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Research article

# Circle-seq based method for eccDNA synthesis and its application as a canonical promoter independent vector for robust microRNA overexpression





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### ABSTRACT

Extrachromosomal circular DNA (eccDNA) has recently gained increasing attention due to its significant role in cancer and other pathophysiologic states. The majority of circular DNAs detected by Circle-seq are small-size eccDNAs with enigmatic functions. One major technical hurdle is to synthesize eccDNA for functional identification. Here, we describe CAES (Circle-seq based Artificial EccDNA Synthesis), a promising and reliable method for artificial eccDNA synthesis. Eight eccDNAs carrying different microRNA genes (eccMIR) found in gastric cancer tissues, ranging from 329 bp to 2189 bp in size, were created utilizing the CAES method. Exonuclease V and single restriction-endonuclease digestion identified the circular structure of synthetic eccDNAs. The DNA circularization efficiency afforded by CAES ranged from 15.6% to 31.1%, which was negatively correlated with the eccDNA length. In addition, we demonstrated that CAES-synthesized eccMIRs can express both miRNA-3p and - 5p molecules efficiently independent of a canonical promoter in human cell lines. Further assays proved that these eccMIRs were functional as they were able to repress the luciferase gene containing a miRNAtarget sequence in the 3'UTR as well as the endogenous mRNA targets. Finally, kinetics study revealed that eccDNA exhibited a decay rate similar to the standard plasmids and linear DNA in cultured cells. Together, this study offers a rapid and convenient method for Circle-seq users to synthesize artificial eccDNAs. It also demonstrates the promising potential of eccMIR as a bacterial DNA-free vector for safe and robust miRNA overexpression in both basic research and therapeutic applications.

1. Introduction

EccDNA, a mobile and circular DNA molecule derived and independent of linear chromosomes, was initially identified in wheat nuclei and boar sperm in 1965 [1]. Nevertheless, progress in studies exploring the biological role of eccDNA has been exceptionally sluggish. In recent years, owing to the rapid development of high-throughput sequencing technologies, including the next-generation and nanopore sequencing,

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Abbreviations: EccDNA, Extrachromosomal circular DNA; EOI, EccDNA Of Interest; Exo V,, Exonuclease V; FBS,, Fetal Bovine Serum; GCT,, Gastric Cancer Tissue; HFF, Human Foreskin Fibroblast; LAMA,, Ligase-Assisted Minicircle Accumulation; NGS,, Next-Generation-Sequencing; PSD,, Plasmid-Safe ATP-dependent DNase; RAES, RCA-product dependent Artificial EccDNA Synthesis; RCA, Rolling Circle Amplification; ddPCR, Droplet digital PCR; qPCR, Real-time quantitative PCR; RE, Restriction Endonuclease; RT, Reverse Transcription; TE, Transposable Element; SV, Structure Variant; UTR, Untranslated Region.

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the field of eccDNA study is evolving at a fast pace. It has been broadly found in various biospecimens, including somatic tissue [2,3], human sperm [4], cancer tissue [5–7], plasma [8,9], urine [10] and even cerebrospinal fluid [11]. The predominant fraction of identified eccDNAs in tissues or body fluids comprises small-size circular DNA molecules, with length ranging from a few hundred base pairs (bp) to around 2 kg-bp (kb) [2–4,12–15]. Recently, Oxford Nanopore Technology (ONT) has been employed for full-length eccDNA sequencing, enabling precise detection and decoding of eccDNAs characterized by longer lengths, multiple tandem DNA repeats, or chimeric structures [4,13,16]. As eccDNA arises from the linear genome, it has the capacity to carry diverse genomic elements, including entire gene [14,17–20], truncated genic segment [2,7,21], enhancer [22-24], microRNA gene [21,25] and transposable elements [2]. Recently, our research group found that the abundance of eccDNA in gastric cancer tissue (GCT) was significantly higher (~7.25-fold) compared to GCT-adjacent normal tissue. Numerous over-represented eccDNAs were identified in GCT, and we demonstrated that the functionality of several eccMIRs highlighting their potential role in facilitating cancer progression [26]. These cancerous eccDNAs are likely to function as a dynamic reservoir for rapid genome plasticity and the adaptive evolution of cancer [26]. Recent study has demonstrated that propagation of retrotransposons relies on the formation of endogenous eccDNAs [27]. Despite these advances, further studies are necessary to elucidate how the small-size eccDNA interacts with the cellular genome and affects related biological processes.

A significant obstacle in investigating the topic above is the absence of an efficient and cost-effective method to synthesize artificial eccDNA for downstream functional studies. In 2018, motivated by exploring the formation and intracellular fates of endogenous eccDNA, we devised the

