

Functional analysis of *Trichinella spiralis* serine protease 1.2 by siRNA mediated RNA interference

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Received 2 March 2019; received in revised form 16 November 2019; accepted 20 December 2019

Abstract. A *T. spiralis* serine protease 1.2 (TsSP1.2) was identified in the muscle larvae (ML) and intestinal larvae surface/excretory–secretory (ES) proteins by immunoproteomics. The aim of this study was to determine the TsSP1.2 function in the process of *T. spiralis* intrusion, growth and reproduction by using RNA interference (RNAi). RNAi was used to silence the expression of TsSP1.2 mRNA and protein in the nematode. On 2 days after the ML were electroporated with 2 μ M of TsSP1.2-specific siRNA 534, TsSP1.2 mRNA and protein expression declined in 56.44 and 84.48%, respectively, compared with untreated ML. Although TsSP1.2 silencing did not impair worm viability, larval intrusion of intestinal epithelium cells (IEC) was suppressed by 57.18% ($P < 0.01$) and the suppression was siRNA-dose dependent ($r = 0.976$). Infection of mice with siRNA 534 transfected ML produced a 57.16% reduction of enteral adult burden and 71.46% reduction of muscle larva burden ($P < 0.05$). Moreover, silencing of TsSP1.2 gene in ML resulted in worm development impediment and reduction of female fertility. The results showed that silencing of TsSP1.2 by RNAi inhibited larval intrusion and development, and reduced female fecundity. TsSP1.2 plays a crucial role for worm invasion and development in *T. spiralis* life cycle, and is a potential vaccine/drug target against *Trichinella* infection.

INTRODUCTION

Trichinellosis, a major important foodborne zoonosis in the world, is resulted mainly from the tissue-dwelling nematode *Trichinella spiralis* (Murrell and Pozio, 2011). Human *Trichinella* infection is primarily acquired by means of ingesting raw or semi-cooked meat contained infective larvae of this parasite. In China, 12 human trichinellosis outbreaks due to contaminated pork were reported during 2004–2009 (Cui *et al.*, 2011). Domestic porcine is the principal infection source for human trichinellosis in developing countries (Cui *et al.*, 2013b; Jiang *et al.*, 2016; Rostami, 2017). *Trichinella* infection is not only a crucial public health issue, but also a great harm to animal meat safety (Cui and Wang, 2011; Bai *et al.*, 2017).

After being ingested, the muscle larvae (ML) encapsulated in animal meat are liberated in the host's stomach, grow into intestinal L1 larvae (IL1) in intestines 0.9 h post infection. The IL1 larvae intrude into enteral epithelium and grow into adult worms (AW) following four molts (Ren *et al.*, 2011; Liu *et al.*, 2013). However, the mechanism of enteral mucosal penetration by *T. spiralis* is unclear (Song *et al.*, 2018; Xu *et al.*, 2018). Furthermore, it is still hard to control *Trichinella* infection because of the broad distribution of animal reservoirs and scarce of anti-*Trichinella* vaccines (Qi *et al.*, 2018a; Zhang *et al.*, 2018). Although benzimidazole derivatives (i.e., albendazole, flubendazole, mebendazole, and thiabendazole) have most commonly been used for treating human trichinellosis, their effectiveness

is strongly dependent upon the time of administration, these anthelmintics may be useless against chronic trichinellosis. Additional, mebendazole is teratogenic in rat, it is contraindicated for pregnant women and children under two years old (Gottstein *et al.*, 2009). These issues have resulted in the screening and characterizing of *Trichinella* invasive molecules as a potential target for vaccines/drugs against intestinal stage worms of this nematode (Wang *et al.*, 2012b; Ren *et al.*, 2018).

