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Trichinella spiralis: RNAi-mediated silencing of serine protease results in reduction of intrusion, development and fecundity

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Abstract. In previous studies, a *Trichinella spiralis* serine protease (TsSP) was identified in excretion/secretion (ES) products from intestinal infective L1 larvae (IIL1) using immunoproteomics. The complete cDNA sequence of TsSP gene was 1372 bp, which encoded 429 amino acids with 47.55 kDa. The TsSP was transcribed and expressed at all *T. spiralis* life cycle phases, as well as mainly located at the cuticle and stichosome of the parasitic nematode. Recombinant TsSP bind to intestinal epithelial cells (IEC) and promoted larva invasion, however, its exact function in invasion, development and reproduction are still unknown. The aim of this study was to confirm the biological function of TsSP during *T. spiralis* invasion and growth using RNA interference (RNAi) technology. The results showed that on 1 day after electroporation using 2.5 μM siRNA156, TsSP mRNA and protein expression of muscle larvae (ML) was suppressed by 48.35 and 59.98%, respectively. Meanwhile, silencing of TsSP gene by RNAi resulted in a 61.38% decrease of serine protease activity of ML ES proteins, and a significant reduction of the *in vitro* and *in vivo* invasive capacity of IIL1 to intrude into the IEC monolayer and intestinal mucosa. When mice were infected with siRNA 156-transfected larvae, adult worm and muscle larva burdens were decreased by 58.85 and 60.48%, respectively. Moreover, intestinal worm growth and female fecundity were evidently inhibited after TsSP gene was knockdown, it was demonstrated that intestinal adults became smaller and the *in vitro* newborn larval yield of females obviously declined compared with the control siRNA group. The results indicated that knockdown of TsSP gene by RNAi significantly reduced the TsSP expression and enzymatic activity, impaired larvae intrusion and growth, and lowered the female reproductive capacity, further verified that TsSP might participate in diverse processes of *T. spiralis* life cycle, it will be a new prospective candidate molecular target of anti-*Trichinella* vaccines.

INTRODUCTION

Trichinella spiralis (Owen, 1835) is a major enzootic nematode distributed in more than 150 mammals around the world (Pozio, 2007). Humans acquire trichinellosis by consuming raw or semi-raw meat or meat products infected with *Trichinella* muscle larvae (ML) (Cui *et al.*, 2011). *T. spiralis* not only harms human health, but also poses a serious threat to meat food safety (Cui & Wang, 2011; Cui *et al.*, 2013). At the same time, *T. spiralis* is ranked as the seventh more important foodborne parasitic disease in the world (FAO/WHO, 2014). It is an important public health issue on *Trichinella* infection in domestic pigs and a tremendous risk for pork and pork product safety (Rostami, 2017). Due to the widespread distribution of *Trichinella* animal hosts and the lack of practical prophylactic vaccines (Liu *et al.*, 2015a; Qi *et al.*, 2018a), it is therefore difficult to control *Trichinella* infection (Jiang *et al.*, 2016; Bai *et al.*, 2017). These

problems prompted the identification of *T. spiralis* invasion-associated proteins as molecular targets for anti-*Trichinella* vaccines (Wang *et al.*, 2012b; Song *et al.*, 2018a; Xu *et al.*, 2018).

Serine protease is an important family of proteases, which play an important role in host cell and tissue invasion, worm development, anticoagulation and immune evasion (Yang *et al.*, 2015). There are more and more researches on serine proteases of parasitic nematodes, mainly including *Trichuris muris*, *Ascaris lumbricoides*, *Brugia malayi*, and *T. spiralis* (Cui *et al.*, 2015). Some serine proteases have been identified from different *T. spiralis* life cycle stages (Robinson *et al.*, 2005; Wang *et al.*, 2013a, 2013b). Furthermore, serine protease expression at intestinal infective L1 larvae (IIL1) was higher than the muscle larval stage (Ren *et al.*, 2013; Liu *et al.*, 2013, 2015b). Antibodies against *T. spiralis* serine proteases (TspSP1 and TspSP1.2) inhibited the enterocyte invasion (Romaris *et al.*, 2002; Wang *et al.*, 2013c). Vaccination of mice with recombinat (rTsSP) protein or DNA vaccine exhibited a significant immune protection, as demonstrated by an obvious worm burden reduction of enteral adult worms (AW) and mucle larvae (ML) in vaccinated mice after challenge infection (Li *et al.*, 2018; Ren *et al.*, 2018). The results demostrated that serine proteases may extert a main act for *T. spiralis* invasion and development inside the host.

In our previous studies, a serine protease (TsSP, GenBank: ABY60762), which had been screened in excretion/secretion (ES) products from *T. spiralis* IIL1 and adults using immunoproteomics (Liu *et al.*, 2016a, 2016b; Wang *et al.*, 2017), was cloned, expressed and characterized in our laboratory (Sun *et al.*, 2018b). The results showed that the complete cDNA sequence of TsSP gene was 1372 bp and encoded 429 amino acids with 47.55 kDa. The TsSP was transcribed and expressed at all *T. spiralis* life cycle phases, it mainly located at the cuticle and stichosome of the parasitic nematode. The rTsSP could bind with intestinal epithelial cell (IEC) and promoted the larva invasion of IEC. Immunization with rTsSP elicited a significant immune response and protection effect, which hindered the worm development and decreased the female adult fecundity, resulted in a 71.1% adult reduction at 5 days post infection (dpi) and a 62.1% ML reduction at 42 dpi (Sun *et al.*, 2018a; 2019b). Although the TsSP is expected to be a prospective molecular target of anti-*Trichinella* vaccine, the exact role of TsSP in worm invasion, development and survival in host have not been well elucidated.

