GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Bottom-up assembly of RNA nanoparticles containing phi29 motor pRNA to silence the asthma *STAT5b* gene

C. Qiu, W.K. Peng, F. Shi and T. Zhang

Department of Respiratory Diseases, Second Clinical Medical College, Jinan University, Shenzhen, Guangdong, P.R. China

Corresponding author: C. Qiu
E-mail: dalyschloe@yahoo.cn

Genet. Mol. Res. 11 (3): 3236-3245 (2012)
Received October 13, 2011
Accepted June 2, 2012
Published September 12, 2012
DOI http://dx.doi.org/10.4238/2012.September.12.6

ABSTRACT. Activation of the transcription factor signal transducer and activator of transcription 5b (STAT5b) is a key event in the development of asthma. The potent ability of small interfering RNA (siRNA) to inhibit the expression of *STAT5b* mRNA has provided a new class of therapeutics for asthma. However, efficient delivery of siRNAs remains a key obstacle to their successful application. A targeted intracellular delivery approach for siRNA to specific cell types would be highly desirable. We used packaging RNA (pRNA), a component of the bacteriophage phi29-packaging motor, to deliver STAT5b siRNA to asthmatic spleen lymphocytes. This pRNA was able to spontaneously carry siRNA/STAT5b and aptamer/CD4, which is a ligand to CD4 molecule. Based on RT-PCR data, the pRNA dimer effectively inhibited *STAT5b* gene mRNA expression of asthmatic spleen lymphocytes, without the need for additional transfections. We conclude that the pRNA dimer carrying both siRNA and aptamer can deliver functional siRNA to cells; possibly, the aptamer acts as a ligand to interact with specific receptors. The pRNAs were evaluated with a CCK-8 kit and were found to have little cytotoxicity. We conclude that

pRNA as a novel nanovehicle for RNA worth further study.

Key words: pRNA; Asthma; STAT5b; siRNA; Aptamer; Nanotechnology

INTRODUCTION

Epidemiological studies have shown that asthma causes a huge burden on health care. Although conventional therapy can control the symptoms of the majority of asthmatic patients, it may not be sufficient in some cases. Therefore, a new class of therapeutics for asthma is desirable. Asthma is an inflammatory airway disease involving a variety of cells, among which lymphocytes play a critical role. The activation of the transcription factor signal transducer and activator of transcription 5b (STAT5b) is known to play a important role in lymphocyte proliferation and apoptosis; in fact, the gene *STAT5b* is one of the targets in gene therapy for asthma (Zhu et al., 2003; Zhu, 2010).

The potent ability of small interfering RNA (siRNA) to inhibit the expression of complementary RNA transcripts is being exploited as a new approach for treating diseases, including asthma (Maes et al., 2011). siRNA is a short, double-stranded RNA (dsRNA) molecule that can target a specific sequence of mRNA for degradation via a cellular process known as RNA interference (RNAi) (Dorsett and Tuschl, 2004). In this process, siRNA is incorporated into an RNA-induced silencing complex (RISC) that recognizes and cleaves the target sequence (Elbashir et al., 2001). In particular, a targeted approach for intracellular delivery of siRNA to specific cell populations or tissues is highly desirable for the safety and efficacy of RNAi-based therapeutics (Castanotto and Rossi, 2009). Moreover, the advent of nanotechnology has greatly accelerated the development of drug delivery, and the field of RNA-based nanotechnology is now emerging (Guo, 2010).

The packaging RNA (pRNA), one of the 6 copies of the RNA components within the nanomotor of the bacteriophage phi29, is 117 nucleotide (nt) long and has been developed and manipulated to produce chimeric RNAs that form dimers by interlocking of the right- and left-hand loops (Shu et al., 2003; Lee et al., 2009). pRNA monomers can fold into a stable and unique secondary structure that serves as the building block to form nanostructures by bottom-up assembly. The pRNA molecule contains 2 independent folding domains with distinct functions: 5'-/3'-double-stranded helical domain and intermolecular interaction domain (loop-loop region). Replacement or insertion of oligonucleotides preceding nt #23 or following nt #97 does not interfere with the formation of dimmers as long as the strands are paired (Shu et al., 2009). Therefore, the 5'/3'-proximate double-stranded helical region of the pRNA can be redesigned to carry additional sequences without altering its secondary structure or intermolecular interactions. On the basis of these characteristics, incubation of cells with pRNA dimmers, one subunit of which carried siRNA and the other, a receptor-binding moiety, resulted in successful binding, entry, and silencing of target genes (Guo et al., 2005; Zhang et al., 2009; Zhou et al., 2011).

