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Bioengineering of a single long noncoding RNA molecule that carries multiple small RNAs

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Abstract

Noncoding RNAs (ncRNAs), including microRNAs (miRNAs), small interfering RNAs (siRNAs), and long noncoding RNAs (lncRNAs), regulate target gene expression and can be used as tools for understanding biological processes and identifying new therapeutic targets. Currently, ncRNA molecules for research and therapeutic use are limited to ncRNA mimics made by chemical synthesis. We have recently established a high-yield and cost-effective method of producing bioengineered or biologic ncRNA agents (BERAs) through bacterial fermentation, which is based on a stable tRNA/pre-miR-34a carrier (~ 180 nt) that accommodates target small RNAs. Nevertheless, it remains a challenge to heterogeneously express longer ncRNAs (e.g., > 260 nt), and it is unknown if single BERA may carry multiple small RNAs. To address this issue, we hypothesized that an additional human pre-miR-34a could be attached to the tRNA/pre-miR-34a scaffold to offer a new tRNA/pre-miR-34a/pre-miR-34a carrier (~ 296 nt) for the accommodation of multiple small RNAs. We thus designed ten different combinatorial BERAs (CO-BERAs) that include different combinations of miRNAs, siRNAs, and antagomirs. Our data showed that all target CO-BERAs were successfully expressed in *Escherichia coli* at high levels, greater than 40% in total bacterial RNAs. Furthermore, recombinant CO-BERAs were purified to a high degree of homogeneity by fast protein liquid chromatography methods. In addition, CO-BERAs exhibited strong anti-proliferative activities against a variety of human non-small cell lung cancer cell lines. These results support the production of long ncRNA molecules carrying different warhead small RNAs for multi-targeting which may open avenues for developing new biologic RNAs as experimental, diagnostic, and therapeutic tools.

Keywords

Noncoding RNA; microRNA; siRNA; Bioengineering; Lung cancer

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Ethical statement The authors confirm that the article does not contain any studies with human participants or animals.

Conflict of interest The authors are named inventors of patent applications related to RNA bioengineering technology and therapeutics that are owned by the UC Davis, and Dr. Yu is a founder of AimRNA, Inc., which has an agreement to license the intellectual property. All other authors declare that they have no conflict of interest.

Introduction

Small noncoding RNAs, including miRNAs derived from the genome and siRNAs introduced exogenously, can govern target gene expression through translational inhibition and/or mRNA degradation mechanisms (Ambros 2004; Esteller 2011). Further research on functional ncRNAs supports their important roles in the etiology and progression of diseases which may be translated into novel diagnostics and therapeutics (Levin 2019; Yu et al. 2019). Currently, ncRNA agents used for research and development are mainly produced by chemical synthesis or in vitro transcription (Bramsen and Kjems 2012; Khvorova and Watts 2017; Yu et al. 2019), besides those using plasmid- or virus-based materials (Liu and Berkhout 2011). However, the latter consists of DNA molecules that depend upon host cell integration and procession to generate target ncRNAs. Synthetic RNA molecules are readily accessible, whereas chemo-engineered RNA mimics contain large amounts and various types of chemical modifications (Bramsen and Kjems 2012; Khvorova and Watts 2017; Yu et al. 2019). It is also noteworthy that chemo-engineered RNA molecules with the same sequences of nucleobases obtained from different companies are actually distinct molecules as each preferably carries different degrees and/or types of chemical modifications at different positions. These modifications might not even be disclosed to investigators, except those made through custom synthesis. By contrast, in vitro transcription with a DNA template and RNA polymerase follows natural means to produce RNA molecules, whereas it lacks other cellular machineries required for post-processing and modification of the resulting RNAs. As a result, synthetic RNA molecules made in test tubes via chemical or enzymatic reactions undoubtedly exhibit their own physicochemical and biological properties and differ explicitly from natural RNAs transcribed from the genome and folded in living cells (Ho and Yu 2016; Yu et al. 2019).

With the demand for biologic RNA molecules that resemble natural RNAs formed and folded in living cells, production of ncRNAs via microbial fermentation holds great potential for a wide variety of applications. This process may revolutionize biological and medical research as well as drug development similar to how recombinant DNA and protein engineering technologies did (Leader et al. 2008; Rosano and Ceccarelli 2014). There is also growing interest in fermentation production or combinatorial biosynthesis of small molecules for the development of new drugs (Hutchinson 1998; Knight et al. 2003) and biofuels (Zhang et al. 2019). However, heterogeneous overexpression of target RNAs has been challenging because RNA molecules are highly susceptible to RNases within host microorganisms or cells and thus becomes hard to accumulate significant levels. Using a tRNA (Gaudin et al. 2003; Ponchon et al. 2009; Ponchon and Dardel 2007) or rRNA (Liu et al. 2010; Zhang et al. 2009) scaffold has proved to mitigate some challenges and permit heterogeneous expression of a number of recombinant RNA molecules through microbial fermentation (Chen et al. 2015; Gaudin et al. 2003; Li et al. 2015; Nelissen et al. 2012; Paige et al. 2011, 2012; Pitulle et al. 1995), whereas most target chimeric RNAs and the tRNA itself are revealed to be unexpressed or accumulated to a negligible level (Chen et al. 2015; Ho et al. 2018). Following the identification of unique hybrid tRNA/pre-miRNA molecules, a novel tRNA/pre-miRNA-based technology has been established for a consistent, high-yield, and large-scale production of bioengineered or biologic RNA agents (BERAs) that

