SCIENCE CHINA
Life Sciences

• RESEARCH PAPER •

April 2013 Vol.56 No.4: 360–365
doi: 10.1007/s11427-013-4459-3

BmCyclin B and *BmCyclin B3* are required for cell cycle progression in the silkworm, *Bombyx mori*

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Received August 14, 2012; accepted January 4, 2013; published online March 15, 2013

Cyclin B is an important regulator of the cell cycle G2 to M phase transition. The silkworm genomic database shows that there are two Cyclin B genes in the silkworm (*Bombyx mori*), *BmCyclin B* and *BmCyclin B3*. Using silkworm EST data, the *cyclin B3* (EU074796) gene was cloned. Its complete cDNA was 1665 bp with an ORF of 1536 bp derived from seven exons and six introns. The *BmCyclin B3* gene encodes 511 amino acids, and the predicted molecular weight is 57.8 kD with an isoelectric point of 9.18. The protein contains one protein damage box and two cyclin boxes. RNA interference-mediated reduction of *BmCyclin B* and *BmCyclin B3* expression induced cell cycle arrest in G2 or M phase in BmN-SWU1 cells, thus inhibiting cell proliferation. These results suggest that *BmCyclin B* and *BmCyclin B3* are necessary for completing the cell cycle in silkworm cells.

***Bombyx mori*, cell cycle, *BmCyclin B*, *BmCyclin B3*, cell proliferation**

Citation: Pan M H, Hong K L, Chen X Y, et al. *BmCyclin B* and *BmCyclin B3* are required for cell cycle progression in the silkworm, *Bombyx mori*. Sci China Life Sci, 2013, 56: 360−365, doi: 10.1007/s11427-013-4459-3

Cyclin B is a mitotic cyclin protein. It is expressed and gradually accumulates from the G1 late phase, reaches a maximum level at the G2 late phase, continues to be maintained to the middle of metaphase, and is then immediately degraded. The accumulation and degradation of cyclin B plays a critical role in mitosis and karyokinesis [1–3]. A cyclin box and conserved cdk-binding domains are present in all cyclins. A- and B-type cyclins possess a destruction box, a motif containing ~nine amino acids required to target cyclins to ubiquitin-dependent degradation during mitosis [4–6].

Cyclin B genes can be placed in two evolutionary branches, the B-type and B3-type cyclin families [7]. B-type cyclins are present in both yeast and higher eukaryotes. Although a single *Cyclin B* gene has been found in invertebrate species, a pair of very closely related B-type cyclins is present in vertebrates. These vertebrate cyclin B1 and B2 proteins differ in their subcellular localization [8,9]. The *Cyclin B3*-type family is conserved in metazoans and has a similarity of 39%–46% in the cyclin protein box [10–12].

Both cyclin B and cyclin B3 have a conserved amino acid sequence, which is known as the cell cyclin box. This domain can combine with CDKI to form complexes, which, when activated by CDK-activating kinase, drives cells from the G2 phase limit point to the M phase. The *Cyclin B* gene has previously been cloned from silkworm eggs [13]. The expression of *Cyclin B* was reduced in silkworm ovarian BmN cells, 24 h after infection by nucleopolyhedrovirus (BmNPV), and the cells then arrested in G2/M phase [14].

The silkworm undergoes complete metamorphosis, and is a good model organism of Lepidoptera. A full understanding of its cell-cycle regulation mechanism would improve

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its use in biological research. Using the *B. mori* ovarian cell line, BmN-SWU1, we cloned the *Cyclin B3* gene and analyzed the function of *BmCyclin B* and *BmCyclin B3* in the cell cycle.

1 Materials and methods

1.1 Cell lines and *B. mori* insects

The *B. mori* ovary cell lines BmN-SWU1 [15] and BmSG-SWU1 were cultured in Grace medium (Gibco) containing 10% fetal bovine serum (FBS) at 27°C. *B. mori* DaZao strain larvae were bred with fresh mulberry leaves at 25°C with a 12 h:12 h photoperiod.

