International Research Journal of Basic and Clinical Studies Vol. 7(4) pp. 1-5, August, 2022
Available online http://www.interesjournals.org/IRJBCS

Review Article

Adenoviral Vectors as Versatile Tools for miRNA Delivery and Expression

Mohammad Khalid Parvez*

Department Of Health and Clinical Research, Saudi Arabia

*Corresponding Author's E-mail: parvez321@gmail.com

Received: 01-Aug-2022, Manuscript No. irjbcs-22-73623; **Editor assigned:** 05-Aug-2022, Pre-QC No. irjbcs-22-73623 (PQ); **Reviewed:** 19-Aug-2022, QC No. irjbcs-22-73623; **Revised:** 24-Aug-2022, Manuscript No. irjbcs-22-73623 (R); **Published:** 30-Aug-2022, DOI: 10.14303/irjbcs.2022.16

Abstract

Only 20 years after the discovery of miRNAs, rapid progress has been made in understanding the functional impact of deregulated miRNAs on disease development, especially cancer. These observations and functional studies form the basis for the development of miRNA-based diagnostic markers or new therapeutic strategies. Adenoviral (Ad) vectors are one of the most commonly used vector types in gene therapy and are suitable for high-level, short-term transgene expression in a variety of cells. Here we report the setup and functionality of an Ad-based miRNA vector platform that can be deployed to efficiently deliver and express high levels of miRNAs. This vector platform enables rapid and efficient production of high-titer vectors and expression of pri-miRNA precursors under the control of the polymerase II promoter. Unlike non-viral miRNA delivery systems, this Ad-based miRNA vector platform enables precise dosing of the delivered miRNA. Using a two-vector model, we have shown that Ad-driven miRNA expression is sufficient to downregulate the expression of overexpressed and highly stable proteins. Additional data confirmed the downregulation of several endogenous target RNAs using the system presented here. Furthermore, we report some unexpected synergistic effects on transduction efficiency in vitro when cells are sequentially transduced with two different Ad vectors. This effect is possible in protocols that use two or more different Ad vectors simultaneously.

Keywords: miRNA, delivery, adenoviral vector, expression platform

INTRODUCTION

MicroRNAs (miRNAs) are a class of endogenous small RNAs (~22 bp). These small RNA molecules, which target the 3'-UTR of mRNAs, can function as post-transcriptional regulators of gene expression, but can also suppress transcriptional gene silencing in yeast Arabidopsis thaliana, Drosophila, and mammalian cells. It has also been shown to be involved in Thing. In 1993, the nematode Caenorhabditis elegans was discovered. We found that the downregulation of the heterochronous gene lin-14 was dependent on the transcription of her second gene (lin-4), which itself was not translated. Genes encoding miRNAs are transcribed by RNA polymerase II [1]. As a result, the primary miRNA (pri-miRNA) has a 5'-7-methylguanosine cap and a 3'-poly(A) tail. A multiprotein complex containing the RNase III enzyme Drosha cleaves pri-miRNAs at the base of a characteristic hairpin structure. This results in a two nucleotide 3'-overhangThe pre-miRNA is subsequently exported to the cytoplasm, where it is further processed to 18–23 bp long duplexes by the RNAse III endonuclease Dicer This processing step includes the removal of the terminal loop, resulting in a mature miRNA duplex Finally, the two miRNA strands are separated. The guiding strand associates with a member of the argonaute (Ago) family of proteins, forming an RNA-induced silencing complex (RISC) The human genome encodes four different Argonaut proteins (Ago 1–4) capable of loading miRNAs. Of those, Ago2 and, recently, Ago3 are described to possess endonuclease activity resulting in mRNA target cleavage A full complementary miRNA: mRNA interaction induces Ago2 endonuclease activity leading to mRNA cleavage However, interaction with perfectly complementary target sites destabilises the miRNA: RISC complex, promoting miRNA degradation Most endogenous miRNA: mRNA interactions are not entirely complementary, [2] thus preventing the endonuclease

activity of Ago2. Consequently, Ago2 mediates downstream effects similar to those induced by the non-endonucleolytic Ago family members, including the repression of translation or poly(A)-deadenylation and decapping of the target [3].

