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1 **Title page**

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3 Synonymous SNPs of viral genes facilitate virus to escape host 4 antiviral RNAi immunity

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23 **Abstract**

24 Synonymous single nucleotide polymorphisms (SNPs) are involved in codon
25 usage preference or mRNA splicing. Up to date, however, the role of synonymous
26 SNPs in immunity remains unclear. To address this issue, the SNPs of white spot
27 syndrome virus (WSSV) were characterized in shrimp in the present study. Our
28 results indicated that there existed synonymous SNPs in the mRNAs of wsv151 and
29 wsv226, two viral genes of WSSV. In the presence of SNP siRNA, wild-type siRNA,
30 wild-type mRNA and SNP mRNA of wsv151 or wsv226, RNAi was significantly
31 suppressed, showing that the synonymous SNPs of wsv151 and wsv226 played
32 negative roles in host siRNA pathway due to mismatch of siRNA with its target. In
33 insect cells, the mismatch, caused by synonymous SNPs of wsv151 or wsv226,
34 between siRNA and its target inhibited the host RNAi. Furthermore, the data revealed
35 that the co-injection of SNP siRNA and wild-type siRNA of wsv151 or wsv226 into
36 WSSV-infected shrimp led to a significant increase of WSSV copies compared with
37 that of SNP siRNA alone or wild-type siRNA alone, indicating that the synonymous
38 SNPs of viral genes could be a strategy of virus escaping host siRNA pathway in
39 shrimp *in vivo*. Therefore, our study provided novel insights into the underlying
40 mechanism of virus escaping host antiviral RNAi immunity by synonymous SNPs of
41 viral genes.

42 **Author Summary:** Our results indicated that there existed synonymous SNPs in the
43 mRNAs of wsv151 and wsv226, two viral genes of WSSV. In the presence of
44 SNP siRNA, wild-type siRNA, wild-type mRNA and SNP mRNA of wsv151 or

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45 wsv226, RNAi was significantly suppressed, showing that the synonymous SNPs of
46 wsv151 and wsv226 played negative roles in host siRNA pathway due to mismatch
47 of siRNA with its target. In insect cells, the mismatch, caused by synonymous SNPs
48 of wsv151 or wsv226, between siRNA and its target inhibited the host RNAi.
49 Furthermore, the data revealed that the co-injection of
50 SNP siRNA and wild-type siRNA of wsv151 or wsv226 into WSSV-infected shrimp
51 led to a significant increase of WSSV copies compared with that of SNP siRNA alone
52 or wild-type siRNA alone, indicating that the synonymous SNPs of viral genes could
53 be a strategy of virus escaping host siRNA pathway in shrimp in vivo.

54 **Key words：** synonymous single nucleotide polymorphism; WSSV; siRNA; shrimp

55

56 **Introduction**

57 Single nucleotide polymorphism (SNP) mainly refers to a DNA or RNA sequence
58 polymorphism caused by the variation of a single nucleotide (1). Generally, SNPs are
59 substitutions, insertions, or deletions of individual bases in the genetic sequence or in
60 non-coding sequences of a DNA. According to the locations, SNPs can be divided
61 into coding SNP and non-coding SNP (2). Based on the change of amino acid or not,
62 the coding SNPs consist of synonymous SNP and non-synonymous SNP (2). The
63 synonymous SNP means that the changed base of a coding sequence does not change
64 its coding protein sequence. At present, SNPs have been widely used as genetic
65 markers to distinguish species, to assess sex ratio of fish populations and to calculate
66 the genomic breeding value in aquaculture (1-4). Recently, it is found that the coding

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67 SNPs can influence the health of human being (5, 6). In hepatitis C, the IFNL3
68 (rs4803217) SNP affects virus infection (6). Many promoter-related SNPs have a
69 relationship with human health (7, 8). As reported, the microRNA (miRNA) pathway
70 can also be influenced by the miRNA-related SNPs (9, 10). The 3' untranslation
71 region (3'UTR) SNP (rs1048638) of carbonic anhydrase IX gene, the target of
72 miR-34a, can promote hepatocellular carcinoma (9). The bipolar disorder is
73 influenced by SNP rs3749034 which is associated with the hsa-miR-504 pathway (10).
74 In contrast to non-synonymous SNPs, the synonymous SNPs have not been
75 extensively characterized. Although some investigations reveal that synonymous
76 SNPs are associated with diseases (8, 9, 11), the mechanism of synonymous SNPs in
77 diseases remains unclear.

78 It has been found that a base mutation of a siRNA has a great effect on antiviral
79 RNAi (RNA interference) response of host by impairing the base pair (12, 13),
80 implying that synonymous SNPs may play important roles in the siRNA pathway.
81 RNAi is a vital strategy of animal immune responses to viruses and other
82 foreign genetic materials, especially in invertebrates which lack adaptive immunity
83 (12-18). In shrimp, the vp28-siRNA, which targets the vp28 gene of white spot
84 syndrome virus (WSSV), is capable of mediating sequence-specific gene silencing
85 (12). However, a base mutation of vp28-siRNA suppresses the silencing capacity of
86 vp28-siRNA. It is found that the target recognition by siRNA proceeds via 5' to 3'
87 base-pairing propagation with proofreading (14). A base change in the 5' seed region
88 of a siRNA affects the initial target recognition, while a base change in the 3'

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89 supplementary region of a siRNA promotes the dissociation of RISC (RNA induced
90 silencing complex) before target cleavage (14). These findings suggest that
91 synonymous SNPs can take great effects on the siRNA-mediated RNAi pathway.
92 RNAi, an evolutionarily conserved mechanism in eukaryotes, functions in the
93 antiviral defense of animals through the cleavage and degradation of target viral
94 mRNAs (15-18). In this context, synonymous SNPs may affect the antiviral siRNA
95 pathway of animals. However, this issue is not addressed at present.

96 To explore the role of synonymous SNPs in antiviral RNAi response, the
97 synonymous SNPs of WSSV were characterized in shrimp in the present study. The
98 results indicated that the synonymous SNPs could be employed by virus to suppress
99 the host's antiviral RNAi.

100 Materials and methods

101 Shrimp culture and WSSV challenge

102 Shrimp (*Marsupenaeus japonicus*), 10 to 12 cm in length, were cultured in groups
103 of 20 individuals in a tank filled with seawater at 25°C. To make sure that shrimp
104 were virus-free before experiments, PCR was conducted to detect WSSV in shrimp
105 using WSSV-specific primers (5'-TATTGTCTCTCCTGACGTAC-3' and 5'-CACAT
106 TCTTCACGAGTCTAC-3'). DNA was extracted from shrimp with the SQ tissue
107 DNA kit (Omega-Bio-Tek, USA) according to the manufacturer's instructions. The
108 virus-free shrimp were infected with WSSV (10^5 copies/ml) by injection (100 μl
109 WSSV inoculum/shrimp) into the lateral area of the fourth abdominal segment. The
110 WSSV isolate used in this study was the Chinese mainland strain (WSSV-CN,

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111 GenBank accession no. AF332093.1). At different times post-infection (0, 2, 4, 6, 12,
112 24, 36, 48, and 72 h), hemocytes of three shrimp, randomly collected for each
113 treatment, were collected for later use.

114 **Isolation of cytoplasm from shrimp hemocytes**

115 Shrimp homocytes were collected. After centrifugation at 1000×g (4°C) for 10
116 min, the homocytes were incubated with lysis buffer [10 mM HEPES (2-[4-(2-
117 hydroxyethyl)-1-piperazinyl] ethane sulfonic acid)-KOH, 10 mM $MgCl_2$, 10 mM KCl,
118 1 mM DTT (dithiothreitol), pH 7.9] for 10 min at 4℃. Subsequently the lysate was
119 centrifuged at 4000×g for 10 min at 4°C. The supernatant (cytoplasm) was collected.

120 **Western blot analysis**

121 The proteins were separated by 15% SDS-polyacrylamide gelelectrophoresis and
122 then transferred onto a nitrocellulose membrane. The membrane was blocked with 5%
123 nonfat milk in Tris-buffered saline (TBST) (10 mM Tris-HCl, 150 mM NaCl, 20%
124 Tween 20, pH7.5) for 2 h at room temperature, followed by incubation with a primary
125 antibody overnight. After washes with TBST, the membrane was incubated with
126 horseradish peroxidase-conjugated secondary antibody (Bio-Rad, USA) for 2 h at
127 room temperature. The membrane was detected by using a Western Lightning
128 Plus-ECL kit (Perkin Elmer, USA). The antibody against Histone 3 was purchased
129 from Beyotime Biotechnology (China).

130 **RNA extraction and complementary DNA (cDNA) synthesis**

131 Total RNAs were extracted using the mirVana^{TM} RNA isolation kit according to
132 the manufacturer's instructions (Ambion, Foster City, USA). To exclude any DNA

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133 contamination, the RNAs were treated with RNase-free DNase I (Takara, Shiga,
134 Japan) at 37°C for 30 min. First-strand cDNA synthesis was performed using total
135 RNAs according to the manufacturer's guidelines for the PrimeScript™ 1st strand
136 cDNA Synthesis kit (Takara, Shiga, Japan).

