

Full Length Research Paper

Subcellular localization of *Bombyx mori* ribosomal protein S3a and effect of its over-expression on BmNPV infection

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In the present study, using a BV/PH-Bms3a-EGFP, we found that *Bombyx mori* ribosomal protein S3a (BmS3a) with EGFP fused to its C-terminal, was predominantly localized in the cytoplasm of *B. mori* cells. Subsequently, to investigate the effect of BmS3a over-expression on BmNPV infection both at the cellular level and *in vivo*, a transgenic BmN cell line expressing BmS3a was constructed using a piggybac-A3-EGFP and recombinant baculovirus expressing BmS3a-EGFP fusion protein (BV/IE1-Bms3a-EGFP) or EGFP (BV/EGFP) were produced using BmNPV/Bac-to-Bac expression system. Results showed that the number of polyhedra in the transgenic cells of BmS3a was much smaller than that in non-transgenic cells, and that silkworms injected with BV/IE1-Bms3a-EGFP survived much longer than those injected with BV/EGFP. Taken together, we speculated that BmS3a might be capable of inhibiting BmNPV replication through its activities in the cytoplasm.

Key words: BmS3a, subcellular localization, over-expression, effect.

INTRODUCTION

Ribosomal proteins have been highly conserved during evolution, reflecting their critical functions in ribosome biogenesis or mature ribosome function. However, in recent years, a growing number of findings from research groups have shown that several ribosomal proteins possess "extra-ribosomal" functions in cellular apoptosis, transcription/ translation mRNA processing, DNA repair development and tumorigenesis (Lindstrom, 2009).

Ribosomal protein S3a is a component of the 40S small

subunit (Lutsch et al., 1990) and binds to 3'-OH end of 18S rRNA (Svoboda and McConkey, 1978). S3a is known to be involved in interaction of 40S subunit with initiation factors (Nygard et al., 1981; Tolan and Traut, 1981; Westermann et al., 1979; Westermann and Nygard, 1983), initiator-tRNA (Westermann et al., 1981), or mRNA (Stahl and Kobets, 1981), and also in the interaction of the 80S subunit with elongation factor-2 (EF-2) (Nagahisa et al., 1996; Nygard et al., 1987), which implies its role in regulation of translation. However, recent studies have revealed that S3a is involved in cell growth, transformation, and apoptosis (Kho et al., 1996; Kho and Zarbl, 1992; Naora et al., 1998; Russell et al., 2000). S3a was first identified as v-fos transformation effector (Fte-1) (Kho and Zarbl, 1992), but Fte-1 was later proved to be identical to S3a (Kho, 1996). Ruggero and Pandolfi (2003) showed that over-expression of Fte-1/S3a is associated with a rapid cell proliferation, maintenance of undifferentiated state, and proneness for apoptosis.

Using fluorescent differential display (FDD) technique, we analyzed differential expression of genes related to *Bombyx mori* nuclear polyhedrosis virus (BmNPV) resis-

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Abbreviations: FDD, Fluorescent differential display; Fte-1, v-fos transformation effector; BmNPV, *Bombyx mori* nuclear polyhedrosis virus; NB, resistant silkworm strain; 306, highly susceptible silkworm strain; 306NNZZ, near isogenic line; Bms3a, *Bombyx mori* S3a; BmN cell, *Bombyx mori* cell line; QRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; FBS, fetal calf serum; pSK, pBluescript II SK (+); PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

