Fabiana Carreira Fernandes

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Establishment and evaluation of flexible insect cell lines for rapid production of recombinant proteins

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Orientador: Paula Marques Alves, PhD, ITQB-UNL
Co-orientador: Ana P. Teixeira, PhD, ITQB-UNL
Co-orientador: Kristala J. Prather, Professor, MIT

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Fabiana Carreira Fernandes

Faculdade de Ciências e Tecnologia – Universidade Nova de Lisboa

à minha mãe e madrinhas,

por todo o vosso amor e apoio

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RESUMO

O sistema de infeção por baculovírus em células de insecto é uma plataforma predileta para a produção de proteínas recombinantes devido às suas capacidades eucarióticas de processamento de proteínas bem como os seus tempos relativamente curtos para o desenvolvimento do processo. Contudo, a natureza lítica da infeção afecta negativamente a qualidade do produto final e aumenta os desafios do processo de purificação. Desta forma, a grande motivação por detrás deste trabalho foi desenvolver uma plataforma alternativa a este sistema de infeção que também consiga explorar e beneficiar do potencial de produção das células de insecto. A estratégia adoptada é baseada na tecnologia de troca de cassetes mediada por recombinases (RMCE) de forma a potenciar o rápido e flexível desenvolvimento de linhas celulares. Esta tecnologia consiste em etiquetar aleatoriamente o genoma da célula com um gene repórter e fazer a triagem dos loci de maior expressão de forma a depois re-usar esses mesmos loci para a expressão de diferentes proteínas de interesse. Este sistema de recombinação foi implementado pela primeira vez em células *Sf9* no âmbito desta tese, usando a recombinase Flipase (*Flp*), derivada de leveduras, para promover a recombinação através de alvos especificamente reconhecidos por ela. A prova deste conceito foi feita através do uso de proteínas repórter. A identificação dos clones cuja etiquetagem foi feita em loci favoráveis à recombinação mediada pela *Flp,* permite a sua re-utilização para expressão de EGFP a níveis semelhantes aos obtidos pela proteína repórter vermelha que tinha sido previamente codificada nos respectivos loci em questão. A melhor linha celular RMCE-*Sf9* foi avaliada em relação à produção de proteínas complexas, nomeadamente partículas semelhantes a rotavírus. Apesar de produtividades especificas serem menores em relação às apresentadas pela infeção de baculovírus, os títulos da proteína final foram comparáveis. Para além disto, um protocolo para o sorteamento de células através de fluorescência ativa foi desenvolvido de forma a promover a seleção de células *Sf9* e High Five com maior expressão de dsRed etiquetada no genoma, de forma a acelerar o desenvolvimento de linhas celulares baseadas em RMCE. Resumindo, as linhas celulares de insecto RMCE desenvolvidas representam plataformas poderosas para a producao de proteínas recombinantes.

Termos chave: sistema de troca de cassete mediado pela flipase, (*Flp*) expressão estável em células de insecto, infeção por baculovíus e integração localizada.

ABSCTRACT

The insect cell/baculovirus infection system is a preferred platform for the production of recombinant proteins due to its eukaryotic protein processing capabilities and relatively short process development timelines. However, the lytic nature of virus infection can negatively impact final product quality and increase the challenges of the downstream process. Therefore, the main motivation of this work was to develop an alternative platform to the infection system that would likewise explore and benefit from the manufacturing potential of insect cells. The adopted strategy relies on recombinase cassette exchange (RMCE) technology to enable flexible and fast establishment of production cell lines. This technology consists in tagging randomly the cell genome with a reporter gene and screen for high expressing *loci* and then, to re-use these loci for expression of different proteins of interest. The recombination system implemented in *Sf9* cells for the first time in the scope of this thesis was based on the yeast-derived Flipase (*Flp*) recombinase, which promotes recombination between *Flp* recognition target sites. The proof of concept was performed with reporter proteins. The identification of clones tagged in loci supporting *Flp*-mediated recombination allowed their re-use for expression of EGFP at levels correlated to those of the red reporter protein previously encoded in the corresponding loci. The best RMCE-*Sf9* cell line was evaluated regarding the production of complex proteins, namely rotavirus core-like particles. Albeit lower cell specific productivities with respect to those presented by baculovirus infection, the protein titers achieved were comparable. Furthermore, a fluorescence-activated cell sorting protocol was developed enabling to sort high-expressing dsRed-tagged *Sf9* and High Five cells, contributing to speed up RMCE-based cell line development. Overall, the developed RMCE-insect cell lines represent powerful platforms for fast production of recombinant proteins.

Keywords: Flipase-mediated cassette exchange system, stable expression in insect cells, baculovirus infection and targeted integration

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ABBREVIATIONS

Abbreviation	Full form
AcMNPV	Autographa California multiple nucleopolyhedrovirus
BEVS	Baculovirus expression vector system
BmNPV	*Bombyx mori* nucleopolyhedrovirus
CCI	Concentration at infection
CHO	Chinese hamster ovary
CO_2	Carbon dioxide
$CuSO_4$	Copper (II) sulfate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dsDNA	Double strand DNA
dsRed	Discosoma sp. Red fluorescent protein
ECACC	European Collection of Cell Cultures
EGFP	Enhanced green fluorescent protein
EMA	European Medicine Agency
FACS	Fluorescent activated cell sorter
Flp	Flipase
Flpe	Flipase enhanced recombinase
Flpo	Codon optimized version of Flpe
FRT	Flipase recombination target
GOI	Gene of interest
H^1-NMR	Hydrogen – Nuclear Magnetic Resonance
Hi5/High Five	*Trichoplusia ni* cell lines
HPV	Human papillomavirus
HR	Homologous recombination
HSP70	Heat shock protein 70
IE	Immediate early promoter
IR	Illegitimate recombination
IRES	Internal ribosome entry site
JE	Japanese encephalitis
MOI	Multiplicity of infection
Mtn	Metallotienin
O_2	Oxygen
OpMNPV	Orgyia pseudotsugata multicapsid nucleopolyhedrovirus
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
polh	Polyhedrin promoter
rBVs	Recombinant baculovirus vectors
RLPs	Rotavirus like particles
RMCE	Recombinase mediated cassette exchange
RTs	Recombination target site
Sf9	*Spodoptera frugiperda*
SSR	Site specific recombinase
TALEN	Transcription activator-like effector nucleases
TOH	Time of harvest
VLP	Virus like particle
VP	Viral particle
ZFNs	Zinc finger nucleases

