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Chapter 20

RNA Interference – A Powerful Functional Analysis Tool for Studying Tick Biology and its Control

Remil Linggatong Galay, Rika Umemiya-Shirafuji, Masami Mochizuki, Kozo Fujisaki and Tetsuya Tanaka

Additional information is available at the end of the chapter

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Abstract

Ticks (Acari: Ixodida) are blood-sucking arthropods globally recognized as vectors of numerous diseases. They are primarily responsible for the transmission of various pathogens, including viruses, rickettsiae, and blood parasites of animals. Ticks are second to mosquitoes in terms of disease transmission to humans. The continuous emergence of tick-borne diseases and acaricide resistance of ticks necessitates the development of new and more effective control agents and strategies; therefore, understanding of different aspects of tick biology and their interaction with pathogens is very crucial in developing effective control strategies. RNA interference (RNAi) has been widely used in the area of tick research as a versatile reverse genetic tool to elucidate the functions of various tick proteins. During the past decade, numerous studies on ticks utilized RNAi to evaluate potentially key tick proteins involved in blood feeding, reproduction, evasion of host immune response, interaction with pathogens, and pathogen transmission that may be targeted for tick and pathogen control. This chapter reviewed the application of RNAi in tick research over the past decade, focusing on the impact of this technique in the advancement of knowledge on tick and pathogen biology.

Keywords: Acari, ticks, Ixodidae, RNA interference, tick-borne diseases

1. Introduction

Ticks belong to the class of Arachnida together with spiders, scorpions, and mites. To date, there are about 900 species of ticks, majority of which are hard ticks belonging to the Ixodidae family, as well as about 200 species are soft ticks belonging to the Argasidae family, and a single species belonging to the Nuttalliellidae family [1]. Most of the ticks of medical and veterinary importance are hard ticks. Through their blood-feeding behavior, ticks can directly

affect their host by causing anemia, irritation, and allergic reactions particularly in heavy infestation. The saliva of some tick species may also contain neurotoxic substances that may cause the condition termed "tick paralysis" [2]. Additionally, the transmission of pathogens including viruses, bacteria, and parasitic protozoa also occurs during blood feeding [1]. Ticks are considered second to mosquitoes in terms of their impact on public health, but they are the most important vectors of different pathogens in both domestic and wild animals [3]. Tick infestation and tick-borne diseases (TBDs) continue to have great economic impact on livestock production, particularly on cattle and small ruminants, in several continents [2]. The annual loss in cattle production worldwide due to ticks and TBDs has been estimated to be worth billions of USD [4].

The complete dependence of ticks to host blood for the completion of their life cycle and generation of offspring is the reason for their notoriety as vectors of several diseases. Depending on the species, a tick may utilize one to three hosts during their life cycle. Most of the pathogens they transmit can be carried on throughout their life cycle through transstadial (from one stage to the next) transmission and to the next generation through transovarial (from adults to eggs) transmission [5]. A single tick may carry multiple pathogens [6], thereby having the potential of infecting a host with a cocktail of pathogens. Most tick-borne infections are zoonotic in nature, and more of these are being recognized in recent years [1, 7]. Among the TBDs that are well-known in the veterinary and medical field are anaplasmosis, borreliosis, rickettsiosis, ehrlichiosis, babesiosis, theileriosis, and tick-borne encephalitis.

The significant impact of ticks and TBDs underscores the importance of tick control. For several decades, the application of chemical acaricides has been the primary tick control method, and acaricides were used extensively in livestock production. However, the continuous emergence of resistant tick strains makes most chemical acaricides ineffective [8]. Moreover, the increasing concerns for animal product and environmental contamination set limitations for this control method. To search for new and more effective means of controlling ticks and TBDs, researchers have actively expanded the understanding on tick biology.

RNA interference (RNAi) is a reverse genetic approach for manipulation of genes that commonly utilizes double-stranded RNA (dsRNA) to induce post-transcriptional gene-specific silencing [9]. RNAi has been extensively employed in many studies on tick biology and pathogen interaction since the first report of RNAi application in the hard tick *Amblyomma americanum* [10]. In fact, it is evident that a number of laboratories in different countries working on tick research are routinely performing RNAi, as shown by an increasing number of recent publications utilizing this technique. Typically, functional studies using RNAi involve gene knockdown with subsequent infestation and evaluation of phenotypes, such as blood feeding and reproduction success (Figure 1). Indeed, RNAi has been particularly useful in searching for tick proteins that can be targeted for control of tick development and TBDs [11].

This chapter aims to show the extent of RNAi application in tick research, emphasizing the progress of advanced knowledge on tick biology and tick-pathogen interaction. We first discussed so far known RNAi mechanisms and the current RNAi inducing methods in ticks; then, briefly described the studies on tick physiology, immunity, and pathogen interaction that employed RNAi, highlighting the prospects of applications of RNAi in tick research.

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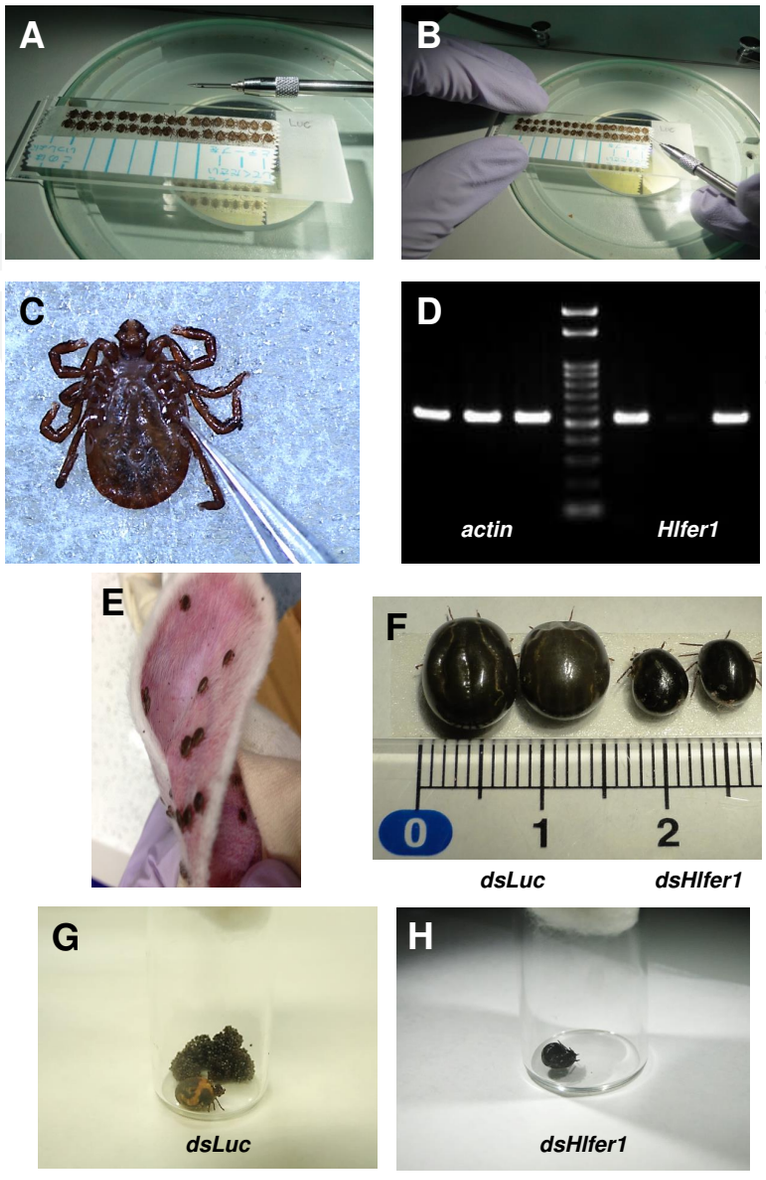