carry specific miRNAs, siRNAs, or other small RNA warheads. For example, tRNA/pre-miR-34a, in which miR-34a duplexes can be substituted by another miRNA or siRNA of interest, achieves a surprisingly high-level expression and accumulation of target BERAs in *Escherichia coli* (Chen et al. 2015; Ho et al. 2018). Further studies have demonstrated that BERAs, purified from total bacterial RNAs by spin columns or anion exchange fast protein liquid chromatography (FPLC) methods, are functional in human cells to specifically release target miRNA or siRNA for the modulation of gene expression and cellular processes including cancer cell proliferation, tumor progression, and metastasis (Alegre et al. 2018; Chen et al. 2015; Ho et al. 2018; Jian et al. 2017; Jilek et al. 2019; Li et al. 2018,2019; Tu et al. 2019; Zhao et al. 2016).

As novel ncRNA bioengineering technology opens new avenues for basic and translational research (Ho and Yu 2016; Pereira et al. 2017; Yu et al. 2019), high-yield expression of target RNAs (e.g., accounting for > 20% of total bacterial RNA) has been limited to those shorter than 260 nt. This is likely attributed to the increase of unstructured regions within a long ncRNA molecule (e.g., > 260 nt) that are misfolded and readily degraded by bacterial RNases. Furthermore, it is unknown whether a single BERA is able to carry multiple small RNAs. Therefore, in this study, we aimed to challenge the tRNA/pre-miRNA-based biotechnology by incorporating another pre-miR-34a into the tRNA/pre-miR-34a carrier, which led to a new tRNA/pre-miR-34a/pre-miR-34a entity, approximately 300 nt in length, for the accommodation of multiple small RNAs. After designing ten different combinatorial BERAs (CO-BERAs) that are comprised of various combinations of miRNAs, siRNAs, and antagomirs, we constructed individual CO-BERA expression plasmids. Further studies demonstrated that all CO-BERAs were successfully overexpressed in *E. coli* at extraordinarily high levels, greater than 40% of total bacterial RNAs. After being purified to a high degree of homogeneity by FPLC methods and introduced to human carcinoma cells, recombinant CO-BERAs exhibited potent anti-proliferative activities against a panel of human non-small cell lung cancer (NSCLC) cell lines. These findings establish the feasibility of fermentation production of a single biologic RNA molecule containing two warhead small RNAs that hold promise for multi-targeting.

Materials and methods

Bacterial culture

E. coli strains DH5 α (Thermo Fisher Scientific, Waltham, MA) and HST08 (Clontech Laboratories, Mountain View, CA) were grown in Luria broth (LB) for plasmid preparation and 2XYT media for RNA production, respectively. The media were supplemented with 100 μ g/ml ampicillin.

Human cell culture

Human lung carcinoma cell lines A549 (ATCC: CRM-CCL-185), H1975 (ATCC: CRL-5908), H23 (ATCC: CRL-5800), H1650 (ATCC: CRL-5883), and H1299 (ATCC: 5803) were purchased from American Type Culture Collection (Manassas, VA). Cell lines were maintained in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% fetal

bovine serum, 100 U/ml penicillin sodium, and 100 µg/ml streptomycin sulfate (Thermo Fisher Scientific) grown at 37 °C in a humidified atmosphere with 5% CO₂.

Construction of CO-BERA expression plasmids

The sequences of all miRNAs were obtained from miRBase (<http://www.mirbase.org/>), and the optimized pre-miR-34a as well as anti-miR-21, NRF2-siRNA, let-7c, and miR-124 sequences were adopted from our recently study (Ho et al. 2018; Li et al. 2018). CO-BERA sequences (Table 1) were generated by substituting miR-34a duplexes with target miRNA or siRNA sequences, as illustrated in Fig. 1a, and corresponding coding sequences were synthesized in the pUC57 vector by GenScript Corporation (Piscataway, NJ). Target inserts were released from the plasmids after digestion with *EcoRI* and *PstI* (New England Biolabs, Ipswich, MA). Following gel purification using USB PrepEase Gel Extraction Kit (Affymetrix, Inc. Cleveland, OH), each insert was ligated to the *EcoRI*- and *PstI*-digested vector pBSTNAV (Ho et al. 2018; Ponchon et al. 2009) with T4 Rapid Ligation Kit (Thermo Fisher Scientific). The plasmids were then transformed into DH5α competent cells and selected with ampicillin. Colonies were expanded, and CO-BERA expression plasmids were isolated with a Miniprep Kit (Qiagen, Hilden, Germany). All target CO-BERA expression plasmids were confirmed by sequencing analysis (GenScript, Piscataway, NJ).

