

## RESEARCH REPORT

**Establishment and characterization of a new embryonic cell line from the silkworm, *Bombyx mori*****M Xu<sup>#</sup>, J Tan<sup>#</sup>, X Wang, X Zhong, H Cui**

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**Abstract**

Insect cell lines are widely used for basic and applied research in the fields of insect pathology, genetics, and molecular biology. In the present study, a new continuous cell line designated BmE-SWU3 was established from blastokinesis-stage embryos of the silkworm *Bombyx mori* (Furong strain). The primary culture was initially performed using Grace's medium supplemented with 20 % foetal bovine serum (FBS) at a constant temperature of 27 °C. The dominant cell type was round and spindle-shaped. Thus far, this cell line has been cultured continuously for 60 passages. The cell doubling time was approximately 3.0 days. The SSR profile of BmE-SWU3 differs from those of the silkworm BmE and BmN-SWU1 cell lines and from those of the *Spodoptera frugiperda* cell line Sf9 and the *Drosophila* cell line S2. However, the SSR profiles among the various passages of BmE-SWU3 were stable and identical. This new cell line was highly susceptible to *Bombyx mori* nucleopolyhedrovirus (BmNPV). Semi-quantitative RT-PCR indicated that the tissue-specific gene expression patterns were completely distinct from those of BmE and BmN-SWU1.

**Key Words:** embryonic cell line; BmE-SWU3; DNA fingerprinting; BmNPV infection; *Bombyx mori***Introduction**

As an experimental tool, cell lines offer great advantages due to their easy handling and amenability to manipulation. Ever since Thomas Grace and Shangyin Gao established cell lines from *Antheraea eucalypti* and *Bombyx mori*, respectively, the development of insect cell lines has progressed rapidly (Grace, 1962; Gaw *et al.*, 1958). Indeed, approximately 800 types of continuous cell lines have been developed from over 100 insect species, including Coleoptera, Hymenoptera, Orthoptera, Homoptera, and Hemiptera, with the majority derived from Lepidopteron and Diptera (Lynn, 1999, 2002; Zhang *et al.*, 2007; Shao *et al.*, 2008).

Insect cell lines have been widely applied to produce certain virus species for use as biopesticides and also can be used as a tool to generate recombinant proteins. In addition, certain lines are used to investigate specific virus pathogenic mechanisms in basic research (Blissard, 1996). Several types of insect cell lines have been widely used to date. The S2 (Schneider 2) cell line,

derived from late embryonic-stage *Drosophila melanogaster*, is one of the most commonly used cell lines (Schneider, 1972). The Lepidopteron cell line Sf9 (*Spodoptera frugiperda* 9) is commonly used and was isolated from the Sf21 (*Spodoptera frugiperda* 21) cell line, which was originally established from ovarian tissue (Vaughn *et al.*, 1977).

Cell lines established from embryonic, larval, pupal or adult stages have become useful tools for basic and applied research. Over 30 cell lines have been established from the silkworm, and most of them are derived from the embryonic or ovarian tissues of larvae or pupae (Lynn, 2001; Pan *et al.*, 2007; Khurad *et al.*, 2006, 2009). For example, BmE, reported in 2007, was established from silkworm embryonic tissue of the reversion phase (Pan *et al.*, 2007). BmN-SWU1 was established from the ovarian tissue of 3-day-old fourth-instar silkworm larvae of the 21 - 872nlw strain (Pan *et al.*, 2010). As the most commonly used silkworm cell lines were established decades ago, new practical and affordable cell lines are needed to facilitate the study of silkworm functional genomics.

In this report, we present a new embryonic cell line, BmE-SWU3, which was isolated from blastokinesis-stage embryos of the silkworm *Bombyx mori* (*Lepidoptera*: Bombycidae). Its distinct