Figure 1. A typical RNAi experiment in the hard tick *Haemaphysalis longicornis*. The double-stranded RNA (dsRNA) is introduced to adult ticks by microinjection. Unfed adult ticks, placed on a double adhesive tape attached on a glass slide (A), are injected with dsRNA using a pointed microcapillary glass attached to a microinjector (B) through the membrane of the fourth coxa under a stereomicroscope (C). Successful silencing, as shown by the absence of a band for the target gene, such as *Hlfer1*, is confirmed around 4 d post-injection through RT-PCR after adjusting the cDNA level using an internal control, such as actin (D). Ticks were infested on a host and allowed to feed to repletion (E) and after dropping, parameters such as engorged body weight (F), survival, egg laying (G, H), and hatch were compared.

2. RNAi pathway in ticks

The mechanism of RNAi has been well studied in the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* [9, 12]. RNAi begins with the uptake of dsRNA by the cell, followed by its cleavage to produce small interfering RNAs (siRNAs). Cleavage of dsRNA is

accomplished by an RNAse III enzyme called Dicer. The siRNAs are then incorporated into RNA-induced silencing complex (RISC), which then drives the degradation or translational inhibition of the target mRNA that results to gene silencing. This silencing signal may spread among the cells and different tissues, leading to systemic gene silencing in the whole organism [13]. The mechanism of RNAi in ticks has not been fully elucidated, but the study of Kurscheid et al. [14] revealed that several components of RNAi machinery in other invertebrates are also present in the ticks, and they proposed a putative tick RNAi pathway. Here, we briefly describe the available knowledge on key components of RNAi machinery in ticks, in comparison of other invertebrates.

2.1. dsRNA uptake

There are two recognized dsRNA uptake mechanisms in invertebrates: a transmembrane channel-mediated uptake through systemic RNA interference defective (SID) transmembrane proteins described in *C. elegans*, and an endocytosis-mediated uptake described in most arthropods [15, 16]. Several SIDs identified in *C. elegans* have been shown to be involved in the spread of RNAi [17]. SID-1, SID-3, and SID-5, which have wide tissue distribution, are involved in the systemic spread of RNAi [18–20], whereas SID-2, localized mainly in the gut, is involved primarily in the intestinal uptake of ingested dsRNA [21]. The multi-domain SID-1 along the plasma membrane facilitates the traffic of dsRNA into and out of the cells. Homologs of SID-1 are present in some arthropods and vertebrates [18]. Both SID-3, a conserved tyrosine kinase, and SID-5 have intracytoplasmic localization, the latter being associated with late endosomes [19, 20]. SID-2 has luminal localization in the intestinal cells, and it was also found in the lower levels of excretory duct cells [21]. In addition to SIDs, endocytosis has also been also implicated as a dsRNA uptake mechanism in *C. elegans* through a protein containing an epsin N-terminal homology (ENTH) domain [22]. In *D. melanogaster*, dsRNA uptake in cells is facilitated mainly by scavenger receptor-mediated endocytosis [23]. Two scavenger receptors, Eater and Sr-CI, have been identified to be responsible for the majority of dsRNA uptake. These scavenger receptors are mainly expressed in the plasmatocytes and have a primary role in the phagocytosis of bacterial pathogens [24, 25].

SID homologues have not been identified in ticks. However, a homologue of ENTH, Epn-I, has been identified in the hard ticks *Rhiphicephalus* (*Boophilus*) *microplus* and *Ixodes scapularis* [14]. A class B scavenger receptor identified in *Haemaphysalis longicornis* (HlSRB) has been demonstrated to mediate systemic RNAi in this tick [26, 27]. Combined injection of dsRNA against *HlSRB* and other target genes, *Vitellogenin*-1 (*HlVg-1*) and *Vitellogenin Receptor* (*HlVgR*) effectively silenced these genes. However, silencing *HlSRB* prior to injection of dsRNA against *HlVg-1* and *HlVgR* inhibited the silencing of the latter two genes, suggesting that the uptake of the injected dsRNA is dependent on HlSRB in ticks. Similar to *D. melanogaster* scavenger receptors, HlSRB is also involved in the phagocytosis of bacteria [28], but it is expressed not only in the hemocytes but also in the other organs such as midguts, salivary glands, and ovary [26]. The presence of ENTH homologue and scavenger receptor indicates that the uptake of dsRNA in ticks is through endocytosis. Additionally, the presence of scavenger receptor in different tick tissues strongly implies its involvement in systemic RNAi, particularly after

dsRNA injection. Introduction of dsRNA into the hemocoel of ticks directly exposes the different tick organs to dsRNA, and the scavenger receptor in these organs most likely mediates the entry of dsRNA into the cells.

2.2. dsRNA processing and RISC assembly

The recommended length of dsRNA to effectively induce silencing of the target gene in non-mammalian systems is more than 200 bp [15]. A study in *R.* (*B.*) *microplus* showed, however, that short dsRNAs between 100 and 200 bp were also effective in inducing silencing of *Ubiquitin-63E* homologue, with minimal off-target effects, but short hairpin dsRNAs were not able to induce silencing effects [29]. After cellular uptake, dsRNAs are cleaved into 21–25 nt siRNAs by an RNAse III enzyme called Dicer. In contrast to *C. elegans* and mammals that have only one Dicer, *D. melanogaster* and mosquitoes have two Dicers [15]. Dicer-2 is the one involved in the generation of siRNA, whereas Dicer-1 acts on stem loop RNA precursors to generate micro RNA (miRNA). Both, however, are required for siRNA-induced gene silencing due to their distinct roles in siRISC assembly [30]. Only a single putative Dicer has been identified so far in the hard tick *I. scapularis*, which is more similar to mammalian Dicer-1 [14].

The RNAi inhibition of a target mRNA is accomplished by RISC formed by siRNAs and Argonaute (AGO) proteins. AGO proteins are highly conserved between species, encoded by multiple genes in most organisms. All AGO proteins are characterized by two domains: the PAZ domain and the PIWI domain [31]. Upon ATP activation, AGO mediates RISC recognition of mRNA target that are homologous to siRNAs, subsequently leading to the cleavage of the mRNA target [9]. In most insects, including *D. melanogaster* and mosquitoes, five AGO genes have been identified [15, 31]. In ticks, a homologue of AGO-1 has been identified in *I. scapularis* and *R.* (*B.*) *microplus,* and a homologue of AGO-2 has been identified in *I. scapularis* [14]. However, the functions of these tick AGOs remains to be confirmed.

