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ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Molecular characterization, tissue-specific expression, and RNA knockdown of the putative heat shock cognate 70 protein from *Rhipicephalus haemaphysaloides*

Fangfang Wang[1,2] · Haiyan Gong[1] · Houshuang Zhang[1] · Yongzhi Zhou[1] · Jie Cao[1] · Jinlin Zhou[1]

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Abstract

Heat shock cognate 70-kDa protein (RH-Hsc70) was identified from a cDNA library synthesized from the sialotranscriptomes of unfed and fed *Rhipicephalus haemaphysaloides*. The RH-Hsc70 open reading frame is 1950 bp long and encodes a protein that is 649 amino acids in length, with a predicted molecular weight of 71.1 kDa and a theoretical pI of 5.43. RH-Hsc70 exhibits 98% amino acid identity with Hsc70 in *Haemaphysalis flava* and 83% identity with Hsc70 in arthropods and mammals. RH-Hsc70 was mainly expressed in nymphs and adult ticks, not in larvae. Real-time quantitative PCR analysis indicated that RH-Hsc70 mRNA expression was induced by blood feeding in adult ticks. In addition, RH-Hsc70 gene expression was higher in the ovaries of fed adult ticks than that in the midguts, salivary glands, and fat bodies of unfed or fed adult ticks. RH-Hsc70 gene knockdown inhibited tick blood feeding, significantly decreased tick engorgement rate, and increased tick death rate. These data illustrate the importance of RH-Hsc70 in tick blood feeding and aging, which makes it a promising candidate for the development of anti-tick vaccines.

Keywords Tick · Heat shock cognate 70 kDa · RNA interference

Introduction

Ticks are responsible for the transmission of several zoonotic pathogens that cause economically important diseases (Sanchez et al. 2016; Schnittger et al. 2012; Sivakumar et al. 2016). In addition, due to the widespread use of chemicals like insecticides, it is common for ticks to develop drug resistance (Reck et al. 2014). Thus, a new method of controlling tick infections is needed, such as developing biologically based treatments, including anti-tick vaccines. After blood feeding, nutrient endocytosis and lysosome maturation facilitate blood digestion and salivary gland maturation (Gulia-Nuss et al. 2016; Moreira et al. 2017), thus integrating reproduction and nutrition. After engorgement, the tick salivary gland ages, and adult female ticks die after oviposition. So, genes relating to blood feeding and aging would be a promising candidate for the development of anti-tick vaccines (de la Fuente et al. 2016). Genome and proteome analyses have provided insight into parasitic processes that are unique to ticks (Aguilar-Diaz et al. 2018; Barrero et al. 2017; Cafarchia et al. 2015; Charrier et al. 2018; Gulia-Nuss et al. 2016), including transmission, immunity, and detoxification. Another study investigated the midgut, ovary, and salivary gland transcriptomes of the *Amblyomma sculptum* tick and found that transcripts of genes involved in nutrient assimilation processes and metabolic processes, such as transportation and sugar, protein, and lipid digestion, were abundant in the tick midgut and ovary transcriptomes (Moreira et al. 2017).

Heat shock cognate 70-kDa protein (Hsc70) gene is a molecular chaperone that plays a critical role in protein folding and is expressed under normal physiological and heat stress

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✉ Jinlin Zhou
jinlinzhou@hotmail.com

[1] Key Laboratory of Animal Parasitology of Ministry of Agriculture, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, China