Chapter I

Introduction

This Chapter is adapted from the manuscript

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and book chapter

Fernandes F, Teixeira AP, Carinhas N, Carrondo MJT, Alves PM. Production of complex virus-like particles in insect cells. Future Medicine, *in press*

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1. Market of insect cell expression

The rising demand for highly purified proteins in enough quantity for therapeutic use, and for functional and structural studies outweighed the need for recombinant production in living systems, since the isolation of proteins derived from natural sources is often scarce. Advances in recombinant DNA technology allowed for significant improvements on recombinant production capacity and the development of production processes that ultimately permitting the production of large amounts of high quality recombinant proteins in a safer, purer and more effective manner using for that purpose different expression systems. Although many refinements have been implemented into these systems towards protein expression optimization, still it is not of usual practice to have a single cell host for recombinant protein production (Palomares, Estrada-Mondaca et al. 2004; Hunt 2005). Despite prevalently presented as a universal host for heterologous protein expression, prokaryotic expression systems still fail to fold properly a number of biomedical relevant proteins, limiting its potential endeavors at the biopharmaceutical field. Alternatively, in order to produce such elaborated proteins that usually contain complex post-translational modifications the reinforced development of eukaryotic expression systems has proceeded in the last decades to cope with the fast growing biopharmaceutical market requirements. Although protein production in eukaryotic systems is generally more time-consuming and expensive, such constraints were obviated with the profitable and exclusive post-translational machinery. The most common eukaryotic expression platforms currently include yeast, baculovirus expression vector systems (BEVS), and mammalian cell systems. Yeasts, such as *Pichia pastoris* and *Saccharomyces cerevisiae,* offer some advantages comparable to prokaryotic systems, namely easy genetic manipulation and ability to perform high-density cultures while also being relatively inexpensive and time efficient; in addition to an eukaryotic secretory pathway (Mattanovich, Branduardi et al. 2012). Still, the quality of glycosylated proteins recombinantly produced in yeasts is adversely affected by the occurrence of hyperglycosylation, limiting their use as potential therapeutic proteins, as well as towards more complex crystallization endeavors (Darby, Cartwright et al. 2012). As a consequence, mammalian cell expression has become the dominant recombinant protein production system for clinical applications because of its capacity for post-translational modification and human protein-like molecular structure assembly, accounting for the biggest share of today's biopharmaceutical market. In particular, Chinese hamster ovary (CHO) cells are the most dependable host cells for the industrial production of recombinant proteins because of their advantages in producing complex therapeutics and manufacturing capability. Furthermore, CHO cells have an established history of regulatory

approval for recombinant protein expression. Most importantly, these cells perform human-compatible, post-translational modifications, thereby improving therapeutic efficacy, protein longevity and reducing safety concerns.

The BEVS is the most prominent eukaryotic system for the production of challenging proteins that are unable to be effectively synthesized in prokaryotic hosts (Figure 1). As a result, more than 500 recombinant proteins from different species have been expressed using the BEVS, with yields ranging between 5 to 70% of the total intracellular protein content (O'Reilly, Miller et al. 1994) and final titers obtained up to 500 mg/L (Summers and Smith 1987). The simple and homogeneous glycosylation pattern is particularly suitable for production of proteins for crystallization studies (Tomiya, Narang et al. 2004). Noteworthy, its capacity to express multiple proteins that assemble into multi-protein complexes (Hu 2005; Kost, Condreay et al. 2005), makes this system a fundamental tool for protein-protein interactions research or virus-like particle- (VLP), -based vaccines development (Roldao, Mellado et al. 2010; Fernandes, Teixeira et al. 2013). However, while the simple glycosylation provided is adequate for the production of proteins for structural biology, can still pose some obstacle for the production of biopharmaceuticals, namely its biomedical use. Insect cells mostly produce simpler N-glycans with terminal mannose residues, whereas mammalian cells produce complex N-glycans containing terminal sialic acids. This structural difference compromises the in vivo bioactivity of glycoproteins and can potentially induce allergenic reactions in humans (Harrison and Jarvis 2006). Thus, only a small percentage of licensed protein-based biopharmaceuticals are produced using the baculovirus/insect cell system. The first licensed human vaccine produced in insect cells was Cervarix^{TM}, a human papillomavirus (HPV) VLP vaccine against cervical cancer, commercialized by GlaxoSmithKline.

