ISJ 14: 190-198, 2017 ISSN 1824-307X

RESARCH REPORT

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

**L Yang*, Z Wei*, M Xu*, Y Zhao, H Liang, P Tan, H Ma, T Lu, Z Wang, H Cui**

*State Key Laboratory of Silkworm Genome Biology (Southwest University), 400715 China*

*These authors contributed equally to this work

*Accepted May 4, 2017*

## 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 LRP5/6 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

*Corresponding author*:
Hongjuan Cui
State Key Laboratory of Silkworm Genome Biology Southwest University
400715 China
E-mail: hcui@swu.edu.cn

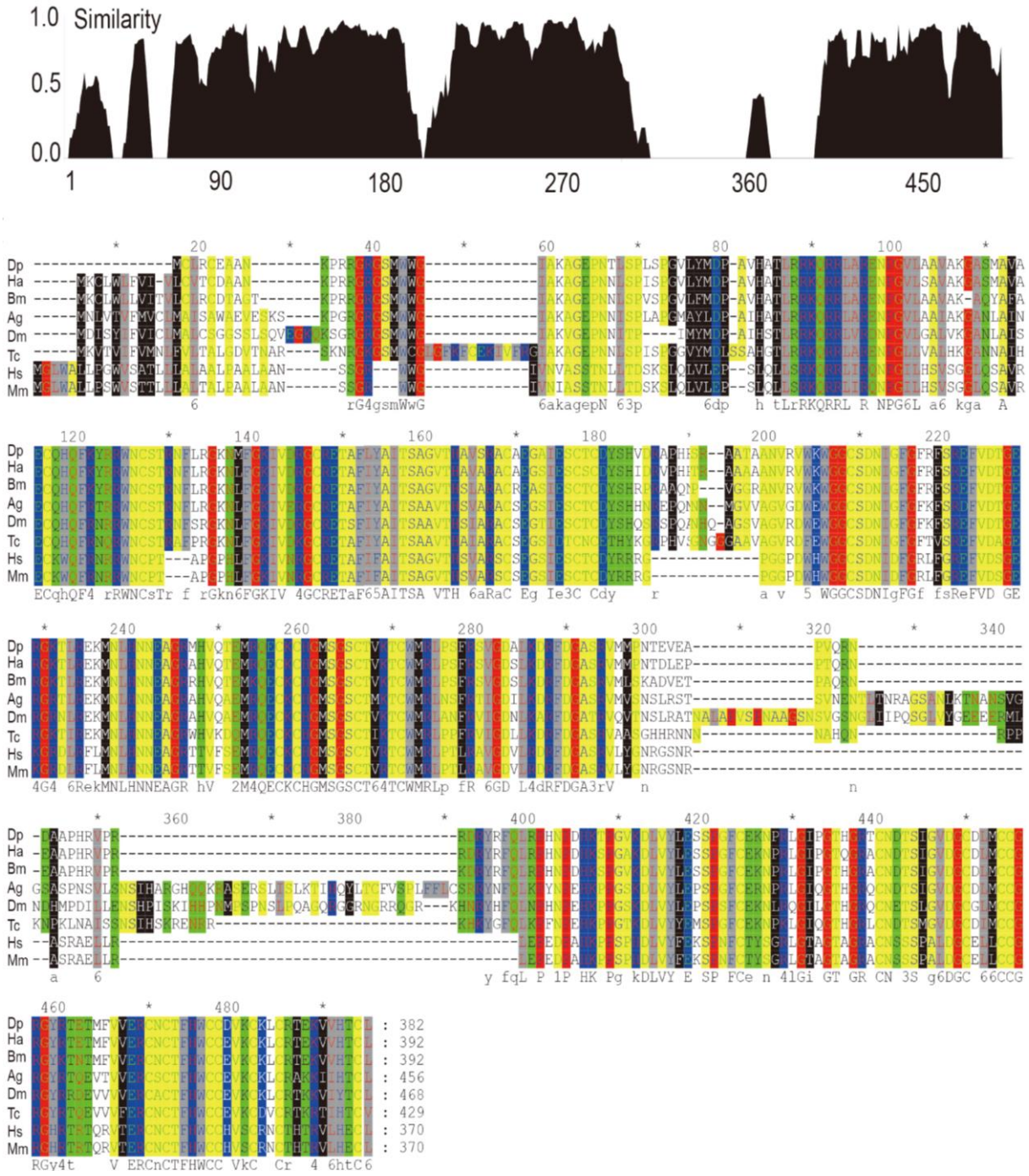


**Fig. 1** Multiple alignment of Wnt1/Wingless amino acid sequences from *D. plexippus* (EHJ69660.1), *H. armigera* (AHN95659.1), *B. mori* (NM_001043850), *A. gambiae* (AAT92559.1), *D. melanogaster* (NP 523502.1), *T. castaneum* (EFA04660.1), *H. sapiens* (NP 005421.1) and *M. musculus* (NP 067254.1).

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'-TTTGCACTTGACCTCGCA-3' and *BmWnt1*-R 5'- GGCAAGAACTTGTTCGGAA-3'. The housekeeping gene *BmActin3* was used as an internal control to standardize the variance among the different templates as described (Jiang et al., 2012). The primer sequences were as follows: *BmActin3*-F 5'-TTCGTACTGGCTCTTC TCGT -3' and *BmActin3*-R 5'-CAAAGTTGATAGCAATTCCCT-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*-qRT-F 5'-GGAACTGCTCCACGAGAAACT-3' and *BmWnt1*-qRT-R 5'-CTCACGGCAACCTCTATCCA-3'. The housekeeping gene *BmGAPDH* was used as an internal control, and the sequences of primers were *BmGAPDH*-F 5'-CATTCCGCGTCCCTGTTGCTAAT-3' and *BmGAPDH*-R 5'-GCTGCCTCCTTGACCTTTTGC-3' (Jin *et al.