METHOD FOR CHIMERIC EXPRESSION OF RECOMBINANT SMALL RNA IN ESCHERICHIA COLI USING ACTIVE TRNA, AND USE THEREOF

TECHNICAL FIELD

[0001] This disclosure relates to the technical field of molecular biology and medicine and, in particular, to a method for chimeric expression of a recombinant small RNA in *Escherichia coli* using an active tRNA, and use thereof.

BACKGROUND

[0002] In recent years, RNA therapy, owing to its advantages such as simple design, short R&D cycle, and abundant candidate targets, has emerged as a novel therapeutic modality attracting attention from both scientific research and industry. An increasing number of studies have found that tRNAs not only participate in protein synthesis but are also widely involved in the occurrence and development of various diseases. Cleavage of mature or precursor tRNAs can generate tRNA-derived small RNAs (tsRNAs), and the tsRNAs have been detected to be aberrantly expressed in various diseases, suggesting the potential role of tRNAs and tsRNAs in disease treatment.

[0003] Previous studies have shown that tRNAs can be successfully used as a scaffold to produce small non-coding RNAs (ncRNAs) in *Escherichia coli* (Luc Ponchon, et al., Recombinant RNA technology: the tRNA scaffold [J]. Nature Methods, 2007, 4(7): 571-576; Luc Ponchon, et al., A generic protocol for the expression and purification of recombinant RNA in *Escherichia coli* using a tRNA scaffold [J]. Nature Protocols, 2007, 4(6): 947-959). The method of Ponchon et al. uses methionyl-tRNA and human lysyl-tRNA to directly chimerize a small ncRNA precursor for expression, and after the recombinant RNA is cleaved by RNase H, the mature small ncRNA fragment is purified using ion exchange chromatography. However, this biological expression method has proven to have limited applicability, with various small RNAs failing to be expressed or being expressed at very low levels under this strategy. Qian et al. from Northwestern Polytechnical University provided a method for producing a recombinant small RNA, which uses human seryl-tRNA to chimerize a precursor microRNA for expressing short RNA fragments (CN110964726B). The method of Qian et al. is more versatile than that

of Luc Ponchon et al. Human seryl-tRNA is a human tRNA, and recombinant small RNAs produced using it as a scaffold have better development prospects because human cells naturally contain seryl-tRNA but lack bacteria-derived methionyl-tRNA. Therefore, this method reduces toxicity and immunogenicity. For multiple small RNAs that cannot be expressed by directly chimerizing a precursor small RNA with a tRNA, the method of Qian et al. enables expression. In the above methods, the tRNA acts as a scaffold to improve the expression level and stability of recombinant small RNAs, but does not possess biological activity.

SUMMARY

[0004] In view of the aforementioned deficiencies of the prior art, this disclosure provides a method for chimeric expression of a recombinant small RNA in *Escherichia coli* using an active tRNA, and use thereof to solve the technical problem.

[0005] This disclosure uses a human active tRNA to chimerize an hsa-miR-34a precursor. Recombinant small RNAs produced using a human tRNA as a scaffold have better development prospects, because human cells naturally contain this tRNA, and thus using the human tRNA as a scaffold may result in lower toxicity and immunogenicity. Moreover, using the active tRNA as a scaffold may achieve a therapeutic effect targeting diseases and act synergistically with carried small RNAs. This disclosure not only reduces cytotoxicity and increases the expression level, but also obtains an active tRNA scaffold that synergizes with chimeric small RNAs to exert a therapeutic effect.

[0006] To solve the above technical problem, the technical solution adopted by this disclosure is: a method for chimeric expression of a recombinant small RNA in *Escherichia coli* using an active tRNA, the method including: using the active tRNA chimerized with a functional miRNA precursor as a scaffold to express a target small RNA in *Escherichia coli*.

[0007] In the method of this disclosure, the small RNA has a length of less than 200 nt.

[0008] In the method of this disclosure, a sequence of the tRNA has at least 90% similarity to a human glutamate tRNA sequence, and the human glutamate tRNA sequence is SEQ ID NO: 2.

[0009] In the method of this disclosure, a sequence of the miRNA precursor has at least 90% similarity to an hsa-miR-34a precursor sequence, and the hsa-miR-34a precursor sequence is SEQ ID NO: 3.

[0010] In the method of this disclosure, the miRNA precursor is chimerized at an anticodon loop of the tRNA, and a chimeric sequence has at least 90% similarity to SEQ ID NO: 1.

[0011] Specifically, the method of this disclosure includes the following steps:

[0012] S1: using a restriction site present in the anticodon loop of the tRNA in a pBSMrnaSeph plasmid to insert an expression sequence of the hsa-miR-34a precursor into the pBSMrnaSeph plasmid, thereby constructing an expression vector;

[0013] S2: replacing a mature sequence portion of miR-34a in the constructed expression vector with a target small RNA sequence to construct an expression vector chimerized with the target sequence; transforming the expression vector chimerized with the target sequence into competent *Escherichia coli*; and

[0014] S3: after culturing and amplifying the *Escherichia coli*, extracting total bacterial RNA using a saturated phenol method, and purifying the target small RNA by FPLC.

[0015] In the method of this disclosure, the small RNA is an shRNA, miRNA, siRNA, or RNA aptamer.

[0016] In the method of this disclosure, in S2, the *Escherichia coli* is an HST08 strain or a DH5α strain.