In this study, we constructed pRNA-siRNA/STAT5b monomers and pRNA-aptamer/CD4 monomers and then formed heterodimers. One subunit of the dimer contained an aptamer molecule at its 5'-end, which was used to target cell recognition, and the other harbored a moiety of therapeutic siRNA for gene therapy. Since CD4+ T lymphocytes are the most important cells in the development of asthma and they are present at a high percentage in asthmatic spleen lymphocytes, we chose aptamer/CD4 as a ligand to interact with the CD4 molecule in

CD4+ T lymphocytes. Without any other transfection, the pRNA dimer strongly inhibited the mRNA expression of *STAT5b*, while none of the pRNA monomers had any effect. Further, we found that pRNAs had little cytotoxicity. These data indicate that pRNA is a promising candidate for targeted gene therapy in asthma.

MATERIAL AND METHODS

Preparation of pRNA-siRNA/STAT5b and pRNA-aptamer/CD4 and pRNA heterodimer formation

pRNA-siRNA/STAT5b and pRNA-aptamer/CD4 were synthesized by *in vitro* transcription of cDNA fragments encoding the chimeric pRNA-siRNA/STAT5b or pRNA-aptamer/CD4. The cDNA fragments were produced by polymerase chain reaction (PCR) according to standard procedures. The template sequences and primer sequences for PCR were specifically designed according to the previous reports (Zhang et al., 2009) and synthesized (Sangon, China). All the template and primer sequences used are listed (Figure 1).

DNA	Sequences(5'→3')
A-b' pRNA-siRNA/STAT5b (129 nt)	Forward: 5'-TAATACGACTCACTATAGGG**TTATCACAGTGG AT GAAA**TTTGTATGTTGGGGATTA Reverse: 5'-AAGGTTATCACAGTGGATCGAAAAAATTGACA CG C AATCAAC Template: 5'-TGTATGTTGGGGATTAGGACCTGATTGAGTTC AG CCCACATACTTTGTTGATTGCGTGTC AAT
B-a' pRNA-aptamer/CD4 (173 nt)	Forward: 5'-TAATACGACTCACTATAGGG**CTCAGAGACAGA GC AGAAAC GACAGTTCAAGCCGAA**GGAATGGTACGG Reverse: 5'-GGAAA GTAGC GTGCAC Template: 5'-GGAATGGTACGGTACTTCCATTGTCATGTGTATG TTGGGGATTAACGCCTGATTGAGTTCAGCCCACATACTTTG TTGATTGTCCGTCAATCATGGCAAAAGTGCACGCTACTTTCC

Figure 1. Template and primer sequences for synthesis of cDNA fragments encoding the pRNA chimera. The underlined sequences are T7 phage 2.5 promoters. The bold sequences are siRNA sequences or aptamer sequences.

Extensive studies of pRNA have revealed that 2 pRNA monomers form a dimer through the interaction of the complementary left- and right-hand loops. In this study, we previously designed pRNA-siRNA/STAT5b (A-b'), which contains a right-hand loop A (5'-$G_{38}G_{39}A_{40}C_{41}$) and a left-hand loop b' (3'-$U_{74}G_{75}C_{76}G_{77}$); these can together pair with the left-hand loop a' (3'-$C_{121}C_{120}U_{119}G_{118}$) and the right-hand loop B (5'-$A_{81}C_{82}G_{83}C_{84}$) of pRNA-aptamer/CD4 (B-a'), respectively.

