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Supporting Information

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Synthetic θ-Defensin Antibacterial Peptide as a Highly Efficient Nonviral Vector for Redox-Responsive miRNA Delivery

Meng Yu, Jin Yan, Wangxiao He, Chenyu Li, Peter X. Ma, and Bo Lei**

Supporting Information

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Meng Yu [a#], Jin Yan [a#], Wangxiao He [b#*], Chenyu Li [c], Peter X Ma [d], Bo Lei [a*]

[a] *Frontier Institute of Science and Technology, State Key Laboratory for Mechanical Behavior of Materials, Xi'an Jiaotong University, Xi'an 710054, China*

[b] *Center for Translational Medicine, Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology and Frontier Institute of Science and Technology, Xi'an Jiaotong University, Xi'an 710049, China*

[c] *School of Science, Xi'an Jiaotong University, Xi'an 710054, China*

[d] *Department of Biologic and Materials Sciences, Department of Biomedical Engineering, Macromolecular Science and Engineering Center, Department of Materials Science and Engineering, University of Michigan, Ann Arbor 48109, USA*

Contents

Materials and methods

1.1. Materials

Polyethylenimine (PEI, Mw=25 kD) was purchase from Sigma Aldrich. Lipofectamine 3000 (Lipo) were purchased from Life Sciences (Thermo Fisher Scientific). All chemicals were used as received without further purification. 5-Carboxyfluorescein (FAM) labeled miR-5106 were purchased from GenePharma (Shanghai, China). All the synthetic peptide source obtain from CS Bio (Shanghai) Ltd. Other biological chemicals were obtained from Life Sciences if not specified.

1.2. Synthesis and characterizations of RC101 and reduced RC101

RC101 were synthesized on MBHA resin in a CS bio 336X automated peptide synthesizer using the optimized HBTU activation/DIEA in situ neutralization protocol developed by ABI-supplied HBTU/HOBt protocol for Fmoc-chemistry SPPS. After cleavage and deprotection in a reagent cocktail containing 88% TFA, 5% phenol, 5% H_2O and 2% TIPS, crude products were precipitated with cold ether and purified to homogeneity by preparative C18 reversed-phase HPLC (E2695, Waters). The molecular masses were ascertained by electrospray ionization mass spectrometry (ESI-MS) (SQD2, Waters). Native chemical ligation reactions were carried out in 0.1 M phosphate buffer containing 6 M guanidine hydrochloride (GuHCl), 200 mM MPAA and 20 mM TCEP, pH 7.4. The ligation products were purified to homogeneity by preparative C18 RP-HPLC. To cyclize the defensin peptide, native chemical ligation reactions were carried out in 0.1 M phosphate buffer containing 6 M GuHCl, 200 mM MPAA and 20 mM TCEP, pH 7.4. The ligation products were purified to homogeneity by preparative C18 RP-HPLC. The as-prepared θ-defensin peptide was denoted as reduced RC101. The RC101 was obtained by the further oxidation of reduced RC101. The chemical structure of synthetic RC101 and reduced RC101 was analyzed through circular dichroism spectrum (CD, J-810, Jasco).

1.3. Simulation for RC101 structure

The structure of RC101and reduced RC101 were simulated by the Discovery Studio 2.5. Bond lengths were constrained using SHAKE, with a geometric tolerance of 0.0001. Nonbonded interactions were calculated using a twin-range cutoff, with short- and long-range cutoffs of 8 A° and 14 A°, respectively. In the water simulations, the SPC water model was used, and a reaction field correction was applied to electrostatic interactions. This parameterization considers partial charges of zero in all CHn atoms. Molecular dynamics simulations were performed using heat baths. Unless stated otherwise, the temperature coupling constant used is 0.1 ps. The time step used in the integration of equations of

motion was 0.002 ps. Simulations were run in the canonical ensemble. The simulated model was then subjected to MolProbity analysis (http://molprobity.biochem.duke.edu/index.php) to test its rationalization.

1.4. Zeta potential determination of RC101 and reduced RC101

The Zeta potential of samples was determined by a potential analyzer (Zetasizer Nano ZS, Malvern). Briefly, the RC101 and reduced RC101 was dissolved in phosphate buffer solution (PBS, PH 7.4), and the solution with a concentration of 10μM was transferred into a low volume Sarstedt UV-transparent disposable cuvette. Then the samples solution was then inserted into a Zetasizer Nano ZS for measurement.

