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Research review paper

RNA interference technology to improve the baculovirus-insect cell expression system

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A B S T R A C T

The baculovirus expression vector system (BEVS) is a popular manufacturing platform for the production of recombinant proteins, antiviral vaccines, gene therapy vectors, and biopesticides. Besides its successful applications in the industrial sector, the system has also played a significant role within the academic community given its extensive use in the production of hard-to-express eukaryotic multiprotein complexes for structural characterization for example. However, as other expression platforms, BEVS has to be continually improved to overcome its limitation and adapt to the constant demand for manufacturing processes that provide recombinant products with improved quality at higher yields and lower production cost.

RNA interference, or RNAi, is a relatively recent technology that has revolutionized how scientist study gene function. Originally introduced as a tool to study biological and disease-related processes it has recently been applied to improve the yield and quality of recombinant proteins produced in several expression systems. In this review, we provide a comprehensive summary of the impact that RNAi-mediated silencing of cellular or viral genes in the BEVS has on the production of recombinant products. We also propose a critical analysis of several aspects of the methodologies described in the literature for the use of RNAi technology in the BEVS with the intent to provide the reader with eventually useful guidance for designing experiments.

1. Introduction

BEVS is a powerful tool for the production of recombinant proteins widely used in several areas of research and industry (Palomares et al., 2005). It is particularly favored over others platforms for the manufacturing of vaccines (Mena and Kamen, 2011), gene therapy vectors (Carinhas et al., 2009; Cecchini et al., 2008) and biopesticides (Reid et al., 2013). Like other platforms for recombinant protein expression, BEVS has been improved through the years thanks to the efforts of many research groups. From a bioprocess standpoint, most of those efforts have been directed to increase the production capabilities of the system. Research focusing on this goal is still ongoing, and revolves around several aspects including, mode of operation of the cultures (Elias et al., 2000; Petricevich et al., 2001; Wu et al., 1989), development of cell culture media (Chan and Reid, 2016) and understanding the biology and engineering host cells and viruses (Hitchman et al., 2010a, 2010b; van Oers, 2011).

Prior to the establishment of BEVS as an expression system, the molecular biology of both insect cells and baculovirus was an active area of research, which has been crucial for the inception and evolution of the system. Noteworthy, while working on baculovirus deletion mutants, Gale Smith and Max D. Summers came with the idea of replacing the coding sequence of the non-essential viral gene polyhedrin with the nucleotide sequence for a protein of interest, a concept that constitutes the core of the BEVS technology (Smith et al., 1983). Thenceforth, significant work has been focused, on the one hand, in modifying the backbone of the baculovirus to optimize recombinant protein production (Hitchman et al., 2010a, 2010b), achieve expression of multiple recombinant proteins from a single virus (Bieniossek et al., 2012) or enhance the stability of the vector. On the other hand, several groups have genetically modified insect host cells to improve the quality of the recombinant proteins by, for example, modifying the expression of cellular genes involved in the posttranslational processing of proteins (Harrison and Jarvis, 2006) or apoptotic responses (Lin et al., 2007).

Attempts to change the expression of cellular or viral genes in the BEVS are routinely accomplished by inserting or deleting gene coding sequences or modifying their regulatory units and evaluating the

Abbreviations: BEVS, Baculovirus expression vector system; CHO cells, Chinese hamster ovary cells; HEK, Human embryonic kidney cells; MIARE, Minimum Information About an RNAi Experiment; miRNA, Micro RNA; RNAi, RNA interference; shRNA, Short hairpin RNA; siRNA, Small interfering RNA

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impact that such gene has on the baculovirus infection process or production of recombinant proteins. Although reliable, this approach can be laborious, expensive and unsuitable for high throughput screenings (Novina and Sharp, 2004). Fortunately, during the last few years, new tools for the study of genes functions have emerged which represent valuable alternatives to the more traditional approach previously described.

Of particular interest is **RNA interference**(RNAi), a tool derived from the 1998 report by Fire and Mello where they described that injection of exogenous double-stranded RNA (dsRNA) into *Caenorhabditis elegans* led to the silencing of the endogenous homolog mRNA (Fire et al., 1998). The discovery of RNAi did not only changed the paradigm of the biological role of RNA but soon proved to be a powerful tool in several research areas including developmental biology, medicine, and biotechnology. RNAi pathway is well conserved among eukaryotes; hence, it has been possible to expand its use to several eukaryotic-based expression systems, such as Chinese Hamster Ovary (CHO cells) or Human Embryonic Kidney(HEK) cell lines (Hebert et al., 2009a, 2009b). In those, it has been shown that production of recombinant proteins can be enhanced by introducing or expressing dsRNA with a homologous sequence to a specific gene, for example, transcriptional regulators or apoptosis mediators.

Gene silencing through RNA interference has been extensively used in lepidopteran cell lines and baculovirus for different purposes. For example, to explore and better understand the different processes involved in the viral infection (Terenius et al., 2011); to protect insects of commercial importance from viral infections (Valdes et al., 2003); to construct baculovirus-based vectors for delivery of dsRNA in mammalian cells (Suzuki et al., 2008) and as a metabolic engineering tool to increase yields in BEVS. In this review, we focus on those reports were RNA interference was used as a mean to improve the quality or quantity of recombinant proteins produced in the BEVS.

