. After growing overnight at 37°C, a single colony was picked up to inoculate an overnight culture with 5 ml of LB media containing 100 μg/ml of ampicillin at 37°C. For large-scale RNA expression, fresh transformants were directly incubated in 1 l of LB medium containing 100 μg/ml of ampicillin at 37°C overnight. Total RNAs were isolated from bacteria using the Tris-HCl–saturated phenol extraction method, and were quantitated using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and analyzed by denaturing urea (8 M) polyacrylamide (8%) gel electrophoresis to examine recombinant ncRNA expression.

Purification of Recombinant ncRNAs. Purification of recombinant ncRNAs consisting of Sephadex aptamer tag was conducted as reported (Ponchon and Dardel, 2007; Ponchon et al., 2009) with minor modifications. In brief, 1 g of Sephadex G-100 beads (Sigma-Aldrich) were incubated in 10 ml of buffer A consisting of 50 mM potassium phosphate, 5 mM $MgCl_2$, and 100 mM NaCl, pH 7.5, at 90°C for 5 hours, and then washed twice with buffer A before use. The *E. coli* cell pellets were sonicated and cellular debris was removed by centrifugation at 10,000*g* for 10 minutes. The supernatant was loaded onto the Sephadex column, washed three times with buffer A, and eluted with buffer A consisting of 50 mg/ml soluble dextran B512 (average molecular mass 9000–11,000 Da; Sigma-Aldrich). The dextran was removed through buffer exchange using Ultra-0.5 ml Centrifugal Filters (30 kilodaltons (kD) for tRNA/MSA, 50 kD for tRNA/mir-1291; Millipore, Billerica, MA). The purity of isolated RNAs was estimated based on the band intensity after resolving on denaturing PAGE gels, and the quantity was determined by NanoDrop. Purified recombinant RNAs were stored in diethylpyrocarbonate (DEPC)-treated water at −80°C before further analyses.

Analysis of Intact Recombinant ncRNA by Electrospray Ionization–Mass Spectrometry. The procedure described previously (Taucher and Breuker, 2010) was followed for electrospray ionization (ESI)–MS analysis of intact ncRNAs. In particular, the electrospray solution consisted of 1 μM ncRNA, 25 mM imidazole, and 25 mM piperidine in 1:1 water/methanol. The flow rate for direct infusion was 3.0 μl/min. The mass spectra were acquired in negative ion mode using a Thermo LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific Inc.) over an *m/z* range of 600–2000. The spray voltage was 4.2 kV with a capillary temperature at 275°C, capillary voltage of −38 V, and tube lens voltage of −95 V. Sheath gas, auxiliary gas, and sweep gas were set at 20, 5, and 2 arbitrary units, respectively. The instrumental settings were optimized by automatic tuning with poly $d(T)_{80}$ (Sigma-Aldrich). The instrument was calibrated as per the manufacturer's instructions (error ~100 ppm). ESI mass spectra of intact ncRNAs were deconvoluted using ProMass software for Xcalibur (Novatia LLC, www.enovatia.com) to determine the average molecular weights (MWs) of recombinant RNAs.

Nucleoside Analysis of Recombinant ncRNAs by Liquid Chromatography Coupled with UV and Mass Spectrometry Detection. The hydrolysates of recombinant RNAs were prepared by heating the RNAs at 95°C for 5 minutes followed by chilling on ice for 3 minutes. The RNA was initially digested, as described (Russell and Limbach, 2013), with Nuclease P1 (2 U/0.5 A_{260} unit; Sigma-Aldrich) in the presence of 1/10 volume of 0.1 M ammonium acetate (pH 5.3) at 45°C for 2 hours. The mixture was further treated with 0.002 units of snake venom phosphodiesterase (Worthington Biochemicals Lakewood, Lakewood, NJ) and 0.5 units of antarctic phosphatase (New England Biolabs) in 1/10 volume of 1 M ammonium bicarbonate at 37°C for 2 hours to release nucleosides from oligonucleotides. These nucleosides were resolved on