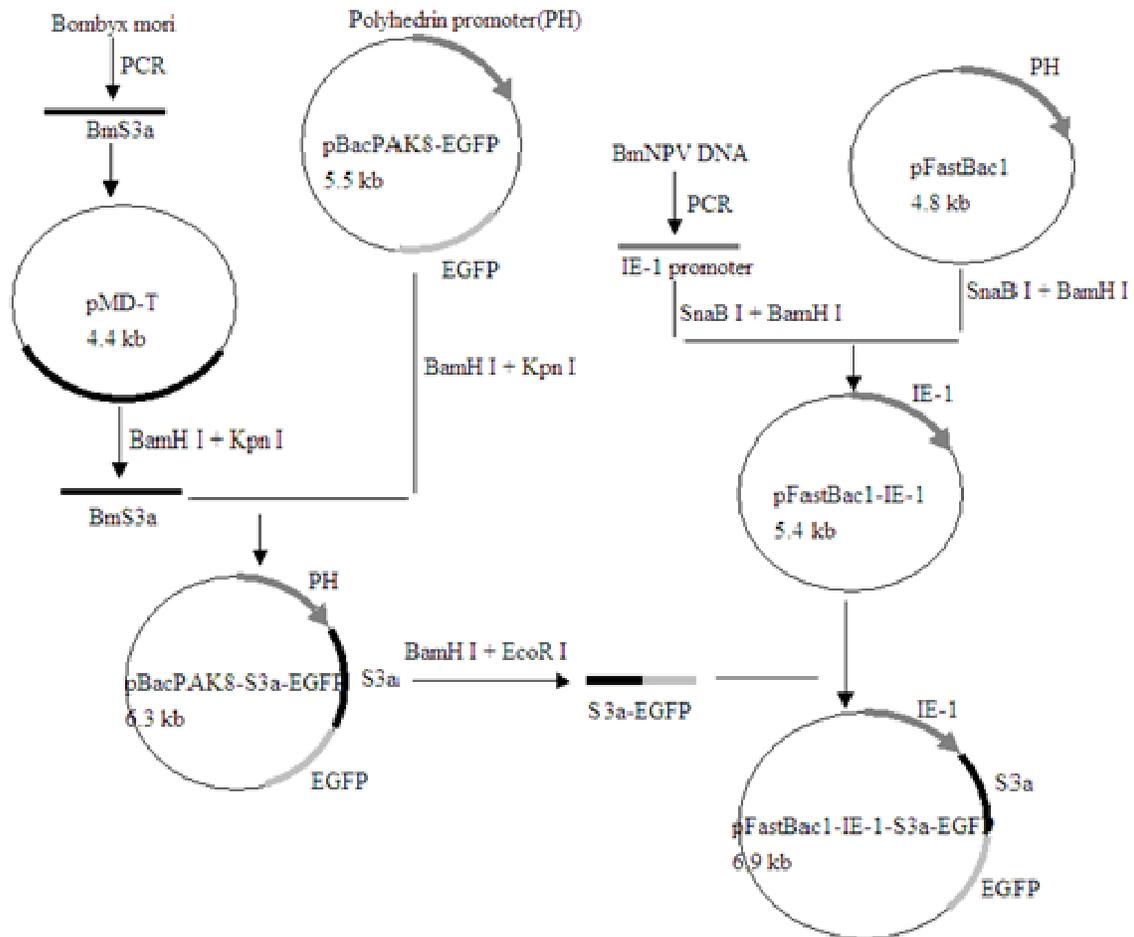


Figure 1. Construction of pBacPAK8-PH-Bms3a-EGFP and pFastBac1-IE1-Bms3a-EGFP.

tance among highly resistant silkworm strain NB, highly susceptible silkworm strain 306 and near isogenic line 306NNZZ. A gene encoding a putative *B. mori s3a* (*Bms3a*) was found to be expressed differently among these strains. More specifically, its transcript level was much more abundant in BmNPV-infected strain NB than in BmNPV-infected strain 306. Therefore, we speculate that *Bms3a* might be involved in the resistance of silkworm to BmNPV (Xu et al., 2008).

In this study, by over-expressing Bms3a in a *B. mori* cell line (BmN cell) and silkworm, we investigated its subcellular localization and attempted to find some evidence for the effect of its over-expression on BmNPV infection.

MATERIALS AND METHODS

Materials

The plasmid pFastBac1 was supplied by Prof. K. Maenaka. pBacPAK-EGFP and recombinant bacmid expressing EGFP was constructed by our laboratory. FuGENe 6 transfection reagent was the product of Roche Applied Science, USA. The TC-100 insect cell culture medium (GIBCO) was purchased from Invitrogen company.

