University of South Bohemia
Faculty of Science
Department of Parasitology

Ph.D. thesis

Functional analysis of metabolic and immune proteins in the tick *Ixodes ricinus* by RNA interference

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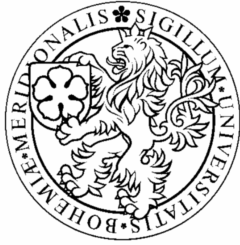

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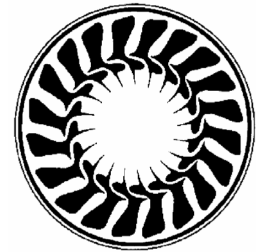

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Cover picture: Microinjection of the double-stranded RNA into the tick *Ixodes ricinus*.

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ANNOTATION

Ticks are obligate hematophagous ectoparasites of vertebrate hosts. *Ixodes ricinus* is the most common tick in the central Europe, transmitting important human diseases including tick-borne encephalitis and Lyme disease. The RNA interference (RNAi) became a valuable method for the functional description of tick genes and evaluation of targets for an efficient anti-tick vaccine. Here I summarize knowledge about the RNAi in ticks and demonstrate how this method can be employed to characterize the gene function. By using RNAi, we have characterized two ferritins and an iron-regulatory protein in the tick iron metabolism pathway, and showed that knock-down of these genes dramatically impaired the ability of ticks to feed and develop. Further, we have characterized fibrinogen-related proteins and thioester-containing proteins, and confirmed their roles in the tick innate immunity.

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DECLARATION

I hereby declare that all the work in this thesis was done on my own or in collaboration with co-authors of published articles, and using only the cited literature. Further, I declare that in accordance with the Czech legal code § 47b law No. 111/1998 in its valid version, I consent to the publication of my Ph.D. thesis (in an edition made by removing marked parts archived by the Faculty of Science) in an electronic way in the public access to the STAG database run by the University of South Bohemia in České Budějovice on its web pages.

České Budějovice, March 17, 2009 Ondřej Hajdušek

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Last but not least, I would like to thank my wife Martina and my family for endless support.

ABBREVIATIONS

α2M	alpha-2-macroglobulin
AGO	argonaute
DCR	dicer
DMT	divalent metal transporter
DS_RBD	double-stranded RNA binding domain
dsRNA	double-stranded RNA
EST	expressed sequence tag
FER	ferritin
FMR	fragile X mental retardation
FREP	fibrinogen-related protein
IRP	iron-regulatory protein
IXO	ixoderin
miRNA	micro RNA
PAZ	piwi/argonaute/zwille
PIWI	P-element induced wimpy testis
PTGS	post-transcriptional gene silencing
rasiRNA	repeat-associated small RNA
RdRP	RNA-dependent RNA polymerase
RMSF	Rocky Mountain spotted fever
RT-PCR	reverse transcription polymerase chain reaction
RNAi	RNA interference
siRNA	short-interfering RNA
TBE	tick-borne encephalitis
TEP	thioester-containing protein
TF	transferrin
UDC	undifferentiated digestive cells
DDC	differentiated digestive cells
WGS	whole-genome sequencing

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RESEARCH OBJECTIVES

The main objectives of my Ph.D. study were to set-up and standardize the method of RNA interference in the tick *Ixodes ricinus* and use it for a determination of gene functions, characterization of biochemical pathways, and testing of anti-tick vaccine candidate antigens. Among proteins of interest were components of the iron metabolism pathway, fibrinogen-related proteins (FREP), thioester-containing proteins (α2M), and digestive peptidases. These proteins have a long research history in our laboratories (Department of Molecular Ecology of Parasites) and many of them have been characterized at the biochemical and molecular level. However, the biological function of these proteins in ticks has never been determined and it was the aim of my work. The results of these efforts have been summarized and published in five international peer-reviewed journals and presented at several conferences.

CHAPTER 1

GENERAL INTRODUCTION

IMPORTANCE OF TICKS

Ticks are blood-feeding ectoparasites and vectors of various pathogens, affecting humans and animals worldwide. Their importance was already depictured by ancient Greek and Roman writers. Carolus Linnaeus (1758) included ticks in his *Systema Naturae*, but the first serious classification of ticks in the animal kingdom was done by Jean-Babtiste Lamarck in 1815. The era of modern tick research began in the 19th and expanded in the second half of the 20th century. Till now, many excellent books and scientific publications have contributed to the detail description of tick evolution, morphology, physiology, and disease association (e.g., Balashov (1972); Sonenshine (1991); Coons and Alberti (1999); Bowman and Nuttal (2008)).

The tick feeding is often painful, irritating, could evoke allergy, and cause toxicoses, paralysis, or secondary infections. Importantly, ticks transmit the greatest variety of infectious diseases among arthropods including viruses, bacteria, fungi and protozoa (Pagel Van Zee et al., 2007; Jongejan and Uilenberg, 2004). After mosquitoes, they are the most important vectors of life-threatening diseases of man and animals. In the Czech Republic, numbers of cases of human tick-borne encephalitis (TBE) and Lyme disease are increasing every year (The National Institute of Public Health, Prague, Czech Republic). A vaccine against TBE is on the market, but despite several attempts, no vaccine against Lyme disease is currently available. A strong dose of antibiotics applied immediately after the appearance of the first symptoms usually eliminates most of the bacteria, but because of the ability of Borrelia to hide in various tissues, the infection often persists and borreliosis becomes chronic.

In addition to the human health, ticks and tick-borne diseases greatly affect livestock production. Protection against ticks (mostly by acaricides) and economical losses on the animals are estimated to amount billions of US dollars spent and lost every year and ticks are classified as the major factor limiting livestock production in many areas around the world.

SYSTEMATIC AND EVOLUTION OF TICKS

In the phylum *Chelicerata*, two classes can be distinguished: *Merostomata* and *Arachnida*. The class *Merostomata* comprises giant “horseshoe crabs” from the subclass *Xiphosura*. These species have many characteristics of primitive ancestral marine arthropods and are often used as experimental animals (large volume of hemolymph in a single organism) for the biochemical identification of immune cascades and proteins in the arthropod hemolymph (Kurata et al, 2006).

The *Arachnida* (including mites, spiders, scorpions, harvestmen, etc.) contains the second class of chelicerates, where *Acari* are the most dominant subclass. The *Acari* comprise a vast assemblage of species proliferating throughout many terrestrial, marine and fresh-water habitats. Many of them are free-living, including herbivores, fungivores, and predators, while others are ecto- and endoparasites. The *Acari* are subdivided into 2 major orders: *Parasitiformes* and *Acariformes*. Ticks (suborder *Ixodida*), members of the order *Parasitiformes*, are obligate blood-sucking parasites and comprise three families - *Ixodidae*, *Argasidae* and *Nutalliellidae*. Today, 907 valid tick genera and species exist, 186 in *Argasidae*, 720 in *Ixodidae*. The family *Nutalliellidae* is represented only by a single species (Barker and Murrel, 2009).

The *Acari* are believed to have evolved in the late Proterozoic or early Paleozoic eras, when chelicerates and trilobites were the dominant arthropods. However, little is known about the evolution of ticks. Species of the group *Parasitiformes*, including ticks, have evolved probably during the late Paleozoic or early Mesozoic eras and their appearance might be tightly connected with the spread of reptiles (Sonenshine, 1991). It is also possible that the first ticks were feeding on amphibians (Oliver, 1989). Radiation of the family *Ixodidae* occurred in the more recent time and is connected with the expansion of mammals and birds. Nevertheless, the oldest tick preserved in amber found in New Jersey, NY, USA (genus *Carios*, *Argasidae*) is dated 90-94 millions years back (Klompen and Grimaldi, 2001).

BASIC CHARACTERISTICS OF TICKS

The life cycles of ticks involve three active developmental stages: the larva, the nymph, and the adult. In contrast to *Ixodidae* (hard ticks), *Argasidae* (soft ticks) usually posses multiple nymphal stages (multi-host life cycle). With the exception of few species, where the fed juveniles remain and develop on the host (1-host and 2-host ticks), most ticks follow the 3-host life cycle and drop from the hosts after each blood feeding. The bloodmeal is necessary for the tick molting and passage into the next stage.

The most characteristic sign of ticks is their mouthpart. It is composed of the paired leg-like palps, the paired chelicerae (for cutting, ripping and tearing the skin), and the hypostome with numerous dentices (for fixation in the skin). These structures penetrate the host skin and/or fixate the tick in the wound to ensure a non-problematic blood flow.

During feeding, the hard or soft ticks excrete the excess watery fluid originating from the bloodmeal. Coxal glands are used for this purpose in the Argasid ticks, while the Ixodid ticks use their salivary glands to periodically secrete the liquids back into the host. Thanks to the water elimination, the tick could concentrate blood and ingest more food (Fig. 1). The female of *I. persulcatus* could take more than 730 µl of blood, while its final weight after repletion (full engorgement) is about 260 mg (2 mg before repletion; Balashov, 1972).

Fig. 1. Appearance of the tick *I. ricinus* before and after feeding. *Upper:* Unfed tick life stages (*From left:* Adult female; Male; Nymph; Larva); *Lower:* Fully fed female. Horizontal bar indicates 1 cm.

A sclerotized plate called *scutum* is placed on the dorsal side of the hard ticks, serving as site for the muscle attachment. The remainder of the body is expansible and increases enormously by the fresh cuticle synthesis during feeding. For this purpose, the hard ticks must remain attached on the host for many days (slow feeding). In contrast, the soft ticks have a leathery highly folded cuticle, which does not grow but only expand during feeding. This predetermines the soft ticks for fast, rapid feeding (minutes or at most a few hours).

IXODES RICINUS

The hard tick *I. ricinus*, the most common tick in the Czech Republic, is a typical 3-host tick (Fig. 2). It can be easily found from early spring until late fall in humid sheltered environments, forests or meadows. The larvae and nymphs usually feed on the small vertebrates, mainly on rodents. Adults then feed on the bigger vertebrates, e.g., deer. After the repletion, tick falls off the host and begins molting (larva or nymph) or oviposition (adult female). The males of *I. ricinus* do not feed in the adult stages. A successful feeding of the adult females requires mating, which can be done directly on the host during feeding or less often off the host before feeding. Under favorable conditions in the natural environment, the life cycle can be completed in one year.

Because ticks have evolved a great ability to survive a long time of starvation, their life cycles can be easily extended to several years when the hosts are not available.

Fig. 2. Appearance of the individual life stages of *I. ricinus* (unfed). *From left:* Adult female; Male; Nymph; Larva. Not in the scale (for the comparison see Fig. 1).

The typical feature of *I. ricinus* (and of some other hard ticks) is its questing behavior (seeking the host). During the day, the tick climbs the vegetation and awaits for the host. An apparatus called Haller´s organ (similar to the insect antennae) allows the tick to sense the host from a long distance. If unlucky, the desiccated tick retreat to the more favorable, hydrating environment to remain there until it has absorbed sufficient atmospheric humidity. Then, the hydrated tick climbs back to seek for the host again. This process can be repeated several times during the day.

INTERNAL ORGANS OF *IXODES RICINUS*

Most experiments in ticks are traditionally focused on the organs that can be easily dissected from the feeding tick female - the midgut, salivary glands and the ovary. These organs, which are also of the big interest for their role in the tick physiology or/and pathogen transmission, will be described in the next paragraphs. Detailed morphological and functional descriptions of other important tick organs can be found elsewhere (e.g., Sonenshine, 1991).

The Midgut

The midgut is the most prominent organ in the tick body. During feeding, it occupies almost the whole body cavity of the tick (Fig. 3). It consists of a central ventriculus (=stomach),

numerous broad diverticula and a small intestine, which directs the waste residues to the hindgut (rectal sac).