1.5. Preparation of RC101@miRNA and reduced RC101@miRNA complexes

Before preparing miRNA complexes, RC101 and reduced RC101 were dissolved in deionized H_2O. RC101@miRNA complexes at different weight ratios were formulated by adding different volumes of RC101 solution into an equal volume of a defined miRNA solution, vortexed for 60 s, and incubated for 30 min at 37^{o}C. In the following experiments, the complex ratio was expressed as the weight ratio of RC101/miRNA. The commercial transfection agents PEI 25K@miRNA and Lipo@miRNA complexes were used as controls. The nanoscale morphology of RC101@miRNA complexes was evaluated by transmitted electronic microscopy (TEM, TEM, HT-7700, Hitachi). The hydrodynamic size distribution of samples was measured by the Zetasizer Nano ZS.

1.6. Loading efficiency evaluation of miRNA

The miRNA loading ability of samples was determined through the gel retardation method. Briefly, RC101 and reduced RC101@miRNA complexes solution at different weight ratios were loaded onto 2 % agarose gel (120 V) with ethidium bromide (EB) staining. These gel assays were performed in 1× Tris-acetate-EDTA (TAE) solution for 15 min. Then the gels were imaged and analyzed by a gel imaging system (Gel DOC^{TM} XR^{+}, Bio-Rad). The miRNA loading efficiency was calculated ImageJ software。The PEI 25K and Lipo were used as controls.

1.7. Polyanion competition and serum stability analysis

The stability of RC101/miRNA complexes was evaluated through a heparin polyanion competition and a serum stability assay. For the heparin polyanion competition assay, synthesized by RC101@miRNA and reduced RC101@miRNA complexes were incubated with different concentrations of heparin (3.12-100 μg/mL) for 1 h at 37^{o}C and subjected to 2% agarose gel electrophoresis. The

dissociate miRNA were stained by the Nucleic Acid Gel Stain (Genecopoeia). The released miRNA was calculated by ImageJ software (National Institutes of Health of USA). For the serum stability study, naked miRNA and miRNA nanocomplexes were incubated in 25% fetal bovine serum (FBS) at 37 ^{o}C for 0-48h. The samples were collected at different time points, added RNase inhibitors and froze in -20^{o}C. After collected all samples, the samples were mixed with loading buffer and loaded on gel. The resulted samples were stained and analyzed by 4% agarose gel electrophoresis and pictured. The intact miRNA percentage was determined by ImageJ software. The commercial transfection agents PEI 25K@miRNA and Lipo@miRNA complexes were used as controls.

1.8. Cells culture and cytotoxicity evaluation

Rat bone marrow derived mesenchymal stem cells (BMSCs, MT-BIO) were cultured in the minimum essential medium (MEM, Life technologies) containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) at 37^{o}C in a 5% CO_2 humidified atmosphere. BMSCs were seeded in a 96-well plate at a density of 10^4 cells/well and incubated for 24 h. The cytotoxicity of samples was carried out through incubating them with cells for 1, 3 and 5 days. The live cell attachment and cell viability was tested by alamar blue kit respectively (Invitrogen). Briefly, the cells were cultured with various concentrations of RC101 (30-240 μg/mL) with or without miRNAs. To analyze the cell viability, the cells were incubated with alamar blue kit for 4 h and the fluorescent intensity was measured using a microreader (SpectraMax$^{@}$, Molecular Devices). The reduced RC101, PEI 25K and Lipo was employed as controls. At least five species per sample were tested.

1.9. Intracellular miRNA transfection analysis

1.9.1 Confocal Laser Scanning Microscopy

Transfection studies were carried out in BMSCs using fluorescence-labelled miRNA. In brief, cells were seed at the density of 5×10^4 /well in 24-well plates and incubated for 24 h at 37^{o}C in 5% CO_2 atmosphere. The PEI/Rc101/Lipo/reduced RC101/miRNA complexes were formulated and incubated for 30 min at 37^{o}C before transfection. The cell culture medium in each well was removed and replaced with serum-free Opti-MEM (Life technologies), and 500 μL complexed solution contained PEI, Lipo, Rc101, reduced RC101 and miRNAs with different weight ratios were added into each well (1 mg miRNA/well). After incubating at 37^{o}C in 5% CO_2 for 24 h, the complexes were replaced with 500 μL of fresh complete medium. The transfection efficiency of Rc101, PEI and defensin was analyzed by Laser confocal microscopy (FV1200, Olympus).