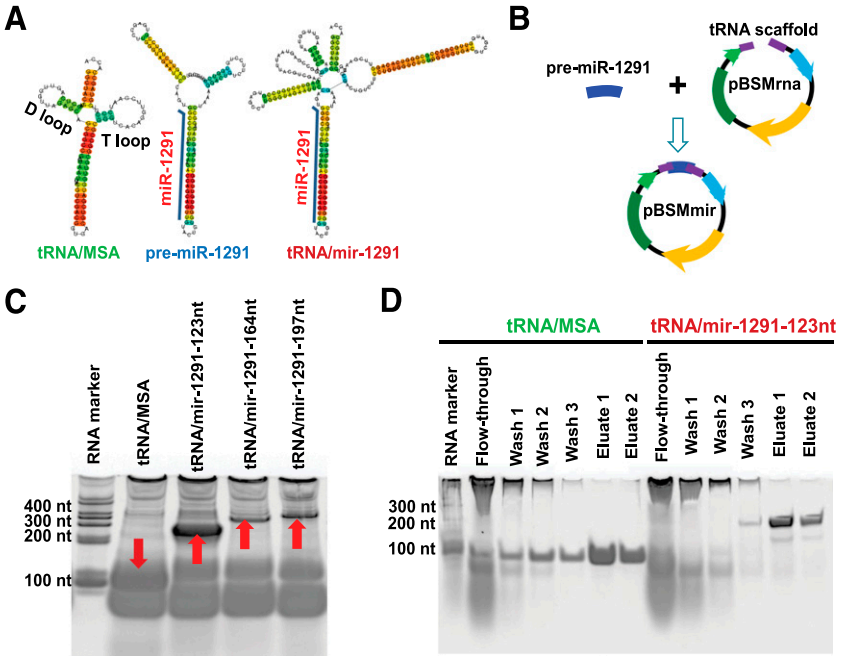


Fig. 1. Design, expression, and purification of recombinant chimeric miR-1291. (A) Secondary structures of tRNA/MSA, pre-miR-1291, and chimeric tRNA/mir-1291–123nt were predicted with CentroidFold. (B) Target pre-miR-1291 inserts were cloned into the pBSMrna vector linearized with endonucleases SalI and *Aat*II. (C) Successful expression of recombinant tRNA/mir-1291 in *E. coli* was demonstrated by the appearance of a new RNA band (indicated by the arrows) at expected size/mobility after urea-PAGE separation. (D) Recombinant ncRNAs bearing a Sephadex aptamer were purified by affinity chromatography. The cell lysate flow-through and individual fractions were analyzed by urea-PAGE. The flow-through and washes 1–3 mainly comprised the unbound RNAs. Eluate 1 and eluate 2 contained >85% pure recombinant ncRNAs.

a 2.1 × 250–mm Supelcosil LC-18-S (5-μm particle) reversed-phase column fitted with a 2.1 × 20–mm Supelguard LC-18-S guard column (Sigma-Aldrich) at a flow rate of 250 μl/min using Hitachi D-7000 high-performance liquid chromatography equipped with a diode array detector (Hitachi High-Technologies Corporation, Tokyo, Japan). The postcolumn flow was split between UV detector (2/3 volume) and mass spectrometer (1/3 volume) to record the UV trace and *m/z* values of analyte ions, respectively. Mobile phases were 5 mM ammonium acetate (pH 5.3) (mobile phase A) and 40% aqueous acetonitrile (mobile phase B) with multilinear gradients, as described (Pomerantz and McCloskey, 1990). Mass spectra were recorded using a Thermo LTQ-XL ion trap mass spectrometer equipped with an ion max electrospray source (Thermo Fisher Scientific, Inc.) in the positive ion mode over an *m/z* range of 100–1000. The electrospray conditions included a capillary temperature of 275°C, spray voltage of 4.2 kV, capillary voltage of 35 V, tube lens voltage of 85 V, and sheath gas, auxiliary gas, and sweep gas of 25, 15, and 10 arbitrary units, respectively. Data-dependent collision-induced dissociation tandem mass spectrometry (MS/MS) was performed on the two most abundant ions observed at a given time during the entire chromatographic run.

Mapping and Sequencing of ncRNAs by Liquid Chromatography–MS/MS Analysis. Mapping of recombinant ncRNAs and identification of nucleoside modifications were achieved by liquid chromatography (LC)–MS/MS analysis of RNase T1-digested RNA fragments (Krivos et al., 2011). In brief, the ammonium acetate–precipitated recombinant ncRNAs (3 μg) were treated with RNase T1 (25 U/μg RNA; Roche Molecular Biochemicals, Indianapolis, IN) and bacterial alkaline phosphatase (0.0001 U/μg RNA; Worthington Biochemical Corporation) at 37°C for 2 hours. The digests were subsequently dried in a SpeedVac (Thermo Fisher Scientific, Inc.) and stored at 4°C. Just before LC-MS/MS, the sample was resuspended with mobile phase C (400 mM hexafluoroisopropanol and 16.3 mM triethylamine in water, pH 7.0). The RNA digestion products were resolved on an XBridge C18 column (1.0 × 150 mm; Waters, Milford, MA) by a 70-minute gradient elution with mobile phase C and mobile phase D (50% mobile phase C, 50% methanol) at a flow rate of 30 μl/min using a Thermo Surveyor high-performance liquid chromatography system and a Thermo Micro AS autosampler. The tuning and MS methods were essentially the same as previously described (Wong et al., 2013). Mass values were restricted to a scan range of *m/z* 600–2000, and data-dependent collision-induced dissociation MS/MS was performed for the top four most abundant ions before keeping them in the exclusion list for 30 seconds.

An in silico analysis of RNase T1 digestion of the unmodified version of each recombinant ncRNA was performed using the Mongo Oligo Mass Calculator (http://mods.rna.albany.edu/masspec/Mongo-Oligo). This helped in identifying the appropriate *m/z* values that are common to tRNA/MSA and tRNA/mir-1291, as well as those unique to tRNA/mir-1291. The observed deviation of *m/z* values from the predicted values was recorded following manual data analysis.

Human Cell Culture and Transfection. Human pancreatic carcinoma PANC-1 and breast cancer MCF-7 cells were purchased from American Type Culture Collection (Manassas, VA). PANC-1 and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium and RPMI 1640 medium, respectively, containing 10% fetal bovine serum, 100 U/ml penicillin sodium, and 100 mg/ml streptomycin sulfate at 37°C in a humidified atmosphere of 5% carbon dioxide. Cells were transfected with purified recombinant ncRNAs using Lipofectamine 2000.