The aim of the present study was to confirm the biological function of TsSP during *T. spiralis* invasion, development and fecundity by using RNA interference (RNAi) technology. Three kinds of TsSP-specific small interfering RNA (siRNAs) were used to silence the expression of TsSP in *T. spiralis* ML. The TsSP enzyme activity and *in vitro* larval invasive capacity were observed after silencing TsSP. Moreover, the development, morphology and fecundity of siRNA-treated worms from the infected mice were also investigated.

MATERIALS AND METHODS

Worm and mice

The *T. spiralis i*solate (ISS534) was recovered from a naturally infected pig in Henan Province of China and kept by serial passage in BALB/c mice in our laboratory (Wang *et al.*, 2012a). BABL/c mice (female, 15-20 g) were obtained from the animal center of Zhengzhou University. Animal experiment was conducted according to the National Guidelines for Experimental Animal Welfare (Minister of Science and Technology, the People's Republic of China, 2006). Experiment protocols were approved by the Institutional Life Science Ethics Committee, Zhengzhou University (No. SCXK 2017–0001).

siRNA preparation

Complete cDNA encoding TsSP was utilized to design the siRNA sequences by using siDirect version 2.0 (Naito *et al.*, 2009). Synthesis of TsSP-specific 21 bp siRNAs

was done by Sangon Biotech (Shanghai, China). To select the better performance of TsSP-specific siRNA in silencing the TsSP mRNA expression and the time of transfection, three TsSP-specific siRNAs, siRNA-156 (5'-UUUCUGAACACUAAUCUU GTT-3'), siRNA-171 (5'-GAAAAG CGGGUAA UAGAAUTT-3'), and siRNA-436 (5'-UACAU AUUCUUUAAG CAUCTT-3') were used in the present study. A control siRNA carrying a scrambled sequence (5'-AUCGGCUACC AAGUCAUACTT-3') was served as a control. The control siRNA was fluorescently labeled with FAM (Sangon Biotech, Shanghai, China) and used to assess the transfection efficiency. The specific siRNA and polyclonal antibodies of *T. spiralis* glutathione S-transferase (TsGST) was also prepared and used for control of the specificity (Wang *et al.*, 2015; Yang *et al.*, 2019).

Delivery of siRNA into ML

The ML were obtained via artificial digestion of experimentally infected mouse muscles at 35 dpi (Jiang *et al.*, 2012). Following washes with electroporation buffer, 5000 fresh larvae were suspended in 100 µl of electroporation buffer contained 1 µM one siRNA of three TsSP-specific siRNAs (Zhang *et al.*, 2016). The worms were transfected by siRNA through electroporation, and cultivated in RPMI 1640 culture medium at 37°C for 1-7 days (Chen *et al.*, 2012).

qPCR

Total RNA from siRNA-transfected and control worms was extracted using Trizol reagent (Invitrogen), transcribed into the first-strand cDNA with PrimeScript RT reagent Kit (TaKaRa, Japan) (Ren *et al.*, 2013). The TsSPI mRNA expression levels were ascertained using qPCR (Song *et al.*, 2018b). The specific primers of qPCR amplifying TsSP gene were 5'-CAGAATTT CAGGAGGCTCTGTTG-3', and 5'-ACCACGT CCACCTTGATATGT-3'. A β-actin gene was used as a housekeeping gene control (Martinez *et al.*, 2001; Sun *et al.*, 2018a). Each sample has three repeats. The quantitative datum was analyzed by comparative Ct ($2^{-\Delta\Delta Ct}$) method (Liu *et al.*, 2018).

Western blotting of TsSP protein expression in siRNA-transfected ML somatic proteins

Somatic soluble worm proteins of siRNA-treated ML were prepared as reported before (Wang *et al.*, 2011; Li *et al.*, 2015). Worm proteins (10 µg/lane) were separated using SDS-PAGE, transferred onto poly-vinylidene difluoride (PVDF) membrane at 18V for 35 min *via* semidry transferred cell (Bio-Rad) (Song *et al.*, 2018c). The membrane was cut into strips, which were blocked for 2 h at 37 °C in TBST with 5% nonfat milk, and incubated for 1 h at 37°C with anti-TsSP antibodies (1:100) prepared in our previous study (Sun *et al.*, 2019a). After washing, the strips were incubated with HRP-conjugated-anti-mouse IgG (1:10000; Sigma-Aldrich, USA). β-actin expression was also determined by using anti-β-Actin antibody (1:1000). The strips were stained with an enhanced chemiluminescent kit (CWBIO, Beijing, China) (Wang *et al.*, 2014). The protein bands were analyzed with the AlphaView Software, and relative protein expression level was assessed based on densitometry (Sun *et al.*, 2018b).

Enzymatic activity assay of worm native serine protease after TsSP gene silencing

After being electroporated with 2.5 µM siRNA 156, the MLs were cultivated at 37°C in a 5% CO_2 for 24 h. The parasites were removed from the medium by centrifugation (100 × g for 5 min). The ES proteins were collected after the cultured ML (Zhang *et al.*, 2013). And then, 100 µl of treated-ML ES proteins co-incubated with 200 µl of 1 mg ml^{-1} azocasein (Sigma-Aldrich, USA) at 37°C for 60 min. Reactions were stopped by adding 10% trichloroacetic acid (TCA), the mixtures were left for 15 min and then centrifuged at 600 *g* for 10 min, and finally, 1 M NaOH was added to 1 ml of the supernatant. Absorbance at 440 nm was determined (Ramírez-Flores *et al.*, 2019). Every group had three independent repeats.

