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Adventitious viruses in insect cell lines used for recombinant protein expression

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ABSTRACT

Insect cells are widely used for recombinant protein expression, typically as hosts for recombinant baculovirus vectors, but also for plasmid-mediated transient transfection or stable genetic transformation. Insect cells are used to express proteins for research, as well as to manufacture biologicals for human and veterinary medicine. Recently, several insect cell lines used for recombinant protein expression were found to be persistently infected with adventitious viruses. This has raised questions about how these infections might affect research performed using those cell lines. Furthermore, these findings raised serious concerns about the safety of biologicals produced using those cell lines. In response, new insect cell lines lacking adventitious viruses have been isolated for use as improved research tools and safer biological manufacturing platforms. Here, we review the scientific and patent literature on adventitious viruses found in insect cell lines, affected cell lines, and new virus-free cell lines.

1. Introduction

The first immortalized insect cell lines were established in the 1960s [1]. Since then, hundreds of insect cell lines have been established from insects in many different Orders, with 940 different cell lines listed in the ExPASy Cellosaurus database at the time of this writing (http://web.expasy.org/cellosaurus). Several of these cell lines are routinely used to express recombinant proteins for basic research. In addition, some insect cell lines are now used to manufacture biologicals approved for use in human or veterinary medicine (see Ref. [2] for a recent list). The insect cell lines most commonly used for recombinant protein expression are derived from the Orders Lepidoptera (moths and butterflies) and Diptera (flies).

Lepidopteran cell lines are typically used as hosts for recombinant baculoviruses encoding the protein(s) of interest (recently reviewed by Ref. [3]). Alternatively, they can express recombinant proteins following transient transfection or stable genetic transformation with insect-specific expression plasmids encoding the protein(s) of interest (reviewed by Refs. [4,5]). Lepidopteran cell lines used for recombinant protein expression include lines derived from *Trichoplusia ni* (Tn), such as TN-368 [6], BTI-Tn5B1-4 (commercialized as High Five™; [7]), and Tni PRO™ (Expression Systems, LLC), lines derived from *Spodoptera frugiperda* (Sf), such as IPLB-Sf21AE (Sf21; [8]), Sf9 [9], and Sf900+ (commercialized as *expres*SF+™; [10]), and a few lines derived from *Bombyx mori* (Bm), such as Bm-N [11].

Dipteran insect cell lines are typically used for plasmid-mediated recombinant protein expression [4,5]. Like mammalian cells, they are not susceptible to baculovirus infection, but can be transduced with baculovirus vectors [12,13]. The dipteran cell lines most commonly used for recombinant protein expression are S2 and S2R+, both derived from *Drosophila melanogaster* (Dm) [14,15].

A problem with insect cell-based recombinant protein expression is most of the relevant cell lines are persistently infected with various adventitious viruses (see below). The absence of obvious cytopathic effects (CPEs), such as syncytia formation, nuclear hypertrophy, apoptosis, or inclusion body formation allowed these infections to go undetected for decades. The specific questions raised by the presence of adventitious viral contaminants in these cell lines focus on the validity of conclusions obtained using these lines for basic research and the potential biosafety hazards associated with their use as substrates for biologicals manufacturing.

In this review, we have compiled information available on persistent viral infections in insect cell lines used to produce recombinant proteins, which is scattered throughout the scientific and patent literature. In addition, we discuss new insect cell lines that are not contaminated with adventitious viruses. Finally, we briefly discuss

Abbreviations: BICS, Baculovirus insect cell system; Bm, *Bombyx mori* (silkworm); CPE, Cytopathic effects; Dm, *Drosophila melanogaster* (fruit fly); DES, *Drosophila* expression system; EST, Expressed sequence tag; Sf, *Spodoptera frugiperda* (fall armyworm); TEM, Transmission electron microscopy; Tn, *Trichoplusia ni* (cabbage looper); Hz, *Helicoverpa zea* (corn earworm)

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measures to prevent contaminating virus-free cell lines.