The 5'-end primer contained the T7 phage 2.5 promoter sequence followed by a sense sequence of siRNA/STAT5b and the 3'-end primer contained the antisense sequence of siRNA/STAT5b. With this, the sequence of siRNA/STAT5b was added to the 5'/3' double-stranded helical region of the pRNA after PCR, thereby resulting in the formation of pRNA-siRNA/STAT5b. Similarly, in order to construct pRNA-aptamer/CD4, the 5'-end primer contained the T7 phage 2.5 promoter sequence followed by the aptamer/CD4 sequence. The siRNA/STAT5b sequence was designed (Ambion, USA). A previous report has indicated that 10 copies of

aptamer/CD4 interact with CD4 molecule specifically and that each of them can inhibit CD4+ lymphocyte proliferation differently (Kraus et al., 1998). In this study, we chose the copy of aptamer/CD4, which has the minimum inhibiting capacity.

pRNA-siRNA/STAT5b and pRNA-aptamer/CD4 were designed previously, and their structure were evaluated through a software application (http://novacripta.cbm.uam.es/bioweb/courses/UFV0708/tema08/index.html) in order to ensure the correct secondary structure (Figure 2A and B). Then, the secondary structure of the pRNA dimer was predicted (Figure 2C).

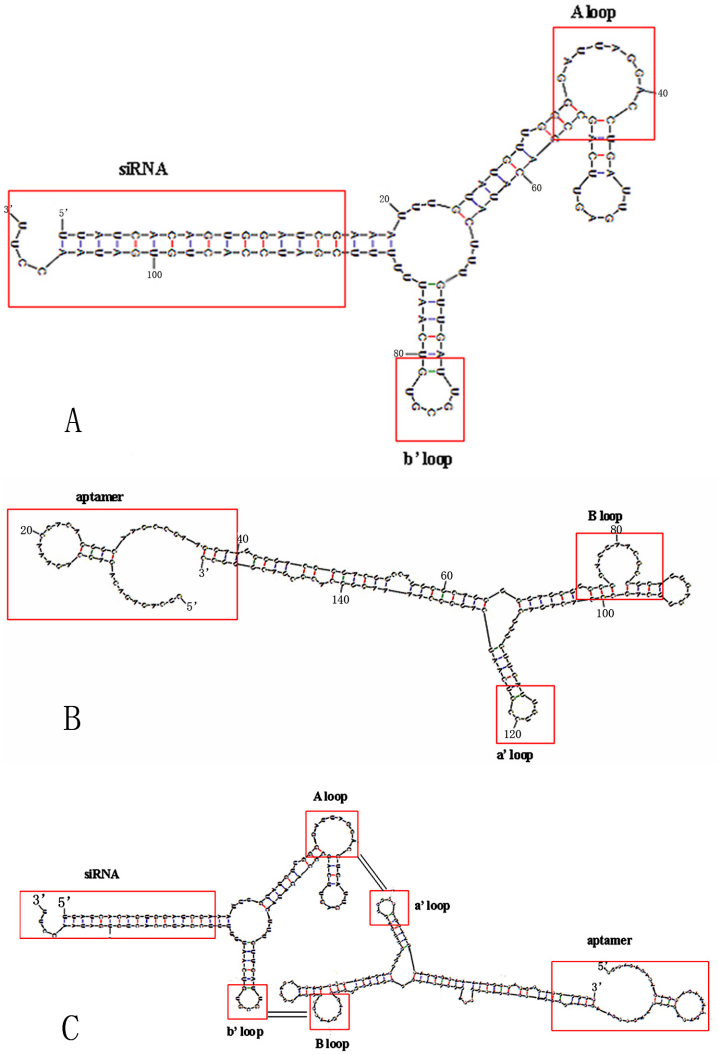

Figure 2. Secondary structure of the pRNA chimera. **A.** Secondary structure of pRNA-siRNA/STAT5b. The siRNA sequence and the domain for dimer formation (right- and left-hand) are marked by red borders. **B.** Secondary structure of pRNA-aptamer/CD4. Similarly, the aptamer sequence and the domain for dimer formation are marked by red borders. **C.** Expected structure of pRNA dimer. Two chimeric pRNA monomers form dimer via the base-pairing between two loops (A-a' and B-b').

To synthesize pRNA-siRNA/STAT5b and pRNA-aptamer/CD4, in vitro transcription was conducted using Riboprobe System-T7 (Promega, USA) according to manufacturer instructions. Briefly, the PCR-amplified cDNA fragments containing the T7 phage 2.5 promoter were transcribed with T7 RNA polymerase in the presence of rATP, rCTP, rGTP, and rUTP. Transcription products were digested by DNase and were then precipitated with ethanol and resuspended in sterilized DEPC-treated water. The concentration of the pRNA chimera products was measured using a spectrophotometer. Then, the products were confirmed by 8% native polyacrylamide gel electrophoresis (PAGE) using TBM running buffer (89 mM Tris-base, 200 mM boric acid, and 5 mM $MgCl_2$, pH 7.6) (Chen et al., 2000), in which the polyacrylamide gel was poured without sodium dodecyl sulfate.