[2] College of Life Science and Food Engineering, Hebei University of Engineering, Hanshan District, Handan 056038, China

conditions (Gonzalez-Aravena et al. 2018). The expression of Hsp70 gene was closely relating to blood meal. In *Aedes aegypti*, Hsp70 gene knockdown impaired protein digestion of blood meal and reduced the production of eggs (Benoit et al. 2011). In *Rhodnius prolixus*, the physiological effects of Hsc70/Hsp70 were more prominent and the insects died even earlier by impairing blood processing and digestion and energetic metabolism (Paim et al. 2016). In tick *Ixodes scapularis*, Hsp70 and Hsp20 genes were both involved in the tick response to blood-feeding stress (Busby et al. 2012). The gene encoding heat shock protein 70 (Hsp70) was identified in a cDNA library derived from *Haemaphysalis longicornis* eggs, and this protein contains the highly conserved Hsp70 family functional motif and the specific endoplasmic reticulum retention signal KDEL (Tian et al. 2011). The tick Hsc70 gene, which was also identified in the salivary gland of *Haemaphysalis flava*, encodes a cytosolic localization signal, which suggests that it may play a role in blood feeding (Liu et al. 2017). However, the RH-Hsc70 cDNA sequence, RH-Hsc70 expression pattern, and effect of RNA interference-mediated knockdown of RH-Hsc70 have not yet been characterized in *Rhipicephalus haemaphysaloides*.

R. haemaphysaloides is a common three-host ixodid species in China, India, and other south Asian countries, which is a major vector of bovine babesiosis in China (Zhou et al. 2006). In this study, we detected RH-Hsc70 expression from the sialotranscriptomes of unfed and fed *R. haemaphysaloides*, determined its expression patterns by real-time quantitative PCR, and, for the first time, showed by RNA interference that RH-Hsc70 is involved in tick blood feeding. This study helps to better understand the physiological processes that are crucial for tick blood feeding, with the goal of developing drug targets for ticks and the control of tick-borne diseases.

Materials and methods

Ticks and sample preparation

Ticks were obtained from a colony of *R. haemaphysaloides* maintained on the ears of New Zealand white rabbits at Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. In detail, a cloth ear bag was fixed on the ear of a rabbit by an adhesive bandage, and ticks were kept in the closed ear bag until they were engorged. Off-host ticks were kept in an incubator at 25 °C with 95% humidity. For gene expression analysis, unfed and fed larvae ($N \geq 10$ per group), nymphs ($N \geq 10$ per group), and adult ticks ($N = 3$ females per group) were collected. Tick ovaries, fat bodies, midguts, and salivary glands from unfed and fed female ticks were removed and washed in PBS to remove hemolymphs, after which RNA was extracted. Three independent samples were collected and processed. Total RNA was extracted using

Table 1 The list of heat shock 70-kDa protein family in the tick sialotranscriptomes by Blastp. The table contained gene name, similar species, identity, gene length, and scores

Gene_ID	Gene_definition	Query_length	Percent_identify	Bit_score	E_value	Blast_OS
comp67592_c0	Heat shock protein Hsp-16.48/Hsp-16.49 (HSP17)	183	31.03	53.5	0.00000002	*Caenorhabditis elegans*
comp80375_c0	Heat shock protein Hsp-16.1/Hsp-16.11 (HSP11)	181	39.78	74.7	1E-15	*Caenorhabditis elegans*
comp81199_c0	Heat shock protein 105 kDa (HSP105)	807	47.45	754	0	*Bos taurus*
comp82371_c0	Heat shock 70-kDa protein cognate 4 (HSP7D)	650	90.36	1158	0	*Manduca sexta*
comp88866_c2	60-kDa heat shock protein(CH60)	576	80.4	884	0	*Drosophila melanogaster*
comp90012_c1	Heat shock protein 67B2 (HSP6B)	253	36.61	75.5	6E-16	*Drosophila melanogaster*
comp91164_c0	Heat shock protein 75 kDa (TRAP1)	693	56.44	721	0	*Mus musculus*
comp92361_c0	Heat shock protein beta-1 (HSPB1)	219	31.34	82.4	8E-18	*Mus musculus*
comp92361_c0	Heat shock protein beta-1 (HSPB1)	201	40.4	80.5		*Gallus gallus*
comp93797_c3	Heat shock 70-kDa protein 12A (HSP12A)	1141	56.88	762	0	*Homo sapiens*
comp95610_c0	Heat shock protein 68 (HSP68)	523	73.04	823	0	*Drosophila melanogaster*
comp95764_c0	Heat shock protein HSP 90-alpha (HS90A)	730	79.95	1176	0	*Oryctolagus cuniculus*
comp95956_c3	Heat shock protein beta-2 (HSPB2)	108	33.73	54.3	5E-09	*Rattus norvegicus*
comp96457_c2	Heat shock 70-kDa protein 14 (HSP7E)	343	41.07	251	4E-77	*Danio rerio*
comp96457_c2	Heat shock 70-kDa protein 14 (HSP7E)	518	38.4	352	3E-113	*Bos taurus*
comp98666_c3	Heat shock protein beta-1 (HSPB1)	193	36.29	68.9	3E-13	*Bos taurus*
comp98672_c0	Heat shock protein 68 (HSP68)	638	76.73	1037	0	*Drosophila melanogaster*