Histologicaly, the midgut wall consists of the epithelium and elongated smooth muscle cells, separated by a thin basal lamina. The inner epithelium possesses numerous microvilli and is covered by the peritrophic matrix (non-membrane structure), which protects the midgut cells from an injury. Two types of the digestive cells can be identified in the epithelium of the feeding female *I. ricinus* - the undifferentiated (UDC) and differentiated digestive cells (DDC). During feeding, the UDC cells, which get to the direct contact with blood, change to DDC and perform the digestion. After DDC are filled with the waste products originated from the hemoglobin digestion (heme), they detach from the basal lamina and are processed to the gut lumen (Coons and Alberti, 1999).

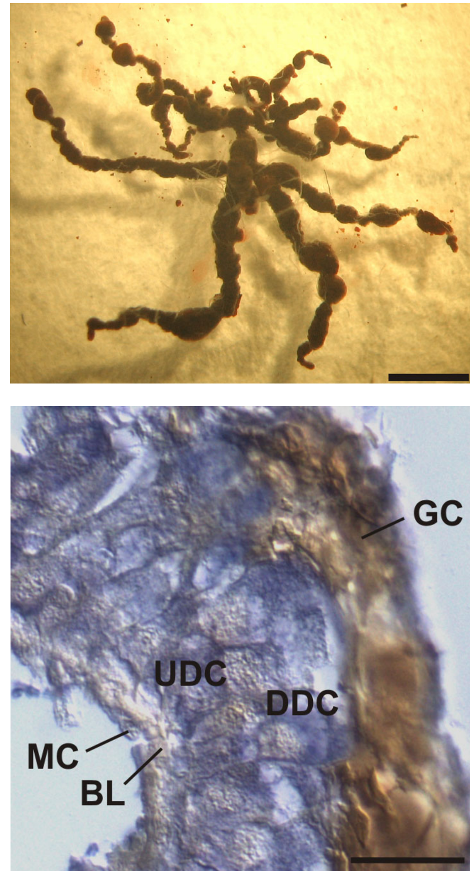

Fig. 3. Tick midgut. *Upper:* Midgut dissected from the half-fed tick female *I. ricinus*. Horizontal bar indicates 2.5 mm. *Lower:* Histological section of the midgut; MC muscle cells; BL basal lamina; UDC undifferentiated digestive cells; DDC differentiated digestive cells; GC gut content. Horizontal bar indicates 100 µm.

After attachment, the whole tick proceeds through a series of well-defined phases. During the first 24-36 hours, no ingestion of blood takes place and there is no significant digestive activity. This time is called the Preparatory phase and tick here secures its attachment to the host by secreting various proteins from the salivary glands. Later, during the Slow feeding phase, tick gradually takes blood for several days and the DGC cells initiate food digestion. Only females that are mated could enter the Rapid feeding phase, known as the "Big sip". During this period (12-36 hours), ticks take up large quantities of blood and expand enormously. The lumen acts as a blood reservoir and digestion is reduced during this time. The engorged tick detaches from the host to continue the digestion, followed by the egg production or molting (Sonenshine, 1991).

Except for the red blood cell hemolysis, the digestion in ticks is solely intracellular and based on a cascade of cysteine and aspartic proteases operating under low pH. This is in a sharp contrast with insects and vertebrates, where digestion is exclusively extracellular and employs serine proteases secreted into the gut lumen. The undigested blood could be stored inside the tick gut for a long time (for years in larvae and nymphs) and it is obvious that such environment is relatively friendly for pathogens taken with the host blood. The processes of hemoglobin degradation and heme or iron detoxification in *I. ricinus* are described in details in the attached publications (Sojka et al., 2007 and 2008; Hajdusek et al., 2009).

Salivary glands

The first days on the host are the most important for the tick attachment and successful feeding. At this moment, the salivary glands increase greatly in size and play a key role in the modification of the host wound. They produce a milky white protein which is the initial component of the cement cone, a structure that fixes tick in the host skin. To ensure continuous and non-problematic feeding, ticks evolved an arsenal of proteins produced into the saliva. This "pharmacy" produces anti-hemostatic, anti-inflammatory, anti-coagulant and immunosuppressive agents that influence the host defense mechanisms in the wound and enable the tick rejection (Maritz-Olivier et al., 2007). The salivary glands also play an important role in the water excretion, concentrating the bloodmeal. At the end of the feeding period, salivary glands of all stages become non-productive, cells undergo autolysis and acini degenerate. This is an important factor, as salivary glands are the primary site for transmission of many pathogens.

The pair salivary glands are located in the lateral regions of the body cavity and consist of grape-like clusters (Fig. 4) composed of the granular and agranular acini. A system of small secondary ducts drains saliva to the main duct towards the opening in the mouthpart.

The agranular acini (Type I) occur along the main salivary gland duct in the anterior and middle regions of the salivary glands, and open directly into the main duct. They lack large secretory granules and comprise of a large central cell and a number of peripheral cells. These cells produce a hygroscopic secretion (salty solution) that enables the unfed ticks to actively take up moisture from the outer environment.

The granular acini (Type II, III, and IV) comprise most of the salivary glands and are characteristic of the dense accumulation of large granules in their cytoplasm. They are often connected to the secondary ducts. In the each type of acinus we can recognize different cell types (A-F), which vary in the granula composition (Vancova et al., 2006). The Types II and III acini secrete the cement proteins, F cells of the Type III acinus secrete the excess water and help to concentrate the blood meal. Type IV can be found only in males.

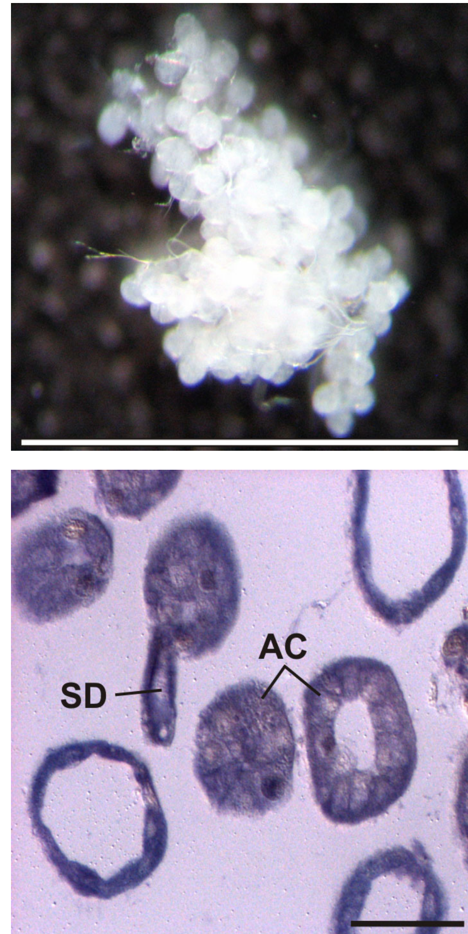

Fig. 4. Tick salivary glands. *Upper:* Salivary gland dissected from the half-fed tick female *I. ricinus*. Horizontal bar indicates 2.5 mm. *Lower:* Histological section of salivary glands; SD salivary duct; AC acini. Horizontal bar indicates 100 µm.

The Ovary

Only few terrestrial organisms equal ticks in number of produced eggs. After engorgement, the female of *I. ricinus* is able to convert over 50% of the replete body weight into the egg mass and lay several thousand eggs in a few days (Sonenshine, 1991). After that, the exhausted female dies.

The reproductive track of the female *I. ricinus* consists of a single U-shaped ovary (Fig. 5), the paired oviducts, the uterus, a muscular connective tube, the paired tubular and lobular accessory glands, and the vagina subdivided into cervical and vestibular regions. In contrast to insect, the tick ovary is not divided into separate zones as the germanium and vitellarium. The tick oocytes remain stationary during formation and yolk deposition takes place *in situ*.

The ovary of feeding female is a tube-like structure of luminal epithelium and developing oocytes connected with an epithelium by a short hollow stalk called *funiculus*. The whole ovary with egg cells is expanded into the hemolymph and surrounded by several layers of acellular *tunica propria*. This layer is permeable for hemolymph proteins, which are taken up by

micropinocytosis and stored inside the oocytes. The brown yolk granules of hemoglycoproteins (vitellogenins) give the developing oocyte its typical brown color. The egg envelope (egg shell), formed by the oocyte itself after mating, is also fully permeable for the hemolymph proteins.

The mature oocytes (still primary oocytes without division) ovulate into the ovarian lumen. After fertilization, the binucleate oocyte undergoes first meiotic division, but oogenesis is not completed until the oviposition, when the nuclei fuse. Embryogenesis thus occur external to the female body. External porose areas on the *basis capituli* and a unique glandular Gené's organ produce antioxidants and wax that protect the eggs after being laid.

Fig. 5. Tick ovary. *Upper:* Ovary dissected from the half-fed tick female *I. ricinus*. Horizontal bar indicates 2.5 mm. *Lower:* Histological section of the ovary; OO oocyte; FC folicular cells; LE luminal epithelium. Horizontal bar indicates 100 µm.

TICK-BORNE DISEASES

Viral diseases

Arboviruses (arthropod-borne viruses) comprise a large group of viruses transmitted by arthropods. At least 500 different arboviruses are registered nowadays (based on Karabatsos, 1985) and the number is increasing continuously. About half of them are mosquito-borne and approximately one third is transmitted by ticks. Many arboviruses infect humans causing systemic febrile illness, encephalitis, and/or hemorrhagic fever. They also cause severe economic problems as a consequence of livestock losses.

Most of the tick arboviruses are RNA viruses. The only exception is a DNA virus causing African Swine Fever. The tick-borne arboviruses are classified into seven families (examples of the most severe diseases are in the brackets) - *Togaviridae*, *Flaviviridae* (tick-borne encephalitis, Powassan Encephalitis, Kyasanur Forest Disease, Louping Ill, Omsk Hemorrhagic Fever), *Bunyaviridae* (Crimean-Congo Hemorrhagic Fever, Nairobi Sheep Disease), *Reoviridae* (Colorado Tick Fever), *Rhabdoviridae*, *Arenaviridae* and *Iridoviridae* (African Swine Fever Virus). From these, *Flaviviridae* and *Bunyaviridae* are the most important agents causing the encephalitic and/or hemorrhagic illness (Calisher and Karabatsos, 1988). In the Czech Republic, the European subtype of tick-borne encephalitis (transmitted by *I. ricinus*) is the most common and most dangerous arbovirus with hundreds of human cases every year (633 cases in 2008; The National Institute of Public Health, Prague, Czech Republic).

Viruses usually persist in the tick body through its whole life (Davies et al., 1986). Because of the longevity, ticks are excellent reservoirs for the viruses they carry. Infection can be passed to the subsequent life stages (transstadially) or following generations (transovarially). However, high level of vertical transmission has not been recorded for any tick-borne virus (Turell, 1988).

Tick borne encephalitis

This disease was originally described in 1932 in the former USSR and termed Russian Spring-Summer Encephalitis because of the seasonal periodicity of its occurrence. Today, three different genetic lineages of the tick-borne encephalitis (TBE) can be distinguished: a European, a Far Eastern and a Siberian subtype (Ecker et al., 1999).

TBE viruses ((+)ssRNA; Fig. 6) are characteristic of their predilection to the central nervous system tissue. After incubation period (about two weeks) the infection expresses as fever, headache, nausea, and vomiting. These early symptoms are followed by neurological symptoms of encephalitis, meningeal irritation, brain dysfunction and paralysis. Several human vaccines directed against TBE have been developed and commercialized. In the Czech Republic, vaccines of the inactivated TBE viruses named FSME-IMMUN (Baxter) and ENCEPUR (Novartis) are available and highly recommended. The treatment of TBE is only supportive and requires hospitalization.