However, the mechanisms of miRNAs in regulating gene expression are far more complex. Besides the canonical mode of action, miRNAs have been shown to interact with other parts of a target mRNA, such as the coding sequence and 5′ UTR, or even with the gene promoter additionally, a single miRNA might impact different pathways and biological processes to regulate one target gene precisely and in a finely nuanced way [5]. Most miRNAs are therefore expressed in a temporal- and tissue-specific manner Recent studies reported that a large number of miRNAs derived from different tissues do exist in various body fluids, such as blood urine or saliva Due to their role in the regulation of numerous physiological processes, miRNAs have been found to be deregulated in various diseases, [6] such as cardiovascular diseases neurodegenerative diseases autoimmune diseases and cancer Given these observations, our understanding of miRNAs as disease markers is rapidly evolving. Understanding the functional effects of deregulated miRNAs on a cellular or systemic level may provide a basis for developing new therapeutic strategies. For example, for the treatment of hepatitis C virus infections, the miRNA-based drug Miravisen is currently in phase-II clinical trials With Patisiran, the first small-interfering RNA was granted by the FDA for the treatment of hereditary transthyretin-mediated (hATTR) amyloidosis and also in the light of the current SARS-CoV-2 pandemic, small RNAs are extensively tested for their therapeutic potential However, highly abundant ribonucleases and sequestration by the reticuloendothelial system hinder the delivery of naked RNA molecules [7]. The combined use of chemical modifications and lipid-based nanocarriers may increase RNA stability, including for in vivo delivery (reviewed input are not applicable for the targeted delivery of miRNAs to specific tissues or organs other than the liver. Thus, viral delivery systems, such as adenoviral (Ad) vectors, represent an attractive alternative. Here, we describe the construction and function of an easily accessible Ad vector-based miRNA expression system [8]. This system combines the ease and efficiency of viral vector-mediated transduction with the strong polymerase II promoter-driven miRNA expression. Importantly, in contrast to non-viral systems, it allows for precise dosing and is suitable for a variety of in vitro research applications. Finally, we present a series of unexpected effects observed after sequential in vitro transduction of cells with two different Ad vectors. These effects may affect the experimental design of assays that generally rely on Ad vector-based transduction [9].

MATERIALS AND METHODS

Adenovirus Vector Purification

Adenoviral vectors were transfected, amplified and purified as described elsewhere Briefly, HEK293 cells, trans complementing E1 were transfected with linearised vector DNA using polyethyleneimine (PEI) and harvested when a cytopathic effect became visible. Subsequently, vectors were amplified by sequential re-infections and purified using CsCl-gradient centrifugation. Two consecutive, discontinuous CsCl gradients were performed, followed by a desalting procedure using PD10 columns (GE Healthcare, Solingen, Germany) For cysteine-carrying vectors, lysis buffers containing 0.1 mM TCEP (Invitrogen, Darmstadt, Germany) as a reducing reagent to prevent the aggregation of particles due to the oxidation of cysteines were used Vectors were lysed with 0.5% sodium dodecyl sulfate and incubated for 10 min at 65 °C to determine physical titers by optical density at 260 nm [10].

PEGylation of Vector Capsids

After the first CsCl gradient, maleimide-activated bifunctional linear polyethylenglycol (PEG, 2 kDa) (MPM2K; IRIS Biotech, Marktredwitz, Germany) was used to PEGylate vector particles. Immediately before mixing with vectors, PEG moieties were dissolved as a 10% (w/v) solution in Ad-buffer (50 mM HEPES, 150 mM NaCl, pH 7.2). Vectors were incubated with PEG solution (final concentration 1.67%; about 2000-fold PEG excess over cysteines), gently rotating for 1 h at room temperature. The Ad-EGFP/MPM2K vector was subsequently purified by a second batch CsCl gradient centrifugation followed by PD10 column desalting to remove unbound PEG moieties [11].

Polymerase chain reaction (PCR)

