Forkhead box transcription factor L2 activates Fcp3C to regulate insect chorion formation

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Received: 15 March 2017
Accepted: 12 May 2017

Subject Area:
developmental biology

Keywords:
forkhead box transcription factor L2, follicle cell protein 3C, follicular cells, chorion formation, female infertility

Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3784568.

1. Introduction

High fecundity based on oogenesis is a typical characteristic of most insects and is also the major cause of pest outbreaks. Oogenesis can be subdivided into three broad developmental periods: pre-vitellogenesis, vitellogenesis and choriogenesis. The chorion in insect eggs is usually synthesized by the follicular cells and carries out the essential function of protecting the embryo from external agents during development [1,2]. The vitelline membrane (VM) is the first egg-shell layer to be synthesized at the end of the vitellogenesis period, dependent upon 20-hydroxyecdysone signalling [3]. After this step, chorionic layers (wax layer, innermost chorionic layer, endochorion and exochorion) are initiated during the choriogenesis period [4]. Chorion genes can be divided into six gene families: ErA/ErB, A/B and HcA/HcB genes are expressed during early, middle and late choriogenesis, respectively [5]. In Bombyx mori, CCAAT/ enhancer-binding proteins (C/EBPs) regulate early and middle chorion genes, while transcription factors of the GATA β family are responsible for late chorion gene expression [6,7]. Despite this accumulating wealth of knowledge, whether other signal pathways and transcript factors also regulate insect egg shell formation remains unclear.

Forkhead box L2 (FoxL2) is a member of the winged helix/forkhead transcription factor family, which has a remarkable functional diversity and is involved in a wide variety of biological processes [8]. In humans, FoxL2 is essential for granulosa cell differentiation and ovary maintenance [9]. Approximately 97% of adult-type granulosa cell tumours of the ovary harbour a missense point mutation in the FoxL2 gene [10,11]. FoxL2 heterozygous mutations result in...
blepharophimosis ptosis epicanthus inversus syndrome, which is characterized by eyelid abnormalities often associated with premature ovarian failure [12–15]. In mice, homozygous FoxL2 mutations resulted in the absence of secondary follicles and oocyte atresia [16]. FoxL2 is also required to prevent the transdifferentiation of an adult ovary to a testis [17]. In addition to humans and mice, FoxL2 has also been studied in numerous species such as fishes, chickens, frogs and goats [18]. In the pea aphid *Acyrthosiphon pisum*, RNA-seq data revealed that FoxL2 is predominantly expressed in sexual females [18]. In the yellow fever mosquito, *Aedes aegypti*, the transcript level of FoxL2 increased after a blood meal. Knockdown of FoxL2 in *Ae. aegypti* resulted in a significant reduction in egg production [19]. However, the actual functions of FoxL2 in ovipara are still not well understood.

To better understand the gene regulating egg shell formation in ovipara and the functions of FoxL2 and its potential application in insect pest control, we used the brown planthopper (BPH) *Nilaparvata lugens* Stål (Hemiptera: Delphacidae), one of the most destructive insect pests of rice [20], as a model to explore the potential transcriptional regulation in ovaries was elevated after a blood meal. Knockdown of FoxL2 in *N. lugens* was also studied in numerous species such as fishes, chickens, frogs and goats [21]. In the pea aphid *Acyrthosiphon pisum*, RNA-seq data revealed that FoxL2 is predominantly expressed in sexual females [22]. In the yellow fever mosquito, *Aedes aegypti*, the transcript level of FoxL2 increased after a blood meal. Knockdown of FoxL2 in *Ae. aegypti* resulted in a significant reduction in egg production [23]. However, the actual functions of FoxL2 in ovipara are still not well understood.

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2. Results

2.1. Sequence analysis

The complete ORF sequence of *NlFoxL2* is 1254 bp in length and has the potential to encode a 417 amino acid residue peptide containing a typical Foxhead domain (97 amino acid DNA-binding domain). Multiple alignments of FoxL2 orthologues from 17 species using the CLUSTALX program showed that FoxL2 was highly conserved from invertebrates to vertebrates (figure 1a). The ORF of *NlFcp3C* is 528 bp long and has the potential to encode a 175 amino acid residue peptide. BLAST analysis for *Nlfcp3C* in NCBI revealed that Fcp3C orthologues exist only in insects. Multiple sequence alignments of the orthologues from 12 species revealed that Fcp3C was well conserved in insects (figure 1b).

