RESARCH REPORT

Wnt1 promotes the proliferation of midgut epithelial cells in silkworm, Bombyx mori

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Abstract

Wnt genes are crucial members of at least three major signaling pathways that control diverse cellular behaviors, such as cell fate decisions, cell migration, and cell proliferation. Wnt genes are involved in many important embryological events, including the development of the gastrula, heart, limb, and nervous system. In this paper, we identified and characterized BmWnt1 in midgut of silkworm larvae, a lepidopteran model insect. The gene expression analysis revealed that BmWnt1 is expressed in most organs although in different patterns. In vivo, the addition of dextran sulfate sodium (DSS) promoted the proliferation of midgut epithelial cells with BmWnt1 up-regulation. In vitro, the down-regulation of BmWnt1 expression by RNA interference significantly inhibited cell proliferation. Taken together, we anticipate that BmWnt1 may contribute to cell proliferation and development in the silkworm midgut.

Key Words: silkworm; BmWnt1; midgut; dextran sulfate sodium; cell proliferation

Introduction

The homeostasis and regeneration of adult tissues require a balance between the production of new cells and the removal of old or damaged cells (Cordero et al., 2012), and several signal transduction pathways, including EGFR and JAK/STAT, Wnt/Wingless and Notch, participate in these processes (Ohlstein and Spradling, 2007; Liu et al., 2010). The Wnt/Wingless and Notch pathways maintain gut progenitors or ISCs in a dividing non-differentiated state (Lin et al., 2008).

Wnt/Wingless proteins are secreted glycoproteins acting as signaling molecules that can trigger a cellular response and activate intracellular signal transduction (Rao and Kuhl, 2010). These proteins bind to receptors of the Frizzled family and LRPS/6 co-receptors and initiate complex signaling cascades (Logan and Nusse, 2004; Kestler and Kuhl, 2008). There are 19 members of the Wnt family and 10 Frizzled receptors in humans (Rao and Kuhl, 2010). The deregulation of the Wnt signaling pathways has been reported to lead to the development of human diseases (Moon et al., 2004; Clevers, 2006). Seven family members of the Wnt family have been identified in Drosophila, and they play a range of roles at multiple stages in the development of the organism (Dhawan and Gopinathan, 2003). Wnt1/Wingless is essential for the development of the wing disc and leg, and it is known to have a crucial role in the patterning of gonads (Campbell et al., 1993; Williams et al., 1993). In addition, Wnt1/Wingless is required for the maintenance and proliferation of Intestinal stem cells (ISCs), although it is not necessary for their survival in Drosophila (Lin et al., 2008).

ISCs that can maintain tissue homeostasis and replenish lost cells in response to tissue damage in Drosophila were identified in the adult midgut (Micchelli and Perrimon, 2006; Ren et al., 2010; Micchelli et al., 2011). However, tissue damage can also induce cell proliferation to replenish damaged cells (Amcheslavsky et al., 2009).

To address the function of Wnt1 in the tissue of midgut, we chose the silkworm Bombyx mori, which is not only an important economic insect for silk production but also a nice research model (Izumi et al., 2016; Matsumoto et al., 2016). BmWnt1 has been reported to be necessary for posterior segmentation in the embryos of B. mori, and the loss of the function of BmWnt1 results in severe defects in body segmentation and pigmentation (Nakao, 2010; Yamaguchi et al., 2011, 2013; Zhang et al., 2015). However, few reports have focused on this gene in the silkworm midgut. In this paper, we identified and characterized BmWnt1 in midgut of silkworm larvae, a lepidopteran model insect we
anticipate that BmWnt1 may contribute to cell proliferation and development in the silkworm midgut.

Materials and Methods

Silkworm strain and cell culture
Silkworm strain Dazao (p50) and silkworm BmE cells were obtained from the State Key Laboratory of Silkworm Genome Biology (Southwest University, China). Various larval tissues in different stages were dissected and stored at -80 °C. Eggs were collected during different embryonic stages and stored as described above. The cells were cultured at 27 °C in Grace medium (Gibco) supplemented with 10 % (v/v) FBS (Gibco) (Xu et al., 2015).
RNA extraction

Total RNA was extracted with TRIzol reagent (TaKaRa) according to the manufacturer’s protocol. After the digestion of the residual genomic DNA using RNase-free DNase I (TaKaRa) for 30 min at 37 °C, 1 μg RNA was reverse transcribed using M-MLV Reverse Transcriptase (Promega) according to the protocol provided by the manufacturer. The cDNA product was stored at -30 °C.

