Insect Biochemistry and Molecular Biology 113 (2019) 103205

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

# *Bmsage* is involved in the determination of cell number in the silk gland of *Bombyx mori*

Sihan Hou[a], Yan Sun[b], Yangchun Wu[c,d], Tingcai Cheng[a], Chun Liu[a,*]

[a] State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing, 400716, China
[b] Biotechnology College, Southwest University, Chongqing, 400716, China
[c] Jiangsu Key Laboratory of Sericultural Biology and Biotechnology, School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu, 212018, China
[d] The Key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture, Sericultural Research Institute, Chinese Academy of Agricultural Science, Zhenjiang, Jiangsu, 212018, China

ARTICLE INFO

*Keywords:*
*Bmsage*
*Bombyx mori*
Silk gland development
Cell number
Cyclin pathway

ABSTRACT

The number of cells in tissues is under strict genetic control, and research on the determination of cell number is of great importance to understand the growth and development of organs. *Bmsage*, a bHLH transcription factor, is involved in the development of the silk gland during the embryonic stage in *Bombyx mori*. However, the mechanism by which it influences silk gland development is unclear. In the present study, we determined via immunofluorescence staining during the embryonic stage of *Bombyx mori* that *Bmsage* is expressed in silk gland cells from the beginning of development of the silk gland until its complete formation. By comparing different silkworm strains, we found that *Bmsage* expression is positively correlated with the number of silk gland cells. *Bmsage* knockdown by RNAi resulted in shorter silk glands and lower cell numbers, especially in the posterior silk gland. The silk gland lumen also shriveled, and the silk protein content was significantly lower than that in the control. Further investigation revealed that all cyclins decreased after knock down of *Bmsage*, and *cyclin B* and *cyclin 3* were significantly down-regulated. *Bmsage* may be involved in the regulation of the cyclin pathway to control silk gland development. Taken together, it can be concluded from our results that *Bmsage* is involved in the determination of cell number in silk glands. Our results help clarify the process of cell number determination in silk gland and identify a potential target for silkworm breeding.

## 1. Introduction

In nature, the cell is the basic unit of life (Mazzarello, 1999). The number of cells in different organisms is generally subject to strict genetic control. For example, a bacterium is composed of a single cell, and *Caenorhabditis elegans* is composed of approximately 1000 somatic cells (Alberts, 2002). However, the mechanism of cell number determination in higher organisms is not fully understood. Research on the mechanism of cell number control will remarkably help in understanding both development and disease occurrence.

As an economically important insect, *Bombyx mori* has been reared by humans for a long time. Its most important economic trait is the ability to spin silk, and the silk gland is the main organ that carries out the biological processes of synthesis, secretion, and spinning of silk. The silk gland can be divided into three parts based on morphological characteristics: the anterior silk gland (ASG), middle silk gland (MSG), and posterior silk gland (PSG). The ASG is a duct connecting the spinning tube and the MSG. It has no function in secreting protein but plays an important role in the crystallization of silk protein (Asakura et al., 2007). The MSG is the thickest part of the whole silk gland, and it mainly secretes the silk protein sericin. The PSG is the longest part of the silk gland; it is coiled and has a powerful ability to synthesize silk fibroin (Inoue et al., 2000; Sprague, 1975; Yamaguchi et al., 1989). To complete the spinning process, all the three parts are essential.

The silk gland of *B. mori* originates from the ectoderm and forms a paired tubular organ, which is composed of silk gland cells and lumen. Silk gland cells are known to complete mitosis at the embryonic stage (Dhawan and Gopinathan, 2003), increase their cell volume, and replicate their nuclei, but no cell division occurs during the larval stage (Niranjanakumari and Gopinathan, 1991, 1993; Perdrix-Gillot, 1979). Finally, at the fifth instar, the DNA content of the MSG and PSG increases by $2^{17}$–$2^{19}$ times (Gage, 1974; Niranjanakumari and Gopinathan, 1992; Perdrix-Gillot, 1979); this high DNA content creates favorable conditions for the mass transcription of silk proteins (Patel

---

* Corresponding author.
*E-mail address:* mlliuchun@163.com (C. Liu).

https://doi.org/10.1016/j.ibmb.2019.103205
Received 26 April 2019; Received in revised form 10 July 2019; Accepted 15 July 2019

and Gopinathan, 1991).

The initial formation of the silk gland begins approximately 36 h before the embryonic reversal stage. The silk gland cells are generated by the two silk gland disc in the base of the second segment of embryo. After 6 h, cell differentiation is extensive, and after 60 h, the MSG becomes S-shaped (Matsunami et al., 1999; Suzuki, 1977). At this point, the ASG, MSG, and PSG are clearly distinguishable. There are approximately 1000 silk gland cells, with the ASG, MSG, and PSG comprising approximately 300, 260, and 500 cells, respectively (Perdrix-Gillot, 1979).

However, only few studies have focused on the molecular mechanism of silk gland development at the embryonic stage. Nagata found that the time of *Scr* expression was consistent with the start of silk gland development, and ectopic expression of *Scr* could induce the development of additional silk glands, indicating that Scr is involved in their initation (Nagata et al., 1996). Salivary gland production in *Drosophila melanogaster* is also controlled by Scr (Andrew et al., 1994; Panzer et al., 1992). Furthermore, the development of the silk gland in the embryo is mainly a process of increasing the number of cells. Although several factors affect cell growth, the final determination of cell number is mainly through apoptosis, autophagy, and division. *cyclin B* has long been considered the main gene controlling mitosis in the silk gland; when silk gland cells stop dividing, *cyclin B* expression ceases (Dhawan and Gopinathan, 2003). This indicates that the cyclin pathway plays an important role in the development of embryonic silk glands.

*Bmsage*, encoding a silk gland-expressed basic helix-loop-helix (bHLH), is homologous to the gene *Dmsage* in *D. melanogaster*. *Dmsage* is specifically expressed in the salivary glands (Fox et al., 2013). *B. mori* silk glands and *D. melanogaster* salivary glands share many common features (Ashburner and Novitski, 1976; Martin et al., 1988). Previous studies have shown that Dmsage could regulate *senseless*, while *senseless* could inhibit expression of the apoptosis gene *reaper*, thereby maintaining the survival of salivary gland cells (Chandrasekaran and Beckendorf, 2003). Dmsage also regulates *PH4αSG1* and *PH4αSG2*. These two genes encode α subunits of prolyl hydroxylase. When *PH4αSG1* and *PH4αSG2* are mutated, the resulting salivary lumen shrinks or is blocked (Abrams et al., 2006).