Reverse-Transcription Quantitative Real-Time PCR. MCF-7 and PANC-1 cells were harvested at different time points after transfection with various doses of recombinant ncRNAs using Lipofectamine 2000. Total RNAs were isolated using a Direct-zol RNA extraction kit (Zymo Research, Irvine, CA). Reverse-transcription quantitative real-time PCR (RT-qPCR) analysis was conducted on a CFX96 Touch Real-Time PCR system (Bio-Rad). Quantification of pre-miR-1291 was performed with a GoTaq 2-Step RT-qPCR system (Promega, Madison, WI) using gene-selective primers, and stem-loop RT-qPCR analysis of mature miR-1291 was conducted with the TaqMan small RNA assay kit (Thermo Fisher Scientific), as reported (Li et al., 2011; Pan et al., 2013). The cycle number (C_T) at which the amplicon concentration crossed a defined threshold was determined for each analyte. The relative expression was calculated using the formula $2^{-\Delta CT}$, where ΔC_T was the difference in C_T value between the analyte (pre-miR-1291 or miR-1291) and internal standard (18S and U6), and then normalized to the control treatment.

Immunoblot Analysis. MCF-7 and PANC-1 cells were transfected with 20 nM tRNA/mir-1291 or tRNA/MSA using Lipofectamine 2000 and then harvested after 48 hours. Cell lysates were prepared with radioimmunoprecipitation assay lysis buffer supplemented with the complete protease inhibitor cocktail, and protein concentrations were determined with the BCA protein assay kit. Whole-cell proteins (40 μg per lane) were separated on 10% SDS-PAGE gel and electrophoretically transferred onto polyvinylidene fluoride membranes, which were then incubated with selective antibody against MeCP2, FOXA2, MRP1, or glyceraldehyde-3-phosphate dehydrogenase. After blotting with peroxidase goat anti-rabbit or mouse IgG, the membranes were incubated with enhanced chemiluminescence substrates, and images were acquired with a ChemiDoc MP Imaging System (Bio-Rad). All experiments were conducted in triplicate using different transfections (N = 3) and repeated at least twice.

Cytotoxicity Assays. MCF-7 and PANC-1 cells were seeded at 10,000 cells/well in a 24-well culture plate or 3000 cells/well in a 96-well culture plate. At different time points after transfection with the recombinant tRNA/mir-1291 or tRNA/MSA at specific concentrations using Lipofectamine 2000, cell viability was determined using a 3-(4,5 -dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Sigma-Aldrich) as described (Pan et al., 2009, 2013) using an Infinite M200 Pro Microplate reader (Tecan, Durham, NC). To examine the influence of ncRNA on doxorubicin cytotoxicity, PANC-1 cells were first transfected with 20 nM ncRNAs or just Lipofectamine 2000 (vehicle control) for 48 hours. After incubation with various concentrations of doxorubicin or drug vehicle (0.1% dimethylsulfoxide) for another 48 hours, cell viability was determined by MTT assays. Doxorubicin cytotoxicity data were fit to a normalized, inhibitory dose-response model with variable slope, Y = 100/(1 + 10^[(LogIC$_{50}$ − X) × Hill slope]) (GraphPad Prism, San Diego, CA). The effects of recombinant ncRNAs on cell growth were better estimated by fitting to a normalized, inhibitory dose-response model, Y = bottom + (top-bottom)/(1+10^[(LogEC50-X) × Hill slope]), where the bottom and top were defined as 40% and 100%, respectively.

Statistical Analysis. All values were the mean ± S.D. According to the number of groups and variances, data were compared by unpaired Student's *t* test, one-way, or two-way analysis of variance using GraphPad Prism. Difference was considered as significant when the probability was less than 0.05 ($P < 0.05$).

Results

Design and Construction of tRNA/mir-1291 Expression Plasmids. To better maintain the hairpin structure of pre-miR-1291 (87 nt), we extended both 5′ and 3′ flanking sequences to 123 nt, 164 nt, and 197 nt whose corresponding DNA segments were thus cloned (Fig. 1B). Construction of ncRNA expression plasmids consisting of various lengths of pre-miR-1291 sequences also would allow us to evaluate the impact of length of oligonucleotides on recombinant ncRNA expression. The predicted secondary structures of tRNA/MSA, pre-miR-1291, and tRNA/mir-1291 (Fig. 1A) suggested that tRNA/mir-1291 chimeras containing 164-nt and 197-nt pre-miR-1291 might be able to maintain the tRNA D-loop and T-loop structures (data not shown), and the 123-nt pre-miR-1291 would form a more stable, intramolecular stem-loop structure at the T-loop arm (Fig. 1A). Nevertheless, the basic stem-loop structure of pre-miR-1291 was retained within all three tRNA/mir-1291 chimeras, suggesting that they would be accessible by endoribonucleases for the production of mature miR-1291 in human cells.

Expression and Purification of Recombinant tRNA/mir-1291. To examine the expression of tRNA/mir-1291 chimeras, total RNAs were isolated from *E. coli* and subjected to RNA electrophoretic mobility assay. The appearance of new RNA bands at the expected sizes (200–300 nt; Fig. 1C) in *E. coli* cells transformed with tRNA/mir-1291 expression plasmids, as compared with the cells transformed with tRNA/MSA expression plasmids, indicated a successful expression of recombinant tRNA/mir-1291 agents. It is noted that the electrophoretic mobility of chimeric tRNA/pre-miRNAs looked greater than that indicated by the single-stranded RNA markers. This is likely due to the presence of "double-stranded" stem structures in these ncRNAs (Fig. 1A). It appeared that the tRNA/mir-1291–123nt (227 nt in total length) was accumulated at a much higher level (e.g., >10% in total RNAs) than other longer tRNA/mir-1291–164nt and tRNA/mir-1291–197nt species (268 nt and 301 nt in total, respectively; <2% in total RNAs) under the same conditions (Fig. 1C).

