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Research paper

Function of the *lesswright* (*lwr*) gene in the growth, development, and reproduction of *Schistosoma japonicum*

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ABSTRACT

The *lesswright (lwr)* gene and its products are essential molecules in mitosis, DNA repair, and embryo formation in many eukaryotes. In this study, immunohistochemical analysis revealed that the Lwr protein was located in the internal tissues and the surface layer of the adult *Schistosoma japonicum (Sj)* worms. The mRNA expression levels of *SjLwr* at different points were evaluated by quantitative real-time RT-PCR. The expression of *SjLwr* peaked at 14 days and then decreased thereafter. *SjLwr* expression was relatively more stable in male worms than in female worms. The functions of *SjLwr* were explored by siRNA-based gene silencing with a simple soaking method. The results showed that knockdown of the *SjLwr* gene impaired the growth and development of *S. japonicum* in mice, as well as survival, morphology, reproductive capacity, and egg vitality. These observations imply that SjLwr presents a novel target for the development of immuno- and/or small molecule-based therapeutics for the control and treatment of schistosome infections.

1. Introduction

Schistosomiasis is a parasitic disease caused by blood flukes (trematodes) of the genus *Schistosoma* that is characterized by severe pathological damage to animal and human hosts worldwide, and causes substantial economic losses to livestock (Siddiqui, 2011; van der Werf et al., 2003 Makundi, et al.,1998; Lakshmanan et al., 2018). *Schistosoma japonicum* (*S. japonicum*) has a wide spectrum of definitive hosts, which includes more than 40 mammal species (Liu et al., 2013). This species also produces a huge amount eggs that are responsible for the pathology and dissemination of schistosomiasis. Treatments for schistosomiasis, such as oxamniquine and praziquantel (PZQ), have been available for several decades, but these drugs do not prevent reinfection. Also, the extensive use of these drugs raises serious concerns about tolerance and resistance of the parasites (Campbell, 1986). The control and elimination of most epidemics have benefited from the development and use of vaccines, but for helminthiasis, commercial vaccines are only available against hookworms. The administration of vaccines has achieved extensive clinical control of helminthiasis (Diemert et al., 2012). However, there are no commercial vaccines for the prevention of schistosomiasis. Although many candidate antigens have been suggested by the World Health Organization and numerous research groups, none has demonstrated satisfactory protection in clinical studies (Bergquist and Colley, 1998; Siddiqui, 2011). Therefore, there is an urgent need for the development of new drugs for the control of schistosomiasis.

UBC9 was first identified as a ubiquitin-conjugating (E2) enzyme, although it lacks the function to conjugate ubiquitin to proteins in the ubiquitin-mediated proteolytic pathway. Several eukaryotic studies have shown that UBC9 is a key modulator of SUMOylation, a post-translational modification that plays essential roles in cellular growth, migration, responses to stress, and oncogenic transformation, and is the only known SUMO E2 enzyme (Hoeller et al., 2007; Gareau and Lima, 2010). UBC9 also plays important roles in multiple aspects of mitosis, including the maintenance of chromosome integrity, proper chromosome segregation, cell cycle progression, kinetochore assembly, and cytokinesis (Seufert et al., 1995; al-Khodairy et al., 1995a; Nacerddine et al., 2005; Jiang and Koltin, 1996; Yang et al., 2015). UBC9 also functions in DNA repair. In *Drosophila melanogaster*, the UBC9 homologue *lesswright* (*Lwr*), encoded by the *lwr* gene, mediates the dissociation of the heterochromatic region at the end of meiotic prophase I (Apionishev et al., 2010) and participates in the regulation of

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primordial germ cells and subsequent maintenance of germ-line identity in *Drosophila* embryos (Van Doren et al., 1998; Deshpande et al., 2014). Furthermore, *Lwr* regulates hemocyte production in *D. melanogaster*. *Lwr* mutant larvae produce many melanotic tumors induced by a large number of circular blood cells in the hemolymph at the third instar stage. The loss of the *Lwr* gene results in excessive production of hemolymph (Huang et al., 2005). Therefore, *Lwr* not only plays important roles in spindle formation and chromosome segregation during meiosis, but is also a key factor in hematopoiesis and embryo formation.

In our earlier study, the *SjLwr* gene was upregulated in single-sex infected female worms (SF), as compared with bisexual infected mature female worms (MF) (data not published). In this study, the *SjLwr* gene was cloned and its distribution in *S. japonicum* was determined by immunohistochemical analysis. *SjLwr* gene expression at the transcript level was detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Also, an interfering RNA (iRNA)-based approach was successfully applied to knockdown the *SjLwr* gene in *S. japonicum in vivo*. The results showed that the *SjLwr* gene and its product function in the growth, development, and reproduction of *S. japonicum*.

2. Materials and methods

2.1. Parasites and animals

Six-week-old BALB/C male mice, purchased from the Shanghai Laboratory Animal, Co., Ltd. (Shanghai, China), were used for worm collection. A Chinese strain of *S. japonicum* was maintained in tropical freshwater snails (*Oncomelania hupensis*) at the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Shanghai, China). The numbers of viable cercariae were determined prior to infection using a light microscope. Worms were collected from single-sex or bisexual cercariae-infected mice by hepatic-portal perfusion, as described elsewhere (Smithers and Terry, 1965). The protocols of all animal experiments were approved by the Animal Care and Use Committee of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Shanghai, China; approval no: SYXK 2011–0909).