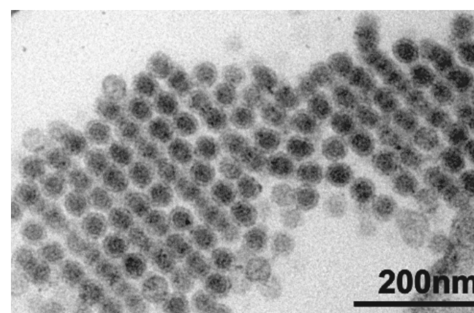

Fig. 6. TBE virions. Electron microphotograph of the TBE virus particles. Source: M. Vancova.

Principal vectors for TBE are *I. ricinus* (European subtype) and *I. persulcatus* (Far Eastern and a Siberian subtype). The estimated natural infections in *I. ricinus* ticks range from 0.1 to 0.9% for nymphs and 0.3 to 6% for adults (Nuttal and Labuda, 1994).

Bacterial diseases

Ticks also transmit several important bacterial diseases. They are traditionally grouped into two groups: tick-borne rickettsioses and other bacterial diseases (Lyme disease, Relapsing Fever, and Tularemia; Sonenshine, 1991). The Lyme disease is now regarded as the most important tick-borne disease affecting humans. All the diseases can be less or more efficiently treated with antibiotics.

Tick-borne Rickettsioses

Rickettsiae (*Alphaproteobateria)* are organisms, which adopted an intracellular lifestyle. Some authors see *Rickettsiales* as a group from which mitochondria arose (Williams et al., 2007). In man, infection with rickettsioses is often characterized by the rash spreading over most of the body (spotted fever). Based on genetic analyses, order *Rickettsiales* comprises two families, *Rickettsiaceae* and *Anaplasmataceae* (Dumler et al., 2001).

The most important tick-borne spotted fevers of the family *Rickettsiaceae* are Mountain spotted fever (*Rickettsia rickettsii*), Boutenneuse Fever (*R. connori*), North Asian Tick Typhus (*R. sibirica*), and Queensland Tick Typhus (*R. australis*). Members of the other family (*Anaplasmataceae*) comprise Anaplasmosis (*Anaplasma* sp.), Ehrlichiosis (*Ehrlichia* sp.), and a disease called Heartwater (*Cowdria ruminantium*).

Rickettsia species parasite generally in the endothelial lining of the vascular system, *Ehrlichia* invades monocytes, *Cowdria* neutrophils, *Anaplasma* erythrocytes. In the tick, rickettsiae first multiply in the midgut cells and than invade other tick tissues. Both the transovarial and transstadial transmission are possible. To better understand the development in the tick vector, two examples of the best studied diseases follow.

Rocky Mountain spotted fever

Rocky Mountain spotted fever (RMSF) is widespread in the western hemisphere. The disease is characterized by fever, headache, and, in most cases, by generalized rash. It is the most severe illness of any rickettsioses in man. The causative agent of RMSF is *R. rickettsii*, which multiplies in the cytoplasm and, occasionally, in the nuclei of the infected cells of the vascular system.

RMSF is a zoonosis, where the tick (primarily *Dermacentor variabilis*) serves both as the vector and the reservoir of the infection. The Rickettsia first invades tick midgut cells, where it multiplies intensively. After that, it escapes into the hemolymph and disseminate into other tissues. Feeding appears to stimulate the intense rickettsial multiplication (Burgdorfer, 1988). The multiplication in salivary glands results in the passage of infection into the host.

The passage of infection from larvae to nymphs and adults ensures the transstadial transmission. The transovarial transmission is often erratic and depends on the developmental stage when the tick acquires infection. The feeding females likely do not pass the bacteria to the eggs, because the rickettsiae are probably excluded by the egg shell of the oocyte (although they appear capable of penetrating *tunica propria*). In contrast, females that have acquired the infection prior to feeding will most likely pass the rickettsiae to their oocytes (Burgdorfer and Brinton, 1975).

Anaplasmosis

Anaplasma marginale is the primary cause of anaplasmosis in livestock. It is considered to be one of the most important diseases of cattle and sheep. In the vertebrate host, the Anaplasma develop in erythrocytes (within the membrane-bound inclusions called colonies), leading to the hemolytic anemia which causes the progresses of the disease. The onset of anaplasmosis is accompanied by high fever. Mortality rate of the acute anaplasmosis ranges from 30 to 50%.

Approximately 20 species of the Ixodid ticks serve as vectors of the anaplasmosis in different part of the world (Kocan et al., 1992a). The pathogen developmental cycle is initiated in the midgut cells and progressed to the gut muscle cells during the next feeding (Kocan et al., 1992b). After that, the colonies are formed in the salivary glands and the pathogen could be transmitted into the host. During feeding, the gut infections remain constant (Kocan et al., 1992a) and the gut cells serve as a reservoir for repeated infection of the salivary glands. This pattern is significantly different from Piroplasms where the gut cells are cleared of the infection as the parasites move to the salivary glands. Infection could be transmitted transstadially, but the transovarial transmission of anaplasmosis does not appear to occur (Kocan et al., 1985).

<u>Other tick-borne bacterial diseases</u>

Excluding the ricketsiae, the tick-borne bacterial pathogens include six major genera: *Borrelia* (Lyme disease and Relapsing fever), *Francisella* (Tularemia), *Klebsiella* (Moose disease), *Staphylocoocus* sp. (Tick Pyaemia of sheep), *Coxiella* (Q-Fever), and *Dermatophilus*. Borreliosis, Relapsing fever, and Tularemia as the most important diseases are described in more detail in next paragraphs.

Borreliosis

Lyme disease (borreliosis) is an emerging disease caused by a coil-shaped Gram-negative spirochete (Fig. 7) belonging to the genus *Borrelia* (Spirochaetaceae). It is the most important human tick-borne disease. In the Czech Republic and other countries, a progressive trend in number of cases is observed every year. In 2008, more than 4.350 new cases of the borreliosis were detected in the Czech Republic (The National Institute of Public Health, Prague, CR), 25.000 cases in the USA in 2007 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Borreliosis affects multiple body systems, producing a range of potential symptoms (Sonenshine, 1991). In humans, it may present abnormalities in the skin, musculoskeletal system, heart, and nervous system. The classical sign of early infection (Stage I) is a circular, expanding, red rash called *erythrema migrans* and the “flu-like symptoms”. After several days or weeks the rash and other symptoms subside and patient may recover or go on to develop a chronic syndrome with associated neurologic, cardiac and arthritic symptoms (Stages II and III). The treatment with antibiotics is effective just in the early stage of infection. In the later stages, when the bacteria disseminate throughout the body or cross the blood-brain barrier, is the infection more difficult to treat.

At least 14 different species of the *Borrelia burgdorferi* sensu lato complex have been described, three of which are Lyme-related (Wang et al., 1999; Rudenko et al., 2009) – *Borrelia burgdorferi* sensu stricto (Eurasia and North America), *B. afzelii*, and *B. garinii* (Eurasia). Most human cases are reported from Europe and North America, where the disease is transmitted mainly by *I. ricinus* or *I. persulcatus* and *I. scapularis* or *I. pacificus*, respectively. A recombinant vaccine against a surface protein OspA has been developed in the USA in 1998 (GlaxoSmithKline), but was withdrawn from the market esentially due to the lack of demand. In Europe, variability of the OspA among different pathogenic species is often used as an argument against the development of an OspA vaccine. New vaccines are being researched using an outer surface protein OspC (Earnhart and Marconi, 2007). However, the personal protection, including wearing protective clothing, use of repellents, and daily checks are highly recommended in endemic areas as a prevention against ticks and Lyme disease.

Initially, the Borrelia is ingested in the bloodmeal of the vector-competent larval or nymphal tick. Spirochetes at first multiply within the midgut fluid, but they soon become sessile and remain attached against the digestive epithelium (Spielman, 1992) as the infected tick digests the bloodmeal and molts to the next stage (transstadial transmission). The transovarial transmission of Borrelia in ticks is not likely, at least for *I. scapularis* (Burgdorfer et al., 1989). Recently, a tick midgut protein called TROSPA has been shown to be involved in the binding of the Borrelia surface protein OspA (Pal et al., 2000 and 2004).

During the next tick feeding, the spirochetes are activated, multiply and penetrate into the hemolymph (expression of OspC instead of OspA (Schwan and Piesman, 2002)). Within 48 hours after the attachment, the spirochetes can be found adjacent to the salivary glands (Zung et

al., 1989). A tick salivary gland protein Salp15 facilitates the Borrelia transmission and initiates their survival inside the host (Ramamoorthi et al., 2005).

A key factor in the transmission dynamics of Borrelia is the duration of attachment required for the efficient transmission of spirochetes to the host. Transmission dynamics of the Borrelia genospecies may differ dramatically (Crippa et al., 2002) and definitely deserve further attention. Kahl et al., 1998 showed that transmission of *B. burgdorferi* sensu lato in *I. ricinus* did not occur at the beginning of the blood uptake, but the hosts became infected after more than 16 hours. Similarly, nymphs of *I. scapularis* have to be attached for >48 hours to efficiently transmit *B. burgdorferii* sensu stricto (Piesman et al., 1991; De Silva and Fikrig, 1995; des Vignes et al., 2001). However, a microscopic examination of the unfed nymphal and adult stages of *I. ricinus* collected in the endemic areas in Switzerland demonstrated that spirochetes may infect salivary glands even before any blood uptake (Lebert and Gern, 1994; Leuba-Garcia et al., 1994; Zhu, 1998).

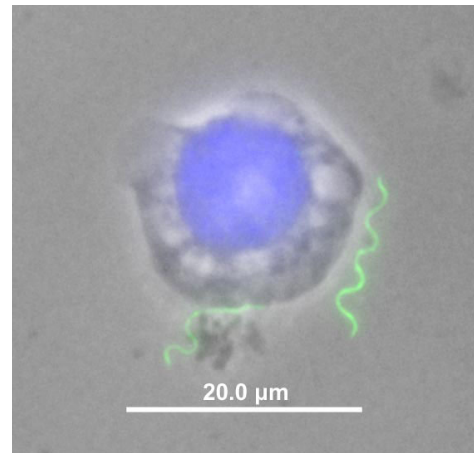

Fig. 7. ***Borrelia burgdorferi.*** *B. burgdorferi* sensu stricto being phagocytosed by the tick hemocyte. Merged image. Phase contrast, bacteria in green (FITC), hemocyte nucleus in blue (DAPI).

Relapsing Fever

Relapsing Fever is caused by numerous spirochetes of the genus *Borrelia*. They are specific to their vectors, species of the family *Argasidae*. Relapsing fever is manifested in the host by high fever with typical febrile relapses occurring at frequent intervals. In contrast to *B. burgdorferi*, spirochetes in the soft ticks disseminate rapidly during feeding (minutes) from the midgut to the internal organs (transstadial transmission), including ovaries (transovarial transmission; not documented in all species). Infection occurs via the bite or coxal fluid emitted during feeding.

Tularemia

Tularemia is caused by Gram-negative coccobacillus *Francisella tularensis* (Gammaproteobacteria). The disease is usually tick-borne, but can be also acquired by the direct contact with the infected host. In most cases, an ulcerated papule develops near the tick bite. Francisella is highly invasive and penetrates most tissues where it rapidly proliferates. The bacteria multiply readily in both the Ixodid and Argasid ticks, where the transstadial and transovarial transmissions have been demonstrated.

Protozoan diseases

Tick-borne protozoan parasites of the phylum *Apicomplexa* are important parasites causing catastrophic effects and enormous financial losses on herds of livestock. In addition, these organisms could infect humans (*Babesia* spp.), pets and other animals (*Hepatozoon* spp.). The most important members, piroplasms Babesia and Theileria, are described in more detail in the following paragraphs.