*, 2014). The relative expression of genes was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The online t-test software GraphPad (http://www.graphpad.com/quickcalcs/ttest1.cfm) was used to evaluate the statistical significance of the results ($p < 0.05$). A semi-quantitative PCR analysis was performed as described previously *BmActin3* was used as the internal reference.

*DSS treatment*

Silkworm larvae at the first day of the 4th instar were used for the feeding experiments. Rounded mulberry leaves with a diameter of 3.5 cm were coated uniformly with 20 μl 3 % or 5 % (m/V) of dextran sulfate sodium (DSS) (Sigma), and 20 μl phosphate buffered saline (PBS) was used for the control group (Tan et al., 2015). After the mulberry leaves were air dried, the silkworms were fed separately in 35 mm×10 mm cell culture dishes.

*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'-TAATACGACTCACTATAGGATGAAGTGTCTGT GGCTGTTAGTGATA-3'and T7-*BmWnt1*-R 5'-TAATACGACTCACTATAGG CTATAAACACGTGTGCACCACTTTTTC-3'. *EGFP* was the control, and the following primers were used: T7-*EGFP*-F 5'-TAATACGACTCACTATAGGACGTAAACGGCCAC AAGTTC-3' and T7-*EGFP*-R 5'-TAATACGACTCACTATAGGTGCTCAGGTAGTGG TTGTCG-3'.

Approximately $1\times10^5$ 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-ethynyl-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 manufacturer'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

