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Methods paper

A novel siRNA validation system for functional screening of effective RNAi targets in mammalian cells and development of a derivative lentivirus delivery system

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ABSTRACT

RNA interference technology is a widely used tool for the regulation of gene expression at the post-transcriptional level. One major challenge is to find the effective short interfering (si)RNA for target gene rapidly and easily, and then to deliver the siRNA into cells or tissues with high efficiency. Here, we designed a novel siRNA validation vector using a dual luciferase reporter system for the functional screening of effective RNAi targets in mammalian cells. Then, based on a siRNA expression cassette, we developed a derivative lentivirus delivery system to infect the appropriate cells or tissues for the efficient knockdown of target gene expression. Based on this system, we used human IRF7 gene, a key regulatory factor for the differentiation of monocytes to macrophages, as an example. We screened for the optimal siRNA, then packaged it into a lentiviral siRNA expression system. Then, monocytes were infected and we confirmed the knockdown of IRF7 expression could inhibit the differentiation of monocytes to macrophages. To validate our method further, we also screened and identified optimal siRNA for human TLR4 gene. In summary, we developed a novel siRNA validation system to identify optimal siRNA to target genes rapidly. In addition, the lentivirus system is an efficient tool for siRNA delivery for the further study of target gene function. Taken together, this represents an efficient and user-friendly strategy to validate and deliver siRNAs.

1. Introduction

RNA interference (RNAi)-mediated gene silencing has become a valuable tool for functional studies, reverse genomics, and drug discovery (Chen and Xie, 2012; Guzman-Villanueva et al., 2012). One major challenge of using RNAi is to identify the effective short interfering RNA (siRNA) target sites of a given gene and transport it efficiently to the appropriate cell or tissue to knockdown the expression of target gene (Chen and Xie, 2012; Guzman-Villanueva et al., 2012; Mohr and Perrimon, 2012). Although several published bioinformatic prediction models (Stormo, 2006; Tilesi et al., 2009) and some fluorescence-based siRNA sequence selection systems (Luo et al., 2007; Zheng et al., 2011) have proven useful, the process to select and validate optimal siRNA sites for a given gene remains empirical and laborious. In addition, introducing siRNAs or expression vectors to primary cells or hard-transfected cell lines such as lymphocytes and PC-12 cells with a high efficiency is still a recognized problem (Aagaard and Rossi, 2007). Here, we developed a reliable and quantitative reporter-based siRNA validation system for the functional screening and delivery of effective RNAi probes in mammalian cells. It is a dual luciferase assay-based selection system, named psiSDR (plasmid for siRNA screening with dual reporters), and is based on the premise that candidate siRNAs would knockdown the chimeric transcript of luciferase and target genes. The expression of siRNA was driven by the opposing convergent H1 and U6 promoters. This configuration simplifies the cloning of duplex siRNA oligonucleotide cassettes. We demonstrated that relative luciferase activity signal reduction was closely correlated with siRNA knockdown efficiency of human interferon regulatory factor 7 (IRF7). It was then packaged into a lentiviral siRNA expression system and used to infect primary monocytes and knockdown of hIRF7 expression inhibited the differentiation of monocytes to macrophages. In addition, we repeated our experiments on human TLR4 (Toll like receptor 4) gene, then validated the efficiency and wide applicability of this system. The

Abbreviations: dsRNA, double-stranded RNA; hRluc, humanized Renilla luciferase; IRF7, interferon regulatory factor 7; NC, negative control; PGK, phosphoglycerate kinase; psiSDR, plasmid for siRNA screening with dual reporters; RNAi, RNA interference; siRNAs, short interfering RNAs; SOE, splicing by overlapping extension; TLR4, Toll like receptor 4.

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use of psiSDR and the lentivirus delivery system should facilitate the selection and validation of candidate siRNA sites and provide efficient tools for delivering siRNAs to mammalian cells via lentiviral vectors. Thus, the psiSDR and corresponding lentivirus delivery system provides a powerful strategy to validate and deliver highly effective siRNAs.

2. Materials and methods

2.1. Cell culture and reagents

Primary human monocytes were obtained from healthy donors with written informed consent and this study was approved by the Medical Ethics Committees of the Third Military Medical University. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. Fresh whole blood was drawn into vacutainer tubes (Becton Dickinson & Co., Franklin Lakes, NJ, USA) containing EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (TBD, Tianjin, China). CD14+ monocytes were isolated from PBMCs by negative selection using Human Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Monocyte purity was verified as >95% by anti-CD14 staining (Cat. #17-0149; eBioscience, San Diego, CA, USA) using flow cytometry. Monocytes were seeded at 1×10^6 cells/well in 12-well plates (Corning Incorporated, Corning, NY, USA) and maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat inactivated human AB serum (Sigma, Batavia, IL, USA). Macrophage differentiation was induced by treatment with 20 ng/ml macrophage colony-stimulating factor (M-CSF, BD Biosciences, San Jose, CA, USA) for 7 days (D'Onofrio and Paradisi, 1983). Every third day, half of the medium was removed and replaced with fresh complete nutrient medium. All monocyte-to-macrophage differentiation experiments were performed in 10% human serum plus 20 ng/ml M-CSF, unless otherwise stated. HEK-293T and COS7 cell lines were purchased from ATCC (ATCC, Rockville, MD, USA). These cells were maintained in complete DMEM containing 10% FBS (Gibco BRL).

