Parasitology Research (2019) 118:2247–2255
https://doi.org/10.1007/s00436-019-06344-4

HELMINTHOLOGY - ORIGINAL PAPER

In vitro silencing of a serine protease inhibitor suppresses *Trichinella spiralis* invasion, development, and fecundity

Fan Yang[1] · Da Qi Yang[1] · Yan Yan Song[1] · Kai Xia Guo[1] · Ya Lan Li[1] · Shao Rong Long[1] · Peng Jiang[1] · Jing Cui[1] · Zhong Quan Wang[1]

Received: 1 March 2019 / Accepted: 29 April 2019 / Published online: 12 May 2019

Abstract

In a previous study, immunoproteomics was used to identify a serine protease inhibitor (TsSPI) of *T. spiralis* excretory/secretory (ES) proteins that exhibited an inhibitory effect on trypsin enzymatic activity, but the precise role of TsSPI on worm infection and development in its host is not well understood. The objective of the present study was to use RNA interference to ascertain the function of TsSPI in larval invasion and growth. TsSPI-specific small interference RNAs (siRNAs) were delivered to muscle larvae (ML) to silence TsSPI expression by electroporation. Four days after electroporation, the ML transfected with 2 μM siRNA-653 exhibited a 75.75% decrease in TsSPI transcription and a 69.23% decrease in TsSPI expression compared with control ML. Although the silencing of TsSPI expression did not decrease worm viability, it significantly suppressed the larval invasion of intestinal epithelium cells (IEC) ($P < 0.01$), and the suppression was siRNA dose-dependent ($r = 0.981$). The infection of mice with siRNA-653-treated ML produced a 63.71% reduction of adult worms and a 72.38% reduction of muscle larvae. In addition, the length of the adults, newborn larvae, and ML and the fecundity of female *T. spiralis* from mice infected with siRNA-treated ML were obviously reduced relative to those in the control siRNA or PBS groups. These results indicated that the silencing of TsSPI by RNAi suppressed larval invasion and development and decreased female fecundity, further confirming that TsSPI plays a crucial role during the *T. spiralis* lifecycle and is a promising molecular target for anti-*Trichinella* vaccines.

Keywords *Trichinella spiralis* · Serine protease inhibitor (SPI) · RNAi · Invasion · Development · Female fecundity

Introduction

Trichinella spiralis is a major foodborne parasitic nematode that is distributed in more than 150 kinds of mammalian species around the world (Pozio 2007). Humans acquire this infection by ingesting raw or semiraw infected meat and meat products contaminated by the muscle larvae (ML) of *T. spiralis* (Cui et al. 2011; Jiang et al. 2016). This parasite is not only a public health hazard but also a serious threat to meat food safety (Cui and Wang 2011; Bai et al. 2017) because it is listed as the 7th most important foodborne parasitic disease in the world (FAO/WHO 2014). It is difficult to control *T. spiralis* infection due to the broad distribution of its animal hosts and the lack of preventive vaccines (Li et al. 2018; Zhang et al. 2018). These problems have prompted the identification and characterization of *Trichinella* invasion- and survival-related proteins as molecular targets for developing anti-*Trichinella* vaccines (Wang et al. 2012, 2013a; Ren et al. 2018).

Parasite serine protease inhibitor (SPI, serpin) is a conserved protein superfamily that has the capacity to suppress the enzymatic activity of the host's serine proteases. SPI exerts a principal function of protecting the parasites from serine protease hydrolysis, helping them break the natural barriers and escape immune attacks in hosts (Dzik 2006; Molehin et al. 2012). In our previous studies, a *T. spiralis* TsSPI (GenBank accession no. XP_003377380.1) was detected in its excretory/secretory (ES) proteins by immunoproteomics

Handling Editor: Una Ryan

✉ Peng Jiang
jpdaisy@126.com

✉ Jing Cui
cuij@zzu.edu.cn

✉ Zhong Quan Wang
wangzq@zzu.edu.cn

[1] Department of Parasitology, Medical College, Zhengzhou University, 40 Daxue Road, Zhengzhou 450052, People's Republic of China

(Wang et al. 2017). The TsSPI was cloned and characterized in our department (Song et al. 2018a). The TsSPI was expressed at all of the various *T. spiralis* stages, and it was a surface and secretory protein of the nematode. Recombinant TsSPI (rTsSPI) possessed an inhibitory role on trypsin enzymatic activity and the capacity to promote the in vitro larval penetration of intestinal epithelium cells (IEC). TsSPI was also observed to induce immune protection against muscle larva (ML) infection (Song et al. 2018b). Although it is promising as a target for an anti-*Trichinella* vaccine, the precise roles of TsSPI in the ability of the worm to intrude, develop, and dwell in the host are not well clarified. Therefore, we used the RNA interference (RNAi) technique to ascertain the biological roles of TsSPI in *T. spiralis* intrusion and growth through the TsSPI gene silencing approach.

