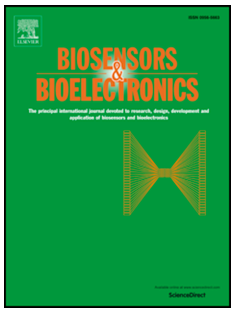

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Wenhao Dai designed the research, performed the experiments, analyzed data, and wrote the manuscript.

Lei Su performed experiments, analyzed data, and wrote the manuscript.

Huiting Lu assisted with the preparation of materials and execution of experiments.

Haifeng Dong designed and supervised the research and wrote the manuscript.

Xueji Zhang supervised the research and wrote the manuscript.

Exosomes-mediated synthetic Dicer substrates delivery for intracellular Dicer imaging detection

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Abstract

Ribonuclease Dicer initiates gene-silencing process by cleaving exogenously long RNA duplexes into small interfering RNA (siRNA) or endogenous precursor microRNAs (pre-miRNAs) into mature miRNAs. It holds great promise in cancer diagnosis and therapeutics due to its molecular ruler role. However, the intracellular Dicer detection remains a key challenge and Dicer related gene therapy has never been explored. In this study, we design a fluorescent labeling Dicer substrate and effectively deliver it into cell by exosomes derived from the target parent cells for intracellular Dicer expression level monitor and gene therapy. Using pre-miRNA let-7a as a model, the Dicer substrates with two terminals labeled with fluorescent and quencher group respectively was obtained by T4 RNA mediated ligase reaction from two short RNA sequences. Then, the substrate was packaged into exosomes by electroporation and delivered to target cells for intracellular dicer imaging detection. After packaging substrates into exosomes with little immunogenicity and good innate biocompatibility by electroporation and delivered to target cells, the Dicer mediated substrate cleavage was effectively monitored by the fluorescence recovery, providing a powerful tool for Dicer analysis. Importantly, the cleaved product exhibited significant suppression toward tumor cell growth and regulated cancer cells cycle. This work might open a new avenue for Dicer analysis and Dicer-related clinical application.