The recombinant tRNA/mir-1291–123nt and tRNA/MSA bearing a Sephadex aptamer tag were isolated by affinity chromatography (Fig. 1D). Other cellular components such as rRNA, tRNA, DNA, and proteins were readily removed during loading and washing processes, and the recombinant ncRNAs bound to Sephadex G-100 were isolated after elution with dextran. A good purity (>85%; based on gel electrophoresis) and reasonable yield (around 2% of recombinant ncRNA/total RNAs or 2–3 mg of ncRNAs from 1 l of bacterial culture; based on the quantitation using NanoDrop) was achieved for tRNA/mir-1291–123nt and tRNA/MSA. In contrast, the purity of tRNA/mir-1291–164nt and tRNA/mir-1291–197nt chimeras was less than 60% (data not shown), which was likely related to their low expression levels. Therefore, only tRNA/mir-1291–123nt was used for the following studies, and was simply referred as tRNA/mir-1291.

Structural Characterization of Recombinant ncRNAs. The sequence of purified tRNA/mir-1291 was initially confirmed by Sanger sequencing after the preparation of cDNAs (data not shown). To directly determine the primary sequence of recombinant tRNA/mir-1291 and possible post-transcriptional modifications, several studies were conducted using MS techniques. First, the intact tRNA/mir-1291 was analyzed by ESI-MS to measure the MW of constituent RNA species. Deconvolution of the multiply charged ESI data indicated the presence of multiple species of RNA. The most abundant (~70%; based on peak areas) experimentally determined MWs were found to be 73,422.4 Da for tRNA/mir-1291 (Fig. 2A). The differences between measured and predicted MWs (162.4 Da) suggest the presence of modified nucleosides. Additional components were also noted, whose MWs correspond to unmodified RNA species or truncated RNA species of lower MWs than tRNA/mir-1291.

To identify possible post-transcriptional modifications which are common for natural RNAs produced in living cells, LC coupled with UV and MS detection analysis was conducted to define the nucleosides in hydrolysates prepared from tRNA/mir-1291 and compared with the scaffold tRNA/MSA. A number of modified nucleosides such as dihydrouridine (D), pseudouridine (ψ), 7-methylguanosine (m^7G), 4-thiouridine, 3-(3-amino-3-carboxypropyl)uridine (acp^3U), 5-methyluridine (m^5U), 2'-*O*-methylguanosine, and 1-methylguanosine (m^1G) were found for both tRNA/mir-1291 and tRNA/MSA samples (Fig. 2B). Whereas some modified nucleosides (e.g., D and ψ) were clearly identified by both the UV and MS data, others (e.g., 2'-*O*-methylguanosine and m^1G in tRNA/mir-1291) were less obviously determined by UV detection but readily discerned by corresponding MS data.

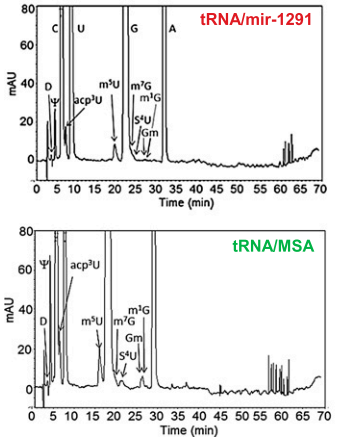
To further localize the nucleoside modifications, the ncRNAs were treated with guanosine-specific ribonuclease T1 and bacterial alkaline phosphatase. The resulting digestion products were resolved on an ion-pairing reversed-phase C18 column, identified and sequenced by tandem MS (Fig. 2C). The majority of modified nucleosides (e.g., D, ψ, m^7G, 4-thiouridine, acp^3U, and m^5U) obviously identified by the LC-UV analysis (Fig. 2B) could be mapped to RNase T1 digestion products and assigned to the tRNA scaffold, whereas the unassigned modification (e.g., m^1G) might be attributed to copurified nucleic acids or prior carryover. These results not only validated the primary sequences of purified tRNA/mir-1291 but also demonstrated the presence of natural modifications of the tRNA scaffold that may be critical for ncRNA stability.

Chimeric tRNA/mir-1291 Is Processed to Mature miR-1291 in Human Carcinoma Cells. To delineate if mature miR-1291 can be produced from recombinant tRNA/mir-1291 chimera in human cells, we used a selective TaqMan stem-loop RT-qPCR small RNA assay kit to quantify mature miR-1291 and regular RT-qPCR to determine pre-miR-1291 levels. Our data showed that pre-miR-1291 levels were sharply increased in human carcinoma MCF-7 cells treated with the tRNA/mir-1291 (Fig. 3A), indicating a successful transfection of tRNA/mir-1291. Meanwhile, the levels of mature miR-1291 were elevated remarkably in a dose-dependent manner (Fig. 3B). Interestingly, an increase of 3 and 2 orders of magnitude in pre-miR-1291 and mature

A

ncRNA	Measured MW (Da)	Predicted MW (Da)	Difference (Da)
tRNA/mir-1291	73422.4	73260.0	+162.4
tRNA/MSA	34765.2	34616.0	+149.2

B



C

Oligo-nucleotide	m/z (Da/e)	Location in tRNA/MSA	Location in tRNA/mir-1291
CUACG	763.1	C3:G7	C3:G7
[s4U]AG	933.1	[s4U]8:G10	[s4U]8:G10
CUCAG	763.1	C11:G15	C11:G15
CUCA[Gm]UUG	1248.7	C11:G18	C11:G18
[D][D]AG	613.4	[D]20:G23	[D]20:G23
CAG	916.2	C26:G28	C26:G28
UAAUUUACG	1398.7	U37:G45	U37:G45
[m7G][acp3U]CACAG	1157.7	[m7G]77:G83	[m7G]:G203
[m5U]ψCG	1213.1	[m5U]85:G88	[m5U]211:G214
AAUCCCG	1080.1	A89:G95	A215:G221
CCACCA	907.5	C102:A107	C228:A233
UUCUG	752.1		U54:G58
CCUUG	752.1		C67:G71
AAUUCCAG	1245.2		A77:G84
ACCAG	774.6		A100:G104
UUUCAAG	1081.4		U126:G132
CCUAAAG	1092.1		C139:G145
UCUUCCUG	1210.1		U151:G158