RNAi has been widely applied for the gene function identification of parasites, including *Entamoeba histolytica* (Aguilar-Díaz et al. 2013), *Schistosoma japonicum* (Cao et al. 2014), *Clonorchis sinensis* (Wang et al. 2014a), *Brugia malayi* (Singh et al. 2012; Kushwaha et al. 2012), and *Setaria digitata* (Somarathne et al. 2018). The function of paramyosin (TsPMY) and nudix hydrolase (TsNd) for *T. spiralis* larval viability was identified using RNAi (Chen et al. 2012; Wang et al. 2015). Although the *T. spiralis* draft genome was published in 2011 and contained 15,808 proteins coding genes (Mitreva et al. 2011), only some genes have been identified and characterized (Nagano et al. 2009).

In the present study, transcription and expression of TsSPI in *T. spiralis* ML were silenced using three small interference RNAs (siRNAs) specific for TsSPI. The infectivity of ML transfected using siRNAs was assessed by means of in vitro penetration and animal infection. The development, morphology, and fecundity of siRNA-treated ML in infected mice were also observed.

Materials and methods

Worm and experimental animal

The *T. spiralis* isolate (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 (Jiang et al. 2012). BALB/c mice (female, 15–20 g) were obtained from the animal center of Zhengzhou University.

SiRNA preparation

Complete cDNA encoding TsSPI was utilized to design the siRNA sequences by using siDirect version 2.0 (Wang et al. 2015). Synthesis of TsSPI-specific 21 bp siRNAs was performed by Sangon Biotech (Shanghai, China). Three TsSPI-specific siRNAs, siRNA-445 (5′-GACGAAUGAUAAGAUACAATT-3′), siRNA-653 (5′-GAAUACGAGCAGUUUCAAAT T-3′), and siRNA-882 (5′-GCUUAACAAGCAUGUUCGATT-3′) were used in the present work. A control siRNA carrying a scrambled sequence (5′-UACAUGCUCGCAAUAA UCATT-3′) was applied as a specificity control. The control siRNA was fluorescently labeled with 5-carboxyfluorescein (FAM; Sangon Biotech, Shanghai, China) and served to detect the transfection efficiency. The siRNA and polyclonal antibodies for *T. spiralis* serine protease 1.2 (TsSP1.2) were also prepared and used for specificity control (Wang et al. 2013b).

Delivery of siRNA into the muscle larvae

The MLs were obtained by artificial digestion of experimentally infected mouse carcasses at 35 days post-infection (dpi) (Li et al. 2010), which were washed with sterile PBS and electroporation buffer. Five thousand fresh larvae were suspended in 100 μl of electroporation buffer supplemented with 2 μM siRNA. The worms were transfected by siRNA through electroporation with a Gene Pulser II System (800 V, 25 μF, 200 Ω; Bio-Rad, USA). Subsequently, 400 μl of RPMI 1640 culture medium was added and cultured at 37 °C and 5% CO_2 for 1–8 days.

Quantitative PCR

The total RNA from siRNA-treated and control larvae was extracted with Trizol reagent (Invitrogen, USA) and subsequently transcribed into first-strand cDNA using the PrimeScript RT reagent Kit (TaKaRa, Japan) (Ren et al. 2013). The TsSPI transcription level was determined by qPCR using SYBR Premix Ex Taq (Takara) (Liu et al. 2015a). The specific primers for qPCR to amplify the TsSPI gene were 5′-TCCAACGTCTTCTTCT CGCC-3′ and 5’-ACAGACTGAACAGGCGATCC-3′. The *T. spiralis* GAPDH gene (GenBank no. AF452239) served as the control of the housekeeping gene (Chen et al. 2012), and the data were analyzed by the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Liu et al. 2013). Three independent assays were conducted and each sample was performed in triplicate.

Western blotting analysis of interference of TsSPI expression with siRNA

The soluble crude proteins of siRNA-treated ML were prepared as reported (Wang et al. 2011; Li et al. 2015). The same amount (10 μg/lane) of larval protein from each group was separated by SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane at 18 V for 35 min via a semidry transfer cell (Bio-Rad) (Cui et al. 2015). The blot was incised into strips, blocked in TBST with 5% skim milk at 4 °C overnight, and incubated for 1 h at 37 °C