Piroplasms

Piroplasms have many characteristics in common with the malaria parasite, especially with regards to their development. These blood-infecting sporozoans possess rhoptries and microspheres (micronemes of Plasmodium), which facilitate infection of the host cells or escape from the encapsulating vacuoles. All piroplasms are tick-borne, their gametocytes develop and fuse in the tick gut. After that, the parasite migrates to the certain tick body tissues where the infectious sporozoites are produced.

Babesiosis

In cattle, babesiosis has been responsible for the worldwide disastrous outbreaks of illness with extremely high mortality. The Babesia parasites develop entirely in erythrocytes. The infected erythrocytes adhere to one another and to the endothelial lining of capillaries, reducing the blood flow. Hemoglobin and other byproducts released from the breakdown of erythrocytes result in the reddish urine of the infected cattle ("Red Water"), high fever, anemia, and eventually death. The Babesia species also infect domestic livestock, pets and wild animals. Humans can be

infected with *B. microti*, the splenectomized individuals with *B. divergens* or *B. major* (vectored by *Ixodes* spp.). Symptoms are similar to malaria, but without the periodicity.

Dipping and spraying with pesticides have been established throughout the world as the preferred techniques for the control of ticks transmitting babesiosis. Vaccination against babesiosis is efficient and widely used, but the heavy use of anti-babesia vaccines exert strong selection pressure on the parasite and select strains capable of evading the host immune response (Sonenshine, 1991).

The life cycle of Babesia includes asexual cycle (schiziogony) in the vertebrate host and sexual cycle (gametogony) with following asexual sporogony in the tick. In vertebrates, sporozoites transmitted during the tick bite initially invade erythrocytes. Meronts multiplicate inside the erythrocytes by binary fission, producing invasive merozoites. Liberated merozoites attach uninfected erythrocytes, disintegrate parasitophorous vacuole and situate themselves freely in their cytoplasm.

In the tick gut content, the predetermined ingested parasites (gametocytes) transform into gamonts with spine-like projections ("ray bodies"; "Strahlenkorper"), leave the erythrocyte and divide into two or four isogamets. Gametes fuse and form an elongated zygote bearing spine-like arrowhead organelle, which facilitates the midgut cell penetration (through the peritrophic matrix and cell membrane). No parasitophorous vacuole is produced; rather the cell membrane is lysed at the point of entry. Zygote undergoes multiple fissions inside the midgut cell and become an elongated kinete. The kinete leaves the midgut and invades other organs (including ovary) of the tick body. There it initiates new cycles of kinete division and spreading (producing sporokinetes). Once in salivary glands, the sporokinetes transform into infectious sporozoites during the next tick feeding. Infection of the ovary results in the transovarial transmission as the sporokinetes invade salivary glands of the newly developed larva and produce sporozoites. In the 3-host ticks, infected as immatures (larva or nymph), the transmission is maintained by a transstadial route.

Theileriosis

Theileriosis is a destructive lympho-proliferative disease with a massive infection of lymphocytes. *Theileria parva*, vectored by *Rhipicephalus appendiculatus*, is widespread in the eastern and central Africa ("East Coast Fever"). In Mediterranean, China, India, and other countries, Theileria infections caused by *T. annulata* also occur (transmitted by *Hyalomma* sp.),

but are not as devastating as in Africa. Infections caused by other Theileria species affect deer, dogs, cats, cattle, and other animals in various parts of the world. In cattle, theileriosis manifests itself by swelling of the lymph glands, anorexia, coughing, edema of the lungs, hemorrhages, fever, and diarrhea. A natural host of this disease is a wild buffalo, which, when infected, does not exhibit any visible signs of the disease. Similar to the babesiosis, pesticides and vaccines are used to reduce the spread of theileriosis.

In the vertebrate host, sporozoites invade lymphoid tissue or lymphocytes. In these cells, parasite escapes into the cytoplasm by dissolving an enclosing vacuolar membrane. Following schizogony gives rise to the generations of schizonts (macroschizonts; "Koch blue bodies"). Macroschizonts divide together with the cell, inducing a clonal proliferation of the infected lymphocytes. Later in the course of the infection, microschizonts appear inside the infected lymphocytes. Merozoites released from the ruptured cells invade erythrocytes and develop into piroplasms.

Inside the tick gut, piroplasms (although most are destroyed) differentiate into macro- and microgametocytes (“ray bodies”). Gametes fuse and zygotes invade the midgut cells, where they transform into elongated kinetes. After the tick molt, the kinetes leave the gut and migrate to the salivary glands where they develop (only in the E cells of Type III acini). They transform into sporoblasts that undergo sporogony, giving rise to numerous sporozoites, ready for a transmission to the vertebrate host when the tick feeds. In contrast to the Babesia parasites, Theilerias are never transmitted transovarially.

CHAPTER 2

RNA INTERFERENCE IN TICKS

INTRODUCTION

The RNA-mediated gene silencing is an ancient mechanism developed for inhibition of the foreign genetic elements and for precise regulation of the endogenous genes during the development of a particular organism (Myers and Ferrell, 2005). The most recent common ancestor of all eukaryotes most likely possessed an RNA interference pathway (RNAi), although the RNAi machinery seems to be entirely absent in *Saccharomyces cerevisiae*, *Trypanosoma cruzi*, *Leishmania major* and *Plasmodium falciparum* (Cerutti and Casas-Mollano, 2006). Some components of the RNAi machinery (Argonaute) can also be found in Archea and some bacteria (Anantharaman et al., 2002).

The post-transcriptional gene silencing (PTGS) was initially described as an unusual phenomenon in plants (Ecker and Davis, 1986; Napoli et al., 1990). Further, a similar effect called "quelling" was discovered in the fungus *Neurospora crassa* (Romano and Macino, 1992). In 1996, Jorgensen et al. showed that introduction of the pigment-producing gene into the petunia genome suppresses the expression of both: the introduced gene and the homologous endogenous gene (the phenomenon called "co-suppression"; Fig. 8). Not long after, plant virologists observed that plants, expressing the viral proteins show an enhanced resistance to the viral infection (Covey et al., 1997) and reversibly, viruses with the introduced plant genes evoke suppression of the targeted plant genes (Ratcliff et al., 1997).

Mystery of the gene silencing was definitely solved when working on the nematode model organism *Caenorhabditis elegans*. In 1992, Fire and Mello injected the double-stranded RNA into the worm (Fire et al., 1998) and detected an efficient gene knock-down (the effect was called the "RNA interference"). Since that, RNAi has become a valuable scientific tool in the cell cultures and living organisms, helping to identify genes involved in various biological processes. RNAi also turned out to be a promising tool in biotechnology and medicine. In 2006, Craig C. Mello and Andrew Fire were awarded the Nobel Prize in Physiology or Medicine for their work, respectivelly.

The RNAi can be further categorized into two partially overlapping pathways - siRNA and miRNA (Meister and Tuschl, 2004; Hammond, 2005; Dykxhoorn et al., 2003; Pasquinelli et al., 2005). The short-interfering RNA (siRNA) pathway utilizes the exogenous or endogenous dsRNA and suppresses the expression of endogenous genes (genes complementary to the dsRNA sequence). It is also involved in the initiation of the heterochromatin formation (Matzke et al.,

2003; Schramke and Alshire, 2003; Sugiyama et al., 2005) and defense against viruses or endogenous transposable elements. On the other hand, the micro-RNA (miRNA) pathway is triggered only by the endogenous dsRNA, which is transcribed from the non-coding genes (pre-miRNA hairpins). It degrades the endogenous target mRNA or blocks its translation (perfect or imperfect complementarity of the miRNAs with the mRNA target).

Fig. 8. Petunia plants in which genes for pigmentation are silenced by RNAi. *Left*: wild-type; *Central and right*: plants containing transgenes that induce suppression of both transgene and endogenous gene expression, resulting in the unpigmented white areas of the flower. Source: Wikipedia.

PATHWAY OF THE dsRNA-MEDIATED GENE SILENCING

Most applications of the RNAi functional genomics have been performed on the common model organisms, namely *C. elegans* (Kamath and Ahringer, 2003) and *Drosophila melanogaster* (Boutros et al., 2004). With respect to the biology and further applications of the RNAi in ticks, I will compare the tick RNAi pathway mostly with the arthropod pathway of *D. melanogaster* (Kavi et al., 2005; Kavi et al., 2008; see review of Meister and Tuschl (2004) for the comparison of the RNAi mechanisms between various organisms).

In the Drosophila siRNA pathway, an exogenous dsRNA is first cleaved by Dicer2 (RNAse III) into small, 20-25 bp long double-stranded fragments with unpaired overhangs on each end (Carmell and Hannon, 2004). The RNA-binding protein named R2D2 than cooperates with Dicer2 and facilitate loading of the single-stranded siRNA onto RISC (RNA-induced silencing complex; Liu et al., 2003). Finally, RISC binds a protein called Argonaute2 (endonuclease) and facilitates a cleavage of the targeted mRNA in a sequence-specific manner (Carmell et al., 2002).

In the miRNA pathway, the pri-miRNA transcribed from a non-coding gene regions form hairpin-like structures with the double-stranded regions inside. The hairpin is later bound by the dsRNA-binding protein called Pasha and processed by an enzyme Drosha (a homolog of Dicer)

to ~70 bp long pre-miRNA (Lee et al., 2004). The dsRNA portion of the pre-miRNA is transported to the cytoplasm, where it is bound by the dsRNA-binding protein called Loquacious and cleaved by Dicer1. Together with Argonaute1, the complex forms miRNPs (miRNA ribonucleoparticles; Meister and Tuschl, 2004), which cleave the target mRNA (perfect complementarity) or block its translation (imperfect complementarity; Carmell et al., 2002).

The RNAi pathway is also important for the genomic organization and structure by the modification of histones, heterochromatin formation and transcriptional silencing (Holmquist and Ashley, 2006). This process is carried out by the RNA-induced transcriptional silencing complex (RITS). In Drosophila, the core components of this complex are members of the Argonaute family (Piwi (P-element induced wimpy testis) and Aubergine), helicase called Spindle-E, and rasiRNA (repeat-associated small RNA of 25-29 bp in length). The transcriptional gene silencing is a result of the histone modifications and heterochromatin formation around the targeted gene, which makes the gene inaccessible to the transcriptional machinery. Such processes play important roles e.g., in the Drosophila germline formation (Saito et al., 2006). In the fission yeasts, a similar mechanism exists, but the spread of the heterochromatic regions acquires RNA-dependent RNA polymerases (RdRP; Irvine et al., 2006).

The systemic RNAi relies on mechanisms of the dsRNA uptake, spread and amplification. Although the systemic RNAi works efficiently in plants, where the siRNA is distributed between cells through plasmodesmata (Lodish et al., 2004), the cell-to-cell spreading has never been reported in any animal except for *C. elegans*. The RdRPs can dramatically enhance the effect of RNAi in plants, *C. elegans*, *N. crassa,* and *Dictyostelium discoideum*, amplifying the amount of siRNA (from the initial dsRNA or homologous mRNA (transient RNAi) (Baulcombe, 2004)). But, RdRPs have not been detected in any metazoan genome yet, with the exception of *C. elegans* and *Brachiostoma floridae* (Cerutti and Casas-Mollano, 2006), so the mechanism of the systemic RNAi in the RNAi-sensitive metazoans remains unknown.

