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Subcellular Localization of *Bombyx mori* CCAAT/enhancer-binding Protein and Its Effect on Cell Proliferation

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The CCAAT/enhancer-binding protein (C/EBP) family of proteins represents an important group of basic leucine zipper (bZIP) transcription factors that are critical to the regulation of essential functions such as energy metabolism, innate and adaptive immunity, inflammation, hematopoiesis, adipogenesis, osteoclastogensis, cell cycle, cell proliferation, and differentiation. Here, we sought to study the potential roles of C/ebp protein in the silkworm, *Bombyx mori* (BmC/ebp). Our findings showed that BmC/ebp with Venus fused to its N-terminus, was localized in the nuclei of the silkworm cells. Subsequently, in order to investigate the effect on cell development, we explored an effective double-stranded RNA (dsRNA) to knock down the expression of *BmC/EBP* both at mRNA and protein levels. The further result demonstrated that cell growth was inhibited following the down-regulation of *BmC/EBP* gene expression. Thus, we speculated that BmC/ebp may be also involved in the regulation of cell proliferation-related proteins in *Bombyx.*

Key words: *Bombyx mori*, CCAAT/enhancer-binding protein, subcellular localization, RNA interference, cell proliferation

INTRODUCTION

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors that all contain a highly conserved, basic leucine zipper domain (bZIP domain) at the C-terminus that is involved in dimerization and DNA binding (RAMJI and FODA, 2002). The function of the C/EBP family has been investigated in detail over the last decade and it has been reported to possess critical roles for regulating energy metabolism, innate and adaptive immunity, inflammation, hematopoiesis, adipogenesis, osteoclastogensis, cell cycle, cell proliferation, differentiation, and so on (NERLOV, 2007; RAMJI and FODA, 2002; TSUKADA et al., 2011).

However, the function of C/EBP in the silkworm, *Bombyx mori*, a model organism for Lepidoptera, is largely unknown. Recently, the first isolation of *C/EBP* gene (*BmC/EBP*) from the silkworm follicular cells was reported by SOURMELI et al. and the research showed that the relative concentration of BmC/ebp, in relation to its differential binding affinity for promoter cis-elements, resulted in activation or repression of silkmoth chorion gene expression (PAPANTONIS et al., 2008; SOURMELI et al., 2005). Therefore, BmC/ebp may function as a transcription factor playing important roles in activating or repressing gene expression in *B. mori*. To explore this possibility, in the present study, we first determined its subcellular localization in cultured silkworm BmN4 cells. We produced a double-stranded RNA (dsRNA) specific for *BmC/EBP* gene that could effectively abolish *BmC/EBP* expression-mediated by RNA interference (RNAi) pathway. Following the down-regulation of *BmC/EBP* gene, cell proliferation was also affected. Our study would provide some basic information for the further investigation on BmC/ebp function.

MATERIALS AND METHODS

Molecular cloning of *BmC/EBP* gene: Based on the reported DNA sequence of *BmC/EBP* gene (SOURMELI et al., 2005), we designed the primers to amplify the open reading frame (ORF) using cDNA from BmN4 cells as a template. The forward and reverse primers were 5'-GAGTCTCCCCAGATGTACGATGCGGCGGCA-3', and 5'- TTTGCGGCCGCTCAAAGCGTGTGGTCCT GGGAG-3', respectively. The PCR products then were digested by *Not*I (shown with an underline), cloned into a pENTR^{TM}11 (Invitrogen) vector, and verified by DNA sequencing. The resulted plasmid was constructed into a destination vector pi2VW (Venus-Dest) by gateway reaction according to the manufacturer's protocol and was named Venus-C/ebp.

Cell culture and transient transfection: The silkworm BmN4 cell line and BmN4-SID1 transgenetic cell line were maintained in our laboratory and cultured at 27 ºC in IPL-41 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco). For transient trans-

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fection assays, BmN4 cells seeded in 24-well plates with a density of $0.5x10^5$ were transfected with the plasmids mentioned above according to our previous method (MISTUNOBU et al., 2011).

Subcellular localization assay: For subcellular localization analysis, 100 ng of expression plasmids for Venus-C/ebp and Venus-Dest were transfected into BmN4 cells, respectively. 72 h of post-transfection, the cells were seeded on a cover slip coated with poly-L-lysine, and then fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The DNA was stained with DAPI (Invitrogen). Fluorescence microscopy images were captured using Biozero BZ-8000 microscope (KEYENCE).