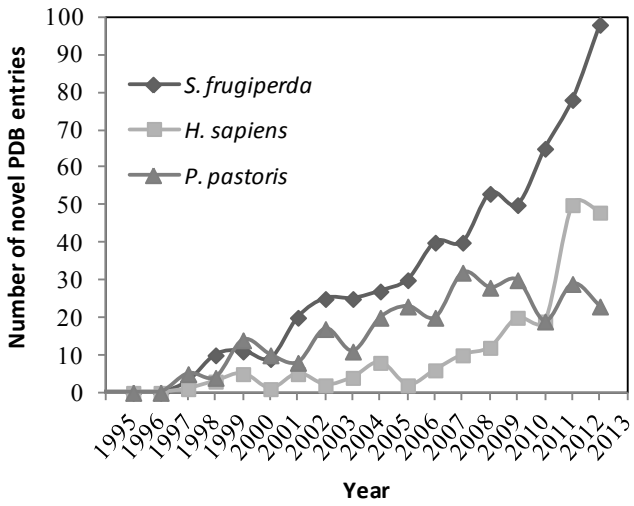

Figure 1. Predominant eukaryotic expression systems aiming determination of protein structure. The graph shows the total number of protein chains deposited in the Protein Data Bank (PDB) per year of deposition, whose expression host has been identified as Mammalian (*H. sapiens*), Insect/Baculovirus (*S. frugiperda*) and Fungi/Yeast (*P. pastoris*). *Adapted* from (Assenberg, Wan et al. 2013).

More recently, another two additional human vaccines produced through this system received market approval: a recombinant trivalent hemagglutinin vaccine against influenza virus, FluBlock®, from Protein Science Corporation (Cox and Hollister 2009), and Provenge®, a vaccine against prostate cancer (Cox 2012). In addition, several BEVS-derived veterinary vaccines are also commercially available (Mena and Kamen 2011). FluBlock® is the world's first recombinant protein-based seasonal influenza vaccine, while Provenge is an autologous vaccine that boosts the immune system to attack prostate cancer cells, due to previous exposure to a recombinant fusion protein produced by the BEVS. Beyond the vaccine field, the BEVS has been widely used to produce several gene therapy vectors in clinical trials; including, the firstly approved gene therapy by European Medicines Agency (EMA), Glybera® developed by UniQure, which is based on a non-replicative adeno-associated viral vector engineered to express lipoprotein lipase in the muscle for the treatment of lipoprotein lipase deficiency (Bryant, Christopher et al. 2013). Despite all these advances within the pharmaceutical regulated market, mammalian cell culture is still the prevailing method for biopharmaceutical protein production. Namely, CHO, HEK-293 and BHK are the preferred hosts for the production of recombinant proteins while VERO, MDCK, MRC-5 and WI-38 have been assessed for their ability as production hosts for vaccines.

2. The baculovirus expression vector system (BEVS) at a glance

Routine insect cell culture has been possible since the middle of the past century, with initial efforts focused on the study of insect physiology, as well as the *in vitro* production of baculoviruses aimed at biological control of insect pests in cereal fields. When it became possible to genetically modify baculoviruses, insect cell culture moved into the mainstream of biotechnology applications. Their use as gene delivery vectors enabled the heterologous expression of proteins in a lytic system involving the infection of specific insect cell lines. Nowadays, the BEVS stands for a powerful and versatile expression platform since it can express gene products from almost any organism and from any cellular location within very short time frames. Unlike mammalian cell culture systems used in biopharmaceutical industry, it is based primarily on engineering the vector and not the host cell line, shortening drastically the time from gene cloning to protein production. Over the years, this expression system has been continuously improved towards more efficient production processes, in order to cope with inherent upstream and downstream processing challenges, as detailed below.

2.1. Baculovirus vector design

Baculoviridae are from a diverse family of arthropod pathogens containing a large dsDNA genome with 90 to 180 genes that varies in size between 88 to 200 kbp (Summers and Anderson 1972; Burgess 1977). Insights into the molecular biology of baculoviruses revealed that not all genes were essential for virus entry and replication in cultured insect cells; such findings have promoted the development of the BEVS in the early 1980s (Smith, Summers et al. 1983). For instance, the matrix proteins polyhedrin and P10, which confer protection to baculoviruses by providing resistance against harsh environmental conditions (extreme temperatures and UV light) (Rohrmann 2008), are not required for baculovirus replication in cell culture. As the promoters of the genes coding for these matrix proteins are very strong, the target recombinant genes are commonly placed under the control of the *polyhedrin (polh)* or *p10* promoters (Kelly and Lescott 1981). The *Autographa california* multiple nucleopolyhedrovirus (AcMNPV) is the prototype baculovirus generally used as vector to produce recombinant proteins. Recombinant baculovirus vectors (rBVs) are simple to construct and have a large packaging capacity (up to 38 kb (Cheshenko, Krougliak et al. 2001), granting the ability to express multi-protein complexes or several distinct proteins using the same vector, in a more rapid and efficient manner in comparison to other available expression systems (Hu 2005; Kost, Condreay et al. 2005). To express multiple genes in the same vector, the stronger promoters *polh* and *p10* are normally

repeated. As the stability of polycistronic vectors may be affected by promoter sequence repetitions, identical promoters have been segregated into different transcription directions to avoid juxtaposition of the same promoters (Belyaev and Roy 1993). Improved stability has also been achieved by coupling the expression of the gene-of-interest to baculovirus essential genes (such as gp64) using an internal ribosome entry site (IRES) element to achieve a single bicistronic transcript (Pijlman, Roode et al. 2006). The emergence of defective viruses along passages was delayed using this strategy. To improve product quality and quantity, deletions to the baculovirus genome have been performed, namely of DNA sequences coding for proteases (eg. *v-cathepsin*) or baculovirus proteins which are thought to obstruct the secretory pathway (eg. *chiA* chitinase), as well as other non-essential genes such as p10, p26 and p74 (Kaba, Salcedo et al. 2004; Hitchman, Possee et al. 2010).