RNAi IN TICKS

Aljamali et al. (2002) were the first who proved the presence of RNAi in ticks. Since that time, an increasing number of scientific papers exploiting the RNAi method are being published every year (9 articles in 2008; reviewed by de la Fuente et al., 2007a). In ticks, where other methods of genetic manipulation are rather limited, RNAi represents a godsend. It allows us to

study functions of the tick genes or biochemical pathways, characterize the tick-pathogen interface, and screen and describe tick protective antigens. In the following chapters I would like to summarize all possible methods of the dsRNA delivery in ticks and propose a model of the tick RNAi pathway based on my *in-silico* genomics results.

dsRNA delivery in ticks

In ticks, four different techniques of the dsRNA delivery are known. Theoretically, also siRNA could be used for the knock-down, although Kurscheid et al. (2008, TTP-6 conference abstract) reported that no reduction of the transcript was observed in the cell cultures *in vitro* and just a partial reduction was achieved *in vivo* after injection of the siRNA into females. For our experiments, we use exclusively dsRNA, which we prepare from vectors (pll, modified BlueScript; Levashina et al., 2001) with two T7 promoters in reverse orientation. We synthesize sense and antisense ssRNA in separate tubes and subsequently hybridize both strands together.

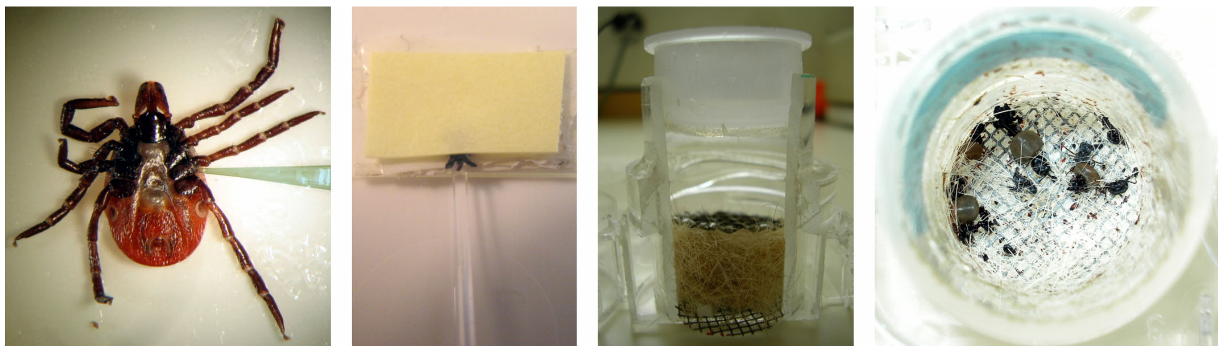

Fig. 9. Demonstration of the dsRNA delivery in ticks. *From left:* Unfed tick female injected with microcapillary; Capillary feeding; A section through the feeding unit used for the artificial membrane feeding; Ticks attached to the artificial membrane.

Injection of the dsRNA

Injection is the most common method of the dsRNA delivery in ticks. Aljamali et al. (2002) were first who injected the dsRNA into the tick females of *Amblyoma americanum*. Since that time, injections by the Hamilton syringe or microcapillary (Narashiman et al., 2004) have become a routine (reviewed by de la Fuente et al., 2007a).

In our laboratory, microinjection (microcapillary connected to the micromanipulator) is the main route of the dsRNA delivery (Fig. 9). In my opinion, it is the most elegant and most efficient method, which does not seriously injure the employed tick. We usually inject 0.5 µl of

the 3 μg/μl dsRNA (100-500 bp long dsRNA) per a single female into the second or third coxa (into hemolymph). After 24 hours of resting in the humid chamber at room temperature, ticks are fed on animals (25 females and 25 males per a single guinea pig). In general, a sufficient knock-down is achieved on the mRNA (RT-PCR) or protein level (Western blot; see e.g., Hajdusek et al., 2009). We also frequently also evaluate tick survival, weight after repletion, oviposition, and larvae hatching to score the effect of gene knock-down on the tick fitness. As seen in our recent paper (Buresova et al., 2009), the microinjection can also be used to knock-down tick immune genes and evaluate their influence on phagocytosis of various pathogens by the tick hemocytes.

Although we have never followed a gene knock-down in the progeny, it has recently been shown that injection of the dsRNA (through the spiracle of fed ticks) could induce RNAi in eggs and larvae of *Rhipicephalus (Boophilus) microplus* (Nijhof et al., 2007; Kocan et al., 2007). The authors detected high levels of the injected (or amplified) full-length dsRNA in the laid eggs, which probably spread over the tick tissues during the embryo development and evoked the gene knock-down in the progeny. However, the silencing was not transferred into adult ticks.

Theoretically, to get an efficient knock-down in the progeny, females could be injected with a vector DNA, which could disperse through the tick cells and continuously produce dsRNA (for the tick cell culture transfections see Kurtii, et al., 2008). Although this method is commonly used in the model organisms (Tavernarakis et al., 2000), it has never been tried in ticks.

Soaking

This application represents a great potential in the tick cell cultures, which have been proved to actively intake the dsRNA and induce RNAi (Blouin et al., 2008). This method allows high throughput screening and functional genomics studies in ticks. Blouin et al. (2008) showed an efficient knock-down of the subolesin expression in the *I. scapularis* IDE8 tick cell line treated with the dsRNA for 24 hours.

The *ex vivo* soaking of dissected tick organs turned out as an effective method to study physiology of salivary glands and protein production (Aljamali et al., 2003; Bowman and Sauer, 2004; Karim et al., 2004). In these experiments, 6-12 hour incubation of the dissected salivary glands with the dsRNA led to the significant (~50 to 90%) reduction of the gene transcript. It is obvious, that a similar knock-down can be done in various tissues, but the application has the greatest biological potential on salivary glands.

The whole tick soaking could induce RNAi in a large number of individuals and could produce an efficient knock-down without an injury resulting from the microinjection. In our experiments we have tried the *in vivo* soaking with nymphs of *I. ricinus*. These nymphs are too small to survive microinjection. About 30 nymphs were incubated in 200 µl of the dsRNA (0.75 µg/µl). After 4 hours, nymphs were dried on the filter paper and allowed to rest for 24 hours in the humid chamber at room temperature. Treated nymphs were fed on mice with ~70% efficiency. As a result, the studied gene was successfully knocked-down (data not shown).

Feeding of the dsRNA

The RNAi-inducing capillary feeding in ticks was demonstrated by Soarez et al., (2005), who showed an efficient gene silencing in *I. scapularis* nymphs. In this experiment, the pre-blood-fed nymphs (fed for 18 hours) were allowed to capillary feed (hypostome in the capillary) on the dsRNA for 4 hours. After 24 hours, nymphs were returned back on the mice to finish blood feeding. The delivered dsRNA was able to silence the studied gene in salivary glands. The authors thus suggest that the dsRNA penetrated the gut and was delivered to salivary glands and other organs by the hemolymph.

We have tried the capillary feeding once in our laboratory (Sojka, Ph.D. thesis, 2007). Alternatively, I have tried an artificial membrane feeding (Krober and Guerin, 2007) with blood mixed with dsRNA (Fig. 10). The aim of this experiment was to enhance the efficiency of knock-down in the gut tissue, which is quite problematic in females of *I. ricinus*. 50 µl of the dsRNA (3 µg/µl; ferritin) were mixed with 3 ml of blood and used for feeding of ticks at day 2. After 12 hours, fresh blood was mixed with the same volume of dsRNA and used for next feeding (12 hours). RT-PCR and Western blot showed the silencing of ferritin expression in all tissues (gut, salivary glands and ovary) with the most successful knock-down in the ovaries. Similarly as seen in Soarez et al., (2005), the dsRNA penetrated the gut wall, spread over the tick body and induced knock-down in various tissues. Although successful, this method as well as capillary feeding is more laborious and less efficient than microinjection.

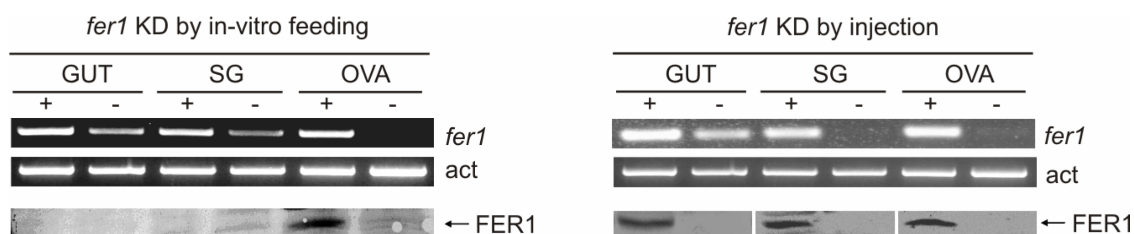

Fig. 10. RNAi silencing of *fer1* in half-fed ticks. *Left:* Artificial *in-vitro* feeding on blood with dsRNA; *Right:* Microinjection of the dsRNA and feeding on guinea pig. Silencing of *fer1* decreased *fer1* mRNA levels (RT-PCRs in rows *fer1*) and translation (Western blots in rows FER1) whereas actin mRNA levels were unchanged (row *act*).

Production of the dsRNA by viral infection

Infection of tick cells with the recombinant RNA virus (Semliki Forest virus) containing the tick-borne Hazara virus (HAZV) nucleoprotein gene in sense or antisense orientation, efficiently inhibited HAZV replication (Garcia et al., 2005). This provides evidence that dsRNA can be introduced into ticks by the recombinant virus and that RNAi might be the basic antiviral response in ticks, similarly as seen in insects (Keene et al., 2004).

The discovery that plant viruses encode suppressors of the gene silencing machinery provided a strong support that RNAi functions as a natural defense mechanism against viruses (Lindbo et al., 1993; Ratcliff et al., 1999). Garcia et al. (2006) showed that viral proteins identified as suppressors in plants and insect cells were able to abrogate RNA silencing also in tick cells.

A MODEL OF RNAi PATHWAY IN *IXODES SCAPULARIS*

The sequencing project of the black-legged tick *Ixodes scapularis* marks the beginning of the genomics era in ticks. As proposed, the whole genome shotgun sequencing (WGS) should include at least six-fold coverage of the genome (Pagel Van Zee et al., 2007). The mitochondrial genome of *I. scapularis* has already been assembled and annotated (Shao et al., 2005).

The genome size of *Ixodes scapularis* is ~2.1 Gbp (two-thirds of the human genome size (Ullmann et al., 2005)) and repetitive DNA constitutes about 21.5% of the *I. scapularis* sequences (Pagel Van Zee et al., 2007). Recently, 19,457,209 traces and 193,772 ESTs (expressed sequence tags) can be accessed and blasted through the NCBI database (http://www.ncbi.nlm.nih.gov/), TIGR Gene Index (http://compbio.dfci.harvard.edu/tgi/tgipage.html) or Vector Base (http://iscapularis.vectorbase.org/index.php). The second version of the *I. scapularis* gene set (IscaW1.1) was released in December 2008. Although the genomic and EST data are often

incomplete, they allow us to screen for genes of interest or even for the whole biochemical pathways.

Results of the *I. scapularis* RNAi pathway genomics screen

In this chapter I would like to present the result of my *in silico* screen for RNAi pathway in *I. scapularis*. To date, the only sequence associated with the RNAi machinery found in ticks is Argonaute-2 from *B. microplus* (de la Fuente et al., 2007a). Using tBLASTn algorithm in *I. scapularis* VectorBase (IscaW0.5) I was able to find more than 40 orthologous genes with *D. melanogaster*, *C. elegans* or *Homo sapiens* and propose a basic model for the RNAi pathway in *I. scapularis* (Fig. 11). Domain structures were predicted by ScanProsite (http://www.expasy.ch/tools/scanprosite/) or NCBI CDS (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Summary

From the results summarized herein, it is obvious that ticks possess a complex RNAi mechanism, which involves both siRNA and miRNA pathways. Ticks probably also use the RNAi pathway for the transcriptional gene silencing through the DNA methylation and formation of heterochromatin. I conclude that RNAi seems to be systemic in ticks, similarly as seen in Tribolium and *C. elegans*, and it works well in all tissues except the gut, where the knock-downs are substantially less efficient. The involvement of RdRPs and other components in the RNAi pathway remains to be investigated more deeply and it definitely represents a big challenge for next years.