2.2. Developmental expression patterns

The developmental expression patterns of *NlfoxL2* and *Nlfcp3C* were assayed by real-time qPCR. Total RNA was extracted from various developmental samples containing all life stages of BPHs (eggs, nymphs in five different instars, male adults and female adults). The results showed that *NlfoxL2* and *Nlfcp3C* transcripts had similar expression patterns, with transcripts peaking approximately 48 h after female adult eclosion and then being maintained at a high level thereafter. The transcripts of *NlfCp3C* in eggs, nymphs and male adults were maintained at a relatively low level, while *Nlfcp3C* was expressed nearly exclusively in female adults (figure 2). As ovaries usually reach maturity 2–3 days after female emergence, the expression profiles of *NlfFoxL2* and *Nlfcp3C* were in line with ovary development.

2.3. Tissue-specific expression patterns

The tissue-specific expression patterns of *NlfFoxL2* and *Nlfcp3C* were assayed by real-time qPCR in various tissues of the female *N. lugens* (48–72 h after adult emergence): integument, gut, Malpighian tube, fat body, salivary gland and ovary. Both *NlfFoxL2* and *Nlfcp3C* were mainly expressed in the ovaries (figure 3a-c), suggesting specific functions for these genes in female reproduction. To further stratify the
gene expression in the ovaries, total RNAs isolated from different parts of ovaries, including oocyte, ooecium wall, ovipositor, terminal filament and fallopian tube, were used for the qPCR analyses. The results showed that both \textit{NlFoxL2} and \textit{NlFcp3C} were highly expressed in the ooecium wall, which mainly contains follicular cells (figure 3b,c).

2.4. Phenotypes of RNA interference

To determine the possible functions of \textit{NlFoxL2} and \textit{NlFcp3C}, double-stranded RNAs (dsRNAs) targeting each of these genes were injected into newly emerged female adults (within 2 h). Real-time qPCR analysis showed that each dsRNA efficiently suppressed the transcript levels of their target genes 3 days after injection (figure 4). Starting from the third day after injection, the body size of the insects injected with dsRNA targeting \textit{NlFoxL2} or \textit{NlFcp3C} became substantially larger than that of the insects in the dsGFP group, with lateral membranes of each segment and inter-segmental membranes in the abdomen obviously stretched, indicating obesity (figure 5a). The average weight of the BPHs injected with ds\textit{NlFoxL2} or ds\textit{NlFcp3C} was significantly heavier than that of the dsGFP group from the third day after injection. On the fifth day, BPHs knocked down for \textit{NlFoxL2} or \textit{NlFcp3C} were approximately 33% heavier than those in the dsGFP group (figure 6a).
Figure 3. Expression of *NlFoxL2* and *NlFcp3C* in different tissues of *Nilaparvata lugens* by qPCR. (a) Expression of *NlFoxL2* in different tissues; (b) expression of *NlFoxL2* in different parts of the ovaries; (c) expression of *NlFcp3C* in different tissues; (d) expression of *NlFcp3C* in different parts of the ovaries. Each total RNA sample used for (a) and (c) was extracted from different tissues including the gut, Malpighian tube, salivary glands, fat body, integument and ovary, which were dissected from 50 female adults 48 – 72 h after eclosion. Each total RNA sample used for (b) and (d) was extracted from different parts of the telotrophic meroistic ovaries of 50 female adults 48 – 72 h after eclosion including oocyte, ooecium wall, ovipositor, terminal filament and fallopian tube. *Nl18S* was used as an internal control gene. Values are means ± s.e.m. from three experiments.

Figure 4. RNAi efficiencies of *NlFoxL2* and *NlFcp3C* and influences on each other. (a) Relative transcripts of *NlFoxL2*; (b) relative transcripts of *NlFcp3C*. dsRNA (50 ng per insect) for *NlFoxL2* or *NlFcp3C* was injected into newly emerged female adults (within 2 h). RNAi efficiency was investigated using real-time qPCR. Each total RNA sample for these two genes was extracted from 10 BPHs 3 days after injection. dsGFP was injected as negative control for the non-specific effects of dsRNA. Values are means ± s.e.m. from three experiments. **p < 0.01 (Student’s *t*-test), difference from dsGFP.
Every ovariole on BPHs injected with only dsGFP for 5 days contained one or, at most, two fully developed banana-shaped oocytes. However, knockdown of \textit{NlFoxL2} or \textit{NlFcp3C} resulted in oocyte dysplasia. Upon dissection of the ovaries, no fully developed oocytes were observed on the fifth day after injection of ds\textit{NlFoxL2} or ds\textit{NlFcp3C}: the short oocytes formed near-spherical shapes rather than the normal banana shapes (figure 5b). As a result, female BPHs treated with ds\textit{NlFoxL2} or ds\textit{NlFcp3C} were unable to lay eggs, though the number of oocytes longer than 0.4 mm in the ovaries greatly increased (figure 6b). A large accumulation of the abnormal oocytes eventually led to an increase in the ovarian volume (figure 5b).