Keywords: Dicer detection, miRNA let-7a, exosomes, cell imaging, gene therapy

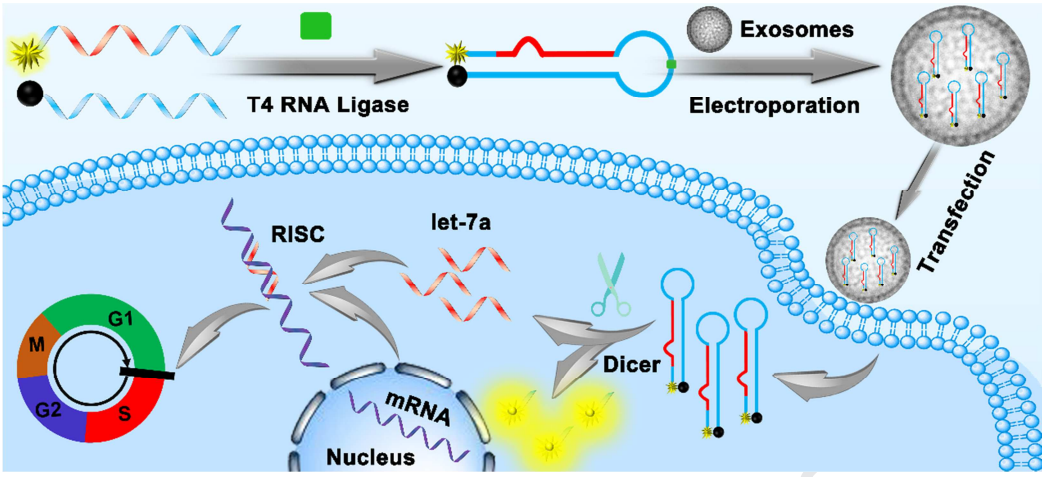
1. Introduction

Dicer is an endoribonuclease in the RNase III family, mainly exists in cytoplasm, and highly conserved in evolution (Lee et al., 2004; Park et al., 2011). It cleaves the precursor microRNAs (pre-miRNAs) to mature miRNAs and exogenously long RNA duplexes into small interfering RNA (siRNA) to mediate gene silence (Hoehener et al., 2018; Hyun et al., 2014; Macrae et al., 2006). Thus, it plays key regulatory roles in a variety of biological processes and pathological processes. Especially, impaired Dicer has been confirmed in many kinds of tumors and it could act as an important tumor inhibitor (Kumar et al., 2009; Pampalakis et al., 2010; Prodromaki et al., 2015). As a cleaved product of Dicer enzyme, miRNAs are small, endogenous and noncoding regulatory RNAs that regulate gene expression at the post-transcriptional level to induce either translational repression or mRNA degradation or sequestration of mRNA from translational machinery (Ambros, 2004; Bartel, 2004; Dong et al., 2013). Recent studies have shown that impaired Dicer function induced a decline in mature miRNAs expressional level, and indirectly influenced the process of tumor occurrence, development and metastasis (Iliou et al., 2013; Kumar et al., 2007). Traditionally, the Dicer detection is involved of complex cell lysis and detection by western blot, real time PCR, ELISA or electrochemistry, and they suffered from limited sensitivity, complicated operation process and quantitative detection (Perron et al., 2011; Passon et al., 2011; Zhao et al., 2017; Prodromaki et al., 2015). The expression levels of the Dicer related miRNAs remains unexplored. Therefore, efficient intracellular real-time monitor probe of Dicer is urgently needed for cancer early clinical diagnosis or prognosis, and the Dicer substrates of pre-miRNAs hold great promise for probe design.

Besides detection probe, the other significant biological barrier for intracellular Dicer detection is the natural cell membrane, which required efficient gene vector due to the low transfection efficiency of the free gene probe. Commercial liposomes are artificial lipoid bodies prepared by arrayed phospholipid biomolecules and widely used as carriers in the gene transfection (Felgner and Ringold, 1989; Malone et al.,

1989). However, their potential cytotoxicity would seriously influence the cell status and induce false information, which has attracted intense attention (Lv et al., 2006). Recently, exosomes derived from the membranous vesicle of late endocytosis in living cells cytoplasm have emerged as promising drug and gene delivery vectors for diagnostic and therapeutic (Alvarez-Erviti et al., 2011; Oh et al., 2019; Xitong and Xiaorong, 2016). It is nano-sized membrane vesicles secreted by virtually all cells with a mean diameter of about 40-100 nm for intercellular communication (An et al., 2019; Mathieu et al., 2019; Milane et al., 2015; Yang et al., 2019) . In comparison with traditional commercial liposomes, it exhibits multiple advantages such as little immunogenicity, good cell fusion ability, high abundance in biological fluids and especially innate biocompatibility (Heusermann et al., 2016; Tian et al., 2013). Thus, the natural exosomes are promising tools for gene delivery across membrane barriers (Alvarez-Erviti et al., 2011; Pitt et al., 2016; Usman et al., 2018; Wang et al., 2017).

Herein, we design a Dicer substrate of pre-miRNA let-7a, a hairpin sequence with two terminals labeled with fluorescent dye and quencher, respectively, and package it into exosomes for intracellular Dicer detection and gene therapy. As shown in Scheme 1 and Fig. S1, the synthetic Dicer substrate was originally in native hairpin formation, and the 5’ terminal fluorophore was in close proximity to the 3’ terminal quencher, leading to fluorescence quenching. The Dicer substrate was packaged into exosomes through electroporation, and it promoted the cell internalization of the Dicer substrate due to the good cell fusing capability. After the Dicer substrate was recognized and cleaved by intracellular Dicer, the fluorophore separated from the quencher to produce strong fluorescence for Dicer detection. Notably, the cleavage products maintain the intrinsic gene silence ability to regulate the cell proliferation and cycle. To our best of knowledge, this is the first report about the intracellular Dicer detection and biomedical applications, and it may open a new avenue for Dicer analysis and Dicer-based clinical diagnosis and therapeutics.



Scheme 1. Schematic presentation of exosomes-mediated synthetic Dicer substrate for intercellular Dicer detection and gene therapy.

2. Materials and methods

2.1. Materials

Tris(hydroxymethyl)aminomethane (Tris), DL-Dithiothreitol (DTT) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (China). Hydrochloric acid (HCl), Sodium chloride (NaCl), magnesium chloride ($MgCl_2$), and dimethyl sulphoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Hoechst 33342 was obtained from Yeasen Biotech. Co., Ltd. (Shanghai, China). One-stop miRNA urea-PAGE pack, column polyaerylamide (PAGE) $miRNA_{BACK}$ and Recombinant Dicer 1 were obtained from Kalang Biotech. Co., Ltd. (Shanghai, China). T4 RNA ligase, M-PER Reagent, phosphate buffer saline (PBS, pH 7.4), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA and penicillin-streptomycin (PS) were all purchased from Gibco Life Technologies (AG, Switzerland). Exosomes Isolation Kit was purchased from Umibio Co., Ltd.

