Enhanced Myc Expression in Silkworm Silk Gland Promotes DNA Replication and Silk Production

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Abstract: Silkworm is an economically important insect that synthesizes silk proteins for silk production in the silkworm posterior silk gland (PSG), which was driven by the promoter of the fibroin heavy chain (FibH) gene, was performed for investigating the biological functions of Myc in silk gland. Enhanced Myc expression elevated the cocoon size. This elevation might be resulted from the increasing of FibH expression and DNA content in the PSG cells by promoting the transcription of the genes that are involved in DNA replication.

Keywords: silkworm; silk gland; Myc overexpression; DNA replication; silk production

1. Introduction

The silkworm (Bombyx mori) is an economically important insect that synthesizes silk proteins for silk production in the silk gland. The silk gland comprises three parts, namely, anterior (ASG), middle (MSG), and posterior (PSG). Cell numbers in the silk gland are determined by mitosis during the late embryonic stage [1]. During the larval stage, silk gland cells stop the mitotic cell cycle and enter into endoreplication. After approximately 17–19 rounds of endoreplicating cell cycles, also called the endocycle, the DNA content in Drosophila salivary gland and ovary, two tissues with an endoreplicating cell cycle, reveal
that blocking Fzr expression results in an arrest of DNA replication and the failure of mitotic-to-endocycle transition [8–10]. Notably, the initiation of DNA replication depends on the assembling of the pre-replication complex (preRC) on the origin of DNA replication [11,12]. The mini-chromosome maintenance proteins 2–7 (MCM2-7), which are identified as preRC subunits, form a hexameric complex during the G1 phase and functions as a DNA helicase to unwind genomic DNA bidirectionally during the S phase; then, they initiate DNA replication [12–14]. In silkworm silk gland, oncogene Ras(CA), insulin, andecdysone have been shown to be involved in DNA replication [15–17]. Undoubtedly, decoding endoreplication of silk gland cells should be helpful for better understanding silk gland growth and silk protein synthesis. Actually, PSG-specific overexpression of some growth-related regulators, such as Ras and Yorkie, can elevate silk protein genes transcription and silk production by promoting endoreplication progression and increasing DNA content in the PSG cells [15,18]. On the contrary, PSG-specific knockout of the LaminA/C gene, which is involved in maintaining the chromatin structure, causes a decrease in DNA content, silk protein gene transcriptions, and silk production [19].

Transcription factor Myc has been primarily identified as an oncogene in mammalian tumor cells and belongs to leucine zipper transcription factor family [20]. Previous reports in animals and plants have demonstrated that Myc is involved in regulating multiple physiological processes, such as cell proliferation and differentiation [21,22], cell growth [23], and cell self-renewal [24,25]. Enhanced Myc expression promotes tumorigenesis, while Myc deletion strongly inhibits cell activity and leads to proliferative arrest [22,26]. Increasing evidence demonstrated that Myc is involved in cell-cycle progression mainly by the initiating DNA replication and G1-S phase transition [27,28]. The observation in Drosophila salivary gland reveals that Myc heterozygous mutation induces continuous segregation of mitotic cells and prevents the entrance of endoreplication progression [29].

Previous reports in silkworm have demonstrated that silencing Myc expression in ovary-derived BmN4 cells causes an arrest in cell-cycle progression, and Myc is also involved in ecdysteroid regulation of cell-cycle progression in wing disc [30,31]. However, the function of Myc in silkworm silk gland with endoreplicating cell cycle remains unclear. In the present study, based on a transgenic approach, we used the promoter of the PSG-specific FibH gene to drive Myc overexpression in the PSG. PSG-specific overexpression of the Myc gene not only increased the size and DNA content of PSG cells but also elevated the weight and shell rate of cocoon. Mechanistically, in addition to silk protein gene FibH, Myc overexpression also upregulated the transcription of the MCM genes that are involved in DNA replication. These data suggest that enhanced Myc expression in silkworm silk gland promotes DNA replication and silk production.