**Figure 5.** Phenotypes of insects injected with dsRNA for \textit{NlFoxL2} or \textit{NlFcp3C}. (a) Severely obese female adults 5 days after injection; (b) ovaries dissected from female adults 5 days after injection. dsRNA (50 ng per insect) for \textit{NlFoxL2} or \textit{NlFcp3C} was injected into newly emerged female adults (within 2 h). dsGFP was injected as negative control for the non-specific effects of dsRNA.

**Figure 6.** Knockdown of \textit{NlFoxL2} or \textit{NlFcp3C} increased body weight and number of eggs. (a) Body weights of 20 female adults 1–5 days after injection, respectively; (b) numbers of eggs in the ovaries 5 days after injection. dsRNA (50 ng per insect) for \textit{NlFoxL2} or \textit{NlFcp3C} was injected into newly emerged female adults (within 2 h). dsGFP was injected as negative control for the non-specific effects of dsRNA. Values are means ± s.e.m. from three experiments. **p < 0.01 and ***p < 0.001 (Student’s t-test), difference from dsGFP.
In further experiments, we found that NilFoxL2 or NilFcp3C depletion suppressed the expression of genes encoding high-cysteine chorion proteins (NilHCA and NilHCB) (figure 8).

2.6. NilFoxL2 activates the NilFcp3C promoter

Experimental results of RNAi efficiency by qPCR revealed that NilFcp3C is a downstream gene of NilFoxL2: knockdown of NilFoxL2 efficiently suppressed the transcript levels of NilFcp3C, but knockdown of NilFoxL2 did not affect expression levels of NilFoxL2 (figure 4). Transient expression assays were performed to test whether NilFoxL2 was a direct activator of the NilFcp3C promoter.

Forty-eight hours after the transfection of HEK-293T cells, an enhanced green fluorescent protein (EGFP) signal was detected. As positive control, HEK-293T cells transfected with pN1-NilFoxL2-EGFP showed extensive EGFP fluorescence, suggesting that the plasmid pN1-NilFoxL2, which had a termination codon (TGA) between the NilFoxL2 ORF and EGFP ORF, could work well in HEK-293T cells to express NilFoxL2. No detectable EGFP signal was observed after transfection with the pN1-NilFoxL2 or the pT1-prom3C-EGFP. By contrast, cells transfected with pT1-prom3C-EGFP showed detectable EGFP signal when co-transfected with pN1-NilFoxL2. This result demonstrated that NilFoxL2 could activate the NilFcp3C promoter to drive EGFP expression in HEK-293T cells (figure 9).

3. Discussion

Our analysis showed that the sequences of FoxL2 are highly conserved in vertebrates and invertebrates, and the roles of FoxL2 in ovarian development also seem somewhat conserved. FoxL2 in mammals mainly affects the differentiation of granulosa cells [9], while our results showed that FoxL2 played an important role in chorion formation. This difference could be because mammals are viviparous and do not have chorions, thus leading to the differentiation of FoxL2 target genes. Our research first discovered that NilFoxL2 played an important role in chorion formation by activating NilFcp3C directly.

Fcp3C was first identified in Drosophila melanogaster, encoding a protein involved in the formation of the VM, the first secreted chorion layer [21]. In Blatella germanica, the mRNAs of Fcp3C appeared in 3-day-old females and peaked at choriogenesis [22]. In Diploptera punctata, the expression patterns also suggested that Fcp3C plays a role in chorion formation [22,23]. Furthermore, failure in forming chorion layers caused by depletion of BgWindei by RNAi was coupled with significant underexpression of Fcp3C [24]. However, there is no direct evidence to prove the function of Fcp3C. Our results gave the direct evidence that Fcp3C played an important role in chorion formation.

