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Tick subolesin is an ortholog of the akirins described in insects and vertebrates

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ABSTRACT

The tick protective antigen, subolesin, is a regulatory protein involved in the control of multiple cellular pathways. Subolesin is evolutionary conserved in invertebrates and vertebrates with sequence homology to akirins, a recently renamed group of proteins that were proposed to function as transcription factors in *Drosophila* and mice. The objective of this research was to provide evidence of the sequence and functional homology between tick subolesin and akirins. The phylogenetic analysis of subolesin and akirins showed that they are evolutionary conserved. The effect of subolesin and akirin2 knockdown was compared in adult ticks and mice, respectively. The results demonstrated that tick subolesin is an ortholog of insect and vertebrate akirins and suggested that these proteins function in the regulation of NF-κB-dependent and independent expression of signal transduction and innate immune response genes. These results suggest that these proteins have an important role in host–pathogen interactions.

1. Introduction

Akirins constitute a recently renamed group of evolutionary conserved proteins in insects and vertebrates that were proposed to function as transcription factors required for NF-κB-dependent gene expression in *Drosophila* and mice [1]. Goto et al. [1] also provided evidence of the role of akirin in *Drosophila melanogaster* innate immune response. However, they failed to recognize that akirins may be homologous to tick subolesin [2]. Ticks are vectors to a large number of pathogens and may constitute a good system to study the role of these molecules in innate immune response in acari.

Tick subolesin was discovered as a tick protective antigen in *Ixodes scapularis* [3]. Subolesin was shown by both RNA interference (RNAi) gene knockdown and immunization trials using the recombinant protein to protect hosts against tick infestations, reduce tick survival and reproduction, and cause degeneration of gut, salivary gland, reproductive tissues and embryos [2,4–8]. The targeting of tick subolesin by RNAi or immunization was also shown to decrease vector capacity of ticks for *Anaplasma marginale* and *A. phagocytophilum*, respectively [9]. In addition, subolesin was shown to be differentially expressed in *Anaplasma*-infected ticks and cultured tick cells [10,11] and to function in the control of gene expression in ticks through the interaction with other regulatory proteins [12]. These studies demonstrated a role of subolesin in the control of multiple cellular pathways by exerting a regulatory function on global gene expression in ticks. Subolesin was also shown to be conserved in different tick species as well as in other invertebrate and vertebrate organisms [2,3]. These results suggested that subolesin and akirins may be structurally and

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functionally homologous but direct comparison of their role in ticks and vertebrates has not been conducted.

The objective of this research was to provide evidence of the sequence and functional homology between tick subolesin and akirins. Herein, the phylogenetic analysis of subolesin and akirins was conducted and the effect of akirin knockdown in adult mice was compared with the results of subolesin knockdown in adult ticks using RNAi and RT-PCR. The results demonstrated that tick subolesin is an ortholog of the akirins described in insects and vertebrates.

2. Materials and methods

2.1. Phylogenetic analysis of akirin and subolesin sequences

The evolutionary history of akirins and tick subolesin was inferred using the minimum evolution (ME) method [13]. Stability or accuracy of inferred topology(ies) were assessed via bootstrap analysis of 1000 replications [14]. The evolutionary distances were computed using the Maximum Composite Likelihood method [15]. The ME tree was searched using the Close-Neighbor-Interchange algorithm [16] at a search level of 1. The Neighbor-joining algorithm [17] was used to generate the initial tree. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). A total of 2750 positions were present in the final dataset used in the phylogenetic analysis conducted using MEGA4 [18].