In silkworm, *Bmsage* has been found to regulate the transcription of *fibroin-H* via interaction with *SGF1* (Zhao et al., 2014). When *Bmsage* was deleted, the silk gland did not fully develop, and the middle and posterior parts of the gland were almost completely absent (Xin et al., 2015). *Bmsage* is not only involved in the transcription of silk protein but also plays an important role in the development of the silk gland at the embryonic stage; however, its molecular mechanism in silk gland development is unknown. In the present study, we determined the expression of *Bmsage* in embryonic silk glands and explored its function by RNA interference (RNAi). We found that compared to *Dmsage* in fruit flies, *Bmsage* in silkworm plays a completely different role. Our findings aid further understanding of the development of embryonic silk glands and have the potential to be used for increasing silk production.

## 2. Material and methods

### 2.1. Silkworm strains

The silkworm strains, DaZao (DZ) and ZhongYe (ZY) were provided by the silkworm gene bank of Southwest University and Biotechnology College of Jiangsu University of Science and Technology, respectively. The silkworm eggs were cultured at a standard temperature of 25 °C under a photoperiod of 12 h light and 12 h dark and the larvae were reared on fresh mulberry leaves.

### 2.2. Western blot analysis

RIPA Lysis Buffer (Beyotime, China) was used to extract protein from the silkworm eggs. The lysate was divided evenly and then centrifuged for 5 min at 12000 × *g*. The protein concentrations were measured using a BCA Protein Assay Kit (Beyotime, China). Anti-Bmsage antibody was prepared following a previous report (Zhao et al., 2014). SDS-PAGE through 10% polyacrylamide gels was used to separate the proteins, followed by transfer onto polyvinylidene difluoride (PVDF) membranes (Roche, Switzerland). Each PVDF membrane was blocked with skim milk overnight and incubated with rabbit anti-Bmsage antibody (1:10000) for 2 h at 37 °C. After washing, the membranes were incubated with goat anti-rabbit IgG secondary antibody labeled with horseradish peroxidase (HRP) (Sigma, USA) for 1.5 h at 37 °C and visualized with SuperSignal™ WestFemto Maximum Sensitivity Substrate (Thermo Scientific, USA).

### 2.3. RNA preparation, reverse transcription PCR (RT-PCR), and quantitative reverse transcription-PCR (RT-qPCR)

Total RNA from the eggs and silk glands was extracted using TRIzol™ reagent (Invitrogen, USA) and reverse transcription was performed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Japan). RT-PCR was carried out with Thermus aquaticus DNA polymerase (Takara, Japan). *Bmsage* was amplified with the primers listed in Supplementary Table 1. Quantitative PCR(qPCR) was performed using SYBR® Premix Ex Taq™ II (Takara) under the following conditions: 95 °C for 10 s, then 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Silkworm ribosomal protein L3 (BmRpl3) mRNA was used as a normalizing gene. Three independent replicates were performed.

### 2.4. Immunofluorescence staining, counting length and cell number of silk glands, statistical method for cocoon layer rate

Immunofluorescence staining of embryos was performed as previously described (Liu et al., 2000). Rabbit anti-Bmsage antibody and FITC-labeled goat anti-rabbit IgG were all used at a 1:500 dilution. Silk glands from third instar and fifth instar larvae were dissected in phosphate buffered saline (PBS, pH 7.4). Fresh silk glands were fixed in methanol and dried, and this was repeated three times. Then, the tissues were stained with 4′,6′-diamidino-2-phenylindole (DAPI) for 10 min and washed away with deionized water. Adobe Photoshop CC 2015 software was used to calculate the number of silk gland cells, Adobe Illustrator CS6 was used to splice images, Image-Pro Plus was used to measure the length of the silk gland, and GraphPad Prism 6 was used for statistical analysis. The whole cocoon was weighed and then gently peeled; the pupa was removed, the epidermis shed, and the remaining cocoon was weighed. The ratio of this weight to the whole cocoon weight was deemed the cocoon layer ratio, which was measured in fifteen RNAi and control individuals.

### 2.5. RNA interference (RNAi)

The full-length gene of *Bmsage* was selected as a template to synthesize dsRNA. The dsRNA was synthesized using RiboMAX Large Scale RNA Production Systems-T7 and SP6 (Promega, Madison, WI, USA) according to the manufacturer's instructions. Approximately 3 nl of 3 mg/ml dsRNA was injected into each silkworm egg within 2 h of oviposition. Similarly, EGFP dsRNA was injected into the control eggs.

### 2.6. Statistical analysis

Statistical differences were evaluated using Student's *t*-tests for unpaired samples. The level of statistical significance was set as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