Zymography

SDS-PAGE gel containing gelatin (1 mg ml^{-1}) were used for zymography of *T. spiralis* ML somatic and ES proteins after larval

transfection with siRNA 156 (Lorenzo-Morales *et al.*, 2005). Briefly, 20 µl of somatic or ES proteins were loaded onto an SDS-PAGE gel containing gelatin (1 mg ml^{-1}). Following electrophoresis, SDS was removed by washing twice with 2.5% Triton solution, and the gel was incubated at 37°C for 36 h in 0.1 M Tris base buffer solution (pH 8.0) containing 2mM NaN_3. Then the gel was put into Coomassie Brilliant Blue R-250 Decolorizer for 3 h and scanned. The experiment was repeated three times.

The *in vitro* survival rate of siRNA-156 transfected ML

The ML were treated with 2.5 µM siRNA-156 and cultured in RPMI 1640 medium at 37°C and 5% CO_2 for 1-7 days. The survival rates of siRNA-156 transfected ML were evaluated in light of larval morphology and motility by microscopy. The live worms are mobile and exhibit a wriggling movement, whereas the dead parasite is "C" shaped or straight and inactive (Liu *et al.*, 2018). The result was presented as the percent of dead worms to all of the worms observed in each group.

***In vitro* larval intrusion test**

To assess the silencing TsSP on *in vitro* larva intrusion of IEC, the ML were transfected with 2.5 µM siRNA-156 and cultivated for 1 day. Then, the siRNA-treated ML were activated into the IIL1 larvae using 5% mouse bile, and used in the intrusion test (ManWarren *et al.*, 1997; Long *et al.*, 2015). The IECs were cultured in a 6-well culture plate and the cell monolayer was covered with 100 IIL1 larvae in 2 ml of DMEM semisolid medium (Qi *et al.*, 2018b; Xu *et al.*, 2018). After culture at 37°C for 2 h, the IIL 1 that invaded into the IEC were examined and numbered under microscopy. The larvae that were motile and migrated within the IEC monolayer were taken as invaded larvae, whereas the larvae that was suspended in the culture medium and coiled were regarded as non-invaded larvae (McVay *et al.*, 1998; Xu *et al.*, 2020). Three repeats were performed to determine the percentage of the invaded larvae in each group (Ren *et al.*, 2018).

***The in vitro* larval invasion of enteral epithelium of isolated intestine**

The siRNA 156- treated ML were activated into the IIL1 using 5% mouse bile at 37°C for 2 h, and used for enteral epithelium invasion test. The mouse small intestine was cut into 2-3 cm, two ends of intestinal segment were clamped and ligated to form an intestinal pouch, and each segment were perfused with 100 siRNA 156- treated IIL larvae. After being incubated in Tyrode's solution at 37°C for 2 h, the intestine was cut off, compressed on grass side and examined by microscopy, the worms remaining in enteral lumen were assessed as non-invaded ones (Long *et al.*, 2015; Yang *et al.*, 2020). Three repeats were applied in each test.

Assessment of infectivity and fecundity of siRNA-treated larvae

To assess the infectivity, developmental capacity and fecundity of siRNA-treated larvae, 60 mice were equally divided into three groups (20 mice/group). Each mouse was orally administrated by gavage with 300 ML treated using siRNA-156, control siRNA or PBS. Intestinal adult worms (AW) were collected from 10 mice of each group at 6 dpi (Cui *et al.*, 2013; Sun *et al.*, 2015). The remaining 10 mice of each group were euthanized at 35 dpi, and the ML were obtained by artificial digestion of mouse carcasses as previously described (Jiang *et al.*, 2012). The parasite burden reduction was estimated by means of gut AW burden and larvae per gram (LPG) of muscles collected from siRNA-156 group relative to those from the PBS group (Cui *et al.*, 2019). Furthermore, the reproductive capacity of adult females was determined according in the light of the *in vitro* NBL yield of each female in 72 h (Yang *et al.*, 2019). The length of various stage worms from infected mice was assessed under microscopy.

Statistical Analysis

The data were analyzed by SPSS 22.0 software, and shown as means ± standard deviation (SD). Differences of relative TsSP expression level, parasite burden and

length, and NBL yield among various groups were analyzed with one-way ANOVA, $P < 0.05$ was assessed as statistically significant difference.

RESULTS

Transfection of siRNA into ML

At 18 h following electroporation with control siRNA-labeled fluorescein, 91% of the ML showed fluorescence staining in different tissues under a fluorescent microscopy (Figure 1), suggested that siRNA has been delivered into the ML with electroporation.

Decrease of TsSP mRNA expression in *T. spiralis* ML with siRNAs

After being transfected with 1 μM of siRNA 156 and siRNA 171 for 24 h, the TsSP mRNA expression level in the ML electropporated by two siRNAs resulted in 50.54 and 36.36% reduction compared with those of the PBS group, respectively ($P < 0.01$). When the siRNA 436 and control siRNA were used, no evidently inhibition of TsSP mRNA expression was observed relative to the PBS group ($P > 0.05$(Figure 2A). While the ML were transfected with 1.5, 2.0, 2.5, 3.0 and 3.5 μM siRNA 156, TsSP mRNA expression reduced by 50.49, 51.49, 55.48, 50.14 and 48.47%, respectively, compared with the PBS group ($F = 12.261$, $P < 0.0001$) (Figure 2B). On 1, 3, 5 and 7 days after being transfected by 2.5 μM of siRNA 156, the worm TsSP mRNA expression decreased by 48.35, 36.68, 35.01 and 21.75% respectively ($F = 26.195$, $P = 0.001$). Nevertheless, the TsSP mRNA expression in control siRNA- treated ML group had no distinct decrease ($P > 0.05$) (Figure 2C).