Several commercial baculovirus vectors are commercial available to attain simultaneous expression of multiple recombinant proteins (Possee and King 2007; Trowitzsch, Bieniossek et al. 2010). One of the most recently developed expression system is MultiBac, which is an extension of the polycistronic vector concept. While previous systems integrate the recombinant genes into the polyhedrin locus, the MultiBac system allows for additional integration in a second locus formed by the replacement of two genes (*v-cath* and *chiA*) by a Cre-loxP site-specific recombination sequence. Therefore, this system has an increased packaging capacity and less proteolytic activity (Senger, Schadlich et al. 2009; Bieniossek, Imasaki et al. 2011). For complex glycosylated protein targets, a more sophisticated version of the MultiBac was created by integrating sequences of the *N*-acetylglucosaminyltransferase II and β1,4-galactosyltransferase I enzymes into the baculovirus genome (Palmberger, Wilson et al. 2012).

2.2. Insect cell culture and infection: bioengineering issues and caveats

Lepidopteran cell lines are the natural hosts for viruses from the *baculoviridae* family. The most used insect cell lines include *Sf9* (derived from *Spodoptera frugiperda*) and BTI-Tn5B1-4 (or High Five™, derived from *Trichoplusia ni*). *Sf9* are preferentially used for the generation and propagation of rBVs, while High Five™ cells have been reported to attain higher specific recombinant protein yields (Wang, Granados et al. 1992; Rhiel, Mitchell-Logean et al. 1997; Krammer, Schinko et al. 2010), most probably due to their larger cell size. Recent reports claim that High Five™ cells stocks were contaminated with latent nodavirus, questioning the integrity of the original stock of these cells. Thus, for safety concerns such cells should not be further considered for production of biological substances for human use (Merten 2007). Comparing to

mammalian cell lines, insect cells present a more efficient metabolism, with much lower secretion of metabolic by-products; only at high cell densities with poor oxygen supply some lactate can start to be released (Bedard, Tom et al. 1993). In addition, insect cells can be easily adapted to suspension cultures, either in serum-containing or serum-free media, they do not require to be cultured in CO_2 incubators, and can resist to fluctuations in temperature (between 18 ºC and 35 ºC) and osmolarity (between 250 and 450 mOsm) (Kurtti, Chaudhary et al. 1975; Wilkie, Stockdale et al. 1980; Hu and Oberg 1990). Despite of their sensitiveness to shear stress mainly caused by sparging or bubble entrainment during agitation (Murhammer and Goochee 1990), the addition of non-ionic copolymers such as pluronic F-68 guarantees a lower culture medium surface tension, allowing the cells to increase their cell membrane rigidity and resistance to hydrodynamic forces. Finally, in terms of product quality, insect cells are not naturally capable to perform human-like glycosylation; their N-glycosylation pathway differs significantly from that found in mammalian cells, which compromises the *in vivo* bioactivity of glycoproteins, hence reducing their therapeutic value. Over the past decade, several attempts have been made to bring closer the N-glycan profiles provided by insect and mammalian cells (Jarvis and Finn 1996; Harrison and Jarvis 2006). In fact, a commercially modified *Sf9* cell line is now available (Mimic Sf9, Invitrogen), obviating its use for the production of therapeutic proteins.

In the scope of BEVS bioprocesses, different culture strategies have been explored aiming at improving final product yields (Caron, Archambault et al. 1990; Caron, Tom et al. 1994; Ikonomou, Schneider et al. 2003). In particular, fed-batch cultures have allowed more productive infections by supplementing nutrients such as glucose, amino acid sources, lipids and vitamins that could otherwise become limiting (Chan, Greenfield et al. 1998; Elias, Zeiser et al. 2000). For instance, Bédard et al. (Bérard, Perret et al. 1997) reported that high specific protein yields obtained at lower cell concentration at infection (CCIs) could be restored when Sf9 cells were infected at up to 11.5 $\times 10^6$cell/mL by the addition of yeastolates and a cocktail of amino acids, thus leading to higher volumetric yields. This result suggested that it is nutrient depletion and not the accumulation of inhibitory byproducts that limits protein production in batch cultures of Sf9 cells. Operation under perfusion mode allows pushing even further CCIs and the total product yield given the continued renewal of "exhausted medium", but it also stretches further the cost for large scale production (Maranga, Cruz et al. 2002). Moreover, its scalability is dependent on efficient cell retention devices that often damage cellular integrity with direct consequences on product yields and costs (Caron, Tom et al. 1994).

The multiplicity of infection (MOI), CCIs and time of harvest (TOH) are key bioprocess parameters in baculovirus infection with influence on productivity and quality of protein targets. Infection at low CCIs with MOIs lower than 1 is the strategy most often employed to produce recombinant proteins using the BEVS. As not all cells are infected after virus addition (asynchronous infection), non-infected cells continue to grow until they are infected by the first progeny viruses, thus permitting multiple cycles of infection and longer bioreaction times (Wong, Peter et al. 1996; Roldao, Vieira et al. 2007). Low MOIs are also employed when the aim is to amplify the baculovirus stock as this strategy keeps the accumulation of defective viral particles to a minimum (Zwart, Erro et al. 2008). However, long bioreaction times can compromise the integrity of the final protein products due to increased exposure to proteases, which can be mitigated by addition of anti-protease cocktails. In contrast, high MOIs demand large viral stocks and are associated with shorter productive periods and lower volumetric yields (Radford, Cavegn et al. 1997).