(Shanghai, China) to extract exosomes from different cell lines culture media. The RNA sequences purified using high-performance liquid chromatography were obtained from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

RNA sequence-1:

5’ FAM-UGG GAU GAG GUA GUA GGU UGU AUA GUU UUA GGG UCA CAC CCA CCA 3’

RNA sequence-2:

5’ Phosphate-CUG GGA GAU AAC UAU ACA AUC UAC UGU CUU UCC UA-BHQ1 3’

2.2. Instruments

The gel electrophoresis images were captured with an Alliance Ld2 (Uvitec, Cambridge, U.K.) with a UV lamp at 365 nm. All fluorescence measurements were performed on a Hitachi F-7000 fluorescence spectrofluorometer (Tokyo, Japan). The morphology of exosomes was examined with transmission electron microscopy (TEM) (using a JEM 2100 TEM microscope) at an acceleration voltage of 200 kV. The concentration and size were measured by nanoparticle tracking analysis (NTA) (ZetaView, Germany). The size distribution and zeta potential analysis were performed using a Zetasizer Nano ZS system (Malvern, UK), and a 633 nm laser was used for the dynamic Light Scattering (DLS). The intracellular Dicer imaging was conducted by a laser confocal microscope. (CLS, FV1200, Olympus, Japan).

2.3. Fabrication of Dicer substrate

The sequence of pre-miRNA let-7a is queried from miRNA database miRBase. According to the manufacturer's protocol of T4 RNA ligase, the RNA sequence-1 with 5'-labled FAM was added to RNA sequence-2 with 5'-labled phosphate group and 3'-modified BHQ1 in Tris-HCl (50 mM pH 7.5) containing 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 0.1 mg/mL BSA, and 200 U T4 ligase and reacted for 18 h at 37 ^{o}C (Davies and Arenz, 2008). The target Dicer substrates were purified by denaturing 15% PAGE gel electrophoresis according to the protocol of column PAGE $miRNA_{BACK}$.

7 M urea denaturing 15 % PAGE gel was prepared at room temperature according to the protocol of One-stop miRNA urea-PAGE pack to confirm the successful formation of the Dicer substrate. Before sample analysis, the prepared gel should be pre-run in order to remove the adverse effect of ammonium persulfate and reached the desired temperature. The analytical samples were mixed with loading buffer and added to the corresponding cleared hole, and run for 2 h at a constant voltage of 200 V and then analyzed with a gel-imaging system (Alliance Ld2).

2.4. Fluorescence spectrometry assays

To investigate the cleavage performance of Dicer toward Dicer substrate, the synthetic Dicer substrate (50 nM) was incubated together with recombinant Dicer (0.5 U) or Dicer from cell lysate (0.5 μL) for different times to monitor the fluorescence intensity.

2.5. Exosomes extract

One human cancer cell line of A549 and two normal cell lines of human embryonic lung fibroblast (MRC-5) and normal human dermal fibroblasts (NHDF) were cultured with DMEM media. When the cell fusion reached approximately 60%-70%, the DMEM media were replaced by fresh opti-MEM, and continued cultured for 36 h. Finally, the media were collected and processed to extract the exosomes through polymer-based precipitation according to the protocol of Exosomes isolation kit. The exosomes were stored in EP tubes at -80 ^{o}C.

2.6. The cytotoxicity of exosomes

The cytotoxicity of exosomes was evaluated by MTT, cell apoptosis and cycle experiments, and the commercial cationic liposome was also employed as a control. The MTT assay was carried out following our previous report (Dong et al., 2015). Cell apoptosis and cell cycle were operated according to the protocols of cell apoptosis detection kit, and analyzed by BD AriaII flow cytometry.

2.7. Packaging of Dicer substrate into exosomes by electroporation

The packaging of Dicer substrate into exosomes by electroporation was performed according to the typical procedure with brief modification (El-Andaloussi et al., 2012). The Dicer substrate (10 μM) with fluorescence labeling was mixed with exosomes (1 ×10^{10} particles/mL) in electroporation buffer (PBS buffer and 21% OptiPrep (v/v)) at 200 V using EBXP-H1 electroporation system (Etta BioTech). Then, the resulting

solution was centrifuged through polymer-based precipitation, and the precipitate was collected and re-suspended by PBS buffer for further use.