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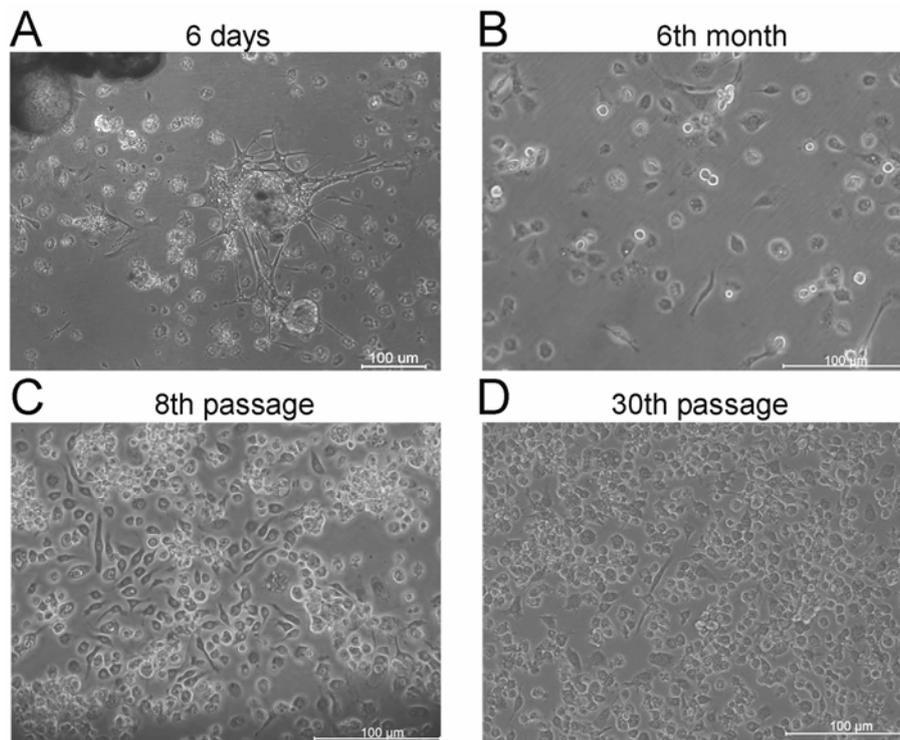
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**Fig. 1** Cell morphology of the BmE-SWU3 cell line established from silkworm embryos. (A) and (B) show cells cultured for 3 days and 6 months, respectively. (C) and (D) show cells at the 8<sup>th</sup> and 30<sup>th</sup> passages, respectively. Scale bars = 100  $\mu$ m.

SSR DNA and mRNA expression profiles indicate that BmE-SWU3 is a novel cell line and distinct from the BmE, BmN-SWU1, Sf9 or S2 cell lines, which are either from the silkworm or other insect species. Due to its high susceptibility to viral infection, BmE-SWU3 can be used to study the BmNPV baculovirus expression system and the mechanism of virus replication.

## Materials and Methods

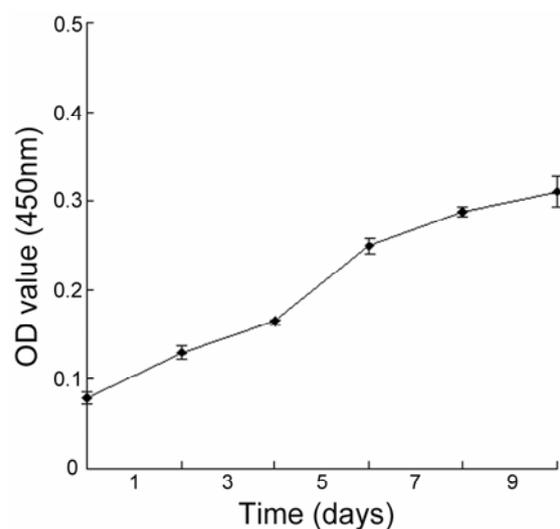
### Insects

All experiments were conducted with embryos of the Furong strain, which is a pure strain that is widely used in research. Eggs from the Silkworm Gene Bank of Southwest University were incubated at 28 °C with a 16:8 h light:dark photoperiod for 3 days and then collected immediately after they entered the blastokinesis stage.