All DNA fragments used for homologous recombination were amplified using Q5 high-fidelity polymerase (New England Biolabs, Frankfurt, Germany) and primers sharing 50 bp of homology with the insertion site. Each reaction contained 0.2 mM dNTPs (Thermo Fisher, Karlsruhe, Germany). , 0.5 µM primers forward and reverse, 0.02 U/µL Q5 high-fidelity polymerase (New England Biolabs, Frankfurt, Germany) and 1–5 ng of template DNA. PCR cycles were performed as follows. 26 cycles: 10 s 98 °C (denaturation) - 30 s X °C (annealing, 3 min 72 °C (elongation); 1 cycle: 10 min 72 °C (elongation). Selected miRNA sequences were analyzed by colony PCR [12]. Clones were pre-incubated for 3 h at 37 °C in 100 µL LB medium (Invitrogen, Darmstadt, Germany) supplemented with the appropriate selection marker. Then, 3 µL of bacterial suspension was mixed with 0.2 µM primer forward and reverse (5'-GGATCACTCTCGGCATGGAC-3' and 5'-ATTGCCGTCATAGCGCGGGT-3'), 0.2 mM dNTPs, and 0.025 U/µL polymerase. PCR cycles were performed as follows. 1 cycle: 2 minutes at 98°C (denaturation). 33 cycles: 30 sec 98 °C (denaturation) - 45 sec 58 °C (annealing) - 2 min 72 °C (extension); 1 cycle: 10 min 72 °C (extension) [13].

Transduction assay

Transduction assays were performed using A549 cells. To analyze the effect of miRNA expression on targets at the protein level, 1 × 105 cells/well were seeded in 24-well

plates and cultured overnight. The next day, cells were transfected with the miRNA expression vectors Ad-miRscr, Ad-miRTom1, or Ad-miRTom2 at a multiplicity of infection (MOI) of 2000 in triplicate and incubated at 37 °C, 5% CO2 for 12 hours. . Cells were then transduced with Ad-tdTomato (MOI 500 expressing the target gene) and incubated at 37 °C, 5% CO2. Cells were then washed three times with phosphate-buffered saline (PBS) and analyzed 24 days post-transduction (h.p.t.) for fluorescent gene expression using flow cytometry (CytoFlex, Beckman Coulter, Munich, Germany). , 48, 72, and 96 hours were analyzed. A 488 nm laser was used for excitation, EGFP expression was analyzed in the FITC channel (525/40 nm) and tdTomato expression was analyzed in the PE channel (585/42 nm). Cells/well were seeded in 6-well plates and cultured overnight to monitor Ad-driven miRNA expression over time. The next day, cells were transfected with miRNA expression vectors Ad-miRscr, Ad-miRTom1 and Ad-miRTom2 (MOI 500) three times. Total RNA was measured at 24, 48, 72, and 96 h.p.t. Extracted. To analyze mRNA levels of target genes, 1×10 6 cells/well were seeded in 6-well plates and cultured overnight. The next day, cells were transduced with a total MOI of 2500, containing varying amounts of Ad-miRTom1 (MOI 10, 100, 1000, and 2500) supplemented with a non-expressing vector (Ad-blank). Six hours after the first transduction, cells were transduced with Ad-tdTomato at MOI 100, incubated for 18 hours, and total RNA was extracted [14].

Isolation of total genomic DNA

Cells were detached with trypsin and collected by centrifugation at 300 xg for 10 minutes. The pellet was resuspended in 200 µl PBS and mixed with 200 µl lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% SDS). Proteinase digestion was performed overnight at 50°C in the presence of 0.9% SDS and 400 µg proteinase K. The next day, samples were treated with 200 µg RNAse A (30 min, 37 °C). DNA was then isolated using phenol-chloroform extraction followed by ethanol precipitation. DNA concentration was estimated by measuring absorbance at 260 nm and the concentration was adjusted to 5 ng/µl before being subjected to qPCR analysis [15].

Statistical analysis

Experiments were performed with a minimum of n=3 independent replicates. Statistical analysis was performed using RStudio. First, the data were tested for normality using the Shapiro-Wilk test. Levene's test was used to test for homogeneity of variance. If the data meet the criterion for homogeneity of variance, test the means for significant differences using either a one-way ANOVA followed by the TukeyHSD post hoc test or Dunnett's test for many-to-one comparisons. Did. For heterogeneity, ANOVA with Welch correction and Games-Howell post hoc test were applied.

RESULT

In this study, we establish an accessible and efficient adenoviral vector platform for miRNA expression. A bi-cistronic expression cassette encoding for a double-stranded pri-miRNA of interest and a reporter gene (EGFP) under the control of a constitutive polymerase II promoter (CMV) was inserted into the E1-deleted region of Ad5 the sequence encoding for nonsense miRNA (miRscr) was replaced with a counter-selection marker (rpsl/neo). This construct served as a platform for the insertion of different miRNAs. To demonstrate the straightforwardness of this cloning strategy, we have inserted 13 different miRNAs and screened for positive clones using diagnostic primers located within the 5′- and 3′-miRNA-flanking regions (5′-/3′-miR FR, In contrast to screening approaches based on restriction enzymes, the PCR screening assay enabled the fast and accurate identification of correct clones and revealed a high insertion efficiency.