Fig. 2. Structural characterization of recombinant ncRNAs. (A) MWs of intact ncRNAs were determined by ESI-MS analyses, and the differences between measured and predicted MWs suggest the presence of post-transcriptionally modified nucleosides. (B) Post-transcriptionally modified nucleosides were identified by LC coupled with UV and MS detection analyses of the hydrolysates of recombinant ncRNAs. Shown are LC-UV traces of the hydrolysates of tRNA/MSA and tRNA/mir-1291, and individual peaks were annotated according to their retention times and mass spectra. C, cytidine; U, uridine; G, guanosine; S^4U, 4-thiouridine; Gm, 2'-*O*-methylguanosine; A, adenosine. (C) Mapping and sequencing of tRNA/MSA and tRNA/mir-1291 was achieved by LC-MS/MS analyses of RNase T1 digestions. Modified nucleosides were also localized, based upon their MS/MS fragmentations.

miR-1291, respectively, persisted in MCF-7 cells for 72 hours after transfection (Fig. 3, C and D). Similarly, there were increases of around 3 orders of magnitude in pre-miR-1291 and 2 orders of magnitude in mature miR-1291 in PANC-1 cells at 24 and 72 hours post-transfection of 20 nM recombinant tRNA/mir-1291 (data not shown). These results suggest that recombinant tRNA/mir-1291 is readily processed to mature miR-1291 within human carcinoma cells.

Recombinant tRNA/mir-1291 Is Effective to Control miR-1291 Target Gene Expression in Human Carcinoma Cells. To evaluate whether chimeric tRNA/mir-1291 is pharmacologically active in the modulation of miR-1291 target gene expression in human carcinoma cells, we first examined the impact of recombinant tRNA/mir-1291 on the protein levels of transporter ABCC1, a validated target for miR-1291 (Pan et al., 2013). Immunoblot analysis using protein-selective antibody revealed that 20 nM tRNA/mir-1291 reduced ABCC1 protein expression by 90% in PANC-1 cells, as compared with control tRNA/MSA treatment (Fig. 4A). We thus evaluated the effects of tRNA/mir-1291 and tRNA/MSA on two other targets, MeCP2 and FOXA2, which were identified by TargetScan algorithm (http://www.targetscan.org/) and validated in our laboratory (unpublished data). MeCP2 and FOXA2 are overexpressed in MCF-7 (Lin and Nelson, 2003) and PANC-1 cells (Song et al., 2010), respectively. Both cell lines had relatively lower levels of miR-1291, as compared with other cell lines such as MCF-7/MX100 and HepG2 (unpublished data). Our data showed that tRNA/mir-1291 led to a 30% reduction of FOXA2 protein levels in PANC-1 cells (Fig. 4B) and 50%

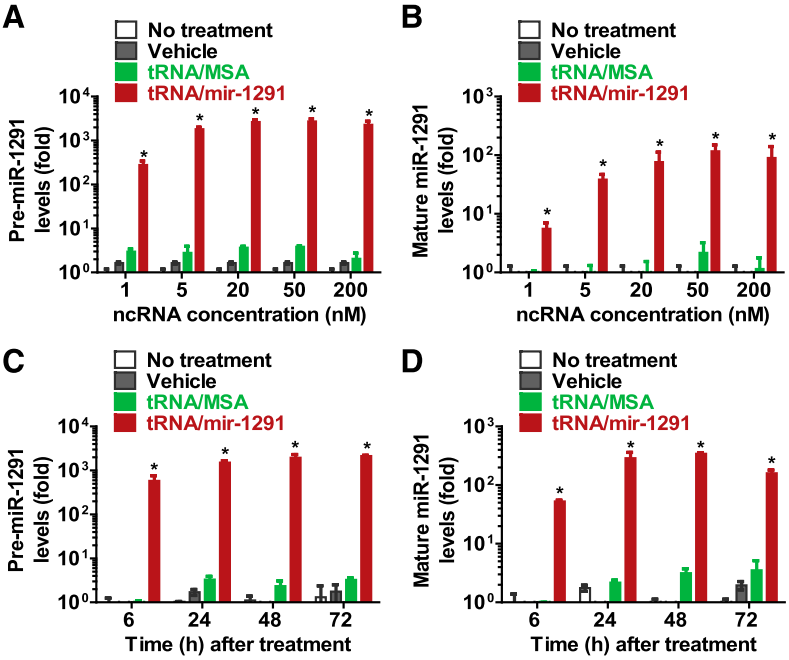


Fig. 3. tRNA-carried pre-miR-1291 is processed to mature miR-1291 in in human MCF-7 breast cancer cells. The levels of pre-miR-1291 (A) and mature miR-1291 (B) were increased in a dose-dependent manner in MCF-7 cells after transfection with purified tRNA/mir-1291. The time courses of pre-miR-1291 (C) and mature miR-1291 (D) were monitored in MCF-7 cells at 6, 24, 48, and 72 hours post-transfection with 20 nM recombinant tRNA/mir-1291. Cells without treatment or treated with the same doses of tRNA/MSA or vehicle were used as controls. RNA levels were determined by selective qPCR assays. Values are the mean ± S.D. of triplicate treatments. $^*P < 0.05$, compared with cells treated with the same doses of tRNA/MSA or vehicle or cells without treatment that were harvested at the same time points.