To quantify the Dicer substrate, the Dicer substrate-loaded exosomes (1 ml) were placed into a quartz cuvette and fluorescence was detected using a Hitachi F-7000 fluorescence spectrofluorometer (Tokyo, Japan) with excitation/emission at 488 nm/523 nm.

2.8. Intercellular Dicer detection

Three different cell lines were cultivated in confocal dish containing DMEM (1 mL) for 12 h. Then, the medium was replaced with Opti-MEM containing exosomes (50 nM loaded Dicer substrate), which was extracted from parent cells, and cultivated for 4 h. After washing with PBS, the fresh DMEM was added and cultured for another 36 h before detecting by a laser scanning microscope.

2.9. Gene therapy

The exosome (5×10^8 particles/mL) loaded with different concentrations of Dicer substrate (10, 25, 50, 75, 100 nM) were prepared for gene therapy by MTT. Briefly, the A549 and MCF-7 cells (5.0×10^4) were cultured for 12 h in a 96-well plate containing DMEM (100 μL) in each well. Then the medium was replaced with fresh serum-free medium (Opti-MEM) alone or medium containing the Dicer substrate-loaded exosomes. 4 h later, the medium was replaced with fresh DMEM (100 μL) and incubated for another 12 h. Next, MTT (20 μL, 5 mg/mL) was added to

each well. The media was removed 4 h later, and DMSO (100 μL) was added to each wells to solubilize the formazan dye. After shocking (37 °C, 120 rpm) for 15 min, the absorbance of each well was measured using a Tecan Sunrise at 492 nm. The anti-tumor rate of the Dicer substrate was estimated by the percentage of growth inhibition calculated with the formula.

Anti-tumor rate % = $(1 - A_{text}/A_{control})$ 100%

The exosome (5×10^8 particles/mL) loaded with 100 nM Dicer substrate was measured by cell apoptosis and cycle according to the protocols of cell apoptosis and cycle detection kit, and analyzed by BD AriaII flow cytometry.

3. Rssults and discussion

3.1. Feasibility of the Dicer substrate for Dicer detection in vitro

miRNA let-7a is one of the most significant cancer-related miRNAs that down-regulated in various cancers and validated as a key anti-oncogene for tumor metastasis and cell cycle regulation (Han et al., 2019; Li et al., 2018). Using pre-miRNA let-7a in A549 cells as a model, a Dicer hairpin detection probe with two terminals labeled with 6-carboxy-fluorescein and BHQ1 quencher respectively was obtained by T4 RNA mediated ligase reaction from two short RNA sequences (Fig. S1). Denaturing polyacrylamide gel electrophoresis (PAGE) experiment was performed to evaluate the formation of the Dicer substrate. As shown in Fig. 1A, both of RNA sequence-1 (45 nt, lane 2) and RNA sequence-2 (35 nt, lane 3) exhibited a single obvious electrophoresis band, the relative lower electrophoretic mobility of both two RNA sequences than miRNA marker were observed due to fluorescein or quencher group modification. A new strong band was appeared in the linked product (lane 4), suggesting Dicer substrate was successfully formed. We further investigated

the Dicer cleaved ability toward the resulting Dicer substrate by electrophoresis and fluorescence experiments. As shown in Fig. 1B, Dicer substrate (pre-miRNA let-7a) could be efficiently recognized and cleaved by recombinant Dicer (lane 3) and cell lysate (lane 4) to produce 22 nucleotides miRNA and other nucleotides fragments. The resulting Dicer substrate was first purified by PAGE to decrease the background signal and operated Dicer cleavage fluorescence experiment. The fluorescence intensity increased as a function of time, and a three-fold enhancement in fluorescent intensity was observed after 3 h, indicating efficient cleavage of Dicer substrate by the recombinant Dicer (Fig. 1C). Furthermore, the cell lysate also showed a fast fluorescence intensity recovery dynamic model toward the Dicer substrate (Fig. 1D). These results suggested the synthetic Dicer substrate was reliable and efficient for Dicer enzyme detection.