Expression of recombinant CO-BERA in *E. coli*

Plasmids with confirmed sequences were transformed into HST08 as previously described (Ho et al. 2018; Li et al. 2014; Wang et al. 2015). Total RNAs were extracted by a Tris-HCl-saturated phenol extraction method, quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and analyzed for recombinant CO-BERA expression by separating 0.1 µg total bacterial RNA per lane on a denaturing urea (8 M) PAGE (8%) gel with a RiboRuler low-range RNA ladder (Thermo Fisher Scientific) for reference. PAGE gels were stained with ethidium bromide and imaged using the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

Purification of CO-BERAs by fast protein liquid chromatography

CO-BERAs were purified from total RNAs using an NGC Quest 10 Plus Chromatography fast protein liquid chromatography (FPLC) system (Bio-Rad, Hercules, CA). All CO-BERAs were initially purified using ENrich-Q 10 × 100 (Bio-Rad, Hercules, CA). FPLC fractions were analyzed by urea-PAGE to verify RNA separation and purity. Fractions containing target CO-BERA were pooled, precipitated in ethanol, and desalted/concentrated in nuclease-free water using an Amicon Ultra 2-ml centrifugal filter (30 kDa; EMD Millipore, Billerica, MA). In some cases, the concentrated CO-BERAs contained some impurities and were subjected for a second purification using either ENrich-Q 10 × 100, Bio-Scale Mini Macro-Prep DEAE, or Bio-Scale Mini CHT Type II (Bio-Rad, Hercules, CA) depending on which column yielded the purest product. Likewise, the fractions were assessed by urea-PAGE analysis, and target fractions were thus combined, desalted, and concentrated.

RNA separation on ENrich-Q 10 × 100 and Bio-Scale Mini Macro-Prep DEAE was conducted with buffer A (10 mM sodium phosphate, pH 7.0) and buffer B (buffer A + 1 M

sodium chloride, pH 7.0) while Bio-Scale Mini CHT Type II was achieved by using buffer C (5 mM sodium phosphate, pH 7.0) and buffer D (150 mM sodium phosphate, pH 7.0). FPLC traces were monitored at 260/280 nm using a UV-visible detector, and fractions were collected accordingly. Specifically, around 5–10 mg of RNA was loaded onto the ENrich-Q column and separated through a gradient elution at a flow rate of 2 ml/min, i.e., 100% buffer A for 4 min, followed by 55% buffer B for 10 min, a gradient of 55–72% buffer B for 20 min, 72–74% buffer B for 8 min, 100% buffer B for 10 min, and then 100% buffer A for 10 min. Around 5 mg of RNA was loaded onto the Bio-Scale Mini Macro-Prep DEAE for separation by gradient elution at 2 ml/min, i.e., 100% buffer A for 12 min, then switched to 50% buffer B for 5 min, a gradient of 50–60% buffer B for 25 min, 60–75% buffer B for 10 min, 100% buffer B for 5 min, and finally 100% buffer A for 5 min. Lastly, around 0.5 mg RNA was loaded onto the Bio-Scale Mini CHT Type II for separation by gradient elution at 2.5 ml/min, i.e., 100% buffer C for 2 min, followed by 73–90% buffer D for 18 min, 100% buffer D for 5 min, then 100% buffer C for 5 min.

Quantitative measurement of the purity of FPLC-isolated CO-BERA

The purity of FPLC-isolated RNA was quantitatively determined by an optimal high-performance liquid chromatography (HPLC) method with a XBridge® Oligonucleotide BEH C₁₈ column (2.1 × 50 mm, 2.5-μm particle size; Waters, Milford, MA) on a Shimadzu LC-20AD HPLC system, as we described previously (Wang et al. 2015).

Endotoxin quantification

Endotoxin levels in FPLC-purified CO-BERAs were quantitated by using Limulus Amebocyte Lysate Pyrogen-5000 kinetic assay (Lonza, Walkersville, MD), following the manufacturer's instructions. In brief, a SpectraMax3 plate reader (Molecular Devices, Sunnyvale, CA) was used to measure the absorbance at a 340-nm wavelength. Endotoxin standards provided in the kit were used to generate a standard curve, and endotoxin levels were calculated as endotoxin units (EU)/microgram RNA.

Cell viability assay

Cells were seeded at 3000 or 5000 cells per well in a 96-well plate, and after overnight incubation, cells were transfected with 15 nM ncRNA or control tRNA using Lipofectamine 3000 (Thermo Fisher Scientific) as well as empty Lipofectamine 3000 as vehicle control. Cell viability was measured using MTT assay 72 h post-transfection, as we described (Ho et al. 2018; Jilek et al. 2019; Wang et al. 2015). All experiments were carried out in triplicate and repeated at least once in separate cultures.

Results

Design of CO-BERAs and construction of plasmids

Using the novel tRNA/pre-miRNA-based ncRNA bioengineering technology, we were able to produce a variety of target BERAs in milligram quantities from 1 liter of bacterial culture (Chen et al. 2015; Ho et al. 2018; Li et al. 2014, 2019; Wang et al. 2015). However, it was unknown if this technology would allow us to produce an RNA molecule longer than 260 nt that carries multiple-target small RNAs. To address this issue, we designed ten new CO-

BERA molecules (Table 1) based on our BERA technology, where another pre-miR-34a was fused consecutively onto the tRNA/pre-miR-34a carrier, and the resulting tRNA/pre-miR-34a/pre-miR-34a carrier, around 296 nt in length, permitted the loading of two target small RNAs through substituting intrinsic miR-34a duplexes (Fig. 1a). To increase the diversity of CO-BERAs, our designs included either a human serine or leucine tRNA followed by two optimized human pre-miR-34a (Ho et al. 2018) carrying different combinations of NRF2-siRNA, anti-miR-21–5p, let-7c-5p, miR-124–3p, and miR-34a-3p (Table 1). Corresponding coding sequences were synthesized and cloned into the pUC57 vector and consequently subcloned into the target pBSTNAV vector (Ho et al. 2018; Li et al. 2014; Ponchon et al. 2009; Ponchon and Dardel 2007), which were confirmed by sequencing before proceeding to heterogeneous expression in HST08 *E. coli*.