As noted previously, the use of polycistronic vectors has become popular to express different protein subunits in the same infected cell in opposition to the use of multiple monocistronic vectors that normally result in an uneven distribution of viruses entering each cell. In this sense, avian influenza VLPs are a good example of how the use of a tricistronic baculovirus was able to produce correctly formed VLPs in contrast to the co-infection methodology (Pushko, Tumpey et al. 2005; Prel, Le Gall-Recule et al. 2007). Nevertheless, more recent strategies have used co-infection either with a cocktail of mono and bicistronic baculoviruses (Wen, Ye et al. 2009) or individual monocistronic baculoviruses (Krammer, Schinko et al. 2010), and both have shown successful production of recombinant influenza VLPs. In the literature, extensive argumentation has been presented focusing on failures, optimization and effectiveness of both strategies (Vieira, Estevao et al. 2005; Roldao, Vieira et al. 2007).

Another problem affecting the expression of recombinant proteins is the low fraction of soluble and/or correctly assembled protein. This issue has been addressed by co-expressing chaperones or foldases that enable correct folding and post-translational processing, increasing the secretion of correctly folded proteins. The co-expression of chaperones is reported to be more efficient when using a rBV than when stably integrated in the cell genome (Kato, Murata et al. 2005), most likely because expression from the host cell genome is inhibited during the late stages of infection when rBV-driven expression takes place. In some cases, the use of baculovirus early promoters (eg. ie-1, ie-2) have been shown to produce higher quantities of biologically active proteins, as they are expressed at earlier stages in infection when the cellular protein processing machinery has not yet

been compromised (Jarvis and Summers 1989). Although these promoters are intrinsically weaker, the addition of baculovirus regulatory elements upstream or downstream to the promoter sequence can be a solution to enhance expression rate ((Guarino, Gonzalez et al. 1986; Ishiyama and Ikeda 2010). Still another alternative is activation of transcription at earlier times post-infection with the use of synthetic versions of very late promoters. These synthetic promoters are obtained with the insertion of specific mutations into very late baculovirus promoter sequences (Rankin, Ooi et al. 1988). In fact, these hybrid promoters revealed to be quite useful for the production of enveloped VLPs with yields superior to those obtained using very late promoters (Guo, Lu et al. 2003; Kang, Quan et al. 2005; Ye, Lin et al. 2006).

2.3. Major challenges in downstream processing

One of the critical aspects of using the BEVS for cost-effective production of biopharmaceuticals, such as vaccines, is the co-production of high titers of rBVs as reviewed in (Vicente, Mota et al. 2011). Baculovirus vectors cannot effectively replicate in mammalian cells and are found in numerous vegetables that are regularly ingested by humans without known adverse effects, thus they are considered relatively safe. However, although the genomic integration of rBVs into the genome of animal cells has only been reported under selection conditions, the spontaneous integration of rBVs into the genome of a patient cannot be ruled out (Jarvis and Summers 1989). Furthermore, rBVs have shown adjuvant activity: if not removed/inactivated they may induce unwanted immunological activity (Guarino, Gonzalez et al. 1986). Thus, rBV as well as host cell DNA contaminations need to be efficiently addressed in order to obtain vaccines of clinical grade. Conventional ultracentrifugation techniques based on density gradients (eg. sucrose) are unable to provide VLPs-based vaccines with acceptable rBV removal as their densities are very similar. Moreover, these techniques are time- and labor-intensive which hampers straightforward scale up. Novavax developed a typical scalable purification process for BEVS-derived influenza VLPs. It includes tangential flow filtration for cell removal followed by concentration/diafiltration to remove media components and cell debris. Based on charge differences between VLPs, rBVs and contaminant DNA, ion exchange chromatography was used for their separation. Residual rBVs are inactivated by treatment with beta-propiolactone and the final polishing is performed with size-exclusion chromatography to remove residual host contaminants (Pincus, Boddapati et al. 2010). Although this purification process inactivates rBVs, it does not completely remove rBVs particles. Also, because the VLPs bud from the insect cells, baculovirus envelope proteins (e.g. gp64) and Sf9 cell proteins (e.g. tubulin, actin, Hsp70 and several housekeeping proteins) can in some cases

represent 25% of the total protein content of the Novavax vaccines in development (Pincus, Boddapati et al. 2010).

To prevent the appearance of rBVs in the VLP bulk, a novel non-replicative baculovirus vector was engineered by deleting a gene (*vp80*) that is essential for viral protein cleavage, maturation and assembly, as well as release of BVs from infected cells (Marek, van Oers et al. 2010). The full development of this system also needs a vp80-transcomplementing cell line for the efficient propagation of the replication-defective viruses. In time, this could represent a viable strategy for virus-free production of target proteins and VLPs.

Finally, to further increase vaccine quality, reprocessing of VLPs after purification is a common practice towards uniform particle morphology and maximized stability; both insect cell- and yeast-derived HPV VLPs approved for human use include *in vitro* disassembly and reassembly as post-purification processing steps (Josefsberg and Buckland 2012), allowing also the elimination of eventual contaminating baculovirus or cellular DNA fragments that are incorporated during intracellular assembly of VLPs.

3. Insect cell platforms bypassing baculovirus infection

Stable protein expression by engineered insect cell lines has been increasingly explored, constituting a powerful alternative to address the issues associated to the baculovirus/insect cell technology, highlighted in the above sections. Targeting production of HIV VLPs, two different stable cell lines were recently established: a High Five- and a *Drosophila melanogaster* S2-derived cell line, which allowed similar amounts of VLP to those produced by insect cells infected with baculoviruses (Tagliamonte, Visciano et al. 2010) (Yang, Song et al. 2012). In another application, a bicistronic expression system using an IRES element was stably integrated in the S2 cell line, resulting in efficient production of VP2-VP6 double-layered RLPs (Lee, Chung et al. 2011). More recently, it was reported the production of Japanese encephalitis (JE) complex VLPs in stably transformed lepidopteran cells with relevant expression yields (approximately 30 μg/ml) (Yamaji, Nakamura et al. 2012). A previously developed high-level expression vector was used, employing the *Bombyx mori* nucleopolyhedrovirus (BmNPV) IE-1 transactivator, the BmNPV HR3 enhancer, and the *Bm* cytoplasmic actin promoter (Farrell, Lu et al. 1998). Alternatively, the company Aldevron and partner Altravax have adopted a transient transfection method for rapid expression of Influenza VLPs in Sf9 cells (Loomis, Yaeger et al. 2005). This methodology has proved to possess some benefits in comparison to the BEVS in terms of time and effort without