CRISPR-C strategy [28]. Endogenous eccDNAs of any length could be generated through dual-CRISPR cleaving on both the start and end sites of eccDNA on a linear chromosome. In 2019, building upon the CRISPR-C method, we developed the HapC approach for genome haplotype phasing through CRISPR-mediated DNA circularization [29]. As both methods rely on cultured live cells, neither CRISPR-C nor HapC is applicable for artificial eccDNA synthesis in vitro. Minicircle DNA vectors, which contain the transgene expression cassette devoid of plasmid backbone, offer an alternative for eccDNA synthesis [30]. Nevertheless, its application in the field is constrained by several drawbacks, including the time-consuming and labor-intensive production process [30]; it is challenging to entirely eliminate the parental plasmid contaminants from the final product, and it includes a recombination site (attR) at the junction region of the minicircle. To address these disadvantages, Quan Du et al. introduced the Ligase-Assisted Minicircle Accumulation (LAMA) method [31], which was subsequently employed by Paulsen et al. to construct synthetic eccMIR and explore its regulatory function [21]. LAMA necessitates two previously synthesized double-stranded DNAs (dsDNA) fragments that are complementary to each other in an "inversed and half-way" manner (Fig. 1, step 3, middle) [21]. This approach has two significant limitations: 1) it is relatively expensive and time-consuming to synthesize the two linear dsDNA fragments before conducting the LAMA reaction; 2) the target eccDNA sequence is predicted based on the reference genome rather than the true sequence of sample from which the eccDNA was detected. Hence, there could be a discordance in DNA sequence between the actual eccDNA and the predicted one.

The critical step for successful LAMA synthesis involves the preparation of two required dsDNA fragments (linear A and B). Circle-seq is a widely employed method for genome-wide eccDNA profiling, involving



Fig. 1. Principle of the CAES technique. Step ①: Workflow of eccDNA extraction, purification and amplification from biospecimen. Total genomic DNA consisting of both circular and linear DNAs were extracted from the samples, linear DNA fragments were removed by exonuclease hydrolyzation and circle DNA were then amplified by rolling circle amplification (RCA). Step ②: Schematic of eccDNA sequencing and identification. Circle-Map software was used for eccDNAs calling based on split read and discordant read pairs and bedtools 2.29.2 was used for eccDNA annotation. Then eccDNA of interest (EOI) can be selected for subsequent experiment. Step ③: Key procedures of EOI synthesis. First, two half-complemental linear-DNA fragments (linear A and linear B) for each eccDNA synthesis were prepared by PCR. Second, LAMA reaction was performed to produce eccDNA. Third, residual linear DNA in LAMA reaction product was removed by Exonuclease digestion.

the elimination of linear genomic DNA and the rolling circle amplification (RCA) of retained circular DNA [2]. The RCA process of eccDNAs inherently forms a pool of tandem repeats of linearized eccDNA sequence. It can serve as a DNA template for cloning the two split-and-reversed DNA fragments of a target eccDNA (Fig. 1, step 3, left). Building on this principle, we devised the CAES method, which requires only two simple PCRs to amplify the linear A and B fragments for LAMA reaction. As the fragments were cloned directly from the original sample, this method is also characterized by high fidelity.

In this study, we constructed eight eccMIRs identified in gastric cancers [26] using CAES and verified their circular structure through exonuclease V and single-restriction enzyme digestion. These eccMIRs demonstrated efficient expression of both miRNA-3p and - 5p in various human cell lines, and this process was independent of a canonical promoter. We further proved that these eccMIRs were functional as they were capable of suppressing the luciferase reporter gene containing a miRNA target sequence at the 3'UTR, as well as the endogenous target mRNA of each miRNA. These results show that CAES is a rapid and reliable method for Circle-seq users to generate artificial eccDNA, underscoring the significant potential of eccMIR as a bacterial DNA-free vector for safe and robust miRNA overexpression in both fundamental research and clinical therapeutics.

### 2. Materials and methods

# 2.1. EccDNA purification, amplification, eccDNA NGS and decipherment

Based on the description of previous study, eccDNA was purified and amplified from the gastric cancer tissue samples [26]. In detail, tissue samples ( $\sim 10$  mg) were cut into small pieces on a 10 cm cell culture dish and the whole genomic DNA was extracted with the MagAttract HMW DNA Kit (QIAGEN) according to the manufacturer's instructions. Linear DNA removal in the genomic DNA of each sample was performed separately by the treatment of plasmid-safe ATP-dependent DNase (PSD) (Lucigen). Briefly, a mixture of 900 ng genomic DNA,0.02 ng spike in plasmids, 1 ng 7 kb linear DNA fragment amplified from the dCas9-VP64 plasmid, 5 µL 10X Reaction Buffer, 5 µL ATP solution (25 mM) and ddH2O was incubated in a 50  $\mu$ L reaction system at 37 °C for 7-12 days with additional ATP solution and PSD added every 24 h according to the manufacturer's suggestion. Polymerase Chain Reaction on the external (spike-in plasmid and 7 kb linear fragment) and internal reference (Cox5b gene) was performed to evaluate the clearance of linear DNA and the retention of circle DNA in each sample. VAHTS DNA Clean Beads (Vazyme) were used to purified the circle DNA in the PSD reaction system after the PCR validation and eluted in 30 µL ddH2O. 14 µL purified product of each sample was subjected to subsequent Rolling Circle Amplification (RCA) by the Phi29 Polymerase (Thermo Scientific), which was conducted at 30 °C continuously for 48  $\sim$ 72 h. The RCA products were recovered by VAHTS DNA Clean Beads, eluted in 80 µL ddH2O and qualified by Qubit 4.0.

For eccDNA Next Generation Sequencing (NGS), first, a total of 1  $\mu$ g beads-purified RCA product of each sample was cut into small pieces (about 400 bp in length) by sonication (Covaris LE220), and 500 ng of sheared DNA was added to library construction according to the instruction of MGIEasy DNA Library Preparation Kit (MGI-BGI, China). The length distribution and quality of the libraries were analyzed by Bioanalyzer 2100 (Agilent). Then Samples' libraries were sequenced (PE150) at the MGISeq-2000 platform (QingDao, China).