2.3. Amplification of RNAi signal

The ability to spread throughout the whole organism, inducing total systemic silencing of the target gene in spite of introducing only a relatively small amount of dsRNA, is an important aspect of RNAi observed in plants and invertebrates. This systemic RNAi-induced gene silencing in both plants and *C. elegans* involves RNA-directed RNA polymerase (RdRP) that amplifies the RNAi signal [9]. RdRP function in RNAi has not been found in arthropods, but a putative homologue of RdRP EGO-1 protein of *C. elegans* has been identified in the hard tick *I. scapularis*, and a partial sequence was also identified from *R.* (*B.*) *microplus* [14].

3. Methods of introducing dsRNA in ticks

3.1. Injection

Direct injection is the most widely used technique for introducing dsRNA for in vivo gene silencing, not only in ticks but also in insects [11, 32]. Through this method, dsRNA is usually

introduced directly into the hemocoel of ticks allowing the dsRNA to circulate within the hemolymph. In most reports, a high concentration of at least 1 μg dsRNA per tick has been shown to be effective in inducing gene silencing [33], but in some reports, lower concentration has been found to be similarly effective [34–36]. Injection has been accomplished using a 33–36-gauge needle attached to a Hamilton syringe particularly in large tick species, such as *Amblyomma americanum*[37], *Dermacentor variabilis*, and *D. marginatus* [38], while microinjection using a microcapillary drawn to a fine point needle and inserted to a micromanipulator has been commonly employed in smaller tick species, including *Ixodes* [39], and *Haemaphysalis* [26] ticks. Different injection sites include the lower right quadrant of the ventral surface of the exoskeleton [40], the groove between the basis capituli and the scutum [37], the ventral torso of the idiosoma, away from the anal opening [39], and the coxal membrane in the fourth coxae [26, 41]. In some reports, dsRNA was injected through the spiracle [29, 42, 43] and anal pore; the latter inducing midgut-specific silencing of the target gene [44]. Injection of dsRNA has been commonly performed in unfed adult ticks, subsequently allowed to recover for at least 24 h before infestation or use in succeeding experiments. Exceptionally, dsRNA has been also injected in engorged *R.* (*B.*) *microplus* [42, 45-47], *A. americanum, I. scapularis*, and *D. variabilis* adults [43], which produced significant effects on the eggs and larvae, and microinjection has also been accomplished in *I. scapularis* [48-52] and engorged *O. moubata* nymphs [53].

3.2. Soaking

Soaking in dsRNA has been previously employed to study RNAi in the cell lines of *D. melanogaster* [23, 54]. In tick research, this method has been applied to induce in vitro RNAi not only in cell cultures [55-57], but also in some organs including whole salivary glands [36, 58-60] and midguts [61]. Soaking live *Varroa destructor* [62] and *Dermanyssus gallinae* [63] mites, as well as *Aedes aegypti* larvae [64] in a solution of dsRNA has been already demonstrated in producing effective silencing of the target genes in these organisms. However, soaking whole ticks in a solution of dsRNA has not been commonly performed. Soaking *Haemaphysalis longicornis* nymphs in a solution of dsRNA for 24 h resulted to significant transcript reduction of the target gene, although the effect on the phenotype was not observed in all the nymphs [65]. In our laboratory, we have attempted to soak *H. longicornis* larvae, nymphs, and adults in a dsRNA solution overnight, which resulted to a significant decrease in the mRNA level of a targeted gene (Galay et al., unpublished results). Soaking offers a simpler and less invasive method of introducing dsRNA without injuring the ticks and is applicable to immature tick stages. Furthermore, it does not require injection equipment; therefore, it is less laborious.

3.3. Electroporation

Electroporation is a technique that employs electric impulses to promote DNA uptake of cells and has been primarily used with in vitro cell transfection [66]. In tick research, this technique has been first applied to facilitate the introduction of dsRNA in *I. scapularis* eggs and nymphs [67]. After electroporation, fluorescein-labeled dsRNA was visualized all over the nymph's body and eggs, indicating the successful entry of dsRNA. In a more recent report, the wax coating of the eggs was first removed using heptane and hypochlorite prior to electroporation

[68]. Using heptane alone did not significantly decrease the hatching rate. Thus, heptane may be more helpful in evaluating the effect of a particular dsRNA to egg hatching. This technique also offers a less invasive method of dsRNA introduction that can be applied to immature tick stages and eggs.

3.4. Feeding

Feeding dsRNA in insects has been achieved in different species using diets mixed with dsRNA, liposome-embedded or lipophilic siRNAs, and bacteria and transgenic plants that can synthesize dsRNA [32, 69]. Although in vitro feeding assays have been shown to be useful in studying different tick molecules and tick-pathogen interaction [70], its application in RNAi study in ticks has been limited. A study on the Lyme disease vector *I. scapularis* employed capillary feeding of dsRNA to nymphs to suppress anticomplement gene *isac* [71]. In another study, adult *R.* (*B.*) *microplus* ticks were capillary fed with *ubiquitin* dsRNA mixed in whole blood or *Bm86* dsRNA mixed in bovine serum [72]. In both cases, ticks were pre-fed in an animal host before capillary feeding was performed. While this method may be advantageous over injection due to very minimal injury, drawbacks may arise from the uncertainty whether an individual tick will ingest the amount of dsRNA that will effectively induce silencing, and the possibility of variation in the amount of dsRNA ingested by the ticks within a treatment group. Furthermore, capillary feeding is difficult to perform and may not be applicable in ticks with short hypostome.

4. RNAi and study of tick physiology

4.1. Genes related to salivary functions

The saliva is an important arsenal of ticks containing hundreds of pharmacologically potent substances that facilitate attachment to their hosts and blood-sucking [73]. Different salivary proteins have redundant functions in counteracting the hemostatic [74], inflammatory, and immune mechanisms [75] of the host. Aside from its function in tick feeding, the salivary glands are also involved in osmoregulation and transmission of pathogens [76].

Many studies on characterization of salivary proteins in the recent years employed RNAi (Table 1). In fact, the first report on the application of RNAi in tick research described a tick inhibitor of inflammatory mediator, a salivary histamine-binding protein, wherein researchers induced in vitro RNAi by soaking salivary glands in dsRNA [10, 58]. Soluble N-ethylmaleimide-sensitive factor attachment receptors (SNARE) complex proteins, which mediate exocytosis in secretory pathways of the salivary glands, have been characterized in *Amblyomma* ticks. These include N-ethylmaleimide-sensitive fusion (NSF) protein, Synaptosomal Associated Protein of 25 kDa (SNAP-25) [77], Ykt6 [65], and vesicle transport through interaction with t-SNAREs (Vti) [78]. Silencing various genes such as Salp14, Salp9pac [39], Neuronal isoform munc18-1 (nSec1) [59], and synaptobrevin [36] affected the secretion of

anticoagulant or the anticoagulant activity of salivary gland extracts, indicating that these genes are important in tick anti-hemostatic mechanism.