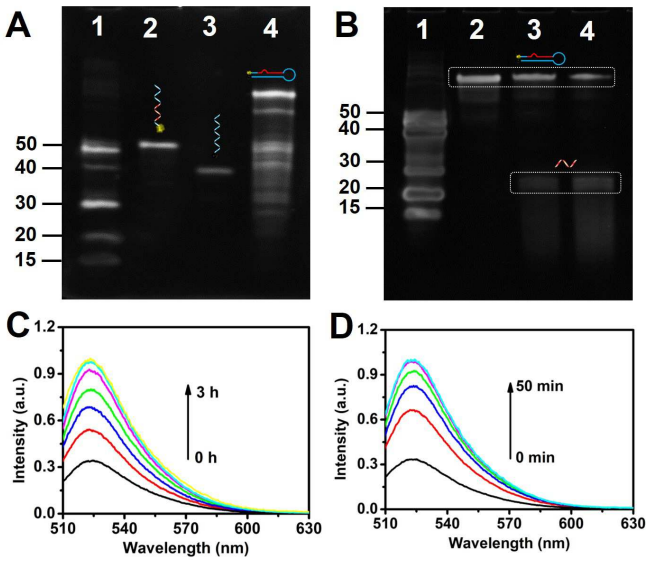


Fig. 1. (A) Verification of the formation of Dicer substrate by denaturing PAGE: lane 1: miRNA marker; lane 2: RNA sequence-1 (2 μM); lane 3: RNA sequence-2 (5 μM); lane 4: linked product (5 μM). (B) PAGE analysis of Dicer substrate cleaved by Dicer: lane 1: miRNA marker; lane 2: Dicer substrate (5 μM); lane 3: Dicer substrate (5 μM) and recombinant Dicer; lane 4: Dicer substrate (5 μM) and cell lysate. The fluorescent intensity of mixture containing Dicer substrate with recombinant Dicer (C) and cell lysate (D) at different time.

3.2. Characterization of exosomes

The resulting Dicer substrates were packaged into exosomes extracted and purified

from human A549 cells through polymer-based precipitation for cellular delivery to avoid degradation during transfection process and improve internalization efficiency. TEM and NTA were first used to characterize the extracted exosomes from A549 cells. Negative staining of exosomes showed classical round-shaped double membrane morphology of exosomes with an anticipated size distribution of 50-100 nm (Fig. 2A and Fig. S2). According to the NTA measurements from Fig. 2B, the exosomes profile exhibited an average aqueous diameter of 110 nm, which was slight larger than the result of TEM due to the existence of hydration layer, in consistent with previously reports (Li et al., 2017; Liang et al., 2019). These results confirmed the successful generation of exosomes.

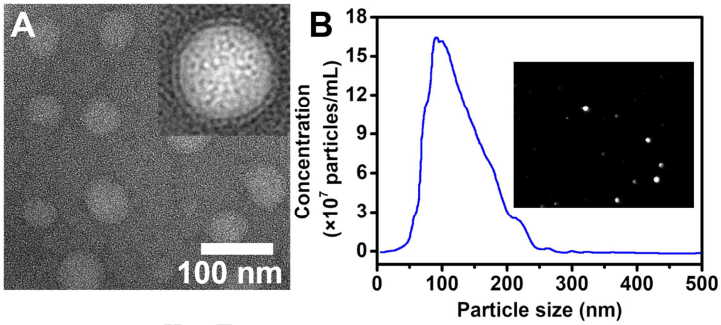


Fig. 2. (A) TEM image of exosomes extracted from A549 cells visualized by negative staining. (B) NTA profiles of exosomes extracted from A549 cells. Inset A: high-resolution TEM image of exosomes. Inset B: the corresponding exosomes with scattered light.

3.3. The Cytotoxicity of exosomes

The cytotoxicity of exosomes to target cells was further investigated by MTT assay and flow cytometry analysis, and the cytotoxicity of commercial cationic liposome 2000 was also measured as a control. As shown in Fig. 3A, the exosomes exhibited almost no toxicity to parent A549 cells even at high concentrations (7.5×10^8 particles/mL), while liposome has obvious toxicity toward A549 cells with approximately 25-30% suppression at the same concentration, indicating the good biocompatibility of the exosomes. Similar results were observed for the exosomes derived from MRC-5 and NHDF cells (Fig. S3). As for the apoptotic analysis, the exosomes-transfected A549 cells exhibited an apoptosis rate of 7.59% and a death rate

of 1.82% (Fig. 3C), being similar to the control group (Fig. 3B). The apoptotic and necrotic rate for liposome-transfected A549 cells was 2-folds higher than that of exosomes-transfected A549 cells. The influence of exosomes to cell cycle was also investigated. As shown in Fig. 3E-G, neither exosomes nor liposomes would cause significant influence on cell cycle compared to the control group. These results suggested the liposome might induce cell apoptosis and necrosis by influencing on the integrity and function of cell membrane and subcellular membrane structure (Xu and Szoka, 1996), and the biodegradation product of liposomes may also produce cytotoxicity (Lv et al., 2006). The natural exosomes delivery was excellent biocompatible and showed negligible influence on the biological processes of target cells, which could be vital for intracellular molecule analysis with little false signal.