To decipher eccDNA from the PE150 high-throughput sequencing data, we used the same methods described by the previous study [10]. In detail, reads were mapped to the human reference genome GRCh38 (UCSC) using Burrows Wheeler Aligner MEM v0.7.15 with default parameters to find chimeric reads. Then, two aligned BAM files were sorted by read names and coordinates to detect the exact eccDNA regions. To validate the accuracy of eccDNA detection, some filtering steps were conducted as follows: (1) The split reads  $\geq$  2, (2) Circle score  $\geq$  200, (3)

Coverage increase in the start coordinate  $\geq 0.33$ , (4) Coverage increase in the end coordinate  $\geq 0.33$ , (5) Coverage continuity = 0.2, (6) The SD of coverage smaller than the mean coverage over the whole eccDNA region, and (7) If the eccDNA sequence is longer than 2Kb, the eccDNA should have more than one inconsistent read. more details can be found in the method of our previous published study [10].

#### 2.2. PCR and Sanger sequencing of eccDNA junction region

To validate the eight eccDNAs, outward PCR and Sanger sequencing were conducted on the eccDNA junction sites. Firstly, we designed forward primers located at 200–300 bp upstream and reverse primers positioned at 200–300 bp downstream of each eccDNA junction region. 2x Rapid Taq Master Mix (Vazyme) was applied to perform standard PCR. The PCR system involved 12.5  $\mu$ L 2x Rapid Taq Master Mix, 0.2  $\mu$ L RCA product, 0.6  $\mu$ L forward primer (10  $\mu$ M), 0.6  $\mu$ L reverse primer (10  $\mu$ M) and supplemented with ddH2O to the volume of 20  $\mu$ L. The thermocycling PCR was performed by ProFlex<sup>TM</sup> PCR system, and the program was as follows: pre-denaturation at 95 °C for 2 min, (95 °C for 15 s, 55 °C for 15 s, 72 °C for 5 s) with 35 cycles, 72 °C for 2 min and keeping at 4 °C saved. Primers used for junction-sequencing are identical to the primers used for linear B fragment amplification (Table S1).

### 2.3. Artificial eccDNA synthesis by CAES

To synthesize the eight interest eccMIR found in this study, we introduced the LAMA strategy as previously reported (Paulsen T, 2019). To be specific, we firstly generated two half-complemental linear-DNA fragments (linear A and linear B) for each eccDNA synthesis by PCR on the eccMIR predicted regions. All primers used here were ordered from BGI-Shenzhen and are listed in the Table S1. PCR system consisted of 10 µL 2x Keypo Master Mix (Vazyme), 0.2 µL RCA product, 0.6 µL forward primer (10 µM), 0.6 µL reverse primer (10 µM) and ddH2O to a volume of 20 µL. Then, the reaction was conducted by the following program in a ProFlexTM system: 95 °C for 2 min; 35 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 3-11 s (extension time); 72 °C for 2 min and 4 °C saved. PCR products were confirmed by gel electrophoresis and purified by Cycle Pure Kit (Omega) according to the manufacturer's protocol. For LAMA reaction, 500 ng linear A and 500 ng linear B for each eccMIR were mixed with 1  $\mu$ L Taq DNA Ligase (NEB), 10 µL corresponding reaction buffer and supplementary ddH2O to a total volume of 100 µL. The reaction was performed in the ProFlex<sup>TM</sup> system by 95 °C for 5 min, (95 °C for 20 s,4 °C for 1 min and 65 °C for 20 min) with10 cycles. Next, to remove the residual linear DNA fragments after LAMA reaction, 1 µL Exonuclease V (NEB), 5 µL ATP solution (10 mM) and 10 µL NEB Buffer 4 were added to the LAMA products and left at 37 °C overnight. The next day, eccMIRs were purified from the hydrolyzed products by VAHTS DNA Clean Beads (Vazyme) and eluted in 20 µL DNase free water. To identify the circular structures of eccMIR and verified the clearance of linear DNA, we digested the eight eccMIR with different restriction enzymes (RE) and confirmed the digested samples by gel electrophoresis. Only those that had one linear band after single RE digestion were selected for subsequent experiments.