Recombinant baculovirus constructs

With BmNPV genomic DNA as template, IE1 promoter region was polymerase chain reaction (PCR)-amplified using the following primers: Forward: 5'-TCTTACGTAGATTTGCAGTTCG GGACATAA-3'; reverse: 5'-GTTCCAGGATCCTGTCGCCGCAATGTCA-3' (*SnaB* I and *BamH* I sites were underlined). A cDNA fragment encoding Bms3a was PCR-amplified using the following primers: Forward: 5'-GGATCCATGGCGGTCGGGAAAAATA-3'; reverse: 5'-GGTACCAACTCTCTTG AACGGGA-3' (*BamH* I and *Kpn* I sites were underlined), and then cloned into a pMD 18-T vector. The cDNA was subcloned into pBacPAK-EGFP and pFastBac1. Figure 1 shows the construction of pBacPAK8-PH-Bms3a-EGFP and pFastBac1-IE1-Bms3a-EGFP. pBacPAK8-PH-Bms3a-EGFP was co-transfected with linearization virus BmPak6 into BmN cells to generate recombinant baculoviruses BV/PH-Bms3a-EGFP. pFastBac1-IE1-Bms3a-EGFP was transformed into *Escherichia coli* DH10Bac/BmNPV where transposition occurred to obtain recombinant bacmid. Recombinant bacmid was transfected into BmN cells to produce recombinant baculovirus BV/IE1-Bms3a-EGFP (Miao et al., 2006).

Cell culture and transfection

BmN cells were cultured in TC-100 (GIBCO) supplemented with 10% fetal calf serum (FCS) at 27°C. Transfections were performed

by lipofectin method.

Transgenic cells

Full length *Bms3a* cDNA was isolated by PCR amplification using specific primers designed. IE1 promoter and SV40pA were also PCR-amplified using appropriate primers. The resulting PCR fragments were inserted into pBluescript II SK (+) (pSK) cloning vector to generate pSK-IE1-Bms3a-SV40pA. Insert-containing clones were selected. Resulting plasmids were confirmed by bidirectional sequencing. IE1-Bms3a-SV40pA was excised from pSK-IE1-Bms3a-SV40pA by digestion with appropriate restriction enzymes and then cloned into piggybac-A3-EGFP (pigA3) to generate pigA3-IE1-Bms3a-SV40pA. The vector containing *Bms3a* was co-transfected with helper vector into BmN cells with Fugene according to manufacturer's protocol. The cells were then grown on selective media.

Quantitative determination of Bms3a expression in transgenic cells by quantitative reverse transcriptase- polymerase chain reaction (QRT-PCR) technique

BmN cells were transfected with either pigA3-IE1-Bms3a-SV40 or pig-A3-EGFP. At 72 h post transfection, the cells were observed under fluorescent microscope. When fluorescence was observed in 40% cells, the transfected cells and normal control cells were harvested for extraction of RNA. QRT-PCR technique was used for quantitative determination of *Bms3a* expression, with *BmA3* as internal control and *Bms3a* in normal cells as a control.

Western blot analysis of Bms3a-EGFP fusion protein

BmN Cells transfected with recombinant baculoviruses expressing BmS3a-EGFP fusion protein, were harvested at 72 h post infection, washed twice in 1× phosphate-buffered saline (PBS) and lysed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer by boiling for 5 min. The cellular debris was removed by centrifugation at 12,000 rpm for 10 min. The samples were separated by SDS-PAGE using 10% polyacrylamide and analyzed by Western blot with antibody against EGFP.

BmNPV-infected transgenic cells

Transgenic cells were infected with BmNPV at an MOI of 5. At 72 h post infection, the number of polyhedral in transgenic cells and normal cells was counted.

Injection of baculoviruses into silkworm

Virus titers of BV/IE1-Bms3a-EGFP and BV/EGFP were determined by Plaque assay. Newly molted fifth-instar larvae were randomly divided into three groups of 20 each. Larvae were injected with a virus at a concentration of 10^7 pfu/ml in 5 μ L. There was a normal group of larvae injected with 5 μ L of sterile water, which was used to investigate incidence of disease. Each group experiment was repeated three times.