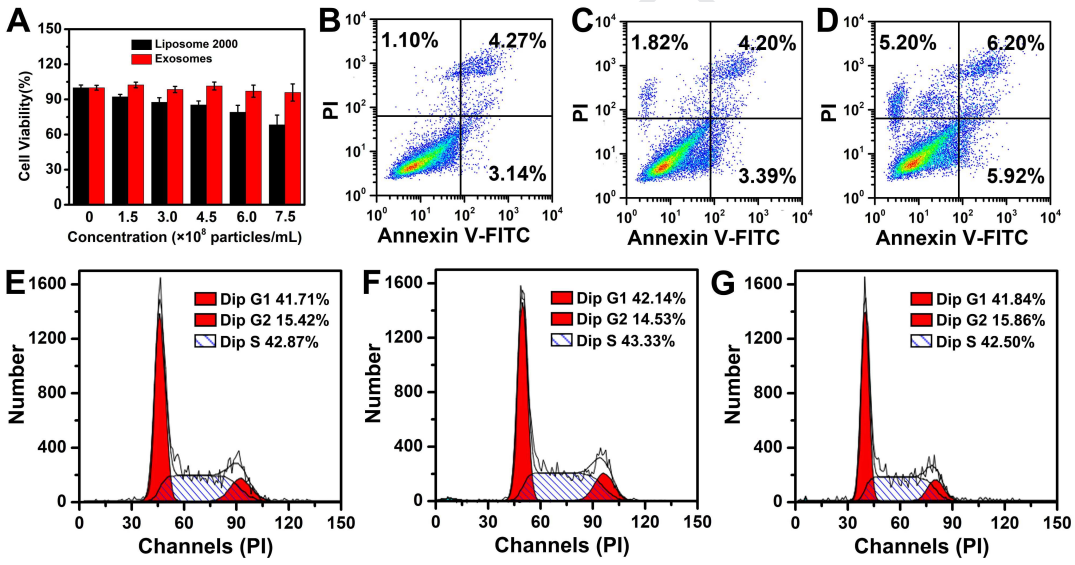


Fig. 3. (A) Cell viability after incubation of A549 cells with exosomes and liposome at different concentration; Flow cytometric analysis of A549 cells apoptosis transfected with (B) cell medium, (C) A549 exosomes and (D) liposome; Flow cytometric analysis of A549 cell cycle transfected with (E) cell medium, (F) A549 exosomes and (G) liposome.

3.4. Intracellular Dicer imaging detection

It was reported that the impaired Dicer enzyme would induced low expression of miRNA let-7a in A549 cells and cause carcinogenesis (Pampalakis et al., 2010). Therefore, it is imperative for real-time monitoring the expression level of intracellular Dicer in the early clinical diagnosis of cancer. The synthetic Dicer

substrates were packaged into exosomes by electroporation, and the zeta potential measurements showed little change of the exosomes before and after electroporation (Fig. S5), indicating the internalization of the Dicer substrates rather than membrane absorption (Lunavat et al., 2016). As shown in Fig. 4, the green fluorescence related to the Dicer cleavage indicated that exosomes vector derived from the parent cells could be efficiently internalized and released the Dicer substrate in the corresponding target cell line. Compared to the strong fluorescence signals observed in MRC-5 and NHDF cells, weak fluorescence signal was presented in A549 cells, suggesting the expression of Dicer in A549 cells was lower than the two other normal cells, in agreement with previous report (Pampalakis et al., 2010; Prodromaki et al., 2015). As shown in Fig. S7 and Fig. S8, the A549 cells were transfected with Dicer substrate-loaded liposomes and exosomes, and the green fluorescence assigned to intracellular Dicer was similar to each other, which is further verified that biocompatible exosomes are better delivery tools than liposomes. It provides a facile and powerful tool to monitor the intracellular Dicer expression level, which could be used to distinguish the cancer cells and normal cell for cancer diagnosis.