For further characterization of this two-vector system and to demonstrate its scalability, miRNA expression and target mRNA suppression were analysed in a dose-dependent manner. Although it had a comparable low expression level miRTom1 proved to most efficiently suppress tdTomato expression at the protein level and was thus employed for further characterization. Since miRTom1 exhibited a full complementary miRTom1: tdTomato interaction, it is conceivable that upon binding to its target, Ago2 endonuclease activity was induced, resulting in tdTomato mRNA cleavage. Therefore, the A549 cells were transduced with different MOIs of Ad-miRTom1, followed by transduction with Ad-tdTomato. As expected, miRTom1 expression was scalable and correlated with applied particle titers, but with tdTomato mRNA levels. Surprisingly, it increased with increasing dose of Ad-miRTom1. A 44.5 ± 39.9-fold ($p = 0.006$) increase in tdTomato mRNA levels was observed. Cells were pretreated with Ad-miRTom1 containing varying amounts of Ad-miRTom1 supplemented with a non-expressing vector (Ad -blank) followed by delayed transduction. Ad-tdTomato. Again, miRTom1 expression levels were scalable and correlated with applied particle titer ($r2 = 0.98$). Furthermore, mRNA levels decreased in a dose-dependent manner, with Ad-miRTom1-treated cells (0.5 ± 0.07, MOI 1000) lowering those in untreated cells (1, 0 ± 0.33). was sufficient for the accompanying targeted silencing.

DISCUSSION

Nearly 20 years after RNA interference was reported in mammalian cells, three siRNA drugs have been approved, and he has seven more in clinical trials. Moreover, small RNAs are increasingly recognized as prognostic markers in cancer or as putative therapeutic agents in preclinical studies. Small RNA delivery in vitro and in vivo usually relies on lipid- or polymer-based delivery systems. It offers several advantages such as biocompatibility, high packaging capacity and low immunogenicity. Due to its location in the liver, disease targets are usually restricted to the liver. Site-

specific delivery to organs other than liver remains a major challenge in siRNA drug development. Therefore, a delivery system with the ability to efficiently target different cell and tissue types would be beneficial. In this study, we constructed an adenoviral vector platform for small RNA delivery and expression. Based on homologous recombination, this vector platform enabled fast and highly efficient insertion of miRNAs of interest. Thus, this time-saving approach reduced production time and enabled miRNA insertion, vector amplification, and purification within 4 weeks. RNAi molecules can be delivered in a variety of precursor formats. For cytoplasmic processing into mature silencing duplexes, RNA molecules must be transcribed with at least one short hairpin-like structure that can be recognized by exportin 5 and Dicer (e.g. shRNA or pre-miRNA precursor). Alternatively, RNAi molecules are transcribed as long primary miRNAs (pri-miRNAs) and processed by DROSHA. Before nuclear export. While pre-miRNAs and pri-miRNAs typically contain one or more mismatches in their double-stranded stems, designed shRNAs or miRNA mimetics typically have perfect sequence complementarity in their stems. Brachtlova et al. Recently, different formats of RNAi precursors were shown to have different expression levels of mature miR-1. Here, delivery in the pri-miRNA precursor format yielded on average more than two orders of magnitude higher levels of mature miR-1. The miRNA expression vector platform used in this study provided miRNAs in a pri-miRNA-like precursor format. RNA was transcribed with an embedded stem-loop structure containing mismatches in the stem. Furthermore, this pre-miRNA sequence was flanked by sequences derived from mouse miR-155 transcripts that mimic the pri-miRNA structure. Combined with a heterologous pol II promoter ensures high-level expression of the miRNA of interest.