All target CO-BERAs are highly expressed in *E. coli*

To determine if long ncRNA CO-BERA can be overexpressed heterogeneously, total RNA was extracted from *E. coli* transformed with individual CO-BERA expression plasmids and analyzed by urea-PAGE. The results (Fig. 1b) showed that all ten CO-BERAs were successfully expressed in bacteria, as manifested by the appearance of new corresponding RNA bands when compared with the wild-type bacteria. Interestingly, these CO-BERAs in similar lengths (Table 2) undoubtedly contain different secondary and higher-order structures as exhibited by variable levels of PAGE mobility (Fig. 1a), similar to our findings on BERAs (Ho et al. 2018; Jilek et al. 2019). While the expression levels of these CO-BERAs also varied slightly, each accounted for over 40% of total bacterial RNAs, as estimated from the intensities of RNA bands (Fig. 1b) as well as more quantitatively from the FPLC peak areas (Fig. 2a). In addition, the amounts of total RNAs extracted from 1 liter of bacterial culture were variable between CO-BERAs, ranging from approximately 50 mg for htRNA^{Leu}/miR-124/miR-34a to 12.6 mg for htRNA^{Ser}/let-7c/miR-124 (Table 2).

CO-BERAs are purified to a high degree of homogeneity by FPLC methods

To isolate recombinant CO-BERAs from bacterial RNAs, we sought to optimize the anion exchange FPLC method utilized for the purification of BERAs (Ho et al. 2018). Through elution with a refined salt gradient for a longer period of time (Fig. 2a), we were able to purify target CO-BERAs from bacterial RNAs to a high degree of homogeneity in a single run with six out of ten CO-BERAs greater than or equal to 99.0% pure (Table 2), as quantitatively determined by HPLC method and evaluated by urea-PAGE analysis (Fig. 3). Nevertheless, the other four CO-BERAs were less than 90% pure after single-run FPLC separation with the strong anion exchange column and thus processed for further purification. Re-purification using either the same strong anion exchange column or a weak anion exchange column (Fig. 2b, c) offered satisfactory 95–97% pure CO-BERAs (Table 2). CO-BERAs htRNA^{Ser}/let-7c/miR-124 and htRNA^{Leu}/let-7c/miR-124 turned out to be extremely hard to purify; and re-purification with a strong anion exchange (Fig. 2b) or ceramic hydroxyapatite column (Fig. 2d) resulted in 96.7% pure htRNA^{Leu}/let-7c/miR-124 and 92.3% pure htRNA^{Ser}/let-7c/miR-124 molecules (Table 2). Meanwhile, overall yields were also lower following two-column purifications, in addition to its association with a lower amount of total RNA from 1 liter of bacterial fermentation (Table 2). By contrast, the majority of target CO-BERAs purified with single-run strong anion exchange FPLC method

were over 10 mg from 1 liter of bacterial culture, and 4–7 mg was usually obtained when re-purification was conducted (Table 2). In addition, FPLC-purified CO-BERAs exhibited low endotoxin activities (Table 2), as measured by the Limulus Amebocyte Lysate Pyrogen-5000 kinetic assay. As one CO-BERA htRNA^{Ser}/NRF2-siRNA/miR-34a had an endotoxin level of 6.32 EU/μg RNA, all other CO-BERAs showed an endotoxin activity less than 4 EU/μg RNA. Together, these results demonstrated a successful large-scale purification of recombinant CO-BERAs by single- or multi-column FPLC methods that generally offer multi-milligrams of over 95% pure CO-BERAs with minimal endotoxin activities from 1 liter of bacterial fermentation.

Biologic CO-BERA molecules inhibit human NSCLC cell viability

We thus assessed and compared the anti-proliferation activities of these recombinant CO-BERAs against human NSCLC cells. A panel of five different NSCLC cell lines, namely, A549, H1975, H23, H1650, and H1299, was chosen to represent a variety of genetic backgrounds and simulate the heterogeneity of NSCLC. Cell variability was determined at 72 h after transfection with 15 nM of individual CO-BERAs, control tRNA (namely, LSA and SSA), or vehicle. Our data (Fig. 4a–e) showed that all CO-BERAs showed remarkable anti-proliferation activities in these NSCLC cell lines, as compared with vehicle and tRNA controls. The same CO-BERA exhibited variable levels of suppression of the viability of different cell lines, while different CO-BERAs demonstrated variable degrees of inhibition of the same cell line (Fig. 4). Among them, htRNA^{Leu}/miR-34a/miR-124 and htRNA^{Leu}/let-7c/miR-124 consistently exhibited the greatest extents of anti-proliferation activities against all NSCLC cell lines (e.g., > 80% inhibition of A549 cells and > 50% suppression of all others), which may be pursued for future studies.