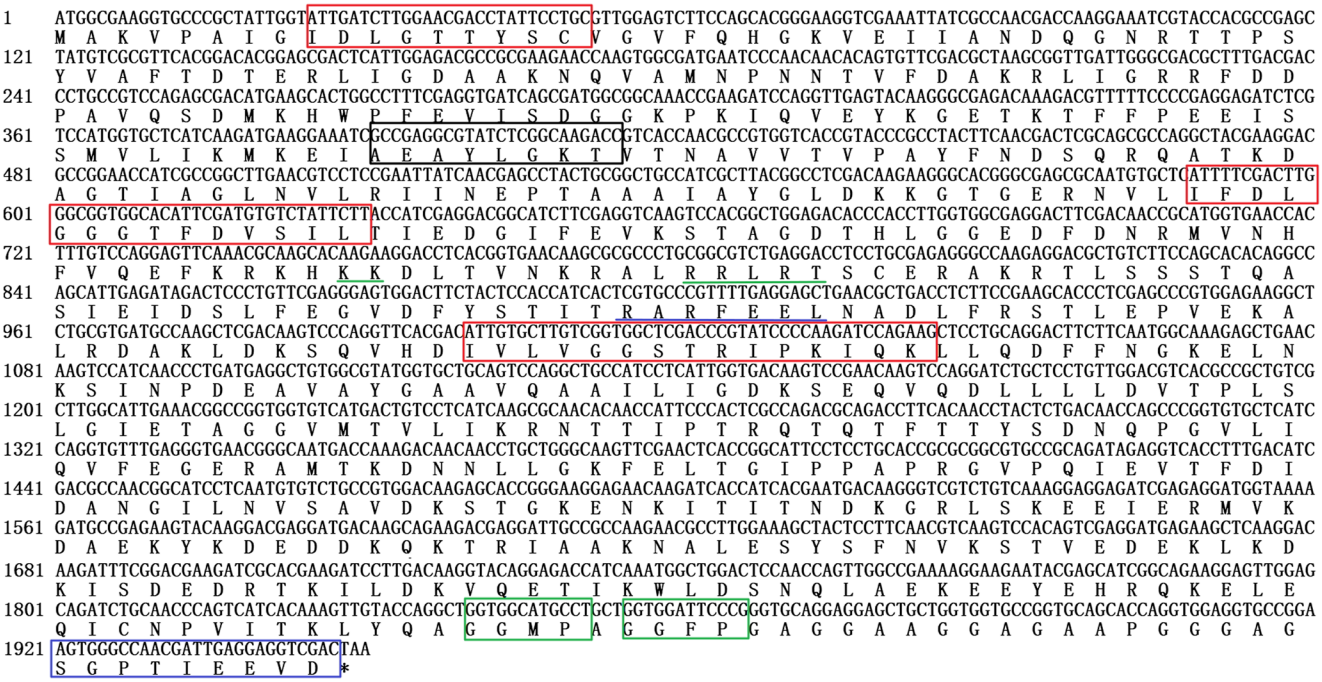

Fig. 1 Structural analysis of the RH-Hsc70 gene from ticks *R. haemaphysaloides*. Nucleotide and predicted amino acid sequences of the RH-Hsc70 gene were analyzed with motif

TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of the total RNA was evaluated using an Agilent 2100 Bioanalyzer RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA). The RNA samples were stored at − 80 °C until use.