2.2. RNA interference in mice

Two groups of five 5-week-old male BALB/c mice (Harlan, Barcelona, Spain) each were injected intraperitoneally every 3 days with 600 μl of siRNA–jetPEI complex during 2 weeks [19]. The treated group was injected with 200 μl of each predesigned siRNA–jetPEI complex to knockdown akirin2 expression (Genbank accession number NM_001007589; siRNAs nos. 217834 (GCACGGAUGCAGUGAAUAGtt), 217835 (GCCUGCUAGUUAUGUUUCAtt) and 217836 (GGUAAUAACCCUUAAUAAGtt), Ambion Applied Biosystems, Austin, TX, USA). These siRNAs were optimized for mouse akirin2 and presumably did not affect akirin1 expression in treated mice. The control group was injected with 600 μl of control siRNA–jetPEI complex (Silencer® negative control no. 1 siRNA; Ambion). siRNAs were resuspended in 400 μl of RNAse free water at a concentration of 50 pmol/μl each. One microliter (50 pmol) of each siRNA was diluted in 50 μl of 150 mM NaCl and added to 50 μl of jetPEI DNA transfection reagent (Polyplus Transfection SA, Illkirch, France) dilution (2 μl of 7.5 mM jetPEI in 48 μl of 150 mM NaCl). Mice were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals. Mice were weighted daily and euthanized 17 days after first siRNA injection. Samples of lung, heart, skeletal muscle, stomach, liver, spleen and kidney were taken for macroscopic and histopathology analysis and for protein and RNA extraction.

2.3. RNA interference in ticks

Adult *I. scapularis* ticks were obtained from the laboratory colony maintained at the Oklahoma State University Tick Rearing Facility. Animals was housed and cared with the approval and supervision of the OSU Institutional Animal Care and Use Committee. Off-host ticks were maintained in a 12 h light:12 h dark photoperiod at 22–25 °C and 95% relative humidity. Subolesin, NF-κB and Rs86 control dsRNA were generated with oligonucleotide primers containing T7 promoter sequences for *in vitro* transcription and synthesis of dsRNA using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion) [2,6]. *I. scapularis* subolesin and control Rs86 dsRNA were synthesized as reported previously [2,20]. For *I. scapularis* NF-κB dsRNA, an EST (EST G893P513RK17.T0; EW791150) was identified encoding for a region of 134 amino acids with 71% homology to *Carcinoscorpius rotundicauda* NF-κB protein Relish factor (REL; DQ345784) and used to synthesize the dsRNA as described above using oligonucleotide primers RelfT75: 5′-TAATACGACTCACTATAGGGTACTAACAGGACCTCTGGTTCGTG-3′ and RelfT73: 5′-TAATACGACTCACTATAGGGTACTCTCGGAGAGACCAGCTTCC-3′ and 62 °C for 1 min annealing conditions. Three groups of 20 adult female ticks each were injected with approximately 0.5 μl of dsRNA (6×10^{10} to 2×10^{11} molecules/μl) in the lower right quadrant of the ventral surface of the exoskeleton of ticks [2,6]. The injections were done with a Hamilton syringe with a 1 in., 33 gauge needle. Control ticks were injected with the Rs86 unrelated dsRNA control. The ticks were held in a humidity chamber for 1 day after injection and then they were allowed to feed on a calf. Female ticks were removed from the calf after 8 days of feeding (9 days post-injection) and 5 ticks from each group were dissected for total RNA extraction.

2.4. Real-time RT-PCR analysis

Total RNA was isolated using TriReagent (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. The mRNA levels were characterized in mouse and tick tissue samples using oligonucleotide primers and conditions described in Table 1 and normalizing against mouse beta-actin and tick 16S rRNA, respectively. The mRNA levels were analyzed by real-time RT-PCR using the iScript One-Step RT-PCR Kit with SYBR Green and an iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA). Control reactions were performed using the same procedures but without RT to test for DNA contamination in the RNA preparations and without RNA added to detect contamination of the PCR reaction. The mRNA levels were compared between treated and control individuals by Student's *t*-test with unequal variance ($P = 0.05$).