2. Materials and Methods
2.1. Insect Strain

The silkworm strain D9L (non-diapause strain) was stocked in the State Key Laboratory of Silkworm Genome Biology of Southwest University and reared with fresh mulberry leaves at 25 °C using a biochemical incubator with a cycle of 12 h light/12 h dark. The non-diapause eggs that were generated from D9L adults were used for germ-line transformation. Following the co-injection of recombinant Myc overexpression plasmid with helper DNA, the eggs were cultured at 25 °C with a humidity of 95–100% until hatching.

2.2. Construction of Recombinant Plasmid

For the construction of recombinant Myc overexpression plasmid pBac (3×P3-EGFP-SV40; FibH-Myc-SV40) (FibH-Myc), the opening reading frame of the silkworm Myc gene, FibH promoter, and SV40 transcription terminator with specific restriction enzyme cutting sites were cloned into the T-simple vector. Following the digesting with corresponding restriction enzymes, the DNA fragments were successively subcloned into the pBac (3×P3-EGFP-SV40) empty vector to generate the FibH-Myc recombinant plasmid, in which PiggyBac was used as the transposable element and EGFP signal in the eyes was used for
positive screening. All primers used for construction of recombinant plasmid are listed in Supplementary Table S1.

2.3. DNA Quantification

For DNA content quantification, 10 silk glands from the early wandering stage silkworm larvae were dissected and subsequently lysed in DNA SDS lysis-phenol supplemented with proteinase K. Following the digestion with RNAase, total genomic DNA from the middle silk gland (internal control) and the posterior silk gland were purified, separately. The DNA content was quantified spectrophotometrically at OD 260 nm using an Agilent 2100 Bioanalyzer System (Agilent, Palo Alto, CA, USA).

2.4. Immunostaining

After washing with PBS, the silk glands from the early wandering stage silkworm larvae were fixed with 4% paraformaldehyde for 30 min and sliced with a thickness of 10 um using a freezing microtome. Then, the slices were stained with DAPI (1:1000, Life Technologies, Carlsbad, CA, USA) for 30 min. After washing with PBS for three times, the slices were mounted with coverslips and the fluorescence signals were captured using confocal microscope Zeiss LSM 880 (Carl Zeiss, Jena, Germany) with excitation wavelengths of 341 nm.

2.5. RNA Extraction and Quantitative Real-Time RT-PCR (RT-qPCR)

Total RNA was extracted from the MSGs and the PSGs at the third day of the last larval instar (L5D3) using Trizol reagent (Invitrogen, Carlsbad, CA, USA), as described previously [32], and used for synthesizing cDNA templates with the M-MLV Reverse Transcriptase Kit (Promega). RT-qPCR reaction systems were prepared with a SYBR Premix ExTaq Kit (Takara, Kyoto, Japan) and performed with a qTower 2.2 Real-time PCR Detection System (Analytik Jena Biometra, Jena, Germany). The silkworm ribosomal protein L3 (RpL3) gene was used as the internal control. The relative mRNA expression levels were calculated using the 2$^{-\Delta\Delta CT}$. All primers used for RT-qPCR are listed in Supplementary Table S1.

2.6. Statistical Methods

The values from three independent biological replicates are presented as the mean $\pm$ SE. Statistical significance ($p$-value) was calculated by spss t-test. For the significance test: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