Cloning the open reading frame of RH-Hsc70 gene

A sequence of Hsc70 was found from the sialotranscriptomes of unfed and fed *R. haemaphysaloides*, named RH-Hsc70 gene. Complementary DNAs (cDNAs) were synthesized by Reverse Transcription System Kit (TaKaRa, Dalian, China) from total RNA of *R. haemaphysaloides* ticks. Oligonucleotide primers (Hsc70-F: 5′-CGG ATT TCG GAC ACA GGC ATG-3′ and Hsc70-R: 5′-GCT TAG TCG ACC TCC TCA ATC-3′) were designed to amplify the ORF sequence of RH-Hsc70, the sequence was routinely cloned into the pMD-18 T easy vector using TaKaRa Premix Taq™ (TaKaRa), and the obtained clones were sequenced. The encoding protein sequence of RH-HSC70 was analyzed by software GENETYX and ExPASy prosite (https://prosite.expasy.org/prosite.html). A phylogenetic tree was performed using software MEGA5.1 with Neighbor-joining tree and 100 of bootstrap replications based on the amino acid sequences in Genbank.

Determination of tick mRNA levels by real-time quantitative PCR

Primers (Hsc70-qRTPCR-F: 5′-CAT TGT GCT TGT CGG TGG CTC-3′ and Hsc70-qRTPCR-R: 5′-GCA CCA TAC

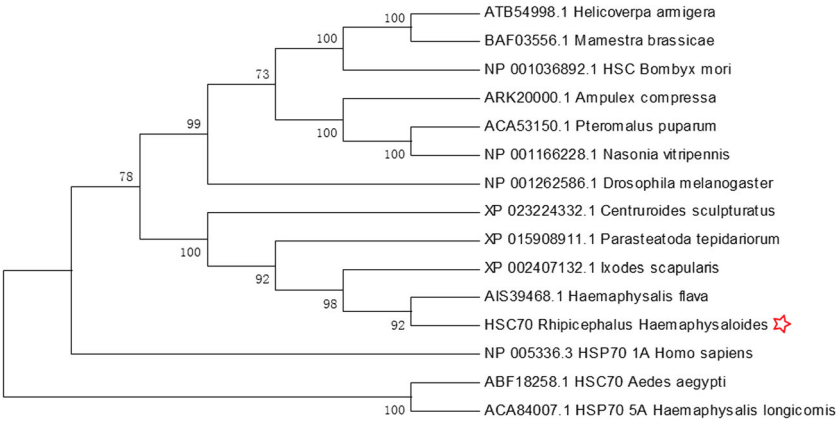

Fig. 2 Phylogenetic analysis of RH-Hsc70 genes from representative species. A phylogenetic tree of RH-Hsc70 genes from representative species with the accession numbers was constructed by software MEGA 5.1 with Neighbor-joining tree and 100 bootstrap replications based on available amino acid sequences of typical species