In a proof-of-concept study, we inserted two different miRNAs targeting the fluorescent protein tdTomato and analyzed their effects on protein expression at both protein and mRNA levels. A significant reduction in tdTomato protein was detected within 72 hpt, which was most pronounced in miRTom1. Surprisingly, the combination of both miRNAs (“combined”) did not lead to more efficient downregulation than miRNA alone. Fluorescently labeled Ad particles reach the nuclear pore within 1 hpt and gene expression is typically observed after 1–2 hpt. Therefore, we can hypothesize that the retarding effect of miRNAs on tdTomato gene expression is due to both the high level of expression of tdTomato and the long half-life of the fluorescent protein. Involving the cutting of or including the dismantling of Treatment of cells with Ad-miRTom1 resulted in a dose-dependent 50% decrease in tdTomato mRNA. Thus, miRNAs transcribed from the polymerase II-controlled co-cistronic expression cassette were properly processed and presumably loaded into miRNA:RISC complexes. However, the Ad vector genome encodes virus-associated RNA (vaRNA). These non-coding RNAs are transcribed by polymerase III and deregulate endogenous miRNA processing by saturating Dicer and exportin-5. vaRNA has also been shown to be transcribed from E1-depleted vectors, suggesting interference with vector-encoded RNAi. Nevertheless, miRNA expression and targeted downregulation were detected up to 96 hpt, probably due to higher CMV-induced expression levels. However, vaRNA not only disrupts the miRNA processing machinery, but also targets cellular genes.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, et al (2021).Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 71: 209–249.
2. Dillekas H, Rogers MS, Straume O (2019). Are 90% of deaths from cancer caused by metastases? Cancer Med. 8: 5574–5576.
3. Castro-Giner F, Aceto N (2019). Tracking cancer progression: From circulating tumor cells to metastasis. Genome Med. 12: 31.
4. Cristofanilli M, Pierga JY, Reuben J, Rademaker A, Davis AA, et al (2019). The clinical use of circulating tumor cells (CTCs) enumeration for staging of metastatic breast cancer (MBC): International expert consensus paper. Crit Rev Oncol Hematol. 134: 39–45.
5. Larsson AM, Jansson S, Bendahl PO, Levin Tykjaer Jorgensen C, Loman N et al (2018). Longitudinal enumeration and cluster evaluation of circulating tumor cells improve prognostication for patients with newly diagnosed metastatic breast cancer in a prospective observational trial. Breast Cancer Res. 20: 48.
6. Bidard FC, Jacot W, Kiavue N, Dureau S, Kadi A, et al (2021). Efficacy of Circulating Tumor Cell Count-Driven vs Clinician-Driven First-line Therapy Choice in Hormone Receptor-Positive, ERBB2-Negative Metastatic Breast Cancer: The STIC CTC Randomized Clinical Trial. JAMA Oncol. 7: 34–41.
7. Baccelli I, Schneeweiss A, Riethdorf S, Stenzinger A, Schillert A, et al (2013). Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay .Nat Biotechnol. 31: 539–544.
8. Shibue T, Weinberg RA (2017). EMT, CSCs, and drug resistance: The mechanistic link and clinical implications. Nat Re Clin Oncol. 14: 611–629.
9. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, et al (2013). Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science. 339: 580–584.
10. Theodoropoulos PA, Polioudaki H, Agelaki S, Kallergi G, Saridaki Z, et al (2010). Circulating tumor cells with a putative stem cell phenotype in peripheral blood of patients with breast cancer. Cancer Lett. 288: 99–106.
11. Papadaki MA, Kallergi G, Zafeiriou Z, Manouras L, Theodoropoulos PA, et al (2014). Co-expression of putative stemness and epithelial-to-mesenchymal transition markers on single circulating tumour cells from patients with early and metastatic breast cancer. BMC Cancer. 14: 651.
12. Papadaki MA, Aggouraki D, Vetsika EK, Xenidis N, Kallergi G, et al (2021). Epithelial-to-mesenchymal Transition Heterogeneity

of Circulating Tumor Cells and Their Correlation with MDSCs and Tregs in HER2-negative Metastatic Breast Cancer Patients. Anticancer Res. 41: 661–670.

13. Kallergi G, Papadaki MA, Politaki E, Mavroudis D, Georgoulias V, et al (2011). Agelaki, S. Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. Breast Cancer Res. 13: R59.
14. Papadaki MA, Stoupis G, Theodoropoulos PA, Mavroudis D, Georgoulias V, et al (2019). Circulating Tumor Cells with Stemness and Epithelial-to-Mesenchymal Transition Features Are Chemoresistant and Predictive of Poor Outcome in Metastatic Breast Cancer. Mol Cancer Ther. 18: 437–447.
15. Yoshida T, Ozawa Y, Kimura T, Sato Y, Kuznetsov G, et al (2014). Eribulin mesilate suppresses experimental metastasis of breast cancer cells by reversing phenotype from epithelial-mesenchymal transition (EMT) to mesenchymal-epithelial transition (MET) states. Br J Cancer. 110: 1497–1505.