Decrease of worm TsSP protein expression following transfection with siRNA

Western blotting results showed that larval TsSP protein expression was 54.55, 46.84 and 30.06% reduction after transfection using siRNA-156, siRNA-171 or siRNA-436, respectively. The siRNA-156 treated worms had a significant reduction relative to the control siRNA and PBS groups ($P < 0.05$) (Figure 3A). When various siRNA-156 concentrations (1.5, 2.0, 2.5, 3.0, and 3.5 μM) were used, TsSP protein expression levels reduced by 49.62, 44.38, 58.63, 51.91 and 54.38%, respectively. While 2.5 μM siRNA-156 was used for 1 day, it resulted in the highest inhibition of TsSP protein expression ($P < 0.05$) (Figure 3B). At 1, 3, 5 and 7 days after treatment with 2.5 μM siRNA-156, the TsSP protein expression decreased by 59.98, 37.23, 36.22 and 8.71%, respectively, compared with the control siRNA group (Figure 3C). The results indicated that 2.5 μM siRNA-156 had the best knockdown effectiveness on the first day. No obvious

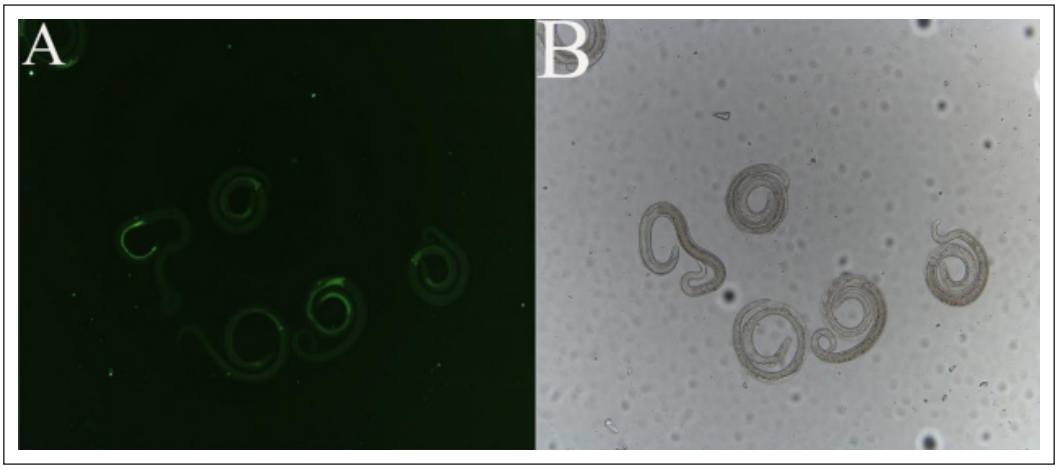

Figure 1. Delivery of FAM-conjugated control siRNA into *T. spiralis* muscle larvae through electroporation. **A:** Uptake of siRNA labeled with FAM into the worm after following transfection (100 ×). **B:** Corresponding white light photo (100 ×) of Figure 1A.

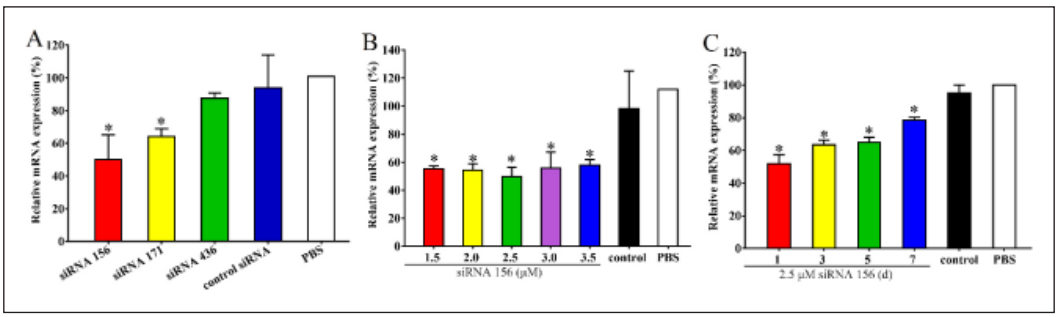

Figure 2. qPCR assay of TsSP mRNA expression level in *T. spiralis* ML treated with siRNAs. **A:** Larva TsSP mRNA expression level at 1 day after transfection using diverse siRNAs. **B:** Larva TsSP mRNA expression level at 1 day after transfection with diverse concentrations of siRNA 156. **C:** Larva TsSP mRNA expression level at diverse times after transfection with 2.5 μM of siRNA 156. Each sample had three repeats, and the results were shown as mean ± SD. **P* < 0.05 relative to the control siRNA and PBS group.

