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REPORT

A new insect cell line from pupal ovary of *Spodoptera exigua* established by stimulation with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)

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Abstract A continuous cell line derived from the pupal ovary of *Spodoptera exigua* was established by treating primary cells with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). Three days after treating cells with 3.0 μg/ml of MNNG, the cells formed a monolayer and were initially subcultured 60 d after the MNNG was removed, followed by subculturing for 30 passages. The established cell line, designated IOZCAS-Spex 12, consisted of a mixture of three types of cells, including spherical, spindle-shaped, and oval cells. The population doubling time of the cell line during its logarithmic growth phase was found to be 71 h. DNA amplification fingerprinting polymerase chain reaction analysis confirmed that the new cell line originated from *S. exigua*. Susceptibility of IOZCAS-Spex 12 cells to infection by certain nucleopolyhedroviruses was investigated. The results showed that the cell line was highly susceptible to infection by *S. exigua* nucleopolyhedrovirus and *Autographa californica* multiple nucleopolyhedrovirus, slightly susceptible to infection by *Spodoptera litura* nucleopolyhedrovirus, and not susceptible to infection by *Helicoverpa armigera* nucleopolyhedroviruses or *Hyphantria cunea* nucleopolyhedroviruses. The results of this study suggest that MNNG treatment may overcome existing limitations to obtaining continually proliferating cells and may open up the possibilities for immortalizing isolated insect cells.

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The use of insect cell lines is becoming more common in the fields of agriculture and biotechnology (Aucoin et al. 2010). Since the first insect cell line was established in the 1960s, more than 500 insect cell lines have been developed from approximate 120 different insect species (Gaw et al. 1959; Grace 1962; Yeh et al. 2007). However, compared to mammalian cell lines, methods for establishing insect cell lines are still in their infancy (Simcox et al. 2008). Methods of in vitro chemical transformation in various mammalian cells are well established. For instance, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), a monofunctional alkylating agent that causes chromosomal DNA damage, has been used in in vitro cell culture systems to transform various types of normal or healthy cells into tumor cells (Gichner and Veleminsky 1982; Schar 2001). Carcinogenesis and the mechanisms of MNNG transformation have been extensively investigated through both in vitro and in vivo studies (Du et al. 1984; Su et al. 1995; Bunton and Wolfe 1996; Schar 2001; Izyumov and Talikina 2007).

However, few similar studies have examined the use of MNNG to establish insect cell lines. Although insect cells treated with carcinogens can survive for a certain time period, cell multiplication has not been observed, and attempts to establish a continuous insect cell line have failed (Mitsuhashi 2002). However, progress in gene technology has enabled the immortalization of cells by the introduction of oncogenes into other invertebrates. Tapay et al. (1995) obtained a continuous cell line from shrimp by transforming primary cultured lymphoid cells with simian virus (SV)-40 (T) antigen. It has been established that its development and

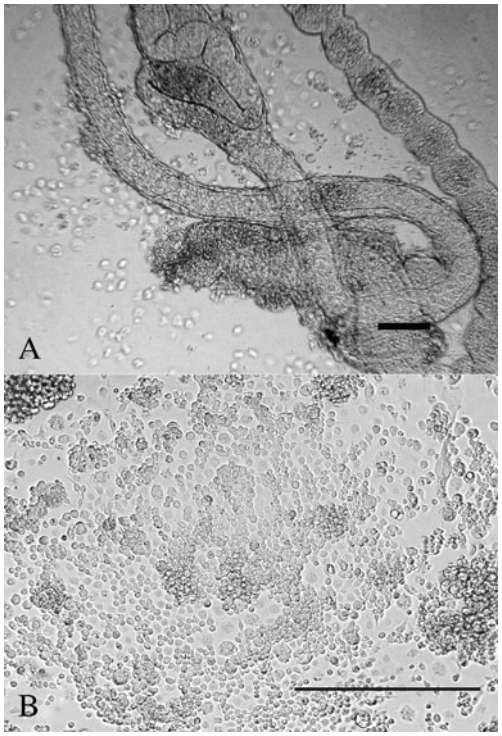

Figure 1. (*A*) Cell migration that occurred within 2 d of cell culturing. The *scale bar* is 100 μm. (*B*) Cell monolayer of IOZCAS-Spex 12. The *scale bar* is 400 μm.