137 **Detection of U6 with PCR**

138 PCR was conducted to detect U6 using U6-specific primers (5'-GGTCTCACTG
139 ACTGTGAC-3' and 5'-AGTAGCAGTCT CACGAGTCTAC-3'). PCR condition
140 was 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 54°C for 40 s and 72°C for
141 1 min, with a final elongation at 72°C for 10 min.

142 **Analysis of WSSV mRNA SNPs**

143 To obtain a global insight into the characteristics of WSSV mRNA SNPs, total
144 RNAs were extracted from cytoplasm of WSSV-infected shrimp hemocytes.
145 Subsequently RNAs were sequenced by Biomarker Technologies (Beijing, China)
146 with a GA-I genome analyser (Illumina, San Diego, CA, USA). RNA assembly was
147 conducted using TRINITY software (Biomarker Technologies, China). After assembly
148 of RNA-seq data, 7.7 GB raw data was processed to remove the sequences of adapter,
149 ploy-N and low-quality reads. Then the clean reads were analyzed to discover SNPs
150 with the reference sequence of WSSV (GenBank accession no. AF332093.1). The
151 prediction of small interference RNAs (siRNAs) was conducted (https://rnaidesigner.
152 lifetechnologies.com/rnaiexpress/sort.do).

153 **Northern blot**

154 After separation of small RNAs on a denaturing 15% polyacrylamide gel

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155 containing 7 M urea or mRNAs on a 1% agarose gel, RNAs were transferred to a
156 Hybond N+ nylon membrane (Amersham Biosciences) for 1 h at 400 mA, followed
157 by ultraviolet cross-linking. The membrane was prehybridized in DIG (digoxigenin)
158 Easy Hyb granule buffer (Roche, Basel, Switzerland) for 0.5 h at 42 ℃ and then
159 hybridized with DIG-labeled wsv151 siRNA probe (5'-TCAGCAAGTATGCAGTGT
160 C-3'), wsv226 siRNA probe (5'-GCTACACACAGTATTGTAC-3'), wsv151 mRNA
161 probe (5'-TTCACTTTCAATCTTGGTTCTGC-3'), wsv226 mRNA probe (5'-TCGC
162 TTCAATACCAGATCGTGGAC-3') or U6 probe
163 (5'-GGGCCATGCTAATCTTCTC TGTATCGTT-3') at 42 ℃ overnight.
164 Subsequently the detection was performed with the DIG High Prime DNA labeling
165 and detection starter kit II (Roche).

166 **SiRNA-mediated cleavage of mRNA *in vitro***

167 Total RNAs were extracted from shrimp lymphoid organs or hemocytes at 48 h
168 after WSSV infection using a mirVanaTM miRNA isolation kit (Ambion, USA)
169 according to the manufacturer's instructions. To remove any genomic DNA
170 contamination, total RNA extracts were treated with RNase-free DNase I (Takara,
171 Shiga, Japan) at 37℃ for 30 min. First-strand cDNA synthesis was performed using
172 total RNAs according to the manufacturer's guidelines for PrimeScript 1st strand
173 cDNA Synthesis Kit (Takara, Japan). The first-strand cDNA was used as a template
174 for PCR amplification of wsv151 or wsv226 with sequence-specific primers (wsv151,
175 5'-GAGGAAGAAAATGTCTACCCCAAA-3' and 5'-CTGCGTCTCAATTGAAG
176 TGATGTAAAAAT-3'; wsv226, 5'-GTGCGTTTTCAAAGTATAAGAAA-3' and

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177 5'- CGAGAGAATAAGAATAAAGTGTAT-3'). The SNPs of wsv151 and wsv226
178 were obtained by PCR using sequence-specific primers (wsv151,
179 5'-TATGCAGTGACAA TGAGTATCAAAAA-3' and
180 5'-ACTCATTGTCACTGCATACTTGCCTC-3'; wsv226,
181 5'-TAAGCTACATACAGTATTGTACGATAAAG-3' and 5'-CAATACTGTA
182 TGTAGCTTACATTTAGGTATTA-3'). The PCR products were cloned into
183 pEASY-Blunt Simple vector (TransGen Biotech, China). Then mRNAs were
184 synthesized *in vitro* using a commercial T7 kit according to the manufacturer's
185 instructions (TaKaRa, Japan).

186 SiRNAs used were wsv151-siRNA (5'-CGUUCAUACGUCACAGUUA-3'),
187 wsv151-SNP-siRNA (5'-CGUUCAUACGUCACUGUUA-3'), wsv226-siRNA (5'-C
188 GAUGUGUGUCAUAACAUG-3') and wsv226-SNP-siRNA (5'-CGAUGUAUGU
189 CAUAACAUG-3'). The siRNAs were synthesized using T7 kit according to the
190 manufacturer's manual (TaKaRa, Japan).

191 To conduct the siRNA-mediated cleavage of mRNA, the shrimp Ago2 complex
192 was prepared by immunoprecipitation using Ago2-specific antibody in lysis buffer
193 (30 mM HEPES-KOH, pH 7.4), followed by incubation with protein A (Bio-Rad,
194 USA) at 4 ℃ overnight. The isolated Ago2 complex was dissolved in 500 μl reaction
195 buffer [100 mM KOAc, 2 mM $Mg(OAc)_2$] containing 1 mM DTT and protease
196 inhibitor cocktail (Roche, Switzerland) at 4 ℃, followed by the addition of 12.5 μl
197 40×reaction mix [20 μl 500 mM creatine monophosphate, 20 μl amino acid stock
198 (Sigma,USA), 2 μl 1M DTT, 2 μl 20 U/μl RNasin (Promega, USA), 4 μl 100 mM

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199 ATP (TaKaRa, Japan), 1 μl 100 mM GTP (TaKaRa), 6 μl 2 U/μl creatine
200 phosphokinase (Cal-Biochem, Germany)]. Then 50 nM siRNA, 100 nM mRNA and
201 the isolated Ago2 complex were incubated at 37 ℃ for different time (0, 5, 10 and
202 15min). The reaction was stopped using proteinase K (Shanghai Generay Biotech Co,
203 Ltd. China) by incubation at 37 ℃ for 5 min. The mRNAs were separated on 1%
204 agarose gel.

205 **Cell culture, transfection and fluorescence assays**

206 Insect High Five cells (Invitrogen, USA) were cultured in Express Five serum-free
207 medium (Invitrogen) containing L -glutamine (Invitrogen) at 27 ℃. At 70%
208 confluence, the insect cells were co-transfected with wild-type siRNA (100 pM) or
209 SNP siRNA (100 pM) and EGFP-wild-type-wsv151/wsv226 (6 μg/ml) or
210 EGFP-SNP-wsv151/wsv226 (6 μg/ml). The constructs were generated by cloning
211 wsv151 and wsv226 mRNAs into the pIZ/V5-His vector (Invitrogen). The
212 recombinant plasmids were confirmed by sequencing. All transfections were carried
213 out in triplicate with Cellfectin transfection reagent (Invitrogen) according to the
214 manufacturer's protocol. At 36h after co-transfection, the fluorescence intensity of
215 cells was evaluated with a Flex Station II microplate reader (Molecular Devices, USA)
216 at 490/510 nm excitation/emission (Ex/Em). The experiments were biologically
217 repeated three times.

218 **RNAi assay in shrimp**

219 The small interfering RNA (siRNA) specifically targeting wsv151 (wsv151-
220 siRNA, 5'-CGUUCAUACGUCACAGUUA-3') or wsv226 (wsv226- siRNA, 5'-CG

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221 AUGUGUGUCAUAACAUG-3') was used in the RNAi assay in shrimp. The
222 synonymous SNP siRNAs (wsv151-SNP-siRNA,
223 5'-CGUUCAUACGUCACUGUUA -3'; wsv226-SNP-siRNA,
224 5'-CGAUGUAUGUCAUAACAUG-3') were included in the RNAi assay. The
225 siRNAs were synthesized using a T7 kit according to the manufacturer's manual
226 (TaKaRa, Japan). The synthesized siRNAs were quantified
227 by spectrophotometry. Shrimp were co-injected with WSSV (10^4 copies/shrimp) and
228 siRNA (4nM). At different time after injection, three shrimp were randomly selected
229 from each treatment. The shrimp hemocytes from each treatment were collected for
230 later use.

231 **Quantification of WSSV copies**

232 Quantitative real-time PCR was used to examine the WSSV copies in shrimp. The
233 genomic DNA of WSSV was extracted with a SQ tissue DNA kit (Omega Bio-tek,
234 Norcross, GA, USA) according to the manufacturer's instruction. The extracted DNA
235 was analyzed by quantitative real-time PCR with WSSV-specific primers and
236 WSSV-specific TaqMan probe (5'-FAM-TGCTGCCGTCTCCAA-TAMRA-3') as
237 described previously (19). The linearized plasmid containing a 1400-bp DNA
238 fragment from the WSSV genome was used as the internal standard of quantitative
239 real-time PCR (19). The PCR procedure was 95°C for 1 min, followed by 40 cycles of
240 95°C for 30 s, 52°C of 30 s, and 72°C for 30 s.

241 **Statistical analysis**

242 To calculate the mean and standard deviation, the numerical data from three

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243 independent experiments were analyzed by one-way analysis of variance (ANOVA).
244 The differences between the different treatments were analyzed by t-test.