compromising the quality of the final product (www.aldevron.com). Despite these efforts, the establishment of conventional stable insect cell lines expressing recombinant proteins in a non-lytic system does not seem to go along industry preference, since normally these insect cell lines express less protein and require longer development times, incrementing the final costs when compared with the BEVS.

3.1. Traditional tools for cell line development

The efficient production of recombinant proteins by stable cell lines requires the integration of the gene of interest (GOI) into the host cell genome, which is a random event. Expression levels of transfected cells can significantly differ within a population mostly due to gene copy variations and neighboring genetic elements that can either support (e.g. enhancers) or block (e.g. silencers or heterochromatin) expression (Festenstein, Tolaini et al. 1996; Bell and Felsenfeld 1999). This phenomenon is called "position effect" and constitutes a major drawback of random integration – based approaches. In addition, higher copy number might not necessarily support stronger expression levels. In fact, further complication arises from the presence of multiple integrated gene copies at different *loci* that tends to cause recombination over time accompanied by chromosomal aberration or silencing (Coroadinha, Schucht et al. 2006; Schucht, Coroadinha et al. 2006). Conventional selection methods rely on gaining resistance to an antibiotic agent or on the complementation of inefficiencies present within vital metabolic pathways of the cell. In this manner, after transfection, cells are kept in selective pressure to guarantee only the propagation of the cells that constitutively express the GOI. However, such procedures introduce prokaryotic sequences that due to their abnormally divergent nucleotide composition might influence transgene transcriptional activity (Scrable and Stambrook 1997). Additionally, the withdrawal of antibiotic selective pressure during cell propagation frequently hampers protein yields caused by mosaic gene silencing (Liu, Xiong et al. 2006). High-producer clones are a minor portion of successfully transfected cells, underlining the need for an extensive clonal screening and a subsequent evaluation of production stability over numerous passages. Despite advances on high-throughput screening technologies, overall this process takes several months and has to be repeated whenever a new expression construct is required. In this sense, the ability to repeatedly reuse a specific chromosomal locus that supports the desired expression characteristics is highly advantageous and useful for the isolation of high-producers in a timeline-efficient, cost-effective and high-throughput manner.

3.2. Cell line development by Tagging: exploitation of a chromosomal integration site

As mentioned before, the integration site impacts both expression level and stability of the producer cell clone. In order to enable the reuse of a favorable chromosomal site, a primary genomic modification is necessary to mark (tag) that particular site, i.e., tagging is a prerequisite to generate a genomic platform that can eventually support subsequent site modifications. The integration of a tag can be accomplished either by: (i) exploiting a characterized genomic site using homologous recombination (HR) or (ii) functional screening of several unknown loci through random integration. The first approach requires genome sequence data and properties of chromosomal sites which is not available for the majority of transformed cell lines, with the exception of CHO cell lines, the "workhorses" for monoclonal antibody production (Wurm and Hacker 2011; Lewis, Liu et al. 2013). As a consequence, the second approach is most often adopted to develop master producer cell lines, encompassing an exhaustive screening procedure to identify clones tagged in single locus with optimal expression patterns. In contrast, using HR the tag is integrated within predefined chromosomal loci, exploiting the cell recombination apparatus; a certain DNA sequence is substituted by the construct of interest as long as the same homologous ends of the endogenous locus flank the target construct. Nevertheless, the inefficiency of HR (with a ratio of HR/illegimate recombination (IR) of 1×10^{-6}), compromises its application to eukaryotic cells (Glaser, Anastassiadis et al. 2005).

3.2.1. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)

In the past years, it was reported that targeted cleavage of chromosomal sequences and enhanced HR could be within reach using chimeric molecules composed of a nuclease domain and tailored DNA-recognition domains (Rouet, Smih et al. 1994; Porteus and Baltimore 2003). Zinc finger nucleases (ZFNs) have emerged aiming targeted genome modifications (Porteus and Carroll 2005), consisting of DNA sequences capable of recognizing zinc finger domains, fused to an endonuclease (Kim, Cha et al. 1996; Cathomen and Joung 2008). They can be designed to generate sequence-specific double-strand DNA breaks which are then repaired by the natural homology-directed DNA repair machinery. Originally developed to repair point mutations, these ZFNs have been applied in animal cell biotechnology for gene addition into a pre-characterized locus (eg. human cells) (Moehle, Rock et al. 2007). Initial limitations concerning specificity (Bibikova, Golic et al. 2002) can now be bypassed by newly engineered variants (Miller, Holmes et al. 2007) which have facilitated the maturation of ZFN technology, allowing to pursuit a broader range of applications (e.g. gene therapy) (Rahman, Maeder et al. 2011). To our

knowledge, none of these technologies have ever been applied in the development of producer insect cell lines; its application has been restricted to targeted mutagenesis in living organisms over transformed insect cell lines; initially developed for *Drosophila*, now it has been introduced and adapted to other insect organism/embryos (Beumer, Trautman et al. 2008; Takasu, Kobayashi et al. 2010; Takasu, Sajwan et al. 2013).