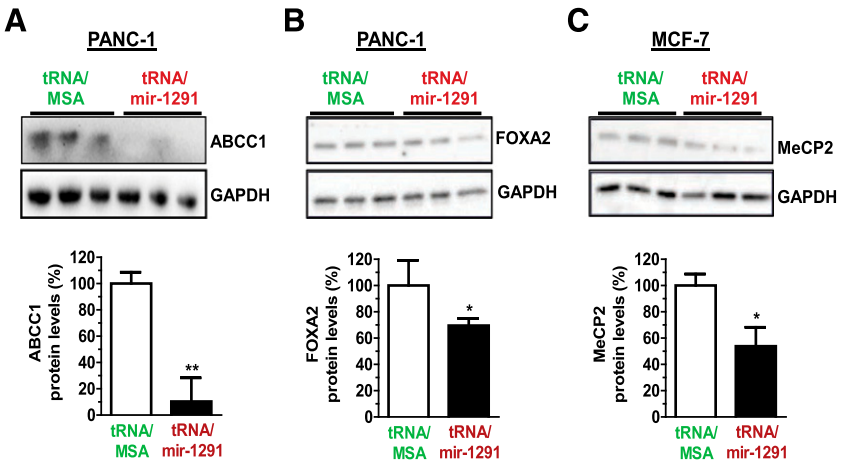


Fig. 4. Recombinant tRNA/mir-1291 is effective in regulating miR-1291 target gene expression in human carcinoma cells. ABCC1 (A), FOXA2 (B), and MeCP2 (C) protein levels were significantly reduced in human carcinoma cells at 48 hours post-transfection with 20 nM tRNA/mir-1291. Western blot analyses were conducted with selective antibodies. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Values are the mean ± S.D. of triplicate treatments. *$P < 0.05$ and **$P < 0.01$, compared with the control tRNA/MSA treatment.

decrease of MeCP2 in MCF-7 cells (Fig. 4C), as compared with control tRNA/MSA treatments. These results demonstrate that recombinant tRNA/mir-1291 is effective in regulating miR-1291 target gene expression in human carcinoma cells, which may be attributable to the actions of mature miR-1291 produced from chimeric tRNA/mir-1291 in the cells (Fig. 3).

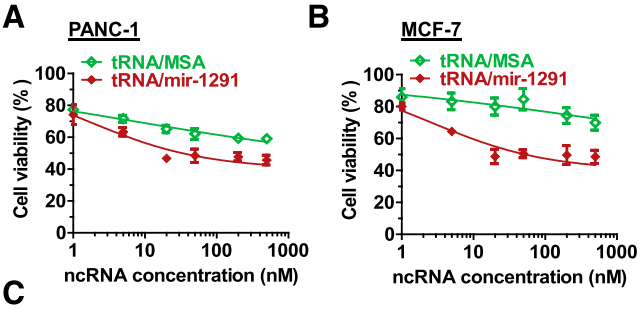
tRNA-Carried Pre-miR-1291 Suppresses the Growth of Human Carcinoma Cells. We thus assessed the impact of chimeric miR-1291 on cancer cell growth, because miR-1291 has been revealed to act as a tumor suppressor by our studies (Yu et al., 2013; Bi et al., 2014) and others (Hidaka et al., 2012; Yamasaki et al., 2013). Our pilot studies showed that, compared with the control tRNA/MSA, a greater extent of inhibition by tRNA/mir-1291 was shown at 48 hours post-transfection in MCF-7 cells and 72 hours post-treatment in PANC-1 cells. Thus, we examined the dose-response relationship of tRNA/mir-1291 in the suppression of MCF-7 and PANC-1 cell growth at 48 and 72 hours, respectively. Our data showed that recombinant tRNA/mir-1291 remarkably inhibited PANC-1 and MCF-7 cell proliferation in a dose-dependent manner and to a significantly ($P < 0.01$, two-way analysis of variance) greater degree than the control tRNA/MSA (Fig. 5, A and B). This was also manifested by lower EC_{50} values for tRNA/mir-1291 (2.59 ± 1.30 nM in MCF-7 cells and 1.61 ± 1.29 nM in PANC-1 cells) than those for tRNA/MSA (1160 ± 1026 nM in MCF-7 cells and 7.09 ± 1.22 nM in PANC-1 cells) (Fig. 5C). These results indicate that tRNA-carried pre-miR-1291 is functional to inhibit the proliferation of human cancer cells.

Chimeric tRNA/mir-1291 Sensitizes Human Carcinoma PANC-1 Cells to Doxorubicin. Because miR-1291 is able to enhance chemosensitivity through downregulation of ABCC1 transporter (Pan et al., 2013), and tRNA-carried pre-miR-1291 is effective in reducing ABCC1 protein levels in human carcinoma PANC-1 cells (Fig. 4A), we investigated if recombinant tRNA/mir-1291 could alter the sensitivity of ABCC1-overexpressing PANC-1 cells to doxorubicin, an ABCC1 substrate anticancer drug. Doxorubicin cytotoxicity against PANC-1 cells was determined by MTT assays after transfection with tRNA/mir-1291, the control tRNA/MSA, and vehicle. Our data showed that tRNA/mir-1291–transfected cells were more sensitive to doxorubicin than the control tRNA/MSA- and vehicle-treated cells (Fig. 6A). The improved sensitivity was also indicated by a significantly lower EC_{50} value in cells transfected with tRNA/mir-1291 (133 ± 21 nM) than tRNA/MSA (297 ± 42 nM) and vehicle control (325 ± 50 nM) (Fig. 6B). These results suggest that coadministration of recombinant tRNA/mir-1291 enhances the antiproliferative effects of doxorubicin.