RNA interference: The synthesis of dsRNAs that was specific for *BmC/EBP*, as well as control genes *EGFP* and *LUC*, and RNAi experiment were performed according to our previous procedure (LI et al., 2011). The specific primers for each gene were as follows: dsC/EBP(5'-CCGAGACGATCCTCAGGCAAAGTAATAG-3' and 5'-TTTGCGGCCGCTCAAAGCGTGTGGTCCTGGGAG-3'), dsEGFP (5'-ATTTGCACTACTGGAAAACTACCTG-3' and 5'-CAGTTACAAACTCAAGAAGGACCAT-3'), dsLUC (5'-GAAGCGACCAACGCCTTGATTGACAAGGAT-3' and 5'-TTACAATTTGGACTTTCCGC-3').

Cell proliferation assay: For cell proliferation assay, $3.0\ x10^3$ BmN4-SID1 cells were seeded in 96-well plates and cultured in a final volume of 100 μL. dsRNA for *EGFP*, or *BmC/EBP* was added into the medium with a final concentration of 0.5 μg/mL. The cells were labeled with 10 μL WST-8 solution (Cell counting Kit-8; Dojindo) for 12 h before the indicated time points, such as 1st day, 3rd day, 5th day, and 7th day. The absorbance was measured at 450 nm in a 96-well spectrophotometric plate reader according to the manufacturer's protocol, and the proliferation curves were plotted using the absorbance at each time point. All of the experiments were performed in triplicate. Statistical significance of difference between the dsC/EBP treated and the corresponding control dsEGFP treated was evaluated by the Student's t-test, and a P-value <0.05 was considered statistically significant.

RESULTS

Subcellular localization of BmC/ebp fusion protein

To study subcellular localization of BmC/ebp protein in *Bombyx*, BmN4 cells transfected with recombinant plasmid expressing Venus-C/ebp fusion protein were detected by a fluorescence microscope at 72 h post transfection. As shown in Fig. 1, green fluorescence of BmC/ebp fused with Venus was detected in the nucleus. The parental Venus control construct (Venus-Dest) was evenly expressed both in the cytoplasm and nucleus. This result implied that BmC/ebp localizes to the nucleus.

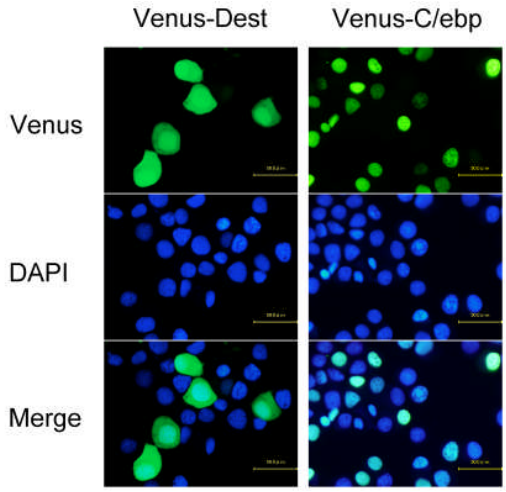

Fig. 1. Subcellular localization of BmC/ebp fusion protein. Subcellular localization of transiently expressed Venus-C/ebp fusion protein in the silkworm BmN4 cells was determined by fluorescence (green) and the nuclei DNA was counterstained with DAPI (blue).

Effective knockdown of *BmC/EBP* expression by dsRNA-mediated RNA interference

To explore potential roles of BmC/ebp in the silkworm cells, we first sought to knock down the expression of *BmC/EBP* gene. Here, we adopted the silkworm BmN4-SID1 cell line with an advantage of high efficiency of soaking the extracellular dsRNA (MON et al., 2012). We produced the dsRNA targeting to *BmC/EBP* and added into the medium pre-cultured BmN4-SID1 cells for 7 days. Knockdown efficiency for *BmC/EBP* gene was assessed by RT-PCR experiment. The results showed that the mRNA transcripts of *BmC/EBP* gene were obviously attenuated after RNAi (Fig. 2A). To check whether the protein levels of *BmC/EBP* expression were also affected upon the dsRNA treatment, we used the Venus-C/ebp fusion protein to monitor the protein changes. As shown in Fig. 2B, green fluorescence of BmC/ebp fused with Venus could not be detected after treatment with dsEGFP against Venus or dsC/EBP against BmC/ebp when compared with the control dsLUC against luciferase gene. These data clearly showed that the dsRNA used in this study could effectively down-regulate the expression of *BmC/EBP* both at mRNA and protein levels.