BmWnt1 cDNA fragment amplification by PCR

Primers based on the predicted EST sequences in the SilkDB were used to amplify the DNA fragment of BmWnt1, and the sequences were as follows: BmWnt1-F 5‘-TTGACCTTGACCTGCA-3‘ and BmWnt1-R 5‘-GGCAAGAACTTGGTTCGGA-3‘. The housekeeping gene BmActin3 was used as an internal control to standardize the variance among housekeeping gene BmWnt1 of BmWnt1 cDNA product was stored at the protocol provided by the manufacturer, The PCR product was cloned into a pEASY™ extension at 72 °C under the following conditions: 5‘-BmActin3 3’ and BmActin3-R 5‘-CAAATGTTGAGCAATTTCT-3‘. PCR was performed with HiFi Taq DNA polymerase (TransGen Biotech) under the following conditions: 94 °C for 4 min; followed by 28 cycles of 94 °C for 40 s, 56 °C for 40 s, 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR product was cloned into a pEASY™-T5 Zero Cloning Vector (TransGen Biotech) and sequenced at Invitrogen (Shanghai).

Bioinformatics analysis

Multiple sequence alignments were performed with ClustalX version 1.81 with default gap penalties (Thompson et al., 1997; Tamura et al., 2007).

Quantitative real-time or semi-quantitative PCR analysis of BmWnt1

Quantitative real-time PCR (qRT-PCR) was conducted with SYBR® Premix Ex Taq™ II (TaKaRa) and the ABI 7500 Fast Real-Time PCR System (Applied BioSystems). The sequences of the primers: BmWnt1-3’-F 5′-GGAACTGGCTCCACGAGAAACT-3’ and BmWnt1-3’-R 5′-CTCACGGCAACCTCTATCCA-3’.

The housekeeping gene BmGAPDH was used as an internal control, and the sequences of primers were BmGAPDH-F 5′-T7GTGCGCTGCTCCTTGACCGAACCACTTTCC-3’ and BmGAPDH-R 5′-T7TCTAATATGTAGGACGTAACCATGTAATGTCCTTC-3’.

The dsRNAs for BmWnt1 and EGFP were generated by a RibomAX Large Scale RNA Production System-T7 kit (Promega) (Payungporn et al., 2006). The primers were as follows: T7-BmWnt1-F 5′-TAATACGACTCACTATAGGAATGTTGATGATA-3’and T7-BmWnt1-R 5′-TAATACGACTCACTATAGGGCTGCCTCCTTGACCTTTTTC-3’. EGFP was the control, and the following primers were used: T7-EGFP-F 5′-TAATACGACTCACTATAGGAATGTTGATGATA-3’ and T7-EGFP-R 5′-TAATACGACTCACTATAGGAATGTTGATGATA-3’.

Approximately 1×10⁵ BmE cells were seeded into 24-well plates for 24 h, and 500 ng dsRNA was transfected into cells using X-treme GENE transfection reagent (Roche, Switzerland) following the manufacturer’s instructions. After 3 days of exposure to dsRNA, the cells were harvested for qPCR and further experiments (Xu et al., 2015).

EdU treatment and immunofluorescence staining

The silkworms were fed EdU (5-ethyl-2'-deoxyuridine, Invitrogen) at a concentration of 10 μg/g body weight for 8 h (Tan et al., 2013). The midguts were dissected, fixed, paraffin embedded, sectioned, and stained with EdU antibody for the immunofluorescence analysis according to the manufacurer’s protocol. According to the protocol provided by the manufacturer, the cells were harvested after 2 h incubation with 5 μg EdU and stained with EdU antibody for the immunofluorescence analysis.

Results

BmWnt1 contains a Wnt domain, and is highly conserved with other species.

Using the NCBI GenBank and SilkBD databases, Primers from 1st base and 322nd base were designed and the Wnt1 gene in silkworm was identified and named BmWnt1. The ORF (open reading frame) is 1203 bp. The predicted protein contains 392 amino acids with a conserved Wnt domain.

Paraffin embedding and H&E staining

The midgut was dissected and gently rinsed in PBS, fixed in 4% paraformaldehyde (PFA, Sigma) for 2 h, rinsed in ethanol, embedded in paraffin, and sectioned at 5 μm. The paraffin sections were mounted on polylysine-coated glass slides. After 2 h at 60 °C, the sections were deparaffinized in xylene and subsequently rehydrated prior to staining. Finally, the sections were stained with hematoxylin-eosin (H&E) and examined by microscopy (Nikon 80i). The basal part of 8 silkworms in each group was measured, and the average thickness was obtained.