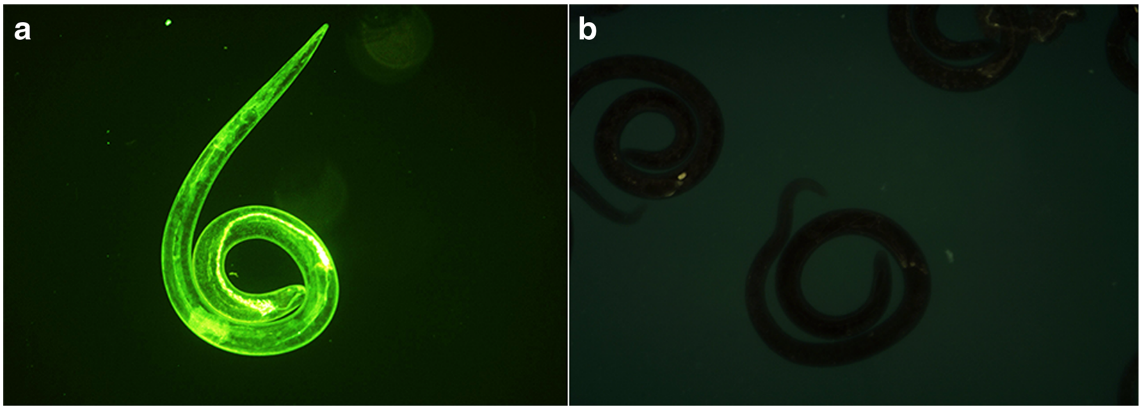


Fig. 1 Delivery of control siRNA labeled with FAM into *T. spiralis* muscle larvae by electroporation. **a** Uptake of siRNA labeled with FAM into the worm at 18 h following transfection. **b** No fluorescent staining was observed in the untreated worms

with anti-TsSPI immune serum (1:100). After washing, the blot was reacted with HRP-labeled anti-mouse IgG (1:10,000; Sigma-Aldrich, USA). A mouse antibody against GAPDH (1:1000) was utilized to determine GAPDH expression as an internal control. Finally, the strip was colored using an enhanced chemiluminescent kit (CWBIO, Beijing, China) (Wang et al. 2014b).

In vitro viability of larvae treated with siRNA

Worms were treated with 2 μM siRNA-653 and cultivated in RPMI 1640 medium at 37 °C and 5% CO_2 for 1–7 days. The siRNA-treated larval viability was evaluated in light of larval morphology and motility by microscopy. The live worm is mobile and exhibits a wriggling movement, whereas the dead parasite is “C” shaped or straight and not active (Liu et al. 2018; Sun et al. 2018). The result was shown as the percentage of dead worms to all of the worms observed in each group.

In vitro larval penetration assay

To assess the suppression of TsSPI silencing on in vitro IEC penetration by *T. spiralis*, the MLs were electroporated using 1, 1.5, 2, 2.5, and 3 μM siRNA-653 and cultured for 18 h. Then, the siRNA-653-treated MLs were activated into the intruding intestinal L1 larvae (IL1) using 5% mouse bile for at 37 °C for 2 h and used in the penetration assay (Liu et al. 2013; Xu et al. 2018). The IECs were cultivated in a 6-well culture plate, and the cell monolayer was capped by 100 infective larvae mixed with 2 ml of DMEM semisolid medium (Ren et al. 2011). After being cultivated at 37 °C for 2 h, the IL1s intruded into the IEC were calculated by microscopy. The worms that penetrated and were motile in the IEC were assessed as invaded worms, while the worms that were still suspended in the culture medium and coiled were considered as unpenetrated worms (Long et al. 2015; Yang et al. 2015). Three independent assays for three groups of larvae were conducted, and three repetitions were performed to ascertain the worm invasion rate for each group (Qi et al. 2018b).