CHAPTER 3

RESEARCH ARTICLES

TICK IRON METABOLISM

The iron metabolism has been thoroughly studied in various organisms, but still some basic questions remain unanswered. *Ixodes ricinus* is a well-known vector for important human and animal diseases. During the blood feeding, when the potential pathogens are transmitted, ticks are exposed to an enormous amount of free (non-heme) iron. In our recent paper we evidence that ticks have developed a unique mechanism for utilization and detoxification of the iron (Hajdusek et al., 2009). Silencing of genes involved in the iron metabolism has an adverse impact on tick feeding, oviposition and hatching. We strongly believe that this work will significantly contribute to the development of an efficient vaccine against ticks and tick-transmitted diseases.

Ferritins

Ferritin is the main protein for intracellular iron storage. It typically consists of two types of subunits: a heavy (ferroxidase sites) and light chain (nucleation sites). Surprisingly, all tick ferritins are composed only from the heavy chain subunits. Ferritin1 is the common intracellular storage ferritin, while ferritin2 is a new secreted ferritin, which functions as a primary iron transporter. Similar heavy-chain ferritin homo-oligomers can be found in plants. Insect and mammalian mitochondrial ferritins are also composed of the heavy chain homo-oligomers.

It is very likely that the light chain ferritin as well as the mitochondrial ferritin is absent from the tick genome. Although, the function of the mitochondrial ferritin is generally unknown (Missirlis et al., 2006), we speculate that its lack in ticks could be connected with the reduction of the heme synthesis pathway.

Iron transport

In vertebrates, the free iron released after the food digestion is actively transported into the gut cells (divalent metal transporter, DMT-1), stored (FER-L and FER-H), and transferred into the blood stream (transferrin). In insects, a similar mechanism exists, but the transport mechanism is unknown, since transferrins are not believed to be involved in the insect iron metabolism (Dunkov and Georgieva, 2006). Insect ferritins (FER-H and FER-L) might serve both the intracellular iron storage and transport (Nichol et al., 2002). In vertebrates and insects, a specific pathway exists also for the exogenous heme, which can be absorbed into the gut cells and

cleaved by heme oxygenases (Hentze et al., 2004; Nichol et al., 2002). The heme oxygenases have not been found in the genome of *I. scapularis*.

Ticks digest blood exclusively intracellularly (Sojka et al., 2008). Our data indicate that the only source of iron in the tick diet might be the host transferrin. We intend to continue our research in setting–up an artificial membrane to perform feeding on the hemoglobin-free host plasma or plasma spiked with the host Fe^{59}-transferrin to confirm our hypothesis. If our anticipation is correct, the non-heme iron released from the host transferrin should be further loaded into the FER2 and transported to the peripheral tissues. A deeper study should also provide detailed insight into the role of tick DMT1 in the iron transportation.

Tick "transferrin"

In the tick genome, a gene similar to the human and insect transferrin has been recently identified (unpublished data). We showed previously that FER2 is the main iron transporter in ticks (Hajdusek et al., 2009). The function of the tick transferrin (TF2) is unknown, but based on our recent data I propose that this protein is not involved in the iron transport.

Vaccine development

The tick control is currently dependent on the employment of chemical pesticides and tick-resistant animals. Because of increased tick resistance to pesticides, their application became problematic. The use of genetically resistant animals, which is a vital component of many tick control strategies, shows also many drawbacks.

Nowadays, scientists are forced to find tick antigens that could be used for a vaccine development, because the production of vaccines is in general not as difficult and expensive as the production of acaricides (Willadsen, 2008). The first vaccine based on the tick gut protein (Bm86; TickGUARD and Gavac) was already developed and commercialized (Willadsen et al., 1995). This vaccine protects cattle not only against ticks, but also against the tick-transmitted diseases (de la Fuente et al., 2007b). However, the protection based solely on the vaccines will require discovery of more efficient vaccines than those currently available.

An option how to screen for the tick vaccine candidates is to employ RNAi screening. These screens can cover thousands of different tick ESTs and could selectively find a single gene, which is responsible for an anti-tick activity (de la Fuente et al., 2005). However, this method

commonly identifies housekeeping genes, which knock-downs have drastic consequences for the tick, but the antigen must be often excluded because of high similarity with the host genes.

An ideal anti-tick target should:

i.) be a unique sequence (not to cross-react with the host antigens)

ii.) be expressed in large quantities in all feeding stages of the tick

iii.) be essential for the tick

iv.) evoke high and stable antibody response of the host

v.) be stable, easily expressed and folded

vi.) be patented for the possible commercialization.

We do hope that the tick FER2 might be such a target. FER2 is a unique ferritin, which does not have any functional orthologs in vertebrates. This protein is expressed in the tick gut before and after feeding across all developmental stages. Further, it has an essential function in the iron metabolism. As proved by RNAi, ticks can not adequately process iron without this protein and die on the host. Importantly, the tick FER2 is easy to express (in *E. coli*), fold, and evokes high antibody response of the host.

As shown by Vaughan et al. (2002), the host antibodies are able to freely cross the tick gut (going through the gut cells) and target proteins in the hemolymph. I believe that host antibodies raised against the tick FER2 might bind the protein in the gut cells and/or hemolymph, and interfere with the iron metabolism pathway. The affected tick should not be able to feed properly and will dry attached on the host. The interference with iron metabolism will block the appropriate transfer of iron into the ovaries, which will result in the hatching defects and reductions of the tick progeny.

Additionally, these antibodies might reduce transmission of pathogens, especially of those, performing at least part of their developmental cycles in the tick gut. *Borrelia burgdorferi* needs at least 16 hours to multiply in the *I. ricinus* gut content, cross the gut, and infect salivary glands (Kahl et al., 1998). This might be sufficient time for the host antibodies to change the tick conditions to unfavorable for the Borrelia development. Borrelia belongs to the few unique organisms on Earth, which do not require iron for their life cycle. Moreover, levels of iron higher than >10 µM inhibit its movement and kill the spirochetes (Posey and Gherardini, 2000). If the antibody against the tick FER2 will interfere with utilization of the iron (resulting from the blood

digestion) inside the tick, the extremely high level of free iron might efficiently block the Borrelia transmission.

ARTICLE I

Knockdown of proteins involved in iron metabolism limits tick reproduction and development.

Hajdusek O, Sojka D, Kopacek P, Buresova V, Franta Z, Sauman I, Winzerling J, Grubhoffer L

Proc Natl Acad Sci U S A (2009) 106:1033-1038.

Ticks are among the most important vectors of a wide range of human and animal diseases. During blood feeding, ticks are exposed to an enormous amount of free iron that must be appropriately used and detoxified. However, the mechanism of iron metabolism in ticks is poorly understood. Here, we show that ticks possess a complex system that efficiently utilizes, stores and transports non-heme iron within the tick body. We have characterized a new secreted ferritin (FER2) and an iron regulatory protein (IRP1) from the sheep tick, *Ixodes ricinus*, and have demonstrated their relationship to a previously described tick intracellular ferritin (FER1). By using RNA interference-mediated gene silencing in the tick, we show that synthesis of FER1, but not of FER2, is subject to IRP1-mediated translational control. Further, we find that depletion of FER2 from the tick plasma leads to a loss of FER1 expression in the salivary glands and ovaries that normally follows blood ingestion. We therefore suggest that secreted FER2 functions as the primary transporter of non-heme iron between the tick gut and the peripheral tissues. Silencing of the fer1, fer2, and irp1 genes by RNAi has an adverse impact on hatching rate and decreases postbloodmeal weight in tick females. Importantly, knockdown of fer2 dramatically impairs the ability of ticks to feed, thus making FER2 a promising candidate for development of an efficient anti-tick vaccine.

TICK IMMUNITY

The innate immunity plays a key role in the transmission of pathogens during the tick feeding. Although several tick immune molecules have already been described, the mechanism of the tick immunity still remains poorly understood (Sonenshine and Hynes, 2008). In the following publications we show that ticks possess a set of thioester-containing proteins (TEP) and fibrinogen-related proteins (FREP) that might play a key role in the pathogen recognition and transmission.

Thioester-containing proteins

In the first publication (Buresova et al., 2009) we determined the structure and function of α_2-macroglobulin (IrAM) in the tick *Ixodes ricinus*. We showed that IrAM is a hemolymph protein that is involved in the phagocytosis of bacteria.

In this article we described for the first time how to knock-down tick immune genes and evaluate their roles in the phagocytosis of various pathogens by the tick hemocytes (Fig. 18). Briefly, ticks were injected with the dsRNA (1.5 μg) and after 24 hours of resting they were fed on guinea pigs. For *ex vivo* experiments, hemolymph was collected from the half-fed ticks (fed for 5 days) and hemocytes were allowed to adhere to the surface of microscopic glass for one hour. After that, suspension of bacteria was added and the glass was incubated for another hour at 28°C. Finally, the preparations were fixed and the bacteria and hemocytes stained with an antibody or DNA-binding dye, respectively. The level of phagocytosis was examined using fluorescence microscope. For *in vivo* experiments, we injected the half fed ticks with the pathogen. After 48 hours we collected the hemolymph and determined the level of phagocytosis.

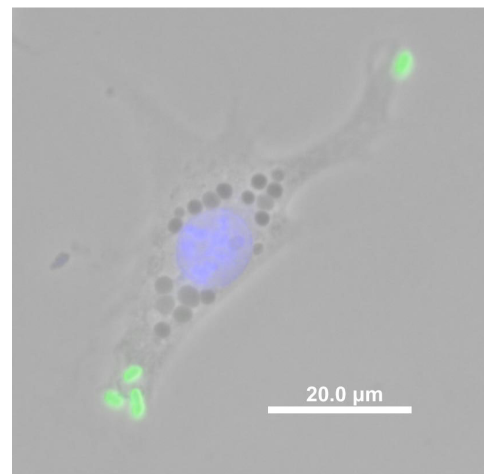

Fig. 18. The test of phagocytosis. *Chryseobacterium indologenes* being phagocytosed by the tick hemocyte after the IrAM knock-down. Merged image. Phase contrast; bacteria in green (FITC); hemocyte nucleus in blue (DAPI).

We routinely use various bacteria and yeast for the phagocytosis, but our goal for the next years is to find a protein that will interfere with the phagocytosis of Borrelia. The identification of such protein might help to develop a vaccine that will disable the transmission of this important pathogen.

Fibrinogen-related proteins

The second article Rego et al., 2005 showed that hard tick *I. ricinus* possessed, similarly to the soft tick *Ornithodoros moubata*, several different fibrinogen-related proteins (FREP). These proteins were related to tachylectins from the horse-shoe crab *Tachypleus tridentatus* and vertebrate ficolins. Although the role of tachylectins in the innate immunity is only suggestive (Zhu et al., 2005), vertebrate ficolins are important immune factors that are involved in the lectin pathway of the vertebrate complement system (Endo et al., 2007).

The tick FREPs are probably pathogen-recognition proteins that might be involved in a complex immune pathway leading to the phagocytosis of a pathogen. Recently, we work on the identification of this pathway and we speculate that some of the tick IXOs and TEPs are involved in a common immune cascade.

ARTICLE II

IrAM-An alpha2-macroglobulin from the hard tick *Ixodes ricinus*: characterization and function in phagocytosis of a potential pathogen *Chryseobacterium indologenes*.

Buresova V, Hajdusek O, Franta Z, Sojka D, Kopacek P

Dev Comp Immunol (2009) 33:489-498.