Serine proteases are a family of enzymes that degrade wide range of proteins and exert the crucial roles in life cycle of parasites. The proteolytic enzymes participated in worm penetration, molt, digestion and degradation, and growth development (Yang *et al.*, 2015b). In parasitic nematodes, it has been known that serine proteases are involved in invasion of host tissues and in nematode molting (Dzik, 2006; Nagano *et al.*, 2003). Mouse intestinal mucus barrier and mucin Muc2 could be hydrolyzed and destroyed by *Trichuris muris* serine proteases (Hasnain *et al.*, 2012). A variety of serine proteases were identified from soluble or ES proteins of various *T. spiralis* stages (Robinson *et al.*, 2005; Wang L *et al.*, 2013c; Wang *et al.*, 2017). While the IL1 penetrated into intestinal epithelial cells (IEC), serine protease expression at IL1 stage was upregulated relative to ML stage (Ren *et al.*, 2013). Antibodies against *T. spiralis* serine proteases (TspSP1 and TspSP1.2) suppressed worm intrusion of IEC (Romaris *et al.*, 2002; Wang B *et al.*, 2013). The results suggested that serine protease might perform a principal role for worm intruding, growing and parasiting in hosts, but its exact function remains to be verified. A serine protease 1.2 of *T. spiralis* (TsSP1.2, GenBank No. EU302800) was found in the ML and IL1 larva surface/excretory–secretory (ES) proteins by immunoproteomics (Cui *et al.*, 2013a; Liu *et al.*, 2015, 2016a; Wang *et al.*, 2014). The TsSP1.2 encoded a 35.5 kDa protein that is a trypsin-like serine protease with an active site of proteolysis. The TsSP1.2 was cloned and identified in our laboratory. Expression of TsSP1.2 was observed at various worm stages and was a cuticle/secretory protein

of this parasite nematode. The *in vitro* *T. spiralis* intrusion of IEC was suppressed by using anti-TsSP1.2 antibodies, vaccination of mice with rTsSP1.2 protein or DNA exhibited an obvious worm burden reduction of adult worm (AW) and ML after larva challenge (Wang B *et al.*, 2013; Li *et al.*, 2018). Though the TsSP1.2 had the immune protection against *Trichinella* infection and a promise as a potential target for the anti-*Trichinella* vaccine, the precise functions of TsSP1.2 in *T. spiralis* life cycle is unclear.

This study aimed to determine the TsSP1.2 roles in the process of *T. spiralis* intrusion, growth and reproduction with the aid of RNA interference (RNAi) method.

MATERIALS AND METHODS

Worm and experimental animal

T. spiralis isolate (ISS534) from a swine of central China was kept by mouse serial passage in our laboratory (Wang *et al.*, 2012a). BALB/c mice (six weeks old female) were purchased from animal center of Zhengzhou University. All animal experimental procedure was approved by the Institutional Life Science Ethics Committee of Zhengzhou University, which is located in central China (No. SCXK 2015–0005).

Synthesis of small interference RNA (siRNA)

Three TsSP1.2-specific siRNAs, siRNA 534 (5'-CUAUUCAUACCAAGAUUATT-3'), siRNA 818 (5'-AUGUUGGUAUCUAUUCAATT-3') and siRNA 303 (5'-UUCAUCACAUUGUGUUAGATT-3') were designed by using siDirect version 2.0 as previously described (Yang *et al.*, 2019). These siRNAs were synthesized by Sangon Biotech (Shanghai, China). Another siRNA with a scrambled sequence (5'-UACAUGCUCGCAAUAAUCAAATT-3') was conjugated with carboxy-fluorescein (FAM) and served as a control of specificity (Sangon Biotech) (Wang *et al.*, 2015).

RNAi treatment of the ML

The ML were collected by artificial digestion of infected mouse muscles at 35 days post

infection (dpi) (Li *et al.*, 2010; Jiang *et al.*, 2012). Transfection of the siRNA to the ML was performed by electroporation (Wang *et al.*, 2015). Briefly, 5000 ML were suspended in 100 μ l of buffer containing 2 μ M siRNA. The electroporation was conducted at 800 V, 25 μ F and 200 Ω using a Gene pulser II System (Bio-Rad, USA), then supplemented with 400 μ l of RPM1640 culture medium containing 25 mM HEPES, 100 μ g/ml streptomycin and 100 U/ml penicillin, and cultivated at 37°C and 5% CO₂ for 1-8 days.

RNA extraction and quantitative PCR (qPCR) amplification

Total RNA from siRNA-transfected ML was isolated using Trizol reagent (Invitrogen, USA), subsequently transcribed into the first-strand cDNA with PrimeScript RT reagent Kit (TaKaRa, Japan) (Ren *et al.*, 2013). The TsSP1.2 mRNA expression level was assessed using qPCR with SYBR Premix Ex Taq (Takara) (Long *et al.*, 2015). Specific primers for qPCR amplifying TsSP1.2 gene were 5'-GTTACGGCCATGCTGGAAAG-3', 5'-CACCGGCTACTGCTGATCTT-3'. *T. spiralis* GAPDH gene (GenBank no. AF452239) was utilized as a house-keeping gene (Chen *et al.*, 2012), and the TsSP1.2 mRNA expression level was calculated using comparative Ct ($2^{-\Delta\Delta Ct}$) method (Liu *et al.*, 2013). Three independent tests were conducted and three replicates were assayed for each sample.