3.1. Construction of Transgenic Silkworm with PSG-Specific Myc Overexpression

To understand the roles of the Myc gene in both silk gland development and silk production, we first constructed transgenic silkworm with PSG-specific Myc overexpression. Based on the full-length sequence of silkworm Myc gene, we cloned the opening reading frame of the Myc gene and constructed recombinant Myc overexpression plasmid driven by FibH promoter, which is specifically activated in the PSG (Figure 1A). In total, 90 non-diapause D9L embryos were microinjected with the FibH-Myc recombinant plasmid, and 72 embryos were allowed to survive to develop to adults. EGFP-positive eggs in G1 generation were screened as positive transgenic strains (Figure 1B,C) and the positive rate was about 5%. Besides, the PSG of transgenic silkworm was isolated to determine whether Myc was specifically overexpressed in the PSG. RT-qPCR analysis confirmed that compared to the control, Myc was highly expressed in the PSG of transgenic silkworm (Figure 1D). These results indicate that Myc was specifically overexpressed in the PSG of transgenic silkworm.
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that Ser1 (Figure S1) as well as MSG-specific sericin 1 (Ser1) gene (Figure 2E). These data suggest that Myc overexpression in the PSG improves silk yield.

3.2. PSG-Specific Myc Overexpression Improves Silk Yield

We next investigated the effects of PSG-specific Myc overexpression on silk yield. The results showed that the cocoons of female transgenic silkworm individuals with PSG-specific Myc overexpression were obviously bigger than that of wild-type silkworm, and the cocoons of male silkworm increased by a small amount compared to the wild-type silkworm (Figure 2A,A'). The cocoon shell rates were elevated by 25% and 22% in female and male transgenic silkworms, respectively (Figure 2B,B'). Further statistics analysis revealed that compared to the control, Myc overexpression led to an increase in the weight of cocoon shell (Figure 2C,C'). Moreover, the transcription of silk protein gene FibH overexpression were obviously bigger than that of wild-type silkworm, and the cocoons of male silkworm increased by a small amount compared to the wild-type silkworm (Figure 2A,A'). The cocoon shell rates were elevated by 25% and 22% in female and male transgenic silkworms, respectively (Figure 2B,B'). Further statistics analysis revealed that compared to the control, Myc overexpression led to an increase in the weight of cocoon shell (Figure 2C,C'). Moreover, the transcription of silk protein gene FibH increased by about 20% following Myc overexpression (Figure 2D), but Myc overexpression did not affect the transcriptions of PSG-specific fibroin light chain (FibL) and P25 genes (Figure S1) as well as MSG-specific sercin 1 (Ser1) gene (Figure 2E). These data suggest that Myc overexpression in the PSG improves silk yield.

Figure 1. Generation of transgenic silkworm with PSG-specific Myc overexpression. (A) Schematic illustration of the vector for Myc overexpression driven by the FibH promoter. (B,C) EGFP-positive eggs (B) and adults (C) were screened in G1 generation. (D) Myc was highly expressed in the PSG of transgenic silkworm. Values are represented as means ± S.E. (error bars). For the significance test: *** p < 0.001 vs. the control.

Figure 2. Enhanced Myc expression in the PSG elevates silk yield. (A,A') Cocoon size of female (A) and male (A') transgenic silkworms with Myc overexpression increased compared with control. Scale bar, 1 cm. (B,C') The cocoon shell rates (B,B') and cocoon weight (C,C') were both largely increased following PSG-specific Myc overexpression. (D,E) Myc overexpression in the PSG promoted the transcription of FibH (D) but had no effect on the transcription of Ser1 (E). Values are represented as means ± S.E. (error bars). For the significance test: ** p < 0.01, *** p < 0.001 vs. the control.
3.3. PSG-specific Myc Overexpression Promotes DNA Replication

To decipher the regulatory mechanism underlying Myc-mediated improvement of silk yield, we dissected the silk gland from the early wandering stage of silkworm larvae and found that the PSG of transgenic silkworm was larger than that of the control (Figure 3A,B). However, the MSG as a negative control had no change in size following PSG-specific Myc overexpression (Figure 3A). Importantly, total DNA content in the PSG of transgenic silkworm was increased by nearly 2.5 times following Myc overexpression (Figure 3C), indicating that PSG-specific Myc overexpression promotes DNA replication during endoreplication in the PSG cells, which subsequently facilitates PSG growth.

