1 Enhancing RNAi efficiency by inserting Nuclear factor- B binding sequence into SiRNA expression

2 cassette

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Abstract

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33 RNAi based applications have been widely used to manipulate cellular phenotypes, analyze gene
34function and as a promising molecular therapy, however, there are rare reports in how to target the SiRNA
35expression constructs from the cytoplasm to nucleus. In this study, we inserted NF- B binding
36sequence into the SiRNA expression cassette to enhance RNAi efficiency, tested this propose by use of
37tRNAval and human H1 promoters, and eGFP and luciferase as reporter protein. The results showed that
38inserting NF- B binding sequence at the 3'-terminal of the SiRNA expression construct could enhance
39RNAi efficiency.

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60 RNA interference(RNAi) is a cellular mechanism by which 21-23 nucleotide RNA duplexes trigger the
61 degradation of cognate mRNAs, RNAi based applications to silence specific genes has become a standard
62 method for manipulating cellular phenotypes, analyzing gene function and as a promising molecular
63 therapy¹⁻⁶. Many efforts have been made to construct efficient SiRNA expression systems since the
64 discovery of the phenomena of RNAi. Synthetic SiRNA duplexes, plasmids and viral vectors are applied to
65 produce SiRNA precursor, such as short hairpin RNAs, the U1, human H1, and tRNA promoters are widely
66 used to drive the expression of SiRNA precursor⁷⁻¹⁰.

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68 All the SiRNA expression constructs have to break through two barrier layers (plasma membrane and
 69 the nucleus) of the cell for the transcription of SiRNA precursor, the critical technical hurdle for RNAi
 70 based applications is the delivery of the constructs across the plasma membrane, a number solutions for the
 71 problem have been reported, including cationic lipids, viral vectors, high pressure injection, electro-
 72 transfection and modified SiRNAs¹¹⁻¹³. However, there are rare reports in targeting the RNAi expression
 73 constructs from the cytoplasm to nucleus passing through the nuclear membrane.

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75 The threshold size for DNA freely passing through the nuclear pore complex (NPC) is a molecular
 76 weight of about 50 kDa, furthermore, DNA fragment is negative charged, which limits its pass through the
 77 nuclear membrane and NPC¹⁴. Therefore, Nuclear translocation has been reported to be the rate-limiting
 78 step in the gene transfer process rather than the cell entry. Lack of an efficient translocation of DNA
 79 fragments into the nucleus results in unacceptably low levels of gene expression by most non-viral
 80 constructs¹⁵⁻¹⁷.

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82 Eukaryotic promoter, enhancer, insulator, or regulator specific sequence plays important roles in the
 83 nuclear targeting of DNA¹⁸⁻²⁰. Following cytoplasmic translocation, DNA is translocated into the nucleus in
 84 association with several types of transcription factor, nuclear factor κB (NF-κB) is one of the most
 85 important transcription factors in the nuclear entry of DNA. NF-κB protein localizes in the cytoplasm
 86 in a bound form with its inhibitory protein, IκB, which shields the nuclear localization signal of NF-
 87 κB, once cells are exposed to any active signal, specific IκB family members rapidly undergo
 88 stimulus-coupled phosphorylation, ubiquitination and proteasome-mediated degradation, resulting in the
 89 liberation of active NF-κB heterodimers, the activated NF-κB can bind specifically to its
 90 corresponding NF-κB binding sequence and translocate the DNA fragments into the nucleus, enhance

the transcription and expression of downstream genes²¹⁻²².

In this study, to enhance the expression of siRNA cassettes, we propose here that the insertion of NF-

B binding sequence into expression cassette is an effective approach to increase SiRNA duplexes

expression in cells. The designed SiRNA expression constructs were generated by inserting NF- B

binding sequences at the terminal of expression cassette.

To assess the efficiency of our constructs, we used the eGFP as reporter protein, different siRNA

expression cassettes against eGFP were designed by insert NF- B binding sequence at the 5' or

3'-terminal of the siRNA expression constructs, The NF- B binding sequence was GGGGACTTTCC

(Figure 1).

NF- B binding sequences were attached to the 5' or 3'-terminal of the siRNA constructs by PCR

method. Same amount of these constructs were co-transfected with pEGFP-C1 plasmid into BHK21 cells,

respectively, the tRNAval-siRNA as control, As shown in Figure 2, cells transfected with expression

cassette attached the NF- B binding sequence exhibited a significant reduction in eGFP expression

compared with cells transfected with control construct, among these constructs, the construct with NF-

B binding sequence at 3'-terminal exhibited the most reduction of eGFP expression.

To quantitatively assess RNAi effect of these constructs, we tested eGFP expression level by GFP

illumination assay, the results further confirmed NF- B binding sequence could enhance the RNAi

efficiency (Figure 3).

114 To assess whether NF- κ B binding sequence is cell limited, we transfected T293 cells with these
115 expression constructs,, the results showed our design was also effective in T293 cells (Figure 4).