The results determined by qPCR and EGFP assay showed that NilFoxL2 directly targets the expression of NilFcp3C, which might explain why knockdown of NilFoxL2 or NilFcp3C had similar results. NilFoxL2 plays an important role in the ovaries by regulating the expression of NilFcp3C. NilFcp3C depletion suppressed the transcript levels of chorion genes (NilHCA and NilHCB expressed during late choriogenesis) and prevented chorion formation. Our TEM observations showed that NilFoxL2- or NilFcp3C-knockdown follicle cells could not form

2.5. Depletion of NilFoxL2 or NilFcp3C prevented chorion formation

As oocyte dysplasia was the major contributor to the abnormal phenotype, we further observed the ultrastructural structure of oocytes in the basal egg chamber of ovarioles using a transmission electron microscope (TEM). The ovarioles were prepared by dissecting the ovaries of adult females 5 days after injection. In the dsGFP group, the oocytes in the basal egg chamber of ovarioles were surrounded by cells of the follicular epithelium on the outside and filled up with protein yolk spheres and lipid droplets. The chorion, sandwiched between the follicular cells and the oocyte protoplasm, had a continuous multilayer structure (figure 7a). After knockdown of NilFoxL2 or NilFcp3C, no structural abnormalities of the follicular cells were observed, but the structure of the chorion had substantial aberrations. The chorion became fractured and could not keep the continuous multilayer structure (figure 7b,c).

However, abnormalities were not observed in nymphs and male adults injected with either dsNilFoxL2 or dsNilFcp3C.
proper chorion layers around the egg. As the abnormal oocytes could not be oviposited, they could only remain stuck in the ovaries. The accumulation of the abnormal oocytes in the ovaries might be the reason for the observed obesity.

In conclusion, our results identified NIFoxL2 as a direct activator of the NIFcp3C promoter. Depletion of NIFcp3C mRNA by RNAi in N. lugens females prevented normal chorion formation. In turn, it impaired oviposition function and eventually prevented reproduction and caused obesity.

4. Experimental procedures

4.1. Insects

The insects used in this study were obtained from local rice fields at Zhejiang University, Hangzhou, Zhejiang, China. The insects were reared on fresh rice seedlings (Xiushui 134) at 25 ± 1°C and 60–70% relative humidity under a 16 L/8 D photoperiod [25].
4.2. Gene identification and sequence analyses

*Nilaparvata lugens* genomic (GenBank accession numbers: AOSB0000000) [26] and transcriptomic databases were screened for genes encoding FoxL2 and Fcp3C against the amino acid sequences from *Drosophila melanogaster*, *Blattella germanica*, *Mus musculus* and *Homo sapiens*, which were obtained from GenBank. The full-length cDNA sequences of the two genes were obtained from transcriptomic databases and confirmed by RT-PCR. Multiple sequence alignments were performed using the ClustalX program [27]. Based on the 5'-untranslated region (5'-UTR) sequence described above, a 1395 bp fragment of the promoter region of *NlFcp3C* was obtained from the genomic database and confirmed by PCR. The primers used here are shown in the electronic supplementary material, table S1.

4.3. Total RNA isolation and first-strand cDNA synthesis

Total RNA from whole insects at various developmental stages or from tissue samples was isolated using a TRIzol Total RNA Isolation Kit (Takara, Kyoto, Japan). Developmental samples were collected from different stages of BPHs (n = 15–20), including eight egg samples (every 24 h after the eggs were laid), 28 nymph samples (every 24 h after moultting for first and second instars; every 12 h after moultting for third, fourth and fifth instars), 11 female adult samples (every 12 h after moultting) and 11 male adult samples (every 12 h after moultting). Similarly, various tissue samples including integument, gut, Malpighian tubule, fat body, salivary gland and ovary were dissected from random female adults 48–72 h after adult emergence. To further characterize the expression in ovaries, the ovaries of female adults 48–60 h after eclosion were dissected and further divided into five parts: oocyte, ooeicum wall, ovipositor, terminal filament and fallopian tube. First-strand cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) using 0.5 μg of total RNA template in a 10 μl reaction, following the manufacturer’s protocol.

4.4. Real-time qRT-PCR analyses

To investigate the developmental and tissue-specific expression patterns, real-time quantitative polymerase chain reaction with reverse transcription (qRT-PCR or qPCR) was conducted using pairs of gene-specific primers designed using the Primer Premier 6 program (electronic supplementary material, table S1) and the cDNA prepared as described. The qPCR reactions (20 μl each) contained 2 μl of cDNA diluted 10-fold, 0.6 μl of each primer and 10 μl of SYBR Premix Ex Taq and were run in a Bio-Rad Real-time PCR system (Bio-Rad, Hercules, CA, USA). The *N. lugens* housekeeping gene for 18S ribosomal RNA (*Nl18S*) (GenBank accession number JN662398.1) was used as an internal control. The qPCR programme consisted of an initial denaturation step at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. A relative quantitative method (2^ΔΔCt) [28] was applied to evaluate the variation in expression among samples.

4.5. RNAi effects on *Nilaparvata lugens*

The double-stranded RNA (dsRNA) was synthesized from the purified DNA templates, which were prepared by RT-PCR amplification using a MEGA script T7 Transcription kit (Ambion, Austin, TX, USA). A unique region of each gene was chosen as a template for dsRNA synthesis. The primers used for dsRNA synthesis are shown in the electronic supplementary material, table S1. The dsRNA for GFP was used as negative control for the non-specific effects of the dsRNA. Microinjection of planthoppers with dsRNA was carried out according to a method described previously [29]. Newly emerged female adults (within 2 h) were chosen to be injected. One hundred and fifty insects were used for each gene treatment, and each treatment was conducted in triplicate. Each insect was injected with 10 nl of dsRNA at a concentration of 5 μg μl⁻¹. Samples were collected from a set of six to ten insects to evaluate the RNAi effects of each gene (sample time: 3 days after injection).

4.6. Determination of BPH weights

The average weight of the female adults (1–5 days after injection) was calculated by weighing BPHs in groups using an AB204-N precision scale (Mettler-Toledo, Ohio, USA).

4.7. Transmission electron microscope observations

Samples for examining the ultrastructure of ovarioles were prepared by dissecting the ovaries of adult females which had been treated with dsRNA for 5 days. Sample treatment procedures were carried out according to a method reported previously [30]. Ultrathin sections were examined with a JEX-1200 TEM (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

4.8. Promoter assay

The full-length *NlFoxL2* ORF (primers: L2-ATG-Xhol-F, L2-CG-BamHI-R) was cloned into the pEGFP-N1 vector with enhanced green fluorescent protein (EGFP) at the C-terminal end (pN1-NlFoxL2-EGFP) via the BamHI/Xhol sites to obtain the fusion protein *NlFoxL2-EGFP*. Similarly, the full-length *NlFcp3C* ORF with a TGA stop codon at the 3' end (primers: L2-ATG-Xhol-F, L2-TGA-BamHI-R) was cloned into the pEGFP-N1 vector (pN1-NlFcp3C). The 1395 bp fragment of the *NlFcp3C* promoter region (primers: prom-3C-F, prom-3C-R) was cloned into the pEASY-T1 vector (TransGen Biotech, Beijing, China) in the forward direction (pT1-prom3C). The EGFP ORF was obtained by PCR (primers: EGFP-ATG-NotI-F, EGFP-TAA-XbaI-R) using pEGFP-N1 as a template and inserted in the pT1-prom3C via NotI/XbaI (pT1-prom3C-EGFP). Cell culture and transfection protocols were previously described by Yang et al. [31]. HEK-293T (human embryonic kidney 293T) cells were co-transfected with pN1-NlFoxL2 and pT1-prom3C-EGFP as an experimental group. pN1-NlFoxL2-EGFP was transfected into HEK-293 cells as positive control. pN1-NlFcp3C or pT1-prom3C-EGFP were transfected into HEK-293 T cells as negative control. For the fluorescence microscopy, cells were observed using a Zeiss LSM510 microscope 48 h post-transfection.

Ethics. Experiments in this study were conducted with approval from Laboratory Animal Center, Zhejiang University, China.

Data accessibility. Sequences of NlFoxL2 and NlFcp3C are available at NCBI, *N. lugens* genome (GenBank accession numbers: AOSB00000000).

Authors' contributions. C.X.Z. and Y.Y.X conceived the experiment. Y. Y.Y performed most experiments, analysed data and drafted the manuscript. P.L.P., J.Y.X., D. K., J.B., Q.L.H., H.J.H. and Y.H. L helped to perform some experiments. Z.F.S. and N.M.Z helped to perform the cell assay. C.X.Z. revised the manuscript.
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Acknowledgements. We thank all other members from the Laboratory of Insect Molecular Biology who are not included in the author list for their help in insect culture and experiments.

Competing interests. We declare we have no competing interests.

Funding. This work was supported by the National Natural Science Foundation of China (grant nos. 31630037 and 31471765).