It has been proven that in a solution containing 5 mM magnesium, 2 pRNA monomers with base-pairing loop (for example, A-b' pRNA and B-a' pRNA) can easily form a dimer through the interaction of the complementary left- and right-hand loops (Shu et al., 2004). In this study, the pRNA heterodimer was prepared by mixing pRNA-siRNA/STAT5b and pRNA-aptamer/CD4 in 1:1 molar ratio in a solution containing 5 mM Mg^{2+} and RNAseOUT RNAse inhibitor.

Murine asthma model and spleen lymphocytes cultivation

Twenty female BALB/c mice (6-8 weeks old, Experimental Animal Center of Guangdong Province, China) were randomly divided into 2 groups: the asthma group and the control group. Mice were sensitized to become asthmatic as described previously (North et al., 2009).

After completing the asthma model protocol, bronchial lung sections were obtained and stained by hematoxylin and eosin to evaluate the model. At the same time, the spleen lymphocytes were obtained using the mouse lymphocyte separation medium (Dakewe Biotech Company, China) according to manufacturer instructions.

The lymphocytes were cultured in six-well plates (1 x 10^6 cells per well) with RPMI1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen, USA). IL-2 (0.02 µg) cytokines (PeproTech, USA) were added to every well, and the lymphocytes were cultured in a 5% CO_2 incubator.

Specific gene silencing by pRNA chimera and dimer

After culturing for 24 h, the lymphocytes were directly incubated with 3 µg pRNA-siRNA/STAT5b, 3 µg pRNA-aptamer/CD4, or 6 µg pRNA dimer without any other transfection for 48 h. In addition, a control group was recruited.

Reverse transcription (RT)-PCR

Total RNA was extracted from cells at the post-incubation indicated time points using Trizol reagent (Invitrogen). RT was conducted according to manufacturer instructions using Quant Reverse Transcriptase (Tiangen Biotech Company, China) and followed by PCR under the standard conditions to amplify *STAT5b* and cDNA of *GAPDH*. The primer sequences of the gene *STAT5b* are as follows: forward 5'-CAACCGGGAGAATTTGCCAG-3', reverse 5'-CCATCATTCCAGTGAGGCTTGA-3', and the product was 102 nt. The primer sequences of the gene *GAPDH* are as follows: forward 5'-AACTTTGGCATTGTGGAAGG-3', reverse

5'-GGATGCAGGGATGATGTTCT-3', and the product was 132 nt. The PCR product from each sample was analyzed by 1.5% agarose gel electrophoresis.

Cytotoxicity test by CCk-8 kit

Safety is very important to a drug. Therefore, we used the CCK-8 kit (Dojindo, Japan) to evaluate the cytotoxicity of the pRNA chimera. In brief, the lymphocytes were incubated with the CCK-8 kit for 4 h after incubation with pRNA chimera for the indicated time. The readings were then obtained using a spectrophotometer.

RESULTS

Photomicrographs of lung sections

Asthma is an inflammatory airways disease. Photomicrographs of lung sections obtained from the asthma group showed that there was an apparent increase in the thickness of the airway epithelium and obvious submucosal cell infiltration (Figure 3). On the other hand, no signs of inflammation were evident in the lung sections of the control group. These data proved that asthma group mice had serious airway inflammation. Thus, a murine asthma model was successfully established. Additionally, although not an objective evidence, asthma symptoms such as shortness of breath were also present in the mice of the asthma group mice, but absent in the control group mice during the asthma modeling process.

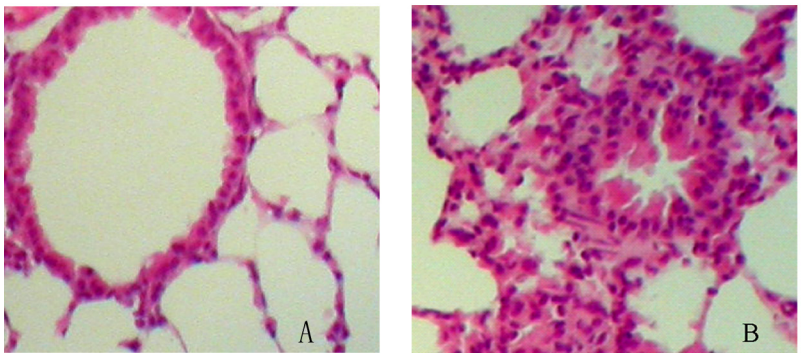

Figure 3. Representative photomicrographs of lung sections from each group (HE staining, original magnification 40X). **A.** The control group. **B.** The asthma group. There is an apparent increase in the thickness of the airway epithelium and submucosal cell infiltration is obvious.