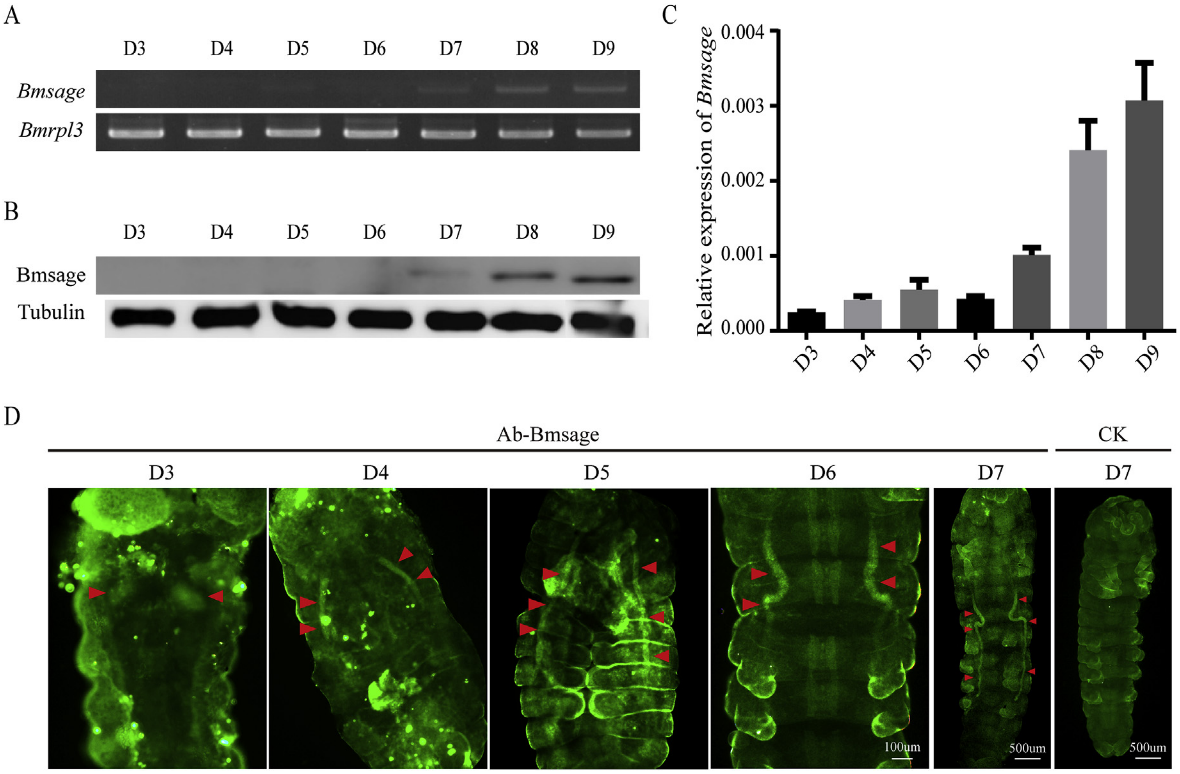

**Fig. 1. Expression pattern of *Bmsage* during the embryonic stage**. A. RT-PCR detection of *Bmsage* expression from the third to ninth days of the embryonic stage. B. Western blot of embryo protein from the third to ninth day of the embryonic stage using anti-Bmsage antibody; three independent experiments were repeated. C. RT-qPCR analysis of the expression profile of *Bmsage* from the third to ninth day of the embryo. The results are expressed as means ± SD of three independent experiments. D. Localization of Bmsage in the PSG and MSG of embryo (arrow). The silk gland of silkworm embryos (arrow) was stained with anti-Bmsage antibody.

## 3. Results

### 3.1. *Bmsage* is expressed in silk glands during the embryonic stage

To explore the role of *Bmsage* in the embryonic stage, first, the expression pattern of *Bmsage* was examined from the third to ninth day of the embryonic stage. RT-PCR showed that *Bmsage* was initially transcribed at low amounts from the fifth day; its level increased gradually until the seventh day and was the highest on the ninth day (Fig. 1A). In order to confirm this expression pattern, RT-qPCR and Western blot analysis were performed and consistent results were obtained (Fig. 1B and C).

In the fifth instar larvae, *Bmsage* is specifically expressed in the silk gland (Zhao et al., 2014). We verified whether this is also true for the embryonic stage. Immunofluorescence staining was performed using an anti-Bmsage antibody. Fluorescence signals were detected from the beginning of cell invaginations to the formation of a complete silk gland (Fig. 1D). During the entire process, Bmsage was specifically localized in the cells that differentiated into MSG and PSG; there was no fluorescence signal in the ASG. Therefore, until the completion of silk gland development, Bmsage is continually expressed in the MSG and PSG.

### 3.2. *Bmsage* is highly expressed in a high silk yielding strain

Through population evolution analysis, previous studies have shown that *Bmsage* is a candidate gene for silkworm domestication (Xia et al., 2009). In the present study, we chose two strains, the high silk-yielding strain ZY and the low-silk yielding strain DZ (Fig. 2A and Fig. S1). Then, we investigated the number of cells in the silk gland. Compared to DZ, ZY was found to have more cells, particularly in the PSG, and no significant difference was observed in the cell number and morphology in the MSG between DZ and ZY (Fig. 2C and B). Subsequently, the expression level of *Bmsage* was compared between the two strains on the sixth and seventh days, which were the key stages when the PSG was being extended in the embryo. The RT-qPCR results show that the expression level of *Bmsage* was higher in ZY than in DZ (Fig. 2D). These results suggest that the expression of *Bmsage* is positively correlated with the cell number of PSG.

### 3.3. Knockdown of *Bmsage* decreases cell number in the silk gland

Although previous studies have shown that *Bmsage* is important for silk gland development (Xin et al., 2015), it is unknown which aspects of the silk gland are affected by *Bmsage*. Thus, we carried out an RNAi experiment of *Bmsage* in the early embryonic stage. *Bmsage* was significantly down-regulated via dsRNA injection on the seventh days compared with that in the control, which was injected with *EGFP* dsRNA (Fig. 3A). To examine the effect of decreasing *Bmsage* expression in the embryonic stage, the morphology of the silk gland was observed at the third instar. Surprisingly, the length of the silk gland with *Bmsage* knockdown was much shorter than that with control RNAi, even without a developed PSG in some individuals (Fig. 3B and D). Statistical analysis revealed that the length of the PSG with *Bmsage* RNAi sample was only ~50% that of the control ($P = 0.0004$), but there was no significant change in the length of the MSG ($P = 0.2133$) (Fig. 3B). To understand why the PSG became shorter after *Bmsage* RNAi, these silk glands were stained with DAPI. The results showed that the nuclei of silk glands were clear, but there were fewer cells in the *Bmsage* RNAi individual. In addition, after counting the number of silk gland cells, it was found that the number of RNAi PSG cells was significantly lower than that of the control ($P = 0.0058$), but no significant difference was obtained with the MSG cells ($P = 0.3038$) (Fig. 3C). Furthermore, the PSGs of the *Bmsage* RNAi individuals were disordered, no longer neatly arranged side-by-side, and with loose nuclei as in the control