2. Sf-rhabdovirus (Mononegavirales; Rhabdoviridae)

2.1. Discovery and characteristics

Sf-rhabdovirus was independently discovered in various Sf cell lines at about the same time by three different groups. One group at the FDA's Center for Biologicals Evaluation and Research (CBER) discovered Sf-rhabdovirus in Sf9 and Sf21 cells by using a combination of degenerate PCR and massively parallel sequencing [16]. A separate group at Takeda Vaccines discovered Sf-rhabdovirus in a noroviral vaccine candidate produced by Sf9 cells infected with a recombinant baculovirus encoding a norovirus capsid protein. They found these cells produced not only the expected norovirus-like and baculovirus particles, but also a distinct, unexpected type of particles. These were subsequently identified as Sf-rhabdovirus by sequencing cDNA clones derived from particle RNA. A third group at the Scripps Research Institute found Sf-rhabdovirus sequences in virus-like particles produced by Sf cells infected with a recombinant baculovirus encoding the *Nudaurelia capensis* omega virus (NωV) capsid protein [17]. This group did not investigate further, but noted some assembled contigs "bore weak protein homology to other Rhabdoviridae and so may reflect a low-level infection with an unknown insect virus." Our follow-up indicated these assembled contigs were in fact derived from Sf-rhabdovirus.

Sf-rhabdovirus is a typical rhabdovirus with a single stranded, negative sense genome of approximately 13.5 kb encoding canonical rhabdoviral nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G), and RNA-dependent RNA polymerase (L), in this order. Some isolates encode an additional ORF, designated "X", which is located between the G and L ORFs and encodes a putative viroporin [18]. The X gene can be lost upon continuous passage of infected Sf cells and is not required for Sf-rhabdovirus infectivity *in vitro* [87]. In Sf9 cells, the Sf-rhabdovirus genome copy number ranges from 1.5 to 3.3 × 10^3 RNA molecules/cell. In addition, the virus is shed into the supernatant and Sf9 cell conditioned medium contains 0.9–2.9 × 10^7 Sf-rhabdovirus RNA genomes/mL [19].

2.1.1. Sf-rhabdovirus is likely derived from Sf caterpillars

Endogenous viral elements (EVE's) are virus-derived DNA fragments embedded in germline chromosomes. Sf cells contain 4 EVE's that are closely related to Sf-rhabdovirus, suggesting this virus was historically associated with Sf populations [2]. To determine if Sf-rhabdovirus is still associated with fall armyworm populations, we searched the transcriptome of Sf adults reared at Frontier Agricultural Sciences in Newark, DE, USA [20] for Sf-rhabdovirus sequences. We found sequences covering the entire Sf-rhabdovirus genome, including the X gene. This 'wild-type' Sf-rhabdovirus is 98.0% identical across 13238 nucleotides to the reference sequence (GenBank Acc. No. KF947078) reported by Ma et al. in 2014 (C. Geisler, unpublished). This new finding supports the ideas that (i) Sf-rhabdovirus can persistently infect Sf populations, and (ii) persistent infection of Sf caterpillars was the ultimate source of the infections recently detected in Sf cell lines.

2.2. Contaminated, susceptible, and refractory cell lines

The surprising discovery of Sf-rhabdovirus in cell lines used for biologicals manufacturing and basic research has stimulated interest in determining if other cell types are contaminated with and/or susceptible to Sf-rhabdovirus infection. The main experimental approach used to test for Sf-rhabdovirus contamination has been by probing for Sf-rhabdovirus RNA using RT-PCR. The approach used to assess susceptibility to Sf-rhabdovirus infection has been to perform infectivity assays, which involve adding conditioned cell medium containing infectious virus to cell cultures, allowing time for viral adsorption, removing the inoculum, and then serially passaging the cells and periodically assaying cell extracts and/or cell-free media for Sf-rhabdovirus RNA by RT-PCR. If a virus-specific PCR signal is observed and its intensity increases with time after inoculation, this is taken as evidence the virus has infected and replicated in the cells and they are susceptible. If the intensity of the virus-specific PCR signal decreases and eventually disappears, this is taken as evidence the cells are not susceptible to infection.