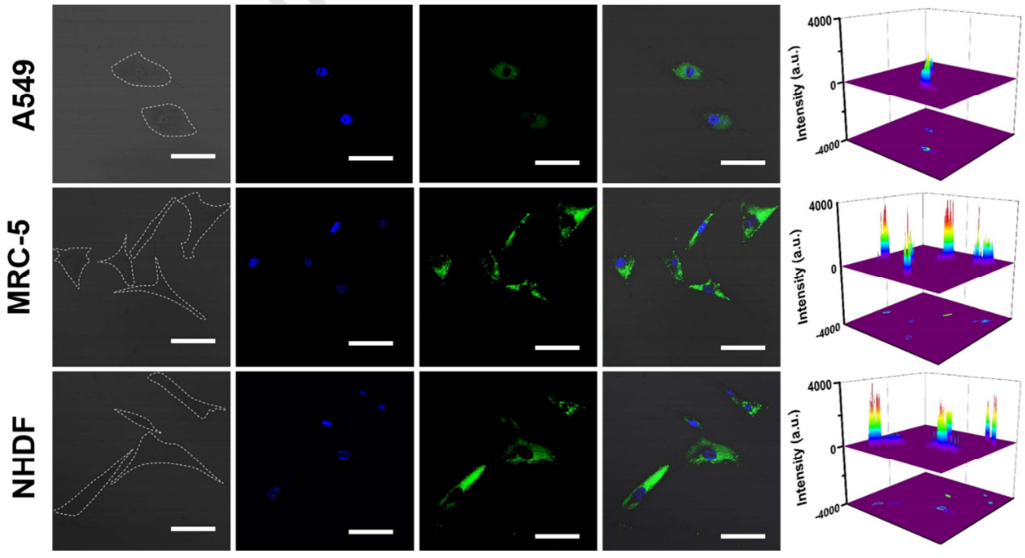


Fig. 4. Confocal images of intracellular Dicer of different cell lines transfected exosomes with Dicer substrates. Scale bar: 50 μm.

3.5. Gene therapy

As we known, miRNA let-7a could act as tumor suppressor miRNAs to target various proto-oncogenes or oncogenes at both transcriptional and post-transcriptional levels (Chen et al., 2017; Roush and Slack, 2008). The down-regulation of miRNA let-7a leads to the occurrence of oncogenesis, development and metastasis, which could be used as a significant gene therapy target (Marques et al., 2018; Shekari et al., 2019). In principle, the synthetic Dicer substrate was cleaved by intracellular Dicer and produced mature miRNA let-7a, which could influence target cell proliferation and progression, and inhibit tumor cell growth. Thus, we further studied the gene therapy performance of the proposed system. It was demonstrated that the synthetic Dicer substrates enabled significantly influence the cell viability of the tumor cell after transfected with exosomes loaded with Dicer substrates (Fig. 5A). The anti-tumor rate was high to 44% when the Dicer substrate at the concentration of 100 nM (Fig. 5A), indicating efficient tumor suppression efficiency. The cell transfected with exosomes loaded with Dicer substrates exhibited an apoptosis rate of 17.73% and a death rate of 6.42%, being higher than that of control group (Fig. 5B-D). As for the cell cycle regulation, the A549 cells transfected with the proposed exosomes increased by 10.82% in stage G1, whereas the cells in G2 stage was slight decreased and the cells in S stage decreased by 8.09%, indicating significant G1/S block (Fig. 5E-G). The significant cancer cells growth suppression and G1/S block performance of the proposed system provides a promising gene therapy strategy. Another tumor cell line MCF-7 was also carried out for verify the effect of the developed gene therapy strategy, which demonstrated that the anti-tumor rate was high to 46% when the Dicer substrate at the concentration of 100 nM (Fig. S6). These results confirmed the proposed strategy provided a powerful tool for tumor Dicer-related gene therapy.