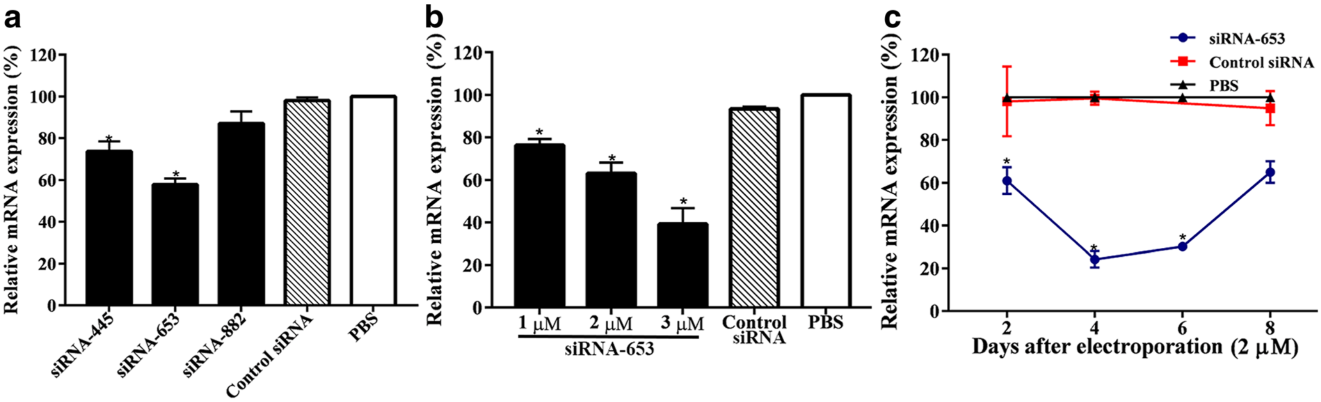


Fig. 2 qPCR analysis of TsSPI transcription level in *T. spiralis* larvae with siRNA. **a** Larval TsSPI transcription level 1 day following electroporation with 2 μM of various siRNAs. **b** Larval TsSPI transcription level 1 day after electroporation with various concentrations of siRNA-653. **c** Larval TsSPI transcription level at 2–8 days after transfection with 2 μM of siRNA-653. Each sample was performed in triplicate, and the data were expressed as the mean ± SD. *$P < 0.05$ relative to the control siRNA and PBS groups

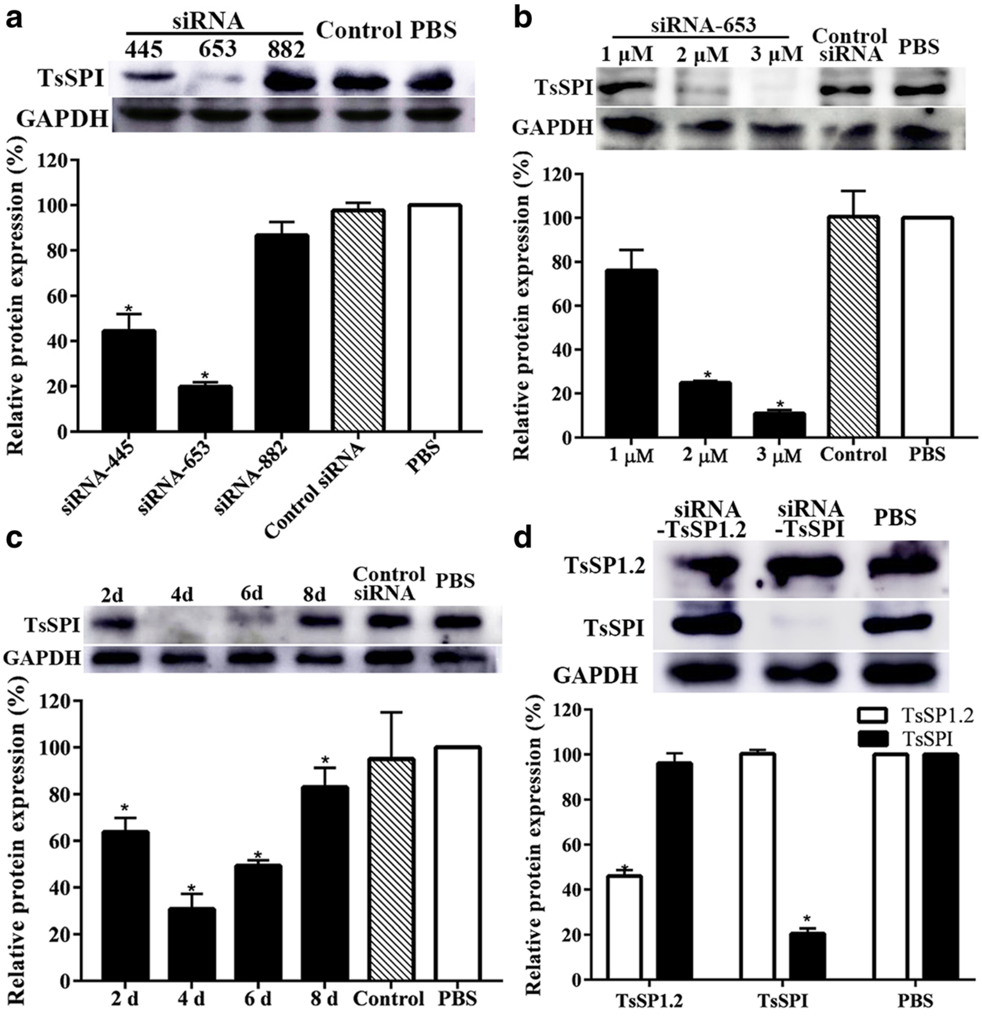


Fig. 3 Silencing of TsSPI protein expression in *T. spiralis* larvae treated with siRNAs. Western blots with specific antibodies exhibiting suppression of TsSPI protein expression in crude proteins of the larvae transfected with three TsSPI siRNAs (**a**) at various concentrations (**b**) for 2–8 days of culture time (**c**). **d** Western blot analysis of the expression levels of TsSPI and TsSP1.2 in ML transfected using TsSPI siRNA-653 or TsSP1.2 siRNA. Graphs indicate the relative protein levels determined by densitometry of the three independent assays. *$P < 0.05$ relative to control siRNA and PBS group