2.5. PCR array analysis of signal transduction and NF-κB signaling pathway genes

Total RNA from spleen samples of treated and control mice were analyzed using the Signal Transduction PathwayFinder (PAMM-014) and NF-κB signaling pathway (PAMM-025) PCR arrays (SABiosciences Co., MD, USA). First strand cDNAs were prepared from 0.5 μg total RNA of each sample using the RT^2 First Strand Kit (SABiosciences Co.). The experiments were done using the iQ5 thermal cycler (Bio-Rad). The mRNA levels were normalized against housekeeping genes included in the PCR array (beta glucuronidase; NM_010368, hypoxanthine guanine phosphoribosyl transferase 1; NM_013556, heat shock protein 90 kDa alpha; NM_008302, glyceraldehyde-3-phosphate dehydrogenase; NM_008084 and beta actin; NM_007393). Fold changes in gene expression were calculated using the DDCt method in the PCR array data analysis template version 3 (http://www.superarray.com/pcr/arrayanalysis.php).

3. Results and discussion

3.1. Subolesin and akirin sequences are homologous and evolutionary conserved

The phylogenetic analysis of akirin and subolesin sequences showed a high degree of conservation between tick subolesin and insect and vertebrate akirins (Fig. 1). In vertebrates, two closely related akirin homologues exist, akirin1 and akirin2 [1]. In insects,

Table 1
RT-PCR oligonucleotide primers and conditions for the characterization of the mRNA levels by real-time RT-PCR.

Gene (Genbank accession no.)	Upstream/downstream primer sequences (5′–3′)	PCR annealing conditions
Mouse		
Akirin2 (NM_001007589)	ATCTCCTGCAAGGCTTCTG TTCCAAAGACAAGGCAAACC	54 °C, 30 s
Cu–Zn superoxide dismutase (SOD) (M35725)	CCAGTGCAGGACCTCATTTT TTGTTTCTCATGGACCACCA	50 °C, 30 s
von Willebrand factor (vWF) (AY208897)	CAGCATCTCTGTGGTCCTGA GATGTTGTTGTGGCAAGTGG	50 °C, 30 s
Beta actin (NM_007393)	AGCCATGTACGTAGCCATCC CTCTCAGCTGTGGTGGTGAA	50 °C, 30 s
I. scapularis		
Subolesin (AY652654)	AGCAGCTCTGCTTCTCGTCT TCGTACTCGTCGCGTATCTG	54 °C, 30 s
NF-κB (EW791150)	TTCTTTGAGGAGGACGAGGA TGAACTCGTTGTCCGACTTG	53 °C, 30 s
Cu–Zn superoxide dismutase (SOD) (FD482629)	ATGACGCAGCAGGCAATG TGACCTGGGCAACGTTGA	53 °C, 30 s
Putative secreted salivary WC peptide (WCP) (FD482665)	ATGTCGTCCCTCCATTGTGT GATATTGATCCAGCCGGAGA	53 °C, 30 s
Putative secreted protein (SecP) (FD482812)	ATTGATGGCAATCCTGTGGA TGAAGGCAACCATTGCAGTT	53 °C, 30 s
Hemelipoglycoprotein precursor (Vit) (FD482835)	CTTGAGGAGGTCCTTGGTGA AGAGGTCGATCTCGGCTACA	53 °C, 30 s
16S rRNA (L34293)	GACAAGAAGACCCTA ATCCAACATCGAGGT	42 °C, 30 s

only one akirin has been identified which agrees with the finding of a single subolesin gene in ticks [1 and unpublished results]. These results expanded previous reports [1,2] and supported that subolesin and akirins are structurally homologous and evolutionary conserved in different tick species and in other invertebrate and vertebrate organisms. Goto et al. [1] proposed that akirin2 is more closely related to invertebrate akirins than akirin1. However, the phylogenetic analysis of akirin and subolesin sequences suggested that tick subolesin is closer to mammalian akirin1 than akirin2 sequences (Fig. 1). Nevertheless, despite differences inferred from phylogenetic trees, the results of gene knockdown on embryo development suggest that tick subolesin