The universal protease inhibitors of the α_2-macroglobulin (α_2-M) family are evolutionarily conserved constituents of innate immunity, presumably because they guard organisms against undesired proteolytic attacks of a different origin. Here, we determined the primary structure of α_2-M from the hard tick *Ixodes ricinus* (IrAM) by sequencing of overlapping PCR products. Predicted disulfide and glycosylation patterns, post-translational cleavage and alternative splicing within its 'bait region' (Figure 1A) demonstrate that IrAM is closely related to the α_2-M from the soft tick *Ornithodoros moubata*. The IrAM message is expressed in all tick developmental stages and tissues, except for the gut, and the protein was detected to be mainly present in the hemolymph. Silencing of IrAM by dsRNA interference markedly reduced the phagocytosis of a potential pathogen, *Chryseobacterium indologenes*, by tick hemocytes both in vitro and in vivo. In contrast, phagocytosis of the Lyme disease spirochete *Borrelia burgdorferi* or a commensal bacteria *Staphylococcus xylosus* was not affected by the IrAM knock-down. Similar results were obtained upon deactivation of all thioester proteins in tick hemolymph by methylamine. We have further demonstrated that phagocytosis of *C. indologenes* is dependent on an active metalloprotease secreted by the bacteria. These data indicate that interaction of tick α_2-M with a protease of an invading pathogen is linked with cellular immune response.

ARTICLE III

Molecular cloning and comparative analysis of fibrinogen-related proteins from the soft tick *Ornithodoros moubata* and the hard tick *Ixodes ricinus*.

Rego RO, Hajdusek O, Kovar V, Kopacek P, Grubhoffer L, Hypsa V

Insect Biochem Mol Biol (2005) 35:991-1004.

Among disease-vectors, the evolution of the tick innate immune system is still lagging when compared to insects. Such an investigation, which was initiated, by first cloning and sequencing lectins associated in the innate immunity of invertebrates and having fibrinogen related domains, helped in the sequencing of cDNA encoding for OMFREP from the soft tick, *Ornithodoros moubata*. Also obtained were Ixoderin A and Ixoderin B cDNA sequences from the hard tick Ixodes ricinus. Tissue-specific expression of OMFREP showed that it was present primarily in the hemocytes and salivary glands. Ixoderin A besides sharing a similar expression profile was also expressed in the midgut. Both showed significantly high homology to the lectin Dorin M, from *O. moubata*. Further, phylogenetic comparisons between these molecules of the soft and hard ticks showed their relatedness to Tachylectins 5A and 5B, involved in the innate immunity of *Tachypleus tridentatus* and ficolins from both vertebrates and invertebrates. Ixoderin B showing tissue-specific expression only in the salivary glands and the sequence displaying certain motif differences in homology point towards a possible function different from the other two molecules. This is the first report of lectin-like sequences, with a fibrinogen-domain, from the hard tick *I. ricinus* and a preliminary phylogenetic study of these tick sequences with related fibrinogen-domain containing sequences highlights a possible role for them in the innate immunity of the ticks.

TICK DIGESTION

Ticks feed solely on blood. Unlike other blood-feeding arthropods, they are believed to digest blood intracellularly in the lysosomal vesicles of gut cells at acidic pH. Although feeding and digestion are the key processes which determine the further tick reproduction capacity, the knowledge of molecular mechanisms involved are rather limited. In the attached publications (Sojka et al., 2007 and 2008) we described cysteine peptidases (cathepsin B, L and C), an asparaginyl endopeptidase (legumain), and an aspartic peptidase (cathepsin D) of the tick *Ixodes ricinus* and demonstrated their involvement in the blood processing.

ARTICLE IV

IrAE: an asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*.

Sojka D, Hajdusek O, Dvorak J, Sajid M, Franta Z, Schneider EL, Craik CS, Vancova M, Buresova V, Bogyo M, Sexton KB, McKerrow JH, Caffrey CR, Kopacek P

Int J Parasitol (2007) 37:713-724.

Ticks are ectoparasitic blood-feeders and important vectors for pathogens including arboviruses, rickettsiae, spirochetes and protozoa. As obligate blood-feeders, one possible strategy to retard disease transmission is disruption of the parasite's ability to digest host proteins. However, the constituent peptidases in the parasite gut and their potential interplay in the digestion of the blood meal are poorly understood. We have characterised a novel asparaginyl endopeptidase (legumain) from the hard tick *Ixodes ricinus* (termed IrAE), which we believe is the first such characterisation of a clan CD family C13 cysteine peptidase (protease) in arthropods. By RT-PCR of different tissues, IrAE mRNA was only expressed in the tick gut. Indirect immunofluorescence and EM localised IrAE in the digestive vesicles of gut cells and within the peritrophic matrix. IrAE was functionally expressed in *Pichia pastoris* and reacted with a specific peptidyl fluorogenic substrate, and acyloxymethyl ketone and aza-asparagine Michael acceptor inhibitors. IrAE activity was unstable at pH P 6.0 and was shown to have a strict specificity for asparagine at P1 using a positional scanning synthetic combinatorial library. The enzyme hydrolyzed protein substrates with a pH optimum of 4.5, consistent with the pH of gut cell digestive vesicles. Thus, IrAE cleaved the major protein of the blood meal, hemoglobin, to a predominant peptide of 4 kDa. Also, IrAE trans-processed and activated the zymogen form of *Schistosoma mansoni* cathepsin B1 – an enzyme contributing to hemoglobin digestion in the gut of that bloodfluke. The possible functions of IrAE in the gut digestive processes of *I. ricinus* are compared with those suggested for other hematophagous parasites.

ARTICLE V

Profiling of proteolytic enzymes in the gut of the tick *Ixodes ricinus* reveals an evolutionarily conserved network of aspartic and cysteine peptidases.

Sojka D, Franta Z, Horn M, Hajdusek O, Caffrey CR, Mares M, Kopacek P

Parasit Vectors (2008) 1:7.

Background: Ticks are vectors for a variety of viral, bacterial and parasitic diseases in human and domestic animals. To survive and reproduce ticks feed on host blood, yet our understanding of the intestinal proteolytic machinery used to derive absorbable nutrients from the blood meal is poor. Intestinal digestive processes are limiting factors for pathogen transmission since the tick gut presents the primary site of infection. Moreover, digestive enzymes may find practical application as anti-tick vaccine targets.
Results: Using the hard tick, *Ixodes ricinus*, we performed a functional activity scan of the peptidase complement in gut tissue extracts that demonstrated the presence of five types of peptidases of the cysteine and aspartic classes. We followed up with genetic screens of gut-derived cDNA to identify and clone genes encoding the cysteine peptidases cathepsins B, L and C, an asparaginyl endopeptidase (legumain), and the aspartic peptidase, cathepsin D. By RT-PCR, expression of asparaginyl endopeptidase and cathepsins B and D was restricted to gut tissue and to those developmental stages feeding on blood.
Conclusion: Overall, our results demonstrate the presence of a network of cysteine and aspartic peptidases that conceivably operates to digest host blood proteins in a concerted manner. Significantly, the peptidase components of this digestive network are orthologous to those described in other parasites, including nematodes and flatworms. Accordingly, the present data and those available for other tick species support the notion of an evolutionary conservation of a cysteine/aspartic peptidase system for digestion that includes ticks, but differs from that of insects relying on serine peptidases.

CONCLUDING REMARKS

During my Ph.D. study I have established the method of RNA interference in the tick *Ixodes ricinus* in our laboratory. This method has become a valuable tool for our joint research. My Ph.D. thesis should introduce the reader into the field and show possible applications and advantages of using the RNAi in ticks. Most of our results have been published in five international journals and also our unpublished results, mentioned in the Chapters II and III, will hopefully be published soon.

<u>Chapter I</u>:

In this chapter I highlighted the importance of ticks, their ecology, basic physiology, and morphological characteristics. I mainly focused on the tick *I. ricinus*, the most common tick in the Czech Republic. The second part of this chapter describes the tick-transmitted diseases including tick-borne encephalitis and Lyme disease, the most important tick-borne diseases in our country.

Nowadays, it is not clear, if RNAi plays a role in the multiplication of viruses in ticks. It is possible, that the RNAi defense system, which is primarily directed against RNA viruses, could make the dispersion at least more difficult. It remains to reveal a role of the RNAi in the tick vector capacity and consider the involvement of RNAi in the transovarial and transstadial transmission of viruses in ticks.

<u>Chapter II</u>:

Here I summarized recent knowledge on RNAi in ticks. We have tried several methods of the dsRNA delivery in ticks, including microinjection, capillary feeding, membrane feeding, and soaking. The last two have not been described in the literature before. I conclude that all the methods are highly recommendable for being employed in ticks. However, the microinjection seems to be an optimal method for the dsRNA delivery in ticks because of the easy application and high efficiency of the knock-downs.

The RNAi seems to be systemic in ticks, similar to Tribolium and *Caenorhabditis elegans*. Based on my *in silico* genomic search I conclude, that ticks probably possess both the dsRNA and miRNA pathways, because the key genes of these pathways were found in the genome of

I. scapularis. They probably also use the RNAi for the transcriptional gene silencing through the DNA methylation and heterochromatin formation.

Additionally, seven different RNA-dependent RNA polymerases (RdRP) have been found in the genome of *I. scapularis*. They have not been detected in any metazoan genome with the exception of *C. elegans* and *Brachiostoma floridae* (Cerutti and Casas-Mollano, 2006). This is the first report of these enzymes in arthropods. Although the function of the RdRPs in ticks remains unknown, these proteins have an important potential for the development of the anti-tick vaccine.

Chapter III:

Most importantly, in the last chapter I demonstrated a practical application of the RNAi for the tick research. This method was used to dissect genes of the tick iron metabolic pathway and tick innate immunity. By using the RNAi we realized that tick ferritin2 might be an ideal target for an efficient vaccine against ticks and tick-transmitted diseases. Of course, additional vaccination experiments are needed to evaluate the possibility of the host immunization and protection with this protein.

Further, I included some additional unpublished data on fibrinogen-related proteins, which molecular and biochemical characteristics were published previously by our group. Finally, the work contains a published description of the tick digestive protease network, involved in the blood digestion.

SHRNUTÍ (IN CZECH)

Předkládaná dizertační práce je zaměřena na popis a využití metody RNA interference u klíšťat, především u klíštěte obecného, *Ixodes ricinus*. Toto klíště je v České republice přenašečem klíšťové encefalitidy a Lymské boreliózy. Počet případů těchto onemocnění v České republice každým rokem roste. Jen v roce 2008 bylo na našem území ohlášeno 633 případů klíšťové encefalitidy a 4350 případů Lymské boreliózy (Státní zdravotní ústav, Praha). Ačkoliv proti klíšťové encefalitidě se v současné době lze nechat očkovat, proti Lymské borelióze vakcína zatím neexistuje.

Klíšťata jsou velmi významnými přenašeči nejrůznějších onemocnění. Přenášejí řadu různých virů, bakterií a prvoků. Těmito nemocemi ovšem netrpí jen lidé, nakažená mohou být i různá zvířata. U hospodářských zvířat, zvlášť v zemích s intenzivním zemědělstvím, způsobují tyto onemocnění přenášené klíšťaty škody v hodnotě miliard dolarů ročně.

K obraně zvířat před klíšťaty se rutině používají nejrůznější akaricidy, někdy se také přistupuje k chovu geneticky odolných zvířat. Nicméně, proti akaricidům jsou si klíšťata schopna velice rychle vyvinout účinnou rezistenci a chov geneticky odolných zvířat má také své nedostatky. Proto je nyní vyvíjen tlak na výzkumné instituce, aby se pokusily identifikovat klíštěcí proteiny, které by mohli sloužit jako vhodné antigeny pro vývoj vakcín proti klíšťatům a klíšťaty přenášených chorob.