Larval challenge infection experiment

To evaluate the infectivity and developmental capacity of siRNA-treated larvae, 60 mice were equally divided into three groups (20 animals per group). Each mouse was orally administered by gavage treated with 300 ML of siRNA-653, control siRNA, or PBS. Intestinal adult worms (AW) were collected from 10 mice of each group at 6 days post-infection (dpi) (Cui et al. 2013; Liu et al. 2016). The other 10 mice from each group were euthanized at 35 dpi, and the MLs were obtained by artificial digestion of the mouse carcasses as described (Li et al. 2010). The parasite burden reduction was estimated based on the mean intestinal AW burden and larvae per gram (LPG) of muscles recovered from the siRNA-653 group compared with that from the group treated with only PBS (Gu et al. 2017; Liu et al. 2017). Female fecundity was assessed in line with the newborn larvae (NBL) production of each female in vitro for 72 h (Liu et al. 2015b; Qi et al. 2018a). The lengths of the AW, NBL, and ML from each group were measured under microscopy.

Statistical analysis

The data analysis was performed by SPSS 17.0 software. The data are presented as the means ± standard deviation (SD). The differences in the relative TsSPI expression levels, worm burdens and lengths, and NBL production among the groups were analyzed using one-way ANOVA. $P < 0.05$ was regarded as statistically significant.

Results

Delivery of siRNA into the muscle larvae

At 18 h following electroporation by control siRNA labeled with FAM, the fluorescent staining within the larvae was observed by fluorescence microscopy, but no staining was observed in the untreated larvae (Fig. 1), indicating that the siRNA was delivered into the ML by electroporation.

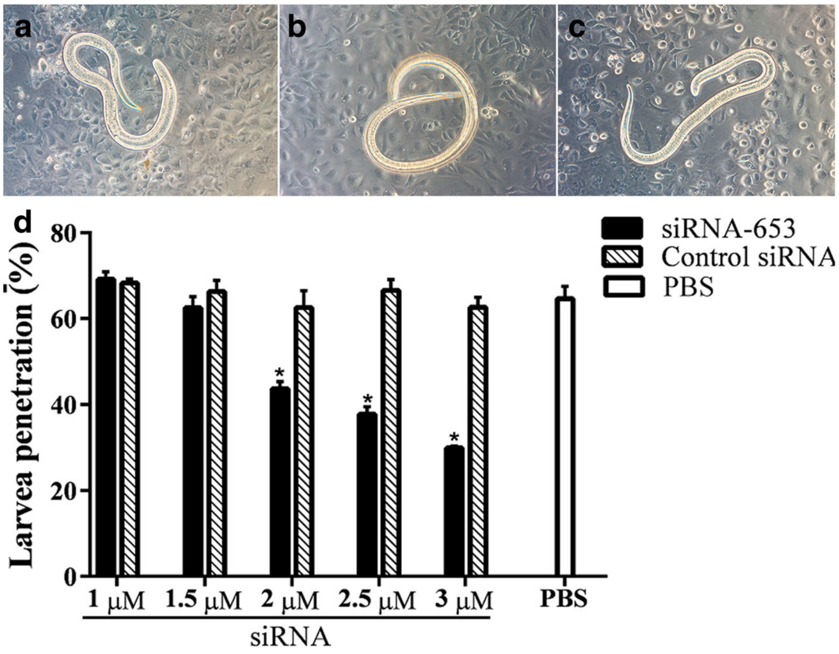


Fig. 4 Inhibition of siRNA-653 on larval penetration of IEC. The larvae were inoculated onto the IEC monolayer, and larval penetration of IEC was examined by microscopy 2 h after culture (200×). **a** siRNA-653-treated larva penetrated into the monolayer. **b** siRNA-653-treated larva did not penetrate into the monolayer. **c** The larva invaded in the monolayer for the PBS group. **d** Inhibition of siRNA-653 on larval penetration of IEC. The data are shown as the percentage of penetrated larvae to all larvae used in each test and are expressed as the mean ± SD of three independent tests. $^{*}P < 0.05$ compared with PBS group