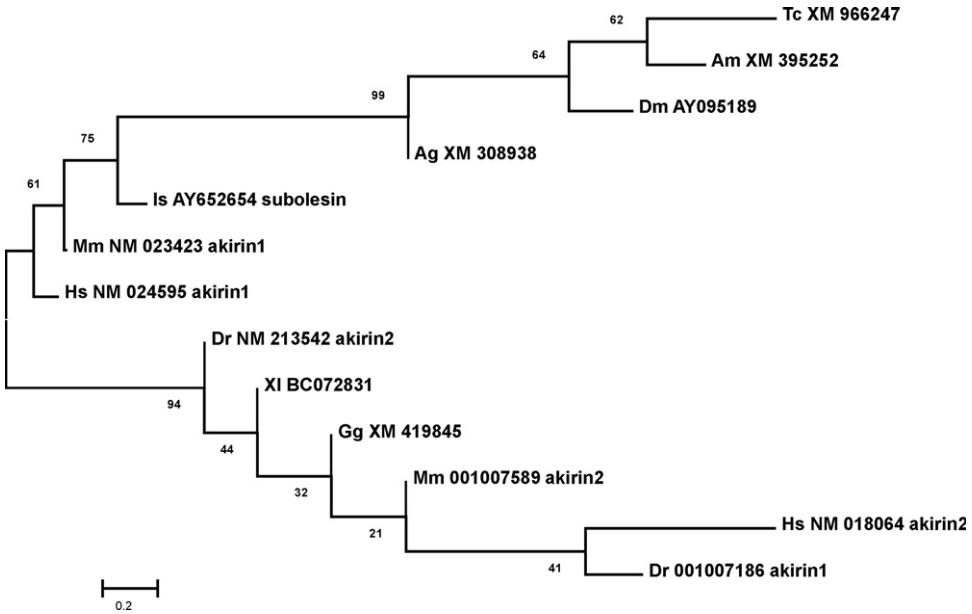

Fig. 1. Unrooted minimum evolution tree showing the high degree of conservation between tick subolesin and insect and vertebrate akirins. The optimal tree with the sum of branch length = 4.80523462 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are represented in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Sequence Genbank accession numbers are shown. The sequences of akirin1 and akirin2 are indicated for vertebrate species in which both genes have been identified. Abbreviations: Dm, *Drosophila melanogaster*; Ag, *Anopheles gambiae*; Am, *Apis mellifera*; Tc, *Tribolium castaneum*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dr, *Danio rerio*; Is, *Ixodes scapularis*.

Table 2
Analysis of signal transduction gene expression in the spleen of mice with akirin2 knockdown.

Gene	Description (biological pathway)	Genbank accession no.	Fold down regulation
Wisp1	WNT1 inducible signaling pathway protein 1 (Wnt pathway)	NM_018865	21.70
Mmp10	Matrix metallopeptidase 10 (Jak-Stat pathway)	NM_019471	16.87
Ccl2	Chemokine (C–C motif) ligand 2 (LDL pathway)	NM_011333	9.78
Ccl20	Chemokine (C–C motif) ligand 20 (NF-κB pathway)	NM_016960	9.00
Csf2	Colony stimulating factor 2 (granulocyte-macrophage) (calcium and PKC pathway)	NM_009969	7.03
IL2	Interleukin 2 (NF-κB pathway)	NM_008366	5.59
Atf2	Activating transcription factor 2 (stress pathway)	NM_001025093	5.34
Fos	FBJ osteosarcoma oncogene (CREB pathway)	NM_010234	4.72
Fasn	Fatty acid synthase (insulin pathway)	NM_007988	4.42
Mdm2	Transformed mouse 3T3 cell double minute 2 (p53 pathway)	NM_010786	2.18

List of signal transduction genes with significant ($P < 0.05$) differential expression in the spleen of mice with akirin2 knockdown when compared to controls. Total RNA samples from spleen of treated and control mice were analyzed using the Signal Transduction PathwayFinder PCR array (PAMM-014; SABiosciences) (http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-014A.html). The mRNA levels were normalized against 5 housekeeping genes included in the PCR array. Fold changes in gene expression were calculated using the DDCt method in the PCR array data analysis template version 3 (http://www.superarray.com/pcr/arrayanalysis.php).

and insect akirins are functionally homologous to mammalian akirin2 [1,2,7,8,10]. Additionally, only akirin2 displayed a role in the NF-κB-mediated innate immune response whereas the role of akirin1 remains unknown [1]. Therefore, we used akirin2 for functional studies in mice.