245 **Results**

246 **Existence of SNP in WSSV**

247 To explore the role of SNP in virus infection, the SNPs in cytoplasm of
248 WSSV-infected shrimp hemocytes were characterized. Western blot analysis showed
249 that the isolated cytoplasm of WSSV-infected shrimp hemocytes had no
250 contamination of nucleus (Fig 1A). Then the extracted RNAs from the isolated
251 cytoplasm, without contamination of nuclear RNAs (Fig 1B), were subjected to
252 RNA-seq analysis.

253 The sequence analysis showed that all the 180 open reading frames (ORFs) of
254 WSSV were found in the RNA-seq analysis. Among them, 16 WSSV mRNAs
255 possessed 47 SNPs (Fig 1C). Of the 47 SNPs, there were 18 synonymous SNPs from
256 10 genes (Fig 1C). As well known, synonymous SNPs of an mRNA could not take
257 effects on its protein-coding sequence. The synonymous SNPs of a viral mRNA might
258 function in antiviral siRNA pathway to escape the targeting by siRNA. The sequence
259 analysis indicated that 4 WSSV mRNAs (wsv465, wsv360, wsv226 and wsv151)
260 containing 4 synonymous SNPs were predicted to potentially generate siRNAs
261 employed by shrimp (Fig 1C).

262 To investigate the existence of the 4 synonymous SNPs, the RNAs extracted from
263 hemocytes of WSSV-infected shrimp were sequenced. The sequence analysis showed
264 that two SNPs (wsv151 and wsv226) existed in WSSV-infected shrimp (Fig 1D).

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265 However, the wsv360 SNP and the wsv465 SNP were not detected (Fig 1D). The
266 sequencing of WSSV genomic DNA generated the same results, confirming the
267 existence of SNPs of wsv151 and wsv226.

268 Taken together, these findings revelaed that SNP existed in WSSV and that 2
269 synonymous SNPs (wsv151 and wsv226) might function in the siRNA pathway of
270 shrimp.

271 **Influence of viral synonymous SNPs on host siRNA pathway**

272 In an attempt to reveal the roles of viral synonymous SNPs in host siRNA
273 pathway, the wsv151 siRNA and wsv226 siRNA of WSSV were examined in
274 hemocytes of WSSV-infected shrimp. Northern blot results indicated that shrimp
275 could generate the wsv151 siRNA and wsv226 siRNA during WSSV infection (Fig
276 2A). To explore the influence of synonymous SNPs of wsv151 and wsv226 mRNAs
277 on shrimp siRNA pathway, the sequences of wild-type siRNAs and mRNAs of
278 wsv151 and wsv226 were mutated, generating SNP-siRNAs and SNP-mRNAs which
279 contained the synonymous SNPs of wsv151 and wsv226, respectively. Then the
280 synthesized wild-type siRNA (WT siRNA) or SNP siRNA and WT mRNA or SNP
281 mRNA were incubated with the isolated shrimp Ago2 complex, followed by
282 separation of mRNAs on agarose gel. The results indicated that wild-type siRNA and
283 SNP-siRNA of wsv226 could mediate the cleavage of wild-type mRNA and
284 SNP-mRNA of wsv226, respectively (Fig 2B). However, the cleavage of wsv226
285 wild-type mRNA mediated by wsv226 SNP-siRNA and the cleavage of wsv226
286 SNP-mRNA mediated by wsv226 wild-type siRNA were inhibited (Fig 2B). For

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287 wsv151, the viral synonymous SNP yielded the similar results (Fig 2C). These data
288 revealed that the viral synonymous SNPs suppressed the host siRNA pathway.

289 To reveal the locations of synonymous SNPs of wsv151 and wsv226, the
290 sequences of wild-type and SNP mRNAs and siRNAs were compared. The results
291 showed that the synonymous SNP of wsv151 was located in the extended 3’
292 supplementary region of siRNA (Fig 2D). For the wsv226 synonymous SNP, it was
293 located in the seed region of siRNA (Fig 2E). As reported, the seed region (positions
294 2-8) and the extended 3’ supplementary region (positions 12-17) of a siRNA were
295 essential for the siRNA-mediated gene silencing (14). Therefore, the synonymous
296 SNPs of wsv151 and wsv226 could inhibit the host siRNA pathway.

297 The sequence showed that the ratio of wild-type mRNA to SNP mRNA of wsv151
298 was 8 to 5, while the ratio of wild-type mRNA to SNP mRNA of wsv226 was 4 to 7.
299 These data indicated the co-existence of wild-type mRNAs and SNP mRNAs of
300 WSSV in natural conditions. Based on prediction, it was found that SNP siRNA could
301 be produced from SNP mRNA of wsv151 or wsv226, showing the co-existence of
302 wild-type siRNA and SNP siRNA. Therefore, wild-type mRNA, SNP mRNA,
303 wild-type siRNA and SNP siRNA of wsv151 or wsv226 simultaneously existed in
304 WSSV-infected shrimp. The results showed that RNAi was significantly suppressed
305 in the presence of SNP siRNA, wild-type siRNA, wild-type mRNA and SNP mRNA
306 (Fig 2F).

307 The above findings indicated that the synonymous SNPs of viral mRNA played
308 negative roles in host siRNA pathway.

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309 **Suppression of siRNA pathway by viral synonymous SNP in insect cells**

310 To explore the effects of synonymous SNPs of wsv151 mRNA and wsv226
311 mRNA on siRNA pathway in cells, the insect High Five cells were co-transfected
312 with wild-type siRNA (WT siRNA) or SNP-siRNA and EGFP-wild-type-wsv151
313 mRNA, EGFP-SNP-wsv151 mRNA, EGFP-wild-type-wsv226 mRNA or EGFP-SNP-
314 wsv226 mRNA. At 36 h after co-transfection, the wsv151 mRNA, the wsv226 mRNA
315 and the fluorescence intensity of insect cells were evaluated.

316 The results of Northern blot showed that when the insect cells were co-transfected
317 with WT siRNA and WT mRNA of wsv226 or SNP siRNA and SNP mRNA of
318 wsv226, the wsv226 mRNA level was significantly decreased (Fig 3A). However, the
319 transfection of insect cells with WT siRNA and SNP mRNA of wsv226 or SNP
320 siRNA and WT mRNA of wsv226 did not change the wsv226 mRNA level (Fig 3A).
321 In the simultaneous existence of WT siRNA, SNP siRNA, WT mRNA and SNP
322 mRNA of wsv226, the siRNA pathway was significantly inhibited compared with the
323 control (Fig 3A). The fluorescence intensity of insect cells generated the similar
324 results (Fig 3B). These data indicated that the viral synonymous SNP of wsv226
325 escaped the siRNA pathway in insect cells.

326 For wsv151, Northern blots indicated that the wsv151 mRNA level was
327 significantly reduced when the insect cells were co-transfected with WT siRNA and
328 WT mRNA of wsv151 or SNP siRNA and SNP mRNA of wsv151 (Fig 3C). However,
329 the co-existence of WT siRNA, SNP siRNA, WT mRNA and SNP mRNA of wsv151,
330 resulted in a significant suppression of RNAi in insect cells (Fig 3A). The

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331 fluorescence intensity of insect cells essentially generated the similar results (Fig 3D).
332 These results revealed that the viral synonymous SNP of wsv151 could inhibit the
333 siRNA pathway.

334 Taken together, the findings showed that the synonymous SNPs of viral mRNAs
335 could escape the siRNA pathway in cells.

336 **Inhibition of siRNA pathway by viral synonymous SNPs in shrimp *in vivo***

337 In order to characterize the influence of viral synonymous SNPs on the host
338 siRNA pathway *in vivo*, the synthesized wild-type (WT) siRNA or/and SNP siRNA of
339 wsv151 or wsv226 and WSSV were co-injected into shrimp and then the wsv151 and
340 wsv226 mRNAs were examined. The results indicated that the mRNA levels of
341 wsv151 and wsv226 were significantly decreased in shrimp treated with WT siRNA
342 or SNP siRNA compared with the control (WSSV alone) (Fig 4A and B). When
343 shrimp were co-injected with WT siRNA and SNP siRNA, the expression profiles of
344 wsv151 and wsv226 in WSSV-infected shrimp were similar to the WT siRNA or SNP
345 siRNA treatment (Fig 4A and B). These findings revealed that SNPs of wsv151 and
346 wsv226 could escape the siRNA pathway of shrimp *in vivo*.

347 To explore the effects of viral synonymous SNPs on virus infection *in vivo*,
348 shrimp were injected with WSSV and wild-type (WT) siRNA or/and SNP siRNA of
349 wsv151 or wsv226, followed by the detection of WSSV copies. The results showed
350 that the silencing of wsv151 and wsv226 by WT siRNAs led to a significant decrease
351 of WSSV copies compared with the control (WSSV alone) (Fig 4C and D). SNP
352 siRNA of wsv151 or wsv226 yielded the similar results (Fig 4C and D). When WT

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353 siRNA and SNP siRNA of wsv151 or wsv226 were co-injected into WSSV-infected
354 shrimp, the WSSV copies were significantly reduced in shrimp compared with the
355 control (WSSV alone) (Fig 4C and D).
356 The above findings revealed that the synonymous SNPs could be a strategy of
357 virus escaping host siRNA pathway in shrimp *in vivo*.