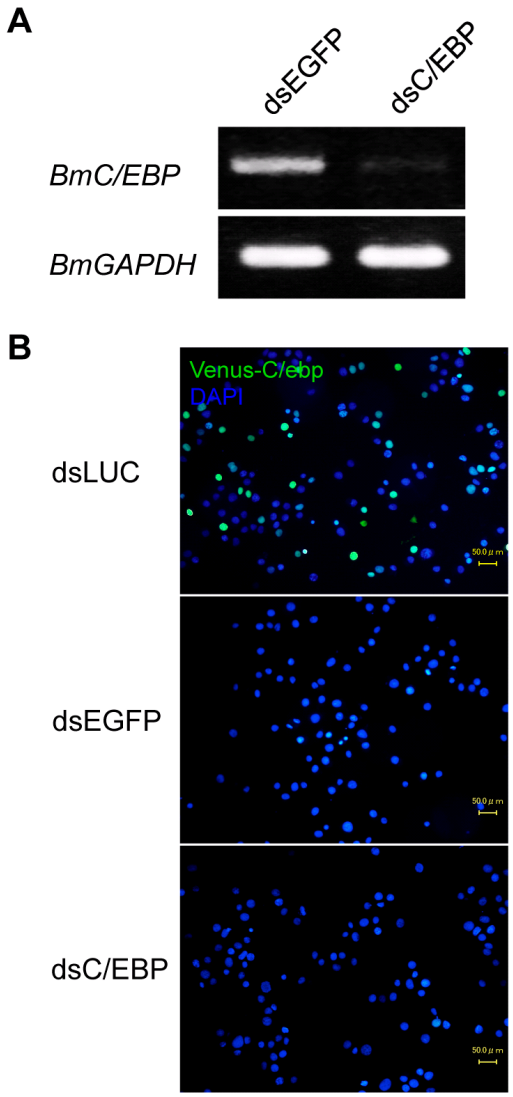

Fig. 2. Effective knockdown of *BmC/EBP* expression by dsRNA-mediated RNA interference. (A) RT-PCR analysis of the knockdown efficiency was performed from BmN4-SID1 cells 7 days after incubating with dsRNAs specific for *BmC/EBP*, or EGFP (control), and the *BmGAPDH* was used as loading control for normalization. (B) Expression of BmC/ebp fused with Venus could be significantly reduced upon treatment with dsEGFP against Venus or dsC/EBP against BmC/ebp compared with the control dsLUC against luciferase gene. The merged graphs representing the Venus and DNA signals were shown in each panel.

Effect of *BmC/EBP* knockdown on cell proliferation

To further investigate the effect of *BmC/EBP* knockdown on the silkworm cells, we analyzed the cell growth by using WST-8 assay. Time course of cell proliferation curve showed the slow increase of cell number after *BmC/EBP* knockdown, especially on the 7th day, compared with dsEGFP treatment (Fig. 3). This finding indicated that the silkworm BmC/ebp could promote cell proliferation.

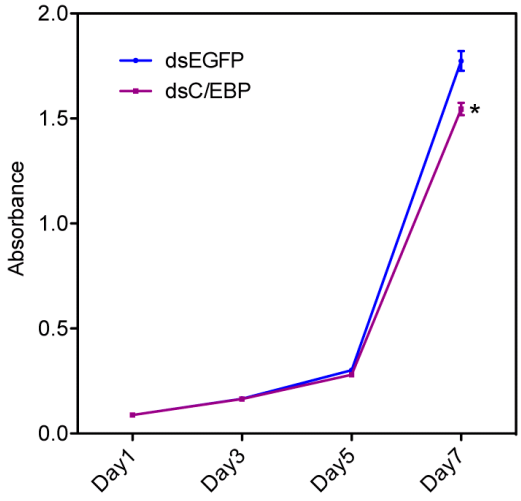

Fig. 3. Effect of *BmC/EBP* knockdown on cell proliferation. Down-regulation of *BmC/EBP* suppressed cell proliferation by using WST-8 assay. *P<0.05, compared to the control of EGFP RNAi cells.

DISCUSSION

In this study, using Venus fused BmC/ebp expression system, we examined its subcellular localization in the silkworm cells and found that BmC/ebp specifically localized to the cell nuclei. In particular, we designed and synthesized a specific dsRNA against *BmC/EBP* that was abled to effectively knock down the expression of *BmC/EBP* gene in the silkworm BmN4-SID1 cells. Furthermore, down-regulation of *BmC/EBP* expression by RNAi resulted in the inhibition of cell growth. Although the present study has not uncovered the regulatory mechanism in which BmC/ebp protein may be involved, our data may give important implications for the regulation of cell proliferation by BmC/ebp in *Bombyx*. The future study, using our available dsRNA targeting to *BmC/EBP* gene for RNAi experiment, will be essential for identifying BmC/ebp target genes on a genome-wide scale, which in turn will also contribute to elucidate its functions as transcriptional activator or repressor.

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