2.2. SjLwr sequence analysis and cloning

The molecular characteristics of *SjLwr* (GenBank accession no: FN321279.1) were analyzed with a bioinformatics approach. The molecular weight (MW) and isoelectric point (pI) were calculated using the Compute pI/Mw tool (http://www.expasy.ch/tools/pitool.html). The SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptides. The NetNGlyc 1.0 server (www.cbs.dtu.dk/services/NetNGlyc/) was used to analyze N-glycosylation sites. The TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used to analyze transmembrane helices. The amino acid sequence was used as a query to identify Lwr orthologues. DNAman software was used to align protein sequences exhibiting sufficient similarity among different species. The stability of the amino acid sequences was predicted with the ProtParam tool (http://web.expasy.org/protparam/).

Total RNA was extracted from adult *S. japonicum* worms using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and cDNA was reverse transcribed from total RNA with the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Shiga, Japan). The primers (forward) 5′−CCGAATTCATGGGTGAGTATGCTGAAG-3′ and (reverse) 5′-AATCTCGAGTTAAGGAAATCACAAGTTTGGG-3′ (the *Eco*RI and *Xho*I restriction sites are underlined) were designed with Primer Premier 5 software to amplify the complete *SjLwr* open reading frame. qRT-PCR was performed with the following cycling conditions: 94 °C for 3 min; 30 cycles at 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 45 s; followed by 72 °C for 5 min. The cDNA product was cloned into the pGEM T-easy vector and sequenced.

2.3. Recombinant protein expression and polyclonal antibody (Ab) production

The full-length cDNA of *SjLwr* was subcloned into the pET28 vector using the restriction enzymes *Eco*RI and *Xho*I to produce a protein that contained an N-terminal hexahistidine tag. The recombinant pET28-SjLwr plasmid construct was overexpressed in *Escherichia coli* BL21 (DE3) cells using 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) at 37 °C. The cells were then harvested by centrifugation at 10,000 rpm for 15 min. The pellet was suspended in 50 mL of phosphate-buffered saline (PBS, pH 7.4). The fusion proteins were extracted using an ultrasonic processor and then purified using nickel column affinity chromatography with the His˙Bind® Purification Kit (Sigma-Aldrich Corporation, St. Louis, MO, USA). Then, 50 μg of purified rSjLwr in Freund's complete adjuvant, followed by Freund's incomplete adjuvant, were used to immunize the BALB/c mice every 2 weeks. One week after the fourth immunization, mouse serum was collected for immunolocalization.

2.4. Western blot analysis

Total extract of a clone before and after IPTG induction were separated with 5% stacking gels and 12% resolving gels, then transferred to 0.45-μm nitrocellulose membranes (Merck Millipore), which were blocked with blocking buffer (5% non-fat milk in PBS) for 2 h at room temperature and then incubated with rabbit polyclonal anti-adult worm protein of *S. japonicum* at 4 °C overnight. The nitrocellulose membranes were washed three times with PBS-Tween 20 (PBST) buffer and probed with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG Ab for 2 h. After three washes, sample detection was performed using an enhanced HRP-3,3′-diaminobenzidine (DAB) chromogenic substrate kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China).

2.5. Immunolocalization of the SjLwr protein

For immunohistochemical analysis, adult *S. japonicum* worms collected from rabbit hosts were fixed in 4% paraformaldehyde overnight at room temperature and then embedded in paraffin. Serial 5 μm-thick sections were cut from the paraffin-embedded blocks, deparaffinized in xylene, and rehydrated with a graded ethanol series. Then, 0.01 M citric acid sodium buffer solution (pH 6.0) was used for antigen retrieval. Endogenous peroxidase activity was quenched with hydrogen peroxidase in methanol (0.3% v/v). Then, the sections were immunolabeled by blocking with 10% goat serum blocking buffer for 45 min at room temperature and incubating with anti-SjLwr serum (dilution, 1:300 in blocking buffer) overnight at 4 °C. Serum from a non-immunized mouse was used as a negative control. The sections were then washed three times with PBST buffer, incubated with HRP-conjugated anti-mouse IgG Ab (dilution, 1:1000 in blocking buffer; Invitrogen Corporation, Carlsbad, CA, USA) for 45 min at room temperature, and then washed again with PBST. After counterstaining the nuclei with hematoxylin, the sections were visualized with DAB at room temperature and then observed under a microscope equipped with a digital camera.

2.6. qRT-PCR analysis

Total RNA was extracted from the collected *S. japonicum* worms using TRIzol reagent and then reverse transcribed using the PrimeScript RT reagent kit (Takara Bio, Inc.). The resulting cDNAs were subjected to qRT-PCR analysis with the primers (forward) 5′-TACGCATTCTTACCG ACCCG-3′ and (reverse) 5′-CATGCTTTTCGCTCCTCAGC-3′ designed with Primer Premier 5.0 software. The amplification products were 111 bp in size. Relative mRNA expression was determined with the relative quantitation method using β-tubulin as an endogenous control. The primers (forward) 5′-ACCTCAACAACCACCACC-3′ and (reverse)

Table 1
Sequence of *SjLwr*-specific siRNA and NC-siRNA.

Name	Sequence	Targeted regions*
S1 siRNA	Sense: 5'-GCGUUAUUCAAUGCCGCAUTT-3' Anti-sense: 5'-AUGCGGCAUUGAAUAACGCTT-3'	500bp–517bp
S2 siRNA	Sense: 5'-GCUACAGUCCAACCACAAATT-3' Anti-sense: 5'-UUUGUGGUUGGACUGUAGGTT-3'	185–202bp
S3 siRNA	Sense: 5'-GCUACUUGACUCCCUACUUTT-3' Anti-sense: 5'-AAGUAGGGAGUCAAGUAGCTT-3'	342–359bp
NC siRNA	Sense: 5'-UUCUCCGAACGUGUCACGUTT-3' Anti-sense: 5'-ACGUGACACGUUCGGAGAATT-3'	Not applicable

* Reference sequence: FN321279.1.