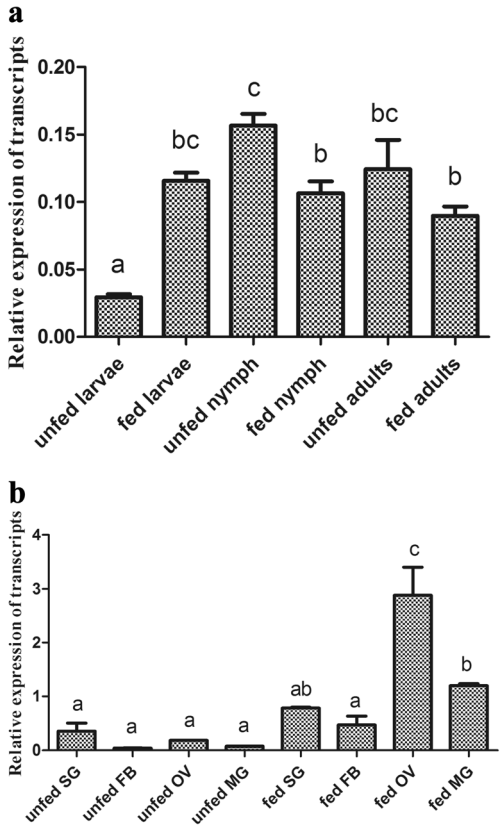

Fig. 3 Expression profile of RH-Hsc70 mRNA in ticks *R. haemaphysaloides*. **a** Real-time quantitative PCR analysis of RH-Hsc70 mRNA expression in larvae, nymphs, and adult ticks at different blood-feeding stages by one-way ANOVA. **b** Relative expression of RH-Hsc70 mRNA versus ELF1α mRNA was investigated in four tick organs of unfed and fed adult ticks by one-way ANOVA. SG, salivary gland; MG, midgut; FB, fat body; OV, ovary. Data not sharing a common letter indicated there was a significant difference ($P < 0.05$)

GCC ACA GCC T-3′) were designed to determine the level of mRNA expression of the RH-Hsc70 gene. PCR was performed on QuantStudio 5 (ABI, USA) using SYBR Premix Ex Taq™II(Tli RNaseH PluS, Takara). Tick elongation factor 1α was used as an internal control because of its stable expression level in *R. microplus* and *R. appendiculatus* (Nijhof et al. 2009). The reactions were incubated for 30 s at 95 °C, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s, followed by analysis of the melting curve. All of the reactions were performed in triplicate. The $2^{-\triangle Ct}$ method was used to calculate the relative expression levels.

RNA interference for gene knockdown in ticks

Double-stranded RNA (dsRNA) was synthesized using the T7 RiboMAX™ RNAi System (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Primers containing T7 promoter sequences (in italics at the 5′-end) (RNAi-Hsc70-1F: 5′*GGATCCTAATACGACTCACT ATAGG*CTCGACAAGAAGGGCACG-3′; RNAi-Hsc70-1R: 5′-GAAGAAGTCCTGCAGGAGC-3′; RNAi-Hsc70-2F: 5′-CTCGACAAGAAGGGCACG-3′; RNAi-Hsc70-2R: 5′-*G G A T C C TA A TA C G A C T C A C TA TA G G* G A A G AAGTCCTGCAGGAGC-3′) were used to amplify the RH-Hsc70 cDNA. A dsRNA targeting luciferase was used as a negative control, and it was subjected to the same PCR amplification protocol using luciferase-specific primers (Yu et al. 2013). The quality of the dsRNA was determined by electrophoresis on a 1.5% agarose gel using a BioPhotometer. The dsRNA was stored at − 80 °C until use. Unfed adult ticks (N = 30 females per group, two independent groups) were microinjected with 0.5 μL dsRNA (about 1 μg) at the base of the fourth right leg of the ventral surface. Ticks in the control group were microinjected with luciferase dsRNA. After microinjection, ticks were maintained in an incubator at 25 °C with 95% humidity for 24 h, after which they were allowed to feed on rabbits. Four female ticks per group were collected after 5 days of feeding for RNA and protein extraction using TRIZOL Reagent (Sigma, USA) following the manufacturer's instructions. Real-time quantitative PCR was then used to characterize gene knockdown relative to the control. The remaining ticks were allowed to feed until full engorgement, and tick mortality, engorgement rate, and engorgement weight were determined for the individual female ticks.

Statistical analysis

The levels of RH-HSC70 mRNA in different organs or life stages were expressed as the mean expression ± SEM and analyzed by one-way ANOVA and the level of RH-HSC70 mRNA downregulation was analyzed by Student's t test. Differences in the 48-h attachment rate, engorgement rate, and death rates of ticks in different groups were tested by chi-square, which was performed using SPSS 20.0 software. A probability P value < 0.05 was considered statistically significant.