Discussion

A new microbial fermentation-based method was established in this study which for the first time achieved high-level heterogeneous expression of novel long ncRNA molecules around 300 nt in length, namely, CO-BERAs, carrying two small RNAs warheads. CO-BERAs were designed by utilizing the unique stable tRNA/pre-miR-34a scaffold, which we identified recently (Chen et al. 2015; Ho et al. 2018), to assemble another human pre-miRNA for the accommodation of additional small RNAs for multi-targeting purposes. All ten CO-BERAs, consisting of different combinations of NRF2-siRNA, miR-34a, miR-124, let-7c, and anti-miR-21, were successfully expressed in the common *E. coli* strain HST08, each accounting for greater than 40% of total bacterial RNA. These small RNAs were chosen for their tumor suppressive properties in NSCLC, and our results suggest that this approach may be employed to accommodate other small RNAs of interest. The majority of recombinant CO-BERAs could be purified to a high degree of homogeneity, generally greater than 99% pure as quantified by HPLC and less than 3 EU/μg RNA endotoxin activity as determined by Limulus Amebocyte Lysate kinetic assay, through the single-run strong anion exchange FPLC method while some others required re-purification and thus showed variable overall yields. While biologic CO-BERAs exhibited potent anti-proliferative activities against a panel of human NSCLC cell lines, further studies are highly warranted to define their multi-targeting mechanisms and effectiveness in controlling tumor progression in animal models.

Bioengineered or recombinant RNA molecules as well as CO-BERAs described in this study are made and folded in living cells, distinguished from chemo-engineered RNA mimics with extensive and various modifications (Bramsen and Kjems 2012; Ho and Yu 2016; Khvorova and Watts 2017; Yu et al. 2019) that have been dominating RNA research and drug development. Interestingly, protein research has been directly ruled by bioengineered or recombinant proteins produced and folded in living cells rather than synthetic polypeptides or proteins, which has proved to be extremely successful in understanding protein structures and functions and developing novel protein therapeutics (Leader et al. 2008). It is also noted that synthetic DNAs or genes, which have become popular in genetic research (Schindler et al. 2018), are actually not comprised of any chemical modifications. Therefore, there is a need to develop novel technologies, especially microbial fermentation-based methods, for the production of biologic RNA molecules (Ho and Yu 2016; Pereira et al. 2017; Yu et al. 2019) that allow for cellular machineries to recognize and perform post-transcriptional modification and processing to necessary structures and folding. Indeed, recombinant RNAs or BERAs have none or just minimal post-transcriptional modifications, such as pseudouridine (Gaudin et al. 2003; Li et al. 2015; Nelissen et al. 2012; Ponchon et al. 2009; Ponchon and Dardel 2007; Ranaei-Siadat et al. 2014; Wang et al. 2015), which are necessary to resemble natural RNAs and pose intrinsic secondary and high-order structures. Furthermore, bioengineered RNA molecules produced heterogeneously in microbial fermentation have been demonstrated to be biologically functional in vitro and in vivo by various studies (Chen et al. 2015; Ho et al. 2018; Jian et al. 2017; Jilek et al. 2019; Li et al. 2014, 2015, 2018, 2019; Liu et al. 2010; Nelissen et al. 2012; Paige et al. 2011, 2012; Pereira et al. 2016a, b; Pitulle et al. 1995; Tu et al. 2019; Wang et al. 2015; Zhang et al. 2009; Zhao et al. 2016). In addition, although we cannot have a direct comparison of the costs in producing the same amounts of equally pure (e.g., > 98%) chemo- and bio-engineered RNA agents, RNA bioengineering technology is proved to be cost-effective in consistent large-scale production of high-purity target RNAi molecules for research and development (Yu et al. 2019).

In this study, we were able to achieve consistent, high-level expression of long ncRNA molecules around 300 nt in length via bacterial fermentation as previous research only offered ncRNAs less than 260 nt. The approach also allowed us to assemble two targeted small RNAs into a single long CO-BERA that may be employed for multi-targeting. Five warhead small RNAs, miR-34a, miR-124, let-7c, NRF2-siRNA, and anti-miR-21, were selected for their anti-tumor activities in NSCLC. Tumor-suppressive miRNAs miR-124, miR-34a and let-7c that target many oncogenes such as STAT3, CDK4/6, and RAS (Hatziaepostolou et al. 2011; Johnson et al. 2005; Sun et al. 2008) are commonly dysregulated in NSCLC tissues or cells due to chromosomal aberrations or methylations (Hermeking 2010; Lin et al. 2010). In contrast, miR-21 that targets tumor suppressive genes such as PTEN and PDCD4 (Asangani et al. 2008; Meng et al. 2007) is usually overexpressed in NSCLC. Nuclear factor erythroid-2-related factor-2 (NRF2) is constitutively activated in NSCLC through a variety of mechanisms and plays an important role in cell proliferation and chemosensitivity (Bar-Peled et al. 2017; Yamadori et al. 2012). Restoration of tumor-suppressive miRNAs and inhibition of tumor-promoting RNAs through miRNA and antagomir agents, respectively, represent new strategies to treat cancer. Indeed, these CO-

BERAs showed strong anti-proliferative activities against all human NSCLC cell lines tested, whereas the underlying multi-targeting mechanisms warrant further verification.