Unless otherwise indicated, all chemicals were purchased from Sangon Biological Engineering Technology Company (Sangon, Shanghai, China). Restriction endonucleases and T4 DNA ligase were purchased from NEB (New England Biolabs, Ipswich, MA, USA), and plasmid DNA miniprep kits and agarose gel DNA fragment recovery kit were from Omega (Omega Bio-Tek, Norcross, GA, USA). Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY, USA). The DNA templates for the IRF7 gene or TLR4 gene siRNAs and PCR primers were synthesized by Sangon. DNA sequencing was conducted by Sangon.

2.2. Design and construction of a novel siRNA validation plasmid psiSDR

Based on the template of plasmid pmirGLO (Promega, Madison, WI, USA), we designed and synthesized three pairs of primers (Table 1) to clone the DNA fragments for luc2 reporter gene, human phosphoglycerate kinase (PGK) promoter and humanized Renilla luciferase (hRluc) reporter gene, respectively. According to the manufacturer's instructions, PCR was performed under the following conditions: plasmid DNA template was denatured at 99 °C for 10 min, followed by 35 cycles of amplification (98 °C for 20 s, 68 °C for 2 min) and 8 min at 72 °C. The anticipated size of PCR products was identified, separated and recovered by electrophoresis using a 1% agarose gel. Based on the 16 nucleotide (nt) complementary overlapping sequence between these three templates (shown in Table 1 as underlined), splicing by overlapping extension (SOE)-PCR (Vallejo et al., 2008) was used to obtain the fused DNA of luc2, PGK promoter and hRluc tandemly. The fused DNA was amplified using primers P1 and P6. PCR was performed as follows: 98 °C for 6 min, then 30 cycles of 98 °C for 20 s and 68 °C for 3 min, followed by 68 °C for 10 min. The anticipated PCR product size recovered was 3681 base pairs (bp). Green fluorescent protein (GFP)-based mammalian siRNA screening vector pSOS-HUS (a kind gift from Dr. Luo Qing, Pediatrics Institution of Chongqing Medical University, Chongqing, China) was digested with *Eco*47 III (blunt end) and *Bgl* II, while the 3681 bp fused DNA was digested with *Bam*H I (*Bam*H I has a compatible end with *Bgl* II) only. After digestion, the fused DNA and the linearized pSOS-HUS vector were recovered, ligated and transformed into *Escherichia coli* DH10B. Transformed positive clones for psiSDR were screened by colony PCR using primers P3 and P4, and further verified by sequencing (Fig. 1).

2.3. Construction of the double-cloned psiSDR-hIRF7i plasmids for screening optimal siRNA

Recombinant hIRF7/pTA2 plasmid containing the human IRF7 intact open reading frame (GenBank ID: U53830.1) was digested with *Eco*R V and *Xba* I, and the siRNA screening plasmid psiSDR was digested with *Pme* I and *Nhe* I. After digestion, the hIRF7 cDNA (1593 bp) and the linearized psiSDR vector were recovered, ligated using T4 DNA ligase, and transformed into *E. coli* DH10B. Transformed positive clones for psiSDR-hIRF7 were screened by colony PCR using primers P7 and P8, and further verified by *Eco*R I digestion (5.9/2.2/1.3 kb). Then psiSDR-hIRF7 vector was digested with *Sfi* I and recovered for the next ligation.

siRNA sequences targeting hIRF7 (GenBank ID: U53830.1) were selected using standard siRNA design algorithms (Moore et al., 2010; Tafer, 2014). We designed three pairs of siRNA template oligonucleotides targeted against hIRF7 gene expression, as shown in Table 2. Eight oligonucleotides were thus synthesized. Five microliters of the complementary oligonucleotides (100 μM) was mixed in a 1.5-ml Eppendorf tube with 4 μl 5× annealing buffer (Beyotime, HaiMen, Jiangsu, China) and distilled water, in a total volume of 20 μl. The Eppendorf tube was place on a 99 °C heat block for 10 min, and then removed and allowed to cool at room temperature. The Eppendorf tube was briefly centrifuged at full speed to recover the reaction solution, which was stored on ice or at 4 °C until ready for use. The construction procedures used standard molecular cloning techniques. Briefly, an annealed oligonucleotide duplex was inserted into the *Sfi* I restriction enzyme site in

Table 1
Primer sequences for SOE-PCR and cloning.

Designation	Primers sequence	Tm	Amplicon size (bp)
luc2 reporter gene	P1: (F) 5′-GCTTGGCAATCCGGTACTGTTGGTAA-3′ P2: (R) 5′-TTGTACGCTTTACCACATTTGTAGAGGTTTTACTTGCT-3′	68 °C	2076
PGK promoter	P3: (F) 5′-GTGGTAAAGCGTACAATTAAGGGATTATGGTA-3′ P4: (R) 5′-GAAGAATCTGGGCTGCAGGTCGAAAGGCC-3′	68 °C	641
hRluc reporter gene	P5: (F) 5′-GCAGCCCAGATTCTTCTGACACAACAGTC-3′ P6: (R) 5′-CGGGATCCTTACTGCTCGTTCTTCAGCAC-3′ (Xba I site)	68 °C	996
hIRF7 cDNA	P7: (F) 5′-GGAATTCACCTGACCGCCACCTAACTG-3′ P8: (R) 5′-GCTCTAGAGTTCTCATTAGACTGGGTTCTAG-3′	68 °C	1583
siRNA expression cassette	P9: (F) 5′-CGGGATCCAGCTTAATTCGAACGCTGACGT-3′ (BamH I site) P10: (R) 5′- CCCTCGAGCAAGGTCGGGCAGGAAGAG-3′ (Xho I site)	68 °C	566

SOE, splicing by overlapping extension; hIRF7, human interferon regulatory factor 7; siRNA, short interfering RNA; PGK, phosphoglycerate kinase; hRluc, humanized Renilla luciferase.