Results

Sequence and phylogenetic analysis of the RH-Hsc70 open reading frame

We isolated the RH-Hsc70 gene (comp82371_c0) from the sialotranscriptomes of unfed and fed *R. haemaphysaloides* (Table 1) (Yu et al. 2015). The open reading frame (ORF) is 1950 bp long and is predicted to encode a protein with a length of 649 amino acids, a molecular mass of 71.1 kDa, six N-glycosylation sites (underlined in red), and a pI of 5.43. The complete amino acid sequence of RH-Hsc70 contains three signature sequences (red boxes): IDLGTTYSC (aa 9–17), IFDLGGGTFDVSIL (aa 197–210), and IVLVGGSTRIPKIQK (aa 334–348). In addition, the predicted RH-Hsc70 protein includes two tetrapeptide motifs

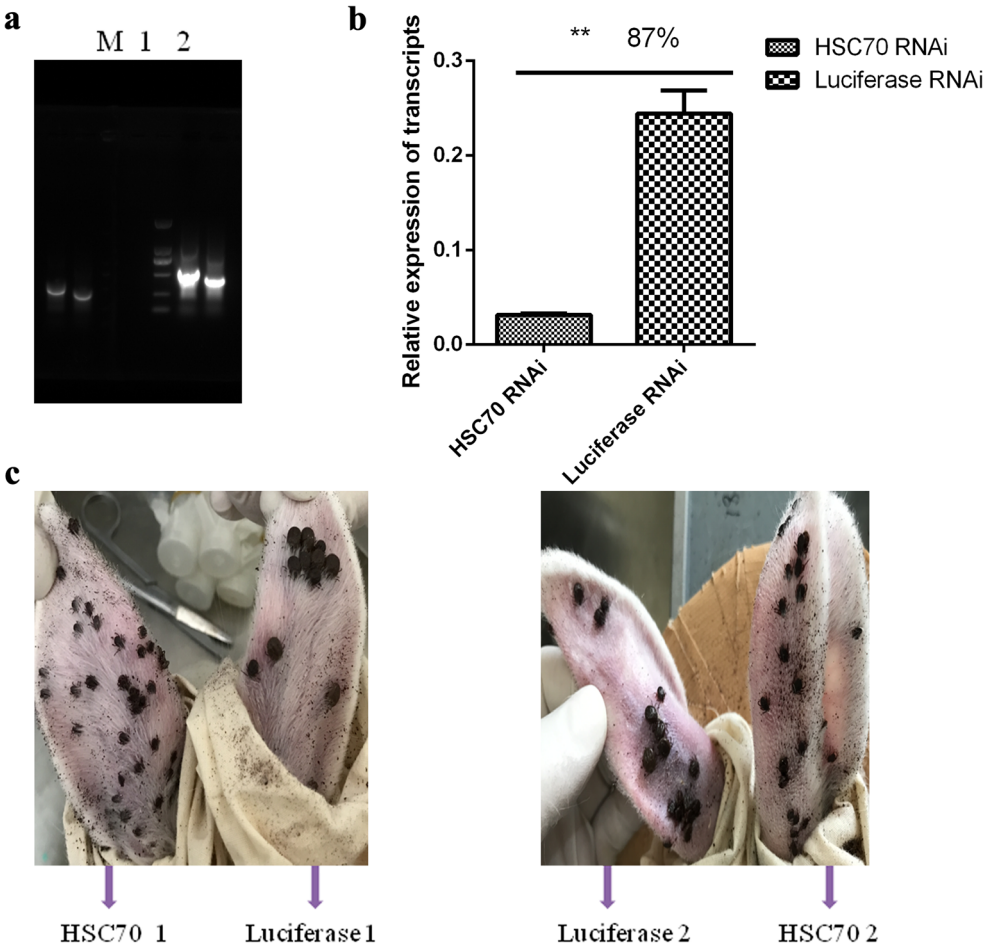

Fig. 4 Effect of the gene knockdown of RH-Hsc70 mRNA on tick blood feeding. **a** The quality of RH-Hsc70 dsRNA was determined by electrophoresis using 1.5% agarose gel. M, marker; 1 represents luciferase dsRNA; 2 means RH-Hsc70 dsRNA. **b** Relative expression of RH-Hsc70 mRNA after interference with RH-Hsc70 dsRNA versus luciferase dsRNA (control group). **c** Tick attachment at 5-day blood feeding between RH-Hsc70 dsRNA interference and luciferase gene knockdown. **Significant difference using Student's *t* test ($P < 0.01$)

(GGMP and GGFP, green boxes), a putative bipartite nuclear location signal (KK and RRLRT, underlined in green), a potential non-organelle eukaryotic consensus motif (RARFEEL, underlined in blue), an ATP/GTP binding site motif A (AEAYLGKT, black box), and a cytosolic localization signal at the extreme C-terminus (SGPTIEEVD, blue box) (Fig. 1), which is consistent with the protein structure of Hf-Hsc70. Based on these structural characteristics, we concluded that the protein was RH-Hsc70.

Gene alignment and phylogenetic analyses performed on available amino acid sequences using MEGA 5.0 software (Fig. 2) showed that RH-Hsc70 is highly conserved in arthropods and mammals, with greater than 83% identity. Notably, it is also highly homologous to Hsc70 in *Haemaphysalis flava* (98%) and *Ixodes scapularis* (97%), which indicates that RH-Hsc70 is an evolutionarily conserved gene.