5′-TTGCGGCTTCTGCTCTTC-3′ were used for amplification of β-tubulin. The amplification products were 234 bp in size. The reaction conditions and components were in accordance with the SYBR Premix EX Taq protocol (Takara Bio, Inc.). All qRT-PCR analyses were conducted using an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Data were collected from three separate experiments performed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression (Livak and Schmittgen, 2001). The results were analyzed with ABI 7500 software v2.0.5.

2.7. RNA-mediated SjLwr knockdown

Three specific siRNAs (S1, S2, and S3) targeting different regions of the *SjLwr* gene and non-*Schistosoma* control siRNA (NC-siRNA) were designed and chemically synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) (Table 1). Ten mice were exposed to approximately 200 cercariae through shaved abdominal skin using the coverglass method and divided into five groups (two mice/group). On day 22 post-infection (pi), the mice were injected via the tail vein with specific siRNA (S1, S2, and S3), NC-siRNA (1 OD siRNA per injection, dissolved in 200 μL of diethyl pyrocarbonate-treated water), or PBS. Worms were collected at 48 h post injection. qRT-PCR was performed to evaluate the interference effects.

After evaluation of the efficacy of *SjLwr* siRNAs, the S2 siRNA was selected for the long-term interference experiments to evaluate the effects of the gene on parasitic development. Nine mice infected with 40 ± 5 cercariae were divided into three groups (three mice/group). Starting on day 11 pi, the mice received eight injections of *SjLwr*-specific S2 siRNA, NC-siRNA (1 OD siRNA per injection, dissolved in 200 μL of diethyl pyrocarbonate-treated water), or PBS via the tail vein every 4 days. On day 42 pi, parasites were recovered from the hepatic vein by perfusion and counted. The mouse livers were also collected. Silencing effects were evaluated by qRT-PCR, electron microscopy, liver egg counts, and calculation of the liver egg hatching rate.

2.8. Worm burden, liver egg count, and hatching rate

The worm burden was calculated using the formula:

$$WB = \left(1 - \frac{WRSG}{WRNG}\right) \times 100\%$$

where WRNG is the number of worms recovered from the NC group and WRSG is the number of worms recovered from the siRNA group.

To evaluate the liver egg burden, the liver of each mouse was removed, weighed, homogenized, and digested with 10% NaOH for 10 min at 56 °C. The number of eggs/g was calculated using the formula:

$$LEB = \left(1 - \frac{NESG}{NENG}\right) \times 100\%$$

where NENG is the number of eggs/g of the NC group and NESG is the number of eggs/g of the siRNA group.

To hatch the miracidia, the liver homogenate was added to a flask filled with chlorine-free water. The neck of the flask was filled with a very thin layer of absorbent cotton (avoiding air bubbles, which could obstruct the miracidia) and kept at 25–30 °C in the light. The supernatant above the cotton, which included the miracidia, was collected at 4 h after hatching. The miracidia were fixed with iodine and collected by centrifugation at 4000 × g for 5 min at 4 °C. The numbers of miracidia were counted under a light microscope to calculate the hatching rate (*i.e.*, number of observed miracidia divided by the number of added eggs).

2.9. Electron microscopy

Adult worms collected from the infected mice were cleaned with PBS (pH 7.2) and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde at 4 °C for 24 h. For scanning electron microscopy, the samples were washed with PBS three times (15 min each), fixed in 1% osmium tetroxide for 2 h, then dehydrated in a graded ethanol series of 30%, 50%, 70%, 80%, 90%, 95%, and 100% for 20 min each. The dehydrated worms were desiccated in a CO_2 critical point drying apparatus, then coated with a gold film in a vacuum-coating device, and examined under a JSM-6380LV scanning electron microscope (JEOL, Ltd., Tokyo, Japan). Micrographs were taken at a magnification of ×4000. For transmission electron microscopy, the worms were cleaned with PBS and then dehydrated in 50% ethanol, 70% ethanol, 90% ethanol, 45% ethanol/45% acetone, 90% acetone, and 100% acetone over a period of 20 min, and then soaked in pure acetone/embedding solution at ratios of 3:1 for 2 h, 1:1 for 4 h, and 1:3 overnight. The embedded specimens were cut into 60–100-nm ultrathin sections using a UC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany), then double stained with uranyl acetate and lead citrate. A Tecnai G2 Spirit BioTWIN transmission electron microscope (Thermo Fisher Scientific) was used to observe the ultrastructural alterations of vitelline cells.

2.10. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). All statistical analyses were performed using the Student's *t*-test or one-way analysis of variance. A probability (p) value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Sequence analysis of SjLwr

The *SjLwr* gene contained an open reading frame of 501 bp that encodes a protein of 137 amino acids, with a predicted molecular mass of 19 kDa and an isoelectric point of 8.54. Prediction of post-translational modifications showed that the SjLwr protein contained no signal peptide or N-glycosylation sites. Comparison of the amino acid sequences showed that the SjLwr sequence (CAX77005.1) shared 96%, 76%, 75%, and 75% identity with its orthologs in *Schistosoma mansoni*, *D. melanogaster*, *Homo sapiens*, and *Rattus norvegicus*, respectively. The consensus sequence showed that the epimerase domain was conserved among these species (Fig. 1).