As each CO-BERA accounted for greater than 40% of total bacterial RNA, individual CO-BERAs led to variable amounts of total RNA from the same volume of microbial fermentation, which may be related to CO-BERAs' structures, stabilities, and biological properties. An interesting observation is that, besides the docked small RNAs, tRNA seems to influence the yield of total RNA. Six CO-BERAs were produced with a leucine tRNA scaffold and four with serine tRNA. The average amount of total RNA extracted for the leucine tRNA-assembled CO-BERAs was 29.8 mg/L bacterial culture while the average amount for the serine tRNA-containing CO-BERAs was 17.5 mg/L. This is presumably due to the difference in their stabilities and/or possible toxicities to host bacteria as CO-BERAs are accumulated in *E. coli*. Furthermore, the order of small RNAs in a CO-BERA does not seem to affect the yield of total RNA as htRNA^{Leu}/miR-34a/miR-124 and htRNA^{Leu}/miR-124/miR-34a offered similar amounts of total RNAs per liter bacterial culture. Understanding the impact of different factors and their underlying mechanisms would facilitate improvement of RNA bioengineering technology and production of CO-BERAs.

Purification of CO-BERAs was achieved by using single or multi-column FPLC methods. Most CO-BERAs were 99% pure after single-run FPLC separation, yielding multi-milligrams of ready-to-use CO-BERAs from 1 liter of bacterial culture. As others required further purification on additional column, their overall yields were also lower. An extra band was obvious in the urea-PAGE gel in some of the less pure CO-BERAs, which was not visible in the untransformed *E. coli*. This band might represent an altered form of CO-BERA such as a truncated, post-transcriptionally modified, or alternately folded species, or simply a bacterial RNA that is upregulated due to the transformation with CO-BERA expression plasmid. Further investigation, such as RNA sequencing, may be needed to identify the nature of such "impurities." Alternative methods may be explored or current methods may be refined to yield purer products required for more extensive structural and functional studies.

In summary, we have established a new approach to produce novel single ncRNA molecule around 300 nt in length bearing multiple warhead small RNAs that holds promise for multi-targeting. This method can be readily adapted for the production of milligram quantities of target CO-BERAs from 1 liter of bacterial culture within a few days. Most importantly, CO-BERAs are produced and folded in living cells and thus may better capture the properties of cellular RNAs. As such, this unique multiplexing of biologic RNAs shall be an invaluable addition to current tools for broad biomedical research including but not limited to the investigation of cellular regulatory mechanisms and development of ncRNA therapeutics.

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Fig. 1.

Novel combinatorial bioengineered RNA agent (CO-BERA) is highly expressed in *E. coli* HST08. **a** Illustration of the designed CO-BERA (~ 297 nt in length) which consists of two optimized pre-miR-34a sequences preceding each of the inserts, different from BERA (~ 180 nt in length) that consists of only one pre-miR-34a. Red represents target sequence and green represents complementary sequence. **b** Denaturing urea PAGE analysis of total RNA extracted from the HST08 cells transformed with plasmids encoding individual CO-BERAs, with a ladder and wild-type (WT) HST08 cells for references. Successful overexpression of each target CO-BERA is demonstrated by the appearance of a new strong band in transformed HST08 cells, as compared with the WT cells

Fig. 2.

FPLC purification of CO-BERAs using one or two columns in sequence. **a** CO-BERAs were separated from total bacterial RNA on an anion exchange ENrich Q column. A few CO-BERAs requiring re-purification were proceeded to further FPLC separation by using either **b** ENrich Q again, **c** DEAE, or **d** CHT Type II. Shown are representative FPLC traces

a) Isolated CO-BERAs **b)** **Fig. 3.**

Verification of the purity of FPLC-isolated CO-BERAs by gel electrophoresis analysis and quantitatively by HPLC method. **a** PAGE analysis showed that majority of the isolated CO-BERAs are very pure with no or minimal impurity. **b** HPLC analysis quantitatively determined the purities of individual CO-BERAs, among which most CO-BERAs are around 99% pure and only one CO-BERA is < 95% pure (see Table 2 for details)

Fig. 4.

Anti-proliferative activities of CO-BERAs against various human NSCLC cell lines. Cell viability of five different NSCLC cell lines (a–e) was reduced by 15 nM of purified CO-BERAs to various degrees, as compared with Lipofectamine 3000 only (vehicle), leucine (LSA), or serine (SSA) tRNA controls. Data were normalized to vehicle control. Values are mean $\pm$ SD ($N = 3$). * $P < 0.05$, as compared with vehicle, LSA, and SSA controls (1-way ANOVA with Bonferroni post-tests)

Table 1

Sequences of individual CO-BERAs designed and heterogeneously expressed in this study