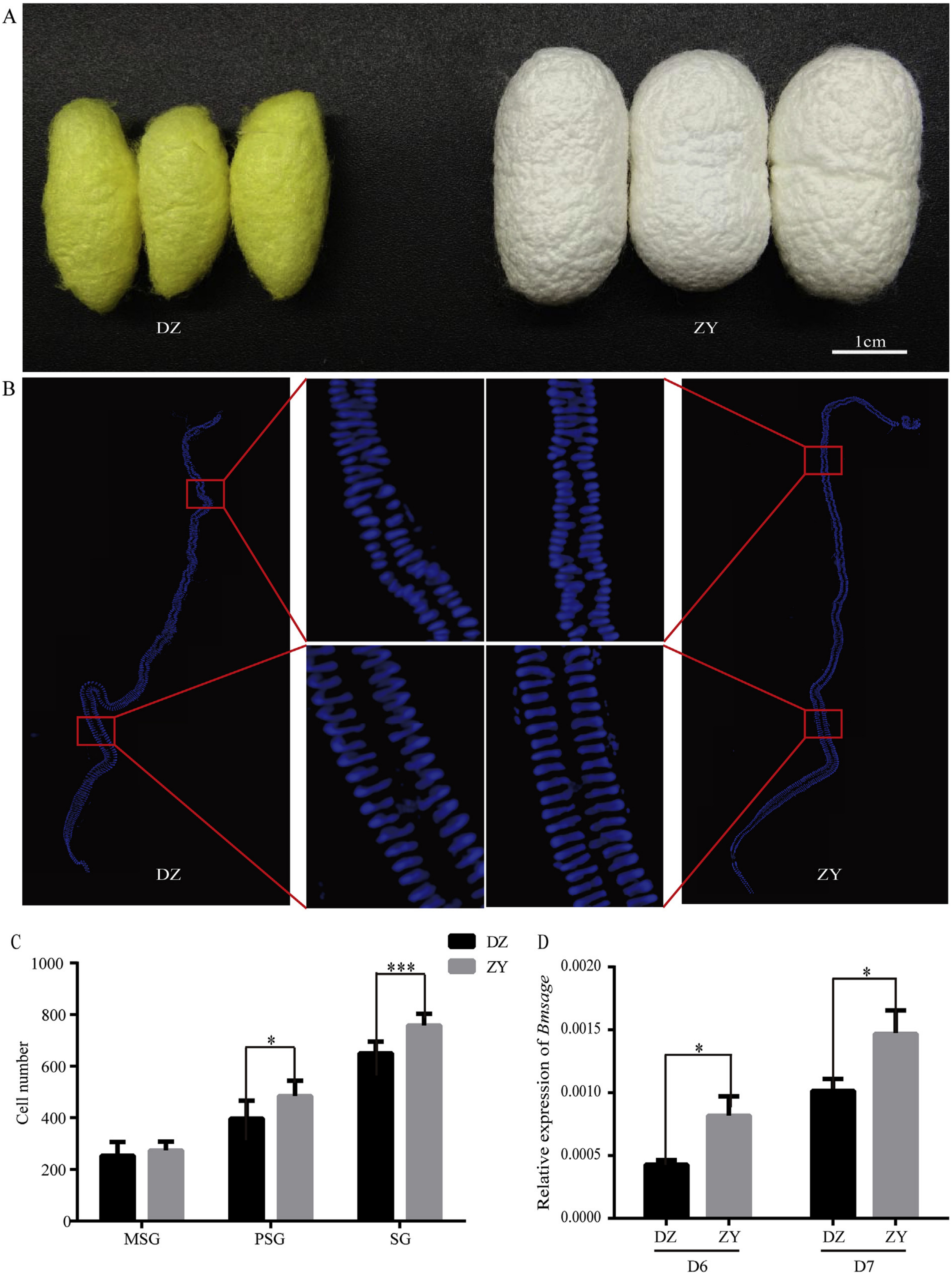

**Fig. 2. Expression of *Bmsage* and silk gland cell number in strains ZY and DZ**. A. Comparison of the cocoons of the high silk-yielding strain ZY and the low silk-yielding strain DZ. B. Comparison of the silk glands of ZY and DZ and comparison of cell arrangement in MSG and PSG. C. Comparison of the number of ZY and DZ silk gland cells. SG means silk gland. D. Comparison of the expression of *Bmsage* between the sixth and seventh day embryos of ZY and DZ.

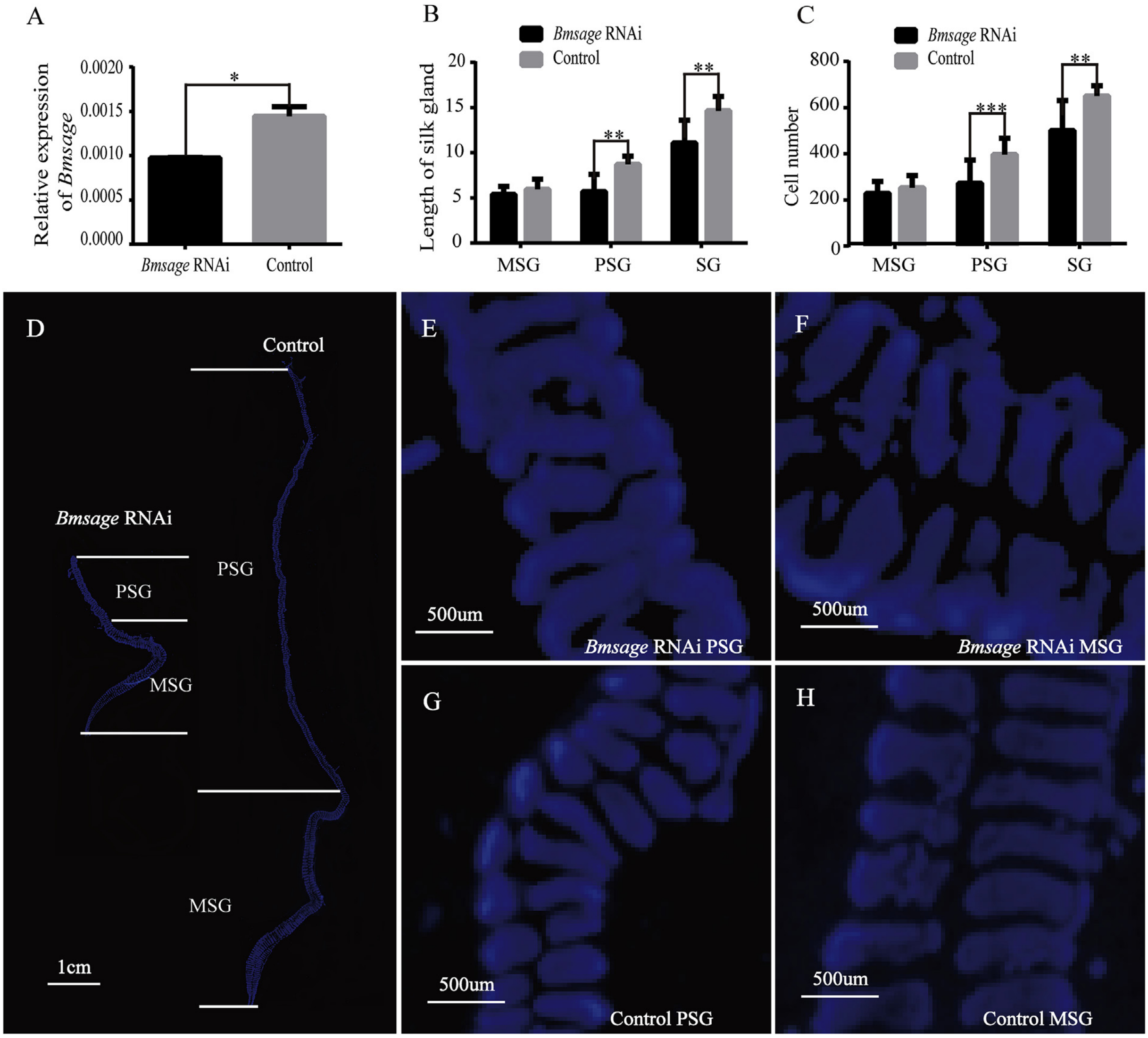

**Fig. 3. Phenotype of silk gland at the third instar of *Bmsage* RNAi individuals**. *Bmsage* and *EGFP* dsRNA were injected at the embryonic stage. The silk glands of the third-instar larvae were observed. A. Relative expression of *Bmsage* in dsRNA-injected seventh-day embryos. Error bars indicate SEM, student's *t*-test, *$P < 0.05$. B. Statistics of the length of 15 RNAi and control silk glands **$0.01 < P < 0.05$. C. Statistics of the cell numbers of 15 RNAi and control silk glands **$0.01 < P < 0.05$, ***$0.001 < P < 0.01$. D. Morphological comparison of *Bmsage* RNAi and control RNAi silk gland. E and G. Comparison of cell arrangement between *Bmsage* RNAi and control RNAi PSG. F and H. Comparison of cell arrangement between *Bmsage* RNAi and control RNAi MSG.

individuals (Fig. 3E and G). However, there were no obvious differences in these parameters in the MSG (Fig. 3F and H). The silk protein-related mRNA, *fibroin-H*, *fibroin-L*, *P25*, and *SGF1*, were detected by RT-qPCR, and no changes were found (Fig. S2). These results show that knock down of *Bmsage* decreased the number of cells and changed the morphology of the silk gland, especially in the PSG, suggesting that *Bmsage* regulates cell division.