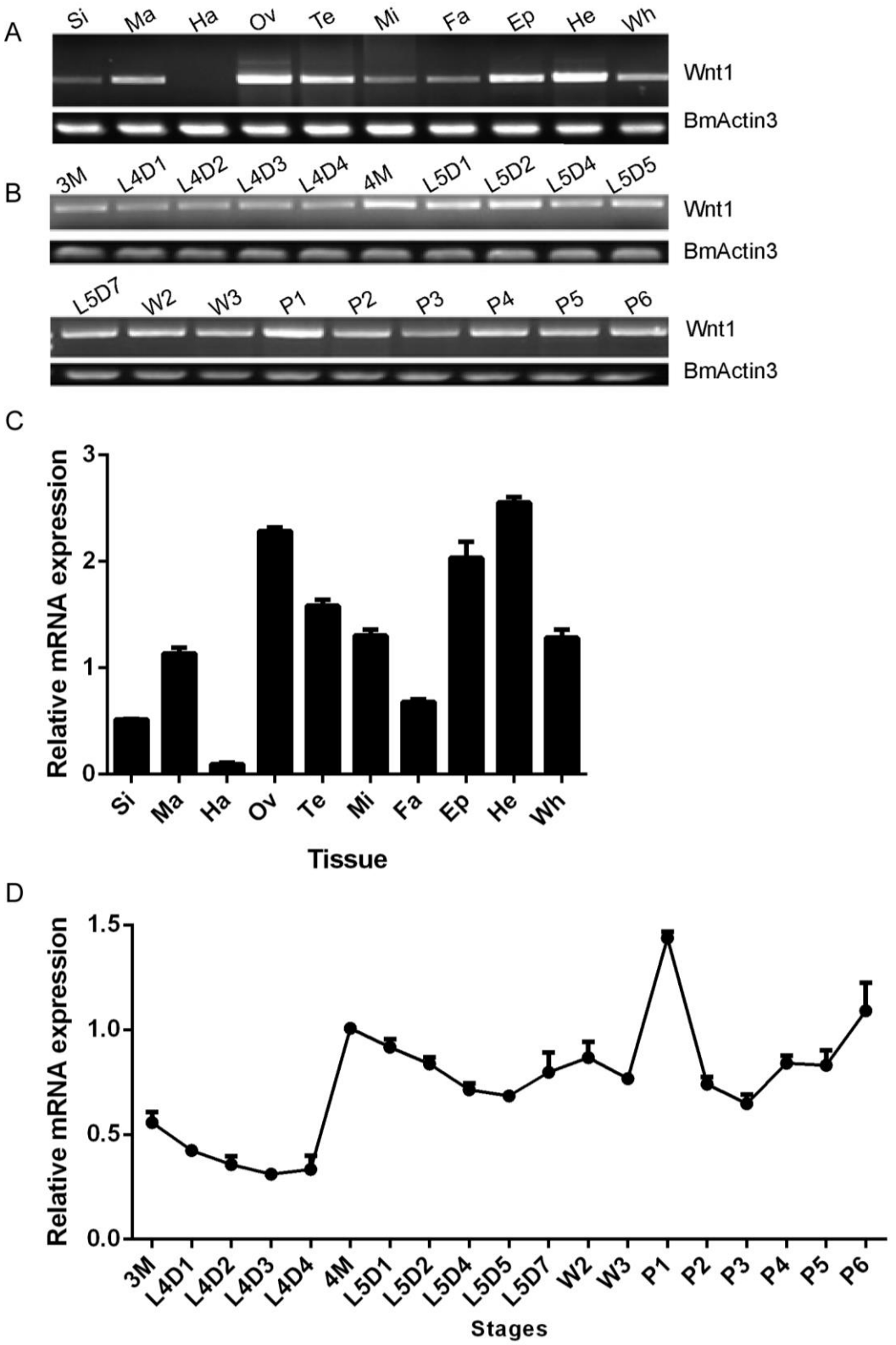


**Fig. 2** Temporal and spatial expression patterns of *BmWnt1* in silkworm analyzed by semi-quantitative PCR and quantitative real-time PCR respectively. (A). Expression of *BmWnt1* in different tissues of L5D3 silkworm. Si: silk gland; Ma: Malpighian tubule; Ha: Hemocyte; Ov: Ovary; Te: Testis; Mi: 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.