[0017] In the method of this disclosure, in S3, the *Escherichia coli* is cultured in a TB medium until an OD of 1.1 to 1.7 is reached, and a ratio of a volume of saturated phenol to a mass of bacterial pellet used in the saturated phenol method for extracting the total bacterial RNA is (2-6):1, where the volume is expressed in mL and the mass is expressed in g.

[0018] Additionally, this disclosure further provides use of a human glutamate tRNA chimerized with an hsa-miR-34a precursor to construct a tRNA scaffold, and use of the scaffold for preparing a recombinant small RNA.

[0019] This disclosure further provides use of a recombinant small RNA produced by the method of this disclosure for preparing diagnostic reagents, research reagents, prodrugs, drugs, active pharmaceutical ingredients, or pharmaceutical compositions.

[0020] The terms and expressions used in this disclosure will be further explained below:

[0021] active tRNA: a tRNA that, in addition to performing transport functions, also possesses other functional activities. The active tRNA referred to in this disclosure means a tRNA that can serve as a source of short non-coding RNAs, i.e., tRNA-derived small RNAs, whereby the tRNA exerts regulatory functions by being degraded into small RNAs.

[0022] Chimeric expression: a method in which a miRNA sequence is inserted into a

certain region of a tRNA and then expressed together.

[0023] Small RNA: a class of non-coding RNA molecules having a length of approximately 20-30 nucleotides.

[0024] Human glutamate tRNA: a human tRNA responsible for transporting glutamate.

[0025] miRNA precursor sequence: a miRNA sequence containing a hairpin structure.

[0026] hsa-miR-34a precursor sequence: a sequence corresponding to a hairpin structure of hsa-miR-34a.

[0027] tRNA anticodon loop: a loop structure generally formed at an anticodon region in a cloverleaf structure of a tRNA, i.e., an anticodon loop.

[0028] pBSMrnaSeph plasmid: the name of a plasmid vector.

[0029] Mature sequence of miR-34a: a sequence obtained after precise cleavage of the hsa-miR-34a precursor sequence by intracellular endonucleases.

[0030] Competent *Escherichia coli*: a specific form of *Escherichia coli* treated with $CaCl_2$ or electroporation, making it highly competent to take up exogenous DNA adsorbed onto a cell surface.

[0031] *Escherichia coli* HST08 strain: a strain deficient in mrr, hsdRMS, mcrBC, and mcrA (genes required by *Escherichia coli* for excising exogenous methylated DNA).

[0032] *Escherichia coli* DH5α strain: a mutagenized strain with the basic characteristics of R^-, M^-, and AMP^-. M^- means that the DH5α strain lacks DNA modification, i.e., its DNA is not modified. R^- means that it lacks an "immunity" mechanism and does not cleave introduced exogenous DNA. AMP^- means that it is sensitive to ampicillin.

[0033] The raw materials and reagents are commercially available, or their sequences can be directly queried from the National Center for Biotechnology Information (NCBI).

[0034] Compared with the prior art, this disclosure has the following advantages:

[0035] 1. The tRNA scaffold for expressing the recombinant small RNA is constructed by chimerizing the human glutamate tRNA with the hsa-miR-34a precursor. The human glutamate tRNA is a human tRNA, and the recombinant small RNA produced using it as a scaffold has better development prospects, because human cells naturally contain glutamate tRNA, and thus using the human glutamate tRNA as a scaffold may result in lower toxicity and immunogenicity, thereby reducing cytotoxicity and improving the expression level. Moreover, the human glutamate tRNA has therapeutic activity against breast cancer. Therefore, this disclosure allows both the recombinant tRNA portion and the small RNA portion it carries to exert biological activity, providing a dual therapeutic effect.

[0036] 2. This disclosure designs and prepares the recombinant small RNA through bioengineering technology, offering advantages such as simple equipment, rapid production, high yield, low cost, and good functionality.

[0037] 3. This disclosure also optimizes *Escherichia coli* culture conditions by replacing an LB or 2×YT medium with the TB medium, which prolongs the time before *Escherichia coli* enters the decline phase and increases the total bacterial mass obtained per unit volume. Furthermore, it determines that the optimal bacterial density for harvesting *Escherichia coli* after culture is an OD of 1.1 to 1.7, under which the maximum amount of total RNA is obtained, thereby increasing the yield of recombinant small RNA.

[0038] 4. This disclosure also optimizes the volume of water-saturated phenol added during extraction of total RNA from bacteria, identifying the minimum saturated phenol volume required to achieve the best RNA extraction efficiency, thereby optimizing the benefit-cost ratio.

[0039] 5. Detection by various technical means indicates that the method of this disclosure can construct an active recombinant small RNA, and that this recombinant small RNA possesses better biological functions. The produced recombinant small RNA meets the demands of scientific research and drug development.

[0040] The technical solution of this disclosure will be further described in detail below with reference to the accompanying drawings and embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

[0041] FIG. 1 shows the detection of recombinant small RNA expression in *Escherichia coli* by denaturing polyacrylamide gel electrophoresis in Example 2 of this disclosure.

[0042] FIG. 2 shows the purification of recombinant small RNA using the Bio-Rad NGC^{TM} Chromatography System and the identification of the purity of collected fractions by denaturing polyacrylamide gel electrophoresis in Example 4 of this disclosure.