1.2 Bioinformatic analysis

B. mori genomic information was obtained from a *B. mori* 9× genomic sequencing database, a *B. mori* EST database, and a predicted protein database (all available at http://silkworm.swu.edu.cn/silkdb/). The homolog sequences of *Cyclin B* (M33192) and *Cyclin B3* (AJ012568) from *Drosophila melanogaster* were obtained from GenBank (http://www.ncbi.nlm.nih.gov) and used as queries. The protein sequences of *Cyclin B3*-related genes were aligned by the BlastP program. Predicted silkworm genes were compared with the NCBI protein database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for further confirmation. The sequences of the conserved domains of the obtained gene were used as queries for BlastP searches against the silkworm predicted protein database and for TBlastN searches against the silkworm 9× genome sequence.

1.3 RNA extraction and cDNA preparation

Total RNA was isolated from BmN-SWU1 cells, BmSG-SWU1 cells and larval tissues (including silk glands and ovaries) on the third day of the fifth instar using Tripure isolation reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration of RNA was calculated by spectrophotometry (Gene Spec V; HITACHI, Japan). cDNA was synthesized from total RNA samples using a cDNA Synthesis Kit (Promega, USA) according to the manufacturer's instructions.

1.4 PCR amplification and cloning of *BmCyclin B* and *BmCyclin B3* genes

PCR primers for *BmCyclin B* were designed based on the coding sequences of the *BmCyclin B* genes [13]. The sequences of the primers were as follows: forward primer (5′-GGAGACAACAACAGCCCTT-3′) and reverse primer (5′-AAACAAAATCGCCAACATC-3′). The *BmCyclin B3* primers were designed based on the coding sequences of putative *BmCyclin B3* genes. The sequences of the primers were as follows: forward primer (5′-AAAAGTAGGTCCGTAATGGCG-3′) and reverse primer (5′-GATCTTGGCGACTAGGCTAACAG-3′). PCR amplification was performed in a total reaction volume of 25 μL containing normalized cDNA (6 μg cDNA per reaction), 1 μL of 15 pmol of each primer, 2 μL of 2 mmol L^{-1} $MgCl_2$, 1 μL of 0.25 mmol L^{-1} dNTP, 2.5 μL of 1× buffer, 2.5 U Taq DNA polymerase, and distilled deionized H_2O. PCR was performed as follows: initial denaturation at 94°C for 5 min, followed by 25 cycles of 50 s at 94°C, 40 s annealing at 55°C, 90 s extension at 72°C, and a final extension at 72°C for 10 min. The amplification products were analyzed on 1% agarose gels. The DNA bands were extracted using a Qiaex II gel extraction kit (Qiagen, USA) and subcloned into the pGEM-T easy vector (Promega, USA). The cloned insert was sequenced on an ABI PRISM™ 310 Genetic Analyzer (PE Applied Biosystems, USA).

1.5 Sequence analysis of *BmCyclin B3*

The exon and intron structures of BmCyclin B3 were predicted by Sim4 (http://gamay.univ-perp.fr/analyse_seq/sim4). The molecular weight, isoelectric point and domains of BmCyclin B3 were predicted by Expasy (http://expasy.org/cgi-bin). Using ClustalW, the BmCyclin B3 protein sequence was aligned with cyclin B sequences from different species available in GenBank. These sequences included *B. mori*, *D. melanogaster*, *Gallus gallus* and *Caenorhabditis elegans*. A neighbor-joining (NJ) phylogenetic tree of Cyclin B protein sequences from different species, including *B. mori*, *D. melanogaster*, *G. gallus*, *C. elegans*, *Homo sapiens*, *Mus musculus* and *Xenopus laevis*, was constructed in MEGA4.0 [16].