### 2.4. EccDNA transfection and real-time quantitative PCR (qPCR)

Human gastric cancer cells MGC-803, human Breast Ductal Cancer Cells T47D and human gastric mucosal epithelial cells GES1 (ATCC) were cultured in RPMI 1640 culture medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (ExCell), 1% GlutaMAX (Gibco), and 1% penicillin/streptomycin (100 units penicillin and 0.1 mg streptomycin/ mL). Human embryonic kidney 239 T HEK293T cells, Human glioma cells U251 and Human gastrointestinal stromal tumor cells GIST-430 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (ExCell), 1% GlutaMAX (Gibco), and 1%penicillin/streptomycin. All cells in this study were cultured in a 37 °C incubator with 5% CO2 atmosphere and maximum humidity. Cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) in 24 well plates following the manufacturer's protocol. Briefly, 60,000 cells were seeded in 24 well plates and medium was changed the next day before transfection. For each transfection, 500 ng of eccMIR and 1 µL Lipofectamine 2000 were diluted separately in Opti-MEM (Gibco) to a total volume of 25 µL, then the diluted DNA was added to the diluted Lipodectamine (1:1 ratio). After the incubation of 15 min, the transfection mixture was homogeneously added to the adherent cells in a dropwise manner. Each experiment in one group was repeated at least three times. Total RNA was extracted from cells after 48 h post-transfection by RNA Extraction Reagent (SeviceBio) based on the instruction of manufacturer and qualified by Qubit 4.0. For miRNA qPCR, firstly,1 ug total RNA was input for miRNA reverse-transcription (RT) by miRNA 1st Strand cDNA Synthesis Kit (Vazyme). U6 acted as internal reference gene and was created in the same RT reaction with target miRNA. The qPCR of miRNA was performed in replicate by miRNA Universal SYBR qPCR Master Mix Kit (Vazyme). For total RNA qPCR, 1 µg of total RNA was input for cDNA creation using HiScript III 1st strand cDNA synthesis kit (+ gDNA wiper) (Vazyme) and gPCR was conducted in replicate by ChamQ Universal SYBR qPCR Master Mix (Vazyme). All primers used in qPCR experiment are listed in the Table S2. The relative expression levels of miRNA and mRNA were calculated by  $2-\Delta\Delta Ct$ .

## 2.5. Droplet digital PCR (ddPCR)

To further confirmed whether the eccMIR could produce miRNA in different cell lines, digital droplet PCR was performed by the QIAcuity Digital PCR System. EccMIR#2, which harbors the most common length of eccDNA, was chosen to be transfected into different cell lines (MGC-803, T47D, U251, HEK293T and GIST-430). cDNA was created by the method mentioned above. Each ddPCR reaction system contained 4 µL 3x EvaGreen PCR Master Mix (FAM channel) (QIAGEN), 0.4 µM forward primer, 0.4 µM reverse primer, 50 ng cDNA, and supplementary RNase free water to 12 µL. The reaction mixture was transferred to the wells of nanoplate, which was then sealed with the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits (QIAGEN). The ddPCR was performed in the QIAcuity instrument by the following program: Activation of QuantiNova® DNA Polymerase (95 °C for 2 min) was followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s, cooling down to 40 °C for 5 min, then held at 12 °C. After the run is completed, the raw data were automatically sent to the QIAcuity Software Suite by which Data analysis was done. All primers used here are the same with those used for miRNA-qPCR (Table S2).

# 2.6. Dual-luciferase reporter gene assay

To validate the function of the eight interest eccMIR, we applied dual-luciferase reporter assay. In this experiment, two DNA oligonucleotides that carry the miRNA target gene sequences for each eccMIR were ordered from BGI (Shenzhen). Oligos are listed in the Table S3. All ordered oligos needed to anneal before being constructed into the pmirGLO plasmid. The Annealing system consisted of 1 µL sense oligo (100  $\mu M$ ), 1ul antisense oligo (100  $\mu M$ ), 2  $\mu L$  10  $\times$  NEB Buffer 2, and ddH2O to a total volume of 20 µL. Then we conducted the following program in a thermocycler: 95 °C for 5 min and ramp to 25 °C at a rate of -5 °C/min. Later, the annealing products with sticky ends were inserted into the 3' untranslated region of the firefly luciferase gene (luc2) in the pmirGLO plasmid. The successful insertion of the eccMIR target sequence in the pmirGLO plasmid was verified by PCR and Sanger sequencing. Firefly luciferase is the primary reporter gene, reduced firefly luciferase expression indicates the binding of introduced miRNAs to the cloned miRNA target sequence. Then the effects of the eight CAESeccMIRs on the relative expression level of firefly luciferase were detected by the Dual-luciferase Reporter Assay System (Promega)

following the manufacturer's protocol.

### 2.7. Time-course monitoring of the eccMIR persistence time in cells

The human foreskin fibroblast cells (HFF-1, ATCC number: SCRC-1041) were cultured in DMEM culture medium supplemented with 10% FBS (ExCell), 1% GlutaMAX (Gibco) and 1%penicillin/streptomycin. It was seeded in 24 well-plate and the transfection was conducted when the cells reached to 60-80% confluence. For each transfection,  $50\,\mu\text{L}$  mixture containing 20 ng linear or circle eccMIR or pmaxGFP plasmid and 0.75 µL lipofectamine 2000 (Invitrogen) supplemented with opti-M (Invitrogen) was prepared individually for each well according to the manufacturer's instruction. For each time point detection, the transfection was performed for triplicate. Cells were harvested for genomic DNA purification at each time point and qPCR detections were conducted in replicate according to the instruction of ChamQ Universal SYBR qPCR Master Mix (Vazyme). For each qPCR detection, 10 ng genomic DNA was input into one reaction and was performed in replicates. Primers for linear and circular eccMIR4535 detection were: eccMIR4535-qP-F: GTACAGGCCTCTGTCCATC; eccMIR4535-qP-R: CTCTTTCACAAACCCACTGTTG. Primers for pmaxGFP detection were: GATTGTCTGTTGTGCCCAGTC; pMaxGFP-oP-F: pMaxGFP-oP-R: CGTATGCAATGGCCAATAGCC. Primers for qPCR of the HBG1 gene locus (HBG1-F: GCTTCTGACACAACTGTGTTCACTAGC; HBG1-R: CAC-CAACTTCATCCACGTTCACC) worked as the internal reference in this study. The relative decay ratio of copy number in each group was calculated using the 2- $\Delta\Delta$ Ct method.