progress are associated with the deregulation of many genes, as well as the mutation of oncogenes and loss of function of tumor suppressor genes (Early et al. 2008). However, the detailed molecular mechanism of establishing a cell line is not fully understood. Many important biological processes are involved in transformation and tumorigenesis, including cell cycle control, DNA damage repair, cell apoptosis, and signal transduction (Zhang et al. 2009a). The present study created a new insect cell line from primary cultured insect tissue using MNNG transformation and potentially describes a new method for establishing insect cell lines.

Successful cultures were initiated in June 2010. Initiation and maintenance of the cell lines were the same as previously described (Zhang et al. 2012). Cell migration occurred within 2 d of initiation of the cell culture. The migrated cells were either fibroblasts or hemocyte-like cells. They initially distributed themselves densely around tissue explants, gradually moved to the surrounding areas, and finally distributed over most of the flask after 30 d of culturing (Fig. 1*A*).

When the cells approached confluence, they were treated with MNNG at a concentration of 1.0, 3.0, or 5.0 μg/ml for 3 d. After incubation with MNNG, the cells were thoroughly washed three times with fresh medium and cultured in growth medium at 27°C (Ming et al. 2006). The cultures were fed with one-half volume of fresh medium every 7 d. In the cultures treated with 3.0 μg/ml MNNG, most cells survived to day 30, although a few cells detached and degenerated. The surviving cells, most of which were polygonal or round, remained healthy, and some cells grew in volume by day 60. Dividing cells were continuously present, and cell survival time was significantly increased by the stimulation of MNNG. The first subculture was carried out 60 d after MNNG treatment and after the cells had reached confluence. The contents of the culture flask were then transferred to a new flask containing 2.0 ml fresh growth medium. The interval of time between the initial subculture to the tenth passage ranged from 10 to 15 d, depending on the growth rate of the cells. After the 11th passage, the cells proliferated more rapidly, and thereafter, the interval of time between passages was 5 to 7 d using a ratio of cell suspension to fresh medium of 1:4 to 1:5 (Fig. 1*B*). The resulting cell line was designated IOZCAS-Spex 12.

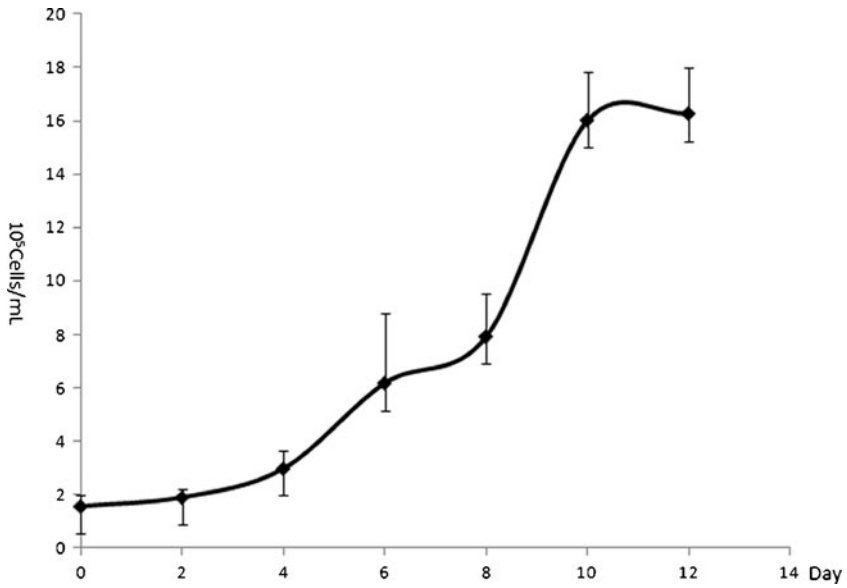

Figure 2. The growth curves of the new cell line, IOZCAS-Spex 12.