[0043] FIG. 3 shows the inhibitory effect of recombinant hsa-tRNA^{Glu}-miR-34a on MDA-MB-231 cells in Example 5 of this disclosure.

[0044] FIG. 4 shows the intracellular levels of corresponding tRNAs detected by qPCR technology after transfection of different recombinant miR-34a into MDA-MB-231 cells in Example 6 of this disclosure.

[0045] FIG. 5 shows the detection of the processing and maturation of recombinant miR-34a in MDA-MB-231 cells by qPCR technology in Example 6 of this disclosure

(values are expressed as “mean ± standard deviation”).

[0046] FIG. 6 shows the IC_{50} values of hsa-tRNA^{Glu}-miR-34a detected by CCK8 assay in Example 7 of this disclosure.

[0047] FIG. 7 shows the synergistic effect of an hsa-tRNA^{Glu} scaffold and miR-34a analyzed by the Chou-Talalay method in Example 8 of this disclosure.

[0048] FIG. 8 shows the effect of hsa-tRNA^{Glu}-miR-34a on the proliferation ability of single MDA-MB-231 cells determined by cell colony formation assay in Example 9 of this disclosure.

[0049] FIG. 9 shows the effect of hsa-tRNA^{Glu}-miR-34a on cell apoptosis detected by immunofluorescence technology in Example 10 of this disclosure.

[0050] FIG. 10 shows the effect of hsa-tRNA^{Glu}-miR-34a on cell migration ability detected by wound healing assay in Example 11 of this disclosure.

[0051] FIG. 11 shows the effect of hsa-tRNA^{Glu}-miR-34a on cell invasion ability detected by Transwell invasion assay in Example 12 of this disclosure.

[0052] FIG. 12 shows the therapeutic effect of hsa-tRNA^{Glu}-miR-34a on a mouse model of breast cancer at the animal level detected by *in vivo* small animal imaging in Example 13 of this disclosure.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[0053] The following embodiments illustrate the implementation of this disclosure. Unless otherwise indicated, the experimental methods disclosed herein all employ conventional techniques in the art.

[0054] The design and expression of recombinant miR-34a will be used as an example for illustration.

[0055] Example 1: a human tRNA^{Glu} sequence with anti-breast cancer activity reported in the literature was retrieved from the GtRNAdb database (http://gtrnadb.ucsc.edu/). A miR-34a precursor was chimerized to design an expression plasmid for recombinant hsa-tRNA^{Glu}-miR-34a (E-miR-34a).

[0056] A secondary structure of the designed recombinant miR-34a chimerically expressed using a human active tRNA^{Glu} scaffold was predicted using the CentroidFold online prediction tool (http://www.ncrna.org/centroidfold). By adjusting the chimerization position between the scaffold and miR-34a and modifying individual nucleotides, a sequence with the optimal secondary structure was selected to construct a

recombinant expression plasmid.

[0057] Example 2: Recombinant Expression of hsa-miR-34a Using an Active tRNA Scaffold

[0058] (1) Mini-preparation of Recombinant E-miR-34a Expression Plasmid

[0059] After transforming 100 ng of the recombinant E-miR-34a expression plasmid into competent HST08 or DH5α bacteria, the bacteria were added to 5 mL of LB medium and cultured overnight at 37℃ with shaking at 200 rpm. The bacterial culture was centrifuged at 10,000 g for 2 min, and the pellet was collected. The pellet was resuspended in 180 μL of 10 mM magnesium acetate-Tris HCl buffer, followed by the addition of 200 μL of saturated phenol, and the mixture was shaken at room temperature for 20-60 min. After centrifugation at 10,000 g for 10 min, the aqueous phase was collected, and 0.1 volume of 5 M NaCl was added to precipitate macromolecular impurities. Two volumes of absolute ethanol were added to the supernatant, followed by centrifugation at 10,000 g for 10 min, and then the supernatant was discarded. Residual ethanol was removed by wicking with absorbent paper. After the RNA had dried, DEPC-treated water was added to dissolve the RNA. The concentration was determined, and the RNA was stored in a freezer at -80℃.

[0060] (2) Identification by Denaturing Polyacrylamide Gel Electrophoresis

[0061] RNA samples were mixed with RNA loading buffer and loaded into the wells of denaturing gel samples. Electrophoresis was performed at 80-100 V for 50-80 min. The gel was then placed in a solution containing 0.5 μg/mL ethidium bromide and gently shaken for 10-30 min. The gel was observed under a gel imaging system, and images were captured and saved.

[0062] FIG. 1 shows the detection of recombinant E-miR-34a expression in *Escherichia coli* by denaturing polyacrylamide gel electrophoresis according to this disclosure. The synthesized recombinant plasmid was transformed into competent *Escherichia coli* HST08 bacteria, and the expression level was identified by urea-denaturing polyacrylamide gel electrophoresis, using recombinant miR-34a expressed with a bacterial methionine tRNA ($btRNA^{Met}$) scaffold ($btRNA^{Met}$-miR-34a, i.e., M-miR-34a) as a positive control. As shown in FIG. 1, if an additional band between 150-300 nt appeared in *Escherichia coli* transformed with the recombinant miR-34a expression plasmid, it indicated successful expression of the recombinant small RNA, and the intensity of the band reflected the expression level. The results showed that expression bands were visible for the E-miR-34a recombinant plasmid in both *Escherichia coli*

HST08 and DH5α, indicating that the recombinant E-miR-34a expression plasmid can efficiently express the recombinant small RNA in *Escherichia coli*. The effective sequence in the recombinant E-miR-34a plasmid is SEQ ID NO: 1.