358 **Discussion**

359 As reported, SNPs in the non-coding regions of a gene can manifest a higher risk
360 of cancer (20) and can affect mRNA structure and disease susceptibility (21, 22). In
361 the coding region, SNPs may result in a premature stop codon, a nonsense codon in
362 the transcribed mRNA or in a truncated, incomplete and nonfunctional protein
363 product (21). In recent years, it is found that synonymous SNPs are involved in codon
364 usage preference or mRNA splicing (23-25). In the genomes of almost all species, one
365 of the most prominent observations on the non-neutrality of synonymous codons is
366 the correlation between synonymous codon usage bias and the level of gene
367 expression (23). Highly expressed genes tend to have a higher preference toward
368 so-called optimal codons than lowly expressed genes (23). The amounts of cognate
369 tRNAs that bind to optimal codons are significantly higher than the amounts of
370 cognate tRNAs that bind to non-optimal codons in genomes (23). In some cases,
371 synonymous SNPs are associated to mRNA splicing (24-26). Based on their impact
372 on mRNA splicing and protein function, it is suggested that the protein structure
373 features offer an added dimension of information while distinguishing disease-causing
374 and neutral synonymous SNPs (26). In this study, the results showed that the viral

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375 synonymous SNPs of wsv1151 and wsv226 played negative roles in shrimp RNAi,
376 indicating the synonymous SNP was employed by virus to escape the host's RNAi
377 immunity during virus infection. Therefore, our findings contributed a novel function
378 of synonymous SNPs.

379 It has been demonstrated that the siRNA-mediated RNAi is the major antiviral
380 defense of invertebrates which lack adaptive immunity (27, 28). In the
381 siRNA-induced silencing complex (RISC), the siRNA uses not only its seed region
382 (the 2nd-7th nt) but also its 3' supplementary region (the 12th-17th nt) (also called
383 extended 3' supplementary region) (14, 29). The target recognition of siRNA depends
384 on base pairing in the seed region but not in the 3' supplementary region of the siRNA,
385 showing the importance of seed pairing for initial target recognition (14). It is also
386 found that the mismatch in the 3' supplementary region of a siRNA prevents RISC
387 from reaching its cleavage-competent conformation and promoting dissociation of
388 RISC without cleavage (14). In the presence of mismatches of siRNA with its target
389 mRNA (wild-type mRNA), the mismatched siRNA and target mRNA can be loaded
390 into RISC. However, the target cleavage does not occur in this RISC (14). In shrimp,
391 the findings show that a single-base mutation in the 6th base of vp28-siRNA leads to
392 the suppression of shrimp antiviral immunity against WSSV (12). In the present study,
393 the results revealed that RNAi was significantly suppressed in the presence of SNP
394 siRNA, wild-type siRNA, wild-type mRNA and SNP mRNA of wsv151 or wsv226.
395 Therefore, in the siRNA-mediated RNAi, a single-base mutation of a siRNA can
396 inactivate the antiviral RNAi immunity. Our *in vitro* and *in vivo* data revealed that

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397 WSSV could escape the shrimp RNAi immunity by the single-base mismatch
398 between the seed region or the 3' supplementary region of a siRNA and its target
399 mRNA during WSSV infection. In this context, our findings presented that the
400 synonymous SNPs played important roles during virus infection, which would be
401 helpful for us to gain insights into the molecular mechanisms in virus-host
402 interactions *in vivo*.
403

404 **Acknowledgements**

405 This work was supported by National Natural Science Foundation of China
406 (31430089) and National Program on the Key Basic Research Project
407 (2015CB755903).
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521 **Figure legends**

522 **Fig 1. Existence of SNP in WSSV.** (A) Western blot analysis of the isolated
523 cytoplasm. Shrimp were infected with WSSV. At 48h post-infection, the shrimp
524 hemocytes were collected and subjected to the isolation of cytoplasm. To exclude the
525 contamination of nucleus, the isolated cytoplasm was detected using Western blot
526 with anti-histone 3 (H3) IgG. (B) PCR detection of RNAs extracted from the isolated
527 cytoplasm RNA. RNAs were extracted from the isolated cytoplasm and then reversely
528 transcribed into cDNAs, followed by PCR using U6-specific primers. The PCR

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529 products were analyzed using agarose gel electrophoresis. (C) The number of
530 synonymous SNPs. (D) Identification of synonymous SNPs of WSSV mRNAs. The
531 RNAs extracted from hemocytes of WSSV-infected shrimp were sequenced. The
532 arrows indicated the SNP sites and the numbers showed the positions of the coding
533 sequences of viral genes.

534 **Fig 2. Influence of viral synonymous SNPs on host siRNA pathway.** (A) The
535 detection of siRNA in WSSV-infected shrimp. Shrimp were infected with WSSV. At
536 different times post-infection, wsv151 siRNA and wsv226 siRNA were detected using
537 hemocytes of WSSV-infected shrimp by Northern blotting. U6 was used as a control.
538 (B) The influence of synonymous SNP of wsv226 mRNA on shrimp siRNA pathway.
539 The wild-type siRNA (WT siRNA) or SNP siRNA and wild-type mRNA (WT mRNA)
540 or SNP mRNA were incubated with the isolated shrimp Ago2 complex. At different
541 times after incubation, the mRNA was separated by agarose gel electrophoresis.
542 Wsv226 mRNA alone was used as a control. (C) The role of synonymous SNP of
543 wsv151 mRNA in shrimp siRNA pathway. The WT siRNA or SNP siRNA, WT
544 mRNA or SNP mRNA and the isolated shrimp Ago2 complex were incubated for
545 different time. The mRNA cleavage was examined using agarose gel electrophoresis.
546 Wsv151 mRNA alone was used as a control. (D) The sequence analysis of wild-type
547 and SNP mRNA and siRNA of wsv151. Boxes indicated the SNPs. The seed region
548 and extended 3' sup (supplementary) region of siRNA were underlined with solid and
549 dashed lines, respectively. (E) The sequence analysis of wild-type and SNP mRNA
550 and siRNA of wsv226. (F) The impact of synonymous SNPs on RNAi in the presence

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551 of SNP mRNA, SNP siRNA, WT mRNA and WT siRNA. SNP mRNA, SNP siRNA,
552 wild-type (WT) mRNA and WT siRNA were mixed and then incubated with the
553 isolated shrimp Ago2 complex. At different times after incubation, the mRNA was
554 separated by agarose gel electrophoresis. As a control, mRNA alone was included in
555 the assays.

556 **Fig 3. Suppression of siRNA pathway by viral synonymous SNP in insect cells.**
557 (A) Influence of synonymous SNP of wsv226 mRNA on siRNA pathway in insect
558 cells. The insect High Five cells were co-transfected with wild-type siRNA (WT
559 siRNA), SNP siRNA, EGFP-WT-mRNA and/or EGFP-SNP-mRNA of wsv226. At 36
560 h after co-transfection, the wsv226 mRNA level was examined by Northern blot. U6
561 was used as a control. Probes were indicated on the left. (B) Evaluation of viral
562 synonymous SNP of wsv226 mRNA on siRNA pathway in insect cells by
563 examination of fluorescence intensity of insect cells. The treatments were indicated on
564 the top. Scale bar, 50 μm. (C) Role of synonymous SNP of wsv151 mRNA in siRNA
565 pathway in insect cells. At 36 h after co-transfection of insect cells with wild-type
566 (WT) siRNA, SNP siRNA, EGFP-WT-mRNA and/or EGFP-SNP-mRNA of wsv151,
567 the wsv151 mRNA level was determined by Northern blot. U6 was used as a control.
568 Probes were indicated on the left. (D) Impact of viral synonymous SNP of wsv151
569 mRNA on siRNA pathway in insect cells by examination of fluorescence intensity of
570 insect cells. The treatments were indicated on the top. Scale bar, 50 μm.

571 **Fig 4. Inhibition of siRNA pathway by viral synonymous SNPs in shrimp *in vivo.***
572 (A) Influence of synonymous SNPs on wsv151 expression in WSSV-infected shrimp.