Synthesis of pRNA chimera

The cDNA fragment of pRNA-siRNA/STAT5b and pRNA-aptamer/CD4 was constructed by PCR. After that, the PCR products were confirmed and purified by agarose electrophoresis (Figure 4A and B). *In vitro* transcription was then conducted, and transcription

products were confirmed by native PAGE (Figure 4C). We found that all the bands were at the expected positions relative to the mark. Thus, pRNA chimera is easy to design and assemble bottom-up.

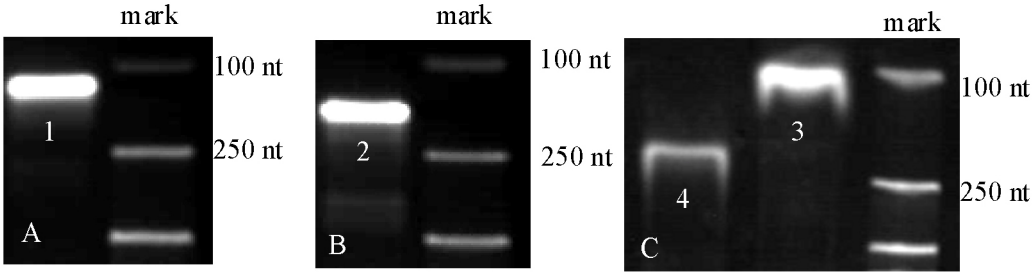

Figure 4. Synthesis of pRNA chimera. **A.** *Lane 1* = cDNA fragment of pRNA-siRNA/STAT5b (129 nt) on agarose gel. **B.** *Lane 2* = cDNA fragment of pRNA-siRNA/aptamer (173 nt) on agarose gel. **C.** *Lane 3* = pRNA-siRNA/STAT5b and *lane 4* = pRNA-aptamer/CD4 on native PAGE gel. All bands are in the expected place comparing to mark.

Inhibition ability of pRNA chimera and dimer

We observed that *STAT5b* expression levels in the pRNA dimer group were less than those in other groups, while those in the pRNA-siRNA/STAT5b group and pRNA-siRNA/CD4 group were similar to that in the control group (Figure 5). Therefore, the data indicate that when incubated with lymphocytes directly, pRNA dimers carrying both siRNA and aptamer play an inhibitory role, while pRNA chimera does not.

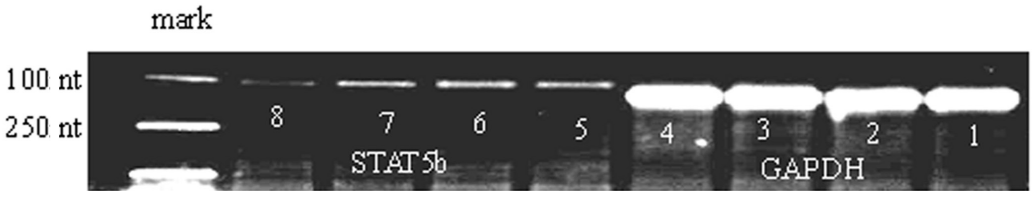

Figure 5. RT-PCR to evaluate the inhibition ability of pRNA chimera and dimer. *Lanes 1* and *5* = Control groups; *lanes 2* and *6* = pRNA-siRNA/STAT5b groups; *lanes 3* and *7* = pRNA-aptamer/CD4 groups; *lanes 4* and *8* = pRNA dimer groups. *STAT5b* gene expression of the pRNA dimer group is less than other groups.

Cytotoxicity of pRNA chimera

We found no significant difference between the control group, pRNA-siRNA/STAT5b group, and pRNA-aptamer/CD4 group (Figure 6). These data proved that pRNA chimera had minimal cytotoxicity.