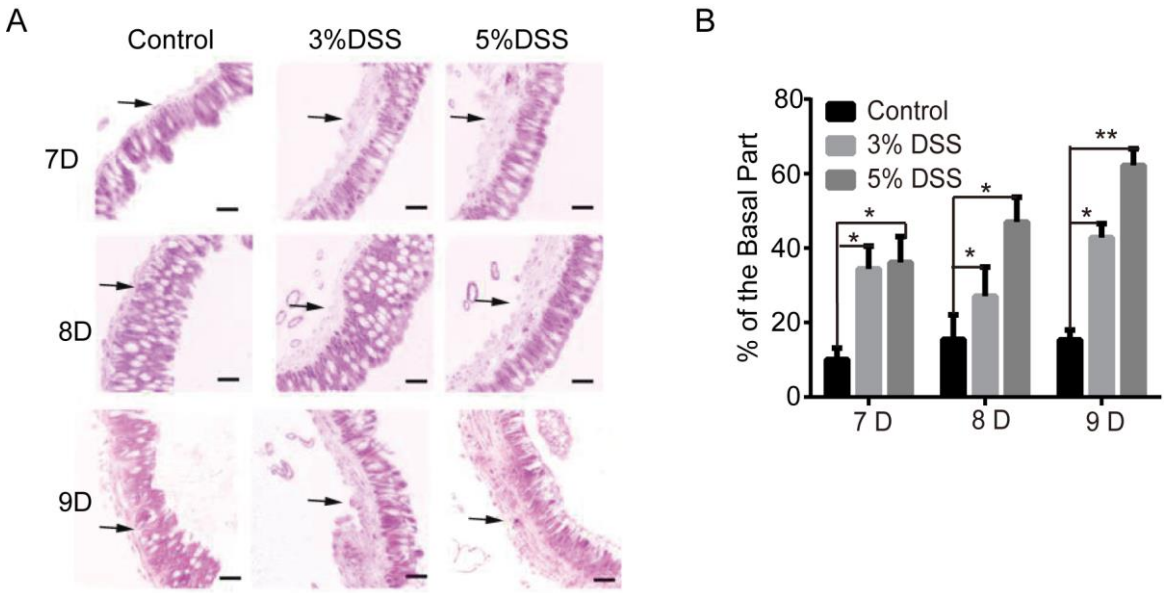


**Fig. 3** Effect of DSS on the silkworm midgut. (A) Basal part was observed by H&E staining after DSS treatment on different days. 8 silkworms in each group were used and the average thickness was obtained. The arrows point to the basal side of the midgut. Scale bar = 25 μm. (B) Thickness of the basal side of the midgut in panel (A) measured after 3 % or 5 % DSS stimulation; the statistical analysis was performed with a two-tailed Student's t-test. PBS was used as the control.

domain. The BmWnt1 protein has a calculated molecular mass of 44,739 Da. The predicted isoelectric point (pI) of its mature peptide is 9.28. Amino acids are highly conserved from invertebrates to vertebrates, especially in the Wnt1 domain from the 57$^{th}$ to 392$^{nd}$ 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 5$^{th}$ (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 3$^{rd}$ molting stage, 4$^{th}$ instar, 4$^{th}$ molting stage, 5$^{th}$ instar, wandering stage, and pupal stage were observed. *BmWnt1* expression was gradually increased and showed three peaks at the 3$^{rd}$ molting stage, 4$^{th}$ 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.