RESULTS

Subcellular localization of Bms3a fusion protein

To study subcellular localization of BmS3a-EGFP fusion

protein, BmN cells infected with recombinant BV/PH-Bms3a-EGFP expressing BmS3a-EGFP fusion protein were examined by a confocal laser scanning microscope at 72 h post infection. As shown in Figure 2a, green fluorescence of ribosomal protein BmS3a fused with EGFP was detected primarily in the cytoplasm. To confirm the correct fusion of BmS3a with EGFP, Western blot analysis was performed using antibody against EGFP. While a band of about 27 KDa was detected in the total proteins of BmN cells infected with recombinant BV/EGFP expressing EGFP (Figure 2b, lane 2), a band of about 57 KDa was detected in the total proteins of BmN cells infected with recombinant BV/PH-Bms3a-EGFP expressing BmS3a-EGFP fusion protein (Figure 2b, lane 1). According to the amino acid sequence of BmS3a, BmS3a has a predicted molecular weight of 30 KDa. Therefore, the result confirmed that the correct fusion products of BmS3a with EGFP. The result suggests that BmS3a was localized predominantly in the cytoplasm of BmN cells.

Effect of BmS3a over-expression in transgenic cells on BmNPV infection

To investigate the effect of BmS3a over-expression on BmNPV infection, we produced transgenic cells of pigA3-IE1-Bms3a-SV40 in which BmS3a was over-expressed. Figure 3 shows that expression level of *Bms3a* in transgenic cells of pigA3-IE1-Bms3a-SV40 was 2.11 times higher than that in normal cells, indicating that *Bms3a* was indeed over-expressed in transgenic cells and thus laying the foundation for further study. Moreover, Figure 3 also shows that expression level of *Bms3a* in transgenic cells of pig-A3-EGFP was a little higher than that in normal cells. We speculated that the increased expression might be caused by cell immune response induced by transfection with the plasmids.

Transgenic cells were infected with BmNPV at an MOI of 5. At 72 h post infection, polyhedral could be observed in both fluorescent transgenic cells and non-fluorescent normal cells (Figures 4B and C). Twenty one fluorescent cells and their adjacent 21 non-fluorescent normal cells were randomly selected for counting the number of polyhedral. Statistical analysis was performed, and results (Figures 4 and 5) showed that the number of polyhedral in fluorescent cells was much lower than that in non-fluorescent normal cells with a highly significant difference ($p = 0.0027$).

Survival assay of silkworms injected with recombinant baculoviruses

To investigate the effect of BmS3a over-expression on silkworms, recombinant baculoviruses BV/IE1-Bms3a-EGFP were injected into newly-molted fifth instar silkworm, with recombinant baculoviruses BV/EGFP and

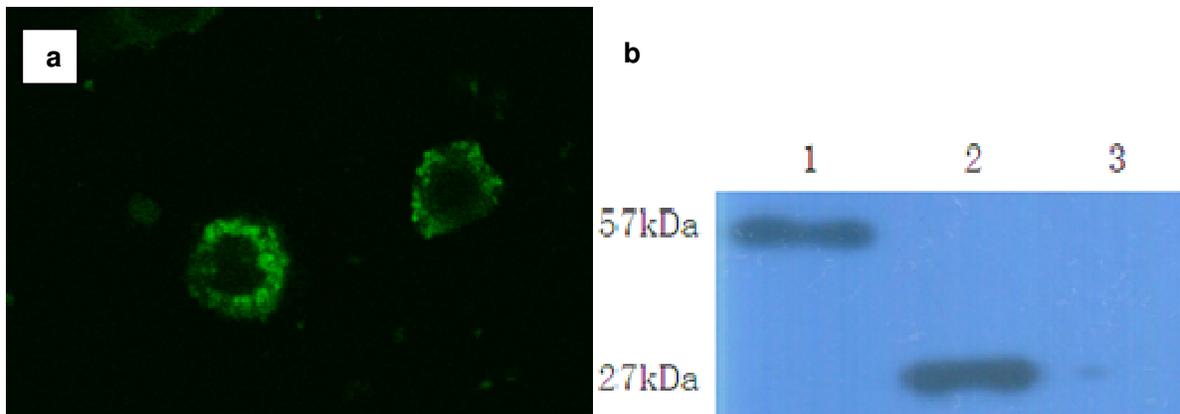


Figure 2. (a) Subcellular localization of BmS3a-EGFP fusion protein. BmN cells were infected with recombinant baculoviruses BV/PH-Bms3a-EGFP at 72 h post infection, cells were observed under confocal laser scanning microscope. (b) Western blot analysis of BmS3a-EGFP fusion protein and EGFP. Lane 1, Total proteins of BmN cells infected with recombinant baculoviruses expressing BmS3a-EGFP fusion protein; lane 2, total proteins of BmN cells infected with recombinant baculoviruses expressing EGFP; lane 3, total proteins of BmN cells transfected with BmPAK6.