[0063] Example 3: Optimization of *Escherichia coli* Culture Conditions and Total RNA Extraction Method

[0064] (1) A TB medium was used to replace the LB medium in "Example 2 (1)". The bacteria were cultured until an OD of 1.1 or higher was reached, and then harvested by centrifugation. Comparison showed that the bacterial yield obtained with this culture protocol was approximately more than 2-fold higher than that obtained with the LB medium culture method.

[0065] (2) The amount of water-saturated phenol used in "Example 2 (1), extraction of total RNA by lysing *Escherichia coli* with water-saturated phenol" was optimized. A more standardized and appropriate lysis buffer ratio was used for *Escherichia coli* cell lysis to determine the minimum volume of water-saturated phenol required for adequate bacterial lysis and to optimize the cost-benefit ratio. Comparison showed that when the ratio of saturated phenol to bacterial pellet was 2-6 (v/w) (e.g., adding 2 mL of water-saturated phenol to 1 g of harvested bacterial pellet), the total RNA extraction rate was increased from 3.5% to more than 7%.

[0066] Example 4: Purification of Recombinant E-miR-34a by FPLC

[0067] (1) The recombinant E-miR-34a was purified using a Bio-Rad NGC^{TM} Chromatography System with an ion exchange column (ENrich^{TM} Q10×100 Column).

[0068] Mobile phase A: 10 mM NaH_2PO_4 solution, pH 7.0.

[0069] Mobile phase B: 10 mM NaH_2PO_4 solution containing 1 M NaCl, pH 7.0.

[0070] The flow rate was 2.0 mL/min. The column was alternately washed with DEPC-treated water, the mobile phase A, and the mobile phase B for approximately 1 h, with 5 column volumes for each wash.

[0071] Total RNA was separated using the following program: 0-8.9 min (0% B), 8.9-13.7 min (55% B), 13.7-53.7 min (55-75% B), 73.7-83.7 min (100% B), and 83.7-93.7 min (0% B). RNA was detected by absorbance at 260 nm, and the peak corresponding to the recombinant RNA was collected. Purity was identified by denaturing polyacrylamide gel electrophoresis.

[0072] (2) RNA Sample Treatment Method

[0073] Total RNA extraction was performed as described above. The extracted total RNA was centrifuged at 13,000 rpm for 10 min at 4℃, and the supernatant was filtered

through a 0.45 μm microporous membrane. Then, 5-10 mg of sample was injected per run.

[0074] (3) FPLC Fraction Collection, Concentration, and Desalting

[0075] The purity of the collected fractions was identified by denaturing polyacrylamide gel electrophoresis. The pooled fractions were mixed, and RNA was precipitated by adding 2 volumes of absolute ethanol, followed by incubation at -80℃ for approximately 1 h. RNA was collected by centrifugation at 10,000 g for 10 min at 4℃. The resulting RNA pellet was dissolved in DEPC-treated water and centrifuged using Ultra-2 mL Centrifugal Filters at 7,500 g for 10 min at 4℃, and then the flow-through was discarded. This step was repeated until all the solution had been centrifuged. The filter was then inverted and centrifuged at 2,000 g for 2 min. The resulting solution was collected and, after concentration determination, stored at -80°C.

[0076] FIG. 2 shows the purification of recombinant miR-34a using the Bio-Rad NGC^{TM} Chromatography System and the identification of the purity of collected fractions by denaturing polyacrylamide gel electrophoresis according to this disclosure. The chromatogram in FIG. 2A indicates the position of the target peak and the collected fractions. The results show that recombinant E-miR-34a was well separated from other endogenous RNAs in *Escherichia coli*. Further identification by urea-denaturing polyacrylamide gel electrophoresis (FIG. 2B) demonstrated that the purified recombinant E-miR-34a exhibited a single band without smearing or contaminating bands compared to unpurified total RNA, confirming the high purity of recombinant E-miR-34a. Thus, pure recombinant E-miR-34a can be obtained after FPLC purification.

[0077] Example 5: Inhibitory Effect of Recombinant hsa-Glu-miR-34a on MDA-MB-231 Cells

[0078] The effect of recombinant E-miR-34a on the proliferation and viability of breast cancer MDA-MB-231 cells was detected using the Cell Counting Kit-8 (CCK8). The specific experimental procedure was as follows:

[0079] (1) cells were seeded into a 96-well plate, with 6 replicate wells per group. After the cells adhered, RNA transfection was performed according to the experimental groups; and

[0080] (2) at 24 h, 48 h, 72 h, and 96 h after transfection, 10% CCK8 detection solution was added, and the absorbance of each well at 450 nm was measured using a microplate reader for data analysis.

[0081] As shown in FIG. 3, recombinant E-miR-34a exhibited a significant inhibitory

effect on cell activity at the 72 h and 96 h time points.