Reduction of TsSPI transcription in *T. spiralis* muscle larvae with siRNA

One day after electroporation using 2 μM siRNA-445, siRNA-653, and siRNA-882, the TsSPI transcription levels in the ML treated with siRNA-445 and siRNA-653 were reduced relative to that of ML treated with control siRNA and PBS ($P < 0.05$) (Fig. 2a). TsSPI transcription levels in ML treated using three siRNAs were reduced by 20.50, 42.09, and 6.55%, respectively, compared with those of the PBS group. The siRNA-882 and control siRNA had no obviously suppressive role in TsSPI mRNA expression. The ML electroporated using 1, 2, and 3 μM siRNA-653 exhibited 23.57, 36.90, and 60.78% reductions in TsSPI transcription levels 1 day after electroporation, which were obviously lower than those of the PBS group ($P < 0.05$) (Fig. 2b). On days 2, 4, 6, and 8 following electroporation using 2 μM of siRNA-653, TsSPI transcription levels were reduced by 38.91, 75.75, 69.79, and 34.92%, respectively ($P < 0.05$). The TsSPI transcription level at 2–8 days after transfection with control siRNA had no distinct decrease ($P > 0.05$) (Fig. 2c).

Reduction of TsSPI protein expression in the muscle larvae transfected with siRNA

The transfection of ML with siRNA-445 and siRNA-653 resulted in 53.52 and 80.26% reductions of TsSPI protein expression, respectively, in comparison with the control siRNA-treated ML or PBS group ($P < 0.05$) (Fig. 3a). Compared with the PBS group, TsSPI expression levels were reduced by 75.13 and 89.18% when the MLs were electroporated with 2 μM and 3 μM siRNA-653, respectively (Fig. 3b) ($P < 0.05$). When the siRNA-653 (2 μM)-treated worms were cultured for 2 and 4 days, the TsSPI expression levels were reduced by 36.19% and 69.23%,

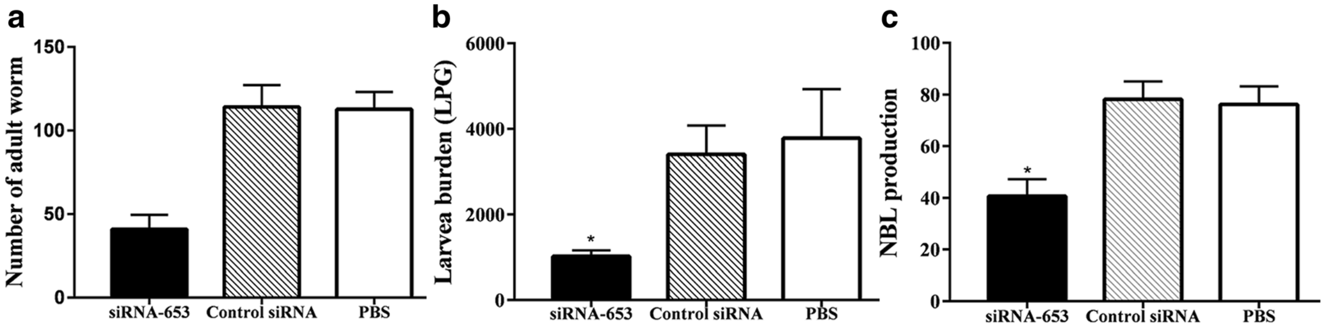
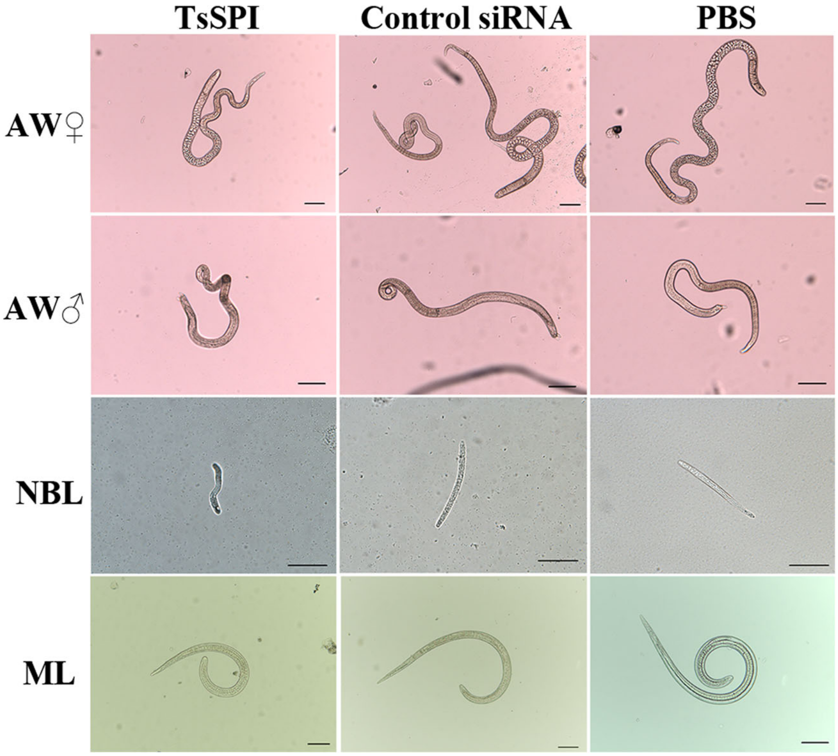