Worm protein preparation and Western blotting

Soluble proteins of siRNA-transfected ML were prepared as reported (Wang L *et al.*, 2013a; Cui *et al.*, 2015). Ten micrograms of worm proteins/lane was separated using SDS-PAGE, subsequently blotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) at 18 V for 35 min (Li *et al.*, 2015; Xu *et al.*, 2017). The membrane was incised into strips, blocked using 5% skim milk in TBST at 4°C overnight (Sun *et al.*, 2019), and probed at 37°C for 1 h with 1:100 dilutions of anti-TsSP1.2 antibody which was prepared in our laboratory (Li *et al.*, 2018). After washes, the strip was incubated with

1:10000 dilutions of anti-mouse IgG-HRP conjugate (Sigma-Aldrich, USA). The GAPDH expression was determined using mouse anti-GAPDH antibody diluted at 1:1000. Finally, coloration was detected by the enhanced chemiluminescent reagent (CW BIO, Beijing, China) (Hu *et al.*, 2014; Wang *et al.*, 2014). The intensities of TsSP1.2 protein expression were analyzed with Image J software (Sun *et al.*, 2018b).

Assessment of worm viability in the medium after siRNA treatment

The ML were transfected using 2 μ M of siRNA 534 and cultured in RPMI-1640 medium at 37°C and 5% CO₂ for 7 days. Viability of the parasite treated with siRNA was assessed in line with worm morphology and motility under the microscope. The live worms are mobile and show wriggling action, whereas the dead worms are straight and not active (Cui *et al.*, 2015; Sun *et al.*, 2018a). The result was presented as percent of dead worms to all worms tested in each group.

***In vitro* larva intrusion test**

To observe the effect of RNAi treatment on *in vitro* larval intrusion of IEC, the ML were transfected using 1, 1.5, 2, 2.5 and 3 μ M of siRNA 534 and then cultivated for 18 h. The siRNA 534 treated ML were activated into the IL1 larvae using 5% mouse bile at 37°C for 2 h, and utilized for larva intrusion test (Liu *et al.*, 2018; Wang L *et al.*, 2013b). The IEC were cultivated in 6-well culture plates and cell monolayer was covered with 2 ml of DMEM semisolid medium supplemented with 100 IL1 larvae (Ren *et al.*, 2011). After incubation for 2 h at 37°C, the IL1 intruded the IEC monolayer were numbered on microscopy. The parasites that intruded and moved in the monolayer were taken as the intruded parasites, whereas the parasite that remained in the medium and coiled were regarded as non-intruded (Long *et al.*, 2015; Yang *et al.*, 2015a). Three independent tests for five groups of the IL1 larvae were performed and each group has three replicates to determine the worm invasion rate (Qi *et al.*, 2018b).

Challenge experiment

To investigate the ability of siRNA treated IL1 larvae to intrude and grow, 60 mice were divided into three groups (20 mice per group). Each group was orally inoculated by 300 IL1 larvae transfected using siRNA 534, control siRNA or PBS. Ten mice of each group were killed at 6 days post infection (dpi), and AW were recovered from infected mouse intestine and counted (Cui *et al.*, 2013c; Liu *et al.*, 2016b). The remaining 10 mice from each group were sacrificed at 35 dpi, and the ML were collected by artificial digestion of mouse carcasses (Jiang *et al.*, 2012). The parasite burden reduction was assessed in accordance with mean AW number and larvae per gram (LPG) of muscles of siRNA 534 group relative to that of PBS group (Gu *et al.*, 2017; Liu *et al.*, 2017). The female fertility was evaluated based on *in vitro* newborn larvae (NBL) production from each female for 72 h (Liu *et al.*, 2015; Qi *et al.*, 2018a). The length of AW, NBL and ML was measured under the microscope.

Statistical analysis

The data was analyzed by SPSS 17.0 software, and shown as means \pm standard deviation (SD). Differences of TsSP1.2 mRNA and protein expression level, parasite burden and length, and NBL production among various

groups was compared with one-way ANOVA. $P < 0.05$ was served as statistically significant. Samples have three replicates and the data are represented as mean \pm SD. * $P < 0.05$ compared with PBS group.