1.9.2 Flow cytometry

BMSCs were transfected with FAM labelled miR-5106 delivered by PEI, Lipo, RC101 and reduced RC101 after cells seeding for 24 h. After that, the culture medium were changed to DMEM+10% FBS and continue culturing for 48 h. After washed by PBS for three times, the cells were typsinized and centrifuged, suspended with 200 μL PBS containing 2% FBS. Flow cytometry is monitoring via the FAM-labelled miRNA, cells were not necessary to be fixed with 4% PFA. The samples were analyzed using a cytoFLEX flow cytometer (Beckman Coulter) which counts 10,000 cells per sample. Data analysis were performed by gating on live cells based on forward versus side scatter profiles with CytExpert software (Beckman Coulter).

1.9.3 Real-time quantitative PCR

To determine the miR-5106 expression of complexes transfected into BMSCs, the Quantitive real-time PCR were applied. Briefly, BMSCs were transfected with RC101 loaded miRNA (weight ratio=30:1), after 4-6 h, the medium changed to fresh culturing medium. At predetermined time points, samples were trypsinized and RNA was collected using Tripure (Roche) according to manufacturer's protocol. Single strand cDNAs were obtained from 0.5 μg RNA using Revert Aid First Strand cDNA Synthesis Kit (Roche). The cycle parameters are: 65^{o}C for 10 min, 25^{o}C for 25 min, 55^{o}C for 30 min and 85^{o}C for 10 min. The qRT-PCR was set up in a 15 μL mixture containing 7.5 μL iTaq™ universal SYBR®Green Supermix (Applied Biosystems® 7500, Bio-rad), 6.4 μL ddH_2O, 0.5 μL cDNA template, 0.3 μL forward primer (0.1 μM) and 0.3 μL reverse primer (0.1 μM). The parameters are: 50 ^{o}C for 2 min, 95 ^{o}C for 2 min, followed by 40 cycles of 95^{o}C for 10 s, 58^{o}C for 30 s. To detect miR-5106 expression, we first reverse transcript miR-5106, the primer is CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCTGCCAA. Then miR-5106 was tested using $2^{-\Delta\Delta Ct}$ method in qRT-PCR by Bio-rad 7500 fast. The primers for qRT-PCR are shown in Table S1 and Table S2. Reduced RC101 and PEI 25K with miRNA (weight ratio=30:1) were utilized as control.

1.10. Von kossa Assay

Cells were seeded at the density of 2×10^5 cells/well in 24-well plate. Cells were transfected with miRNA delivered by PEI 25kD, Lipo, RC101 and reduced RC101 according the biomaterial: miRNA w/w ratio of 30:1. After osteogenic induction for 14 days and 21 days, the cells were analyzed by a von Kossa stain kit (Genmed) for detecting the extracellular matrix mineralization according to the manufacturer's protocol. The principle of von kossa assay was based on the silver nitration solution in this kit. When

insoluble calcium salt was replaced by silver, silver salt was reduced to black metal silver when samples under the light. Therefore, when calcium was generated in cells, the samples were easily stained by this solution. Briefly, at 14 days or 21 days, the cells were fixed with reagent A for 20 min at room temperature. After washed by PBS for three times, the samples were exposed under an ultralight lamp for 1 h. After that, the samples were washed by PBS for three times. The stained pictures were taken by an inverted microscope (IX83, Olympus).

1.11. *In vitro* antibacterial performance

The antibacterial activities of PEI 25K, reduced RC101 and RC101 were evaluated by the spread plate analysis method. Briefly, *Staphylococcus aureus* (ATCC No.29213) were adjusted to a cell density of 1×10^5 CFU/mL in different concentrations of RC101 (0.59 mM, 1.18 mM, 2.95 mM). The mixtures were incubated in 37^{o}C for 2 h. After that, 50 μL of samples were plated in triplicate onto Luria Bertani broth plates and incubated at 37 °C for 24 h. The bacterial growth was observed and captured. *S.aureus* without materials as a control.