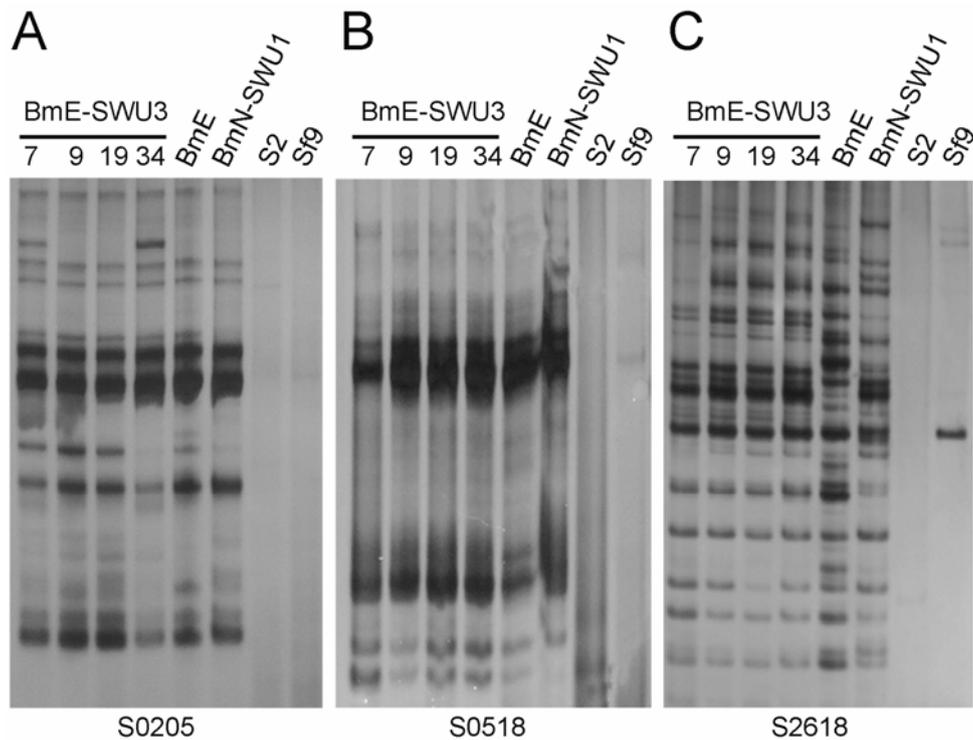
### Primary cultures

The eggs were sterilised by submersion in 70 % ethyl alcohol for 3 min before processing using previously described procedures (Pan *et al.*, 2007). Briefly, the embryos were cut into small pieces and dissociated with 0.25 % trypsin for 5 min; this reaction was stopped by adding FBS (Invitrogen). The pellets were collected, centrifuged at 100g for 5 min, then suspended in 2 ml Grace's medium (pH 6.8) supplemented with 20 % FBS. The suspension was placed into a 25-cm<sup>2</sup> culture flask and cultured

at 28 °C. Fresh medium was added after the majority of the embryonic tissues were attached, and half of the medium was replenished every two weeks until the culture was split.



**Fig. 2** BmE-SWU3 cell line growth curve. Cell growth was assessed using the CCK-8 cell proliferation assay. For each time point, 5 wells were examined. Data are presented as the means  $\pm$  SD.



**Fig. 3** SSR-PCR DNA profile analysis. (A), (B), and (C) show the results of SSR-PCR using primers S0205, S0518, and S2618, respectively. Lanes 1 - 4 represent the PCR results of BmE-SWU3 cells at the 7<sup>th</sup>, 9<sup>th</sup>, 19<sup>th</sup>, and 34<sup>th</sup> passages, and lanes 5-8 represent the PCR results for the BmE, BmN-SWU1, S2, and Sf9 cell lines, respectively.

#### *Subculture and morphological observation*

The cells kept split and were subcultured in half year after the original culture. Briefly, the cells were suspended using a sterile Pasteur pipette and centrifuged at 100g for 5 min; the cell pellets were resuspended in 5 ml medium containing 15 % FBS and then transferred into a new culture flask. The culture medium was replaced every 5 days. The FBS concentration was decreased from 20 % to 15 % at the 10<sup>th</sup> passage and from 15 % to 10 % at the 20<sup>th</sup> passage. The cells were observed under a Nikon TE2000 inverted phase-contrast microscope.

#### *Growth analysis*

Growth curves were determined at the 30<sup>th</sup> passage. A sample of 8,000 cells in 200 µl medium was seeded into a 96-well plate. The cell number was counted every 24 h using Cell Counting Kit-8 (CCK-8) (Beyotime), and the growth curve was plotted as OD values at 450 nm.