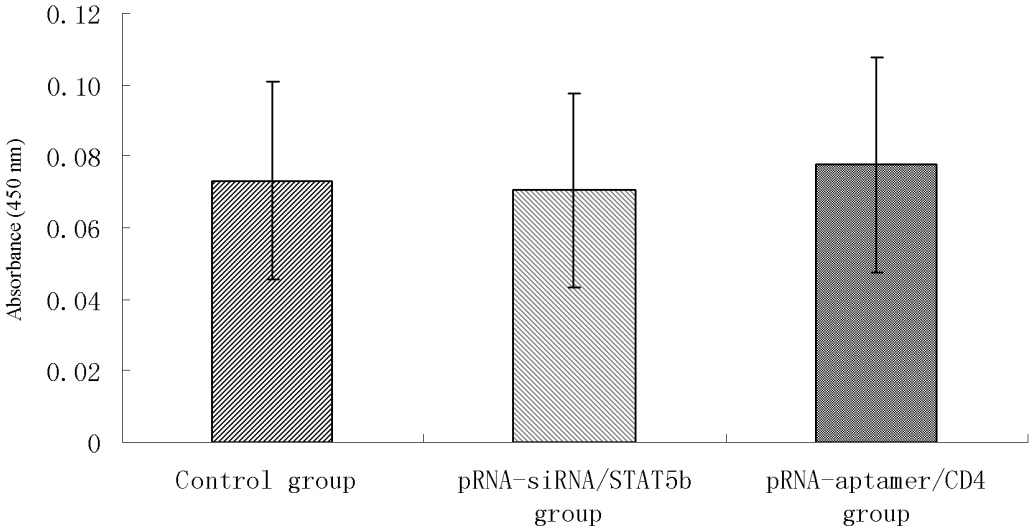

Figure 6. CCK-8 to evaluate cytotoxicity of pRNA chimera. There is no significant difference between the three groups. The values shown are reported as means ± SD of the data from three separated experiments.

DISCUSSION

During the last decade, RNA interference has shown great potential as a therapeutic modality for various diseases, including asthma. However, in drug development, one of the most critical barriers for clinical application is the non-specific distribution of the drug in the body after administration. This non-specific delivery not only reduces the therapeutic efficiency and gives rise to harmful adverse effects but also causes drug wastage, resulting in increased cost. With this in view, the development of an effective system for targeted delivery of therapeutic molecules is the main goal of the present study.

pRNA has been reported to have application in nanotechnology and nanomedicine. Through the interaction of 2 reengineered interlocking loops, pRNAs can form nanoscale dimers, even trimers and hexamers. This unique ability of pRNA makes it a versatile polyvalent vehicle to combine with therapeutic siRNAs for treating and other ligand for cell recognition and maybe applied as a therapeutic nanoplatform, which can improve the targeting, solubility, and pharmacokinetic/distribution properties of drugs (Lee et al., 2009).

Many genes have been reported to be definitively related to asthma. We chose siRNA targeting the *STAT5b* gene, a critical gene involved in lymphocyte proliferation and apoptosis, as an effective therapeutic agent to test the novel delivery system.

The pRNA molecule can be employed as a novel RNA vector to carry siRNA or an aptamer molecule. Previous reports indicate that the pRNA-siRNA chimera exerts the silencing function of siRNA effectively and that the silencing efficiency of this chimera is similar to or even better than that of free siRNA (Zhang et al., 2009; Tarapore et al., 2011). Similarly, the pRNA-aptamer chimera interacts with the specific receptor, as expected (Guo et al., 2005;

Zhou et al., 2011). In this study, we built pRNA chimera using a synthetic PCR template, but not plasmid containing pRNA sequences, and proved that the pRNA chimeras were successfully built. Next, we found that without any other transfection, pRNA dimer carrying both siRNA/STAT5b and aptamer/CD4 strongly inhibited mRNA expression of *STAT5b*. On the other hand, incubation of pRNA-siRNA/STAT5b or pRNA-aptamer/CD4 with lymphocytes did not have any effect on the expression of *STAT5b*. The data indicate that pRNA dimer can deliver siRNA to cells and work as expected, maybe the mechanism is via aptamer as a ligand to interact with specific receptor. Further experiments are needed to confirm the ability of pRNA-aptamer/CD4 to specifically interact with the CD4 molecule.