Longistatin [79] and acidic chitinases [80] have been found to be important in the formation of blood pool and tick cement cone, respectively. The attachment site of *longistatin*-silenced *H. longicornis* ticks did not show pathological changes, such as hemorrhagic lesions corresponding to the blood pool, while the attachment site of ticks simultaneously silenced acidic chitinases exhibited blood leakage and these ticks can be easily removed. Various protease inhibitors that have roles in anti-hemostatic, anti-inflammatory, and immunomodulatory mechanism have been also characterized using RNAi, including a cystatin, sialostatine L [81], a Kunitz type protease inhibitor, rhipilin [82], and serine protease inhibitors (serpin) [83, 84].

Other salivary proteins with immunomodulatory function, such as the anti-complement protein, isac [71], and two proteins that can inhibit neutrophil function, ISL 929 and 1373 [85], have also been knockdowned in *I. scapularis*. Silencing of *isac* in nymphs, induced by capillary feeding of dsRNA, not only reduced blood feeding, but also decreased the load of the spirochete *Borrelia burgdorferi* in the tick. Meanwhile, the saliva of ticks devoid of ISL 929 and 1373 had reduced ability in inhibiting host integrin. An osmoregulatory protein aquaporin, characterized in *I. ricinus* through RNAi, showed that suppression of this protein impaired the concentration of blood meal due to failure in removing water [86].

4.2. Genes related to digestion and midgut function

The midgut of ticks houses various kinds of enzymes that act on a large amount of ingested host blood, which contains great quantities of hemoglobin [151]. Functional studies on these enzymes and other midgut proteins using RNAi have expanded the understanding of tick digestive physiology (Table 1). Silencing hemoglobinolytic enzymes, such as leucine aminopeptidase [91, 92], longipain [95], and cathepsin L [96, 97] had negative impact on tick feeding. Moreover, the longipain of *H. longicornis* was found to have a protective role in *Babesia* infection through its babesiacidal activity [95]. Other proteins important in tick digestion that have been characterized using RNAi are thrombin inhibitors that prevent blood coagulation and serine proteinase, which induce erythrocyte degradation. Silencing of thrombin inhibitor hemalin from *H. longicornis* [93] and boophilin from *R.* (*B.*) *microplus* [47] prolonged the blood feeding period and decreased the oviposition of these ticks, respectively. Silencing serine protease reduced the weight of ticks after blood feeding due to impaired erythrocyte degradation [94].

4.3. Genes related to reproductive function

Ticks are known for their high fecundity, laying hundreds of eggs per batch in the case of soft ticks and up to thousands in the case of hard ticks. A series of physiological events takes place in female ticks during and after blood feeding that initiate ovarian maturation and subsequent oviposition. Vitellogenesis, the synthesis and oocyte deposition of the yolk protein precursor (vitellogenin), is a key process for ovarian development and oocyte maturation induced by blood meal in ticks [152]. Three genes encoding vitellogenin have been identified and characterized in *H. longicornis* [102].

Target gene	Tick species	RNAi Effect	Refs
Salivary proteins			
Histamine-binding protein (HBP)	*Amblyomma americanum*	Altered feeding pattern and longer feeding period; decreased histamine-binding activity in the salivary glands	[10, 58]
Salp14/ Salp9pac	*Ixodes scapularis*	Impaired feeding, decreased post-blood meal weight, decreased anticoagulant activity of salivary gland extract	[39]
Neuronal isoform munc18-1 (nSec1)	*A. americanum*	Decreased post-blood meal weight and prolonged feeding time, decreased anticoagulant secretion of salivary gland	[59]
Synaptobrevin	*A. americanum*	Inhibited secretion of anticoagulant stimulated by PGE	[36]
Cystatin	*A. americanum*	Decreased post-blood meal weight, mortality during feeding, low feeding success rate	[61]
Anticomplement protein (Isac)	*I. scapularis*	Decreased post-blood meal weight, decreased *Borrelia burgdorferi* infection	[71]
Sialostatin L (cystatin)	*I. scapularis*	Failure to feed on the host, decreased post-blood meal weight and failed oviposition	[77]
Aquaporin	*I. ricinus*	Decreased post-blood meal weight, decreased volume of ingested blood	[78]
HlYkt6 (SNARE)	*Haemaphysalis longicornis*	Decreased post-blood meal weight, high mortality, supressed salivary secretion and anticoagulant activity	[65]
ISL 929 and 1373	*I. scapularis*	Supressed PMN inhibitory activity of saliva from knockdowned ticks	[79]
Rhipilin (Kunitz type protease inhibitor)	*Rhipicephalus haemaphysaloides*	Prolonged attachment time, decreased post-blood meal weight	[80]
Longistatin	*H. longicornis*	Mortality after attachment, failure to engorge, poor blood pool formation	[81]
Serine protease inhibitor (serpin)	*A. americanum*	No effect on tick attachment, feeding and oviposition	[82]
	R. haemaphysaloides	Decreased attachment rate and engorgement weight	[83]
Reprolysin	*Rhipicephalus* (*Boophilus*) *microplus*	Decreased egg weight and egg conversion ratio	[84]
N-ethylmaleimide sensitive fusion protein (NSF)	*A. maculatum*	Inhibition of engorgement, failure of oviposition	[85]

Target gene	Tick species	RNAi Effect	Refs
Synaptosomal Associated Protein of 25 kDa (SNAP-25)	*A. maculatum*	Decreased post-blood meal weight, decreased egg weight, failure in hatching	[85]
Vti (SNARE)	*A. americanum*, *A. maculatum*	Decreased post-blood meal weight and survival, failed oviposition	[86]
Glutaminyl cyclase (QC)	*A. maculatum*, *I. scapularis*	Decreased post-blood meal weight, egg weight and hatch	[87]
AV422	*A. americanum*	Decreased post-blood meal weight	[88]
Acidic chitinase (Ach)	*A. americanum*	Leakage of blood from the mouthparts in late feeding phase, loose attachment in the host's skin	[89]
Digestive activity			
Longepsin	*H. longicornis*	No effects reported	[90]
Leucine aminopeptidase	*H. longicornis*	Extended pre-oviposition period, decreased egg weight and egg conversion ratio, morphological abnormalities in the oocytes	[91, 92]
Hemalin (thrombin inhibitor)	*H. longicornis*	Longer blood feeding period, failure to engorge, decreased inhibitory activity of fibrinogen clot formation in the midgut	[93]
Boophilin (thrombin inhibitor)	*R.* (*B.*) *microplus*	Decreased oviposition	[47]
Serine proteinase	*H. longicornis*	Suppressed erythrocyte degradation; decreased post-blood meal weight	[94]
Longipain	*H. longicornis*	Impaired blood feeding, decreased post-blood meal weight, increased *B. gibsoni* infection level and transovarial transmission	[95]
Cathepsin L	*I. ricinus*	Decreased weight gain	[96]
	H. longicornis	Decreased post-blood meal weight	[97]
Astacin	*R.* (*B.*) *microplus*	Decreased egg weight and egg conversion ratio	[84]
Tick reproduction			
Follistatin-related protein (FRP)	*H. longicornis*	Decreased egg conversion ratio	[98]
Vitellogenin receptor (VgR)	*Dermacentor variabilis*	Failure of Vg uptake by oocytes; failed oviposition	[34]
	H. longicornis	Suppressed oocyte maturation and failed oviposition, failure of *B. gibsoni* transovarial transmission	[99]