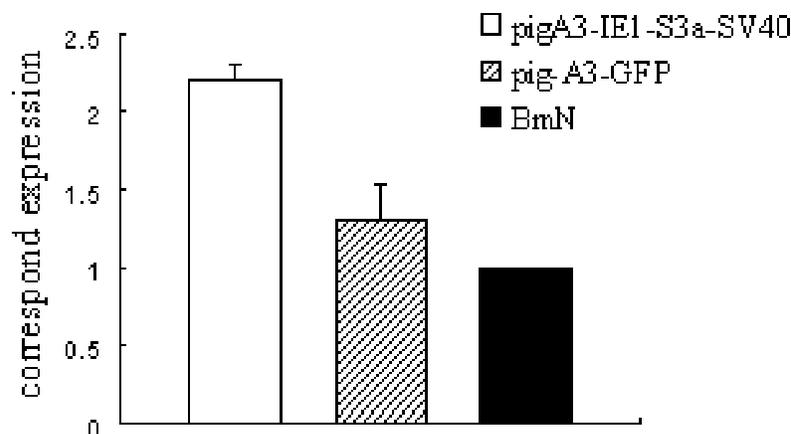


Figure 3. Relative expression of *Bms3a* in transgenic cells of pigA3-IE1-Bms3a-SV40 and pig-A3-EGFP as well as in normal BmN cells determined by QRT-PCR.

sterile water as controls. After injection, silkworms were reared with artificial diet at 25°C. At 80 h post injection, survival rate in each group was 100% and no significant difference was observed. However, from 93 h, significant difference was observed, as shown in Table 1. Analysis of LT50 data showed the group injected with BV/IE1-Bms3a-EGFP, with its LT50 being 123 h, had a highly significant difference with the group injected with BV/EGFP.

DISCUSSION

In this study, using BmNPV baculovirus expression system, a recombinant baculovirus over-expressing BmS3a-EGFP fusion protein was constructed and used to infect BmN

cells. The fusion protein was found to be localized mainly in cytoplasm when infected cells were observed under laser confocal microscope, whereas human endogenous BmS3a is localized in both cytoplasm and nucleus (Kashuba et al., 2005), suggesting that BmS3a might be involved in translation initiation in cytoplasm.

The smaller number of polyhedral in transgenic cells over-expressing BmS3a (Figure 5), together with the longer survival time of silkworms injected with baculoviruses over-expressing BmS3a-EGFP fusion protein (Table 1), suggests that BmS3a might be capable of inhibiting BmNPV replication. We speculated that over-expression of BmS3a might regulate cell apoptosis and thus block BmNPV translation initiation, resulting in the inhibition of BmNPV replication and the resistance to BmNPV.