Name	Sequence (underlined: tRNA, bold: sequence relating to insert #1, green: target miRNA/siRNA, red: complementary sequence)
htRNA ^{Leu} /miR-34a/miR-124	<p>ACCAGGAUGGCCGAGUGGUUAAAGGCGUUGGACUGGCCAGCUGUG</p> <p><u>AGUGUUUCUU</u>UAGGACAGUGUUUAGCUGGUGUUUGUUGAGCGGCCA</p> <p>GCUGAGUGUUUCUUAAAGGACACGCGUGAAUGCCGUUGUGAG</p> <p>CAAUAGUAAGGAAGCGUGUUCGCGUGUGCCUUCUAGAAGUGC</p> <p>UGCACGUUGUGGCCCGUAAGGAAGCAACAGCAAGUAUCUGGCC</p> <p><u>UAGAAGUGCUGACAGUUGUUGGCCCGAUCCAAUGGACAU AUGUC</u></p> <p><u>CGCGUGGUUCGAACCCACUCCUGGUACCA</u></p>
htRNA ^{Leu} /miR-124/miR-34a	<p>ACCAGGAUGGCCGAGUGGUUAAAGGCGUUGGACUGGCCAGCUGUG</p> <p><u>AGUGUUUCUU</u>UAGGACAGUGUUUAGCUGGUGUUUGAGCGGCCA</p> <p>CUGAGUGUUUCUUUAGGACAGUGUUUAGCUGGUGUUUGAGC</p> <p>AAUAGUAAGGAAGCAACAGCAAGUAUACUGCCUUCUAGAAGUCU</p> <p>GCACGUUGUUGGCCCGUAAGGAAGCGUGUUCGCGUGUGGCCU</p> <p><u>UAGAAGUGCUGACAGUUGUUGGCCCGAUCCAAUGGACAU AUGUC</u></p> <p><u>CGCGUGGUUCGAACCCACUCCUGGUACCA</u></p>
htRNA ^{Leu} /let-7c/miR-124	<p>ACCAGGAUGGCCGAGUGGUUAAAGGCGUUGGACUGGCCAGCUGUG</p> <p><u>AGUGUUUCUU</u>UAGGACAGUGUUUAGCUGGUGUUUGAGCGGCCA</p> <p>GCUGAGUGUUUCUUAAAGGACACGCGUGAAUGCCGUUGUGAG</p> <p>CAAUAGUAAGGAAGCGUGUUCGCGUGUGCCUUCUAGAAGUGC</p> <p>UGCACGUUGUGGCCCGUAAGGAAGCAACAGCAAGUAUACUGCCU</p> <p><u>UAGAAGUGCUGACAGUUGUUGGCCCGAUCCAAUGGACAU AUG</u></p> <p><u>UCCGCGUGGUUCGAACCCACUCCUGGUACCA</u></p>
htRNA ^{Leu} /let-7c/miR-34a	<p>ACCAGGAUGGCCGAGUGGUUAAAGGCGUUGGACUGGCCAGCUGUG</p> <p><u>AGUGUUUCUU</u>UAGGACAGUGUUUAGCUGGUGUUUGAGCGGCCA</p> <p>GCUGAGUGUUUCUUUAGGACAGUGUUUAGCUGGUGUUUGAGC</p> <p>CAAUAGUAAGGAAGCAACAGCAAGUAUACUGCCUUCUAGAAGUGC</p> <p>UGCACGUUGUGGCCCGUAAGGAAGCAACAGCAAGUAUACUGCCU</p> <p><u>UUCAGAAGUGCUGACAGUUGUUGGCCCGAUCCAAUGGACAU AUG</u></p> <p><u>UCCGCGUGGUUCGAACCCACUCCUGGUACCA</u></p>
htRNA ^{Ser} /miR-124/miR-34a	<p>GCAGCGAUGGCCGAGUGGUUAAAGGCGUUGGACUGGCCAGCUGUG</p> <p><u>AGUGUUUCUU</u>UAGGACAGUGUUUAGCUGGUGUUUGAGCGGCCA</p> <p>CUGAGUGUUUCUUUAGGACAGUGUUUAGCUGGUGUUUGAGC</p> <p>AAUAGUAAGGAAGCAACAGCAAGUAUACUGCCUUCUAGAAGUCU</p> <p>GCACGUUGUGGCCCGUAAGGAAGCGUGUUCGCGUGUGGCCU</p> <p><u>UAGAAGUGCUGACAGUUGUUGGCCCGAUCCAAUGGAGUUCUCCCG</u></p> <p><u>CGCAGGUUCGAACCCUGCUGCGUGGCCA</u></p>
htRNA ^{Ser} /let-7c/miR-124	<p>GCAGCGAUGGCCGAGUGGUUAAAGGCGUUGGACUGGCCAGCUGUG</p> <p><u>AGUGUUUCUU</u>UAGGACAGUGUUUAGCUGGUGUUUGAGCGGCCA</p> <p>GCUGAGUGUUUCUUUAGGACAGUGUUUAGCUGGUGUUUGAGC</p> <p>CAAUAGUAAGGAAGCGUGUUCGCGUGUGCCUUCUAGAAGUGC</p> <p>UGCACGUUGUGGCCCGUAAGGAAGCAACAGCAAGUAUACUGCCU</p> <p><u>UUCAGAAGUGCUGACAGUUGUUGGCCCGAUCCAAUGGAGUUCUCC</u></p> <p><u>CGCGAGGUUCGAACCCUGCUGCGUGGCCA</u></p>