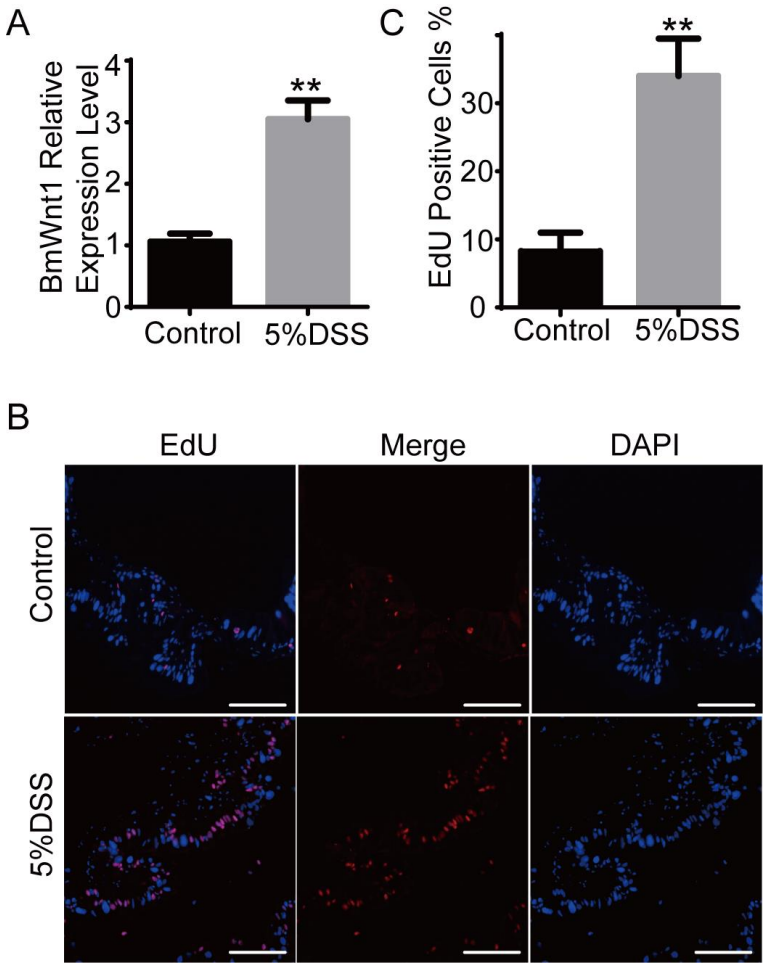


**Fig. 4** Effect of DSS on cell proliferation of silkworm midgut. (A) Expression of *BmWnt1* detected by qRT-PCR in the midgut after feeding with DSS. (B) EdU Immunofluorescence staining of the midgut after EdU treatment for 8 h following 5 % DSS treatment for 8 days. Scale bar = 25 μm. (C) EdU-positive 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). *$p \leq 0.05$ and **$p \leq 0.01$ 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 holometabolic 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