### 2.8. Statistical analysis

All the experimental assays were repeated for three to four times in independent experiments. For statistics data analysis and comparation between two groups, Student's *t*-test was used as instructed in the Graphpad Prism 9 software and P < 0.05 was considered statistically significant.

# 3. Results

## 3.1. CAES workflow description

The workflow of CAES method is illustrated in Fig. 1. Steps 1 and 2 provide a simple overview of the standard processing steps for Circleseq, as described in previous studies [26,32]. In summary, the total DNA was extracted from different sample types, including tissue, body fluid or cultured cells, depending on the specific subjects targeted in a given study. Subsequently, linear DNA fragments were removed from the purified total DNA through exonuclease digestion (researchers can use either plasmid-safe ATP-dependent DNase (PSD) or Exonuclease V (Exo) to achieve the purpose). The remaining circular DNA molecules (including mitochondrial DNA) in the target sample were then subjected to RCA and NGS analysis. Then researchers can analyze this information and search for the specific eccDNA of interest (EOI). In general, the EOI contains potentially functional genomic segments, such as enhancer elements, genic segments (including portions of exons or introns), microRNA genes, transposable elements (TE) and more.

Step 3 is the core process for EOI synthesis. First, design and synthesize two pairs of primer oligos for PCR of linear A and linear B fragments. The RCA product containing the EOI was used as a template for PCR. One pair of primers (blue arrows) amplified the interval DNA between eccDNA-start and -end coordinates (linear A), while another pair (red arrows) amplified the "inversed and half-way" complementary DNA fragment containing the eccDNA junction region (linear B). Second, once the two DNA fragments are prepared, conduct a standard LAMA reaction which requires cycles of denaturation, annealing and nick ligation (see the method). Third, purify the LAMA reaction product and remove the residual linear DNA by Exo digestion.

# 3.2. Junction sequence of endogenous eccDNA is not as accurate as predicted by Circle-seq

To assess the feasibility and reproducibility of CAES, we chose 8 eccDNAs containing different miRNA genes (eccMIR) which were identified in gastric cancer tissues in our previous study [26]. Information on the 8 eccMIRs is presented in Fig. 2A. In the recent study, we observed that the junction region sequences of validated eccDNAs did not precisely match those outputted by Circle-seq, as determined by Sanger sequencing. Typically, small deletions, rather than insertions, occurred in eccDNA junction sites. To confirm this phenomenon, we amplified and sequenced the junction regions of the 8 selected eccMIRs finding deletions of 1–3 bp in length (Fig. 2B). These results indicate that the junction sequence of eccDNA is not as precise as predicted by Circle-seq. This is crucial for ensuring accurate eccDNA synthesis and mimicking the biological role of endogenous eccDNA as faithfully as possible.

### 3.3. Construct eccDNAs with different sizes using CAES

After acquiring the sequence information of eccMIR junction sites, we designed paired primers for precise amplification of linear A and B fragments. The reference sequence of eccDNA and primers are listed in Table S1. To ensure the high-fidelity cloning of eccDNA sequence, the RCA-product from which the corresponding eccDNA was detected, was utilized as a PCR template. For example, in the present study, eccMIR#1 was found to exist in sample "JXM-7" by Circle-map analysis, then the RCA-product of JXM-7 was diluted to  $10-50 \text{ ng/}\mu\text{L}$  for the subsequent PCR reactions. We strongly recommend subcloning the PCR fragment to a cloning vector (e.g. the pCR Blunt II-TOPO vector) for Sanger sequencing and storing the vector as a DNA template backup for future use.

In this study, linear A and B fragments of the eight selected eccMIRs were amplified successfully and accurately from their corresponding RCA templates (Fig. 3A). We then conducted LAMA reaction and treated

Α							
	eccDNA label	Chr	Start	End	RCA label	microRNA	eccDNA size (bp)
	eccMIR#1	chr20	47,166,754	47,167,083	JXM-7	MIR3616	330
	eccMIR#2	chr9	28,888,617	28,889,028	JXM-7	MIR873	412
	eccMIR#3	chr22	48,780,111	48,780,708	JXM-4	MIR4535	598
	eccMIR#4	chr19	53,788,257	53,789,057	JXM-11	MIR373	801
	eccMIR#5	chr17	40,025,720	40,026,798	JXM-3	MIR6884	1,079
	eccMIR#6	chr11	34,941,018	34,942,545	JXM-8	MIR1343	1,528
	eccMIR#7	chr16	69,932,763	69,934,606	JXM-4	MIR140	1,844
	eccMIR#8	chr5	160,484,672	160,486,860	JXM-9	MIR146A	2,189
в				Junction			
-	eccMIR#1						🛆 1 bp del
	eccMIR#2				MMM		3 bp del
	eccMIR#3				MMM	MMMMM/	2 bp del
	eccMIR#4			MANN M	MAAAAA	MANA	1 bp del
	eccMIR#5			MM	MMM	MANA	3 bp del
	eccMIR#6	MA	<u> </u>				1 bp del
	eccMIR#7		XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX				3 bp del
	eccMIR#8		MAAMAAA			MANA A	🛆 2 bp del