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573 Shrimp were co-injected with WSSV and WT siRNA or/and SNP siRNA of wsv151.
574 WSSV alone was included in the injection as a positive control. At different time after
575 injection, the shrimp hemocytes were analyzed by Northern blotting. β-actin was used
576 as a loading control. Numbers indicated the time post-infection. (B) Impact of
577 synonymous SNPs on the expression of wsv226 in WSSV-infected shrimp. WSSV
578 and siRNA or/and SNP siRNA of wsv226 were injected into shrimp. At different time
579 post-infection, the shrimp hemocytes were subjected to Northern blotting. (C)
580 Detection of WSSV copies in shrimp hemocytes by quantitative real-time PCR.
581 Shrimp were injected with WSSV and wsv151 WT siRNA or/and wsv151 SNP
582 siRNA. WSSV alone and PBS were included in the injection as controls. At different
583 time after injection (0, 24, 36 and 48h), the WSSV copies in shrimp were examined (*,
584 $p<0.05$; **, $p<0.01$). (D) Effects of synonymous SNPs of wsv226 on WSSV infection
585 in shrimp. The treatments were indicated on the top. The numbers showed the time
586 post-infection. Significant differences between treatments were indicated with
587 asterisks (*, $p<0.05$; **, $p<0.01$).
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609 Fig 1

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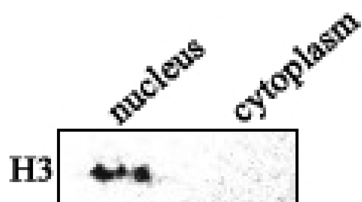

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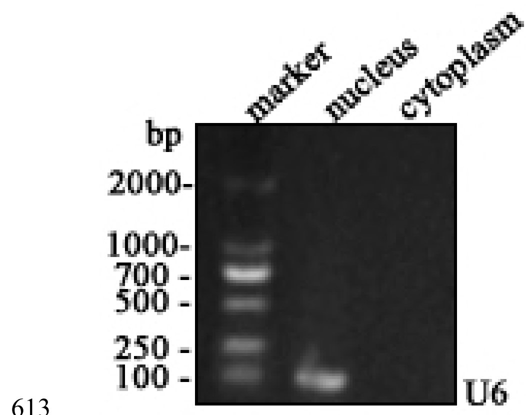

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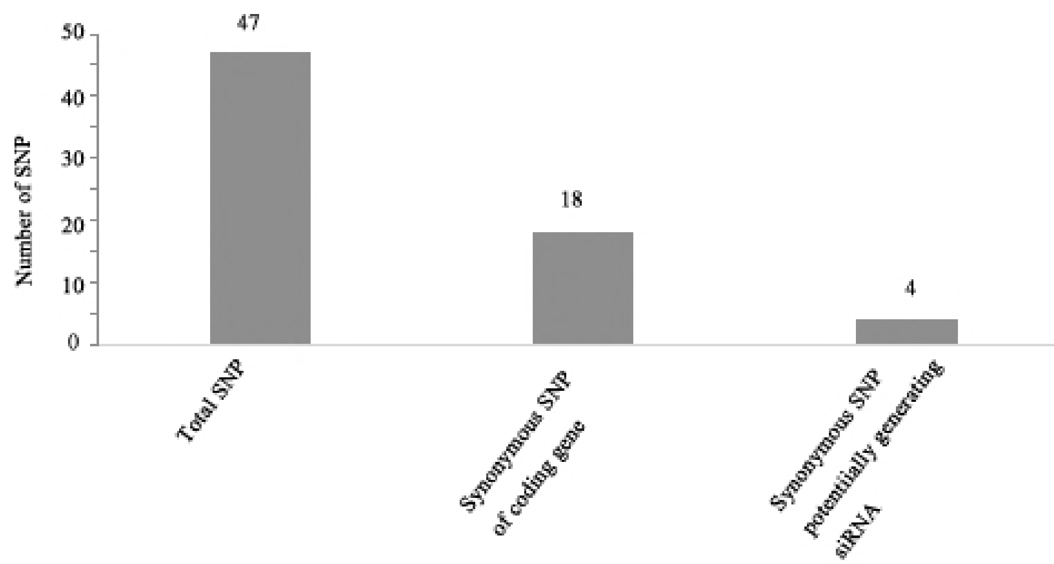

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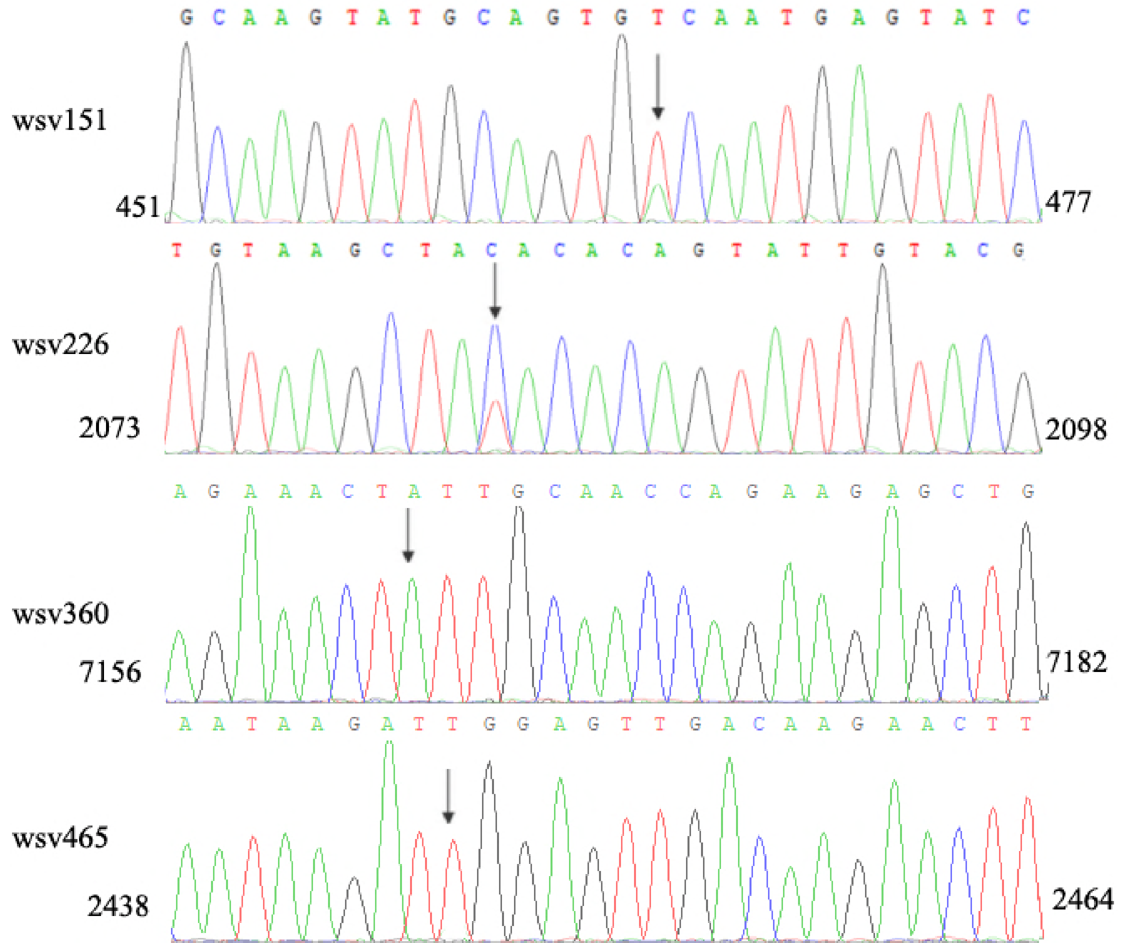

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629 Fig 2

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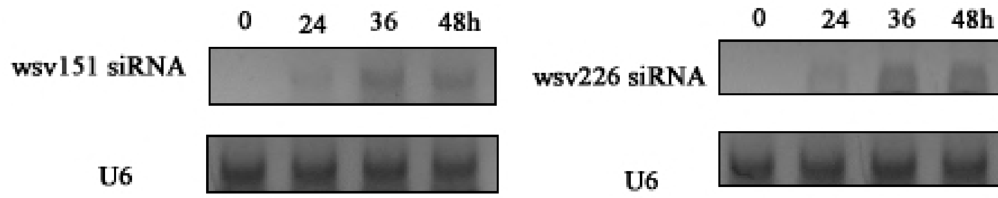

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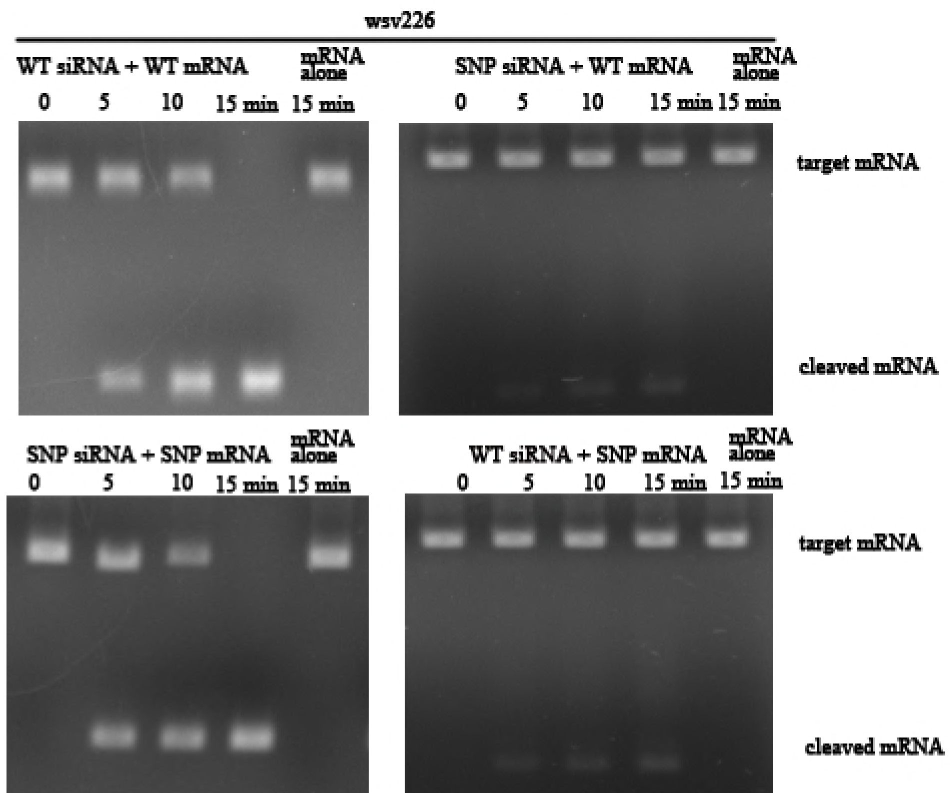