RH-Hsc70 mRNA expression patterns at different developmental stages and in different tick organs

RH-Hsc70 was expressed during multiple developmental stages, and primarily in nymphs and adult ticks. RH-Hsc70 was not expressed in unfed larvae, but was induced in larvae during blood feeding (Fig. 3a). Unfed and fed adult ticks were dissected to obtain the salivary glands, fat bodies, midguts, and ovaries. Real-time quantitative PCR analysis indicated that RH-Hsc70 mRNA expression was higher in all of the organs from fed adult ticks compared with the organs from unfed adult ticks. In addition, the RH-Hsc70 gene was expressed at higher levels in the ovaries (OV) of fed adult ticks than that in midguts (MG), salivary glands (SG), and fat bodies (FB), regardless of the feeding status (Fig. 3b).

Table 2 Effect of RH-Hsc70 gene knockdown on tick feeding and death

Groups	Attachment rate in 48 h	Engorged body weight (mg)	Engorgement rate[a]	Death rate[a]
RH-Hsc70	94.06% (95/101)	–	0%	100%
Luciferase	96% (48/50)	415.47 ± 83.72	80.49% (33/41)	17.07% (7/41)

[a] Significant difference using the chi-square test

Effect of RH-Hsc70 mRNA knockdown on *R. haemaphysaloides* blood feeding

The quality of the isolated RH-Hsc70 dsRNA was determined by electrophoresis on a 1.5% agarose gel, as shown in Fig. 4a. Female ticks that had fed for 5 days were collected to detect the expression level of RH-Hsc70 mRNA. RH-Hsc70 mRNA expression decreased by 87% after microinjection with RH-Hsc70 dsRNA, compared with the luciferase RNAi group (Fig. 4b). When RH-Hsc70 gene expression was silenced, the mean 48-h attachment rate was 94.1%, the death rate reached up to 100%, and no engorged females were observed. In the luciferase RNAi group, the mean 48-h attachment rate was 96%, the death rate was 17%, and the average engorgement rate was 80.48%. These results show that downregulation of RH-Hsc70 expression significantly decreased the tick engorgement rate and increased the tick death rate (Table 2). Although ticks that were microinjected with RH-Hsc70 dsRNA attached to rabbit ears (Fig. 4c), they did not engage in blood feeding or become engorged (Fig. 4d), indicating that RH-Hsc70 expression promotes tick blood feeding.