Knockdown of BmWnt1 in BmE cells

The dsRNAs for BmWnt1 and EGFP were generated by a RibomAX Large Scale RNA Production System-T7 kit (Promega) (Payungporn et al., 2006). The primers were as follows: T7-BmWnt1-F 5′-TAATACGACTCACTATAGGAATGTTGATGATA-3’ and T7-BmWnt1-R 5′-TAATACGACTCACTATAGGGCTGCCTCCTTGACCTTTTTC-3’. EGFP was the control, and the following primers were used: T7-EGFP-F 5′-TAATACGACTCACTATAGGAATGTTGATGATA-3’ and T7-EGFP-R 5′-TAATACGACTCACTATAGGAATGTTGATGATA-3’.

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Fig. 2 Temporal and spatial expression patterns of BmWnt1 in silkworm analyzed by semi-quantitative PCR and quantitative real-time PCR respectively. (A). Expression of BmWnt1 in different tissues of L5D3 silkworm. Si: silk gland; Ma: Malpighian tubule; Ha: Hemocyte; Ov: Ovary; Te: Testis; Mi: Midgut; Fa: Fat body; Ep: Epidermis; He: Head; Wh: Whole body. (B) Expression of BmWnt1 in different stages of silkworm midgut development. 3M: molting larvae of the 3rd instar; L4D1, L4D2, L4D3, L4D4: from day 1 to day 4 of 4th instar larvae; 4M: molting larvae of the 4th instar; L5D1, L5D2, L5D4, L5D5, L5D7: from day 1 to day 7 of 5th instar larvae; W2, W3: from day 2 to day 3 of wandering stage larvae; P1, P2, P3, P4, P5, P6: from day 1 to day 6 of pupation; BmActin3 was used as an internal control. (C) Quantitative real-time PCR analysis of BmWnt1 in different tissues of L5D3 silkworm. (D) Quantitative real-time PCR analysis of BmWnt1 in different stages of silkworm midgut development.
domain. The BmWnt1 protein has a calculated molecular mass of 44,739 Da. The predicted isoelectric point (pl) of its mature peptide is 9.28. Amino acids are highly conserved from invertebrates to vertebrates, especially in the Wnt1 domain from the 57th to 392nd amino acids, which is highly conserved in insects such as Lepidoptera (Fig. 1).

BmWnt1 is showed in differential expression profile

The temporal and spatial expressions of BmWnt1 were analyzed in different tissues on day 3 of the 5th (L5D3) instar larva and different developmental stages by RT-PCR. Agarose gel electrophoresis showed that BmWnt1 was highly expressed in the ovary, epidermis, head, testis, and Malpighian tubule and was detectable in the silk gland, midgut, and fat body, although it was not detectable in the hemocytes. The silkworm expression was used as the control (Fig. 2A). To further confirm this finding, qRT-PCR was employed, which showed similar results (Fig. 2C).

The mRNA expression levels of BmWnt1 in the midgut at different developmental stages were detected by semi-quantitative PCR and real-time PCR. The developmental stages from the 3rd molting stage, 4th instar, 4th molting stage, 5th instar, wandering stage, and pupal stage were observed. BmWnt1 expression was gradually increased and showed three peaks at the 3rd molting stage, 4th molting stage and the first day of pupal stage (Fig. 2B). qRT-PCR confirmed similar results (Fig. 2D).

DSS treatment thickens the midgut basal part

DSS can damage the basement membrane organization (Apidianakis et al., 2009; Ren et al., 2010; Tian et al., 2015). To verify the role of BmWnt1 during the development of the silkworm midgut, 3% or 5% DSS were used and fed to silkworms. In DSS treatment group, the midgut basal part was clearly thicker than the control (Figs 3A, B). The body weight of DSS treatment group had no significant change (data not shown).

DSS treatment promotes the midgut epithelial cell proliferation and BmWnt1 up-regulation

To examine the proliferation of midgut epithelial cells, the silkworm was dissected after treatment with EdU for 8 h following with 5% DSS treatment for 8 days. EdU staining of the midgut was accomplished, and the result showed that the number of EdU-positive cells in 5% DSS was dramatically higher than that in the control (Figs 4B, C). The expression of BmWnt1 was also investigated, and the results showed that the BmWnt1 expression in 5% DSS was higher than that of control (Fig. 4A).