[0082] Recombinant miR-34a carried by two "inert" (i.e., relatively inactive in the context of breast cancer in this disclosure, referred to as inert herein, as no literature has reported that these two tRNAs possess significant anti-breast cancer activity) tRNA scaffolds were used as controls: btRNA^{Met}-miR-34a constructed using a bacterial methionine tRNA validated for efficient recombinant expression of small RNA, and hsa-tRNA^{Ser}-miR-34a constructed using human seryl-tRNA. To investigate whether the tRNA scaffolds exert a synergistic therapeutic effect with the miRNA, tRNA scaffold controls were used. Control recombinant RNAs were constructed with btRNA^{Met}, hsa-tRNA^{Ser}, and hsa-tRNA^{Glu} (NC^{Met}; NC^{Ser}; NC^{Glu}) scaffolds. Therefore, the subsequent experimental groups were set as follows: Blank, Mock, NC^{Met}, btRNA^{Met}-miR-34a (M-miR-34a), NC^{Ser}, hsa-tRNA^{Ser}-miR-34a (S-miR-34a), NC^{Glu} and hsa-tRNA^{Glu}-miR-34a (E-miR-34a).

[0083] Example 6: Intracellular Processing and Maturation of Recombinant miR-34a

[0084] (1) Transfection of MDA-MB-231 Cells

[0085] Cells were seeded into a 24-well plate at 10,000 cells per well. Cell transfection was performed according to the instructions of the Engreen (Entranster^{TM}-R4000) transfection reagent. The transfection concentration of recombinant miR-34a was 50 nM.

[0086] (2) RNA Extraction (E.Z.N.A. miRNA Kit, OMEGA miRNA Extraction Kit)

[0087] The procedure was carried out according to the kit instructions. The obtained miRNA, after concentration determination, was stored at -80℃ for later use.

[0088] (3) Detection of Intracellular Processing and Maturation of Recombinant miR-34a by Fluorescence Quantitative PCR (q-PCR)

[0089] The extracted RNA was reverse transcribed into cDNA using the TAKARA reverse transcription kit. The reverse transcription conditions were: 37ºC for 15 min, and 80ºC for 15 s. Using the cDNA from each group as a template and U6 as an internal control, the expression level of miR-34a in the cells was detected by qPCR. The qPCR reaction conditions were: 95℃ for 30 s, denaturation; 95℃ for 10 s; 60℃ for 30 s, for 44 cycles. The primer sequences used are shown in Table 2 (the sequences are set forth in SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, and SEQ ID NO: 16).

[0090] Table 2: Primer Sequences Used in Reverse Transcription and Q-PCR Detection of Recombinant miR-34a

Prime Name	Sequence (5’ to 3')
Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACACAACC
U6	Fow- ACGTAGCTCAGTTGGTTAGAGCAG
	Rev- AAAATATGGAACGCTTCACGAA
Mature-miR-34a	Fow- CACGCATGGCAGTGTCTT
	Rev- CCAGTGCAGGGTCCGAGGTA
18S	Fow-ACGTAGCTCAGTTGGTTAGAGCAG
	Rev- AAAATATGGAACGCTTCACGAA
$tRNA^{Met}$	Fow-ACGTAGCTCAGTTGGTTAGAGCAG
	Rev-GTGGCTACGACGGGATTCGA
$tRNA^{Ser}$	Fow-GGCAGTGTCTTAGCTGGTTG
	Rev-GCAGCACTTCTAGGGCAGTAT
$tRNA^{Glu}$	Fow-GGCAGTGTCTTAGCTGGTTG
	Rev-GCAGCACTTCTAGGGCAGTAT

[0091] As shown in FIG. 4, compared with the Mock group, the hsa-$tRNA^{Glu}$ scaffold was significantly expressed in both the E-miR-34a and NC^{Glu} groups. The expression of the other two tRNA scaffolds also showed a consistent trend, demonstrating that all three recombinant small RNAs were successfully transfected into the cells.

[0092] FIG. 5 shows the detection of the processing and maturation of recombinant miR-34a in MDA-MB-231 cells by qPCR technology according to this disclosure (values are expressed as “mean ± standard deviation”). Compared with the Mock group, the levels of mature miR-34a were significantly increased in all three recombinant miR-34a groups. Compared with the NC^{Glu} group, the level of mature miR-34a expressed in cells after transfection with E-miR-34a was significantly increased, and it was also significantly higher than that of the other two recombinant miR-34a molecules (M-miR-34a and S-miR-34a). The three tRNA scaffold control groups showed no significant difference compared with the Mock group. Therefore, the results indicate that the recombinant miR-34a constructed using the novel human tRNA-derived scaffold hsa-$tRNA^{Glu}$ carrying miR-34a can be successfully transfected into cells and processed into its mature form within the cells.

[0093] Example 7: Determination of the IC_{50} Value of hsa-$tRNA^{Glu}$-miR-34a for Inhibiting MDA-MB-231 Cells

[0094] The effect of recombinant miR-34a on the proliferation and viability of breast cancer MDA-MB-231 cells was detected using CCK8. The specific experimental procedure was as follows:

[0095] (1) cells were seeded into a 96-well plate at 3.5×10^3 cells per well, with 6 replicate wells per group, and cultured in a cell culture incubator;

[0096] (2) after cell adherence, RNA transfection was performed according to the experimental groups: Blank, Mock, NC^{Glu}, E-miR-34a, NC^{Ser}, S-miR-34a, NC^{Met}, and M-miR-34a;

[0097] (3) after 72 h, 10% CCK8 detection solution was added, and the plate was incubated in a 37℃ incubator for 2 h; and

[0098] (4) the plate was gently shaken, and the absorbance of each well at 450 nm was measured using a microplate reader for data analysis.