3.2. SjLwr clone expression and polyclonal Ab production

The *SjLwr* coding sequence was obtained by PCR amplification with specific oligonucleotides and cloned into the pET28 expression vector. Expression of the recombinant protein in *E. coli* BL21 (DE3) cells was induced by IPTG. The fusion protein product was about 24.5 kDa (rSjLwr) (Fig. 2, lanes 8 and 10). The expression of rSjLwr remained stable at 4 h after IPTG induction (Fig. 2, lanes 1–4). Then, the bacterial cells were extracted using an ultrasonic processor and the lysate was separated into supernatant (containing most of the recombinant

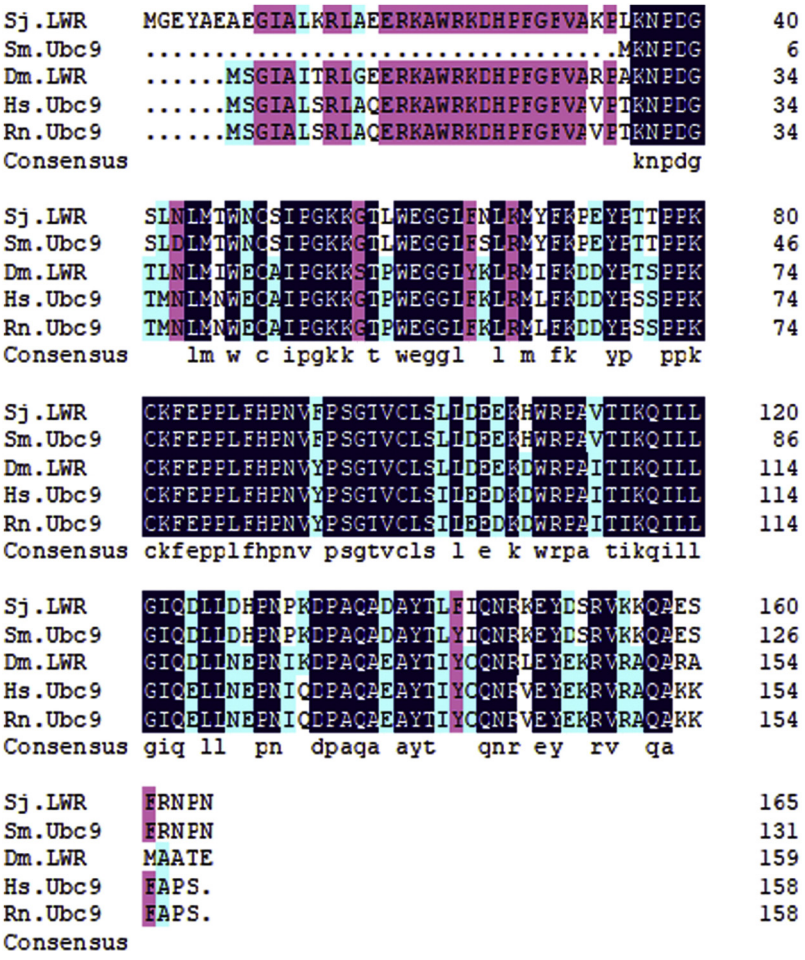

Fig. 1. The complete SjLwr protein sequence in relation to its orthologs in *S. mansoni*, *D. melanogaster*, *H. sapiens*, and *R. norvegicus*. DNAman alignment of the derived amino acid sequences of SmUBC9 (AAX30015.1), DmLwr (NP_476978.1), HsUBC9 (NP919237.1), and RnUBC9 (NP037182.1). The regions with high identity and similarity are shown in color.

protein) and insoluble fractions (Fig. 2, lanes 5 and 6). The recombinant proteins in the supernatant were purified using nickel-charged columns (Fig. 2, lane 8). After purification, the protein was used to immunize mice for the generation of polyclonal Abs. The titer of antiserum used for the immunolocalization analysis was determined using an enzyme-linked immunosorbent assay, which revealed that the titer of antiserum was > 1:32,000.

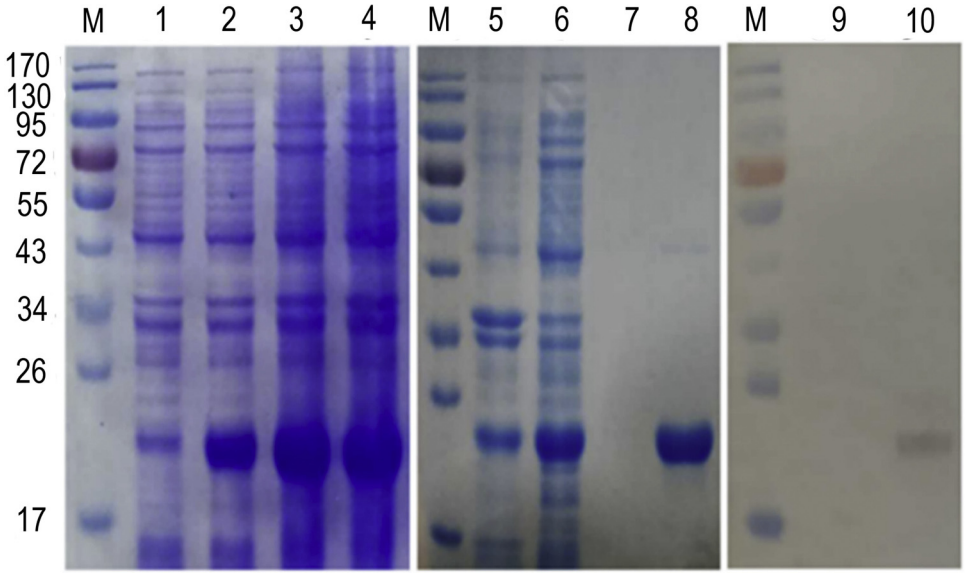

Fig. 2. Expression and purification of SjLwr in *E. coli* BL21 (DE3) cells. Cell extracts and fractions from *E. coli BL21 (DE3)* cells transformed with pET28-SjLwr were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes 1–4: Total extract of clones at 0, 2, 4, and 6 h after induction with 1 mM IPTG. Lanes 5 and 6: Inclusion bodies and supernatant after lysis, respectively. Lane 7: Blank control. Lane 8: The proteins of inclusion bodies purified through Ni-NTA His-Bind resin. Lanes 9 and 10: Western blot profiles (antigen: total extract at 0 and 2 h after induction; primary Ab: polyclonal anti-adult worm protein of *S. japonicum*).