Discussion

A new way to efficiently produce multimilligrams of chimeric pre-miR-1291 agents from 1 l of bacterial culture was demonstrated in this study, which utilizes a tRNA-based recombinant RNA technology. Pre-miR-1291 fused to the tRNA was protected against nucleolytic digestion in bacteria and thus accumulated to a high level for further purification. This stable tRNA scaffold was revealed to comprise a number of post-transcriptionally modified nucleosides, which were directly identified and mapped to specific sites through MS analyses of the purified ncRNAs. Our data also showed that tRNA-carried pre-miR-1291 was readily processed to mature miR-1291 in different types of human carcinoma cells, and consequently suppressed miR-1291 target gene expression (e.g., ABCC1), inhibited human MCF-7 and PANC-1 cell growth, and enhanced the chemosensitivity of PANC-1 cells.

Human miR-1291 is shown to regulate the expression of transporter ABCC1, modulate intracellular drug accumulation, and affect chemosensitivity (Pan et al., 2013). In addition, miR-1291 has been revealed to act as a tumor suppressor in various human carcinoma cell lines by

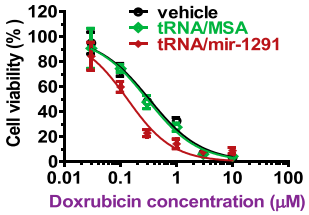


C

Cell line	ncRNA	EC50 (nM)	Hill slope	Goodness of fit (R^2)
PANC-1	tRNA/MSA	7.09 ± 1.22	-0.22 ± 0.02	0.90
	tRNA/mir-1291	1.61 ± 1.29*	-0.53 ± 0.07*	0.85
MCF-7	tRNA/MSA	1164 ± 1026	-0.19 ± 0.05	0.49
	tRNA/mir-1291	2.59 ± 1.30*	-0.53 ± 0.08*	0.83

Fig. 5. Chimeric miR-1291 is effective in suppressing human carcinoma cell proliferation. PANC-1 (A) and MCF-7 (B) cells were sensitive to recombinant tRNA/mir-1291 in a dose-dependent manner, to a significantly ($P < 0.01$, two-way analysis of variance) greater degree than tRNA/MSA. This is also indicated by the estimated EC_{50} and Hill slope values (C). Cell viability was determined with MTT assay. Values are the mean ± S.D. ($N = 3$) of triplicate treatments. *Significantly ($P < 0.05$) different from the control tRNA/MSA in the same cell line.

A



B

ncRNA	vehicle	tRNA/MSA	tRNA/mir-1291
Drug	Doxorubicin	Doxorubicin	Doxorubicin
EC50 (nM)	325 ± 50	297 ± 42	133 ± 21*
Hill slope	-0.94 ± 0.06	-0.96 ± 0.07	-1.07 ± 0.09
Goodness of fit (R^2)	0.97	0.96	0.94

Fig. 6. tRNA-carried pre-miR-1291 enhances the chemosensitivity of PANC-1 cells. (A) tRNA/mir-1291 significantly ($P < 0.01$, two-way analysis of variance) sensitized PANC-1 cells to doxorubicin, as compared with tRNA/MSA or vehicle treatment. (B) Estimated EC_{50} and Hill slope values for doxorubicin cytotoxicity in vehicle-, tRNA/MSA-, and tRNA/mir-1291–transfected PANC-1 cells. Cell viability was determined with a MTT assay. Values are the mean ± S.D. ($N = 3$) of triplicate treatments. *Significantly ($P < 0.05$) different from tRNA/MSA and vehicle treatment.

our studies (Yu et al., 2013; Bi et al., 2014) (unpublished data) as well as others (Hidaka et al., 2012; Yamasaki et al., 2013). Motivated by the idea of producing natural miRNA agents to perform miRNA actions, we made large efforts to biosynthesize pre-miR-1291 agents using the tRNA scaffold to examine miR-1291 biologic functions and therapeutic implications in vitro and in vivo (Li et al., 2014; Chen et al., 2015). The success and efficiency in producing recombinant RNAs relies on the structure and metabolic stability of target RNA in the organism. It is obvious that any target RNAs labile to bacterial RNase digestion are undoubtedly subjected to nucleolytic cleavage, and thus there will be limited or no accumulation of recombinant RNAs (e.g., pre-miR-27b) (Li et al., 2014; Chen et al., 2015). Recombinant pre-miR-1291 chimeras were revealed to be expressed successfully in a number of common *E. coli* strains, and a high-level accumulation was observed in HST08 and DH5α cells, similar to pre-miR-34a (Chen et al., 2015). Consistent with previous findings (Ponchon and Dardel, 2007; Ponchon et al., 2009), lower levels of recombinant RNAs were found for longer pre-miR-1291 species, which is presumably due to an increase of unstructured regions that are misfolded and/or cleaved by bacterial RNases. High-level expression also facilitated the purification of recombinant tRNA/mir-1291–123nt, which was consistently produced in multimilligram quantity from 1-l fermentation that would allow us to assess miR-1291 activities in vivo, as we have done with other efficiently expressed ncRNAs (Chen et al., 2015) (unpublished data). Despite that the purification yields using Sephadex G-100 were relatively low (e.g., 2–3%), this method is simple, and the Sephadex-tagged recombinant ncRNAs that failed to bind to Sephadex in the first round (e.g., flow through and wash 1–3) may be combined for repurification to offer a much improved total yield (e.g., 5–10%) close to anion exchange fast protein liquid chromatography method (Li et al., 2014; Chen et al., 2015).