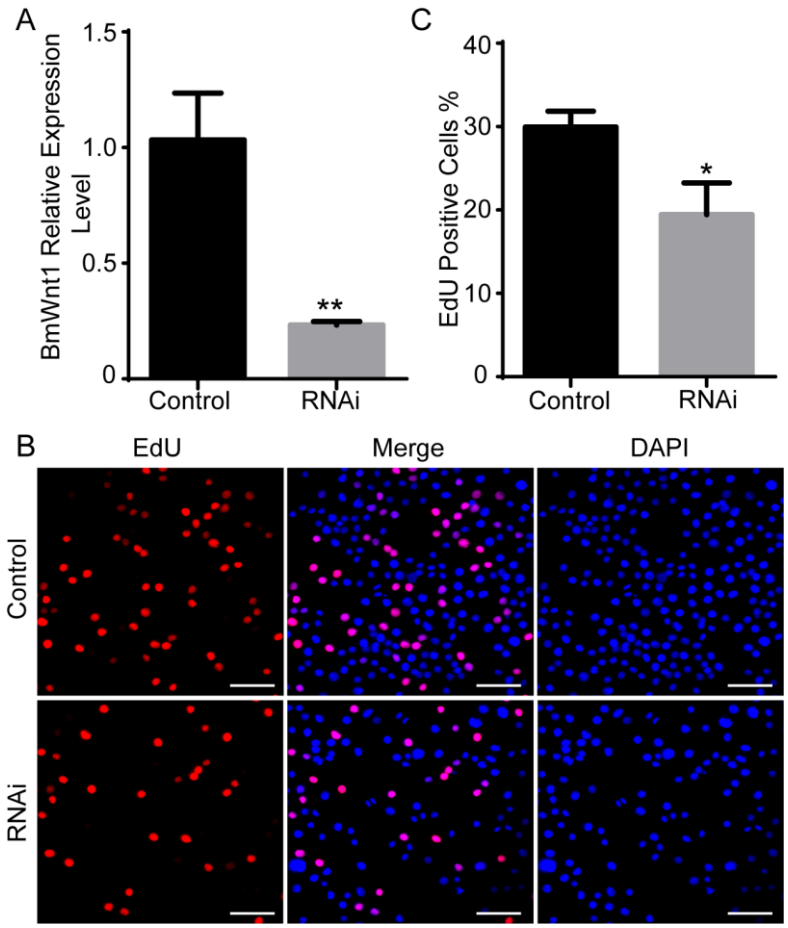


**Fig. 5** Effect of *BmWnt1* knockdown on cell proliferation in BmE cells. (A) Expression level of *BmWnt1* detected by qRT-PCR after RNA interference in BmE cells. EGFP dsRNA was used as the control. (B) EdU immunofluorescence analysis after 2 h incubation with 5 μg EdU following RNA interference for 3 days in BmE cells. EGFP dsRNA was used as the control. Scale bar = 50 μm. (C) EdU-positive cells in panel (B) were counted, and the statistical analysis was performed with a two-tailed Student's t-test. Data are given as the mean ± SD (n ≥ 3). *$p \leq 0.05$ and **$p \leq 0.01$ indicate statistically significant differences.

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 *BmWnt*1 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**


Amcheslavsky A, Jiang J, Ip YT. Tissue damage-induced intestinal stem cell division in *Drosophila*. Cell stem cell 4: 49-61, 2009.

Apidianakis Y, Pitsouli C, Perrimon N, Rahme L. Synergy between bacterial infection and genetic predisposition in intestinal dysplasia. Proc. Natl. Acad. Sci. USA 106: 20883-20888, 2009.

Campbell G, Weaver T, Tomlinson, A., 1993. Axis specification in the developing *Drosophila* appendage - the role of wingless, decapentaplegic, and the homeobox gene aristaless. Cell 74: 1113-1123, 1993.

Clevers H. Wnt/beta-catenin signaling in development and disease. Cell 127: 469-480, 2006.

Cordero JB, Stefanatos RK, Scopelliti A, Vidal, M, Sansom OJ. Inducible progenitor-derived Wingless regulates adult midgut regeneration in *Drosophila*. EMBO J. 31: 3901-3917, 2012.

Dhawan, S., Gopinathan, K.P., 2003. Spatio-temporal expression of wnt-1 during embryonic-, wing- and silkgland development in *Bombyx mori*. Gene Expr. Patterns 3: 559-570.

Franzetti E, Huang ZJ, Shi YX, Xie K, Deng XJ, Li JP, *et al*. Autophagy precedes apoptosis during the remodeling of silkworm larval midgut. Apoptosis 17: 305-324, 2012.

Izumi N, Shoji K, Sakaguchi Y, Honda S, Kirino Y, Suzuki T, *et al*. Identification and Functional Analysis of the Pre-piRNA 3' Trimmer in Silkworms. Cell 164: 962-973, 2016.

Jiang L, Cheng TC, Zhao P, Yang Q, Wang GH, Jin SK, *et al.* Resistance to BmNPV via overexpression of an exogenous gene controlled by an inducible promoter and enhancer in transgenic silkworm, *Bombyx mori*. PLOS ONE 7, e41838, 2012.

Jin SK, Cheng TC, Jiang L, Lin P, Yang Q, Xiao Y, *et al.* Identification of a new sprouty protein responsible for the inhibition of the *Bombyx mori* nucleopolyhedrovirus reproduction. PLOS ONE 9, e99200, 2014.

Kawada M, Arihiro A, Mizoguchi E. Insights from advances in research of chemically induced experimental models of human inflammatory bowel disease. World J. Gastroenterol. 13: 5581-5593, 2007.

Kestler HA, Kuhl M. From individual Wnt pathways towards a Wnt signalling network. Phil. Trans. R. Soc. B 363: 1333-1347, 2008.

Lin G, Xu N, Xi R. Paracrine Wingless signalling controls self-renewal of *Drosophila* intestinal stem cells. Nature 455: 1119-1123, 2008.

Liu W, Singh SR, Hou SX. JAK-STAT is restrained by notch to control cell proliferation of the *Drosophila* intestinal stem cells. J. Cell Biochem. 109: 992-999, 2010.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408, 2001.

Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. 20: 781-810, 2004.

Matsumoto N, Nishimasu H, Sakakibara K., Nishida, K.M., Hirano, T., Ishitani, R.,*et al*. Crystal Structure of Silkworm PIWI-Clade Argonaute Siwi Bound to piRNA. Cell 167: 484-497, 2016.

