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Construction of circular miRNA sponges targeting miR-21 or miR-221 and demonstration of their excellent anticancer effects on malignant melanoma cells

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ABSTRACT

microRNA sponges antagonizing the oncogenic microRNAs are potential candidates for RNA-based cancer therapies. Although the constructed sponges so far are to some extent suitable for biological experiments, they can only express at relative low levels in cells, because they are sensitive to microRNA-mediated activation of deadenylation and subsequent exonucleolytic degradation. Since circular RNA molecules are resistant to exonuclease degradation, we report the production of circular microRNA sponges against miR-21 or miR-221 in cell lines using the self-splicing permuted intron–exon sequences derived from T4 bacteriophage gene *td*. The circularized microRNA sponges withstand enzymatic degradation and are completely resistant to microRNA-mediated degradation. They are more effective than typical linear microRNA sponges and microRNA inhibitors in derepressing microRNA targets. They also display superior anti-cancer activities compared to the linear sponges in malignant melanoma cell lines. We have provided an alternative method for circular microRNA sponge production and malignant melanoma treatment.

1. Introduction

Malignant melanoma represents only 4% of skin cancer cases per year, but accounts for ~74% of all skin cancer deaths (Streicher et al., 2012). Its incidence is continuously increasing during the last decade, but the pathogenesis is still poorly understood (Gray-Schopfer et al., 2007). The most common treatments for malignant melanoma are surgery, chemotherapy, immunotherapy and radiation therapy (Garbe et al., 2012). However, they are far from satisfactory and there is currently no highly effective standard treatment available for melanoma at metastatic stage (Flaherty et al., 2010).microRNAs (miRNAs) are small noncoding RNAs, which have important roles in numerous biological processes in both plants and animals (Chu and Rana, 2007). miRNAs repress the expression levels of many targeted genes by base-pairing interaction (Bartel, 2004). Binding of the miRNA complex to target 3′-untranslated region site removes the poly (A) tail and makes the mRNA susceptible to exonucleolytic degradation (Braun et al., 2011). Deregulated miRNAs have long been shown to be associated with the development and progression of malignant melanoma (Kunz, 2013). Therefore, miRNAs have generated great interest in their possible use as novel biomarkers and therapeutic targets for diagnosis and treatment of this disease.

To achieve miRNA loss-of function, there are three classical methods (Krützfeldt et al., 2005; Meister et al., 2004; Ebert et al., 2007): genetic knockouts, antisense oligonucleotides, and miRNA sponges. Genetic-knockouts allow studying the role of miRNAs in vivo, but the generation of miRNA knockout animals is time consuming, costly and difficult. Chemically modified antisense oligonucleotides are reported useful for short term experiments. However, miRNA loss-of-function phenotypes and anticancer effects induced by these inhibitors are only generated with a high dose. miRNA sponge technology is another approach that have been described as an alternative to the generation of knockouts (Ebert and Sharp, 2010). These competitive inhibitors are expressed RNA transcripts that contain multiple bulged miRNA binding sites to sequester miRNA of interest. When miRNA sponges are transfected into human cells, they depress miRNA targets as strongly as the antisense oligonucleotides. Sponges targeting oncogenic miRNAs

Abbreviations: miRNAs, microRNAs; PIE, permutated intron–exon sequences; TAP, tobacco acid pyrophosphatase; EXO, exonuclease; FITC, fluorescein isothiocyanate; PI, propidium iodide; 5′SS, 5′ splice site; 3′SS, 3′ splice site.

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can reverse the malignant phenotypes of human cancer cells (Lin et al., 2013).

Although artificial sponges are believed to be powerful tools for RNA therapeutics, it has been shown that this technology has its own limitation (Kluiver et al., 2012; Liu et al., 2012).Similar to miRNA targets, sponges are subject to miRNA-mediated transcript deadenylation and degradation. So it is not certain that sponge RNAs will be able to accumulate to levels sufficient to sequester miRNAs in cells.

A method to stabilize sponge transcripts should be very useful for improving miRNA sponge technology and enhancing their anticancer potency. Linear RNA transcripts turnover typically involve 5′ and 3′-exonucleolytic activity at unprotected ends (Schmid and Jensen, 2008), but this mechanism may be insufficient for circular form of RNA, which lack a 5′-cap and 3′-poly (A) tail. Previous work has shown that group I introns in which the 3′-splice site (3′SS) is artificially placed upstream of the 5′-splice site (5′SS) carry out efficient trans-splicing reactions and produce RNA circles both in vitro and in vivo (Umekage and Kikuchi, 2009a,b).