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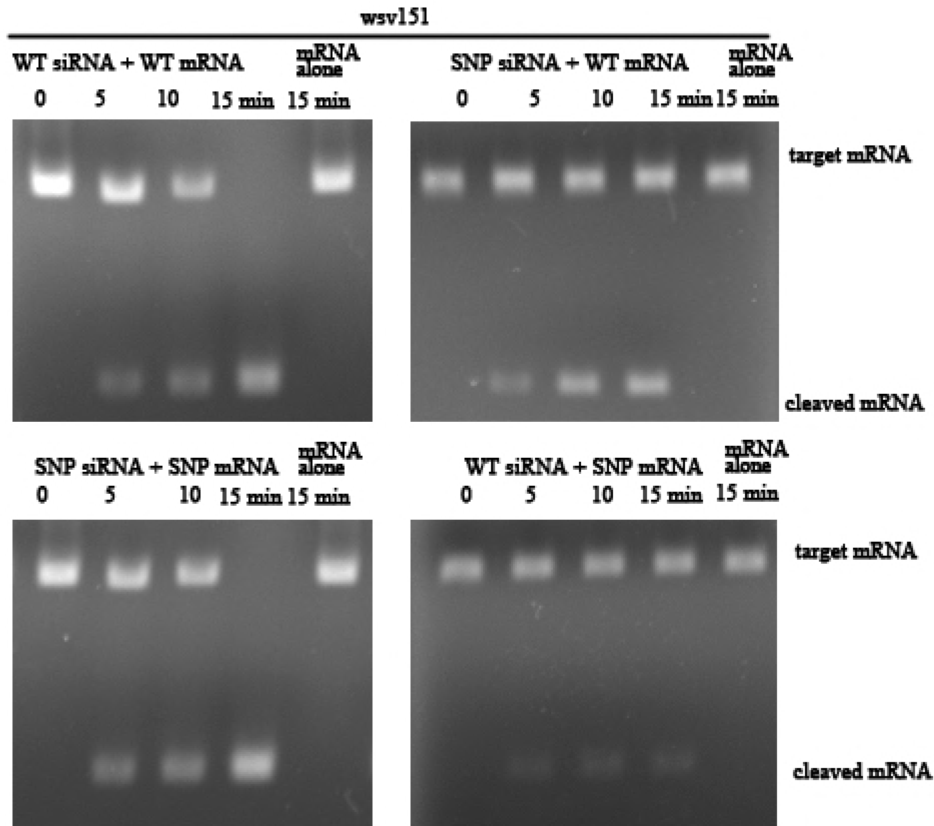

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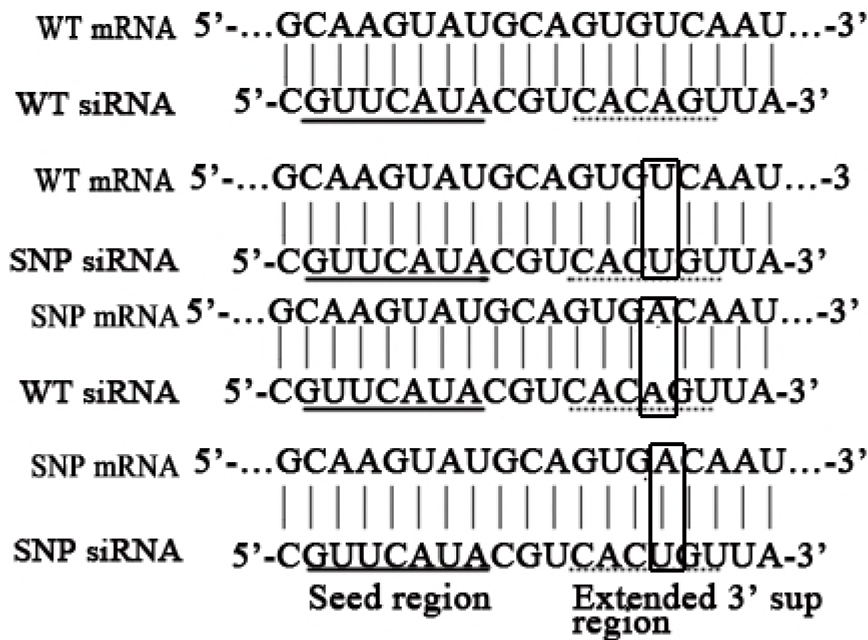

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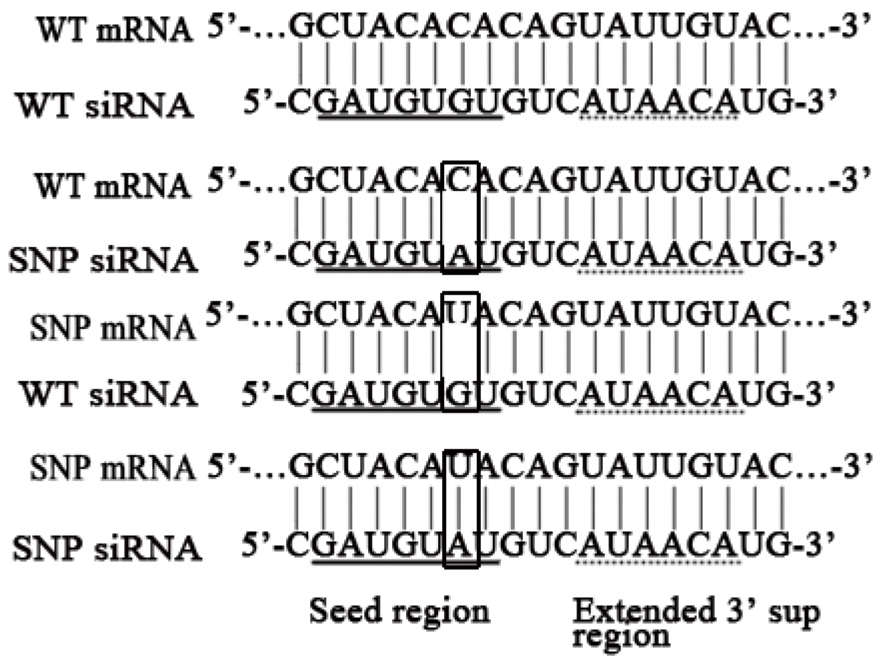

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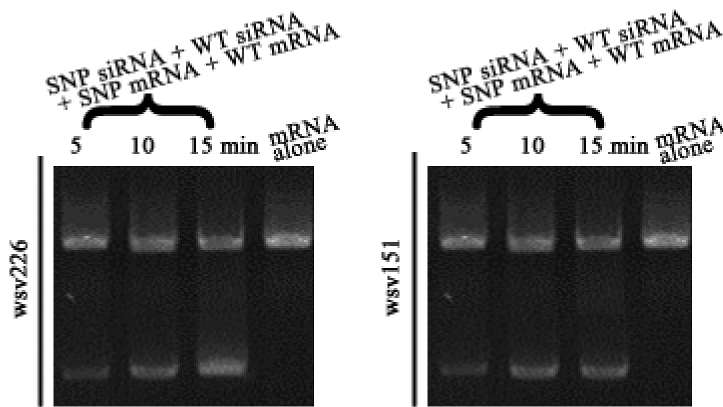

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650 Fig 3

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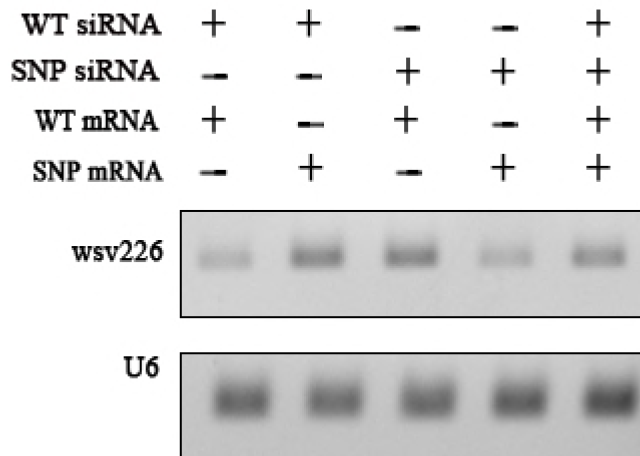