3.2. Akirin knockdown in mice resulted in downregulation of genes that are also downregulated in ticks after subolesin RNAi

The intraperitoneal injection of akirin2 and control siRNAs in mice did not affect the weight of treated and control mice and differences were not observed after macroscopic and microscopic examination of lung, heart, skeletal muscle, stomach, liver, spleen and kidney tissue samples. These results were in contrast to the pronounced effect of subolesin knockdown on tick weight and tissue histopathology that most likely was related to the dramatic weight increase and tissue remodeling which occurs during tick feeding [2]. Additionally, in ticks injected with subolesin dsRNA gene knockdown is observed in several tissues including gut, salivary gland and reproductive tissues [2] while in mice akirin2 knockdown was demonstrated in the spleen only. Akirin mRNA levels were reduced by $90 \pm 16\%$ in the spleen of treated mice when compared to controls ($P < 0.05$). In previous experiments using siRNA–jetPEI complexes to knockdown gene expression in mice [19], siRNA concentrations were higher in muscle cells when compared to liver, kidney, brain and lung but they did not analyze spleen samples.

In the first series of experiments, gene expression was analyzed by real-time RT-PCR in the spleen of treated and control mice using two genes that are known to be downregulated in subolesin-silenced ticks, Cu–Zn superoxide dismutase (SOD) and von Willebrand factor (vWF) [12]. As shown previously in ticks [12], akirin knockdown reduced SOD and vWF expression in the spleen of treated mice by 4.7 ± 1.9 and 20.5 ± 7.4 fold, respectively when compared to controls ($P < 0.05$).

Subolesin knockdown in ticks has been shown to affect different biological processes including signal transduction [12]. Therefore, the effect of akirin2 knockdown on the expression of signal transduction genes was tested in mouse spleen using the signal transduction PCR array. The expression of 10 signal transduction genes from different biological pathways was significantly downregulated in the spleen of mice with akirin2 knockdown when compared to controls (Table 2). In ticks, subolesin knockdown results in both gene down and upregulation but signal transduction genes (e.g. clone LibPlateC3_wellE2 predicted similar to ruby CG11427-PA isoform 2; Ref. [12]) are downregulated after RNAi.

3.3. Subolesin and akirin may be involved in NF-κB-dependent and independent gene expression

The analysis of signal transduction genes in the spleen of mice with akirin2 knockdown showed the downregulation of two genes, Ccl20 and IL2, involved in the NF-κB signaling pathway (Table 2). To further characterize the effect of akirin2 knockdown on the expression of NF-κB signaling pathway genes in mouse spleen, the expression of 84 key genes related to NF-κB-mediated signal transduction were analyzed using a PCR array. The results showed downregulation of NF-κB responsive genes (Icam1, Csf3 and Lta), NF-κB pathway activation genes (Ikbkg, Tnfsf14 and Tlr4) and genes involved in the NF-κB pathway (Tlr7 and IL10) (Table 3). Two genes involved in the NF-κB pathway, Tollip and Nlrp12, were upregulated in the spleen of mice with akirin2 knockdown,

Table 3
Analysis of NF-κB signaling pathway gene expression in the spleen of mice with akirin2 knockdown.

Gene	Description (role in NF-κB pathway)	Genbank accession no.	Fold down regulation
Ikbkg	Inhibitor of κB kinase gamma (activation of NF-κB pathway)	NM_010547	18.35
Tnfsf14	Tumor necrosis factor ligand superfamily, member 14 (activation of NF-κB pathway)	NM_019418	6.95
Icam1	Intercellular adhesion molecule 1 (NF-κB responsive gene)	NM_010493	6.18
Tlr7	Toll-like receptor 7 (involved in NF-κB pathway)	NM_133211	2.79
IL10	Interleukin 10 (involved in NF-κB pathway)	NM_010548	2.75
Tlr4	Toll-like receptor 4 (activation of NF-κB pathway)	NM_021297	2.64
Csf3	Colony stimulating factor 3 (NF-κB responsive gene)	NM_009971	2.53
Lta	Lymphotoxin A (NF-κB responsive gene)	NM_010735	2.50
			Fold up regulation
Tollip	Toll interacting protein (involved in NF-κB pathway)	NM_023764	30.11
Nlrp12	NLR family, pyrin domain containing 12 (involved in NF-κB pathway)	XM_904112	2.83