1.12 Luciferase Reporter Assay

The dual-luciferase reporter gene vectors constructs were generated by cloning the entire 3'UTR of Gsk3a into pmiR-RB-Report™ vector (Ribobio, China) at the site which was digested by NotI and XhoI enzyme. The Firefly luciferase vector was used for internal reference. A total of 50 ng of pmiR-RB-ReportTM-Gsk3a 3'UTR and miR-5106 mimics were co-transfected into BMSCs in a 48-well plate using Lipofectamine 3000™ (Invitrogen). After 48 h, all the target validation assays were performed with the dual-luciferase reporter system (Vigorous Biotech) according to the manufacturer's instructions. The activities were measured by a SpectraMax@ optical analyze reader (Molecular Services).

1.13. Western blot

Total cell extracts were prepared from BMSCs. Proteins in BMSCs were extracted in 1×SDS-PAGE sample loading buffer. Total proteins were resolved by SDS-PAGE, transferred to PVDF membrane and probed with GAPDH (1:1000, Beyotime Biotech), GSK3A (1:1000, Sangon Biotech). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse were used as a secondary antibody (1:1000, Beyotime Biotech). The detection was performed using the ECL Western blotting Substrate (Millipore) and the chemical luminometer (Tanon).

1.13. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using two

population Student's t-test to evaluate the significant difference. The results were considered significantly when *P< 0.05 and **P< 0.01. Every experiment were conducted three times.

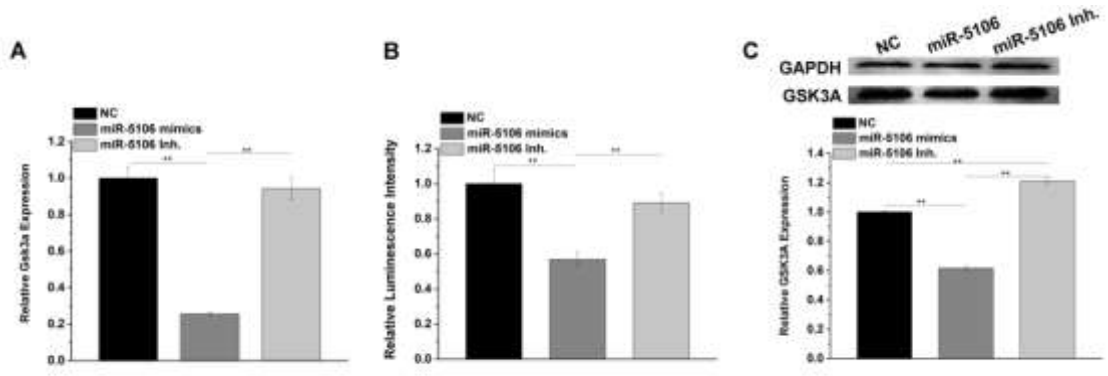

Fig.S1. Target mechanism study of miR-5106 in BMSCs. The effects of miR-5106 on the expression of Gsk3a. (A) Real-time PCR analysis revealed that miR-5106 mimics inhibit the expression of Gsk3a; (B) Relative luminescence intensity detected by a dual-luciferase vectors transfection study, suggesting the strong targeted interaction between miR-5106 and Gsk3a; (C). GSK3a protein levels was also down-regulated after over-expression of miR-5106 in BMSCs. (*P<0.05, **P<0.01).

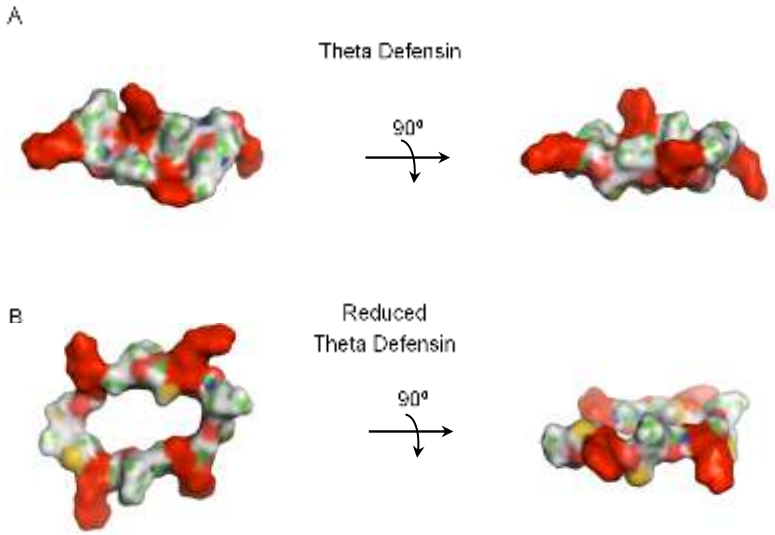

Fig. S2. Electron density pictures of RC101 (A) and reduced RC101 (B). The red represents the positive charges.