#### *Fingerprinting analysis*

DNA was extracted and purified from BmE-SWU3, BmE, BmN-SWU1, S2, and Sf9 cells using the EasyPure Genomic DNA Kit (Transgene Biotechnology, Inc.). Ten pairs of SSR primers (kind gifts from Prof Fangyin Dai in Southwest University) were employed (Miao *et al*, 2005): S0105, S0201, S0205, S0317, S0411, S0518, S0709, S0804, S0818, S2502, S2503, S2612, and S2618. PCR amplification was performed in reaction volumes of

15 µl containing 1X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.6 µM of SSR primers, 0.4 unit of Taq polymerase (Takara), and 20 ng of template DNA. The PCR conditions were as follows: 94 °C for 30 sec, 63 °C for 1 min, and 72 °C for 1 min; 15 cycles of 94 °C for 30 sec, 62.5 °C for 1 min, and 72 °C for 1 min, with the temperature lowered 0.5 °C per cycle; followed by 25 cycles of 94 °C for 30 sec, 56 °C for 1 min, and 72 °C for 1 min. The PCR products were analysed by 12 % polyacrylamide gel electrophoresis (PAGE). Gel images were captured using a Bio-Rad gel documentation system.

#### *Reverse transcription-PCR analysis*

Total RNA from BmE-SWU3, BmE, and BmN-SWU1 cells was isolated using TRIzol (Invitrogen). cDNA was synthesised with 2 µg total RNA using reverse transcriptase and the supplied solutions (Promega). PCR was performed as follows: 94 °C for 3 min; 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min; and 72 °C for 10 min. The products were analysed by 1 % agarose gel electrophoresis, and gel images were captured using a Bio-Rad gel documentation system. The sequences of the primers used in the RT-PCR experiments are as follows: VASA-F, 5' GGAGGAGGCGATAGAAATG 3'; VASA-R, 5' ATGATACACGATTCCTTTCCA 3'; LSP-F, 5' TATCACCCTGCCCATTACAA 3'; LSP-R, 5' GCTTAGCGGGCTTCAGG 3'; FibL-F, 5' TTTTGGTATTACTCGTCGCT 3'; FibL-R, 5'

TCACTGCTGGCTAAGATTGC 3'; APN-F, 5'  
 CAAACAATGGCGTTATCACTT 3'; APN-R, 5'  
 TCCTAAACTGTCTCCATTTCTGA 3'; RPL3-F, 5'  
 CGGTGTTGTTGGATACATTGAG 3'; and RPL3, 5'  
 GCTCATCCTGCCATTTCTTACT 3'.

#### Virus infection

BmE-SWU3 cells or BmN-SWU1 cells were seeded into 96-well plates at  $8 \times 10^3$  cells/well and infected with recombinant BmNPV expressing EGFP (enhanced green fluorescent protein) under the BmNPV 39K promoter (v39Kprm-EGFP) at an MOI (multiplicity of infection) of 20. After 3 days of infection, the titres were determined by TCID50 analysis based on EGFP as described (O'Reilly, 1993).

## Results

#### Establishment of the BmE-SWU3 cell line

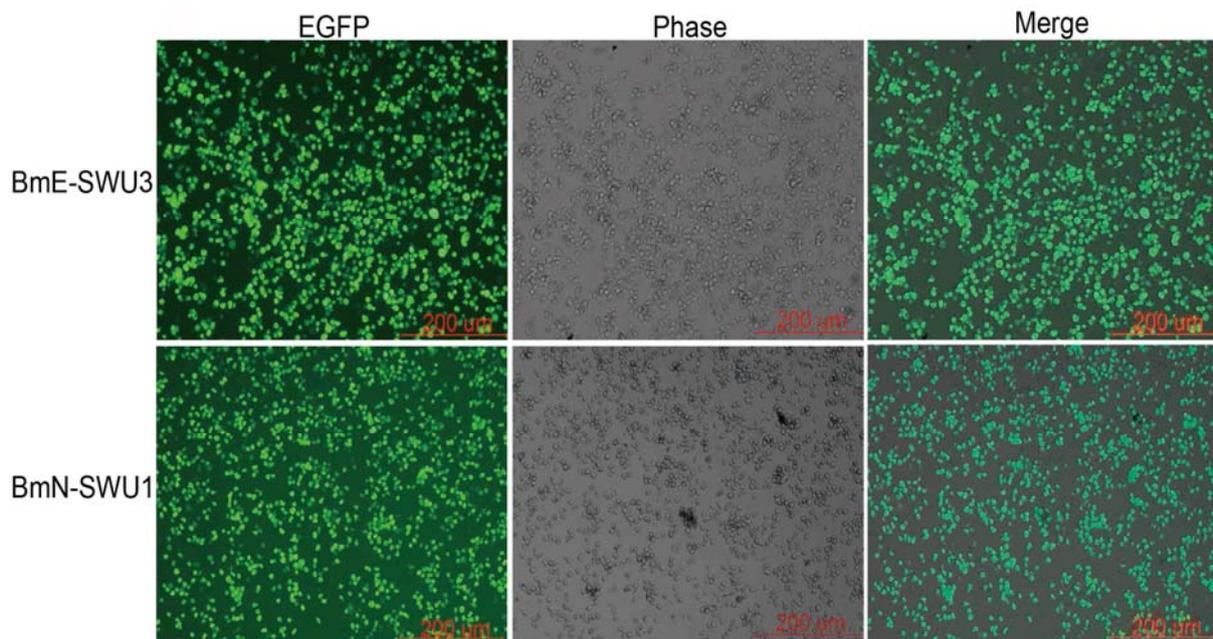
After 6 days of incubation, the cells had migrated from the embryonic tissue sections. The cell number gradually increased, and some cells were de-attached when the cell confluence reached 80 %. The suspended cells were harvested and then re-cultured in 3 ml Grace's medium (pH 6.8) supplemented with 20 % FBS. The BmE-SWU3 line was successfully subcultured after another 6 months of culturing. The interval of subculture for the first five passages was 30 days at a ratio of 1:2, then became 10 days. The morphology of the majority of cells was round and smaller than the cells of the BmE cell line, with a spindle shape that became round at 80 % confluence (Fig. 1). To date, the cells were successfully subcultured for 60 passages at an interval of 5 days.