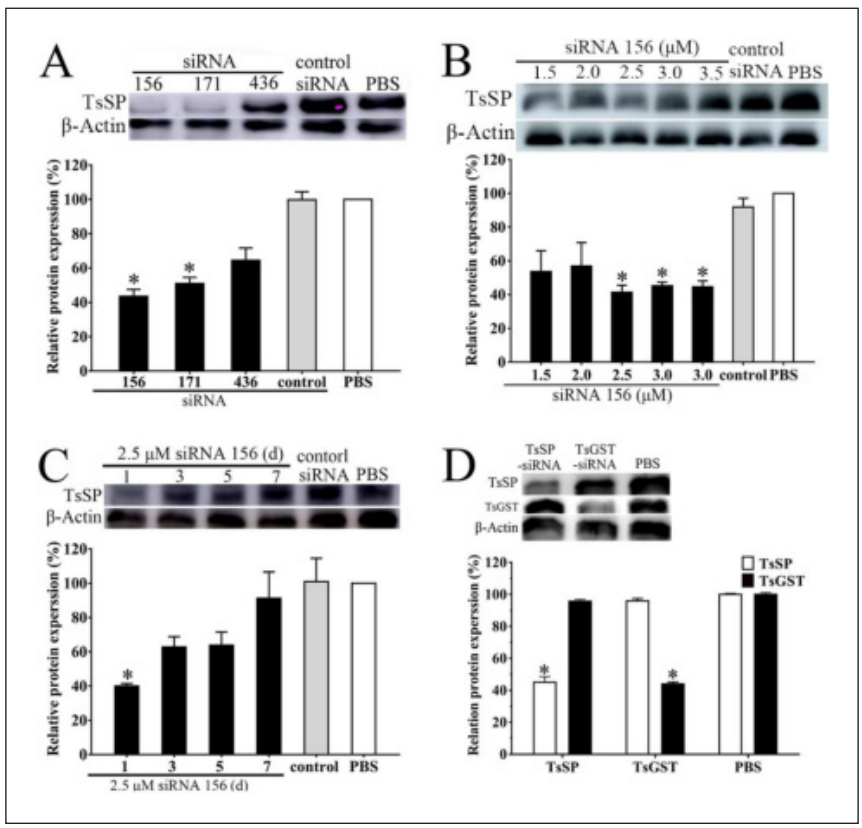

Figure 3. Silencing of TsSP protein expression in *T. spiralis* larvae following transfection with siRNAs. Western blot results showed that TsSP protein expression in worm crude proteins was suppressed by three TsSP siRNAs (**A**) at diverse concentrations (**B**) for various incubation times (**C**). **D:** Western blotting of TsSP and TsGST expression level in larvae treated with TsSP siRNA-156 or TsGST siRNA for one day. The relative protein expression level was assessed based on densitometry. Results showed the relative protein expression assessed in three repeated experiment. * *P* < 0.05 relative with the control siRNA or PBS group.

decrease of β-actin protein expression was seen in muscle larvae transfected using TsSP-siRNA. In the control worm transfected by TsSP-specific siRNA, TsGST protein expression was no suppressed ($P > 0.05$), only TsGST protein expression reduced 57.10% compared with the PBS group ($P < 0.05$) (Figure 3D), certifying the specificity of TsSP gene silencing mediated by siRNA 156.

Suppression of TsSP enzymatic activity after siRNA-156 treatment

The TsSP enzymatic activity following siRNA-156 treatment was detected by degrading azocasein. Among three groups of siRNA-156 treated, control siRNA and PBS control, the serine protease activity of siRNA-156 treated ML ES proteins for degrading azocasein was decreased by 61.38% relative to the PBS group ($F = 1570.157$, $P < 0.05$) (Figure 4).

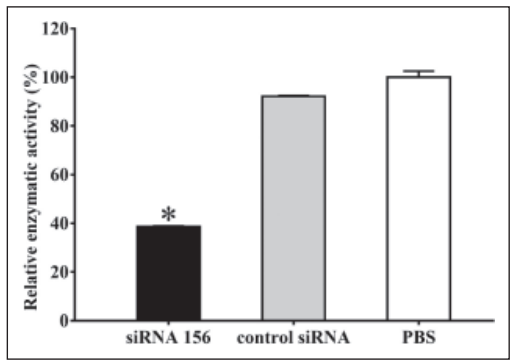

Figure 4. The azocasein degradation by *T. spiralis* larva ES proteins after siRNA 156 treatment. * $P < 0.05$ compared with the control siRNA and PBS group.

Zymography

Zymography profiles showed that the somatic crude proteins from the ML treated with siRNA 156, control siRNA and PBS did not hydrolyze the gelatin. However, after the ML was treated with 2.5 μM siRNA 156, the capacity of the ML ES proteins to hydrolyze the gelatin was obviously reduced in comparison to the control siRNA and PBS groups (Figure 5), suggesting that the TsSP exist principally in the ML ES proteins.

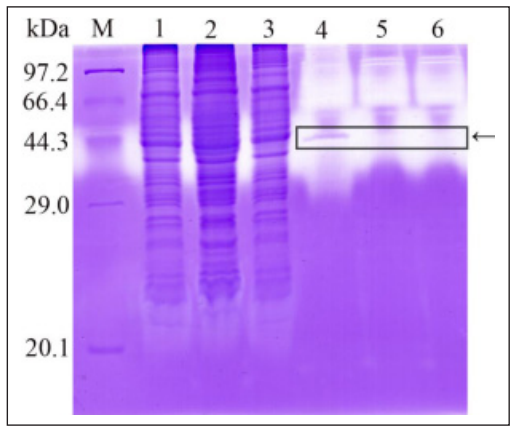

Figure 5. Zymography of *T. spiralis* serine protease. Lane M: Protein marker; Somatic soluble proteins from the ML treated with siRNA 156 (lane 1), control siRNA (lane 2) and PBS (lane 3) did not hydrolyze the gelatin. However, the capacity of the ML ES proteins from ML treated with siRNA 156 (lane 4) to hydrolyze the gelatin was obviously reduced in comparison to the control siRNA (lane 5) and PBS (lane 6) group, demonstrating the capacity of the ML ES proteins to hydrolyze the gelatin was obviously suppressed after the TsST gene was knockdown using siRNA 156. The arrow represents the protein bands hydrolyzed by the ES proteins from the control siRNA- or PBS-treated ML (lane 5 and 6), but not ES proteins from the siRNA 156-treated ML (lane 4).