2. The RNAi pathway

2.1. General considerations

Since the discovery of RNAi, a lot of efforts have been dedicated to understand its biological role and to identify its main components. Enabled by the purification of key enzymes, the development of RNA interference cell-free systems and the mapping of genes associated with RNAi pathway, a broad understanding of RNAi mechanism, function, and limitations has been achieved. This section will briefly cover the general pathway, and basic component associated with the RNA mediated silencing; whereas several excellent reviews are available for a more detailed understanding of the RNA-mediated silencing (Carthew and Sontheimer, 2009; Ghildiyal and Zamore, 2009; Liu and Paroo, 2010).

The canonical RNA interference pathway is shown in Fig. 1. RNAi is triggered in response to exogenously introduced or endogenously transcribed dsRNA; in the first case, dsRNA can be microinjected or transfected into cells, while in the second, dsRNA is synthesized by the cell using as templates genomic sequences from viruses or plasmids, in the form of inverted repeats or self-annealing transcripts. Once dsRNA is in the cytoplasm, **Dicer** - a type III RNA endonuclease- cleaves it to produce short 21–25 nt long fragments, paired in a way that leaves two nucleotide overhangs at the 3′ ends of both strands (Ghildiyal and Zamore, 2009). Dicer family of enzymes can produce two kinds of cleavage products: **micro (mi)RNAs** and **small interfering (si)RNAs**. In some species, a single Dicer can produce both types of short RNAs, while in others the task is divided between two different enzymes: Dicer-1 for miRNAs and Dicer-2 for siRNAs. These short RNA fragments are loaded into the RNA-mediated silencing complex- or *RISC*- and are the active, sequence-specific mediators of RNAi (Elbashir et al., 2001). RISC is a macromolecular complex with endonuclease activity responsible for degrading the targeted transcript; **Ago**, a protein member of the Argonaute superfamily, confers RISC with its catalytic activity. Loading of siRNA or miRNA into RISC requires the participation of several cofactors which varies from species to species and depends on the type of molecule being loaded. In the canonical pathway, protein **R2D2** is responsible for loading siRNAs while another protein, **loquacious**, does it for miRNAs. In both cases, these RNA-binding proteins sense thermodynamic asymmetries in the strands of the siRNA/miRNA and bind to the strand with the more stable 5′-end, thus defining which strand will be loaded into Ago. This strand, called the guide strand, remains bound to Ago, while the other is degraded (Schwarz et al., 2003). The activated RISC targets mRNAs with a complementary sequence to the one in the guide strand. Typically, siRNA-directed RNAi results in degradation of the mRNA, while miRNA-directed gene silencing is established by translational inhibition (Carthew and Sontheimer, 2009).

Although the core components of RNA-mediated gene silencing are well conserved among eukaryotes, there are some species-specific deviations from the canonical pathway. Many are related to the number of paralogs of *ago* and *dicer* genes in each organism and also the extent to which these paralogs specialize in using siRNA or miRNA to guide the silencing. In other cases, the importance of particular RISC-related proteins seems not to be the same across different species. For example, *Drosophila r2d2* null mutants are unable of mounting a strong RNAi interference response, while similar mutants in mouse derived cell lines can effectively silence the expression of a target upon dsRNA treatment. RNAi mechanisms in insect species, and their derived cell lines, used in the baculovirus expression system have its particularities too; knowing and understanding them is essential for the efficient use and further improvement of RNAi in the BEVS.

2.2. Particular aspects of RNAi pathway in cell lines used in the BEVS

Cell lines employed in BEVS originate from *Lepidopterans*, an order of arthropods in which systemic RNAi in-vivo has been difficult to achieve consistently (Terenius et al., 2011), although this is not the case for reports of RNA-mediated silencing performed in vitro in cell cultures. This difficulty has given rise to numerous studies aiming to identify limiting factors of the RNAi pathway in *Lepidopterans* (Swevers et al., 2011; Terenius et al., 2011).

For in-vitro cell cultures, the limiting factor in RNAi experiments remains the delivery of dsRNA into the cell cytoplasm, which means uptake of exogenous RNA from the environment, i.e. the culture media (Yu et al., 2013). The mechanism of cellular uptake of dsRNA has been thoroughly studied in *C. elegans.* The cells of this nematode express a protein called SID-1 which is capable of transmembrane channel-mediated uptake of dsRNA into cells (Wynant et al., 2014). Because of SID-1, dsRNA can enter into *C. elegans* cells without the need of transfection or microinjection; a technique called “RNAi by soaking.” *sid-1* homologous sequences have been identified in several lepidopterans and other insects. However, it is unclear if their expression products are in full involved in the transport of dsRNA since silencing of *sid1*-like sequences in lepidopterans like *T. castaneum* had no adverse effect on the RNAi response (Tomoyasu et al., 2008). An alternative mechanism for the uptake of dsRNA in lepidopterans could be one similar to that found in *D. melanogaster*. Drosophila S2 cells lack *sid-1*-like sequences but can up-take dsRNA from the media by a receptor-mediated endocytosis process, in which a scavenger receptor plays an essential role. Although the actual mechanism of dsRNA uptake in insect cells is not well understood yet, several research groups are attempting to make this step more efficient. In two different reports, the *Spodoptera frugiperda* derived cell line **Sf9** (Xu et al., 2013b), and the *Bombyx mori* derived line cell **Bme21** (Kobayashi et al., 2012) were transformed to express *C. elegans* SID-1 ectopically. Transformed stable cell lines were capable of uptaking “naked” dsRNA from the media without the use of transfection reagents, and silencing efficiencies of exogenous and endogenous genes increased to 90%. However, some of their clones

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