#### Cell growth analysis

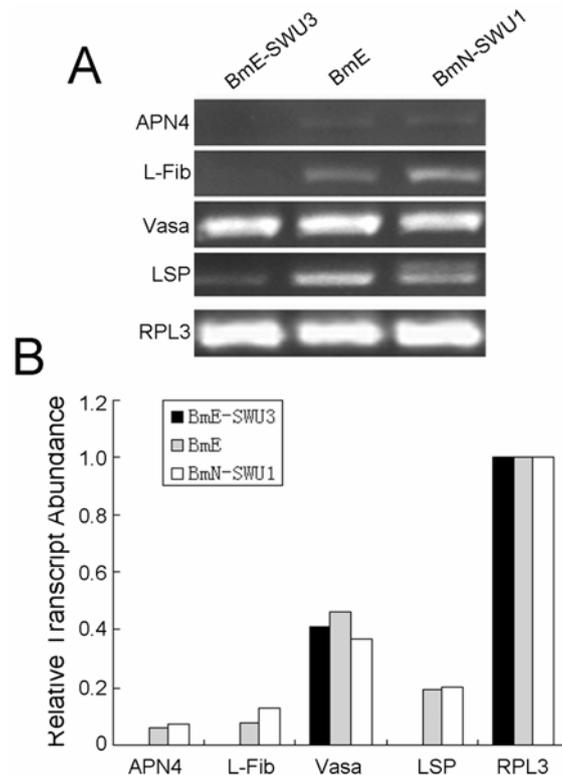
Cell growth was assessed using the CCK-8 cell proliferation assay when the cells reached the 35<sup>th</sup> passage (Fig. 2). The growth curve of BmE-SWU3 cells expressed as OD values showed an obvious "S" shape. The population doubling time was approximately 3 days, which was calculated using the formula provided by Hayflick (1973).

#### DNA profiling

DNA fingerprinting is a valuable and reliable technique for cell line identification (McIntosh *et al.*, 1996). We conducted PCR with 13 primers to analyse the DNA profiles of the BmE-SWU3 cell line in comparison with those of the BmE, BmN-SWU1, Sf9 or S2 cell lines. PCR using 10 primers generated clear DNA fragments in the silkworm cell lines. Because the SSR primers used are specific for *B. mori*, only 4 of them were able to generate DNA fragments in the S2 cell line, with 7 in the Sf9 cell line. However, the DNA banding patterns of the S2 and Sf9 cell lines were highly distinct from those of the silkworm cell lines. The DNA fingerprinting profiles using S0205 (Fig. 3A) and S0518 (Fig. 3B) indicated no difference among the BmE-SWU3, BmE, and BmN-SWU1 cells. In contrast, the DNA fingerprinting profiles with the S2618 primer (Fig. 3C) and another 7 primers were significantly different among the BmE-SWU3, BmE, and BmN-SWU1 cell lines (Fig. 3B). However, no polymorphism was detected between the various passages of the BmE-SWU3 cell line. All of these results confirmed that the BmE-SWU3 line is genetically closer to *B. mori* cell lines than to cells from other insect species.