Target gene	Tick species	RNAi Effect	Refs
	A. hebraeum	Suppressed oocyte maturation, long pre-oviposition period,	[100]
Voraxin	*A. americanum*	Failure to engorge and lay eggs in females fed with males injected with a combination of subolesin and voraxin dsRNA	[101]
Vitellogenin (Vg)	*H. longicornis*	Decreased post-blood meal weight, abnormal oocytes, decreased egg conversion ratio	[102]
GATA factor	*H. longicornis*	Disrupted egg development	[103]
S6 kinase	*H. longicornis*	Disrupted egg development	[103]
Target of rapamycin (TOR)	*H. longicornis*	Decreased post-blood meal weight, mortality after engorgement, Failure of oocytes to mature and failure to lay eggs	[104]
Structural and metabolic function			
Glutamine:fructose-6-phosphate aminotransferase (HlGFAT)	*H. longicornis*	Decreased post-blood meal weight and survival	[105]
β-Actin	*I. scapularis*	Decreased post-blood meal weight and oviposition	[106]
$Na^{+}K^{+}$-ATPase	*I. scapularis*	Decreased post-blood meal weight and oviposition	[106]
Valosin-containing protein (HlVCP)	*H. longicornis*	Decreased post-blood meal weight	[107]
Cyclophilins (Immunophilin)	*H. longicornis*	Lower post-blood meal weight, low survival after blood feeding and failure to lay eggs after silencing cyclophilin A	[108]
Ribosomal protein P0	*H. longicornis*	Decreased post-blood meal weight, low engorgement rate, and high mortality	[109]
Protein disulphide isomerases (PDI)	*H. longicornis*	Mortality after engorgement, leakage of blood from the midgut, little egg output	[110]
Organic anion transporter polypeptide (OATP)	*A. americanum*	Decreased post-blood meal weight, oviposition and egg conversion ratio	[37]
Ferritins (FER)	*I. ricinus*	Decreased post-blood meal weight, oviposition and hatch	[111]
	H. longicornis	Decreased post-blood meal weight, survival, oviposition and hatch	[112, 113]
Iron regulatory protein (IRP1)	*I. ricinus*	Decreased post-blood meal weight and hatching of eggs	[111]

Target gene	Tick species	RNAi Effect	Refs
Elongation factor 1-α	*R. annulatus, R.* (*B.*) *microplus*	High post-blood meal mortality, decreased post-blood meal weight and failure of oviposition	[45]
Lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH)	*H. longicornis*	Longer blood feeding period, decreased post-blood meal weight, longer pre-oviposition period, decreased oviposition and hatch after SDH silencing; higher volume of hemolymph after LKR silencing	[114]
Ubiquitin	*R.* (*B.*) *microplus*	Shorter post-blood meal survival, decreased or absence of egg output, impaired embryogenesis	[14, 29, 45, 72];
	R. annulatus	High mortality	[45]
Glycogen synthase kinase-3 (GSK-3)	*R.* (*B.*) *microplus*	Decreased oviposition and hatching	[115]
CD147 receptor	*A. americanum*	Inhibited feeding, low post-blood meal weight tender cuticle	[116]
Insulin-like growth factor binding protein-related proteins	*A. americanum*	Decreased post-blood meal weight	[117]
Putative 5.8S, ITS2 and 28S rRNA	*A. americanum*	High mortality and very low post-blood meal weight	[118]
Putative 2B7 60S ribosomal protein L13e	*A. americanum*	High mortality and very low post-blood meal weight	[118]
Putative interphase cytoplasm foci protein 45	*A. americanum*	High mortality and very low post-blood meal weight	[118]
Putative threonyl-tRNA synthetase	*A. americanum*	High mortality and very low post-blood meal weight	[118]
Putative 60S ribosomal protein L13a	*A. americanum*	100% mortality	[118]
Putative mitochondrial 12S rRNA	*A. americanum*	High mortality and very low post-blood meal weight	[118]
Chymotrypsin inhibitor (HlChI)	*H. longicornis*	Mortality after attachment, retarded blood feeding and longer feeding period, decreased post-blood meal weight, decreased egg weight and egg conversion ratio	[119]
Scavenger receptor	*H. longicornis*	Decreased post-blood meal weight, mortality after engorgement, decreased oviposition and hatch; inhibited bacterial phagocytosis of granulocytes	[26, 28]

Target gene	Tick species	RNAi Effect	Refs
4E-BP (eIF4E-binding protein)	*H. longicornis*	Decreased lipid accumulation in the midgut and fat bodies after long starvation period	[120]
Protein kinase B (AKT)	*H. longicornis*	Inhibition of engorgement and growth of organs during blood feeding; decreased expression of *longepsin*, *HlMIF* and *HlVg*s	[121]
	R. (*B.*) *microplus*	Decreased cell glycogen content and viability, and altered cell membrane permeability	[57]
Spook (Spo)	*Ornithodoros moubata*	Arrested development and molting	[53]
Shade (Shd)	*O. moubata*	Abnormal ecdysis and delayed molting	[53]
Cystatin (RHCyst)	*R. haemaphysaloides*	Decreased attachment and hatching rate	[122]
Tropomyosin	*H. longicornis*	Longer feeding time, decreased engorgement rate and post-blood meal weight, high mortality after blood feeding, failed oviposition	[123]
Protective antigens			
Subolesin (4D8)	*I. scapularis*	Decreased post-blood meal weight, oviposition and survival; failure of embryogenesis; silencing in eggs and larvae when dsRNA injected to engorged females	[43, 124]
	A. americanum	Decreased post-blood meal weight, oviposition and survival	[43, 101, 124]
	D. marginatus	Decreased post-blood meal weight, oviposition and survival	[124]
	D. variabilis	Decreased post-blood meal weight, oviposition and survival; decreased fertility; silencing in eggs and larvae when dsRNA injected to engorged females	[43, 124]
	R. sanguineus	Decreased post-blood meal weight, oviposition and survival; more dramatic effect when simultaneously silenced with Rs86	[124, 125]
	R. (*B.*) *microplus*	High mortality, decreased post-blood meal weight, oviposition and hatch in dsRNA-injected adults and progeny of dsRNA-injected adults	[42, 45, 46]
	R. annulatus,	Decreased post-blood meal weight	[45, 126]
	O. erraticus	Decreased egg output	[127]
	O. moubata	Decreased egg output	[127]