By acting directly on nucleic acids, MNNG is a strong mutagen that causes chromosomal DNA damage and can successfully induce in vitro cell transformation in several cell lines (Gichner and Veleminsky 1982; Du et al. 1984; Milo et al. 1992; Malik et al. 1997; Ming et al. 2006). Therefore, we used MNNG in our study to induce the division and transformation of cells cultured from the pupal ovary of *Spodoptera exigua*. In the presence of MNNG, the cultured cells grew well, and cells survived more than 30 passages. Both the concentration and timing of MNNG treatment were found to be important factors in determining the rates of proliferation and survival of the cultured cells. Cell division and the number of proliferating cells increased over time when the concentration of MNNG was 3.0 μg/ml. In the groups treated with 1.0 μg/ml MNNG, cells detached from the flasks by 30 d after MNNG treatment. Small rounded cells, but no dividing cells, were observed at day 60, and most cells in these cultures degenerated and eventually died at day 150. When the concentration of MNNG was 5.0 μg/ml or higher, most of the cells detached and degenerated by 3 d after treatment, showing that high concentrations of MNNG had a toxic effect on cells (Ming et al. 2006). In addition, the timing of stimulation seemed to be important. The most appropriate length of time for MNNG treatment in our study was 3 d after isolation. We found that MNNG treatment might overcome previously described limitations to obtaining continually proliferating insect cells and may open up the possibility of immortalizing isolated cells.

The morphology of the different cell types in the IOZCAS cultures was observed under phase contrast microscopy. The growth curve of the cell line was determined as previously described (Zhang et al. 2006a), and the cell population doubling time during the logarithmic growth phase was found to be 71 h (Fig. 2) (McIntosh and Ignoffo 1989). The cell line was found to have originated from *S. exigua* by utilizing the DNA fingerprinting-PCR

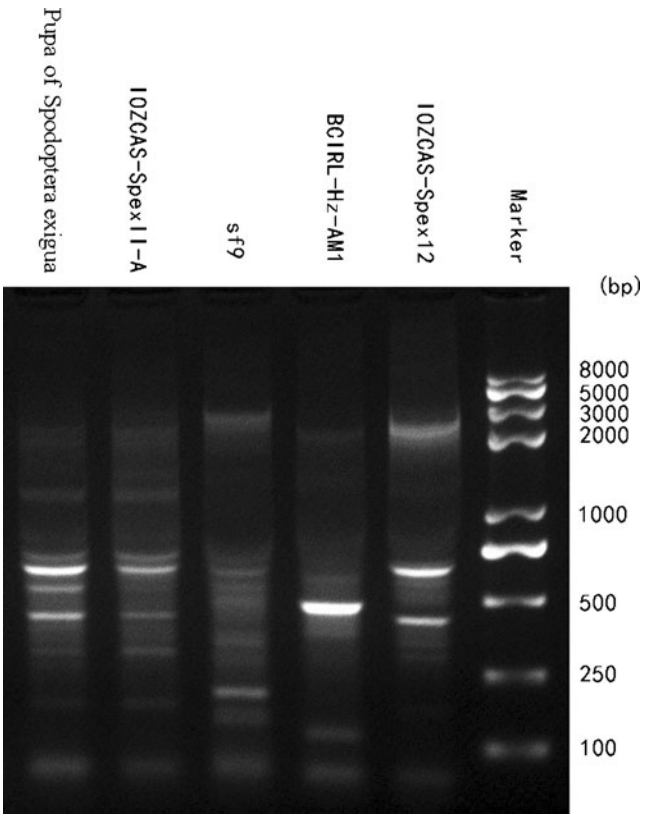

Figure 3. DAF-PCR profiles of IOZCAS-Spex 12 cell lines and other cell lines in our laboratory, including Sf9, BCRIL-HzAM1, and IOZCAS-Spex II-A. The major bands identified are shared between the cell lines and their host, while the other cell lines show different profiles from IOZCAS-Spex 12 cells.