Name	Sequence (underlined: tRNA, bold: sequence relating to insert #1, green: target miRNA/siRNA, red: complementary sequence)
htRNA ^{Ser} /let-7c/miR-34a	GCAGCGAUGGCCGAGUGGUUAAAGGCGUUGGACUGGCCAGCUGUG <u>AGUGUUUUUU</u> UUAUUGUCAACUGAUAAGCUAUGUGAGCGGCCAG GUGUGAGUGUUUUUUGGCAGUGUCUAGCUGGUUGUGAGC CAUAAGUAAGGAAGCAAGCAAGCAAGUAUACUGCCUAGAAAGUC UGACGUUGUGGCCGUAAGGAAGACUGUACACCUUACUACCU <u>UUCAAGUGUGCAGUGUGUUGGCCCAAUCCAAUUGGGGUCUCCCG</u> CGCGAGGUUCGAACCCACUCCUGGUGGCCA
htRNA ^{Leu} /miR-34a/anti-miR-21	ACCAGGAUGGCCGAGUGGUUAAAGGCGUUGGACUGGCCAGCUGUG <u>AGUGUUUUUU</u> UUAUUGUCAACUGAUAAGCUAUGUGAGCGGCCAG CUGUGAGUGUUUUUUGGCAGUGUCUAGCUGGUUGUGAGC AAUAGUAAGGAAGCAACAGCAAGUAUACUGCCUAGAAAGUCU GCACGUUGUGGCCGUAAGGAAGACUGUUAUAAGAAUGAUGUG <u>CAGAGUGUGCAGUGUGUUGGCCGGAUCCAAUGGACAU AUGUCC</u> GCGUGGUGUUCGAACCCACUCCUGGUAACCA
htRNA ^{Leu} /NRF2-siRNA/miR-34a	ACCAGGAUGGCCGAGUGGUUAAAGGCGUUGGACUGGCCAGCUGUG <u>AGUGUUUUUU</u> UUAUUGUCAACUGAUAAGCUAUGUGAGCGGCCAG CUGUGAGUGUUUUUUGGCAGUGUCUAGCUGGUUGUGAGC AAUAGUAAGGAAGCAACAGCAAGUAUACUGCCUAGAAAGUCU GCACGUUGUGGCCGUAAGGAAGACUGAAGACAGUAGACAUUC <u>UAGAAGUGUGCAGUGUGUUGGCCGGAUCCAAUGGACAU AUGUCC</u> GCGUGGUGUUCGAACCCACUCCUGGUAACCA
htRNA ^{Ser} /NRF2-siRNA/miR-34a	GCAGCGAUGGCCGAGUGGUUAAAGGCGUUGGACUGGCCAGCUGUG <u>AGUGUUUUUU</u> UUAUUGUCAACUGAUAAGCUAUGUGAGCGGCCAG CUGUGAGUGUUUUUUGGCAGUGUCUAGCUGGUUGUGAGC AAUAGUAAGGAAGCAACAGCAAGUAUACUGCCUAGAAAGUCU GCACGUUGUGGCCGUAAGGAAGACUGAAGACAGUAGACAUUC <u>UAGAAGUGUGCAGUGUGUUGGCCGGAUCCAAUGGAGUAGACAUUC</u> CGCAGGUUCGAACCCUGCUGGUGGCCA

Underlined sequences represent tRNA, either leucine or serine, followed by two consecutive pre-miR-34a sequences (Ho et al. 2018), where mature miR-34a sequences were replaced with target warhead small RNAs highlighted in red whose complementary sequences are in green

Table 2

Yields, purities, and endotoxin levels of individual FPLC-isolated CO-BERAs. The yield was defined as the amount of purified CO-BERA per liter bacterial culture, and the purity of isolated CO-BERA was quantified by an optimal HPLC method (Wang et al. 2015). The molecular weights of individual CO-BERAs were calculated with OligoCalc (<http://biotools.nubic.northwestern.edu/OligoCalc.html>). Endotoxin levels were determined by using the Limulus Amebocyte Lysate Pyrogen-5000 kinetic assay

Name	Length (nt)	Molecular Weight (g/mol)	Total RNA extracted from 1 L culture (mg)	Target CO-BERA purified from 1 L culture (mg)	Purity (%)	Endotoxin activity (EU/ μ g RNA)
1 htRNA ^{Leu} /miR-34a/miR-124 ^a	297	96,008.1	48.4	21.3	99.0	0.15
2 htRNA ^{Leu} /miR-124/miR-34a ^a	297	96,583.4	50.0	22.0	99.0	3.31
3 htRNA ^{Leu} /let-7c/miR-124 ^b	298	96,300.3	21.6	4.3	96.7	1.60
4 htRNA ^{Leu} /let-7c/miR-34a ^c	298	96,278.3	21.3	10.7	95.6	1.34
5 htRNA ^{Ser} /miR-124/miR-34a ^a	296	95,684.9	22.2	4.4	99.4	1.44
6 htRNA ^{Ser} /let-7c/miR-124 ^d	297	95,977.0	12.6	0.5	92.3	0.33
7 htRNA ^{Ser} /let-7c/miR-34a ^a	297	95,955.1	22.0	13.2	99.6	0.71
8 htRNA ^{Leu} /miR-34a/anti-miR-21 ^c	294	96,010.2	17.6	0.2	95.1	1.11
9 htRNA ^{Leu} /NRF2-siRNA/miR-34a ^a	297	95,931.1	20.0	6.4	99.6	0.19
10 htRNA ^{Ser} /NRF2-siRNA/miR-34a ^a	296	95,607.9	13.3	1.3	99.0	6.32

^aENrich Q only;

^bENrich Q then CHT Type II;

^cENrich Q then DEAE;

^dENrich Q then ENrich Q