In this study, we constructed circular miRNA sponges in human cell lines using the self-splicing permutated intron–exon sequences (PIE) of bacteriophage T4 td gene. We tested their ability to derepress miRNA targets, and investigated their therapeutic effects on the phenotypes of malignant melanoma cells. We have provided a potentially useful platform for miRNA loss-of-function study and cancer treatment.

2. Materials and methods

2.1. Cell lines and cell culture

Human cell lines (HeLa, A-375 and SK-MEL-1) used in this study were all purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 (1640) medium supplemented with 10% fetal bovine serum. Plates were then placed at 37 °C in an atmosphere of 5% CO_2.

2.2. Construction of the circular miRNA sponge

We constructed the circular sponge expression vector by introducing the PIE sequence downstream the CMV promoter of pcDNA3 (Invitrogen). The PIE sequence consisted of three fragments, 3′-half-intron–exon 2 segment of the T4 td gene, bulged miRNA binding region, and exon 1–5′-half-intron segment of the T4 td gene, and each part was chemically synthesized. These synthetic elements were inserted between the XhoI and ApaI restriction sites of the pcDNA3. Linear and polyadenylated miRNA sponges containing all corresponding miRNA binding sites but excluding the splicing elements were generated by using the same vector. As their non-targeting controls, both circular and linear sponges with binding sites complementary to no known miRNAs were also constructed by using the same methods as described above. Thorough descriptions of the related sequences are presented in Table S1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.09.003.

2.3. Cell transfection

1 μg of each constructed vector was transiently transfected into cells using Hiperfect Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. siRNA targeting the head-to-tail splice junction and a control siRNA were designed and chemically synthesized. Cells were transfected with 20 nM siRNA as described above. Transfection of miRNA mimics and miRNA inhibitors were conducted using the same method. The final concentration of miRNA mimic was 100 nM. The final concentration of miRNA inhibitor was 200 nM.

2.4. RNA extraction and Real-Time qPCR

Total RNA was isolated from cells using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from total RNA by SuperScript III® (Invitrogen) according to the supplied protocol. Real-Time qPCR was performed in triplicate with an ABI PRISM 7000 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA, USA) according to the standard procedures. The relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ methods. The forward and reverse primer sequences were listed in Table S2.

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2.5. RNA degradation assay

5 μg total RNA was incubated with or without 10 U of tobacco acid pyrophosphatase (TAP) at 37 °C for 1 h. After incubation, RNA was then treated with 10 U of Terminator 5′-dependent Exonuclease (EXO) (Epicentre Biotechnologies) and subjected to qRT-PCR as described above.

5 μg total RNA was treated with or without 20 U of RNase R (Epicentre Biotechnologies) at 30 °C for 10 min and then subjected to qRT-PCR as described above.

5 μg total RNA was incubated with or without 20% human serum at 37 °C and then subjected to qRT-PCR as described above.

2.6. Circular RNA gel trap

Total RNA (10 μg) was mixed with solidifying agarose and subjected to electrophoresis as described in the previous paper (Schindler et al., 1982). A no-electrophoresis control was included to determine gel-extraction efficiency. The expression levels of circular RNA were assessed by qRT-PCR, normalized to GAPDH and quantified as fold enrichment relative to total input RNA.

2.7. Construction of the luciferase reporters

The luciferase reporter for miR-21 was constructed by inserting the validated miR-21 binding site from 3′-UTR of PTEN mRNA (Dey et al., 2011) into the XhoI and NotI sites of the psiCHECK-2 vector.

The luciferase reporter for miR-221 was created by ligating the validated miR-221 binding sequence from 3′-UTR of TIMP3 mRNA (Zhang et al., 2012) into the XhoI and NotI sites of the psiCHECK-2 vector.

2.8. Dual-luciferase reporter assay

Luciferase activity was assessed using the dual luciferase assay system (Promega, Madison, WI, USA) at 48 h after transfection. The measured Renilla luciferase activity was then normalized to the firefly luciferase activity. The experiments were done in duplicate and repeated at least three times.

2.9. Cell proliferation assay

24, 48 or 72 h after transfection, 20 μl of MTT (5 mg/ml) was added to each well of a 96-well plate and the cells were incubated for 5 h. The MTT medium mixture was then discarded and 100 μl of