RESULTS

Reduction of TsSP1.2 mRNA and protein and expression in ML treated with siRNA

Compared with that of ML treated with PBS, transfection of ML with 2 μ M of siRNA 534, 818 and 303 decreased 57.05, 57.37 and 27.18% of TsSP1.2 mRNA expression level, respectively (Figure 1a); TsSP1.2 protein expression level of treated ML inhibited 69.65%, 68.97% and 37.09%, respectively (Figure 1b). Nevertheless, the control siRNA had no significant inhibition on TsSP1.2 mRNA and protein expression.

When the ML were electroporated with 1, 2 and 3 μ M of siRNA 534, the TsSP1.2 mRNA expression level reduced 44.29%, 53.81% and 79.16%, respectively ($P < 0.05$) (Figure 2); TsSP1.2 protein expression level inhibited 35.83%, 44.05% and 63.28%, respectively, compared with that of PBS group ($P < 0.05$). Although better transcription knockdown was observed when using 3 μ M siRNA concentration, 2 μ M siRNA 534 was used

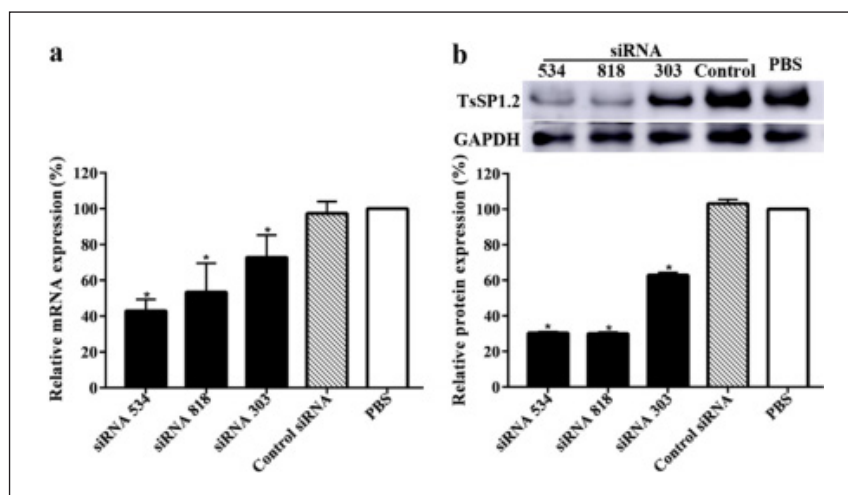


Figure 1. Relative mRNA and protein and expression of TsSP1.2 in *T. spiralis* ML transfected with siRNA. **a:** qPCR analysis of TsSP1.2 mRNA expression level in ML treated with three siRNAs. **b:** Western blot analysis of TsSP1.2 protein expression level in ML treated with three siRNAs. * $P < 0.05$ compared with the PBS group.

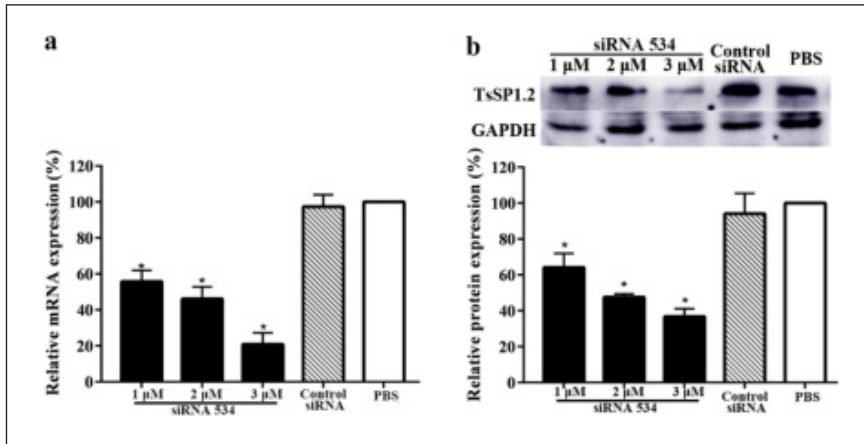


Figure 2. The mRNA and protein expression levels of TsSP1.2 in *T. spiralis* ML treated with various dose of siRNA 534. **a**: The mRNA expression levels of TsSP1.2 assayed by qPCR. **b**: TsSP1.2 protein expression level assayed by Western blotting. * $P < 0.05$ compared with PBS group.