List of NF-κB signaling pathway genes with significant ($P < 0.05$) differential expression ($\geq$2.50-fold) in the spleen of mice with akirin2 knockdown when compared to controls. Total RNA samples from spleen of treated and control mice were analyzed using the NF-κB signaling pathway PCR array (PAMM-025; SABiosciences) (http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-025A.html). Data analysis was conducted as described in Table 2.

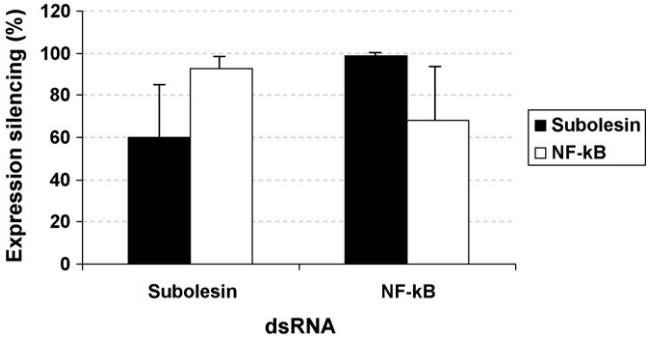

Fig. 2. Subolesin and NF-κB RNAi in ticks. Female *I. scapularis* were injected with subolesin or NF-κB dsRNAs and compared to control ticks injected with the unrelated Rs86 dsRNA. Subolesin and NF-κB mRNA levels were determined by real-time RT-PCR at 9 days post-injection and normalized against tick 16S rRNA. The percent silencing of gene expression was calculated by comparing the normalized Ct values for each treated tick against the average control normalized Ct value and plotted as average + S.D. ($N = 5$). The normalized Ct values were compared between treated and control ticks by Student's *t*-test with unequal variance and were statistically significant ($P < 0.05$).

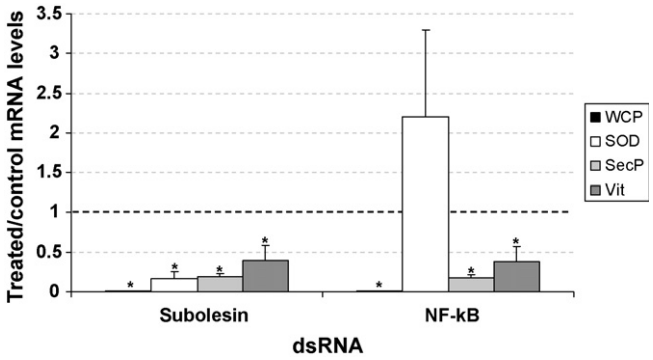

Fig. 3. Gene expression in ticks after subolesin and NF-κB knockdown. The expression of SOD, WCP, SecP and Vit was analyzed in female *I. scapularis* after subolesin and NF-κB knockdown by real-time RT-PCR normalizing against tick 16S rRNA. The treated to control mRNA levels were calculated as the ratio between normalized Ct values in subolesin or NF-κB dsRNA treated ticks and the average normalized Ct value in control ticks injected with the unrelated Rs86 dsRNA. The normalized Ct values were compared between treated and control ticks by Student's *t*-test with unequal variance ($^*P < 0.05$).

suggesting that as previously shown for subolesin [12], silencing of akirin2 results in both gene down and upregulation (Table 3).