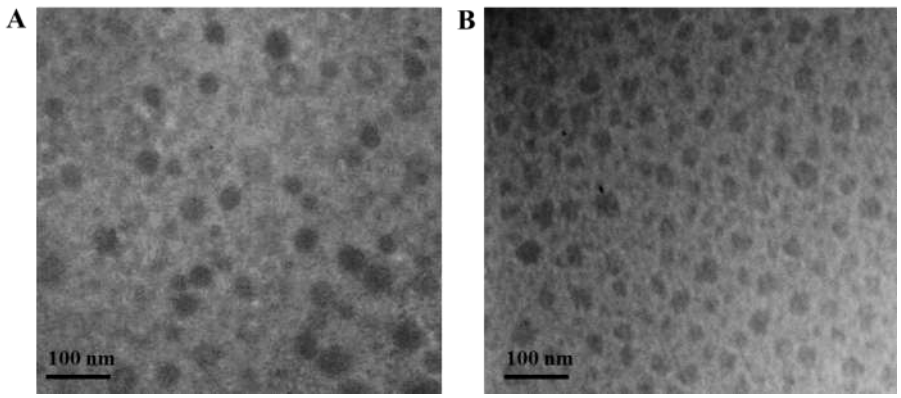

Fig. S3. TEM images and size distribution of RC101@miRNA (A) and reduced RC101@miRNA(B) complex. The TEM images showed the size distribution of 50-70 nm for RC101@miRNA and reduced RC101@miRNA.

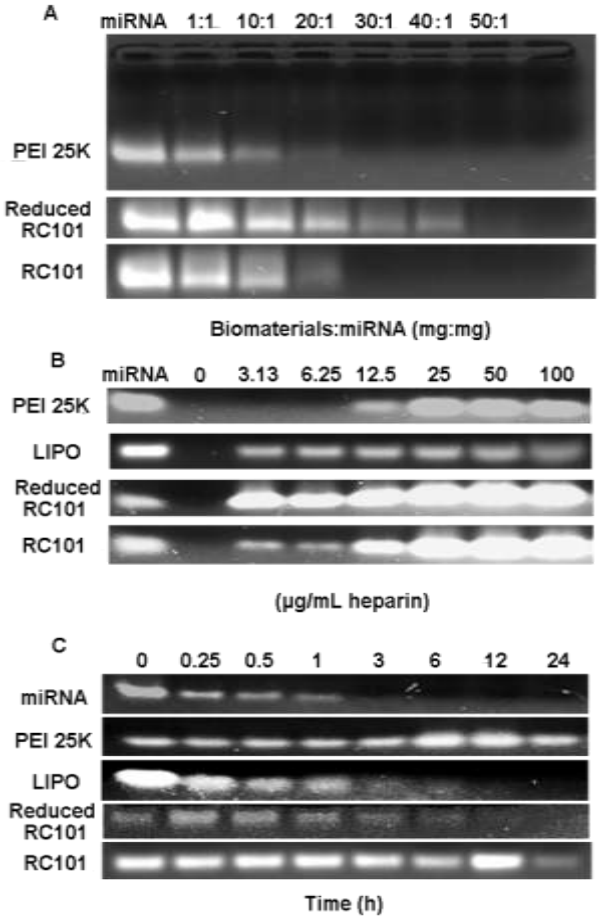

Fig. S4. miRNA loading ability and stability of RC101 and reduced RC101, as compared with commercial transfection agents PEI 25K and Lipo. (A) Electrophoretic retardation analysis of miRNA binding by PEI 25K, RC101, reduced RC101 at different weight ratios; (B) Stability of PEI 25K@miRNA, Lipo@miRNA, reduced RC101@miRNA, RC101@miRNA complexes(samples: miRNA weight ratio of 50:1) with increasing concentrations of heparin; (C) Serum stability of naked miRNA and various miRNA complexes during 48 h incubation.