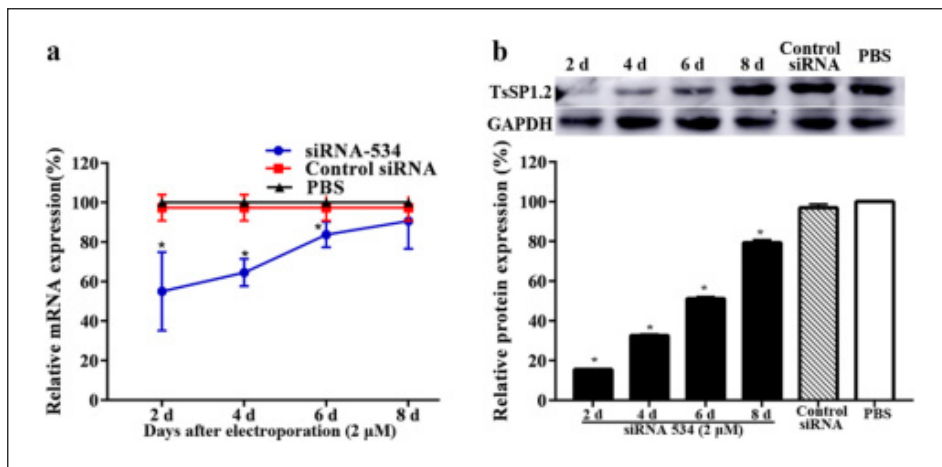


Figure 3. The mRNA and protein expression levels of TsSP1.2 at different times after transfection with 2 μM of siRNA-534. **a**: TsSP1.2 mRNA expression level assayed by qPCR. **b**: TsSP1.2 protein expression level assayed by Western blotting. * $P < 0.05$ compared with PBS group.

in the following experiments in order to reduce its cytotoxicity effect and to make the larvae to survive long.

At 2, 4, 6 and 8 days after transfection with 2 μM of siRNA 534, the TsSP1.2 mRNA expression level inhibited 56.44, 35.46, 16.27 and 9.31%, respectively (Figure 3); The TsSP1.2 mRNA expression level at 2, 4 and 6 days was obviously lower than that of PBS group ($P < 0.05$); TsSP1.2 protein expression level suppressed 84.48, 67.35, 48.83 and 20.62%, respectively, compared

with PBS group ($P < 0.05$). The TsSP1.2 mRNA expression at different times after electroporation with control siRNA had no significant difference ($P > 0.05$).

Worm viability in medium after siRNA treatment

When the worms treated by siRNA 534, control siRNA and PBS were cultured at 37°C for 7 days, the worm death rate of three groups was 37.03, 37.60 and 40%, respectively ($\chi^2 = 0.227$, $P > 0.05$), suggesting

that silencing of TsSP1.2 by siRNA did not impair the worm viability.

Suppression of larval intrusion of IEC by siRNA 534

When the IEC monolayer was covered with siRNA 534-treated IL1 larvae and incubated for 2 h, the larvae intruded the monolayer and migrated in the monolayer (Figure 4). The inferring of TsSP1.2 with siRNA534 reduced larva intrusion of IEC. The intrusion rate of the IL1 transfected with 1, 1.5, 2, 2.5 and 3 μM siRNA-534 was 65.92, 58.64, 42.82, 38.66 and 32.88%, respectively. Compared to PBS group, intrusion rate of larvae treated with three concentration (2, 2.5 and 3 μM) of siRNA 534 suppressed 57.18, 61.34, and 67.12%, respectively ($\chi^2_{2\mu\text{M}} = 11.095$, $\chi^2_{2.5\mu\text{M}} = 14.892$, $\chi^2_{3\mu\text{M}} = 23.878$, $P < 0.01$). The suppressive effect was dose-dependent of siRNA 534 ($r = 0.976$), showing an increasing trend with the elevation of siRNA 534 dose ($F = 61.430$, $P < 0.01$). However, no remarkable

suppression on larva intrusion was detected when control siRNA was used.