More recently, transcription activator-like effector nucleases (TALEN) have emerged; these novel fusion proteins can introduce chromosomal breaks at selected sites. TALEN are artificial restriction enzymes generated by fusing TALEN DNA binding domain to a DNA cleavage domain. As ZFNs, TALENs also stimulate HR in human cells with similar efficiency levels (Hockemeyer, Wang et al. 2011). However, despite the apparent easiness with which TALENs can be engineered, these enzymes may prove more difficult to deliver into cells than ZFNs due to its highly repetitive sequences and large size (Holkers, Maggio et al. 2013). While these site-specific nuclease-based approaches represent a promising tool for targeted genomic editing, their successful application is yet limited by current transfection methods that fail on high throughput deliver of these enzymes into relevant cell types (Hockemeyer, Wang et al. 2011).

3.2.2. Site-specific recombinases (SSR)

Site specific recombination was originally developed to study gene functions as well as regulation and expression characteristics of certain loci (Branda and Dymecki 2004) and it has enabled targeted integration of several genes in a wide range of cell types and organisms. The most commonly used SSRs are the *E. coli* bacteriophage P1-derived Cre (Sternberg, Sauer et al. 1986), the *Saccharomyces cerevisiae*-derived flipase (Flp) (Buchholz, Angrand et al. 1996; Schaft, Ashery-Padan et al. 2001) and the bacteriophage ΦC31-derived integrase (Thorpe and Smith 1998). These SSRs recognize distinct sequence motifs (LoxP, FRT and attP/ attB, respectively) designated as recombination target sites (RTs), and catalyze conservative DNA rearrangements (Branda and Dymecki 2004). Such rearrangements can lead to excision (Cre and Flp), insertion (Cre, Flp, and ΦC31) or inversion (Cre and Flp) (Turan, Galla et al. 2011). When two copies of specific RTs are arranged as direct repeats, the corresponding enzyme excises the DNA segment within the RTs and releases it as circular DNA, whereas the reverse reaction leads to an insertion of a circular DNA. However, a prerequisite for the generation of producer cell lines with these systems is the preceding tagging of specific RT into the host cell genome followed by clonal screening to identify chromosomal loci with suitable expression patterns. Subsequently, targeted integration of the GOI is accomplished and the clonal isolation and propagation of producer cell

lines is feasible in much less time than conventional cell line development based on random integration of the GOI.

3.2.3. Recombinase-Mediated Cassette Exchange (RMCE)

The first generation of SSR-based chromosomal targeting relied on the integration of a single LoxP or FRT site into the genome of a cell line. Since the insertion reaction is thermodynamically unfavoured compared to the excision, a so called "selection trap" is implemented to promote a restricted selection process. The trap relies on the complementation of a non-functional, ATG-deficient resistance gene. The donor vector harbouring the gene of interest comprises a promoter and the missing start codon. Upon integration the resistance gene in the host cell genome is complemented and thereby transcriptionally activated. Consequently, resistance is exclusively conferred to cells that have performed recombination (O'Gorman, Fox et al. 1991). This method has been commercialized by Invitrogen (Flp-In® System). However, this pioneering technology has some important limitations: firstly, due to the reversibility of the reaction, excision is favored over insertion, thus limited temporal expression of the recombinase is advised; secondly, prokaryotic sequences and positive selection marker originally used to screen for successfully tagged cell lines are left behind in the chromosome after the integration, which might jeopardize gene expression (Wirth, Gama-Norton et al. 2007).

A significant improvement came with the development of non-interacting RTs (Schlake and Bode 1994) which has motivated the second-generation chromosomal targeting systems known as recombinase-mediated cassette exchange (RMCE). With the exception of the naturally occurring heterotypic RTs, several mutants have been developed for Cre and Flp-based systems (Branda and Dymecki 2004), providing the required heterospecificity crucial for a functional RMCE approach. Non-interacting RTs are used to flank the initial tagging cassette, thus allowing a precise exchange of the selectable marker with the sequences of an incoming donor vector harbouring the GOI. This reaction is catalyzed by the recombinase via a double crossover recombination event. Its main advantage is the lack of excision reaction and an increased frequency of targeting of up to 100 % due to stringent selection strategies (Schucht, Coroadinha et al. 2006). Despite the required single copy integration of the gene of interest in RMCE systems, the identification of a potent integration site can allow to reach competitive productivity levels (Wiberg, Rasmussen et al. 2006). Additionally, the bacterial vector sequences that potentially limit mammalian gene expression, are not integrated.

Due to the thermolability of Flp at 37°C, Cre has emerged as the most efficient RMCE system in mammalian cells applications. However, advances on the Flp/FRT system have been implemented; a "Flp-enhanced" (Flpe) showed an improved thermostability and Flpe/FRT system was five-fold higher than Flp/FRT system in terms of recombination efficiency in mammalian cells (Buchholz, Angrand et al. 1996). Moreover, a mouse codon-optimized version of Flpe (Flpo) enabled a further increase in recombination efficiencies to levels comparable to those obtained by Cre (Raymond and Soriano 2007). As a consequence, Flp mediated recombination is nowadays considered to be superior to Cre/loxP system since several pseudo-loxP sites are present within the mammalian genome and Cre is referred to potentiate toxic effects on cells (Thyagarajan, Guimaraes et al. 2000). This technology has been successfully applied for the establishment of mammalian cell lines, targeting production of gene therapy vectors (Verhoeyen, Hauser et al. 2001; Coroadinha, Schucht et al. 2006), as well as recombinant proteins (Kim and Lee 2008; Nehlsen, Schucht et al. 2009). Figure 2, schematically depicts the implementation of RMCE strategy for the development of a flexible producer cell line where the reuse of a pre-characterized locus speeds the development of a new producer cell line.