Fig. 5 Parasite burdens of adults (**a**) and muscle larvae (**b**), and newborn larvae (NBL) production of females (**c**) recovered from mice infected with larvae transfected using siRNA-653. The worm burden is presented as the mean ± SD from siRNA-653, control siRNA and PBS group ($n = 10$). $^{*}P < 0.05$ compared with control siRNA and PBS group

Fig. 6 Morphology of various *T. spiralis* stage worms recovered from mice infected with muscle larvae transfected with siRNA-653. Scale bar = 100 μm

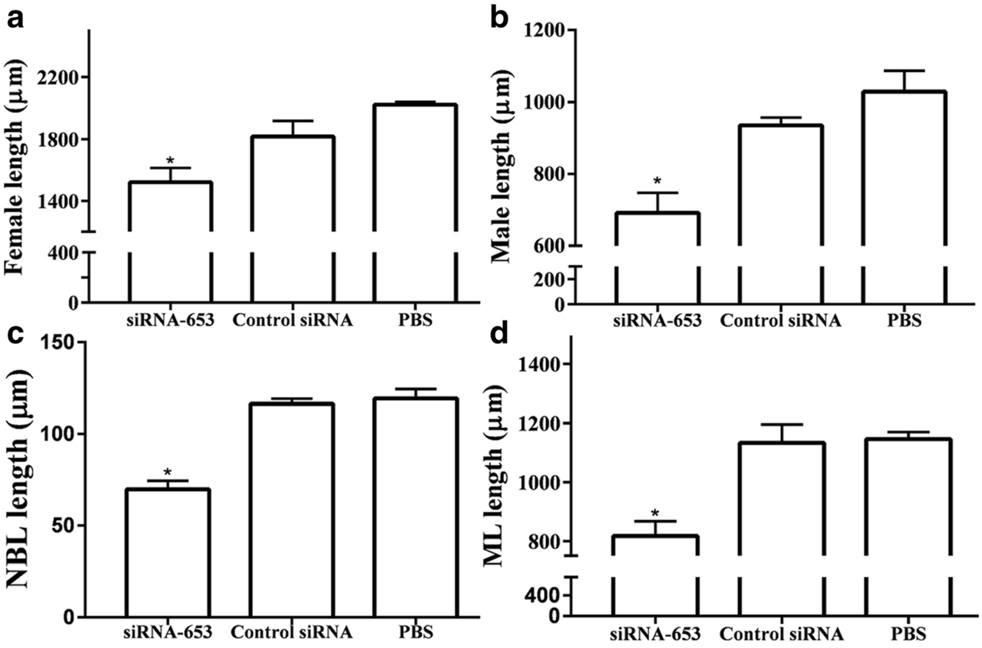


respectively, relative to the PBS group (Fig. 3c) ($P < 0.05$). No evident reduction of housekeeping protein GAPDH expression was observed in the worms treated with any TsSPI siRNA. In control larvae treated with the TsSP1.2-specific siRNA, the TsSPI protein expression was not inhibited ($P > 0.05$), and TsSP1.2 protein expression was reduced only 46.05% compared with the PBS group (Fig. 3d) ($P < 0.05$).

No effect of siRNA on larval viability

When the worms treated with siRNA-653, control siRNA and PBS were cultivated at 37 °C for 1 day, 4.80, 4.49, and 1.14% of the worms of each respective group died ($\chi^2 = 0.316$, $P > 0.05$). When the treated worms were cultivated for 7 days, 34.08, 33.13, and 32.73% of the worms of each respective group died ($\chi^2 = 0.227$, $P > 0.05$),

Fig. 7 The lengths of different stages of *T. spiralis* worms in mice infected with muscle larvae transfected with siRNA-653 (n = 10). **a** Females; **b** males; **c** newborn larvae (NBL); **d** muscle larvae (ML)



demonstrating that silencing TsSPI expression had no obvious inhibitory effect on larval viability.