Inhibition of siRNA 534 on *in vivo* larval infectivity and growth

Inoculation of mice with ML treated using siRNA 534 resulted in a 57.16% AW reduction and 71.46% ML reduction relative to that of PBS treated ML ($F_{\text{adults}} = 162.493$, $F_{\text{larvae}} = 35.530$, $P < 0.001$); but inoculation of mice with ML treated with control siRNA did not show significant parasite burden decrease of AW and ML (Figure 5) ($F_{\text{adults}} = 0.088$, $F_{\text{larvae}} = 0.029$, $P > 0.05$). Furthermore, the ML length of siRNA 534 group was markedly smaller than those of control siRNA or PBS group ($F = 60.914$, $P = 0.000 < 0.001$).

Female fertility and length of NBL and ML

Length of female and male adults recovered from mice infected with siRNA 534-transfected ML distinctly reduced in

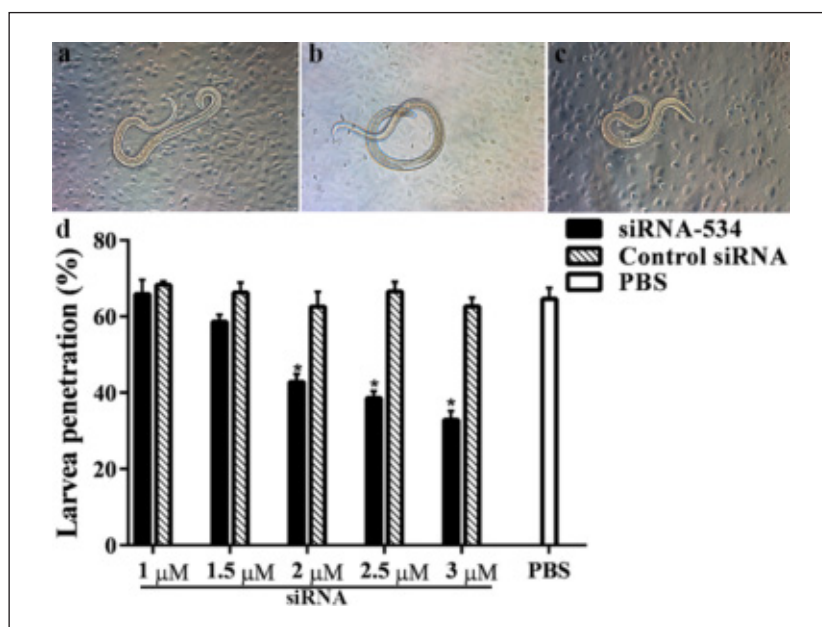


Figure 4. Suppression of siRNA-534 on larva intrusion of IEC. The IL1 larvae were inoculated onto the IEC monolayer and larva intrusion of IEC was investigated on microscopy at 2 h after incubation (200 \times). **a**: siRNA 534-transfected larva intruded the monolayer. **b**: siRNA 534 transfected larva did not intrude the monolayer. **c**: PBS treated larva penetrated the monolayer. **d**: Suppression of larval intrusion of IEC by siRNA 534. The result is expressed as percent of intruded worms to all worms observed in each experiment. * $P < 0.05$ compared with PBS group.

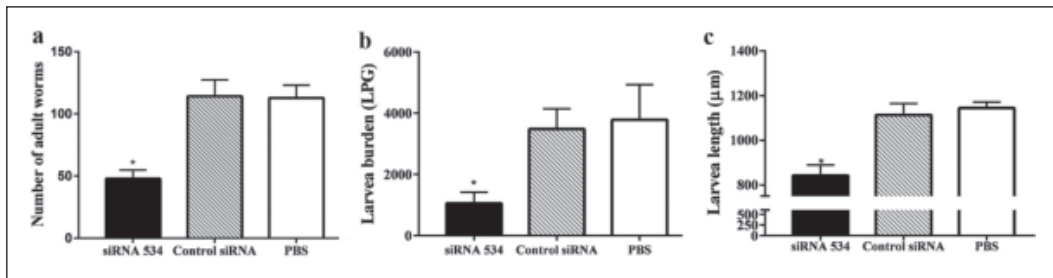


Figure 5. The worm burdens from mice challenged by ML treated with siRNA 534. Parasite burden is shown as mean \pm SD from each group (n=10). **a:** AW burden; **b:** ML burden; **c:** ML length. * $P < 0.05$ compared with PBS group.