1.6 *BmCyclin B* and *BmCyclin B3* expression analysis

mRNA levels of *BmCyclin B3* and *BmCyclin B* were detected on the third day of the fifth instar using silkworm microarray data (http://www.silkdb.org/microarray/Bmarray.php). The expression of *BmCyclin B* (P1: 5′-GGAGACAACAACAGCCCTT-3′; P2: 5′-AAACAAAATCGCCAACATC-3′), *BmCyclin B3* (P1: 5′-AAGACCCACCCAAGCAAGT-3′; P2: 5′-TCGTCAA TAGGGAAAAGGC-3′), *BmCyclin A* (P1: 5′-ACCCTCAGTAATAGCAGCGT-3′; P2: 5′-GCCTTCAAACTTGTTGCTCT-3′), *BmPCNA* (P1: 5′-AAAGTTCTCGGCAACAGGC-3′; P2: 5′-CGCCAGGTAGTAGCGGATGT-3′) and *Bmaction3* (P1: 5′-AACACCCCGTCCTGCTCACTG-3′; P2: 5′-GGGCGAGACGTGTGATTTCCT-3′) were detected by RT-PCR using a PCR kit (TaKaRa, China). RT-PCR was performed using P1 and P2 primer pairs with 300 ng cDNA template from larval tissues on the third day of the fifth instar as described above. RT-PCR was conducted under the following conditions: denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 90 s, and a final

extension at 72°C for 10 min. The silkworm *actin-3* gene was used as an internal control. Products were visualized on a 1% agarose gel stained with ethidium bromide and detected on a Gel Doc 2000 (BIO-RAD, USA).

1.7 Double-stranded RNA preparation

Double-stranded RNA (dsRNA) was synthesized using a Ribomax™ Large Scale RNA Production System-T7 and a Ribomax™ Large Scale RNA Production System-SP6 (Promega) based on *BmCyclin B* (P1: 5′-GGAGACAACAACAGCCCTT-3′; P2: 5′-AAACAAAATCGCCAACATC-3′) and *BmCyclin B3* (P1: 5′-AAGACCCACCCAAGCAAGT-3′; P2: 5′-TCGTCAATAGGGAAAAGGC-3′). The sense and antisense RNA was mixed, digested, and precipitated to obtain dsRNA.

1.8 RNA interference (RNAi)

FuGENE^{R}HD Transfection Reagent (Roche, USA) was used for transfection following the manufacturer's instructions. The dsRNA was diluted to 2 μg/100 mL with dilution reagent, then FuGENE^{R}HD Transfection Reagent was added to form a transfection complex. The mixture was incubated at room temperature for 45 min and then added to cells. Transfected cells were collected and divided into five groups: control 1 (normal cells), control 2 (transfection reagent alone, 18 μL of transfection reagent), *BmCyclin B* (18 μL transfection reagent+3 μL *BmCyclin B* dsRNA), *BmCyclin B3* (18 μL transfection reagent+3 μL *BmCyclin B3* dsRNA), *BmCyclin B/B3* (18 μL transfection reagent+3 μL *BmCyclin B* dsRNA+3 μL *BmCyclin B3* dsRNA cotransfection), with triplicate samples prepared for each group. The transfected cells were collected and analyzed 48 h after transfection.

1.9 Cell cycle analysis

Cells from each group were collected and washed twice with PBS. The supernatant was discarded and 1 mL 70% cold ethanol was added. The cells were gently mixed by pipetting up and down, fixed for at least 12 h at 4°C, and then washed with PBS to remove the ethanol. The cells were centrifuged for 5 min at 1000 r min^{-1} and the washing procedure was repeated. Ten mg mL^{-1} RNase A was then added to a final concentration of 100 μg mL^{-1} and the cells were left to stand at room temperature for 30 min. Propidium iodide (PI) was added to a final concentration of 10 μg mL^{-1} and the cells were incubated for 30 min at 4°C while protected from light. The cell cycle was analyzed by flow cytometry (BD FACSAria, USA).