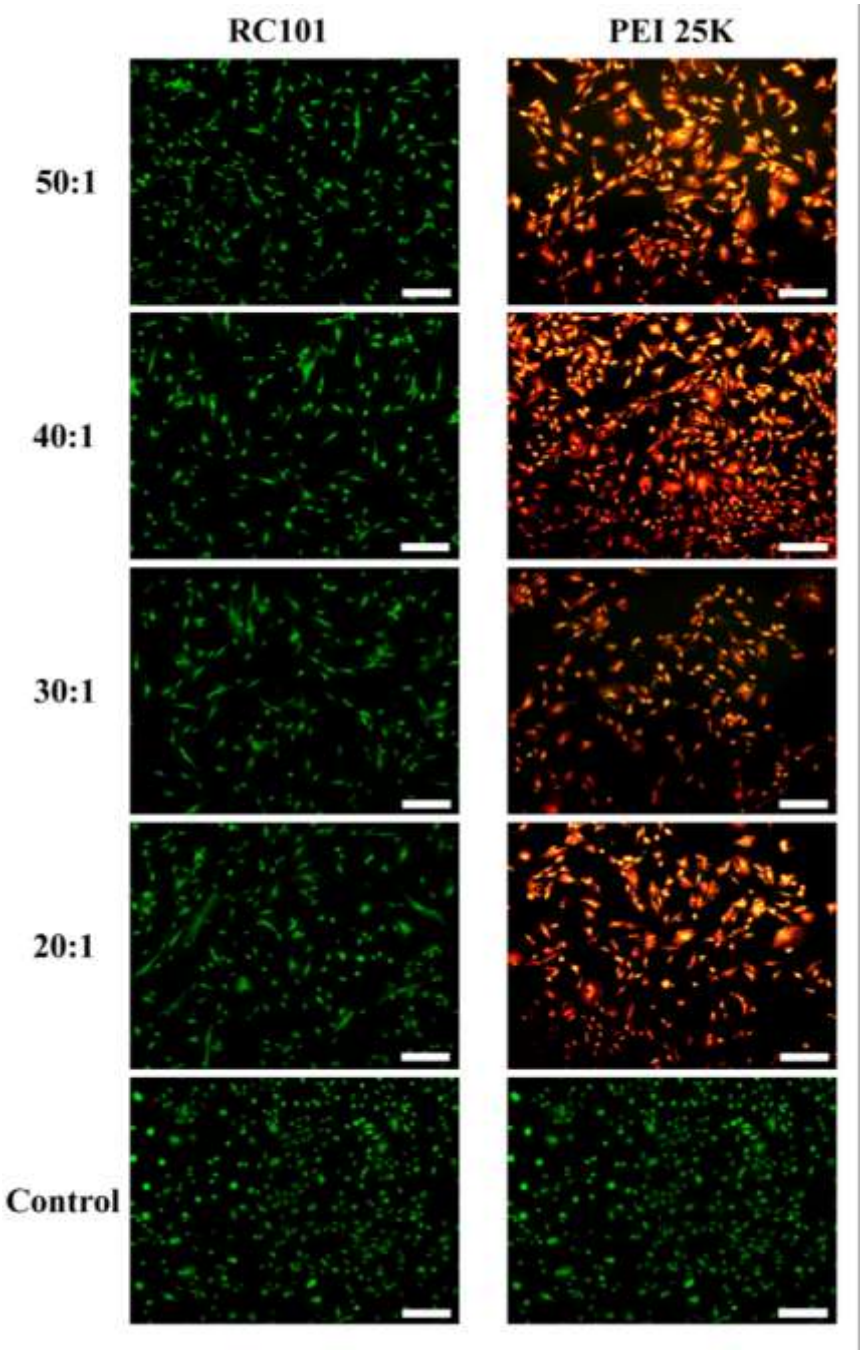

Fig. S5. Live-dead staining evaluation of BMSCs after culture with RC101@miR-5106 complex at different weight ratios for 24 h, PEI 25K and cells not cultured with biomaterials/miRNAs were used as controls. (Scale bar=200 μm).