Target gene	Tick species	RNAi Effect	Refs
Midgut protein Rs86	*R. sanguineus*	Decreased post-blood meal weight and oviposition	[125]
Midgut protein Hl86	*H. longicornis*	Decreased post-blood meal weight	[128]
Midgut protein Bm86	*R.* (*B.*) *microplus*	Decreased number of engorging ticks, lower post-blood meal body weight and survival after feeding in *B. bovis*-infected host, decreased egg weight	[129]
Midgut protein Ree86	*R. evertsi evertsi*	No significant effect	[130]
Midgut protein ReeATAQ	*R. evertsi evertsi*	No significant effect	[130]
Longicin	*H. longicornis*	Decreased post-blood meal weight, increased *B. gibsoni* infection in the midgut and ovary, and transmission in the eggs	[131]
α2-macroglobulin proteins	*I. ricinus*	Decreased phagocytic action of hemocytes	[132, 133]
Macrophage migration inhibitory factor	*A. americanum*	No effect on phenotypes	[134]
Janus kinase (JAK)–signaling transducer activator of transcription (STAT) pathway	*I. scapularis*	Increased *A. phagocytophilum* infection level	[135]
Dual oxidase (Duox)	*I. scapularis*	Decreased level of *B. burgdorferi*	[136]
Peroxidase ISCW017368	*I. scapularis*	Decreased level of *B. burgdorferi*	[136]
Glutathione S-transferase	*R.* (*B.*) *microplus*	Decreased tick attachment and post-blood meal weight	[45]
	R.sanguineus	Increased susceptibility to permethrin	[137]
Selenoprotein W	*R.* (*B.*) *microplus*	Decreased tick attachment and post-blood meal weight	[45]
Selenoprotein K	*A. maculatum*	Decreased oviposition	[138]
Selenoprotein M	*A. maculatum*	Decreased oviposition	[138, 139]
Thioredoxin reductase	*A. maculatum*	Decreased native microbial load in midguts and salivary glands	[139]
Rmcystatin3 (cysteine protease inhibitor)	*R.* (*B.*) *microplus*	Increased resistance to bacteria	[140]
Pathogen acquisition/ transmission			
Subolesin	*D. variabilis*	Inhibited *Anaplasma marginale* infection in salivary glands	[141, 142]

Target gene	Tick species	RNAi Effect	Refs
	R. (*B.*) *microplus*	Decreased *A. marginale* infection level in salivary glands and tick cells	[143]
Salp15	*I. scapularis*	Decreased *Borrelia burgdorferi* transmission to the host	[52]
Salp14	*I. scapularis*	No effect on acquisition of *A. phagocytophilum* and *B. burgdorferi* in nymphs	[49]
Salp16	*I. scapularis*	Reduced *A. phagocytophilum* acquisition	[48]
Salp25D	*I. scapularis*	Decreased acquisition of *B. burgdorferi* after knockdown in salivary glands	[44]
Varisin	*D. variabilis*	Decreased *A. marginale* infection level	[144]
Immunophilin	*R.* (*B.*) *microplus*	Decreased hatch, decreased larval survival, increased *B. bovis* infection in larval progeny	[41]
Kunitz-type serine protease inhibitor (Spi)	*R.* (*B.*) *microplus*	Inhibition of engorgement, decreased egg weight	[41]
Glutathione S-transferase	*D. variabilis*	Inhibited *A. marginale* infection	[142]
H^+ transporting lysosomal vacuolar proton pump (vATPase)	*D. variabilis*	Inhibited *A. marginale* infection in the midgut after acquisition feeding	[142]
Selenoprotein M	*D. variabilis*	Inhibited *A. marginale* infection and multiplication in salivary glands	[142]
Putative von Willebrand factor (94Will)	*R.* (*B.*) *microplus*	Decreased *A. marginale* infection level in salivary glands	[143]
Flagelliform silk protein (100Silk)	*R.* (*B.*) *microplus*	Decreased *A. marginale* infection level in salivary glands and tick cells	[143]
Putative metallothionein (93 Meth)	*R.* (*B.*) *microplus*	Increased *A. marginale* infection level in tick cells	[143]
Tick salivary lectin pathway inhibitor (TSLPI)	*I. scapularis*	Decreased load of *B. burgdorferi* and transmission to host	[145]
Kunitz-type serine protease inhibitor (DvKPI)	*D. variabilis*	Increased rickettsial infection in the midgut	[146]
Kunitz-type protease inhibitor 5 (KTPI)	*R. annulatus*, *R.* (*B.*) *microplus*	Decreased post-blood meal weight	[126]
Histamine release factor	*I. scapularis*	Decreased post-blood meal weight, decreased *B. burgdorferi* transmission	[147]
TROSPA	*R. annulatus*, *R.* (*B.*) *microplus*	Decreased *B. bigemina* infection level; Decreased post-blood meal weight in *R. microplus*	[126]

Target gene	Tick species	RNAi Effect	Refs
Serum amyloid A	*R. annulatus*, *R.* (*B.*) *microplus*	Decreased *B. bigemina* infection level	[126]
Ricinusin	*R. annulatus*, *R.* (*B.*) *microplus*	Decreased post-blood meal weight in *R. annulatus*	[126]
Calreticulin	*R. annulatus*, *R.* (*B.*) *microplus*	Decreased *B. bigemina* infection level in *R. microplus*; decreased post-blood meal weight in *R. annulatus*	[126]
Chitin deacetylase-like protein (IsCDA)	*I. scapularis*	No significant effect on *B. burgdorferi* acquisition or transmission	[50]
Antifreeze glycoprotein	*I. scapularis*	Decreased survival and mobility of ticks in extremely cold temperature; decreased *A. phagocytophilum* infection level	[148]
x-linked inhibitor of apoptosis protein (E3 ubiquitin ligase, XIAP)	*I. scapularis*	Increased *A. phagocytophilum* infection	[149]
Cytochrome c oxidase subunit III	*R.* (*B.*) *microplus*	Failure in transmission of *A. marginale*	[150]

Table 1. Genes functionally characterized through RNAi in different tick species.

Silencing these genes through RNAi greatly reduced the reproductive capacity of female ticks, which showed immature and light-colored oocytes. The uptake of vitellogenin in the oocytes is facilitated by vitellogenin receptor, which has been characterized in *D. variabilis* [34], *H.longicornis* [99], and *A. hebraeum* [100]. Aside from the negative impact in oviposition consistently induced by RNAi in all these studies, silencing of *H. longicornis* vitellogenin receptor also reportedly inhibited the transovarial transmission of *Babesia gibsoni*. Three factors involved in the initiation of vitellogenesis, the GATA factor, S6 kinase [103], and target of rapamycin (TOR) pathway [104], have been also characterized in *H. longicornis* ticks using RNAi. The significance of other proteins to reproduction, such as a tick homologue of the human follistatin-related protein [98] and the engorgement protein voraxin [101] from the male gonad, has been also demonstrated using RNAi.