Post-transcriptional modification of RNA molecules is ubiquitous in a living organism and greatly increases the chemical diversity. As a result, the modified RNAs may exhibit different structures via alternative folding, as well as physicochemical properties and biologic functions. Over 100 different base modifications such as methylation, pseudouridylation, thiolation, and reduction of uracil to dihydrouracil have been identified for various classes of natural RNAs, and around 85% are present in tRNAs (Limbach et al., 1994; Cantara et al., 2011). Using mass spectrometry analyses of isolated tRNA/mir-1291 and tRNA/MSA, we were able to determine the modified nucleosides and map them to the specific sites in the tRNA scaffold. A number of modified nucleosides occurred on the D- and T-loops, including D20, D21, [m^7G]77, [acp^{3}U]78, [m^5U]85, and ψ86, which may be important for the stability (Alexandrov et al., 2006) and function of natural methionyl tRNA. Nevertheless, not all modifications detected by LC coupled with UV and MS detection–based nucleoside analysis could be assigned to the recombinant ncRNAs, which might be attributed to the small fraction of copurified bacterial tRNAs (Hossain and Limbach, 2007, 2009).

In the whole cell system, a 3 orders of magnitude increase of pre-miR-1291 levels was shown at 72 hours post-transfection, highlighting a successful introduction of tRNA/mir-1291 chimera into human cells and most importantly a favorable stability of recombinant ncRNAs within human cells. Consequently, mature miR-1291 levels were increased by 2 orders of magnitude, indicating a successful processing of tRNA/mir-1291 to mature miR-1291. It is noteworthy that all recombinant ncRNAs, such as tRNA/mir-27 (Li et al., 2014), tRNA/mir-1291 (this study), and tRNA/mir-34a (Chen et al., 2015) (unpublished data), showed favorable cellular stability and selective generation of target mature miRNAs, suggesting that tRNA carrier also offers a "stealth delivery" of target miRNAs into human cells beyond the production of chimeric ncRNAs in bacteria. Nevertheless, further studies are warranted to define the specific RNases involved in the processing of recombinant ncRNAs in human cells.

The production of mature miR-1291 from chimeric RNAs led to a significant suppression of protein levels of miR-1291 target genes such as *ABCC1* and *FOXA2* in PANC-1 cells, and *MeCP2* in MCF-7 cells. Overexpression of efflux transporter ABCC1 is associated with multidrug resistance (Filipits et al., 2005; Haber et al., 2006; Faggad et al., 2009), and downregulation or inhibition of such transporters may represent an effective means to overcome multidrug resistance (Choi and Yu, 2014). Consistent with our recent studies (Pan et al., 2013), tRNA-carried pre-miR-1291 was able to suppress ABCC1 protein expression, and consequently led to an improved sensitivity of PANC-1 cells to doxorubicin. On the other hand, MeCP2 is the first methyl-cytosine-phosphate-guanine–binding domain protein discovered in its family which acts as a chromatin regulator of transcription and is encoded by the gene mutated in the neurodevelopmental disorder Rett syndrome (LaSalle and Yasui, 2009). MeCP2, usually overexpressed in human carcinomas, promotes the growth and invasiveness of many types of cancer cells, including breast carcinoma cells (Billard et al., 2002; Ray et al., 2013), and may modulate ABCB1/MDR1 expression (El-Osta and Wolffe, 2001). FOXA2, a transcription factor belonging to the forkhead class of DNA-binding proteins, is revealed as an important regulator in promoting pancreatic/hepatic/colorectal cell differentiation and organ development (Gao et al., 2008; Song et al., 2010) and regulating the expression of some (proto-)oncogenes (Zhang et al., 2010) and a number of ABC transporters (Bochkis et al., 2008, 2012). Therefore, reduction of MeCP2 and FOXA2 protein levels may at least partially provide an explanation for miR-1291 in suppressing cancer cell proliferation (Yu et al., 2013; Bi et al., 2014) and enhancing chemosensitivity. Together, these findings indicate the potential utility of recombinant miR-1291 agent for examining miR-1291 functions and sensitizing human carcinoma cells to anticancer drugs.

In summary, this study demonstrated a rapid and efficient method for the production of multimilligrams of chimeric miR-1291 agents from 1 l of bacterial culture in a research laboratory setting, which was achieved by using a methionyl tRNA scaffold. Our data showed the tRNA scaffold consisted of a number of natural post-transcriptional modifications, and the tRNA-carried pre-miR-1291 was effective in

modulating miR-1291 target gene (e.g., ABCC1) expression in human carcinoma cells and improving chemosensitivity. The results are expected to offer clues to the production of new miRNA agents for studying pharmacoepigenetics and developing ncRNA therapeutics.

Authorship Contributions

Participated in research design: Yu, Li, Addepalli, Tu, Limbach, LaSalle, Zeng, Huang.

Conducted experiments: Li, Addepalli, Tu, Chen, Wang.

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

Performed data analysis: Li, Addepalli, Tu, Chen, Wang, Limbach, LaSalle, Zeng, Huang, Yu.

Wrote or contributed to the writing of the manuscript: Yu, Li, Addepalli, Tu, Chen, Wang, Limbach, LaSalle, Zeng, Huang.

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