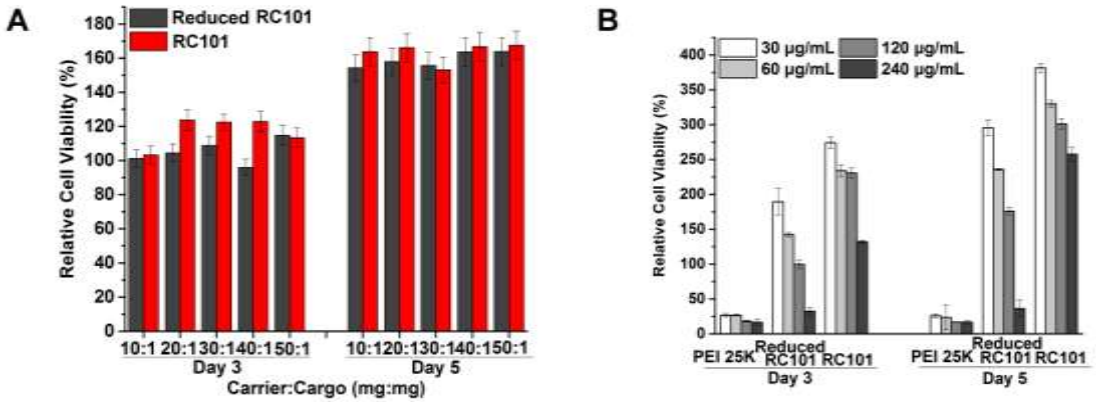

Fig.S6. Cytotoxicity evaluation of the samples (Lipo, PEI 25K, reduced RC101 and RC101) and RC101/reduced RC101@miRNA complexes toward BMSCs after incubated for 3 days and 5 days. (A) Cellular viability after culture with reduced RC101@miRNA and RC101@miRNA with different weight ratios; (B) Cell viability after incubation with different samples concentrations. At least three independent experiments were performed in triplicate.

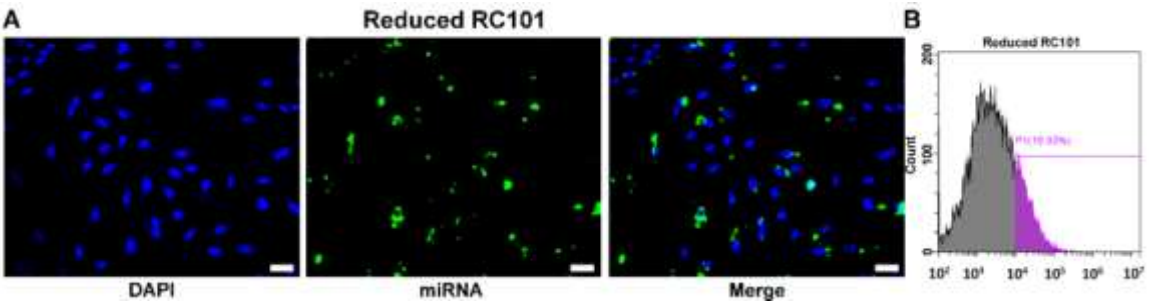

Fig. S7. Transfection efficiency evaluation of reduced RC101@miRNA in BMSCs. (A) CLSM images; (B) flow cytometry analysis after 48 h transfection. (Scale bar=100 μm).

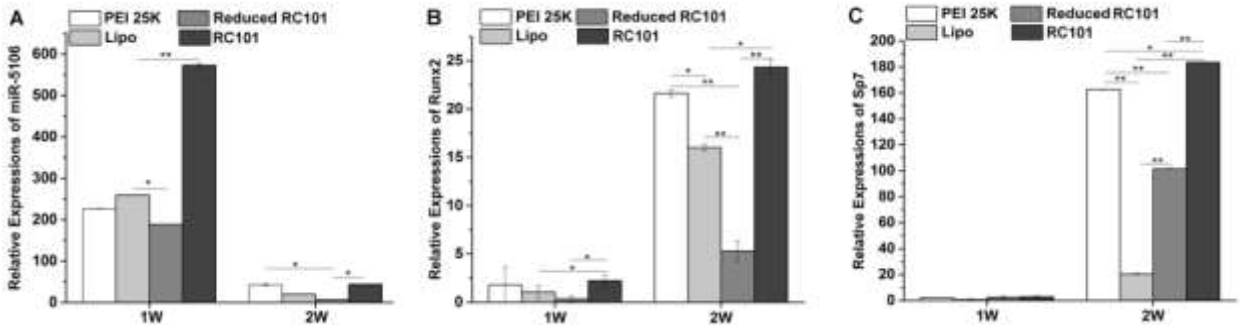

Fig. S8. Gene expressions of BMSCs after treated with PEI 25K, Lipo, reduced RC101 and RC101 for 1W and 2W. (A) miR-5106 ;(B) Runx2; (C) SP7. At least three independent experiments were performed in triplicate. (*P<0.05, **P<0.01)