No effect of siRNA on larval survival

When the ML treated with siRNA-156, control siRNA and PBS were cultured at 37°C for 1 day, 4.67, 4.33 and 3.33% of the ML of each group died ($\chi^2 = 0.039$, $P > 0.05$). When the treated ML were cultured for 7 days, 33.33, 32.00 and 31.67% of the ML of each group died ($\chi^2 = 0.1000$, $P > 0.05$), indicating that silencing of TsSP expression has no obvious impairment effect on the larval survival.

Inhibition of siRNA-156 on the *in vitro* larva invasion of IEC

While the IIL 1 were inoculated onto the IEC monolayer and cultivated for 2 h, the IIL 1 invaded and migrated in the monolayer (Figure 6). The worm invasion rate (40.90%) of siRNA-156 group was significantly lower than the control siRNA (65.76%) and PBS group (64.47%) ($\chi^2 = 20.999$, $P < 0.0001$). The results revealed that the knockdown of TsSP gene by siRNA-156 obviously suppressed the IIL1 invasion of IEC.

Figure 6. *Trichinella spiralis* IIL1 larvae were inoculated onto IEC monolayer and larva intrusion of IEC was observed by microscopy after incubation for 2 h (200 ×). **A**: siRNA 156-treated larva did not invade the IEC monolayer and exhibited the coiled. **B**: PBS-treated larva invaded the monolayer and was motile. **C.** Larval invasive rate of each group was presented as mean ± standard deviation of three independent experiments. * $P < 0.05$ compared with the control siRNA and PBS group.

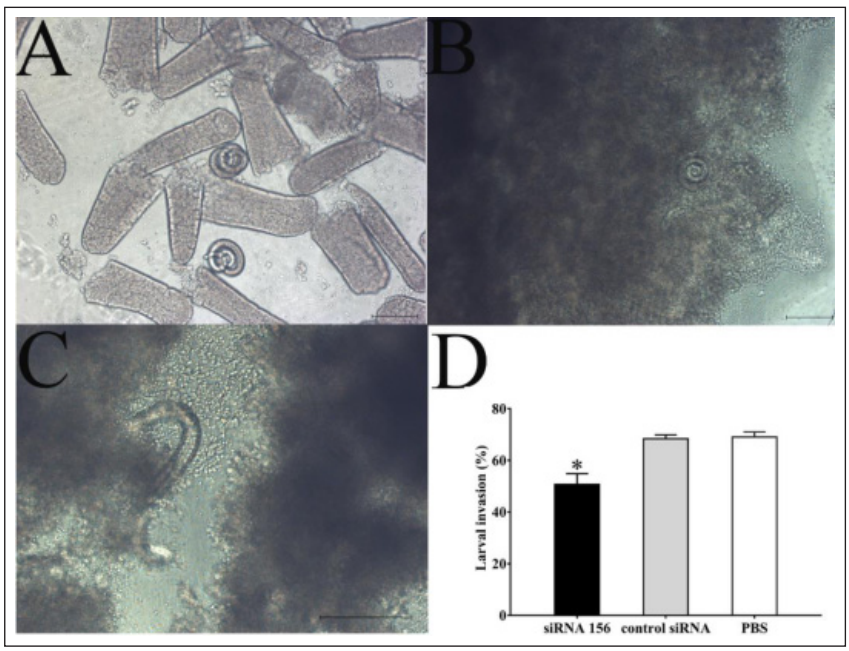

Figure 7. siRNA-156 suppressed on the *in vitro* larval invasion of enteral mucosa. One hundred IIL1 pretreated with siRNA-156, control siRNA or PBS were injected into the isolated and ligated mouse intestine segment, and cultivated in Tyrode's solution at 37°C, 5% CO_2 for 2 h. The intestine segment was cut off and enteral mucosa were examined on microscopy. The larvae invaded into enteral mucosa were observed and counted. **A:** siRNA-156 treated larvae did not invade the intestinal mucosa. **B:** Control siRNA- treated larva invaded into the intestinal mucosa. **C:** PBS-treated larva invaded into the intestinal mucosa. **D:** Larva invasion of intestinal mucosa were presented as mean ± standard deviation of three independent experiments of each group. * $P < 0.05$ compared with the PBS group. Scale-bar: 100 μm.

Suppression of siRNA-156 on the *in vivo* larval invasion of enteral mucosa

After being perfused and incubated in the gut for 2 h with Tyrode's solution, the IIL intruded into intestinal mucosa (Figure 7). The larva invasive rate of siRNA-156, control siRNA and PBS group was 51.00%, 68.00%, and 69.00% ($\chi^2 = 8.748$, $P < 0.05$). The results indicated that siRNA-156 resulted in 26.09% inhibition of larva invasion of enteral mucosa in comparison to the PBS group.