3.4. Myc Positively Regulates the Transcription of the MCM Genes Involving in DNA Replication

Given that enhanced Myc expression in the PSG cells promotes DNA replication, we further investigated whether Myc regulates the expression of the MCM genes, which are required for initiating DNA replication in both endocycling and mitotic cells [12]. RT-qPCR examination showed that three MCM genes, MCM5, MCM6, and MCM7, were significantly upregulated following Myc overexpression in the PSG (Figure 4A). As a negative control, PSG-specific Myc overexpression had no effect on the transcription of the MCM genes in the MSG (Figure 4B). Taken together, we proposed that Myc promotes the DNA replication in the endoreplicating silk gland cells by positively regulating the transcription of the MCM genes, which subsequently enhances the expression of silk protein gene and silk production.

4. Discussion

Silkworm is an economically important insect that produces silk fiber and silk proteins that are synthesized by the silk gland in which the cells undergo endoreplication. It has been demonstrated that the overexpression of Ras1(CA) and Yorkie in the silk gland improved silk yield by promoting DNA replication and increasing protein synthesis [15,18], while PSG-specific knockout of LaminA/C causes a decrease in DNA content, silk protein gene transcription, and silk yield [19]. DNA replication in silk gland cells can also be regulated by ecdysone and insulin [16,17]. Intriguingly, previous studies reported that ecdysone mediated DNA replication and cell proliferation in silkworm wing disc cells by positively regulated Myc transcription, and Myc is required for DNA replication and tissue growth in Drosophila endoreplicating tissues [10,29,30,33]. Accordingly, we here conducted a transgenic overexpression of the Myc gene in the PSG and observed that enhanced Myc expression promotes DNA replication and silk protein synthesis. These data indicate that
Myc plays conserved roles in regulating DNA replication and protein synthesis in different types of endoreplicating cells.

![Figure 4](image)

**Figure 4.** Myc positively regulates the transcription of the MCM genes. (A) RT-qPCR examination showed that PSG-specific Myc overexpression upregulated the transcription of the MCM genes in the PSG. (B) PSG-specific Myc overexpression had no effect on the transcription of the MCM genes in the MSG. Values are represented as means ± S.E. (error bars). For the significance test: * p < 0.05, ** p < 0.01 vs. control.

The members of the MCM family, MCM2-MCM7, interacted physically to form a hexameric complex and colocalized at assembled replication origins to initiate DNA synthesis in endoreplicating cells [12–14]. This hexameric helicase complex is essential for DNA replication by providing a platform for recruitment of other preRC subunits and bidirectionally unwinding genomic DNA [34,35]. Our previous study found that *Drosophila* Myc positively regulated the transcription of the MCM6 gene by directly binding to a specific motif within its promoter during endoreplication [10]. We here observed that Myc overexpression in silkworm PSG can upregulate the transcription of three members of the MCM family, MCM5, MCM6, and MCM7, which is most likely correlated with an increase in DNA content following Myc overexpression. Whether Myc can directly bind to the promoter of silkworm MCM genes needs further investigation.

High expression of silk proteins in silkworm silk gland is required for silk production. We found that enhanced Myc expression in the PSG elevates both the transcription of silk protein gene *FibH* and silk production. This elevation may be associated with a Myc overexpression-caused increase in DNA content. In addition, the transcription of the *FibH* gene can be regulated by other silk gland-specific transcription by its direct binding to the *FibH* promoter, including basic helix-loop-helix transcription factor Sage [36], nuclear hormone receptor FTZ-F1 [37], and fibroin modulator binding protein-1 [38]. It should
be necessary for elucidating whether Myc can directly regulate the transcription of silk protein genes.

5. Conclusions

In conclusion, we suggest that enhanced Myc expression in the PSG can promote silk yield by increasing DNA content and FibH transcription. This provides a novel target for improving silk production in silkworm breeding.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects12040361/s1, Table S1: The primers used in this study.

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