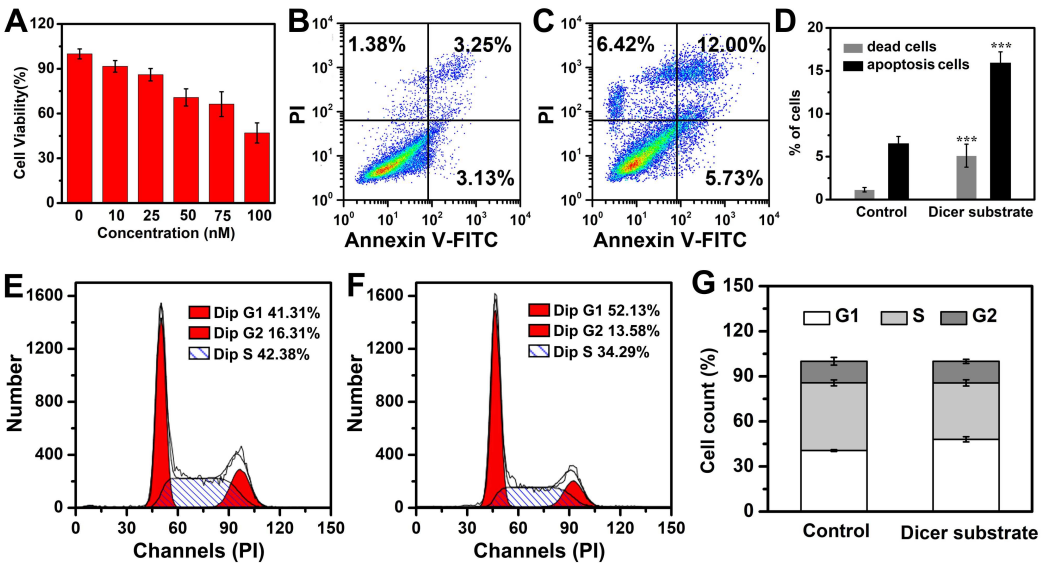


Fig. 5. (A) Cell viability of A549 cells transfected with exosomes loaded with Dicer substrates at different concentration; Apoptosis analysis of A549 cells incubated with (B) exosomes only and (C) exosomes loading with Dicer substrates; (D) Flow cytometric analysis of apoptosis and dead cells; Cell cycle analysis of A549 cells incubated with (E) exosomes only and (F) exosomes loading with Dicer substrates; (G) Flow cytometric analysis of cell cycle.

4. Conclusion

This work develops an intracellular ribonuclease Dicer detection strategy using a synthetic Dicer substrate combined with an exosomes-mediated delivery system. Using pre-miRNA let-7a as a model, the synthetic Dicer substrate hairpin probe with two terminals labeled with dye and quencher group could be effectively recognized and cleaved by commercial recombinant Dicer and cell lysate in vitro, which was confirmed by denaturing PAGE and fluorescence spectrum. After packaging the reliable probe into biocompatible exosomes by electroporation and delivering into cancer cell, the recognition and cleavage of the Dicer substrate by Ribonuclease Dicer induced the separation of the dye from the quencher, producing strong fluorescence signal for Dicer detection. The good biocompatibility of the exosomes facilitated the intracellular Dicer analysis with little false signal. Importantly, the significant tumor suppression effect of Dicer cleavage product was also demonstrated. To our best of knowledge, it is the first exploration of Dicer intracellular detection and gene therapy using Dicer substrate gene probe, which is promising to promote Dicer bioanalysis

and clinical application research.

Acknowledgments

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Conflicts of interest

The authors declare no competing financial interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at.

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Exosomes-mediated synthetic Dicer substrates delivery for intracellular Dicer imaging detection

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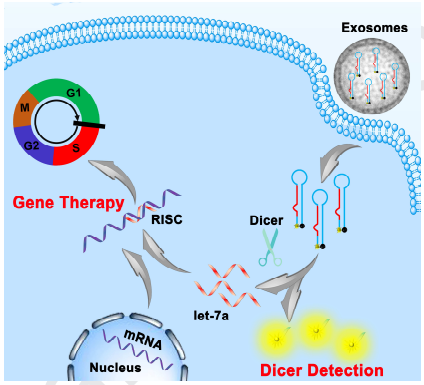
Keywords: Dicer detection, miRNA let-7a, exosomes, cell imaging, gene therapy

A simple strategy is designed for intracellular Dicer expression level monitor and gene therapy. The synthetic Dicer substrates can cleave in cytoplasm result in fluorescent recovery for Dicer analysis by RNase Dicer through exosome mediated delivery. Another cleaved product with intrinsic gene silence ability trigger significant cancer cells growth suppression and G1/S block.



Highlights

1. A fluorescent labeling probe was designed with high selectivity for intracellular Dicer detection.
2. Exosomes with little immunogenicity and good innate biocompatibility were used as nanocarriers for gene probe delivery.
3. After packaging the reliable probe into exosomes by electroporation, the assembled nanosystem could detect intracellular Dicer.

The authors declared that they have no conflicts of interest to this work.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: