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Matrix metalloproteinases promote fat body cell dissociation and ovary development in *Bombyx mori*

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ABSTRACT

Matrix metalloproteinases (Mmps) are pivotal extracellular proteinases participating in tissue remodeling. Three *Mmps* genes have been identified from the silkworm, *Bombyx mori*, and their expression levels and enzyme activity are consistent with progressive fat body cell dissociation during the early pupal stages. Using both loss-of-function and gain-of-function experiments, we have demonstrated that Mmps are functionally required for fat body cell dissociation and ovary development in female pupae. Moderate inhibition of Mmps activity via inhibitor treatments delayed fat body cell dissociation and ovary development, while severe inhibition blocked these developmental processes and eventually led to pupal lethality. Individual RNAi knockdown of each *Mmp* delayed fat body cell dissociation, with the strongest and weakest phenotypes occurring for *Mmp3* and *Mmp1*, respectively. By contrast, overexpression of each *Mmp* promoted fat body cell dissociation and ovary development, with the strongest stimulatory effects for *Mmp3* overexpression and the weakest effects for *Mmp1* overexpression. This is the first time to show that Mmps induce fat body cell dissociation in Lepidoptera, and we also hypothesize that Mmps-induced fat body cell dissociation is required for ovary development in this insect species.

1. Introduction

The insect fat body has been considered as an organ functionally equivalent to the vertebrate adipose tissue and liver, which is known to participate in storage, metabolic, immune, and endocrine functions (Arrese and Soulages, 2010; Costa-Leonardo et al., 2013). In holometabolous insects, fat body undergoes a dramatic remodeling process during metamorphosis (Hoshizaki, 2013). In the fruit fly, *Drosophila melanogaster*, the larval fat body is a single-cell layer of attached polygonal cells, which are held together by DE-cadherin mediated cell-cell junctions and surrounded by a basement membrane (BM) that is attached to the cells by integrin (Nelliot et al., 2006; Jia et al., 2014). During *Drosophila* prepupal-pupal metamorphosis, the larval fat body gradually dissociates from a single layer of attached polygonal cells into individual, spherical, and free-floating cells. This developmental process is referred to as fat body cell dissociation (Jia et al., 2014). Some dissociated fat body cells survive throughout the pupal stages and are present in newly emerged adults. At 3–4 days post-eclosion, these surviving larval fat body cells are completely replaced by adult fat body cells that are differentiated de novo from cells embedded within the larval body wall and from adepithelial cells associated with imaginal discs (Aguila et al., 2007; Hoshizaki, 2013).

Matrix metalloproteinases (MMPs) are extracellular Zn^{2+}-dependent endopeptidases. There are at least 23 members in human and 24 in mice. Most MMPs are expressed as proenzymes that share a common domain organization, consisting of signal peptide, propeptide domain, catalytic domain, hinge domain, and hemopexin-like domain. They are responsible for degrading cell-cell junctions, cell-BM junctions, and BM components. MMPs exhibit many overlapping substrates, showing genetic redundancy and functional compensation, and some MMPs can activate other MMPs, forming a proteolytic network (Page-McCaw et al., 2003; Mason and Joyce, 2011). MMPs are inhibited by specific endogenous proteins called TIMPs (tissue inhibitors of metalloproteinases). TIMPs suppress the activity of MMPs through direct inhibition or by modulating zymogen activation. Four TIMPs were found in human and mice which have overlapping but different abilities to inhibit individual MMPs (Murphy, 2011).

There are only two Mmps (Mmp1 and Mmp2) and one Timp (which inhibit both MMPs) in *Drosophila*. The two Mmps share a conserved domain structure. Both single and double *Mmp* mutants can complete

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embryonic development and progress partially through the larval stages. *Mmp1* strong mutants exhibit defects in larval tracheal remodeling and pupal head eversion, while *Mmp2* strong mutants show defects in larval tissue histolysis and epithelial fusion during metamorphosis (Page-McCaw et al., 2003; Glasheen et al., 2009). Our previous work has demonstrated the collaborative and distinct functions of Mmp1 and Mmp2 in inducing fat body cell dissociation in *Drosophila*. Mmp1 preferentially cleaves DE-cadherin-mediated cell-cell junctions, while Mmp2 preferentially degrades BM components and thus destroys cell-BM junctions, resulting in the complete dissociation of the fat body tissues into individual cells during *Drosophila* metamorphosis (Jia et al., 2014). Besides, juvenile hormone (JH) and 20-hydroxyecdysone (20E) coordinately and precisely control the timing of Mmps-induced fat body cell dissociation by regulating *Mmps* expression (Jia et al., 2017).

Research in other insects indicates cathepsins also participate in fat body cell dissociation. Cathepsins are a group of proteinases located in lysosome and play vital roles in different physiological processes. Cathepsin B in the flesh fly, *Sarcophaga peregrine*, is a 29 kD cysteine proteinases excreted from pupal hemocytes. In the hemolymph, cathepsin B binds to the fat body BM and causes its cell dissociation (Kurata et al., 1989, 1992). *Cathepsin L* in the cotton bollworm, *Helicoverpa armigera*, is highly expressed in the fat body. Cathepsin L is released from the fat body cells into its extracellular matrix, and then causes its dissociation (Zhang et al., 2013). A recent study also showed that cathepsin L is highly expressed in the fat body of the Chinese oak silkworm, *Antheraea pernyi*, and RNAi knockdown of *cathepsin L* expression delays fat body cell dissociation (Sun et al., 2018).

Bombyx mori is a domesticated insect of economic importance and is also used in fundamental research as a Lepidoptera model for understanding other insects, but the regulatory mechanisms of *B. mori* fat body cell dissociation still remain ambiguous at present. Although a recent study speculated that Mmps induce fat body cell dissociation in *B. mori* according to developmental changes of fat body basement membrane and Mmps expression (Kawasaki et al., 2018), it is not definite that Mmps and Timp regulate this developmental process. In this work, we identified three Mmps in *B. mori* and demonstrated that Mmps promote fat body cell dissociation.

2. Materials and methods

2.1. Insects and chemicals

Bombyx mori larvae (p50 strain) were provided by the Sericultural Research Institute, Chinese Academy of Agricultural Sciences (Zhenjiang, China), were reared on fresh mulberry leaves in the laboratory at 25 °C under 14 h light/10 h dark cycles (Li et al., 2016). Female silkworms were used through all experiments.

MMPs broad spectrum inhibitor GM6001 (S7157, Selleck) was dissolved in DMSO (D2650, SIGMA-ALDRICH). Three microliters of DMSO was injected as control, three microliters of GM6001 (10 mM or 20 mM) was injected as treatment.

2.2. Total RNA extraction and quantitative real-time PCR

Total RNA of abdominal fat body was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed by using Reverse Transcriptase M-MLV (Takara). Quantitative real-time PCR (qPCR) was performed using SYBR® Select Master Mix (Applied Biosystems) and the Applied Biosystem™ QuantStudio™ 6 Flex Real-Time PCR System. qPCR was carried out in a 20 μl reaction solution, containing 10 μl of SYBR Green, 2 μl of first strand cDNA template, and 0.5 mM of each primer. Rp49 was chosen as the endogenous control for qPCR analysis (Zhang et al., 2017). The primers used for qPCR are listed in Table S1. Three biological replicates were performed.

2.3. Protein extraction and Western blotting

Fat body tissues collected from different developmental times were homogenized in the RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Shanghai) containing protease inhibitor cocktail (Roche). For Western blotting, the primary antibodies mouse anti-Mmp1 (1:1000, DSHB), mouse anti-HIS tag (1:1000, Yisheng Co. Ltm, Shanghai, China), mouse anti-tubulin (1:3000; Beyotime Institute of Biotechnology, Shanghai) were used. The secondary antibodies donkey anti-mouse IgG-HRP (1:1000 for Mmp1; 1:3000 for tubulin and HIS tag, Santa Cruz Biotechnology, sc-2020) were used (Li et al., 2016).

2.4. Total Mmps enzyme activity assay

The total Mmps enzyme activity from fat body samples was measured using the total MMP Fluorescent Assay Kit (Genmed Scientifics, USA) according to the manufacturer's instructions with a Varioskan Flash Multimode Reader (Thermo Scientific, USA). Using a quench fluorescence method with the polypeptide Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH2 as a fluorescent substrate, the relative fluorescence units were determined, employing an excitation wavelength of 330 nm and an emission wavelength of 400 nm. The consistency of fluorescent polypeptide segments was calculated on the basis of the relative fluorescence units. Total Mmps enzyme activity was expressed as RFU/mg/min (Jia et al., 2014, 2017).

2.5. RNAi in Bombyx

The 300–500 bp fragments of the target genes were first amplified by PCR from the fat body cDNA of P0h (5′ flanked primers and 3′ flanked primers are listed in Table S1). Then the T7 promoter sequence (TAATACGACTCACTATAGG) attached primers were used for PCR amplification of the dsRNA templates. The dsRNA was synthesized using T7 RiboMAX Express RNAi kit (Promega, USA) according to the manufacturer's instructions. The GFP dsRNA was used as a control (Hossain et al., 2013). DsRNAs were injected into the abdomen of silkworm by 15 μg each animal at P0h stage (Li et al., 2016), the degrees of fat body cell dissociation were observed at P60h. All the primers used for dsRNA synthesis in this study are listed in Table S1.

2.6. Baculovirus-mediated overexpression in Bombyx

The full-length cDNA of *B. mori Mmp1*, *Mmp2*, *Mmp3*, and *Timp* was amplified by PCR and cloned into the BamH I-Xho I sites of pFastBac1 plasmid (Invitrogen) separately. Then AcNPVs expressing target genes were obtained in Sf9 cells according to Bac-to Bac® Baculovirus Expression System User Manual (Invitrogen). Three microliters of viruses of P2 generation were injected into *B. mori* pupa on P0 stage, and fat body was collected at the 120 h after pupation (P120h) for further analysis, *GFP* viruses made in our lab previously were used as control (Hossain et al., 2013; Li et al., 2016; Liu et al., 2016).

2.7. Statistical analysis of data

All the data were analyzed using Excel 2010 with the analysis of variance (ANOVA). The analysis results were shown as the bars labeled with different lowercase letters as significantly different ($p < 0.05$). Throughout the study, values are represented as the mean ± standard deviation of 3–10 independent experiments.

3. Results

3.1. Identification and structural analysis of B. mori Mmps

Except in *Drosophila*, there are three *Mmps* genes in an insect genome based on previous predictions (Knorr et al., 2009; Kantor et al.,