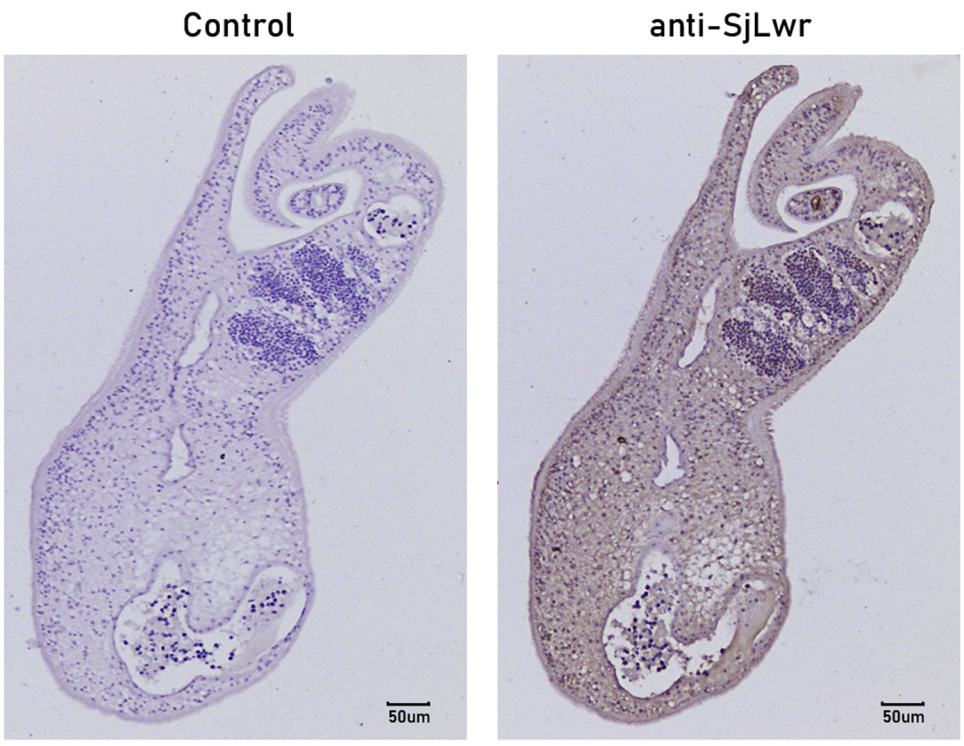

Fig. 3. Distribution of the SjLwr protein in adult *S. japonicum* worms as determined by immunohistochemical analysis using mouse polyclonal anti-SjLwr Abs and HRP-conjugated anti-mouse IgG Ab (brown). Serum from pre-immune mice was used as a control. The nuclei were stained with hematoxylin (blue) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

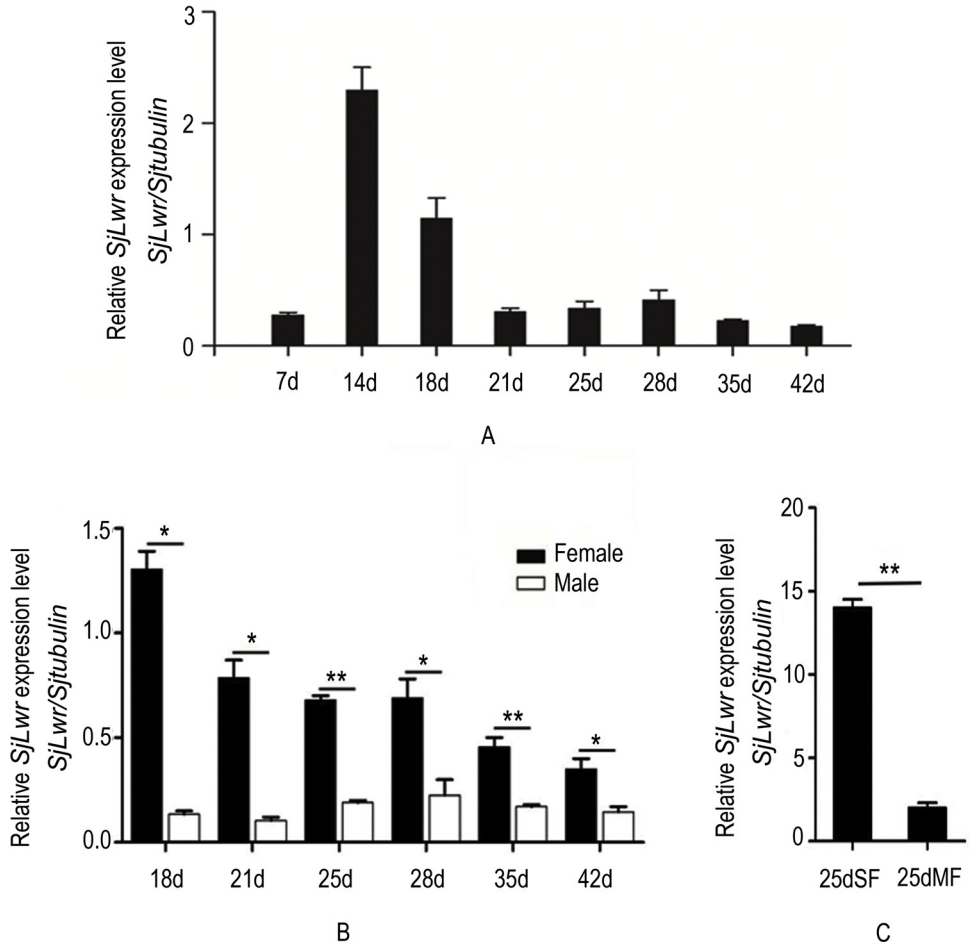

Fig. 4. *SjLwr* mRNA profiles. *SjLwr* transcript levels on days 7, 14, 18, 21, 25, 28, 35, and 42 were compared. The expression of *S. japonicum* β-tubulin was used as an internal control. All experiments were performed in triplicate, $*p < 0.05$, $**p < 0.01$ vs. the control group. SF: single-sex infected female worms; MF: bisexual infected mature female worms.