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117 To test whether NF- κ B binding sequence decreases eGFP expression by virtue of RNAi pathway,
118 Semi-Quantitative RT-PCR assay was used to assess the mRNA level of eGFP and western blot was used to
119 assess protein level, the results proved the NF- κ B sequence could enhance the RNAi efficiency of
120 expression cassettes (figure 5).

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122 To assess whether NF- κ B binding sequence could enhance the expression of SiRNA constructs,
123 Northern-blot was used to test the level of SiRNA fragments. The results show NF- κ B binding
124 sequence greatly enhance the expression of SiRNA precursor (Figure.6).

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126 To assess whether NF- κ B binding sequence is gene specific, we added NF- κ B binding sequence
127 at 3'-terminal of the SiRNA expression construct against luciferase, the results showed this design was also
128 more effective (Figure 7).

129

130 To examine the universality of NF- κ B binding sequence, we attached NF- κ B sequence to the
131 SiRNA expression cassette driven by H1 promoter, As Figure.8 shown, it also enhanced the RNAi
132 efficiency of the H1 promoter expression constructs.

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134 In this report, we inserted NF- κ B binding sequence into SiRNA expression cassette to enhance
135 RNAi efficiency, the propose was tested by use of tRNAval and human H1 promoters, and eGFP and
136 luciferase as reporter protein. The inserting NF- κ B binding sequence at the 3'-terminal, not the 5'

137-terminal of the SiRNA expression construct could enhance RNAi efficiency, this could be explained as
138that NF- B protein binding to 5' -terminal of the SiRNA expression construct block the transcription
139start of the SiRNA duplexes driven by type III RNA polymerase III .

140

141 **Methods**

142 **Construction of human H1,tRNAval core promoter**

143 To construct core promoters, we first synthesized tRNAval promoter,H1 promoter by PCR, two
144overlapping oligonucleotides were synthesized (Takara Biotechnology; HPLC-purified) and suspended to
145100 µM in water: denature at 95 for 3 min, annealing at room temperature for 10min.the 10ul annealing
146oligonucleotides, 5 µl 10×KOD polymerase buffer,2µl dNTP, 10U KOD polymerase(Toybo,Japan);
147incubated at 72 for 10min, The PCR conditions consisted of an initial denaturing incubation of 95°C for 3
148min, 15 cycles of 60°C for 30 s, 68°C for 30 s, 95°C for 45 s, with an additional incubation 70°C for 5 min
149on the final cycle.

150 **tRNAval-**

151 **F:**CCGGAATTCAGGACTAGTCTTTTAGGTCAAAAAGAAGAAGCTTTGTAACCGTTGGTTTCCGTA
152GTGTAGTGGTTAT

153 **tRNAval-**

154 **R:**AGCGCTCTCGAGGTTATATTCCCCGGGAGCCCGGTTTCGAACCGGGGACCTTTCGCGTGTTAG
155GCGAACGTGATAACCAC

156 **H1-F:**

157CTCGAGGCTAGCGAATTCTAAAGATATTTGCAATGTCGCTATGTGTTCTGGGAAATCACCATAAA
158CGTGAAATGTCTTTGG

159**H1-R:**

160AAGCTTGCTAGCGGATCCAGTGGTCTCATACAGAACTTATAAGATTCCCAAATCCAAAGACATTT

161CACG

162

163**Construction of SiRNA expression cassettes**

164 2µl core promoter as template, the overlapping GFP-SiRNA and 5' -1-NF- B oligonucleotides as
165amplification primer for PCR amplification, generated the complete SiRNA expression cassette by one
166round PCR amplification, and then with the PCR product as template, a several oligonucleotides containing
167NF- B binding site as primer, the amplification product was purified for RNAi.

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169**GFP-**

170**siRNA:**GCGGCTCTAGAGTTCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAAGATGAACTTCA

171GGGTCAGCAGCGCTCTCGAGGTTATATTC

172**5' -1-NF- B Primer** AGTTGAGGGGACTTTCCCAGGCGCCGGAATTCAGGACTAGTC

173**3' -1- NF- B Primer** GCCTGGGAAAGTCCCCTCAACTGCGGCTCTAGAGTTCAAAAAA

174**3'-2- NF- B** CTCTCTCGGAAAGTCCCCTCTGCCTGGGAAAGTCCCCT

175

176**Cell culture and transfection**

177 BHK21 cells were cultured in DMEM (GIBCO) containing 10% FBS supplemented with 1% penicillin-
178streptomycin (GIBCO). Cells were cultured in 24-well plates and transfected by lipofectamine 2000(
179Invitrogen,USA). Cells were co-transfected with 0.25 µg pECFP-C1 plasmid and 0.25 µg of siRNA
180expression cassettes.

181

182Reporter gene assay

183 After transfection,cultured BHK21 cells were washed with PBS three times, added 200μl lisisbuffer
184(0.1Mtris,0.1 Triton X-100,2mMEDTA,pH7.8),incubated 5 min, transferred to a 1.5 ml tube and centrifuged
185at 12000g for 8 min. The fluorescence of eGFP are dicrectly tested by luminometer (Modulus,Turner
186Biosystems,USA).

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