[0099] As shown in FIG. 6 and Table 3, the IC_{50} value of the E-miR-34a group (51.44 nM) < the IC_{50} value of S-miR-34a (56.39 nM) < the IC_{50} value of M-miR-34a (60.39 nM). The results indicated that E-miR-34a exerted the strongest inhibitory effect on breast cancer MDA-MB-231 cells. The IC_{50} values of the three tRNA scaffold control groups were all relatively high, with the order as follows: NC^{Met} (111.9 nM) < NC^{Ser} (116 nM) < NC^{Glu} (118.9 nM), indicating that the tRNA scaffolds had weaker effects on cell proliferation than the corresponding recombinant miR-34a molecules. The IC_{50} ratio for each pair of recombinant RNA and its corresponding scaffold control is the IC_{50} shift. An IC_{50} shift greater than 1.5 was considered significant. The IC_{50} shift between E-miR-34a and NC^{Glu} was 2.31, that between S-miR-34a and NC^{Ser} was 2.06, and that between M-miR-34a and NC^{Met} was 1.825. This suggested that each recombinant miR-34a produced a more significant inhibitory effect compared to its corresponding tRNA scaffold control, indicating that miR-34a chimerized with all three tRNA scaffolds retained biological activity. This demonstrated the universality of the biosynthetic recombinant small RNA strategy. Moreover, the IC_{50} shift between E-miR-34a and NC^{Glu} was the largest, confirming that the combination of miR-34a with hsa-$tRNA^{Glu}$ exhibited the best activity and achieved a superior effect.

[0100] Table 3: IC50 Values of Recombinant miR-34a for Inhibiting MDA-MB-231 Cells

	IC_{50} (nM)
E-miR-34a	51.44±1.0261

NC^{Glu}	118.9±1.0556
M-miR -34a	60.39±1.0430
NC^{Met}	111.9±1.0189
S-miR -34a	56.39±1.0337
NC^{Ser}	116.0±1.0260

[0101] Example 8: Synergistic Effect of the hsa-$tRNA^{Glu}$ Scaffold and miR-34a in Recombinant miR-34a

[0102] Chou-Talalay is currently a widely accepted quantitative method for analyzing drug synergy. The combination index formula for two drugs is $CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2}$, where D represents the dose when used alone and Dx represents the dose when used in combination.

[0103] Qualitative and quantitative descriptions of the drug combination effect were performed based on the dose-response relationship data obtained from CCK8 assays. The CompuSyn software was used to calculate the combination index (CI) value according to the formula. The strength and nature of the drug interaction can be quantitatively determined by the CI value (CI > 1 indicates antagonism, CI = 1 indicates additivity, and CI < 1 indicates synergy). Meanwhile, the CompuSyn software generated a Fa-CI plot at an equivalent dose-response ratio based on the obtained data, allowing for an intuitive assessment of the trend in the drug combination effect. The analysis results shown in FIG. 7 demonstrate that the CI value of E-miR-34a was < 1, confirming a strong synergistic effect between the hsa-$tRNA^{Glu}$ scaffold and miR-34a.

[0104] Example 9: Effect of hsa-$tRNA^{Glu}$-miR-34a on the Proliferation Ability of MDA-MB-231 Cells

[0105] The colony formation assay is an experimental method that stains and counts cell clone colonies using crystal violet staining solution, reflecting the proliferation ability of cells. The specific operation steps are as follows:

[0106] (1) RNA transfection was performed according to the experimental groups: Blank, Mock, NC^{Met}, M-miR-34a, NC^{Ser}, S-miR-34a, NC^{Glu}, G-miR-34a, and chemically synthesized miR-34a precursor and mature forms; 12 hours after transfection, the cells were digested, pipetted, and resuspended; and MDA-MB-231 cells were seeded into a 6-well plate at 500 cells per well, with 3 replicate wells per group;

[0107] (2) cell status was observed, and the medium was changed every 3 days until colonies appeared; the process took approximately 14 days;

[0108] (3) after fixing the cells, they were stained with crystal violet solution; and

[0109] (4) the plate was scanned and photographed, and the number of cell colonies was counted.

[0110] The results shown in FIG. 8 indicate that, compared with the chemically synthesized mature miR-34a, all groups of biosynthesized recombinant miR-34a exhibited significantly reduced colony formation, demonstrating that bioactive recombinant miR-34a was more potent than chemically synthesized miR-34a. The order of the number of cell colonies was E-miR-34a < S-miR-34a < M-miR-34a, indicating that E-miR-34a had the strongest inhibitory effect on cell proliferation. NC^{Met}, NC^{Ser}, and NC^{Glu} also showed some ability to inhibit cell colony formation, with NC^{Glu} exhibiting a stronger effect. Furthermore, the results showed that all three recombinant miR-34a molecules were more effective than their corresponding tRNA scaffold control groups, demonstrating that chimerization of miR-34a with the tRNA scaffold synergistically inhibits cell proliferation.