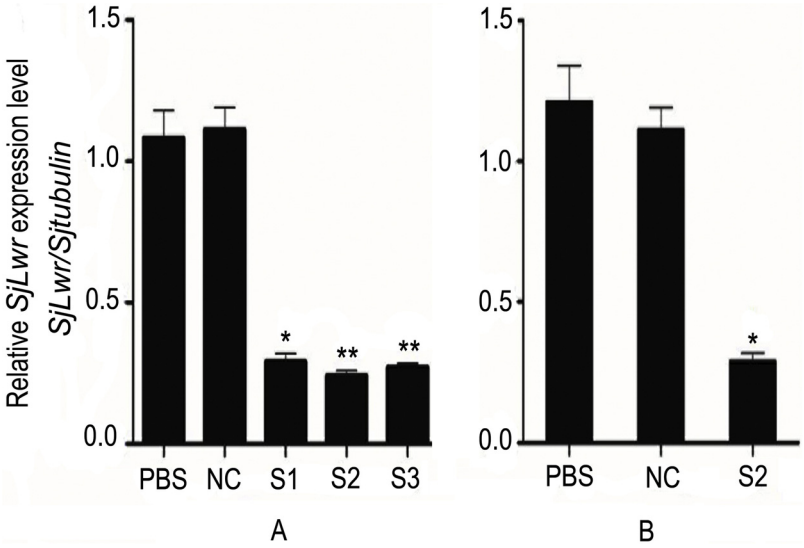

Fig. 5. Effects of siRNA-based gene silencing on the expression of the *SjLwr* gene using β-tubulin mRNA as a control for normalization. A. Comparison on the efficacies of the control (NC) and three SjLwr-specific siRNA species (S1, S2, and S3) on *SjLwr* gene expression. B. Effects of SjLwr-S2 siRNA on *SjLwr* gene expression in adult worms after SjLwr-S2 treatment of mice throughout all developmental stages (eight injections on days 11–42 pi). Data are expressed as the mean ± SD of triplicate experiments. *$p <$ 0.05, **$p <$ 0.01vs. the control group).

Table 2
The effect of gene silencing on the survival and fertility of *S. japonicum* receiving SjLwr-S2 siRNA as a long-term treatment.

Group n = 3	Adult worms Mean ± SD	Liver eggs/Female Mean ± SD	Liver eggs/gram Mean ± SD	Egg hatching rate Mean ± SD
PBS	34.00 ± 5.20	2545.51 ± 207.73	19781.89 ± 1305.32	0.7029 ± 0.0625
NC	36.33 ± 4.04	2465.36 ± 219	22523.00 ± 1422	0.6296 ± 0.0331
S2 siRNA	17.67 ± 2.52	2086.45 ± 213.22	12616.18 ± 844	0.2207 ± 0.0269
Rate reduction (%)	48.04* (PBS) 51.37* (NC)	18.00* (PBS) 15.37* (NC)	36.22* (PBS) 43.98* (NC)	69.61* (PBS) 64.95** (NC)

* $p < 0.05$.
** $p < 0.01$.

3.3. Localization of SjLwr in adult worms

The immunohistochemical approach was used to localize the SjLwr protein in adult *S. japonicum* worms. The use of polyclonal Abs against rSjLwr clearly indicated that this protein was present throughout the entire body of the adult worm. The internal tissues and surface layer of the worms were clearly stained (Fig. 3, labeled as anti-SjLwr). Immunolabeling was specific. Staining of serum collected from naïve mice together with the HRP-conjugated secondary Ab was non-specific (Fig. 3, labeled as control).

3.4. Lwr transcriptional expression levels in S. japonicum

The transcript levels of *SjLwr* at different developmental time points between sexes and statuses were determined by qRT-PCR. Eight stages of development were examined (*i.e.*, days 7, 14, 18, 21, 25, 28, 35, and 42 pi). Female and male worms from day 18 were separated for examination in another experiment. Meanwhile, the transcript levels of *SjLwr* at day 25 pi in SF and MF worms were also examined. Differences in transcription levels were observed by comparisons to the housekeeping gene, β-tubulin. All samples were run three times in triplicate and the Student's *t*-test and one-way analysis of variance followed by the Tukey pairwise comparison were used for data analysis.

The results demonstrated that *SjLwr* mRNA was expressed at all developmental time points examined and exhibited the highest transcription level at day 14 pi, which gradually decreased to the lowest level at day 21 pi and was relatively stable thereafter (Fig. 4A). The results also suggested that the *SjLwr* mRNA expression in male worms was somewhat stabilized, but at far lower levels than in female worms (Fig. 4B). *SjLwr* expression was greater in 25d SF worms than in 25d MF worms (Fig. 4C).

3.5. Selection of effective siRNA of SjLwr

In the present study, three *SjLwr* siRNAs were designed and their efficacies in silencing the *SjLwr* gene were evaluated *in vivo*. At day 22 pi, siRNAs were injected into hosts via the tail vein and the parasites were harvested after 48 h. All three siRNA species effectively reduced *SjLwr* gene expression in the parasites, as determined by qRT-PCR using the β-tubulin gene as an internal control (Fig. 5A). Among them, SjLwr-S2 displayed the highest efficacy (77.7%) in gene silencing, followed by SjLwr-S3 (72.86%) and SjLwr-S1 (72.8%). Based on these observations, SjLwr-S2 was selected in the subsequent long-term gene silencing experiments.