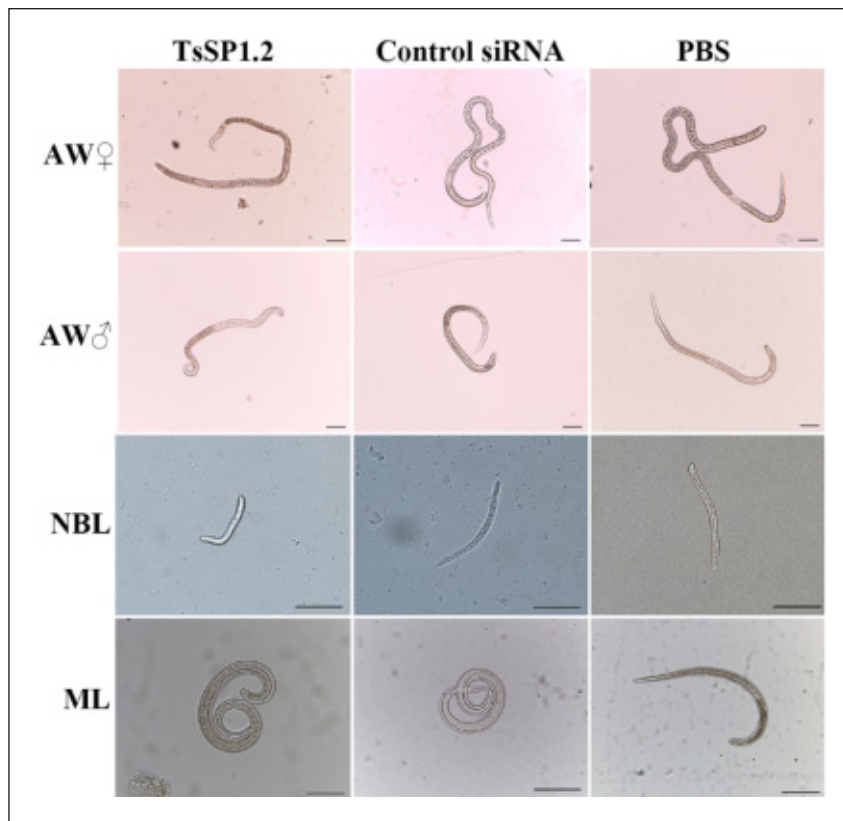


Figure 6. Morphology of different *T. spiralis* stages collected from mice infected with ML transfected with siRNA 534. Scale bar = 100 μ m.

comparison with PBS group ($F_{fAW} = 50.965$, $P < 0.001$, $F_{mAW} = 6.205$, $P < 0.05$) (Figure 6, 7). The *in vitro* NBL production of females from mice infected with siRNA 534-treated ML was significantly lower than those from control siRNA or PBS group (Figure 7c) ($F = 39.547$, $P < 0.001$). Also, the NBL length of siRNA 534 group was also distinctly less

than that of control siRNA or PBS group (Figure 7d) ($F = 57.836$, $P < 0.0001$). Our suggested that interference of TsSP1.2 with siRNA 534 impaired the larval infectivity, invasive and grow development capacity, reduced fecundity of female adults, as a result, lead to burden reduction of AW and ML in challenged mice.