[0111] Example 10: Effect of hsa-$tRNA^{Glu}$-miR-34a on Apoptosis of MDA-MB-231 Cells

[0112] The best recognized biochemical hallmark of both early and late stages of cell apoptosis is the activation of caspases. Caspase-3 is an evolutionarily conserved member of the caspase family and plays a definitive role in cell apoptosis. The molecular mechanism of cell apoptosis is triggered by proteolytic enzymes belonging to caspase-3. When inducible caspase-3 is cleaved and activated into cleaved-caspase-3, it enters the nucleus and cleaves chromosomal DNA into nucleosome-sized ladder-like fragments, leading to the progression of cell apoptosis. Therefore, detection of cleaved-caspase-3 is important for assessing cell apoptosis. After RNA transfection according to the experimental groups, immunofluorescence staining was performed, and the expression of cleaved-caspase-3 protein in cells of each group was detected under a fluorescence microscope (FIG. 9).

[0113] (1) Cell seeding on coverslips: cells were seeded into a 24-well plate containing 14 mm coverslips. After cell adherence and recovery of normal cell morphology, RNA transfection was performed according to the experimental groups. After 48 h of culture, the original medium was removed, and the cells were washed twice with pre-warmed PBS and fixed with pre-warmed 4% paraformaldehyde for 15 min;

[0114] (2) the cells were washed three times with PBS, 3 min each;

[0115] (3) the cells were incubated with 0.5% Triton X-100 for 10 min;

[0116] (4) the cells were washed three times with 0.1% Triton X-100, 5 min each;

[0117] (5) the cells were blocked with a blocking solution for 10 min;

[0118] (6) according to the antibody instructions, the antibody was diluted with the blocking solution at an appropriate ratio, and 80 μL of antibody dilution was added to each well, followed by overnight incubation at 4℃;

[0119] (7) the cells were washed three times with 0.1% Triton X-100, 5 min each;

[0120] (8) according to the instructions, the fluorescently labeled secondary antibody was diluted with the blocking solution at an appropriate ratio, and 80 μL of antibody dilution was added to each well, followed by incubation for 2 h in the dark;

[0121] (9) the cells were washed three times with 0.1% Triton X-100, 5 min each;

[0122] (10) Hochest was diluted with PBS to 5 μg/mL, 100 μL was added to each well, and the cells were stained for 10 min at room temperature;

[0123] (11) the cells were washed three times with 0.1% Triton X-100, 5 min each;

[0124] (12) the coverslips were removed, and with the cell side facing down, they were mounted onto glass slides using a mounting medium; and

[0125] (13) photographs were taken under a fluorescence microscope within two weeks to prevent fluorescence quenching.

[0126] The experimental results showed that both the biosynthesized recombinant miR-34a and the corresponding tRNA scaffold controls exhibited varying degrees of fluorescence intensity. The order of fluorescence intensity from the strongest to the weakest was: E-miR-34a > NC^{Glu} > M-miR-34a > NC^{Met} > S-miR-34a > NC^{Ser}. E-miR-34a and NC^{Glu} showed the strongest fluorescence intensity, which was predominantly localized in the nucleus, indicating that E-miR-34a and NC^{Glu} significantly upregulated caspase-3 and more effectively promoted cell apoptosis. Moreover, the upregulation effect of each recombinant miR-34a was superior to that of its corresponding tRNA scaffold control group, verifying the synergistic therapeutic effect between the tRNA scaffold and miR-34a. In the Blank, Mock, chemically synthesized miR-34a precursor, and chemically synthesized mature miR-34a groups, no obvious fluorescence signal was observed, suggesting that the biosynthesized recombinant miR-34a has a superior ability to upregulate cleaved-caspase-3 compared to chemically synthesized miR-34a.

[0127] Example 11: Effect of hsa-$tRNA^{Glu}$-miR-34a on the Migration of MDA-MB-231 Cells

[0128] The wound healing assay is a simple method used to study cell migration *in vitro*. This assay mimics the migratory process of cells during healing *in vivo*. The basic steps involve creating a "wound" in a cell monolayer, capturing images at regular intervals during cell migration, and comparing the images to determine the cell migration rate.

[0129] (1) A marker pen was used to draw two straight horizontal lines on the bottom of a 24-well plate, evenly dividing the well into three sections for subsequent photographic positioning;

[0130] (2) approximately 70% of cells were seeded into the marked plate, and RNA transfection was performed according to the experimental groups;

[0131] (3) after the cell monolayer had grown to fully cover the bottom of the well plate, a pipette tip was used to draw a line perpendicular to the horizontal lines at the bottom of the well plate; pre-warmed PBS was used to wash away detached cells; a serum-free medium was added; the plate was placed in a cell culture incubator for continued culture; and

[0132] (4) at 0 h and 24 h after scratching, photographs were taken under an optical microscope, and the cell migration rate was calculated using Image J software.

[0133] The experimental results (FIG. 10) showed that, compared with the Blank group, the chemically synthesized miR-34a precursor, chemically synthesized mature miR-34a, NC^{Met}, and M-miR-34a did not significantly inhibit cell migration; compared with NC^{Met}, the other two tRNA scaffold control groups (NC^{Ser} and NC^{Glu}) exhibited a significant inhibitory effect on cell migration, with NC^{Glu} showing the best effect, suggesting that the hsa-$tRNA^{Glu}$ scaffold has a strong inhibitory effect on cell migration; regarding the recombinant miR-34a molecules, the order of migration inhibition ability was E-miR-34a > S-miR-34a > M-miR-34a, with E-miR-34a demonstrating the best migration inhibition ability; furthermore, S-miR-34a showed stronger migration inhibition than NC^{Ser}, and E-miR-34a showed stronger migration inhibition than NC^{Glu}, again indicating that the combination of miR-34a with an active tRNA scaffold can achieve a synergistic effect.