3.6. SjLwr knockdown impaired S. japonicum growth and development

To test the effect of *SjLwr* silencing on parasitic growth and development, SjLwr-S2 siRNA was injected into the tail veins of mice infected with cercariae (days 11–42 pi) with eight injections throughout the 42-day long-term treatment. The long-term treatment reduced *SjLwr* transcript levels by 73.1% (Fig. 5B). Silencing of the *SjLwr* gene negatively affected parasitic growth and development in mice, and resulted in parasite death. As shown in Table 2, as compared with the NC and PBS groups, 51.37% and 48.04% of *S. japonicum* in hosts did not survive to adulthood. In addition, gene knockdown inhibited the reproductive capacity of the surviving adult female worms, as fertility decreased by 15.37% and 18.00%, as compared to worms in the NC and PBS groups, respectively. The loss of reproductive capacity led to a lower liver egg burden. The liver egg burden of the S2 siRNA group was 43.98% and 36.22% of those of the NC and PBS groups. Moreover, 64.95% and 69.61% of liver eggs failed to hatch into miracidia.

Additionally, *SjLwr* gene silencing also significantly affected parasitic morphology in mice, as the adult worms were deformed. As shown

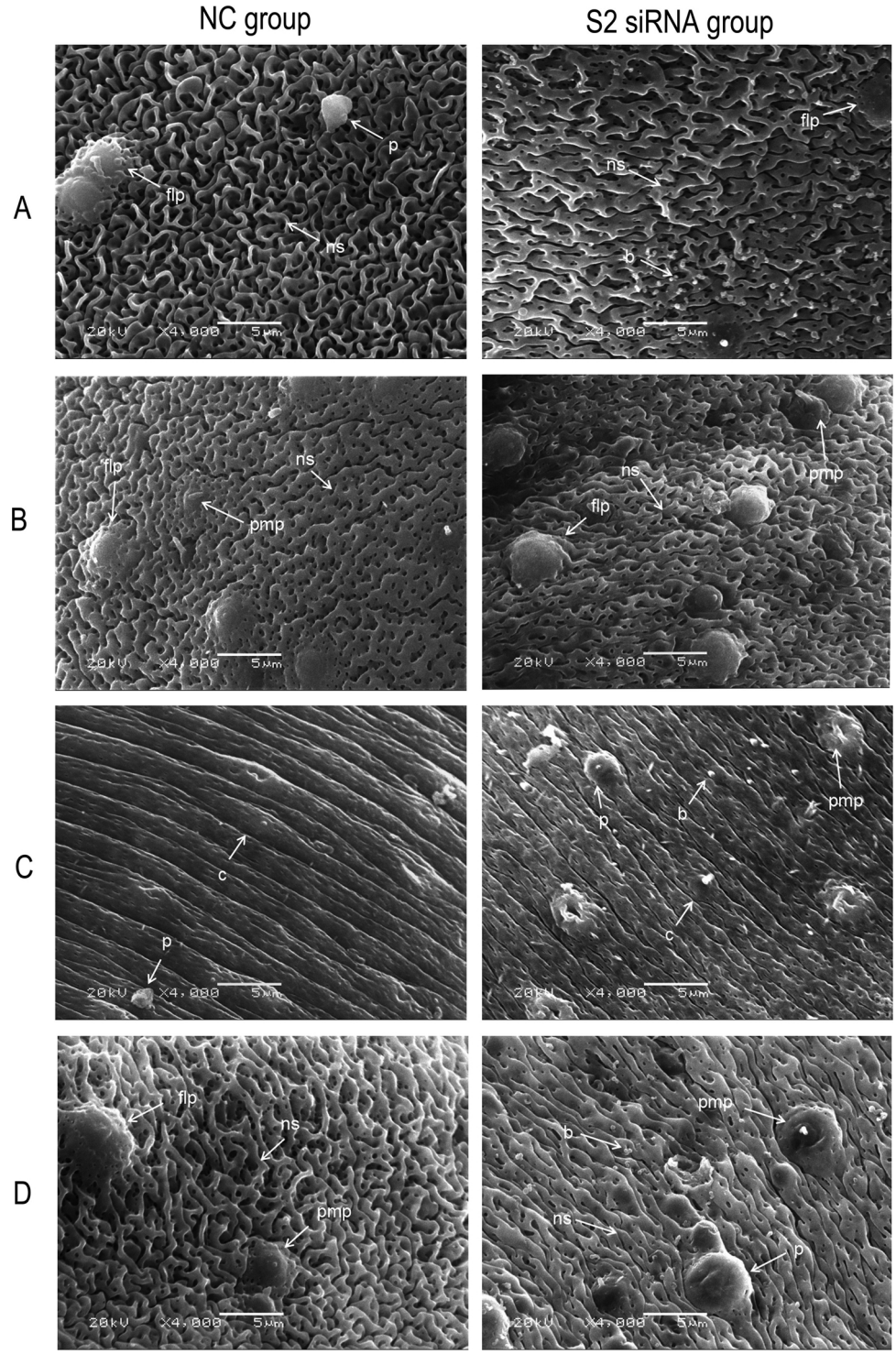

Fig. 6. Comparison of surface structures by scanning electron microscopy between 42-day-old adult *S. japonicum* worms after treatment with control siRNA (NC group) or SjLwr-S2 siRNA (S2 siRNA group) (eight injections via the tail vein on days 14–42 pi). SjLwr-S2-induced alterations of surface structures at the midsection of the body surface of male worms (A) and the anterior portion of the gynecophoral canal (B), the middle portion (C), and the end portion of female worms (D). flp: flower-like papillae; pmp: pedicle mastoid process; p: protrusion; b: bubbles; ns: network structure; c: crest.