### 3.4. *Bmsage RNAi affects silk synthesis and silk yield*

To further observe the effect of *Bmsage* RNAi on the silk gland of fifth instar larvae, the silk gland and silk yield were investigated. *Bmsage* RNAi silk glands were still shorter than those of the control at the fifth instar, including in the MSG and PSG (Fig. 4A). At the same time, an abnormal morphology of the PSG was present, appearing as a knob at the corner (Fig. 4B and C). The secretion of fibroin in the lumen was affected, and it appeared to be loosely packed (Fig. 4D and E). The cells were arranged unevenly, and were absent at some locations (Fig. 4F and G). Moreover, the expression levels of *fibroin-H* ($P = 0.0331$) was affected in the silk gland after *Bmsage* knockdown by RNAi in the embryonic stage (Fig. 4I). However, *Bmsage* expression had returned to normal by this time (Fig. 4H), indicating that these phenomena were caused by embryonic RNAi, and they could not recover during postnatal development. After the cocoons were formed, the silk yield was measured. It was observed that the cocoons with *Bmsage* RNAi were smaller and thinner than those with control RNAi, and their green color had nearly faded (Fig. 4K and L). The cocoon layer ratio also declined significantly (Fig. 4M). These results indicate that *Bmsage* knockdown in the embryonic period resulted in lower silk protein synthesis in the fifth instar.

### 3.5. *Bmsage affects development of the silk gland by affecting expression of cyclin genes*

In *D. melanogaster*, *Dmsage* controls the cell number of the salivary gland by inhibiting the apoptotic gene *reaper* (Chandrasekaran and Beckendorf, 2003). However, there is no homolog of *reaper* in the silkworm genome. Therefore we measured other apoptosis-related pathway genes. The results indicated that, although *senseless* was down-regulated (Fig. S3), no significant change was detected in the expression

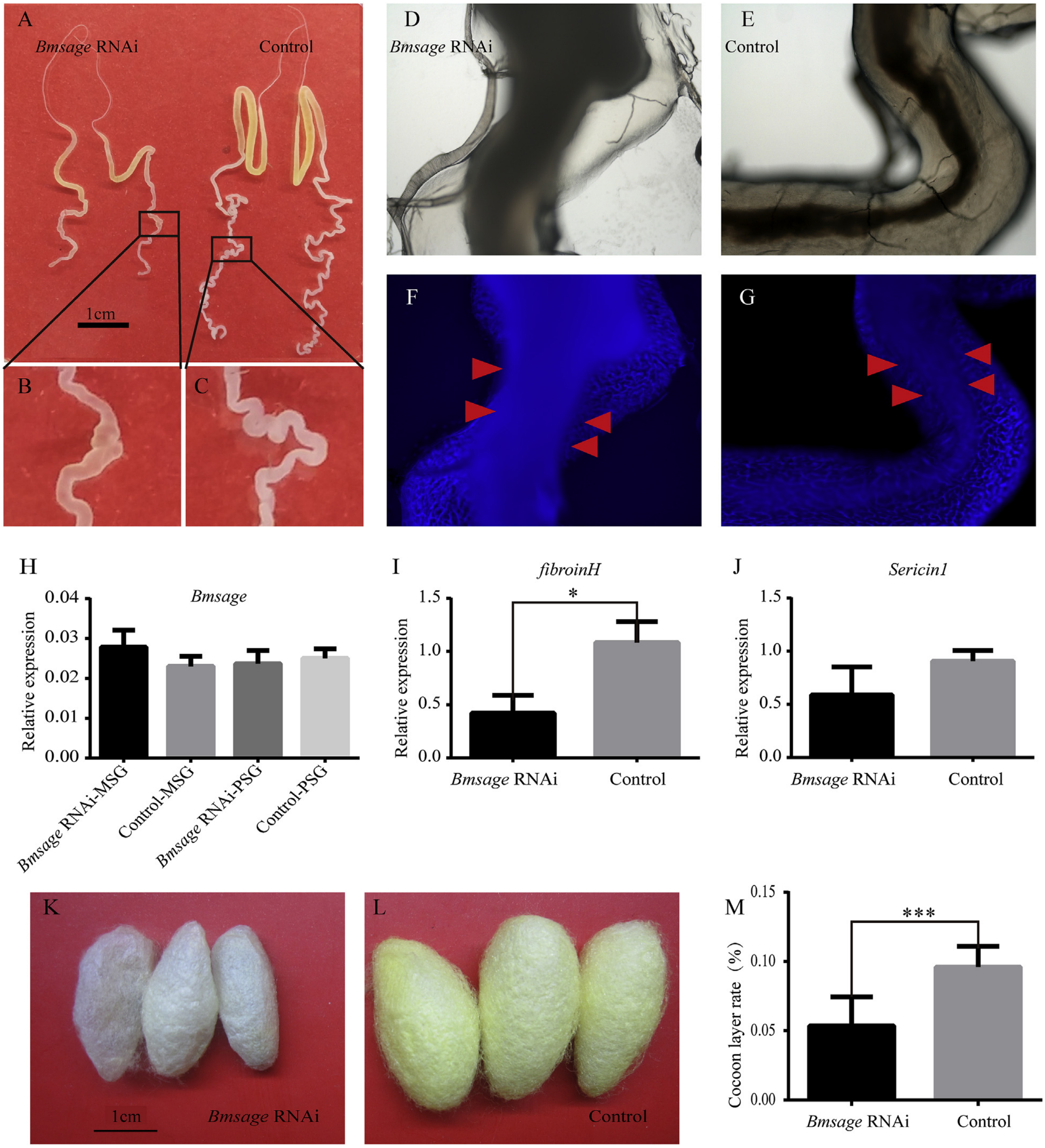

**Fig. 4. Phenotype of silk gland at the fifth instar and cocoon of *Bmsage* RNAi.** A. Fifth-instar larva RNAi and control RNAi silk glands. B and C. Distortion of the PSG. D-G. RNAi defects in the inner cavities of the silk gland. Arrow indicates that cells are missing. H. Relative expression of *Bmsage* in the MSG and PSG of *Bmsage* RNAi and control RNAi fifth-instar larvae. I and J. Relative expression levels of *fibroin-H* and *sericin-1*. K and L. Phenotype of *Bmsage* RNAi and control RNAi cocoons. M. Comparison of cocoon layer ratio between *Bmsage* RNAi and control RNAi.