Biosafety is the most important feature of a drug. If a drug is highly cytotoxic, it cannot have widespread clinical application even if it is highly efficient. We showed that both pRNA-siRNA/STAT5b and pRNA-aptamer/CD4 have minimal cytotoxicity. Therefore, pRNA warrants further investigation as a novel nanovehicle.

ACKNOWLEDGMENTS

Research supported by the Natural Science Foundation of Guangdong Province, China (#10151802001000004). Moreover, we thank Peixuan Guo (Department of Biomedical Engineering, College of Engineering/College of Medicine, University of Cincinnati, USA) for guidance of the pRNA sequence design and providing the structure testing website.

REFERENCES

Castanotto D and Rossi JJ (2009). The promises and pitfalls of RNA-interference-based therapeutics. *Nature* 457: 426-433.

Chen C, Sheng S, Shao Z and Guo P (2000). A dimer as a building block in assembling RNA. A hexamer that gears bacterial virus phi29 DNA-translocating machinery. *J. Biol. Chem.* 275: 17510-17516.

Dorsett Y and Tuschl T (2004). siRNAs: applications in functional genomics and potential as therapeutics. *Nat. Rev. Drug. Discov.* 3: 318-329.

Elbashir SM, Lendeckel W and Tuschl T (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15: 188-200.

Guo P (2010). The emerging field of RNA nanotechnology. *Nat. Nanotechnol.* 5: 833-842.

Guo S, Tschammer N, Mohammed S and Guo P (2005). Specific delivery of therapeutic RNAs to cancer cells via the dimerization mechanism of phi29 motor pRNA. *Hum. Gene Ther.* 16: 1097-1109.

Kraus E, James W and Barclay AN (1998). Cutting edge: novel RNA ligands able to bind CD4 antigen and inhibit CD4+ T lymphocyte function. *J. Immunol.* 160: 5209-5212.

Lee TJ, Schwartz C and Guo P (2009). Construction of bacteriophage phi29 DNA packaging motor and its applications in nanotechnology and therapy. *Ann. Biomed. Eng.* 37: 2064-2081.

Maes T, Tournoy KG and Joos GF (2011). Gene therapy for allergic airway diseases. *Curr. Allergy Asthma Rep.* 11: 163-172.

North ML, Khanna N, Marsden PA, Grasemann H, et al. (2009). Functionally important role for arginase 1 in the airway hyperresponsiveness of asthma. *Am. J. Physiol. Lung Cell Mol. Physiol.* 296: L911-L920.

Shu D, Huang LP, Hoeprich S and Guo P (2003). Construction of phi29 DNA-packaging RNA monomers, dimers, and trimers with variable sizes and shapes as potential parts for nanodevices. *J. Nanosci. Nanotechnol.* 3: 295-302.

Shu D, Moll WD, Deng Z, Mao C, et al. (2004). Bottom-up Assembly of RNA Arrays and Superstructures as Potential Parts in Nanotechnology. *Nano Lett.* 4: 1717-1723.

Shu Y, Shu D, Diao Z, Shen G, et al. (2009). Fabrication of Polyvalent Therapeutic RNA Nanoparticles for Specific Delivery of siRNA, Ribozyme and Drugs to Targeted Cells for Cancer Therapy. *IEEE NIH Life Sci. Syst. Appl. Workshop* 2009: 9-12.

Tarapore P, Shu Y, Guo P and Ho SM (2011). Application of phi29 motor pRNA for targeted therapeutic delivery of siRNA silencing metallothionein-IIA and survivin in ovarian cancers. *Mol. Ther.* 19: 386-394.

Zhang HM, Su Y, Guo S, Yuan J, et al. (2009). Targeted delivery of anti-coxsackievirus siRNAs using ligand-conjugated packaging RNAs. *Antiviral Res.* 83: 307-316.

Zhou J, Shu Y, Guo P, Smith DD, et al. (2011). Dual functional RNA nanoparticles containing phi29 motor pRNA and anti-gp120 aptamer for cell-type specific delivery and HIV-1 inhibition. *Methods* 54: 284-294.

Zhu J (2010). Transcriptional regulation of Th2 cell differentiation. *Immunol. Cell Biol.* 88: 244-249.

Zhu J, Cote-Sierra J, Guo L and Paul WE (2003). Stat5 activation plays a critical role in Th2 differentiation. *Immunity* 19: 739-748.