[0134] Example 12: Regulation of MDA-MB-231 Cell Invasion by hsa-$tRNA^{Glu}$-miR-34a

[0135] The Transwell invasion assay is the most commonly used experimental method to assess the tumor cell invasion ability. The principle involves separating a nutrient-rich culture medium from a low-nutrient culture medium using a polycarbonate membrane. Cells are seeded into the low-nutrient medium, and the membrane pores are coated with Matrigel to mimic the extracellular matrix. Tumor cells must secrete hydrolytic enzymes

and undergo deformability to traverse the Matrigel-coated filter membrane. The number of cells that migrate into the lower chamber is counted to reflect the tumor cell invasion ability.

[0136] (1) Diluted Matrigel was added onto the membrane of the upper Transwell chamber and allowed to solidify by standing in a 37°C incubator for 0.5-1 h; complete medium (500 μL) was added to the lower chamber;

[0137] (2) cells pre-transfected with RNA were digested and resuspended in a serum-free medium; 100 μL of the cell suspension was added to the upper chamber, while taking care to avoid air bubbles between the upper and lower chambers; the chambers were placed in a cell culture incubator for continued culture;

[0138] (3) after 18 h of culture, the chambers were removed, the supernatant was discarded, and a cotton swab was used to gently wipe off the non-invaded cells on the inner side of the upper chamber; the chambers were washed with PBS, and the cells were fixed with 4% paraformaldehyde for 15 min; and

[0139] (4) the fixative was discarded, and the cells on the lower side of the chamber were stained with 0.1% crystal violet staining solution for 10 min, followed by three washes with PBS; after the chambers had dried, photographs were taken under an optical microscope.

[0140] The experimental results (FIG. 11) showed that, compared with the Mock group, the chemically synthesized miR-34a precursor, chemically synthesized mature miR-34a, NC^{Met}, and M-miR-34a did not significantly inhibit cell invasion. The hsa-$tRNA^{Glu}$ (NC^{Glu}) and hsa-$tRNA^{Ser}$ (NC^{Ser}) scaffolds significantly inhibited the invasion of MDA-MB-231 cells, and their corresponding recombinant miR-34a molecules (S-miR-34a and E-miR-34a) exhibited even stronger invasion inhibition, verifying that chimerization of miR-34a with the tRNA scaffold exerts a synergistic therapeutic effect. Furthermore, E-miR-34a and NC^{Glu} showed the best inhibitory effect, also indicating that the biosynthesized miR-34a is more effective than chemically synthesized miR-34a.

[0141] Example 13: Therapeutic Effect of hsa-$tRNA^{Glu}$-miR-34a on a Mouse Model of Orthotopic Breast Cancer

[0142] To further investigate the therapeutic effect of E-miR-34a at the animal level, a mouse model of orthotopic breast cancer was established and treated accordingly.

[0143] MDA-MB-231 cells (5×10^6) stably expressing GFP and luciferase were injected into the mammary fat pads (the fourth pair of mammary glands) of 8-9 week old female BALB/C nu mice to establish an animal model of orthotopic breast cancer. Ten days after

modeling, the mice were randomly divided into 7 groups (saline, NC^{Met}, NC^{Ser}, NC^{Glu}, M-miR-34a, S-miR-34a, and E-miR-34a) based on tumor volume, with 8 mice per group, and each group was treated with the corresponding drug. The drug was administered once every three days for a total of 6 administrations. The dose was 1 mg/kg based on mouse body weight. During the treatment period, the tumor volume was monitored. After the completion of treatment, the fluorescence signal intensity of orthotopic tumors was detected by *in vivo* imaging to evaluate the therapeutic effect of the recombinant small RNA at the animal level. The experimental results (FIG. 12A, *in vivo* small animal imaging; FIG. 12B, statistical analysis of bioluminescence signal intensity) showed that, compared with the saline group, the bioluminescence signal intensity at the tumor site in the NC^{Met} group showed no significant change, whereas the bioluminescence signal intensity in the other five groups was significantly decreased; compared with the NC^{Met} group, the bioluminescence signal intensity at the tumor site in the other two tRNA scaffold control groups (NC^{Ser} and NC^{Glu}) was significantly downregulated, with NC^{Glu} showing the best therapeutic effect, suggesting that the hsa-$tRNA^{Glu}$ scaffold has an inhibitory effect on tumor growth; regarding the recombinant miR-34a molecules, the order of bioluminescence signal intensity at the tumor site was E-miR-34a < S-miR-34a < M-miR-34a, with E-miR-34a showing the best therapeutic effect; furthermore, the bioluminescence signal intensity in the E-miR-34a group was lower than that in the NC^{Glu} group, indicating that the therapeutic effect of E-miR-34a at the animal level was stronger than that of NC^{Glu}. This suggests that at the animal level, the combination of miR-34a with an active tRNA scaffold also achieves a synergistic effect.

[0144] The above merely illustrates exemplary embodiments of this disclosure and is not intended to limit this disclosure in any way. Any simple modifications, changes, and equivalent structural alterations made to the above embodiments based on the technical essence of this disclosure still fall within the scope of protection of the technical solution of this disclosure.