1.10 MTT test

The cells were cultured in normal conditions. Two hundred μL of 0.5% MTT (Sigma, USA) was added per well and the cells were cultured for an additional 4 h at 28°C. The MTT solution was then removed, 200 μL DMSO was added to each well and the plates were shaken at low speed for 10 min. Absorbance (*A* value) was then detected at a wavelength of 492 nm using an automatic microplate reader (iMark, Bio-Rad, USA). Each group was analyzed in triplicate. The cell survival rate was calculated based on the *A* value, where the cell survival rate (%)=(experimental group *A* value/control group *A* value)×100.

2 Results

2.1 Cloning and sequence analysis of the silkworm *Cyclin B3* gene

The silkworm *Cyclin B3* gene contains seven exons and six introns with a coding sequence of 1536 bp (GenBank accession number: EU074796), encoding a 511 amino acid (aa) protein. Online SMART program analysis showed that cyclin B3 contains two cyclin boxes, located at 288–379 aa and at 392–473 aa, respectively. It also possesses a 9 aa cyclin destruction box (D-box), RAAFGDITN (consensus sequence RxxFGxxxN), at the N-terminal, located between 75 and 84 aa.

BLAST alignment of cyclin B3 amino acid sequences demonstrated that silkworm cyclin B3 had the highest protein identities with *D. melanogaster* (CAA10059), *H. sapiens* (CAC40024), *X. laevis* (XP001660237), *M. musculus* (CAD88195), *G. domesticus* (NP990570) and *C. elegans* (NP001024112). The protein sequence similarities were 44%, 50%, 43%, 46%, 51% and 42%, respectively. Multiple sequence alignment analysis by ClustalW demonstrated that the conserved cyclin destruction box of *B. mori* cyclin B3 is similar to that in other species (Figure 1).

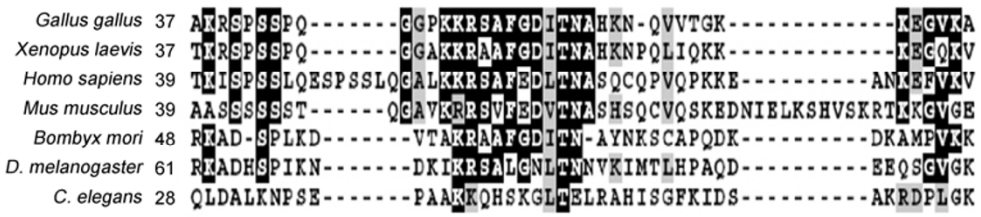

Figure 1 Destruction-box of Cyclin B3 in seven species. Black shading indicates identical amino acids, and gray shading indicates similar amino acids.

The reconstructed phylogenetic tree was clearly divided into two major groups, namely B-type cyclins and B3-type cyclins. The invertebrates and vertebrates were respectively grouped together, while *C. elegans* was the most primitive clade (Figure 2).

2.2 Expression of *cyclin B* and *cyclin B3* in tissues and cell lines of *B. mori*

Based on the silkworm microarray expression database of the third day of the fifth instar, the expression of *cyclin B3* and *cyclin B* in different tissues was analyzed. *Cyclin B3* and *cyclin B* are expressed at high levels in larval ovaries and testes, but were expressed at lower levels in highly differentiated tissues (Figure 3).