levels of multiple genes related to apoptosis and autophagy pathways (Fig. S4). *PH4αSG* expression levels also remained unchanged in our *Bmsage* RNAi individuals (Fig. S3). Therefore, we believe that *Dmsage* regulates salivary gland cell number in *D. melanogaster* and *Bmsage* regulates silk glands in silkworms by different pathways. As the development of silk glands in embryos mainly involves mitosis of silk gland cells, we investigated the expression of cyclin family genes. Our results showed that, in *Bmsage* RNAi individuals, various cyclins were down-regulated, such as *cyclin B* (P = 0.02569) and *cyclin 3* (P = 0.02593), resulting in significant downregulation (Fig. 5). The results suggest that *Bmsage* regulates the development of the silk gland via cyclin pathways.

## 4. Discussion

The silkworm is an important lepidopteran model insect, which is also economically important. Research on development of the silk gland not only helps in revealing the developmental mechanisms in insects but also is of great economic value to the silkworm industry. However, studies on the molecular mechanism of silk gland development are few and difficult to perform. In the present study, we found that *Bmsage* could regulate the number of silk gland cells at the embryonic stage

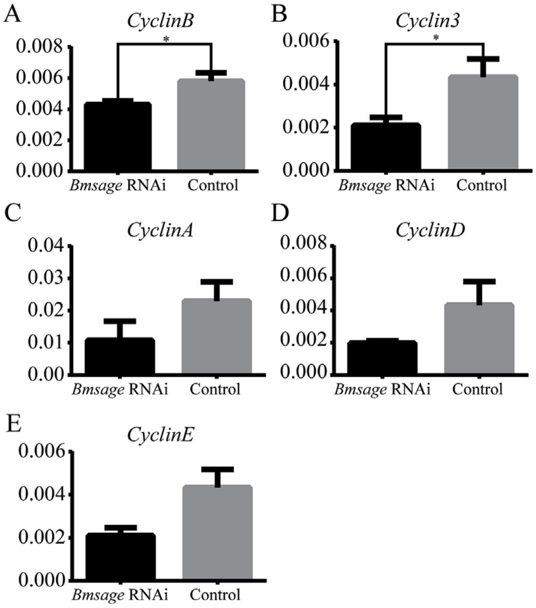

**Fig. 5. Changes in the expression levels of cyclin-related genes in *Bmsage* RNAi individuals at sixth day in embryos**. The following cyclin genes were selected: *cyclinB* (A), *cyclin3* (B), *cyclinA* (C), *cyclinD* (D), and *cyclinE* (E). The silkworm housekeeping gene ribosomal protein L3 (*BmRpl3*) was used as a control. Results are expressed as means ± SD of three independent experiments. Statistically significant differences were evaluated using Student's *t*-test for unpaired samples (*P < 0.05).

through the cyclin pathway, and that *Bmsage* could potentially increase silk production by increasing the number of silk gland cells. It is also worth mentioning that, to the best of our knowledge, *Bmsage* is the first gene reported to be involved in the regulation of the number of silk gland cells.

The salivary glands of *D. melanogaster* and the silk glands of silkworms are believed to be homologous organs. Salivary gland development in *D. melanogaster* is a good reference to understand silk gland development. In these two tissues, *sage* knockdown yielded similar phenotypes, with smaller tissues and warped lumina. In particular, the reduced size of the tissue was caused by missing cells, because they were not produced by cell division or they lost by apoptosis or autophagy. In *D. melanogaster*, *Dmsage* inhibits cell apoptosis by inhibiting *reaper* (Chandrasekaran and Beckendorf, 2003). However, there is no known equivalent of *reaper* in the silkworm genome. The expression of autophagy-related (*ATG8*) and apoptosis-related (*caspase-1*, *caspase-4*, *dredd*, *dronc*, *ICE*) genes, which could decrease cell numbers, was not changed in *Bmsage* RNAi silkworms. Therefore, we believe that apoptosis is not the cause of cell number reduction. Another gene reported in *D. melanogaster* is *PH4αSG1*, which caused abnormalities in the salivary gland cavity when it was mutated. However, the expression levels of *PH4αSG* did not change in *Bmsage* RNAi individuals. More importantly, knockdown of *Bmsage* did not distort the silk gland lumen as severely as in the salivary glands of *D. melanogaster* (Abrams et al., 2006), and the distortion occurred mainly at the locations where the cells were missing (Fig. 4). We speculate that this is caused by the abnormal arrangement of cells. On the basis of these results, we believe that *Bmsage* exerts its biological function via another regulatory mechanism (Fig. 6).

*Bmsage* is expressed in both MSG and PSG. However, knockdown of *Bmsage* led to a significant decline only in the cells in the PSG. We believe that the MSG was also affected, although not as significantly as the PSG. This could be attributed to the cascading effect of RNAi (Mann et al., 2008; Sioud, 2010). The MSG develops early, during which *Bmsage* is not downregulated much, whereas the PSG develops late, during which RNAi signals are amplified. This resulted in an insignificant decrease in the number of cells in the MSG and a significant decrease in the cell number in the PSG. This is also supported by the fact that the MSG are almost absent in the *Bmsage* knockout individuals (Xin et al., 2015).

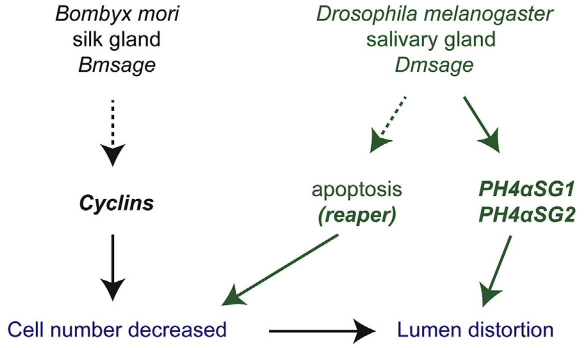

**Fig. 6. Model depicting the functional differences of *sage* in *Bombyx mori* and *Drosophila melanogaster***. Green represents how *sage* functions in *Drosophila*. Black represents how *sage* functions in silkworm. Blue represents the corresponding phenotype. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

During the cell cycle, cyclins regulate the activity of cyclin-dependent kinases (CDKs) through periodic changes and promote cell cycle progression, and this process is highly conserved in biology (Murray, 2004). Cyclin A-cdk2 is mainly concentrated in the replication region of the nucleolus (Cardoso et al., 1993) and is necessary for DNA replication (Labbe et al., 1989). *Cyclin D* is a key gene for cell cycle entry from the resting phase (Zhou et al., 1995). *Cyclin E* is necessary for cells to enter S phase (Ohtsubo et al., 1995). *Cyclin B* is mainly expressed at the end of G2, and it is a key protein for cells to enter division and is necessary for cell proliferation (King et al., 1995). More importantly, previous studies have shown that the expression pattern of *cyclin B* is consistent with mitosis of silk gland cells (Dhawan and Gopinathan, 2003). In the whole silkworm genome, there are only two cyclin B family proteins, cyclin B and cyclin 3 (cyclin B3). They are important regulators of the G2 to M phase transition of the cell cycle in silkworm (Pan et al., 2013). In the present study, in *Bmsage* RNAi individuals, *cyclin B* and *cyclin 3* were significantly down-regulated, and all cyclins were down-regulated to varying degrees. This suggests that *Bmsage* is involved in the mitotic process in silk gland cells (Figs. 5 and 6). Therefore, we believe that the decrease in silk gland cell number is caused by the down-regulation of genes associated with the cyclin pathway.