Inhibition of siRNA-653 on larval penetration of IEC

When the IL1 larvae were added onto the IEC monolayers and cultured for 2 h, the IL1 larvae intruded into the IEC and migrated along the monolayers (Fig. 4a–c). The silencing of TsSPI by siRNA-653 suppressed the larval penetration of the IEC. The penetration rates of worms treated with 1, 1.5, 2, 2.5, and 3 μM siRNA-653 were 69.15, 62.48, 43.68, 36.07, and 30.54%, respectively (Fig. 4d). Compared with the PBS group, the larval invasions of the three siRNA-653 dose groups (2, 2.5, and 3 μM) were significantly inhibited by 38.33, 49.17, and 57.08%, respectively ($\chi^2_{2\mu M} = 12.789$, $\chi^2_{2.5\mu M} = 17.185$, $\chi^2_{3\mu M} = 28.474$, $P < 0.001$). The inhibitory effect was siRNA dose-dependent ($r = 0.981$) and exhibited a positive trend with the increase of the siRNA concentration ($F = 78.440$, $P < 0.001$). No apparent inhibition of larval invasion was observed using control siRNA.

Inhibition of siRNA-653 on larval infectivity and development in vivo

Compared to the PBS group, mice infected with ML transfected with siRNA-653 showed a 63.71% intestinal AW burden reduction (Fig. 5a) and a 72.38% ML burden reduction (Fig. 5b) ($F_{\text{adults}} = 191.887$, $F_{\text{larvae}} = 57.014$, $P < 0.001$); however, the mice inoculated with ML transfected using control siRNA did not exhibit any obvious worm burden reductions for either AW or ML ($F_{\text{adults}} = 0.07$, $F_{\text{larvae}} = 0.520$, $P > 0.05$).

Additionally, the length of females (Fig. 7a) and males (Fig. 7b) collected from the siRNA-653 group at 6 dpi was significantly shorter than those from the control siRNA or PBS groups (Fig. 6) ($F_{\text{fAW}} = 40.683$, $P < 0.001$, $F_{\text{mAW}} = 40.008$, $P < 0.001$). The in vitro NBL production of the females from the siRNA-653 group was also obviously lower than that of the control siRNA or PBS groups (Fig. 5c) ($F = 112.406$, $P < 0.001$). Moreover, NBL (Fig. 7c) and ML (Fig. 7d) lengths from the siRNA-653 group were significantly shorter than that of the control siRNA or PBS groups ($F_{\text{NBL}} = 331.912$, $F_{\text{ML}} = 67.382$, $P < 0.001$). The results indicated that silencing TsSPI by RNAi inhibited larval infectivity, invasion and development capacity, and female fecundity, consequently resulting in a decrease in the AW and ML burdens of infected mice.

Discussion

In this study, we investigated TsSPI function using an RNAi-silencing approach. Four days after ML were transfected with 2 μM siRNA-653, TsSPI transcription and expression levels were reduced by 75.75% and 69.23%, respectively, demonstrating that TsSPI transcription and expression were inhibited by TsSPI siRNA. Although silencing of the TsSPI gene has no inhibitory role on parasite viability, it suppressed the in vitro larval invasion of IEC. The TsSPI investigated in this study was located in the *T. spiralis* epicuticle and secretory organ (stichosome), was a surface and secretory protein, and functioned by inhibiting trypsin enzymatic activity and promoting larval penetration of the host's IEC (Song et al. 2018a, b). Silencing TsSPI by RNAi reduced the ability of the larvae to penetrate into the IEC monolayer. Another study has shown that invasion and metastasis of colon cancer cells could be inhibited by serpin peptidase inhibitor clade A member 3 (serpina3) silencing (Cao et al. 2018).

Our results also showed that mice infected with siRNA-653-treated larvae produced a 63.71% reduction of intestinal adults and a 72.38% reduction of muscle larvae. After the TsSPI gene in the ML was silenced, the enteral worm growth development and female fecundity were obviously suppressed as the adults became shorter and the in vitro NBL production of females declined compared to the siRNA-653 and PBS groups (Fig. 5). Furthermore, the length of other *T. spiralis* developmental stages (NBL and ML) recovered from the siRNA-653 group was also significantly shorter than that of the control siRNA or PBS groups, as shown in Fig. 6. The results indicated that silencing TsSPI by RNAi inhibited the larval infectivity, invasion, and development capacities. Moreover, it is also clear that the intestinal female developmental disability was directly related to a decrease in the ML burden of mice infected with siRNA-653-treated larvae because the uterus length is connected with the female reproductive capacity index, that is, the shorter the uterus, the smaller the reproductive capacity index (Li et al. 2018; Murrell et al. 2000). Our results further confirmed that TsSPI plays a crucial role in *T. spiralis* invasion and development in the host and is a promising molecular target for developing preventive vaccines and new drugs against *Trichinella spiralis* invasive stage worms.

In summary, our results revealed that silencing TsSPI by RNAi suppressed larval invasion and development in the intestine and decreased female fecundity in mice.

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

Compliance with ethical standards

Ethics statement All animal experimental procedure was approved by the Life Science Ethics Committee of Zhengzhou University (No. SCXK 2015–0005).

Conflict of interest The authors declare that they have no conflict of interest.

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