**Fig. 4** BmE-SWU3 and BmN-SWU1 cells were infected with recombinant BmNPV for 72 h. The infected cells were indicated by EGFP signals (green). Scale bars = 200  $\mu$ m.



**Fig. 5** Semi-quantitative RT-PCR of BmE-SWU3, BmE, and BmN-SWU1 cells. (A) APN4, L-Fib, Vasa, and LSP were analysed using semi-quantitative PCR. RPL3 was included as a control. (B) Quantitative analysis of the mRNA expression level.

#### Viral susceptibility

BmE-SWU3 and BmN-SWU1 cells were infected with recombinant BmNPV for 72 h, and EGFP signals were used to monitor viral infection via fluorescence microscopy (Fig. 4). According to the formula described by O'Reilly (O'Reilly, 1993), the virus titres in the BmE-SWU3 and BmN-SWU1 cells were  $1.756 \times 10^8$  TCID50/ml and  $2.338 \times 10^8$  TCID50/ml, respectively. BmN-SWU1 cells have been reported to be highly susceptible to BmNPV (*Bombyx mori* nucleopolyhedrovirus), and the similar TCID50 values indicate that BmE-SWU3 is also highly susceptible to BmNPV.

#### Tissue-specific gene expression patterns in BmE-SWU3, BmE and BmN-SWU1 cells

APN4 (Aminopeptidase N4), L-Fib (L-Fibroin), Vasa, and LSP (Larval Serum Protein) are specifically expressed in the midgut, silk gland, gonad, and fat body, respectively, in silkworm larvae (Hua *et al.*, 1998; Tang *et al.*, 2003; Tomita *et al.*, 2003; Nakao *et al.*, 2006). Thus, we chose these four tissue-specific genes to characterize the BmE-SWU3 cell line. RT-PCR data indicated that the BmE-SWU3 mRNA expression patterns were significantly different from those of BmE or BmN-SWU1 cells (Fig. 5). The BmE-SWU3 cells were negative for APN4, L-Fib, and LSP, but positive for Vasa, confirming that BmE-SWU3 cells are derived from the germ line rather than from other tissues, such as the midgut, silk gland or fat body.

#### Discussion

In this study, we established and characterised a new embryonic cell line, BmE-SWU3, from the silkworm *B. mori*. This is the first cell line cultured from the Furong strain, which is a pure strain that has been commonly applied in many research fields. BmE-SWU3 was initiated from the third-day embryos, the prophase blastokinesis stage, which stage belongs to gastrula and also had the totipotency capability. It's different from BmE which was generated from the fourth-day embryos, which stage belongs to the reversion phase. In this stage, ectoderm, endoderm and mesoblast formed consecutively, all of the organs began differentiate and develop. As we expected, the tissue-specific gene expression patterns in BmE-SWU3 were different from BmE or BmN-SWU1. Derived from different stage, and owned different genetic background, and different gene transcriptional regulation, and that would provide diversity for silkworm basic research.

In addition, The SSR profile of BmE-SWU3 was compared with those of the current silkworm cell lines (BmE-SWU1 and BmN-SWU1) and another two other insect cell lines (Sf9 and S2) at the DNA level, confirming that BmE-SWU3 was derived from the silkworm *B. mori* and is distinct from other cell lines. Moreover, Sf9 cell line is derived from *Spodoptera frugiperda*, and BmE-SWU3 is from *B. mori*, both of them belong to Lepidopteron, but S2

cell line is from the Diptera *Drosophila melanogaster*, the expression profile is totally different from the *B. mori*. As the same Lepidopteron resource, the Sf9 cell line expression profile showed some similarity with BmE-SWU3, both of them shared a clear band with the S2618 primer. These results confirmed that BmE-SWU3 cell line is genetically closer to Sf 9 cell line rather than S2 cell line.

More important, BmE-SWU3 has high susceptibility to BmNPV, and it could be used in research of the mechanism about infection, replication, assembly and release of BmNPV and utilised as a foreign gene-expressing system to produce useful recombinant proteins with BmNPV-derived expression vectors (e.g., Bac-to-Bac baculovirus expression system).

In summary, BmE-SWU3 is a brand new cell line derived from silkworm, and it's very practical and affordable, and it could be widely used in insect gene functions research and application.

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