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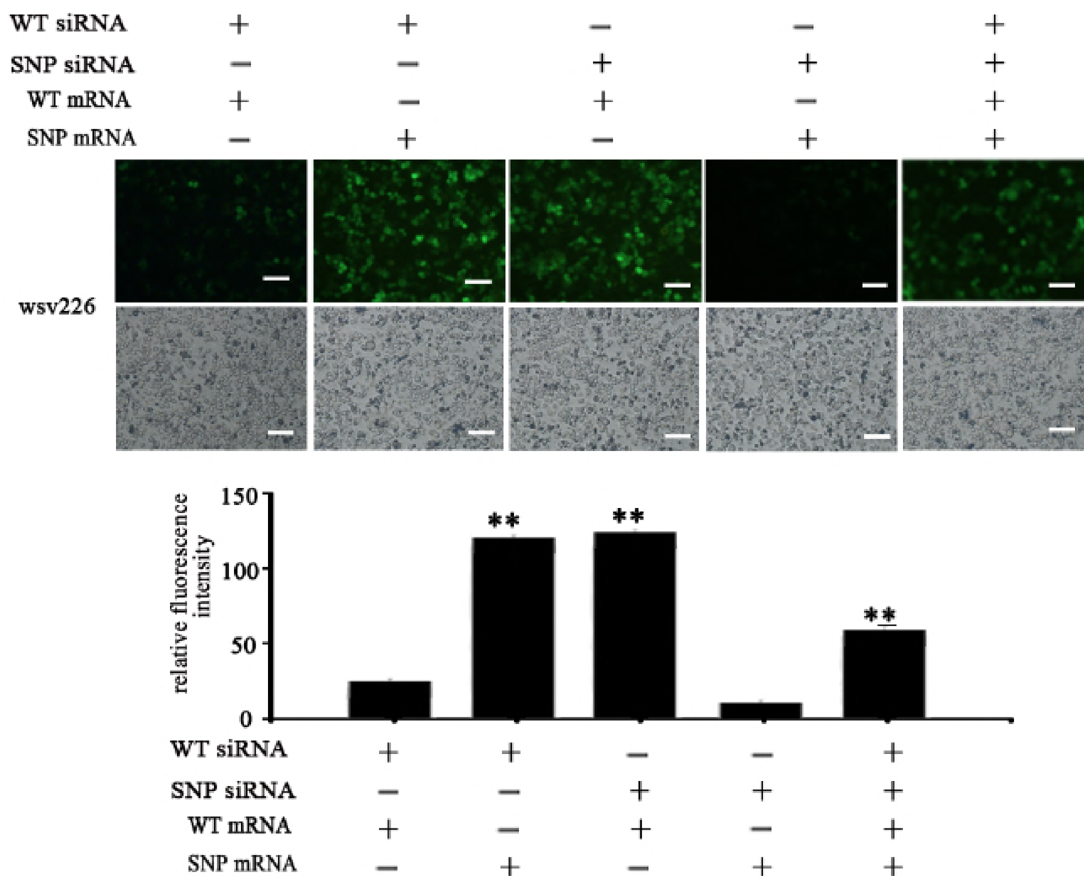

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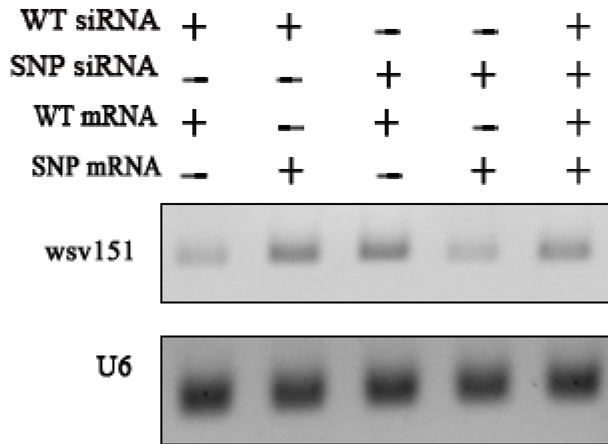

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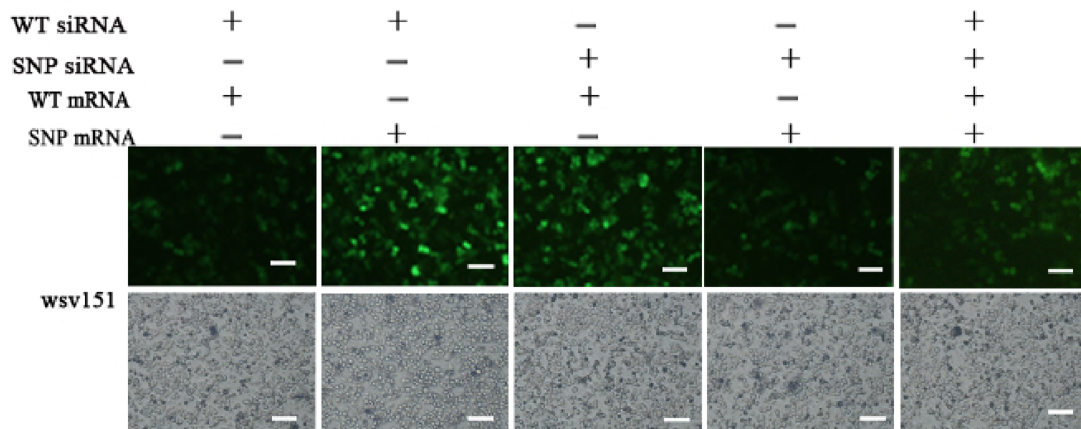
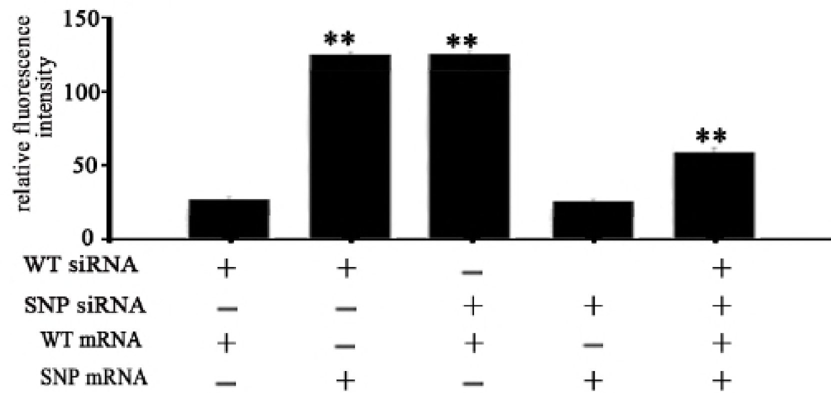
657 D





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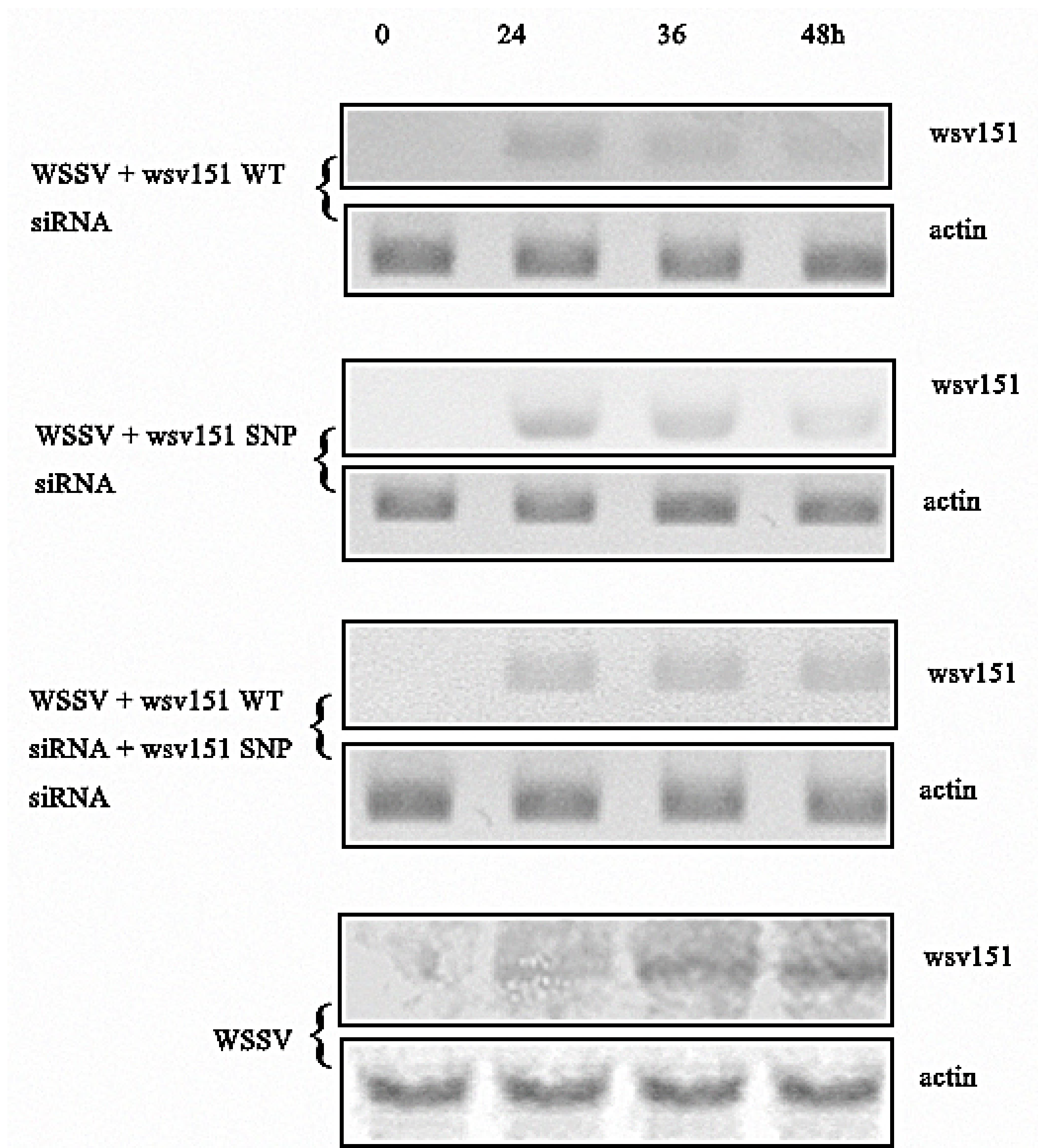
664 Fig 4