**Fig. 2.** Selected eccDNA information and eccDNA-junction sequencing. **(A)** Information of the eight eccDNAs, including eccDNA label in this study, start and end sites of eccDNA on the chromosome, label of RCA sample which the target eccDNA was detected, miRNA gene which the eccDNA contained and eccDNA size. **(B)** Junction sites of the 8 selected eccMIRs were analyzed by Sanger sequencing and found that there were deletions of 1–3 bp in size, which indicated the junction sequence of eccDNA was not precise as predicted by Circle-seq.

both the linear fragments and LAMA-products with Exo digestion to verify the circular structure of the CAES-produced eccMIRs. The results show that all the linear DNA fragments were digested, while the LAMA circle-products were preserved (Fig. 3A). To further confirm the absence of residual linear fragments in the final CAES-products, single restriction endonuclease cleavage was conducted on both the linear fragment and the CAES-circles (Fig. 3B). The results show that the linear A fragments were cleaved into two bands while there was only one band for all the eccDNAs (Fig. 3C), reflecting the circular structure and the high purity of synthetic eccDNAs. Additionally, we assessed the circularization efficiency of CAES method by calculating the DNA weight ratio before and after LAMA reaction (final eccDNA weight divided by linear DNA weight inputted in LAMA reaction). As showcased in Fig. 3D, the yield of ecc-MIRs ranged from 15.60%  $\pm$  0.42% to 31.13%  $\pm$  1.01%, which were negatively correlated to the eccDNA size. These results indicate that CAES can produce eccDNAs with different lengths (up to 2187 bp) rapidly and efficiently.

# 3.4. CAES-prepared eccMIRs transcribed high level of miRNA-3p and – 5p molecules in diverse human cell lines

EccDNAs carrying miRNA genes can be transcribed independent of a canonical promoter and the transcripts will be processed by endogenous Dicer to form mature miRNAs [21]. In our previous study, we demonstrated that LAMA-produced eccMIRs were capable of expressing either miRNA-3p or - 5p in MGC-803 cells [26]. To evaluate the miRNA-transcription capacity of CAES-produced eccMIRs, we transfected the eight eccMIRs into HEK293T cells separately and detected the relative expression level of both miRNA-3p and - 5p by qPCR. It shows that the majority of tested eccMIRs were able to produce both miRNA-3p and - 5p with high efficiency, ranging from several to thousands of fold-increases that of the control group. Nevertheless, not all the ecc-MIRs exhibited high efficacy in miRNA production, likely due to that the fold-change of miRNAs arising from eccMIR was diminished by the basal level of endogenous miRNA level in the control cells. Only the ecc-MIR6884 and eccMIR1343 didn't elevate the miRNA-5p level. We speculate this was attributed to the different preferences of miRNA-3p and - 5p decay in a specific cellular context, influencing the rapid turnover dynamics of miRNA-3p and - 5p molecules in cells [33].

When analyzing the qPCR data for each eccMIR, we observed that neither endogenous hsa-miR873-3p nor - 5p was expressed in HEK293T cells (Ct value > 39). In addition, the size of eccMIR873 is 409 bp, which can represent the typical eccDNA length found in gastric cancer tissues (the highest peak of eccDNA length was 364 bp) [26]. Therefore, to evaluate the expression capability of CAES-produced eccMIR in different cellular contexts, we transfected eccMIR873 into six different human cell lines, including HEK293T, GIST-430, U-251MG, GES1, MGC-803 and T470, and measured the accurate miRNA molecules concentration by ddPCR. The results show that eccMIR873 can express a high concentration of both hsa-miR873–3p and – 5p molecules (Fig. 4B, blue dots showed in the ddPCR 1D plots) in the six cell lines, while the control group expressed no or very low copies of endogenous miR873 molecules (Fig. 4B and C). These results also present the relatively lower expression level of miR873-5p than that of miR873-3p, reflecting the differential endogenous processing pathways of miR-3p and - 5p. These tests and results demonstrate that CAES-produced eccMIRs can express miRNA molecules efficiently and robustly independent of a canonical promoter in human cell lines.

### 3.5. Artificial eccMIRs produced miRNA molecules are functional

To determine the functionality of the miRNAs produced by eccMIRs, we leveraged the dual-luciferase reporter assay in which the pmirGLO plasmid (Promega) contains a target sequence complementary to either the miRNA-3p or -5p at the 3'UTR of firefly luciferase gene, with the renilla gene serving as the internal luciferase control (Fig. 5A). We tested



**Fig. 3.** CAES-construction and verification of the 8 eccMIRs. (**A**) Amplification of linear A and linear B for LAMA reaction. Linear DNA can be digested easily by Exo, while LAMA products were reserved. (**B**) Schematic illustration of single restriction endonuclease cleavage. (**C**) Gel electrophoresis of single restriction endonuclease digestion products. The linear A fragments were cleaved into two bands while there was only one band for all the eccDNAs. (**D**) Measurement of CAES technique efficiency by calculating the DNA weight ratio before and after LAMA reaction (n = 4). The yield of eccMIRs ranged from 15.60%  $\pm$  0.42% to 31.13%  $\pm$  1.01%, which was negatively correlated to the eccDNA size.