Micchelli CA, Perrimon N. Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. Nature 439: 475-479, 2006.

Micchelli CA, Sudmeier L, Perrimon N, Tang S, Beehler-Evans R. Identification of adult midgut precursors in *Drosophila*. Gene Expr. Patterns 11: 12-21, 2011.

Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. Nat. Rev. Genet. 5: 689-699, 2004.

Nakao, H., 2010. Characterization of *Bombyx* embryo segmentation process: expression profiles of engrailed, even-skipped, caudal, and wnt1/wingless homologues. J. Exp. Zool. Part B 314B: 224-231, 2010.

Ohlstein B, Spradling A. Multipotent *Drosophila* intestinal stem cells specify daughter cell fates by differential notch signaling. Science 315: 988-992, 2007.

Payungporn S, Chutinimitkul S, Chaisingh A, Damrongwantanapokin S, Buranathai C, Amonsin A, *et al*. Theamboonlers, A., Poovorawan, Y. Single step multiplex real-time RT-PCR for H5N1 influenza A virus detection. J: Virol. Methods 131: 143-147, 2006.

Rao TP, Kuhl M. An updated overview on Wnt signaling pathways: a prelude for more. Circ. Res. 106: 1798-1806, 2010.

Ren F, Wang B, Yue T, Yun EY, Ip YT, Jiang J. Hippo signaling regulates *Drosophila* intestine stem cell proliferation through multiple pathways. Proc. Natl. Acad. Sci. USA 107: 21064-21069, 2010.

Takashima S, Mkrtchyan M, Younossi-Hartenstein A, Merriam JR, Hartenstein V. The behaviour of *Drosophila* adult hindgut stem cells is controlled by Wnt and Hh signalling. Nature 454: 651-U658, 2008.

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596-1599, 2007.

Tan J, Xu M, Zhang K, Wang X, Chen S, Li T, *et al.* Characterization of hemocytes proliferation in larval silkworm, *Bombyx mori*. J. Insect Physiol. 59: 595-603, 2013.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876-4882, 1997.

Tian A, Shi Q, Jiang A, Li S, Wang B, Jiang J. Injury-stimulated Hedgehog signaling promotes regenerative proliferation of Drosophila intestinal stem cells. J. Cell Biol. 208: 807-819, 2015.

Williams JA, Paddock SW, Carroll SB. Pattern-formation in a secondary field - a hierarchy of regulatory genes subdivides the developing *Drosophila* wing disc into discrete subregions. Development 117: 571-584, 1993.

Xia Q, Zhou Z, Lu C, Cheng D, Dai F, Li B, *et al.* A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). Science 306: 1937-1940, 2004.

Xu M, Wang X, Tan J, Zhang K, Guan X, Patterson LH, *et al.* A novel Lozenge gene in silkworm, *Bombyx mori* regulates the melanization response of hemolymph. Dev. Comp. Immunol. 53: 191-198, 2015.

Xu QY, Lu AR, Xiao GH, Yang B, Zhang J, Li XQ, *et al.* Transcriptional profiling of midgut immunity response and degeneration in the wandering silkworm, *Bombyx mori*. PLOS ONE 7, e43769, 2012.

Yamaguchi J, Banno Y, Mita K, Yamamoto K, Ando T, Fujiwara H. Periodic Wnt1 expression in response to ecdysteroid generates twin-spot markings on caterpillars. Nat. Commun. 4: 1857, 2013.

Yamaguchi J, Mizoguchi T, Fujiwara H. siRNAs induce efficient RNAi response in *Bombyx mori* embryos. PLOS ONE 6, e25469, 2011.

Zhang ZJ, Aslam AM, Liu XJ, Li MW, Huang YP, Tan AJ. Functional analysis of *Bombyx* Wnt1 during embryogenesis using the CRISPR/Cas9 system. J. Insect Physiol. 79: 73-79, 2015.

Tian A, Shi Q, Jiang A, Li S, Wang B, Jiang J. Injury-stimulated Hedgehog signaling promotes regenerative proliferation of *Drosophila* intestinal stem cells. J. Cell Biol. 208 960: 807-819, 2015.