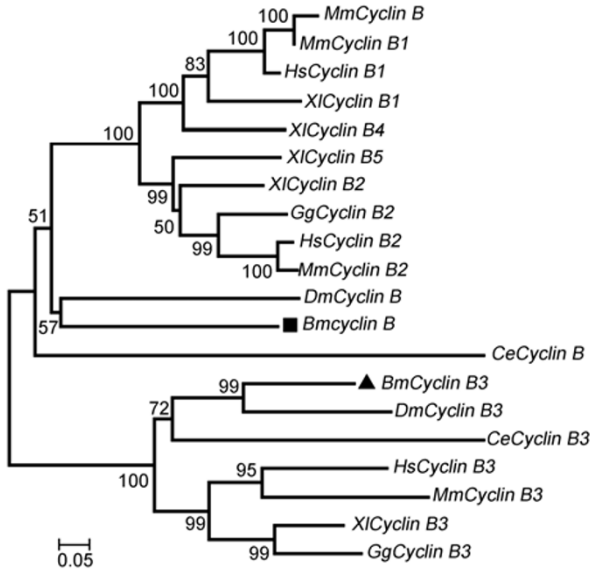

Figure 2 Phylogenetic tree of *Cyclin B* and *Cyclin B3* in different species (neighbor-joining tree reconstructed with MEGA4.0 software). *Mm*, *Mus musculus*; *Hs*, *Homo sapiens*; *Xl*, *Xenopus laevis*; *Gg*, *Gallus gallus*; *Dm*, *Drosophila melanogaster*; *Bm*, *Bombyx mori*; *Ce*, *Caenorhabditis elegans*.

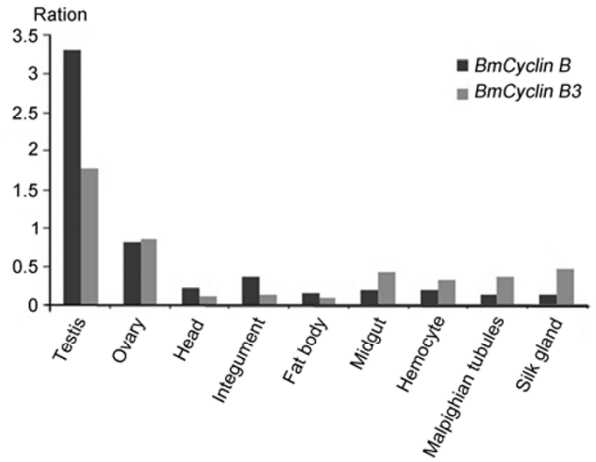

Figure 3 Expression of *BmCyclin B* and *BmCyclin B3* in 3-day larval tissues of *B. mori*.

The expression levels of *BmCyclin A*, *BmCyclin B*, *BmCyclin B3* and *BmPCNA* in silkworm larval ovaries, silk glands, BmN-SWU1 ovarian cells and in the silkworm cell line, BmSG-SWU1, were analyzed by RT-PCR. The genes *BmCyclin A* and *BmPCNA* were expressed in all tested samples. *BmCyclin B* was expressed in all tested tissues except silk glands, whereas *BmCyclin B3* was expressed at a low rate in the silk glands. *BmActin 3* was used as the internal reference (Figure 4).

2.3 The effect of *BmCyclin B* and *BmCyclin B3* knockdown on silkworm cell cycle

Normal BmN-SWU1 cells (control 1), transfection reagent treated cells (control 2), BmCyclin B dsRNA transfected cells (BmCyclin B), BmCyclin B3 dsRNA transfected cells (BmCyclin B3), and BmCyclin B and BmCyclin B3 dsRNA co-transfected cells (BmCyclin B/BmCyclin B3) were used to analyze the cell cycle by flow cytometry 48 h after transfection.

In the BmCyclin B3, BmCyclin B and BmCyclin B/BmCyclin B3 groups, the numbers of G2/M phase cells were significantly increased 48 hpt (hours post transfection). The percentages of G2/M phase cells in the BmCyclin B3, BmCyclin B and BmCyclin B/BmCyclin B3 groups were 63.65%, 65.43% and 69.56%, respectively. In the two control groups, however, the percentages were 36.69% and 32.99%.

There were no significant differences in G0/G1 cell numbers between the control groups 1 and 2, and between the BmCyclin B3 and BmCyclin B/BmCyclin B3 groups. There were, however, significant differences in G0/G1 cell numbers between the BmCyclin B3, BmCyclin B/BmCyclin B3 and BmCyclin B groups ($P<0.05$).

There were no significant differences in the percentage of S phase cells between the control groups 1 and 2, and between the BmCyclin B and BmCyclin B/BmCyclin B3 groups. On the other hand, there were highly significant differences between the BmCyclin B, BmCyclin B/BmCyclin B3 and BmCyclin B3 groups, and between the two control groups and other treatment groups ($P<0.01$).