in Fig. 6, in comparison with worms receiving control siRNA, the bulb-like structure (b) on the midsection of male worms and the flower-like papillae (flp) and pedicle mastoid process (pmp) on the anterior portion of the gynecophoral canal were significantly increased (Fig. 6A and B). On the middle or end of the female worms, there were not only more white bulb-like structures and protrusions, but the crest (c) and network structure (ns) were irregularly distributed (Fig. 6C and D). As compared with the S2 siRNA-treated group (Fig. 7), the vitelline cells were fuller, more regular, and more matured, and contained large amounts of lipid droplets (l) filled with vitelline globules (vg).

4. Discussion

Schistosomiasis, a neglected tropical disease, is the second most

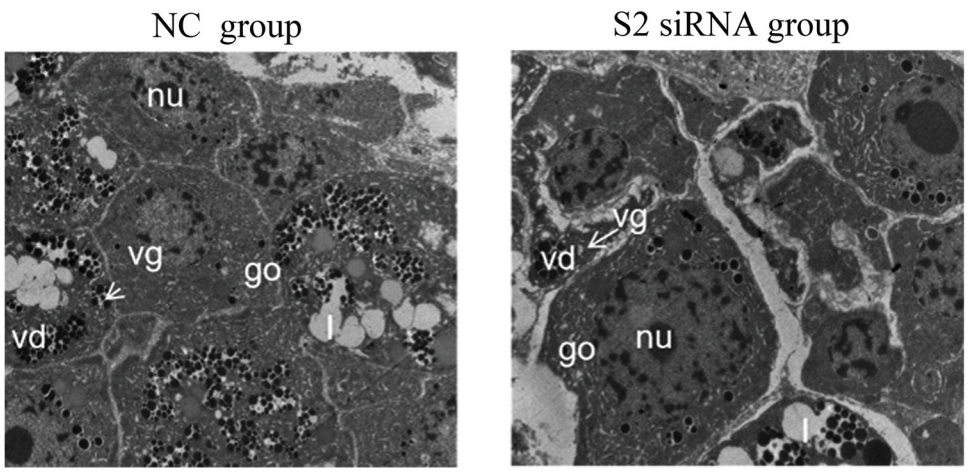

Fig. 7. Comparison of vitelline cells by transmission electron microscopy between 42-day-old adult S. japonicum worms after treatment with control siRNA (NC group) or SjLwr-S2 siRNA (S2 siRNA group) (eight injections via the tail vein on days 14–42 pi). nu: nucleolus; I: lipid droplets; vd: vitelline droplets; vg: vitelline globules; go: Golgi complex.

devastating parasitic disease after malaria and threatens tens of millions people and farm animals in many developing countries. Treatment and control of schistosomiasis relies almost exclusively on a single drug, PZQ, which is a dangerous situation.

UBC9, a post-translational modifier, plays essential roles in spindle formation, meiotic chromosome segregation, hematopoiesis, and embryo formation (Huang et al., 2005; Hoeller et al., 2007; Deshpande et al., 2014; Apionishev et al., 2010). Lwr, the UBC9 homologue, may have similar functions in the growth and development of organisms. A number of eukaryotic studies have reported that the Lwr gene and its products play pivotal roles in mitosis, DNA repair, and embryo formation (Huang et al., 2005; Apionishev et al., 2010). Homologous genes that are highly conserved among different species should have similar conserved functions in the regulation of growth and development. The sequence of Schistosoma Lwr shared high identity with its orthologs in *D. melanogaster*, *Homo sapiens*, and *Rattus norvegicus*, suggesting that Lwr may also play important roles in the growth and development of *S. japonicum*.

For *S. japonicum*, the male and female worms mate at around day 15–16 pi. The qRT-PCR results of this study showed that the highest expression of *SjLwr* mRNA occurred at 14 days pi and expression in male worms was stable and far lower than in female worms, suggesting that the *SjLwr* gene or its products may play important roles in female worms to ensure a smooth mating process. After mating, the gene or its product was not necessary for the maturation of the female worms. Alternatively, a high expression level may inhibit the maturation of females, as the expression level began to decrease, which may explain why *SjLwr* expression was significantly greater in SF worms ready for mating than in MF worms.

To explore the importance of *Lwr* in schistosome development, an RNA interference (RNAi)-based approach was employed to knockdown expression of the *SjLwr* gene. In schistosomes, RNAi can be delivered to the parasite by a simple, but effective, soaking method (Ndegwa et al., 2007; Kumagai et al., 2009; Cao et al., 2014), which is a valuable tool to determine the biological roles of genes, especially in organisms for which knockout is difficult, such as parasitic trematodes. The siRNA-based gene silencing approach used in this study was sufficient to support the notion that the *Lwr* gene is also critical for the growth, development, and reproduction of *S. japonicum*. In this study, silencing of *SjLwr* affected the survival, reproductive capacity, and viable egg production of affected worms. SjLwr gene silencing also caused considerable changes to the tegument tissue and vitelline cells.

Taken together, the *SjLwr* mRNA profile, the results of *SjLwr* silencing in this study with the SjLwr distribution in *S. japonicum* indicates that the location of the SjLwr protein at the surface layer and the internal tissues suggests it affects mutual aspects of the growth and development of *S. japonicum*. Hence, we speculate that the *Lwr* gene presents a novel target for the development of immuno- and/or small molecule-based therapeutics for the control and treatment of schistosome infection.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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