4.4. Genes related to structural and metabolic functions

Various gene encoding proteins important in cellular structure and metabolism have been characterized using RNAi. Due to their wide distribution and systemic function, knockdown of these proteins caused detrimental effects on different tick physiological functions and some even proved to be lethal (Table 1). Among these proteins is the multifunctional ubiquitin, which has been first targeted based on a homologous gene of *D. melanogaster* in a study investigating the components of tick RNAi pathway [14]. Ubiquitin knockdown in *R.* (*B.*)

microplus shortened the post-blood meal survival of ticks and impaired egg viability and hatch. In separate studies, ubiquitin has also been the subject in examining off-target effects of RNAi [29] and the feasibility of dsRNA feeding in *R.* (*B.*) *microplus* [72]. RNAi-mediated silencing of ribosomal proteins in *A. americanum* [118], and ubiquitin, elongation factor-1 alpha and several other proteins in *R.* (*B.*) *microplus* and *R. annulatus* [45] has been employed to screen potential antigens for tick control.

In the hard tick *H. longicornis*, individual knockdown of glutamine:fructose-6-phosphate aminotransferase [105], cyclophilin A [108], the ribosomal protein P0 [109], protein disulphide isomerases [110], and tropomyosin [123] resulted to decreased survival of ticks after engorgement. Two proteins with apparent roles in withstanding long starvation period, lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) [114] and 4E-BP [120], have also been characterized in *H. longicornis*. LKR/SDH mRNA expression is higher in starved ticks than in unfed ticks and knockdown of LKR resulted to high volume of hemolymph after blood feeding, suggesting its role in osmoregulation. Meanwhile, 4E-BP knockdown led to decreased lipid storage in the midguts and fat bodies of ticks during longer starvation period. An interesting report on the application of RNAi in studying tick neurobiology targetedβ-actin and Na^+-K^+-ATPase of *I. scapularis* using fluorescently labeled dsRNAs to monitor the uptake in tick synganglia [106].

The significance of proteins involved in iron metabolism to tick feeding and reproduction has been also demonstrated using RNAi in two hard tick species, *I. ricinus* [111] and *H. longicornis* [112]. Silencing two types of the iron storage protein ferritin greatly reduced the ticks' capacity to engorge and produce eggs, also affecting post-blood meal survival due to occurrence of iron-mediated oxidative stress [113]. An iron regulatory protein responsible for translation of iron binding proteins was characterized in *I. scapularis*, with its knockdown greatly reducing egg hatchability [111]. Two enzymes, spook and shade, were characterized in the soft tick *O. moubata* and were shown to be important in ecdysteroidogenesis through RNAi [53]. Silencing spook protein in nymphs caused arrested development and molting, whereas silencing shade delayed molting and led to abnormal ecdysis.

5. RNAi studies on tick protective antigens and immunity

The immune system of ticks has a vital role of protecting them from harmful substances in the blood, including components of their host's immune system, and from various pathogens that they acquire in their blood feeding activity. Tick protective antigens, therefore, gain wide interest due to their potential as target for tick control. The highly conserved tick protective antigen subolesin, previously known as 4D8, was first identified from *I. scapularis* through cDNA expression library immunization (ELI) [153], after which, it has been also identified in other hard tick species, and using RNAi, was found to be important in the success of blood feeding and reproduction [124]. An ortholog of subolesin has also been characterized in two soft tick species and RNAi demonstrated that subolesin is also important in the reproduction of soft ticks [127]. The function of subolesin is unclear, but a report showed that subolesin

knockdown affected the expression of several genes involved in multiple cellular pathways, suggesting a role in gene expression by interacting with regulatory proteins [154]. Aside from being reported as a promising anti-tick vaccine antigen candidate in many studies, it has been also proposed that subolesin may be targeted in ticks that subsequently will be released for sterile acarine technique (SAT) [38].

The membrane-bound glycoprotein Bm86 expressed mainly in the midgut of *R.* (*B.*) *microplus* [155] is the first, and until recently, the only tick antigen that is commercially available as an anti-tick vaccine in some countries. The exact function of Bm86, however, remains unclear yet. RNAi has been employed to knockdown *Bm86* and its homologues in other tick species, including *R. sanguineus*, *H. longicornis*, and *R. evertsi evertsi*, which in most cases affected the blood feeding and reproduction of adult ticks, except in *R. evertsi evertsi* wherein knockdown of two homologues did not yield significant effects [130]. A study in *R.* (*B.*) *microplus* also showed that knockdown of *Bm86* decreased the blood feeding capacity and survival of ticks after feeding on a *B. bovis*-infected host, suggesting that Bm86 may have a critical role in the fitness of ticks after feeding from an acutely *B. bovis*-infected host [129].

The function of some components of immunity, such as α2-macroglobulin proteins [132, 133], antimicrobial peptides [131], Janus kinase (JAK)-signaling transducer activator of transcription (STAT) pathway [135], dityrosine network [136], and cysteine protease inhibitor in the hemocytes [140] have been analyzed using RNAi. The α2-macroglobulin proteins of *I. ricinus*, related to vertebrate complement system, were shown to be involved in the phagocytic activity of hemocytes against Gram-negative bacteria [132, 133]. In contrast, a cysteine protease Rmcystatin3 identified in *R.* (*B.*) *microplus* was implicated as a negative modulator of tick immune response after its silencing greatly reduced the number of bacterial load in the ticks [140]. The role of a defensin from *H. longicornis*, longicin, in ticks' immune defense against *Babesia* parasites was demonstrated through RNAi, as exhibited by a higher load of *B. gibsoni* in the midgut and ovary of *longicin*-silenced ticks after infestation in an infected host [131]. Meanwhile, JAK-STAT pathway was shown to be important in *Anaplasma phagocytophilum* infection in ticks after its knockdown increased the infection in the salivary glands of nymphs that fed on infected mice [135]. A dual oxidase and a peroxidase, ISCW017368, which together forms a dityrosine network, were separately silenced in *I. scapularis*, both resulting to reduced *Borrelia burgdorferi* persistence in ticks [136].

The obligatory blood feeding lifestyle of ticks exposes them to high levels of pro-oxidants that may trigger oxidative stress. Antioxidant enzymes function to protect them from the harmful effects of oxidative stress. Furthermore, these antioxidant enzymes provide detoxification mechanisms to counteract toxins that they encounter in the environment, such as chemical acaricides. RNAi has been very useful in evaluating the function of these antioxidants. Silencing a selenoprotein in *R.* (*B.*) *microplus* reduced the engorged body weight and egg output [45]. In contrast, a study on *A. maculatum* showed that silencing two selenoproteins did not alter blood feeding, although the egg output was reduced. Interestingly, the total antioxidant capacities of the saliva from knockdowned ticks were higher, indicating that other antioxidant enzymes may have compensated for the absence of selenoproteins [138]. In another study, silencing thioredoxin reductase, another selenoprotein, in *A. maculatum* did not have a negative

impact on blood feeding and reproduction. Likewise, variations in transcriptional expression of some antioxidant enzymes were also observed, suggesting compensatory mechanism in the absence of thioredoxin reductase [139]. However, the more interesting finding in that study was the decreased microbiota population following thioredoxin reductase knockdown, possibly because of disturbed redox homeostasis balance. Meanwhile, silencing a glutathione S-transferase (GST) gene affected the attachment of ticks and reduced the post-blood meal bodyweight of *R.* (*B.*) *microplus* [45]. It also made *R. sanguineus* ticks more susceptible to permethrin, although no significant effects on tick attachment, feeding and reproductive capacity were observed [137].