Generally, studies on Sf-rhabdovirus contamination and susceptibility revealed some lepidopteran insect cell lines in addition to those derived from Sf are contaminated with or susceptible to infection with Sf-rhabdovirus. It should also be noted that Sf-rhabdovirus has not been reported to cause CPEs in any cell line. Furthermore, as of this writing, no mammalian cell lines have been found to be contaminated with or susceptible to infection with Sf-rhabdovirus [16,19,87].

2.2.1. Contaminated insect cell lines

Various groups have now shown Sf-rhabdovirus contaminates Sf21 [8], $Sf9^{L5814}$[10], and Sf9 [9] cells [16,17,19,21,22]. Since $Sf9^{L5814}$ was derived from Sf9, and Sf9 was derived from Sf21, the presence of Sf-rhabdovirus in these daughter and granddaughter cell lines most likely reflects a persistent infection inherited from Sf21. In turn, this most likely reflects persistent infection of the insects from which this cell line was derived, as noted above.

Beyond Sf, it has been reported BCIRL-HS-AM1, a cell line derived from *Heliothis subflexa* [23], is contaminated with Sf-rhabdovirus [19]. In addition, *Bombyx mori* Bm-N cells [11] are contaminated with Sf-rhabdovirus based on the identification of a Bm-N EST (Genbank Acc. No. AK377209.1) identical to a portion of the Sf-rhabdovirus genome (C. Geisler, unpublished). We have extended this result by assaying low passage Bm-N cells obtained from the American Type Culture Collection (ATCC, Manassas, VA) using RT-PCR, which produced strong signals for the entire Sf-rhabdovirus genome (*A. Maghodia*, pers. comm.). A search of the *Spodoptera litura* SL221 cell line transcriptome [24] indicated this cell line is also contamined with Sf-rhabdovirus (*C. Geisler*, unpublished).

2.2.2. Susceptible insect cell lines

Infectivity assays have demonstrated Sf-RVN (Sf-rhabdovirus negative), which is an Sf cell line that is not persistently infected with Sf-rhabdovirus [21], is susceptible to experimental infection with Sf-rhabdovirus [87]. Similarly, BCIRL/AMCY-SE-E1, -E4, and -E5, which are three cell lines derived from *Spodoptera exigua* [25], were shown to be susceptible to experimental infection with Sf-rhabdovirus [19]. Together with the Sf-rhabdovirus sequences in the *Spodoptera litura* SL221 cell transcriptome [24], these observations suggest cell lines derived from *Spodoptera* are likely generally susceptible to Sf-rhabdovirus infection. In addition, the Sf-rhabdovirus contamination in Bm-N and BCIRL-HS-AM1 cells indicates some Bm and Hs cell lines are susceptible, as well.

Finally, considering (i) Sf caterpillars are contaminated with Sf-rhabdovirus, (ii) other lepidopteran rhabdoviruses are only distantly related [26], and (iii) several lepidopteran cell lines are susceptible to Sf-rhabdovirus infection, we conclude Sf-rhabdovirus in lepidopteran cell lines from species other than Sf most likely reflects cross-contamination in facilities where these cell lines were cultivated in proximity to Sf-rhabdovirus-contaminated Sf lines.

2.2.3. Refractory insect cell lines

Sf-rhabdovirus infectivity assays demonstrated *Trichoplusia ni* High Five™ [7], *Heliothis virescens* BCIRL-HV-AM1 [27], *Helicoverpa zea* BCIRL-HZ-AM1 and BCIRL-HZ-FB33 [28], *Anticarsia gemmatalis* BCIRL-AG-AM1 [23], and *Drosophila melanogaster* S2 and S2R+ [14,15] are not susceptible to Sf-rhabdovirus infection [16,19] [87]. These results suggest Sf-rhabdovirus has a relatively narrow host range, which is most likely restricted to a few lepidopteran insect species and cell lines.