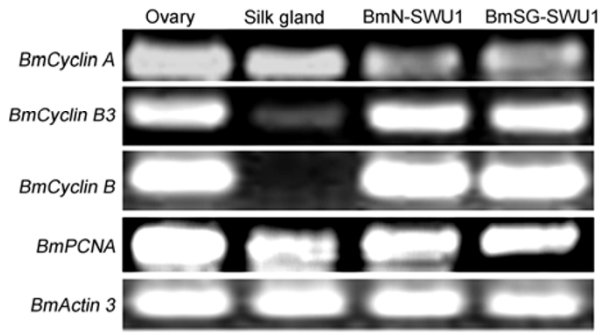

Figure 4 RT-PCR analysis of expression of cyclin genes in larval tissues and cell lines of *B. mori*.

There were no significant differences in the percentage of G2/M phase cells between the control groups 1 and 2, while there were significant differences between the BmCyclin B and BmCyclin B/BmCyclin B3 groups ($P<0.05$). Furthermore, there were highly significant differences between the BmCyclin B3 and BmCyclin B/ BmCyclin B3 groups, and between the two control and other treatment groups ($P<0.01$) (Figure 5).

These results show significant increases in the percentages of G2/M phase cells, indicating that silencing Bm*Cyclin B* and Bm*Cyclin B3* leads to cell cycle arrest at the G2/M phase and that the coordinated work of both cyclins promotes a normal cell cycle.

2.4 The effect of *BmCyclin B* and *BmCyclin B3* knockdown on cell proliferation

Forty-eight hours after transfection, inhibition of cell growth became apparent in the BmCyclin B/BmCyclin B3 group where the cell number was only 48.45% that of the normal cell group (control 1). In the BmCyclin B and BmCyclin B3 groups, the cell numbers were only 58.69% and 63.92% of control 1, respectively. Statistical analysis showed there were significant differences between the BmCyclin B and BmCyclin B3 groups compared to the BmCyclin B/BmCyclin B3 group ($P<0.05$), and between the two controls and other treatment groups ($P<0.01$) (Figure 6).

3 Discussion

In *B. mori* and *Drosophila*, two types of cyclin B protein exist, Cyclin B and Cyclin B3. In *Drosophila* embryonic cells, in which *Cyclin B* was knocked out, the spindle became multipolar and lacked the ability to drive chromosome movement. These cells could divide normally; however, the speed of division was visibly reduced. In *Drosophila* embryonic cells, in which *Cyclin B3* was knocked out, the chromosome did not condense, which would normally occur during the beginning of cytokinesis. These cells could also divide normally, and at a greater speed than cells with knocked out *Cyclin B*. Cells could not pass the G2/M checkpoint properly when both *Cyclin B* and *Cyclin B3* were blocked or knocked out. With a blockage of the G2/M phase, the cell cycle was prolonged and cells underwent apoptosis more easily [7].

In *B. mori*, the expression levels of *BmCyclin* B and *BmCyclin* B3 were very high in BmN-SWU1 cells cultured *in vitro* (Figure 4). With either *BmCyclin B* or *BmCyclin B3* knocked down or with both knocked down, the G2/M phase was blocked (Figure 5) and cell proliferation was significantly inhibited (Figure 6). The effects on cell cycle and proliferation of knocked down *Cyclin B* were greater than those of knocked down *Cyclin B3*, but these differences were not significant. The difference, however, was significant between single and combined knocked down of *Cyclin B* and *Cyclin B3*. These results indicate that *BmCyclin B* and *BmCyclin B3* are essential genes for the cell cycle that act to limit the M phase.