the regulatory activity of either miRNA-3p or -5p expressed by the corresponding eccMIRs, given their relatively higher levels of miR-3p or -5p molecules in our qPCR data (Fig. 4A) and as indicated by miRbase (https://www.mirbase.org/). The results show that transfection of ecc-MIRs in MGC-803 cells repressed the firefly luciferase gene carrying either the miR-3p or -5p target sequence by up to 91.8% (ranging from 46.4% to 91.8%) (Fig. 5B). Furthermore, as shown in Fig. 5C, transfection of eccMIRs in MGC-803 cells repressed their downstream target mRNAs by up to 47.0% (ranging from 13.0% to 47.0%). These miRNA-

target genes were predicted by TargetScanHuman (https://www.targ etscan.org/vert\_80/). Together, both the dual-luciferase reporter assay and endogenous miRNA-targets qPCR assays demonstrate that CAESproduced eccMIR was able to produce functional miRNA molecules and modulate endogenous gene expression.



**Fig. 4.** MiRNA expression ability of the eight eccMIRs. (**A**) Evaluation of eccMIRs relative transcription level by qPCR. The majority of eccMIRs produced by CAES method can transcribe both miRNA-3p and -5p with high efficiency (n = 3 or 4). (**B**, **C**) Evaluation of eccMIR873 expression capacity in different cell lines (HEK293T, GIST-430, U-251MG, GES1, MGC-803 and T470) by ddPCR. EccMIR873 can express high concentration of both has-miR873–3p and -5p molecules (blue dots in Fig. 4B). \* indicated P < 0.05; \*\* P < 0.01; \*\*\*: P < 0.005 and \*\*\* P < 0.0001 in comparison with controls.



**Fig. 5.** Functional verification of the eight eccMIRs and persistence time assay in cells. (**A**) Diagram depicting the principle of eccMIR functional identification by dual-luciferase reporter gene assay system. (**B**) Measurement of eccMIRs target sites repression ability by dual-luciferase assay in MGC-803 cells. EccMIRs can repress the firefly luciferase gene carrying either the miR-3p or -5p target sequence by up to 91.8% (ranging from 46.4% to 91.8%). (**C**) Evaluation of endogenous target mRNA suppression by eccMIRs. Transfection of the eight eccMIRs in MGC-803 cells can separately repress their downstream target mRNAs by up to 47.0% (ranging from 13.0% to 47.0%). The statistic results were presented by Mean and S.E. of three experiments. \* Indicated P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and \*\*\* P < 0.0001. (**D**) Structure and qPCR primers design for copy number detection of linear, circle eccMIR4535 and the standard plasmid (pmaxGFP) transfected in HFF cells. P1 and P2 indicate forward and reverse primers for qPCR of the linear and circle eccMIR4535 junction site; P3 and P4 represent the primers for qPCR of the pmaxGFP plasmid. (**E**) Ct values of linear, circle eccMIR4535 and pmaxGFP plasmid detected by qPCR at different time points. (**F**) Decay kinetics of linear, circle eccMIR4535 and pmaxGFP plasmid in HFF cells 48–144 h post-transfection.

# 3.6. Kinetics of eccDNA decay in cultured cells is similar to standard plasmid and linear DNA

It has been reported that minicircle vector devoid of bacterial sequence displays highly enhanced and persistent transgene expression levels compared to the standard plasmid vector in vivo [30,34]. However, the kinetics of minicircle decay and its comparison with that of the plasmid and linear DNA remain unknown. To determinate the eccMIR decay in cultured cells, we transfected linear, circular eccMIR4535 and pmaxGFP plasmid into the human foreskin fibroblast (HFF-1), a primary cell which can be kept in culturing for more than 7 days without passaging, respectively. Then, the relative copy number of the three molecule forms were detected by qPCR every 24 h until 144 h post-transfection (Fig. 5D). The Ct values of linear, circular eccMIR4535 and pmaxGFP plasmid at different time points ranged from 8 to 13, 8 to 13 and 12 to 16, respectively, while the Ct value of internal reference HBG1 remained at around 26 (10 ng genomic DNA was input for each qPCR reaction) (Fig. 5E), indicating the absolute copy numbers of the three molecules at each time points were far more that the genomic DNA copies (represented by the HBG1 Ct value). Nevertheless, only 31.7% circular eccMIR4535, 33.0% pmaxGFP and 19.2% linear eccMIR4535 retained in the cells at 72 h compared to that of 48 h. And the percentages decreased to 6.3%, 7.0% and 5.2% up to 144 h (Fig. 5F). These results indicate that synthetic eccDNA can persistent at a relatively high level of copy numbers in host cells and the kinetics of artificial eccDNA decay is similar to those of conventional plasmid and linear DNA.

### 4. Discussion

Due to its distinct topological structure and enigmatic functions in cells, eccDNA has attracted increasing research interest in recent years. Although numerous studies suggest its potential role in various pathophysiologic states, including tumorigenesis [7,25,26], drug resistance [14,35], aging [36,37], human reproduction [4,8,9] and immune-response [13,38,39], the detailed molecular role of eccDNA remains to be elucidated. One major hurdle in the field is the lack of a convenient method for eccDNA synthesis. To overcome this limitation, we developed the CAES approach based on Circle-seq method. By integrating the characteristics of eccDNA-RCA products and LAMA protocol, CAES facilitates the rapid, cost-effective and high-fidelity production of tranfection-grade artificial eccDNAs. In the present study, we constructed eight cancerous eccMIRs spanning a wide size range and proved their functionality as miRNA producer in human cells.