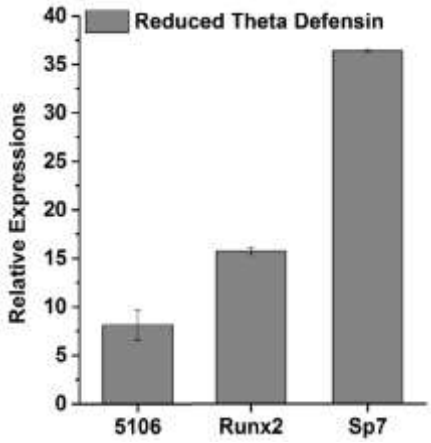

Fig. S9. Gene expressions of 5106, Runx2 and Sp7 of BMSCs treated with reduced RC101@miRNA for 3W. At least three independent experiments were performed in triplicate.

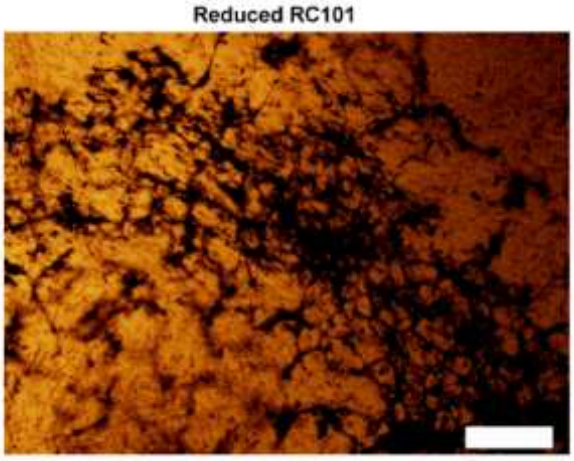

Fig. S10. Von Kossa stain of BMSCs after treated with reduced RC101@miRNAs for 3W (Scale bar=200 μm).

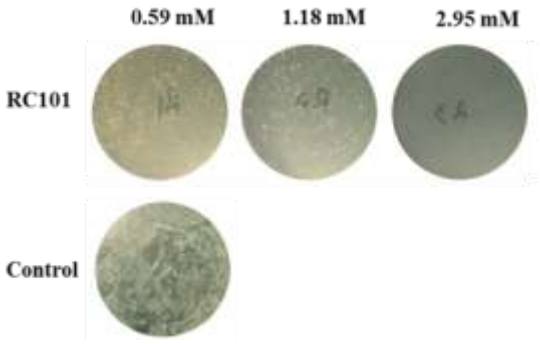

Fig. S11. Antibacterial activity evaluation of RC101. Representative images of viable colonies of *S.aureus* after incubated with different concentrations of RC101 for 24 h.

Table S1. qRT-PCR primers of miR-5106

Gene	Forward Primer & Reverse Primer (5'-3')	Annealing temperature (^{o}C)
U6	F:CTCGCTTCGGCAGCACA	60
	R:ACGCTTCACGAATTTGCGT	
miR-5106	F: ACACTCCAGCTGGGAGGTCTGTAGCTCAGTT	60
	R: TGGTGTCGTGGAGTCG	

Table S2. qRT-PCR primers of osteogenic gene markers

Gene	Forward Primer & Reverse Primer (5'-3')	Annealing temperature (^{o}C)
Gapdh	F: GGGTCCCAGCTTAGGTTCAT	59
	R: TACGGCCAAATCCGTTCACA	
Runx2	F:CCGAGACCAACCGAGTCATT	59
	R:TCACTGCACTGAAGAGGCTG	
Sp7	F:GCCACCCATTGCCAGTAATC	59
	R: AGTGAGCTTCTTCCTGGGGA	
Gsk3a	F: ATTATGCGTAAGCTGGACCAC	60
	R: CGTCTCGGGCACATACTCC	