6. Understanding tick-pathogen interaction through RNAi

RNAi has undoubtedly paved a way to better understand the different aspects of ticks' association with various pathogens. Numerous tick proteins with different functions have been found to be involved in the acquisition, establishment, and transmission of pathogens. Several proteins have been studied through RNAi to determine their importance in the development cycle of different pathogens. The knockdown of subolesin [142, 156], GST, vATPase, and selenoprotein M [142] in *D. variabilis*, and putative von Willebrand factor, flagelliform silk protein and subolesin in *R.* (*B.*) *microplus* [143] decreased the infection level of *A. marginale* in these hard ticks, implying that these proteins are significant in the establishment of infection of this rickettsia.

RNAi also demonstrated that the Lyme disease agent *B. burgdorferi* can utilize several proteins of *I. scapularis* to facilitate its transmission to the host. These include salivary proteins such as tick histamine release factor [147], Salp15 [52], and the lectin complement pathway inhibitor (TSLPI) [145]; the latter two provide protection for *B. burgdorferi* against components of the host immune system. Salivary proteins Salp14 [49], Salp16 [48], and Salp25D [44] have been examined for their function in acquiring *A. phagocytophilum* or *B. burgdorferi* through RNAi. Knockdown of Salp14 did not affect the acquisition of either rickettsiae, whereas the knockdown of Salp16 and Salp25D decreased the infection level of *A. phagocytophilum* and *B. burgdorferi* in the tick, respectively.

An interesting study on *I. scapularis* showed that *A. phagocytophilum* promotes cold tolerance through an antifreeze glycoprotein [148]. In the absence of this antifreeze glycoprotein, the survival rate of ticks after exposure to extremely cold temperature and the infection level of *A. phagocytophilum* following exposure was reduced. Tick defensins, varisin from *D. variabilis* [144], and ricinusin from *Rhipicephalus* ticks [126] have been silenced to examine their functions in pathogen establishment; the former reduced *A. marginale* infection level, while the latter did not have an effect on *B. bigemina* infection.

Several reports also demonstrated the interaction of *Babesia* parasites and tick proteins through RNAi. Knockdown of the immunophilin gene in *R.* (*B.*) *microplus* had negative impact on the reproductive performance of the tick and also increased the infection rate of *B. bovis* in larval progeny [41], while knockdown of TROSPA, serum amyloid A, and calreticulin reduced the

infection level of *B. bigemina* in *Rhipicephalus* ticks [126]. Silencing a Kunitz-type serine protease inhibitor from *D. variabilis* increased the rickettsial infection in the midgut [146], whereas in *R.* (*B.*) *microplus*, silencing a Kunitz-type serine protease inhibitor, Spi, tended to increase the infection rate of *B. bovis* in larval progeny [41], but silencing of another Kunitz-type protease inhibitor 5 (KTPI) did not have any effect on *Babesia* infection [126].

7. Future directions in tick research and application in tick control

Indeed, great progress in understanding tick biology has been already accomplished in the past. However, many aspects of tick physiology and host-tick-pathogen interaction need to be unraveled yet. Moreover, several optimizations can still be done to improve RNAi in tick research. While being the most widely used method of introducing dsRNA, the injection method (particularly microinjection) that requires elaborate equipment may not be accessible to all laboratories. Moreover, injection is mostly applicable to adult and sometimes nymphal stages, and may be injurious to the ticks, especially when performed by an inexperienced researcher. The soaking method is simpler, less invasive, and less laborious. Electroporation has been recently shown to be effective in introducing dsRNA in eggs [66] and may be useful in studying the function of genes that are involved in embryogenesis and physiology of immature tick stages.

RNAi may also prove to be a promising tick control method and not just a research tool. In pest insect management, the possibility of using RNAi as a novel tool of pest control is already being explored by feeding liposome-coated dsRNA or dsRNA expressed in transgenic plants or bacteria [32]. RNAi targeting several genes have been accomplished by feeding plants expressing dsRNA in several species of economically important crop pests [16]. Feeding dsRNA to ticks is still an underdeveloped approach, which has been yet accomplished only by artificial feeding. Coating dsRNAs with liposomes or nanocarriers may increase dsRNA stability that may make it feasible for administration to the host. Genes that are highly conserved across different tick species, and are of importance in tick survival are good candidate targets. These include proteins with structural and metabolic functions, such as ubiquitin, tropomyosin, and ferritin. However, the specificity of dsRNA to the tick gene should be highly considered. Additional consideration would be the establishment of a minimum effective dose, since the synthesis of dsRNA is costly.

Additionally, RNAi has been proposed as an alternative method for the sterile insect technique in blood-sucking mosquitoes that will produce sterile males by feeding dsRNA in mosquito larvae [64]. Quite similarly, the application of RNAi for tick control was also proposed in a single report on *D. variabilis*, wherein the highly conserved subolesin was targeted leading to reproductive incapacity [38]. In conclusion, the authors suggested that RNAi may be used to massively produce sterile ticks (SAT) that may be released in the field. Releasing subolesin-silenced ticks may also aid in the control of *A. marginale*, since it has been reported that subolesin knockdown reduced the infection level of this pathogen [141–143]. Introducing dsRNA to eggs through electroporation described above may be a more convenient way of producing knockdowned ticks.

8. Summary

In this chapter, we have reviewed the application of RNAi in tick research and described the significant contribution of RNAi in advancing our knowledge on tick biology and tick-pathogen interaction. RNAi has revolutionized the advancement of our understanding of various aspects of tick blood feeding and digestion, reproduction, metabolism, and immunity. As a functional analysis tool, RNAi has become very handy in elucidating the functions of different proteins from more than 10 hard tick species and a few soft tick species. It has been particularly helpful in screening potential target antigens for anti-tick and tick-borne pathogen vaccine development [157]. Several methods of introducing dsRNA in ticks have been employed but injection has remained to be the most widely used technique. The number of published research on ticks that involves the application of RNAi has been continuously increasing through the years, and it is expected to continue doing so. A great majority of the published reports focused on hard ticks, but due to some physiological differences, more research using RNAi on soft ticks should be conducted. Finally, with numerous potential target genes already identified, the application of RNAi as a tick control method should be investigated in the future, starting with optimization of dsRNA delivery method for practical use.

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Author details

Remil Linggatong Galay[1,2], Rika Umemiya-Shirafuji[3], Masami Mochizuki[1], Kozo Fujisaki[4] and Tetsuya Tanaka[2*]

*Address all correspondence to: tetsuya@ms.kagoshima-u.ac.jp

1 Department of Veterinary Paraclinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, Los Baños, Philippines

2 Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan

3 National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, Japan

4 National Agricultural and Food Research Organization, Tsukuba, Ibaraki, Japan

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