Since the surge in eccDNA research in recent years, LAMA has gained popularity and is being adopted by an increasing number of researchers [21,24–26]. However, it's both money- and time-consuming to synthesize the required two "inversed and halfway" complementary DNA fragments before LAMA prescribed thermocycles. In the present study, CAES overcomes this limitation. For a researcher with the profiling data and the eccDNA-RCA product derived from a certain sample, it is possible to produce artificial EOIs within 5 workdays: (i) 3 days for paired-primers design and synthesis; (ii) half day for PCR and purification of the linear A and B fragments; (iii) half day for LAMA reaction; (iv) 1 day for Exo digestion and purification, while the original LAMA requires 3-4 weeks: (i) 2-3 weeks for linear A and B synthesis in a biotechnology company; (ii) 1 week for linear A/B amplification and LAMA reaction. In addition, since the linear A and B DNA fragments are cloned from the original eccDNA-RCA product, the sequence of CAES-produced eccDNA is identical to that of the endogenous one, preserving all the SNPs, indels or structural variants (SVs) carried by the EOI. Meanwhile, users can collaboratively adjust the paired primer sequence to match the eccDNA sequence, especially in cases where amplification of the required fragments is challenging due to abnormal DNA sequence composition of primer binding site. In contrast, the primers of LAMA method are fixed. Hence, CAES should be the preferred method for Circle-seq users aiming to investigate the eccDNA function.

On the other hand, CAES does have some limitations. Firstly, amplifying linear fragments longer than 10 kb can be challenging due to PCR capacity constraints. However, it's important to note that the bottleneck of LAMA also lies in obtaining dsDNA fragments with maximum length. Thus, both CAES and LAMA may not be suitable for the synthesis of large-size eccDNAs. Secondly, CAES relies on the Circleseq method, necessitating the availability of eccDNA profiling data and RCA product. Therefore, it is not applicable for individuals attempting to synthesize eccDNA without an actual biological sample. Nevertheless, we believe that the two "weaknesses" of CAES will not impede its applications in the eccDNA research field for two reasons: 1) The predominant eccDNA identified in human biological samples are small in size, typically ranging from a few hundred bp to around 2 kb in length [2-4,12-15]. The standard PCR can readily cover this length interval; 2) Circle-seq has become a common method among researchers studying the profiling of small-size eccDNAs in related samples. It is unlikely that they lack eccDNAs data and the corresponding RCA products.

Besides, this study presents a robust and promising method for achieving miRNA overexpression. It is well-known that endogenous miRNAs play critical roles throughout the whole cell life cycle by targeting and modulating gene expression in a posttranscriptional regulation manner [40]. Overexpression of miRNA has been widely used in both fundamental and clinical therapeutics. This is typically accomplished by utilizing a standard plasmid carrying a miRNA expression cassette or by employing synthetic miRNA/siRNA molecules. The two strategies are well established. However, certain shortcomings impede their progress in clinical applications: (i) plasmid carriers of miRNA necessitate essential prokaryotic DNA elements, including the origin of replication, antibiotic-resistance gene, viral promoter sequence and others. This configuration often leads to substantial transcriptional silencing of the transgene [34,41,42]; (ii) Synthetic RNA molecules are vulnerable to the abundant nucleases present in the bloodstream, resulting in a relatively short half-life and inherent instability in vivo [43]. In contrast, eccDNA carrying miRNA genes, devoid of bacterial DNA, is theoretically more stable with a longer half-life time than synthetic miRNA/siRNAs. This increased stability is attributed to its covalently closed circular structure [44]. Furthermore, it has been demonstrated that minicircle DNA vectors devoid of bacterial backbone can achieve 10-1000-fold enhancement in transgene expression level in vivo compared to standard plasmids [30,34]. This effect may be attributed to the elimination of prokaryotic DNA, which typically induces transgene silencing. Additionally, the smaller size of minicircle contributes to more effective delivery into cells. Prior studies on minicircle vectors, coupled with our own data, underscore the capability of synthetic eccMIR to generate persistent and high levels of miRNAs. This positions it as a novel and promising DNA vector for both fundamental and clinical applications.

### 5. Conclusions

In conclusion, we have introduced a rapid and user-friendly method, CAES, tailored for Circle-seq users to synthesize artificial eccDNA. Our study established the feasibility and reproducibility of the CAES method, showcasing that CAES-produced eccMIRs could express functional miRNA molecules at high levels across various human cell types. Furthermore, we emphasize the utility of synthetic eccDNA as a secure and bacterial DNA-free vector for robust and efficient miRNA overexpression underscoring its significant potential for applications in miRNA-based clinical therapeutics.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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### Author statement

Yonghui Dang and Xi Xiang conceived the idea and oversaw the study. Jiaying Yu, Haoran Zhang and Peng Han contributed equally to the experimental study and data analysis. All other co-authors contributed to the sample collection and performance of the experiments. Jiaying Yu, Yonghui Dang and Xi Xiang drafted the manuscript, and all authors read and revised the manuscript.

### **Declaration of Competing Interest**

The authors declare that they have no competing interests.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2023.12.019.

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