Down-regulation of BmWnt1 by RNA interference reduces cell proliferation in vitro

BmWnt1 was down-regulated in BmE cells under dsRNA interference. The qRT-PCR showed that BmWnt1 expression was down-regulated successfully, and it was dramatically reduced by more than 70% (Fig. 5A). The EdU retention analysis showed that the number of EdU+ cells in the BmWnt1 RNAi group was reduced distinctly relative to that in the control group, and it was reduced by more than 30% (Figs 5B, C). These findings demonstrate that the down-regulation of BmWnt1 inhibited cell proliferation in BmE cells.
Fig. 4 Effect of DSS on cell proliferation of silkworm midgut. (A) Expression of BmWnt1 detected by qRT-PCR in the midgut after feeding with DSS. (B) EdU Immunofluorescence staining of the midgut after EdU treatment for 8 h following 5 % DSS treatment for 8 days. Scale bar = 25 μm. (C) EdU-positive cells in panel (B) were counted, and the statistical analysis was performed with a two-tailed Student’s t-test. PBS was used as the control. Data are presented as the mean±SD (n≥3). *p ≤ 0.05 and **p ≤ 0.01 indicate statistically significant differences.

Discussion

The Wnt family has been extensively studied in mammals as well as in Drosophila which comprising of several paralogous members. Until now, 19 of Wnt ligands in mammals and 7 in Drosophila have been identified. The bombyx genome probably has a single copy of BmWnt1 gene and other paralogous members of the wnt gene family (Dhawan et al., 2003). Wnt1/Wingless, which plays a crucial role in embryo development and tumorigenesis, is a key component in the Wnt pathway, and its function in the silkworm midgut remains to be elucidated. Here we mainly focus on the BmWnt1 gene. Wnt1 is conserved in most insects including Helicoverpa armigera and Danaus plexippus, and BmWnt1 shares 88 % and 79 % identity with them respectively.

As a holometabolous insect, the silkworm undergoes four stages including the egg, larva, pupa, and adult stages. At certain stages of development, internal tissues and organs undergo obvious changes (Xu et al., 2012). Since the midgut is responsible for digesting food and absorbing nutrients, the midgut epithelium is continually remodeled, with degenerated old cells replaced with new cells in the phase of molting (Franzetti et al., 2012). Wnt1 is mainly produced by the intestinal epithelial cells upon damage or stress, and this protein is apparently required for ISC proliferation during tissue regeneration in Drosophila (Cordero et al., 2012). Therefore, BmWnt1 might play a similar role in the silkworm midgut in maintaining tissue homeostasis.

DSS is a compound that can lead to excessive inflammation that resembles ulcerative colitis in humans (Kawada et al., 2007), and it has been reported to promote the proliferation of Drosophila midgut cells (Amcheslavsky et al., 2009). In this paper, 3 % or 5 % DSS were used to feed silkworms. After DSS treatment the silkworm midgut epithelial cell proliferation was obviously promoted with
BmWnt1 up-regulation. Down-regulation of BmWnt1 by siRNA can inhibit cell proliferation, which suggest BmWnt1 may be involved in regulation of silkworm midgut epithelial cell proliferation. DSS has been reported to stimulate Hedgehog (Hh) signaling in Drosophila, which promotes intestinal stem cell proliferation (Apidianakis et al., 2009; Tian et al., 2015). Here, we found that the basal sides of midgut in the DSS-treated group were visibly thicker than those of the control. During the growth process, the expression of BmWnt1 was also up-regulated in the DSS-treated silkworm compared with that in the control and midgut epithelial cell proliferation was promoted, indicating that BmWnt1 may play an important role in maintenance of silkworm midgut homeostasis. Moreover, Wingless has been reported to be required for the proliferation and maintenance of ISCs and HPZ (the hindgut proliferation zone) in Drosophila larvae (Lin et al., 2008; Takashima et al., 2008), which further supporting our findings. Therefore, BmWnt1 may play important roles in the process of cell proliferation in silkworm, as it does in other species.

In summary, our study with DSS treatment of B. mori suggests a injury-stimulated activity for Wnt1 in B. mori and a role for midgut epithelial cell proliferation. Downregulation of BmWnt1 by RNA interference inhibits BmE cell proliferation. This injury-induced midgut basement membrane damage model should enable further analysis of the role of BmWnt1 signaling pathway in B. mori.

Acknowledgments
This work was supported by the Chinese National Natural Science Foundation (31672496), the Natural Science Foundation of Chongqing (cstc2016jcyjA0425), the Research Fund for the Doctoral Program of Higher Education of China (20130182110003), and the Chongqing University Innovation Team Building Program Funded Projects (CXTDX201601010). We are grateful to Prof. Li C of the State Key Laboratory of Silkworm Genome Biology for his help in the preparation of samples.
References