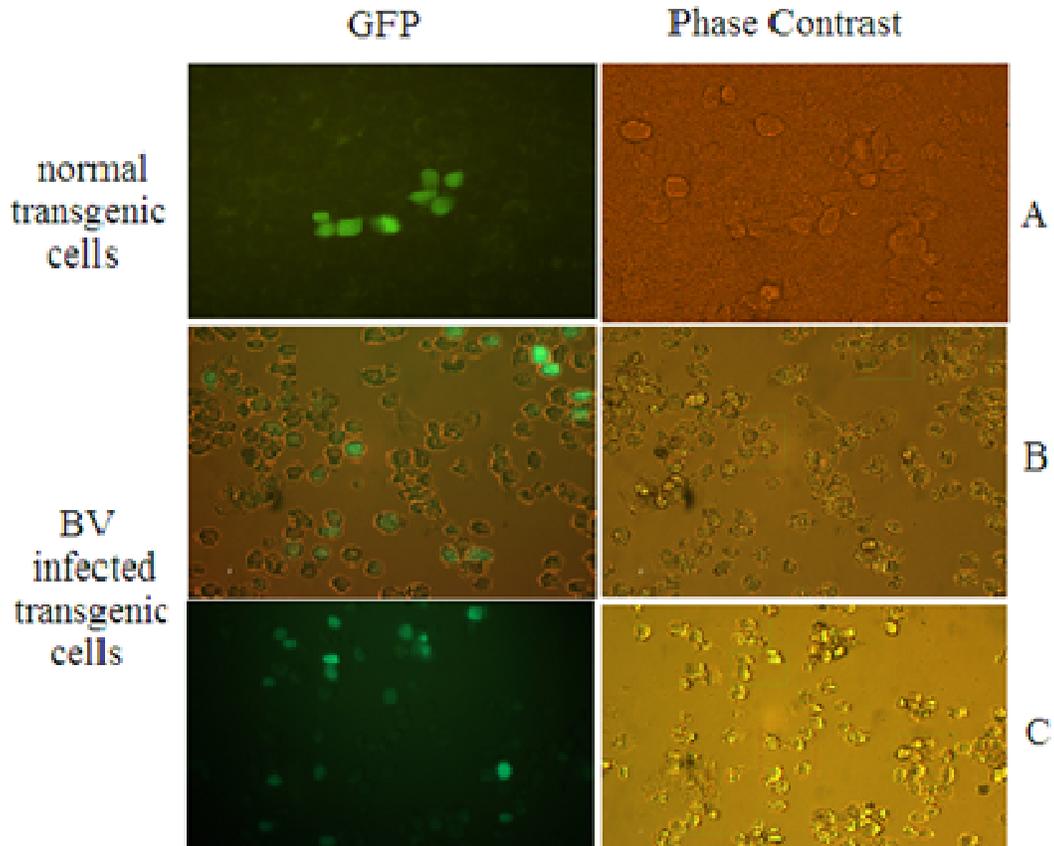


Figure 4. Transgenic cells. (A) Normal transgenic cells cultured in TC-100 supplemented with 10% FCS at 27°C. (B and C) transgenic cells infected with BV. Five cell clusters were randomly selected to count the number of polyhedral.

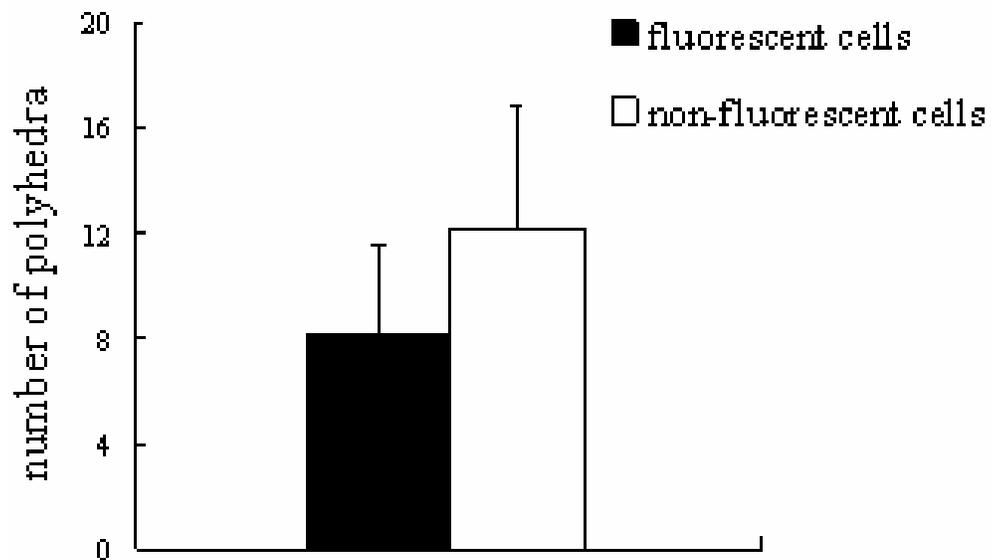


Figure 5. Number of polyhedral in fluorescent and non-fluorescent normal cells.

To conclude, BmS3a plays a role in inhibiting BmNPV replication. However, the mechanism through which it

regulates cell apoptosis and thus inhibits BmNPV replication needs to be further studied.

Table 1. Median lethal time (LT₅₀) of different recombinant baculoviruses.

Virus	LT ₅₀ (h) (mean ± SD)	95% Fiducial limit (h)	
		Lower	Upper
BV/ IE1-s3a-EGFP	123.3 ± 3.8	114.0	132.6
BV/ EGFP	105.0 ± 0.6	103.6	106.4

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