Kapitola I:

V této kapitole jsem se snažil uvést čtenáře do klíštěcí problematiky a ve stručnosti popsat některé morfologické, fyziologické a ekologické znaky klíšťat. Ve více detailech je zde popsána morfologie klíštěcího střeva, slinných žláz a vaječníků. Tyto orgány jsou u klíšťat vědecky nejčastěji studovanými a hrají také významnou roli v přenosech patogenů na hostitele.

Ve druhé části této kapitoly poté předkládám souhrn hlavních patogenů přenášených klíšťaty, s ohledem na jejich vývoj právě v klíštěti. Je známo, že viry se v klíšťatech přenášejí s větší či menší mírou úspěšnosti transstadiálně (mezi klíštěcími stádii) nebo transovariálně (mezi generacemi). V současné době není známo, jakou roli v tomto přenosu hraje RNA interference, která primárně slouží zřejmě právě jako ochrana před RNA viry.

Kapitola II:

Tato kapitola obsahuje souhrn současných znalostí o RNA interferenci u klíšťat. Jsou zde popsány možnosti aplikace RNAi. Většinu těchto metod jsme sami vyzkoušeli v naší laboratoři. Popsaná metoda umělého sání klíšťat přes membránu a metoda "vsakování" dvouvláknové RNA (dsRNA) do klíšťat ještě nebyla doposud v literatuře popsána.

Mohu říci, že všechny uvedené metody RNAi u klíšťat jsou účinné, nicméně metoda mikroinjikace dsRNA do klíštěte se zdá být nejpraktičtější a také nejefektivnější ze všech popsaných metod. RNAi u klíšťat se jeví jako tzv. systémová, stejně jako např. u háďátka *Caenorhabditis elegans* nebo brouka *Tribolium castaneum*. To znamená, že po vpravení dsRNA do těla klíštěte se je schopen efekt RNAi rozšířit do všech tkání a vyvolat silný účinek ztlumení genové exprese.

Díky nedávno zveřejněné genomové sekvenci klíštěte *I. scapularis* jsem byl schopen u tohoto klíštěte identifikovat většinu genů (více než 40), které jsou u ostatních organismů zodpovědné za RNAi. Donedávna byla z RNAi dráhy u klíšťat známa pouze sekvence genu pro Argonaute 2. Na základě mých dat mohu říci, že u klíšťat fungují obě popisované dráhy RNAi (siRNA i miRNA dráha). Dále se zdá, že RNAi je u klíšťat zodpovědná i za tvorbu heterochromatinu a inhibici genové transkripce.

Jedním z nejzajímavějších výsledků v této části je podle mě nález sedmi genů pro RNA-dependentní RNA polymerázy u klíšťat. Tyto polymerázy jsou u některých organismů zodpovědné za amplifikaci RNAi signálu nebo jsou zúčastněny v procesu blokování genové transkripce (hlavně repetitivních DNA sekvencí) a tvorby heterochromatinu. U metazoí byly tyto geny nalezeny pouze u *C. elegans* a kopinatce *Branchiostoma florida*. Co spojuje právě klíšťata s těmito organismy zůstává záhadou. Nicméně faktu, že tyto polymerázy nejsou přítomny u lidí či u jiných obratlovců, by se dalo v budoucnu využít pro tvorbu vakcín proti klíšťatům.

Kapitola III:

V poslední kapitole jsem se pokusil demonstrovat, že metoda RNAi je vhodná pro studium funkce jednotlivých genů nebo dokonce celých metabolických drah. V přiložených publikacích popisujeme, s velkým přispěním metody RNAi, dráhu pro metabolismus železa u klíštěte *I. ricinus* a také některé komponenty fungující v imunitním systému klíšťat. V dráze pro zpracování železa se nám podařilo identifikovat unikátní protein, který jsme nazvali ferritin 2. Tento ferritin

se v klíštěti účastní přenosu železa mezi střevem a jednotlivými tkáněmi. Ztlumení jeho exprese pomocí RNAi má u klíštěte za následek to, že klíšťata špatně sají a zasychají na hostiteli. Věříme, že právě tento protein by mohl být v budoucnu použit pro vývoj vakcín proti klíšťatům nebo klíšťaty přenášeným patogenům.

Dále v této kapitole uvádíme některá dosud nepublikovaná data týkající se proteinů s doménou příbuznou fibrinogenu. Domníváme se, na základě RNAi, že tyto proteiny hrají v klíštěti zásadní roli v identifikaci patogenů a jejich následné fagocytóze hemocyty. Na závěr mé dizertační práce přikládám dvě publikace, ve kterých se nám podařilo popsat klíštěcí střevní cysteinové proteázy, které jsou hlavními složkami procesu trávení hostitelské krve.

Naše výsledky byly celkem publikovány v pěti impaktovaných zahraničních časopisech a prezentovány na několika zahraničních konferencích. Domnívám se, že i zde uvedené nepublikované výsledky mají velký potenciál k tomu, aby byly v budoucnu otištěny v kvalitních vědeckých časopisech.

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SUPPORTING INFORMATIONS

SEQUENCE DESCRIPTIONS

Fig. 12. (Dicers)

DCR-1_H.sapiens (*Homo sapiens* NP_803187), Drosha_H.sapiens (*Homo sapiens* NP_037367), DCR-1_D.melanogaster (*Drosophila melanogaster* NP_524453), DCR-2_D.melanogaster (*Drosophila melanogaster* NP_523778), Drosha_D.melanogaster (*Drosophila melanogaster* NP_477436), DCR-1_C.elegans (*Caenorhabditis elegans* NP_498761), Drosha_C.elegans (*Caenorhabditis elegans* NP_492599).

Fig. 13. (Argonauts)

AGO-1_H.sapiens (*Homo sapiens* NP_036331), AGO-2_H.sapiens (*Homo sapiens* NP_036286), AGO-3_H.sapiens (*Homo sapiens* NP_079128), AGO-4_H.sapiens (*Homo sapiens* NP_060099), Piwi-1_H.sapiens (*Homo sapiens* NP_004755), Piwi-2_H.sapiens (*Homo sapiens* NP_001129193), Piwi-3_H.sapiens (*Homo sapiens* NP_001008496), Piwi-4_H.sapiens (*Homo* sapiens NP_689644), AGO-1_D.melanogaster (*Drosophila melanogaster* NP_725341), AGO-2_D.melanogaster (*Drosophila melanogaster* NP_648775), AGO-3_D.melanogaster (*Drosophila melanogaster* NP_001036627), Aubergine_D.melanogaster (*Drosophila melanogaster* NP_476734), Piwi_D.melanogaster (*Drosophila melanogaster* NP_476875), ALG-1_C.elegans (*Caenorhabditis elegans* NP_510322), ALG-2 (*Caenorhabditis elegans* NP_871992), EGO-1_C.elegans (*Caenorhabditis elegans* NP_492132), RDE-2_C.elegans (*Caenorhabditis elegans* NP_001021398), PRG-1_C.elegans (*Caenorhabditis elegans* NP_492121), PRG-2_C.elegans (*Caenorhabditis elegans* NP_500994), PPW-1_C.elegans (*Caenorhabditis elegans* NP_740835), PPW-2_C.elegans (*Caenorhabditis elegans* NP_491535).

Fig. 14. (dsRNA binding proteins)

PRKRA_H.sapiens (*Homo sapiens* NP_001132989), TARBP2_H.sapiens (*Homo sapiens* NP_599150), Loquacious_D.melanogaster (*Drosophila melanogaster* NP_723813), Pasha_D.melanogaster (*Drosophila melanogaster* NP_651879), R2D2_D.melanogaster (*Drosophila melanogaster* NP_609152), R2D2_T.castaneum (*Tribolium castaneum* NP_001128425).

Fig. 15. (RNA-dependent RNA polymerases)

RRF-1_C.elegans (*Caenorhabditis elegans* NP_492131), RRF-3 (*Caenorhabditis elegans* NP_495713), EGO-1_C.elegans (*Caenorhabditis elegans* NP_492132), RdRP_A.thaliana (*Arabidopsis thaliana* NP_172932), RdRP_P.hybrida (*Petunia hybrida* CAA09896), RdRP_O.sativa (*Oryza sativa* EAY87440), RdRP_B.floridae (*Branchiostoma floridae*, XP_002219358), RdRP_B.malayi (*Brugia malayi* XP_001896001), RdRP_T.thermophila (*Tetrahymena thermophia*, ABH11712), RdRP_N.crassa (*Neurospora crassa* XP_963405), RdRP_N.vectensis (*Nematostella vectensis* XP_001631543).

Fig. 16. (Transferrins)

TF_H.sapiens (*Homo sapiens* NP_001054), LTF_H.sapiens (*Homo sapiens* NP_002334), MFI2_H.sapiens (*Homo sapiens* NP_005920), TF-1_A.aegypti (*Aedes aegypti* XP_001647719), TF-2_A.aegypti (*Aedes aegypti* XP_001662114), TF-3_A.aegypti (*Aedes aegypti* XP_001661801), TF-1_D.melanogaster (*Drosophila melanogaster* AAF48831), TF-2_D.melanogaster (*Drosophila melanogaster* AAF49900), TF-3_D.melanogaster (*Drosophila melanogaster* AAF58039).

CURRICULUM VITAE

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Education:

Bachelor program (graduated 2000) - University of South Bohemia in České Budějovice, Faculty of Biological Sciences, Czech Republic (Biology; Biomedicine laboratory technique)

Masters program (graduated 2003) - University of South Bohemia in České Budějovice, Faculty of Biological Sciences, Czech Republic (Parasitology)

Ph.D. student from 2003 - University of South Bohemia in České Budějovice, Faculty of Science, Czech Republic (Parasitology)

Science visits and workshops:

1998 - PHLS London (UK) - science visit - "Genotyping of *Cryptosporidia* spp." (1 week)

2001 - University of Glasgow (UK) - Socrates Erasmus program - "Genotyping of *Cryptosporidia* spp." (4 months)

2005 - National Research Council of Canada, Ottawa (CAN) - science visit - "Phage display - recombinant antibodies" (3 months, R. MacKenzie)

2006 - Centre National de la Recherche Scientifique (FRA) - science visit - "TEP family in *A. gambiae* and response to malaria" (2 months, E. Levashina)

2008 - University of Neuchatel (SUI) - workshop - "Feeding hard ticks *in vitro*" (1 week)

2008 - University of South Bohemia, České Budějovice (CR) - workshop - "ICTTD Bioinformatics Workshop" (1 week)

Presentations:

2005 - 5th International Conference on Ticks and Tick-borne Pathogens (Neuchatel, SUI) - poster - “IxoderinA and Chitinase1: novel sugar-binding proteins involved in the tick innate immunity”

2006 - Insect immunity - the postgenomic era (Roscof, FRA) - poster - “RNA interference in studies of the tick immune system”

2006 - 5th International Symposium on Molecular Insect Science (Tucson, USA) - poster - “RNA interference in function studies of the tick genes”

2008 - 10th IUBMB Conference: "Infectious Diseases: Biochemistry of Parasites, Vectors and Hosts" (Salvador, BRA) - poster - “Iron metabolism after bloodmeal in the tick *Ixodes ricinus*”

2008 - 6th International Conference on Ticks and Tick-borne Pathogens (Buenos Aires, ARG) - poster - “The role of a novel secreted ferritin in the iron metabolism of the tick *Ixodes ricinus*” (awarded by the committee)

Teaching experience:

2004-2005 - University of South Bohemia in České Budějovice, Faculty of Science, Czech Republic. Training of undergraduate students in practical courses of Biochemistry.

List of publications:

Hajdušek O, Sojka D, Kopáček P, Burešová V, Franta Z, Šauman I, Winzerling J, Grubhoffer L (2009). Knockdown of proteins involved in iron metabolism limits tick reproduction and development. *Proc Natl Acad Sci U S A* 106:1033-1038.

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