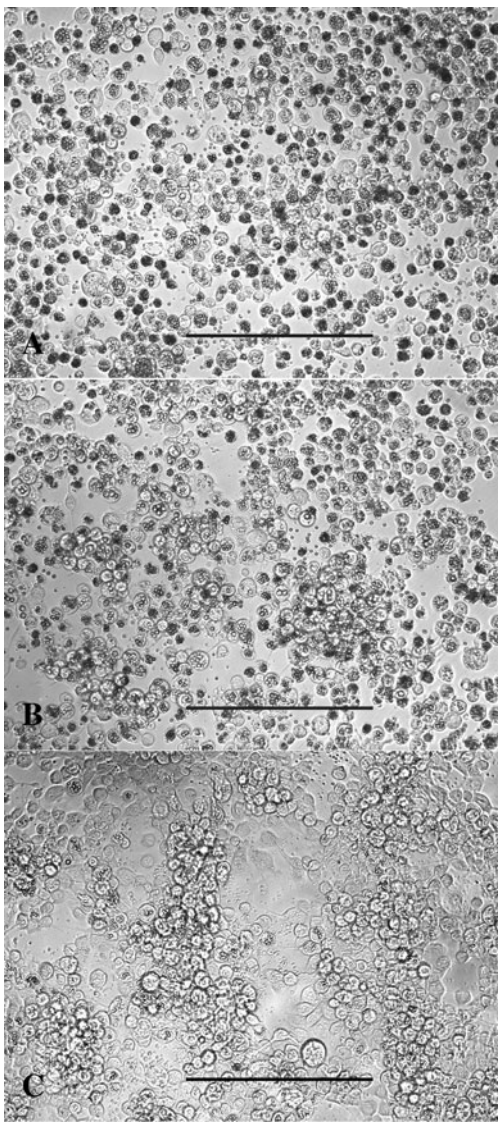

Figure 4. The majority of cells are infected with SeNPV (*A*), AcMNPV (*B*), and SpltNPV (*C*). The *scale bar* is 200 μm.

(DAF-PCR) method and comparing the band profiles of the cell line with its host, using the aldolase sequence as a primer (McIntosh et al. 1996; Liu et al. 2003). The PCR reaction conditions used in this study have been previously described (Zhang et al. 2006a). Three major bands at approximately 350, 450, and 700 bp of identity were shared (Fig. 3) between the new *S. exigua* cell line, another cell line from the same host (IOZCAS-Spex II-A), a cell clone from larval fat bodies of *S. exigua* Zhang et al. 2009b), and the homologous host (*S. exigua* pupa). A distinctive profile was seen compared to other cell lines maintained in the laboratory (Sf9, a cell line from *Spodoptera frugiperda*, Vaughn et al. 1977; BCIRL-Hz-AM1, a cell lines from *Helicoverpa zea*, McIntosh and Ignoffo 1983).

For determining the susceptibility of the cell line to nucleopolyhedroviruses, the viral inocula and inoculating process were as previously described (Zhang et al. 2006a, b). The viruses tested in this study include *S. exigua* nucleopolyhedrovirus (SeNPV), *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), *Spodoptera litura* nucleopolyhedrovirus (SpltNPV), *Helicoverpa armigera* nucleopolyhedrovirus (HaNPV), and *Hyphantria cunea* nucleopolyhedrovirus (HcNPV). Typical cytopathogenic phenomena, such as enlarged nuclei with numerous occlusion bodies formed in the nuclei at the late stage of infection, were observed in the cultures inoculated with SeNPV, AcMNPV, and SpltNPV (Fig. 4 *A–C*). Susceptibility of the cell line to infection by these three viruses was further confirmed using a bioassay. The IOZCAS-Spex 12 cells were not susceptible to infection by HaNPV or HcNPV.

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