Discussion

Heat shock proteins are molecular chaperones that are conserved in *I. scapularis, H. flava,* and *H. longicornis* ticks (Busby et al. 2012; Liu et al. 2017; Tian et al. 2011) and affect stress responses (Lu et al. 2017; Shahein et al. 2010; Villar et al. 2010). However, little is known about the Hsc70 gene in ticks. Here, we found that the RH-Hsc70 gene sequence was highly homologous to Hsc70 in other tick species, as it had 98% identity with *Haemaphysalis flava* Hsc70 and 97% identity with *I. scapularis* Hsc70, indicating that RH-Hsc70 is evolutionarily conserved. RH-Hsc70 mRNA was expressed at higher levels in all of the organs of fed adult ticks compared with organs of unfed adult ticks, indicating that RH-Hsc70 expression is induced by blood feeding. Although we originally isolated the RH-Hsc70 gene from *R. haemaphysaloides* sialotranscriptomes (Yu et al. 2013), we found that RH-Hsc70 was expressed at a significantly higher level in the ovaries than that in the midguts and salivary glands, which is consistent with the expression pattern of Hsc70 in *H. longicornis* (Tian et al. 2011).

Blood feeding is essential for reproduction in adult female ticks (Gulia-Nuss et al. 2016). Although the data are as yet inconclusive, there is increasing evidence that nutrient transportation is critical for ovary development and embryonic development in ticks. In ticks, blood feeding triggers salivary gland degeneration (Friesen and Kaufman 2009) and ovary maturation (Ullah and Kaufman 2014), while oviposition leads to the aging of the ovary and salivary gland tissue, and ultimately death in adult female ticks (Freitas et al. 2007; L'Amoreaux et al. 2003). In this study, we found that RH-Hsc70 gene silencing significantly inhibited blood feeding and increased the tick death rate, which further verified that RH-Hsc70 plays an important role in blood feeding and would be a useful candidate for an anti-tick vaccine. Some *Drosophila melanogaster* HSPs are upregulated following exposure to oxidative stress and during the aging process (Morrow and Tanguay 2003). HSP expression and increased antioxidant activity extend the *D. melanogaster* lifespan by regulating the downstream protein autophagy-related gene 7 (Chen et al. 2012; Liao et al. 2008; Morrow et al. 2004; Sarup et al. 2014; Vos et al. 2016), while HSP depletion reduces the number of viviparous offspring and simultaneously increases the number of premature nymphs developing in the ovaries, suggesting an unexpected role in aphid embryogenesis and eclosion (Will et al. 2017). This shows that heat shock proteins play pleiotropic roles in embryogenesis, longevity, and fecundity, and that slower aging is associated with reproductive dormancy. The Hsc70 gene is highly homologous in invertebrates like ticks (Busby et al. 2012; Liu et al. 2017; Tian et al. 2011), sea urchins (Gonzalez-Aravena et al. 2018), and insects. However, understanding the relationship between RH-Hsc70 expression and tick aging, as well as the mechanism underlying this relationship, requires further study.

In this work, we isolated RH-Hsc70 from the sialotranscriptomes of unfed and fed *R. haemaphysaloides*, analyzed its expression pattern, and found that it played an important role in blood feeding. RH-Hsc70 silencing inhibited tick blood feeding, significantly decreased tick engorgement rate, and increased tick death rate. We believe that this study adds to our current knowledge of tick salivary gland development and blood feeding, and it could help lead to the discovery of novel target molecules for effective anti-tick vaccines.

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Compliance with ethical standards

The protocols (shvri-ra-0268) with New Zealand white rabbits were approved by the Institutional Animal Care and Use Committee of the Shanghai Veterinary Research Institute and authorized by the Animal Ethical Committee of Shanghai Veterinary Research Institute.

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

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