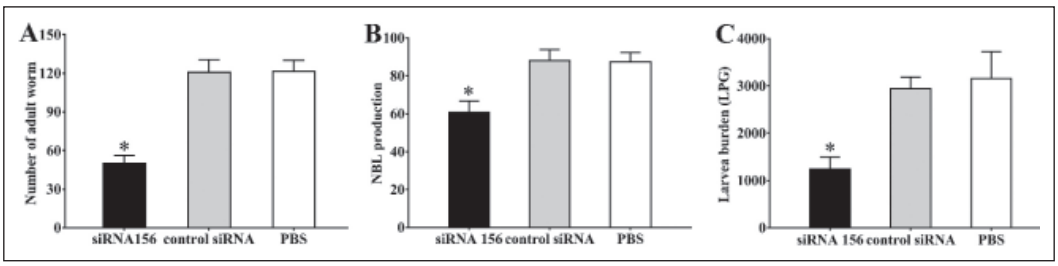

Figure 8. Worm burdens of adults (**A**) as well as larvae per gram (LPG) of muscles (**C**), and newborn larvae (NBL) production of females (**B**) recovered from mice infected with ML electroporated with siRNA-156. The worm burden is shown as mean ± SD of siRNA-156, control siRNA and PBS group (n = 10). * $P < 0.05$ compared with the control siRNA and PBS group.

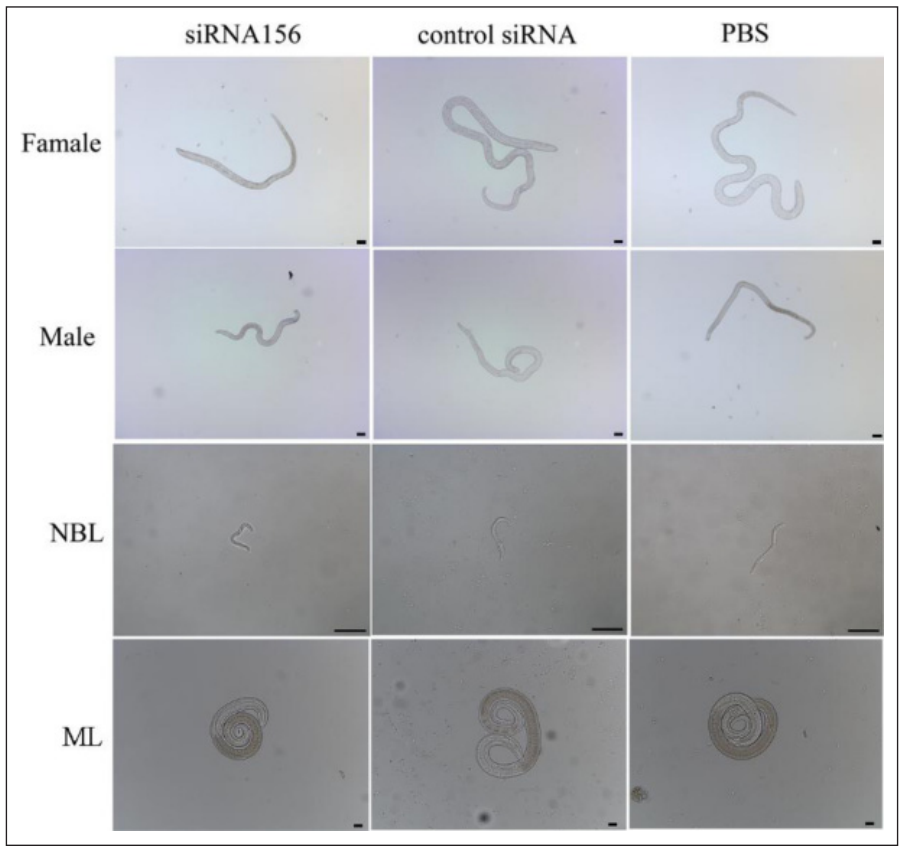

Figure 9. Size of different lifecycle phase worms collected from mice infected with *T. spirali* ML transfected with siRNA-156. Scale bars of AW and ML = 100 μm, and scale bars of NBL = 50 μm.

Inhibition of siRNA-156 on the *in vivo* larval infectivity, growth and fecundity

Compared to the PBS group, mice infected with ML treated using siRNA-156 exhibited a 58.85% intestinal AW reduction and 60.48% ML reduction ($F_{adults} = 256.630$, $F_{larvea} = 81.877$, $P < 0.05$). There was no obvious AW and ML burden reduction in mice infected by ML treated using the control siRNA (Figure 8 A, C) ($F_{adults} = 0.862$, $F_{larvea} = 0.262$, $P > 0.05$).

Moreover, the length of female and male adults recovered from siRNA-156 group was evidently shorter than the control siRNA and PBS groups (Figure 9, 10) ($F_{fAW} = 5.817$, $F_{mAW} = 9.617$, $P < 0.05$). The *in vitro* NBL yield