In ticks, NF-κB knockdown by RNAi resulted in silencing of both NF-κB and subolesin expression (Fig. 2). Similar results were obtained when subolesin dsRNA was used for RNAi (Fig. 2). These results suggested that NF-κB may participate in the transcription of subolesin while subolesin may be involved in the regulation of NF-κB expression. The possibility that subolesin participates in the regulation of NF-κB expression in ticks agreed with results in *D. melanogaster* suggesting that akirins are likely involved in the regulation of NF-κB as well as other transcription factors such as the associated β-catenin and GATA transcription factors [21–23]. Interestingly, akirin2 knockdown downregulated the expression of positive (Atf2) and negative (Mdm2, a p53-interacting protein that represses p53 transcriptional activity) transcriptional regulators in mouse spleen.

To compare the effect on gene expression of subolesin and NF-κB knockdown in ticks, the expression of 4 genes known to be downregulated in *I. scapularis* at 9 days post-injection of subolesin dsRNA was analyzed [12]. As previously shown in ticks after subolesin knockdown [12], the expression of SOD, putative secreted salivary WC peptide (WCP), putative secreted protein (SecP) and hemelipoglycoprotein precursor (Vit) was downregulated in ticks with subolesin knockdown ($P < 0.05$) (Fig. 3). In ticks with NF-κB knockdown, the expression of WCP, SecP and Vit were also downregulated ($P < 0.05$) (Fig. 3). The effect of subolesin or NF-κB knockdown on tick gene expression could be the result of their mutual downregulation (Fig. 2). However, the expression of SOD did not change significantly in NF-κB silenced ticks (Fig. 3), thus suggesting a difference between subolesin and NF-κB-dependent gene expression in ticks. In *D. melanogaster*, akirin is required for the transcription of some genes that are NF-κB-independent [1]. It may be possible that subolesin is not only required for NF-κB-dependent gene expression but is also involved in NF-κB-independent gene expression in ticks. Future experiments would be required to fully address this question in ticks and other organisms.

3.4. Assessing the role of akirin and subolesin in innate immune response

In their study, Goto et al. [1] found that akirin is required for defense against Gram-negative bacteria in flies. In the results reported herein, the expression of innate immune response genes such as Wisp1, Mmp10, Ccl2, Ccl20, Csf2, IL2, Atf2 and Mdm2 was downregulated in the spleen of treated mice ($P < 0.05$) (Table 2). In *I. scapularis*, the immune response gene, clone LibPlateC4_wellB7 ML-domain containing protein, was downregulated after subolesin knockdown [12]. These results supported a role for akirin2 and subolesin in the regulation of innate immune response genes in mice and ticks, respectively. However, subolesin is up-regulated and required for *A. marginale* infection of tick salivary glands and cultured IDE8 cells [9,10]. This difference in the role of akirin and subolesin in defense against Gram-negative bacterial infections may be related to the fact that ticks are natural vectors of *A. marginale* and tick–pathogen interactions may have evolved mechanisms that guarantee pathogen multiplication while preserving vector survival. These mechanisms would not be present in nonvector–pathogen relationships such as the infection of *D. melanogaster* with *Agrobacterium tumefasciens* [1]. In fact, the knockdown of the *D. variabilis* innate immune antimicrobial peptide, varisin, resulted in lower *A. marginale* infection levels in ticks but increased the systemic infection of an unclassified microbe [24]. Future experiments should address the effect of subolesin knockdown on tick infection by several pathogens, including tick-borne and non-tick-borne Gram-negative and Gram-positive bacteria.

4. Conclusions

These results demonstrated that tick subolesin is an ortholog of insect and vertebrate akirins and suggested that these proteins may affect the expression of signal transduction and innate immune response genes as well as positive and negative transcriptional regulators. In ticks, subolesin may function in the regulation of NF-κB-dependent and independent gene expression through interaction with intermediate regulatory proteins such as GI, GII and other as yet unidentified proteins [12]. These intermediate proteins interact with NF-κB, bind DNA or remodel chromatin to regulate gene expression. This broad function of akirins and subolesin as transcription factors would explain the profound effect of their silencing on tick physiology and development and on gene expression in ticks, *D. melanogaster* and mice. The recognition of subolesin as an ortholog of insect and vertebrate akirins supports the need for further research on

the role of these proteins in the evolution of host–pathogen interactions.

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