After *Bmsage* knockdown at the embryonic stage, the cocoons produced by the larvae became smaller and lighter (Fig. 4), and *fibroin-H* was significantly reduced (Fig. 4I). These results indicate that the reduced number of silk gland cells affected the production of silk, and the synthesis of silk protein by silk gland cells was affected. At the fifth instar, the silk glands need to synthesize a large amount of silk protein, and at this time, *Bmsage* mainly interacts with *SGF1* to regulate the transcription of *fibroin-H* (Zhao et al., 2014). However, at the embryonic stage, *sericin-1*, *fibroin-H*, *fibroin-L*, and *P25*, the main silk protein genes, were all unchanged in *Bmsage* RNAi individuals (Fig. S2), which indicates that *Bmsage* may play different roles in different developmental stages of the silkworm.

Silkworms are artificially selected continuously in the process of long-term rearing by human beings. The silk gland is one of the most remarkable organs associated with selective evolution. In previous studies, *Bmsage* was predicted to be a gene subject to evolution by artificial selection (Xia et al., 2009). By comparing DZ and ZY, we found significantly higher number of cells in the silk gland of the high-silk yield strain than of the low silk-yield strain. This is probably the result of domestication.

Improving silk production of the silkworm is a subject of continuous interest for the silkworm mulberry industry. Current studies focus on the transcriptional regulation of silk proteins, and many transcription factors involved in this process have been reported, such as *dimm* (Zhou et al., 2016) and *SGF1* (Zhao et al., 2014). However, silk gland development also has an important effect on silk yield. The silk gland is larger in high silk-yield strains than in low silk-yield strains (Belles, 2011). Previous studies have indicated that overexpression of *Ras1* or *Yorkie* could result in larger silk glands and higher cocoon yields (Ma et al., 2011; Zhang et al., 2017). The size of the silk gland is determined by cell size and cell number. Although we did not obtain transgenic individuals overexpressing *Bmsage*, considering the comparison between the strains ZY and DZ, we believe that *Bmsage* is likely to increase cell numbers in the silk gland. In addition, *Bmsage* determines the number of silk gland cells by controlling cyclins rather than apoptosis, also indicating that it has the potential to increase the number of silk gland cells.

## Footnotes

The abbreviations used are as follow: SG, silk gland; ASG, anterior silk gland; MSG, middle silk gland; PSG, posterior silk gland; ZY, ZhongYe; DZ, DaZao.

## Conflicts of interest

The authors declare that they have no conflicts of interest.

## Author contributions

CL conceived and designed the study. CL, SH, and YS performed the experiments. YW offered the ZY silkworm strain. CL, SH, and YS wrote the manuscript. CL and TC revised the manuscript.

## Acknowledgment

We thank Feng Wang (Biotechnology College, Southwest University, Chongqing, China) for microinjection assistance and Marian R. Goldsmith for the suggestions on writing manuscript. This study was supported by grants from the National Natural Science Foundation of China (Grant No. 31972618), Chongqing Research Program of Basic Research and Frontier Technology (Grant No. CSTC2018JCYJAX0396; CSTC2017JCYJAX0090), and the Fundamental Research Funds for the Central Universities of China (Grant No. XDJK2018AB001).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103205.

## References

Abrams, E.W., Mihoulides, W.K., Andrew, D.J., 2006. Fork head and Sage maintain a uniform and patent salivary gland lumen through regulation of two downstream target genes, PH4alphaSG1 and PH4alphaSG2. Development 133, 3517–3527.

Alberts, B., 2002. Molecular Biology of the Cell, fourth ed. Garland Science, New York.

Andrew, D.J., Horner, M.A., Petitt, M.G., Smolik, S.M., Scott, M.P., 1994. Setting limits on homeotic gene function: restraint of Sex combs reduced activity by teashirt and other homeotic genes. EMBO J. 13, 1132–1144.

Asakura, T., Umemura, K., Nakazawa, Y., Hirose, H., Higham, J., Knight, D., 2007. Some observations on the structure and function of the spinning apparatus in the silkworm Bombyx mori. Biomacromolecules 8, 175–181.

Ashburner, M., Novitski, E., 1976. The Genetics and Biology of Drosophila. Academic Press, London ; New York.

Belles, X., 2011. When inordinate tissue growth is beneficial: improving silk production by increasing silk gland size. Cell Res. 21, 862–863.

Cardoso, M.C., Leonhardt, H., Nadal-Ginard, B., 1993. Reversal of terminal differentiation and control of DNA replication: cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication. Cell 74, 979–992.

Chandrasekaran, V., Beckendorf, S.K., 2003. Senseless is necessary for the survival of embryonic salivary glands in Drosophila. Development 130, 4719–4728.

Dhawan, S., Gopinathan, K.P., 2003. Cell cycle events during the development of the silk glands in the mulberry silkworm Bombyx mori. Dev. Gene. Evol. 213, 435–444.

Fox, R.M., Vaishnavi, A., Maruyama, R., Andrew, D.J., 2013. Organ-specific gene expression: the bHLH protein Sage provides tissue specificity to Drosophila FoxA. Development 140, 2160–2171.

Gage, L.P., 1974. Polyploidization of the silk gland of Bombyx mori. J. Mol. Biol. 86, 97–108.

Inoue, S., Tanaka, K., Arisaka, F., Kimura, S., Ohtomo, K., Mizuno, S., 2000. Silk fibroin of Bombyx mori is secreted, assembling a high molecular mass elementary unit consisting of H-chain, L-chain, and P25, with a 6:6:1 molar ratio. J. Biol. Chem. 275, 40517–40528.

King, R.W., Peters, J.M., Tugendreich, S., Rolfe, M., Hieter, P., Kirschner, M.W., 1995. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell 81, 279–288.