The silk gland cells are terminally differentiated somatic cells that continue to enlarge in size during larval development. However, the number of cells remains unchanged as the cells undergo an endomitotic cell cycle [17–19]. During endomitosis, the G1 and S phases alternate without an intervening mitotic division, leading to an increase in DNA content [20]. During the larval stage, 17–19 rounds of endomitotic DNA replication occur in the middle silk glamd and posterior silk gland resulting in a 2^{17}–2^{19}-fold increase in DNA content, with each cell containing approximately a 400000-fold haploid genomic DNA content [21]. When *B. mori* embryos develop to stage 25, silk glands express only cyclin E. Cyclin B is only expressed when the silk gland cells conduct endomitosis [8,22]. *BmCyclin B* and *BmCyclin B3* were highly expressed in the silk gland cell line, BmSG-SWU1, but at very low levels in silk gland tissue (Figure 4). This indicates that these two cyclins play an important role during the return of highly specialized silk gland cells to the cell cycle. In *Drosophila*, neither *Cyclin B3* nor *Cyclin B* is required for mitosis. However, both are

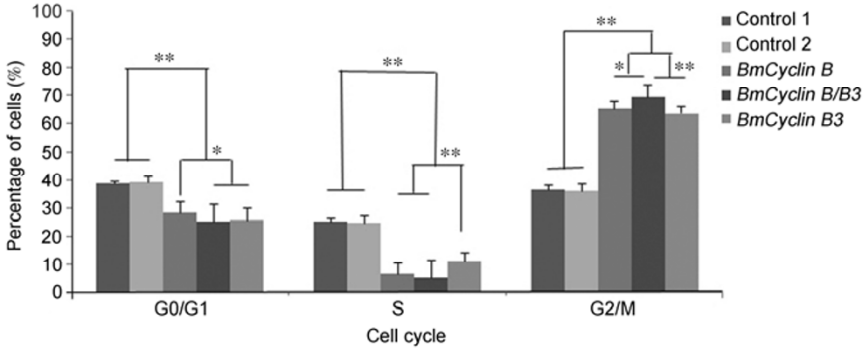

Figure 5 Effect of *BmCyclin B* and *BmCyclin B3* knockdown on cell cycle. Control 1, normal BmN-SWU1 cells; Control 2, transfection reagent-treated cells; *BmCyclin B*, *BmCyclin B* dsRNA-transfected cells; *BmCyclin B3*, *BmCyclin B3* dsRNA-transfected cells; *BmCyclin B/BmCyclin B3*, *BmCyclin B* and *BmCyclin B3* dsRNA co-transfected cells. *, Significant difference ($P<0.05$); **, significant difference ($P<0.01$).

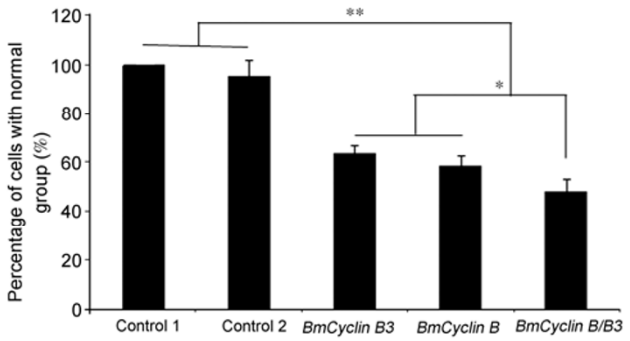

Figure 6 Effect of *BmCyclin B* and *BmCyclin B3* knockdown on cell proliferation. Control 1, normal BmN-SWU1 cells; Control 2, transfection reagent-treated cells; BmCyclin B, BmCyclin B dsRNA-transfected cells; BmCyclin B3, BmCyclin B3 dsRNA-transfected cells; BmCyclin B/BmCyclin B3, BmCyclin B and BmCyclin B3 dsRNA co-transfected cells. *, Significant difference ($P<0.05$); **, significant difference ($P<0.01$).

required for female fertility, and *Cyclin B* is also required for male fertility [7]. Investigations into whether *BmCyclin B* and *BmCyclin B3* have other functions are therefore warranted.

This work was supported by the National Natural Science Foundation of China (31272505, 31172269).

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