665 A



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667 B

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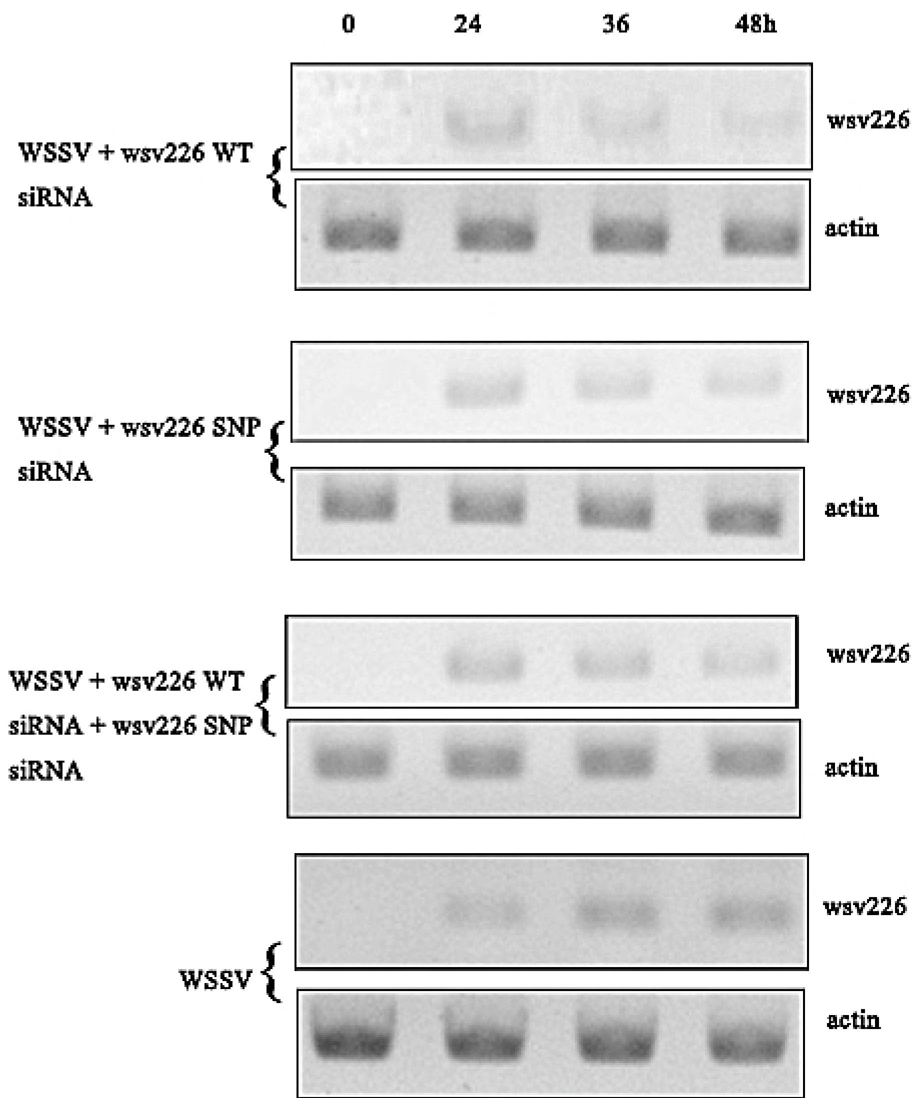

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669 C

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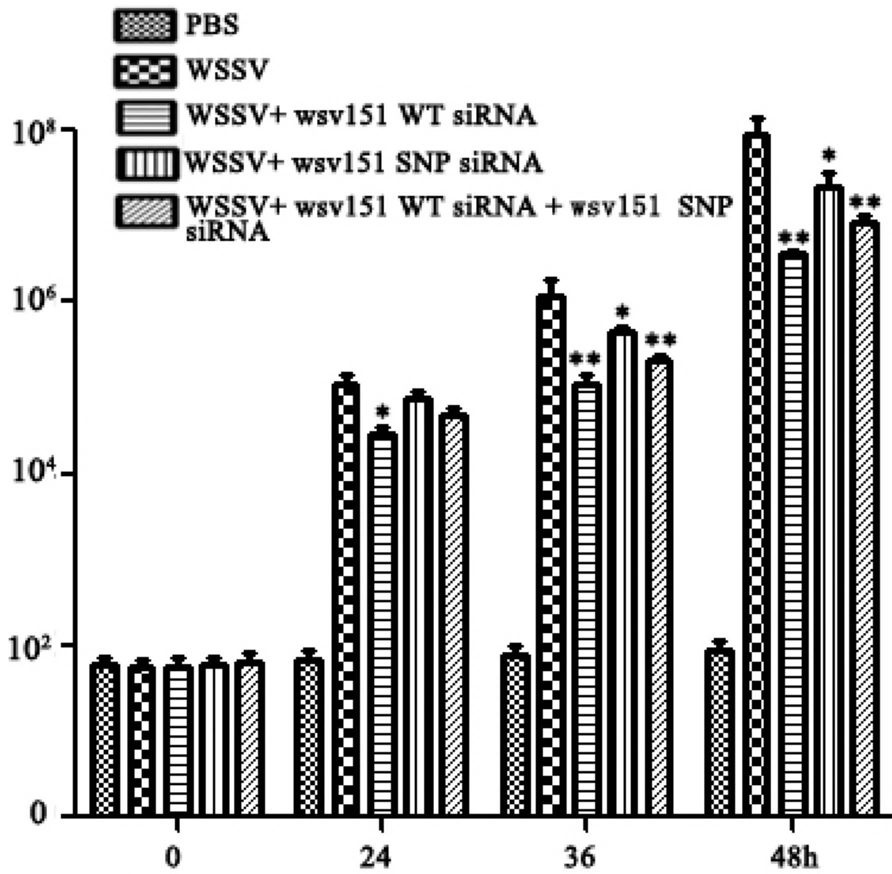

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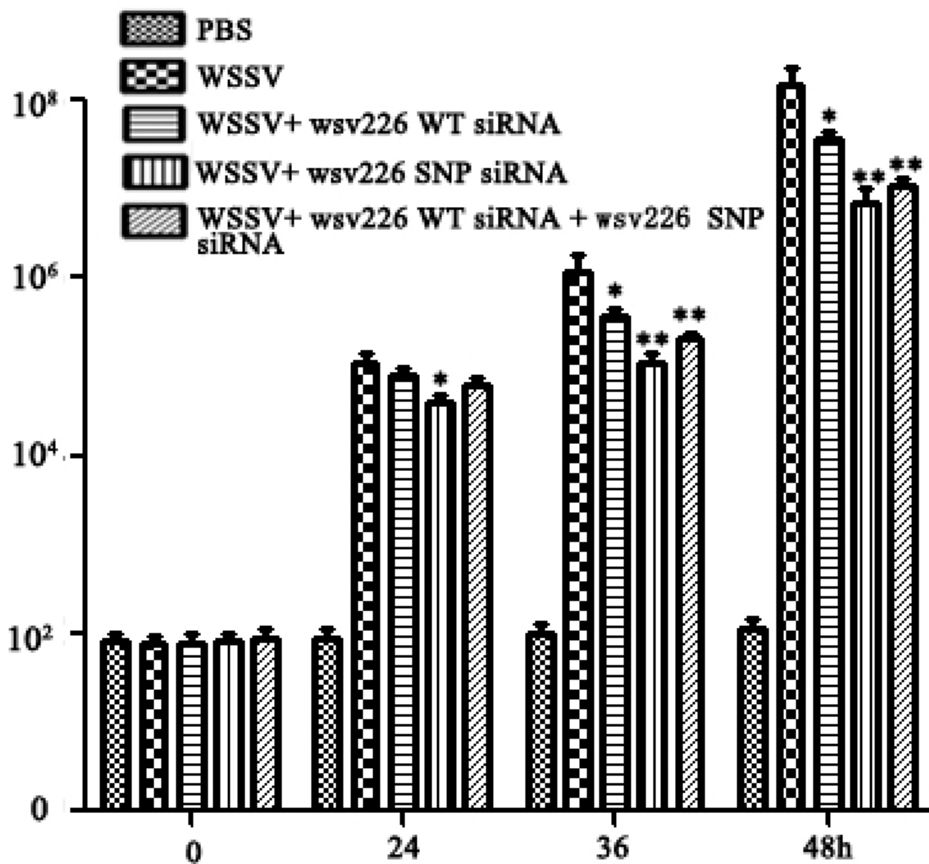

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