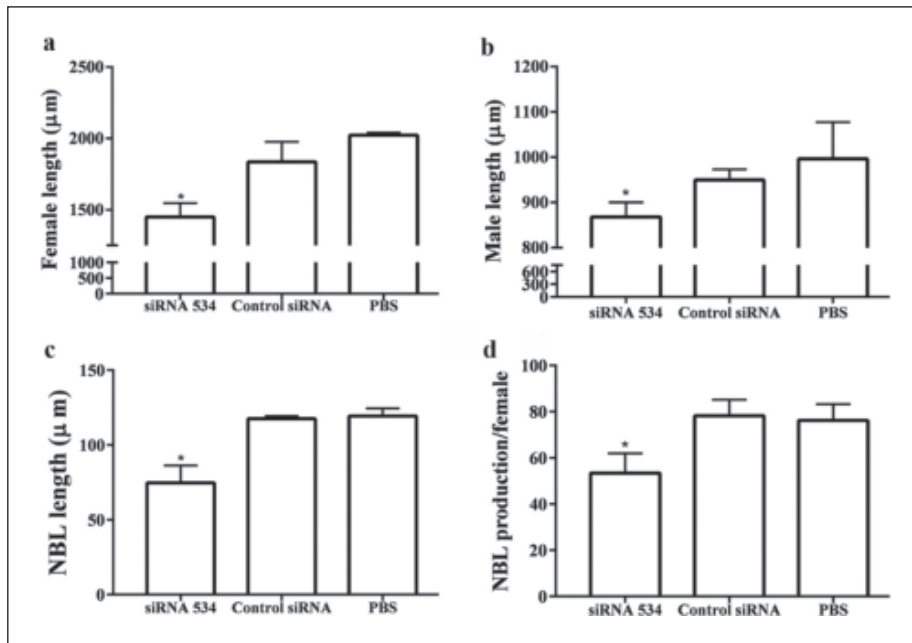


Figure 7. Length of different *T. spiralis* stages and female fecundity from mice infected with ML treated with siRNA 534 (n=10). a: Females; b: Males; c: Newborn larvae (NBL); d: NBL production/female.

DISCUSSION

RNAi is a post-transcriptional gene silencing technique and widely applied for the gene function identification of parasites because this technique degrades RNA in sequence-specific mode. RNAi has been used to screen and identify of specific candidate vaccine/drug targets against parasites (Bartz and Jackson, 2005), such as *Clonorchis sinensis* (Wang *et al.*, 2014), *Brugia malayi* (Singh *et al.*, 2012; Kushwaha *et al.*, 2012) and *Setaria digitata* (Somaratne *et al.*, 2018). In zoonotic medical nematode *T. spiralis*, the gene function of paramyosin and nudix hydrolase for viability and infectivity has been assessed through RNAi method (Chen *et al.*, 2012; Zhang *et al.*, 2016). The *T. spiralis* draft genome has been published in 2011 and contains 15,808 protein coding genes (Mitreva *et al.*, 2011), but the functions of only partial genes have been certified until now (Nagano *et al.*, 2009).

In the present study, the biological function of TsSP1.2 during the process of *T. spiralis* infection was assessed by using RNAi technique. Better transcription knock

down was observed when using siRNA 534. Therefore, siRNA 534 was chosen to continue further testing in this study. On 2 days after the ML were electroporated by 2 μM of siRNA 534, TsSP1.2 mRNA and protein expression declined in 56.44 and 84.48%, respectively, indicating that expression of TsSP1.2 mRNA and protein was suppressed by specific siRNA. Although silencing of TsSP1.2 gene did not impair the *in vitro* worm viability, significantly reduced the worm intrusion of IEC, and the intrusion suppression was siRNA-dose dependent, suggesting that silencing of TsSP1.2 by RNAi reduced the capacity of infective larvae to intrude the IEC. Previous studies revealed extracellular serine protease activity of *Acanthamoeba* was inhibited, and the encystation obviously reduced following treatment with siRNA (Lorenzo-Morales *et al.*, 2005; Moon *et al.*, 2008). RNAi-mediated silencing of a trypsin-like serine-protease from *Spodoptera frugiperda* reduces susceptibility to Cry1Ca1 protoxin of *Bacillus thuringiensis* (Rodríguez-Cabrera *et al.*, 2010). The silencing of urokinase with siRNA suppresses the invasion and migration of

hepatocellular carcinoma cells (Salvi *et al.*, 2004).

Our results also revealed that infection of mice with siRNA 534- transfected ML exhibited a 57.16% AW reduction and 71.46% ML reduction compared with that of mice infected with PBS treated ML. Following the silencing of TsSP1.2 gene in ML, the worm growth, development and female fecundity were significantly impaired as demonstrated that larval developmental impediment (smaller adults), and the decline of female fecundity relative to that from control siRNA and PBS group (as shown in Figure 6 and 7). Moreover, the length of NBL released from females of mice infected with siRNA 534-treated ML was also significantly shorter than that of control siRNA or PBS group. The results demonstrated that interfering of TsSP1.2 with specific siRNA prominently reduced the capacity of the worm to intrude and develop in host. Besides, the decline of adult female fertility is connected with subsequent ML burden reduction in mice infected with siRNA 534 treated larvae, because the uterus length is positively correlated with female fertility, i.e., the shorter the female uterus, the lower female fertility (Murrell *et al.*, 2000; Liu *et al.*, 2015). Our results further verified that TsSP1.2 plays a crucial role for worm invasion and development in *T. spiralis* life cycle, and is a potential vaccine/drug target against *Trichinella* infection.

Conclusively, the results showed that interfering of TsSP1.2 by siRNA impaired *T. spiralis* intrusion and growth development, and reduced the female fertility.

Acknowledgements. This work was supported by the National Natural Science Foundation of China (No. U1704284, 81572024).

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