Labbe, J.C., Picard, A., Peaucellier, G., Cavadore, J.C., Nurse, P., Doree, M., 1989. Purification of MPF from starfish: identification as the H1 histone kinase p34cdc2 and a possible mechanism for its periodic activation. Cell 57, 253–263.

Liu, Q.X., Ueda, H., Hirose, S., 2000. MBF2 is a tissue- and stage-specific coactivator that Is regulated at the step of nuclear transport in the silkworm Bombyx mori. Dev. Biol. 225, 437–446.

Ma, L., Xu, H., Zhu, J., Ma, S., Liu, Y., Jiang, R.J., Xia, Q., Li, S., 2011. Ras1(CA) overexpression in the posterior silk gland improves silk yield. Cell Res. 21, 934–943.

Mann, D.G., McKnight, T.E., McPherson, J.T., Hoyt, P.R., Melechko, A.V., Simpson, M.L., Sayler, G.S., 2008. Inducible RNA interference-mediated gene silencing using nanostructured gene delivery arrays. ACS Nano 2, 69–76.

Martin, C.H., Mayeda, C.A., Meyerowitz, E.M., 1988. Evolution and expression of the Sgs-3 glue gene of Drosophila. J. Mol. Biol. 201, 273–287.

Matsunami, K., Kokubo, H., Ohno, K., Xu, P., Ueno, K., Suzuki, Y., 1999. Embryonic silk gland development in Bombyx: molecular cloning and expression of the Bombyx trachealess gene. Dev. Gene. Evol. 209, 507–514.

Mazzarello, P., 1999. A unifying concept: the history of cell theory. Nat. Cell Biol. 1, E13–E15.

Murray, A.W., 2004. Recycling the cell cycle: cyclins revisited. Cell 116, 221–234.

Nagata, T., Suzuki, Y., Ueno, K., Kokubo, H., Xu, X., Hui, C., Hara, W., Fukuta, M., 1996. Developmental expression of the Bombyx Antennapedia homologue and homeotic changes in the Nc mutant. Genes Cells 1, 555–568.

Niranjanakumari, S., Gopinathan, K.P., 1991. Characterisation of the DNA-polymerase-alpha-primase complex from the silk glands of Bombyx mori. Eur. J. Biochem. 201, 431–438.

Niranjanakumari, S., Gopinathan, K.P., 1992. DNA polymerase-delta from the silk glands of Bombyx mori. J. Biol. Chem. 267, 17531–17539.

Niranjanakumari, S., Gopinathan, K.P., 1993. Isolation and characterization of DNA polymerase epsilon from the silk glands of Bombyx mori. J. Biol. Chem. 268, 15557–15564.

Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M., Pagano, M., 1995. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol. Cell. Biol. 15, 2612–2624.

Pan, M., Hong, K., Chen, X., Pan, C., Chen, X., Kuang, X., Lu, C., 2013. BmCyclin B and BmCyclin B3 are required for cell cycle progression in the silkworm, Bombyx mori. Sci. China Life Sci. 56, 360–365.

Panzer, S., Weigel, D., Beckendorf, S.K., 1992. Organogenesis in Drosophila melanogaster: embryonic salivary gland determination is controlled by homeotic and dorsoventral patterning genes. Development 114, 49–57.

Patel, C.V., Gopinathan, K.P., 1991. Development stage-specific expression of fibroin in the silk worm Bombyx mori is regulated translationally. Indian J. Biochem. Biophys. 28, 521–530.

Perdrix-Gillot, S., 1979. DNA synthesis and endomitoses in the giant nuclei of the silkgland of Bombyx mori. Biochimie 61, 171–204.

Sioud, M., 2010. RNA Therapeutics : Function, Design, and Delivery. Humana Press, New York.

Sprague, K.U., 1975. The Bombyx mori silk proteins: characterization of large polypeptides. Biochemistry 14, 925–931.

Suzuki, Y., 1977. Differentiation of the silk gland. A model system for the study of differential gene action. Results Probl. Cell Differ. 8, 1–44.

Xia, Q., Guo, Y., Zhang, Z., Li, D., Xuan, Z., Li, Z., Dai, F., Li, Y., Cheng, D., Li, R., Cheng, T., Jiang, T., Becquet, C., Xu, X., Liu, C., Zha, X., Fan, W., Lin, Y., Shen, Y., Jiang, L., Jensen, J., Hellmann, I., Tang, S., Zhao, P., Xu, H., Yu, C., Zhang, G., Li, J., Cao, J., Liu, S., He, N., Zhou, Y., Liu, H., Zhao, J., Ye, C., Du, Z., Pan, G., Zhao, A., Shao, H., Zeng, W., Wu, P., Li, C., Pan, M., Li, J., Yin, X., Li, D., Wang, J., Zheng, H., Wang, W., Zhang, X., Li, S., Yang, H., Lu, C., Nielsen, R., Zhou, Z., Wang, J., Xiang, Z., Wang, J., 2009. Complete resequencing of 40 genomes reveals domestication events and genes in silkworm (Bombyx). Science 326, 433–436.

Xin, H.H., Zhang, D.P., Chen, R.T., Cai, Z.Z., Lu, Y., Liang, S., Miao, Y.G., 2015. Transcription factor bmsage plays a crucial role in silk gland generation in silkworm, Bombyx mori. Arch. Insect Biochem. Physiol. 90, 59–69.

Yamaguchi, K., Kikuchi, Y., Takagi, T., Kikuchi, A., Oyama, F., Shimura, K., Mizuno, S., 1989. Primary structure of the silk fibroin light chain determined by cDNA sequencing and peptide analysis. J. Mol. Biol. 210, 127–139.

Zhang, P., Liu, S., Song, H.S., Zhang, G., Jia, Q., Li, S., 2017. Yorkie(CA) overexpression in the posterior silk gland improves silk yield in Bombyx mori. J. Insect Physiol. 100, 93–99.

Zhao, X.M., Liu, C., Li, Q.Y., Hu, W.B., Zhou, M.T., Nie, H.Y., Zhang, Y.X., Peng, Z.C., Zhao, P., Xia, Q.Y., 2014. Basic helix-loop-helix transcription factor Bmsage is involved in regulation of fibroin H-chain gene via interaction with SGF1 in Bombyx mori. PLoS One 9 e94091.

Zhou, C., Zha, X., Shi, P., Wei, S., Wang, H., Zheng, R., Xia, Q., 2016. Multiprotein bridging factor 2 regulates the expression of the fibroin heavy chain gene by interacting with Bmdimmed in the silkworm Bombyx mori. Insect Mol. Biol. 25, 509–518.

Zhou, P., Jiang, W., Zhang, Y.J., Kahn, S.M., Schieren, I., Santella, R.M., Weinstein, I.B., 1995. Antisense to cyclin D1 inhibits growth and reverses the transformed phenotype of human esophageal cancer cells. Oncogene 11, 571–580.