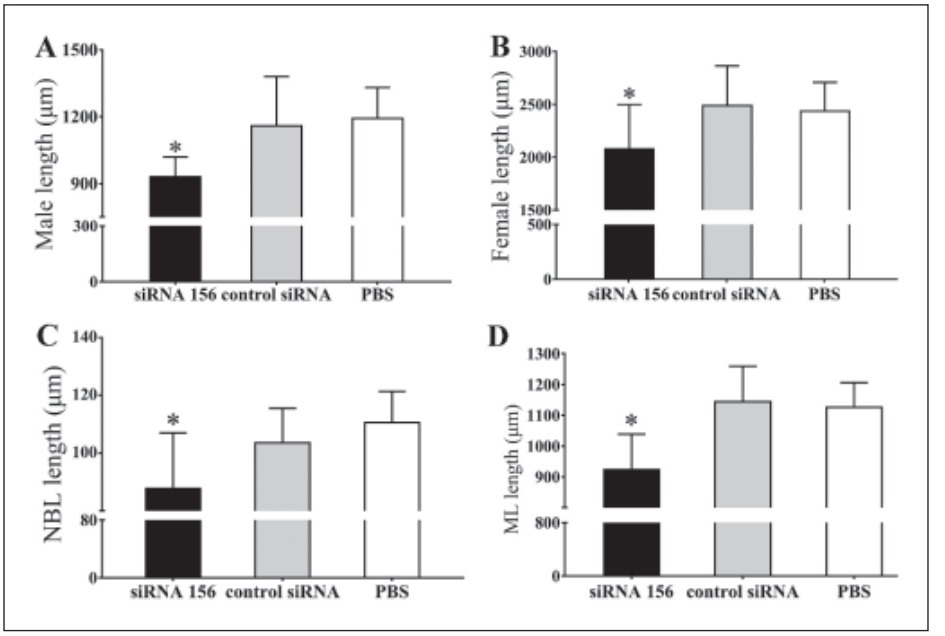

Figure 10. The lengths of diverse phase worms from mice infected with *T. spiralis* larvae transfected using siRNA-156. Ten worms were measured from each mouse. **A**: Female adults; **B**: Male adults; **C**: Newborn larvae (NBL); **D**: Muscle larvae (ML).

of each adult female of siRNA-156 group was also dramatically decreased relative to the control siRNA or PBS group (Figure 8B) ($F = 50.440$, $P < 0.05$). Additionally, the NBL and ML from siRNA-156 group was notably shorten in comparison to the control siRNA or PBS group (Figure 8, 9) ($F_{NBL} = 9.753$, $F_{ML} = 40.933$, $P < 0.05$). The results demonstrated that knockdown of TsSP gene by RNAi inhibited the larval infectivity, invasive and growth, and female reproductive ability, and therefore mitigated the *Trichinella* infection in challenged mice.

DISCUSSION

The *T. spiralis* draft genome contains 15808 protein-coding genes (Mitreva *et al.*, 2011), but only a few of *T. spiralis* gene functions were identified until now (Nagano *et al.*, 2009; Zarlenga *et al.*, 2016). RNAi technique has been widely applied to the function characterization of the nematodes with medical and veterinary importance (e.g., *Brugia malayi* and *Setaria digitate*) (Kushwahae *et al.*, 2012; Somarathne *et al.*, 2018). The acts of *T. spiralis* paratomyosin, nudix hydrolase, and serine protease inhibitor have been also determined through RNAi (Chen *et al.*, 2012; Zhang *et al.*, 2016; Yang *et al.*, 2019). However, the TsSP function in *T. spiralis* lifecycle is still unknown.

In this study, we used the RNAi silencing method to investigate the TsSP function in lifecycle of *T. spiralis*. On 1 day of transfection with 2.5 μM siRNA156, TsSP mRNA and protein expression level was reduced by 48.35 and 59.98%, respectively. Meanwhile, the serine protease activity of siRNA 156 treated ML ES proteins was significantly decreased relative to the control siRNA and PBS group. Previous studies showed that TsSP was located in epi-cuticle and secretory organs of the tissue-parasitizing nematode, it was a surface and secreted protein that facilitated the host' enterocyte intrusion by the IIL1 larvae (Sun *et al.*, 2018a, 2018b). TsSP might exerts an important act during worm invasion by means of hydrolyzing the host's extracellular matrix proteins, adhesion proteins and enteral mucins, helping the parasite to break through the native intestinal mucosal barriers, and escaping the immune damage (Dzik, 2006; Hasnain *et al.*, 2012; Toubarro *et al.*, 2010;

Yang *et al.*, 2015). Our results demonstrated that TsSP-specific siRNA inhibited the expression and enzymatic activity of TsSP.

Additionally, *T. spiralis* serine protease is a family of proteases which contains eight subfamilies and 81 serine proteases, which own high similarity. The knockdown of one serine protease only suppressed partially the expression and enzymatic activity of TsSP (Yang *et al.*, 2020). Moreover, although the silencing of TsSP expression had no obvious impairment effect on the larval survival, the knockdown of TsST resulted in a significant reduction of the *in vitro* and *in vivo* invasive capacity of IIL1 to penetrate into the IEC monolayer and intestinal mucosa.

The results of animal experiment revealed that intestinal adult worm and muscle larval burdens of infected mice with siRNA 156 treated larvae decreased by 58.85 and 60.48%, respectively. When the TsSP gene was knockdown, intestinal worm growth and female reproductive capacity was evidently inhibited, it was demonstrated that adult worms became smaller and the *in vitro* NBL yield of female adults declined compared with the control siRNA and PBS group. Moreover, other development phase worms (NBL and ML) of siRNA 156-treated group was also prominently smaller than the control groups (as shown in Figure 9 and 10). Previous studies showed that RNAi mediated silencing of some genes from *Brugia malayi* impaired the embryogenesis, microfilaria release and infective larva development in jirds (Kushwaha *et al.*, 2012). Our results demonstrated that knockdown of TsSP gene by RNAi significantly suppressed the larvae infectivity, invasive and developmental capacity. Additionally, it is evident that that the enteral worm development disability had a direct relation with muscle larva burden reduction in mice challenged using siRNA156-treated larvae, since the uterus length is positively related to the female fecundity index (Murrell *et al.*, 2000; Yang *et al.*, 2019). Our results further verified that TsSP might participate in diverse processes of *T. spiralis* development, such as intrusion, growth, and reproduction. A further understanding of the TsSP role during a host/nematode interaction might be valuable to screen the new prospective molecular targets of anti-*T. spiralis* vaccines.

In summary, the results indicated that knockdown of TsSP gene by RNAi significantly reduced the TsSP expression and enzymatic activity, impaired larvae intrusion and growth, and lowered the reproductive capacity of female adults in mice.

Conflicts of interest

The authors declare no conflicts of interest with regards to this study or the manuscript prepared for publication.

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