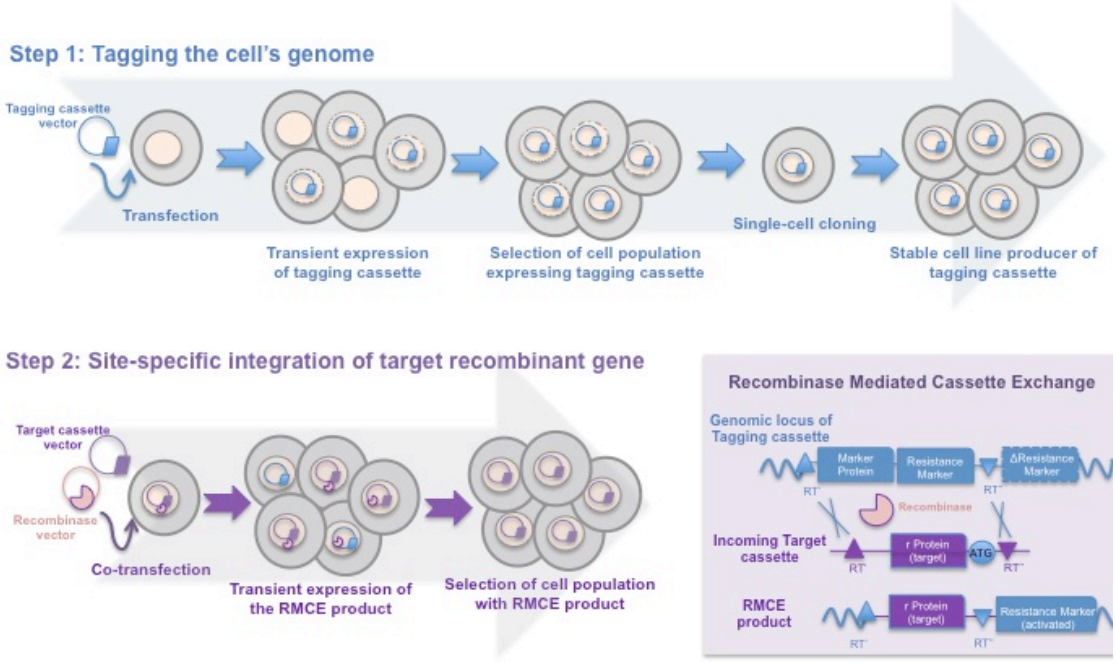

Figure 2. Development of a master cell line through recombinase-mediated cassette exchange. Step 1 is similar to the conventional cell line development process. Cells are transfected with a vector carrying a fluorescent protein and a selection marker flanked by recombinase RT sites (tagging cassette). After selection of a cell population resistant to antibiotic 1 (Resistance Marker), single-cell cloning is performed to screen for high protein producing clones. Selected clones are expanded and stored. The overall procedure requires approximately 3 to 6 months. To express a desired target protein (Step 2), the initial tagging cassette can be replaced by the target cassette containing the gene of interest (flanked by the same pair of RT sites) through a recombinase-mediated process. The defective selection marker within the integrated tagging cassette is activated by an ATG-complementary sequence present in the target vector. Upon 3 weeks in antibiotic 2 selection (ATG-complemented Δ Resistance Marker), a resulting stable cell line producing the target protein is established with similar expression properties to the tagged cell line, thus avoiding repetition of the laborious and time-consuming Step 1.

More recently, it was demonstrated the feasibility of exchanging simultaneously two genomically anchored cassettes, each flanked by a different pair of heterospecific FRT sites that do not recombine with each other (Turan, Kuehle et al. 2010). This incompatibility prevents mistargeting events, as well as unwanted chromosomal rearrangements that would limit the feasibility of the overall process. These authors tested several combinations of mutated FRTs and those that could successfully achieve the "multiplexing" were identified. This discovery is an important milestone since it enables the use of RMCE to a broader range of applications; for instance the co-expression of two distinct subunit proteins, derived from a complex protein, in a stable and fast paced manner.

4. Thesis scope and overview

Up to now the BEVS has overshadowed the potential of insect cell manufacturing capacity regarding the production of heterologous proteins in the absence of baculovirus infection. At the beginning of this PhD thesis, studies aiming transient or stable expression strategies in insect cells were at its infancy by the beginning of this work despite all advances that have boosted the potential application of these expression approaches for protein production. Based on this premise, the main aim of this work was to develop a viable alternative expression platform to the BEVS to produce recombinant proteins, benefiting from the manufacturing capacity of insect cells as well as stable expression productive assets. The main idea was the establishment of a flexible insect cell line, by-passing not only the limitations of the BEVS, but also the tedious and time-consuming procedures inherent to conventional cell line development through the use of innovative technology to circumvent such constraints. Hence, an RMCE system based on Flpe recombinase was pursued to enable rapid, efficient and predictable protein expression. However, due to a substantial lack of knowledge regarding stable expression application in insect cells, before RMCE translation could be performed, preliminary work was conducted to test and evaluate several genetic elements based on its expression performance. After RMCE implementation a proof of concept of the feasibility of the RMCE-based cassette exchange process mediated by Flpe has been successfully delivered and verified since it requires single copy integration for its proper function and robustness. Therefore, in Chapter II the establishment of several *Sf9*-based master cell lines are enlightened and in principle constitute a versatile and re-usable platform to produce recombinant proteins for fundamental and applied research in which predictable expression patterns are expected.

After establishment of the master cell line, its potentiality was further exploited to produce complex protein particles against the BEVS platform (Chapter III). The presented data provide a clear basis to judge both assets and constraints of the different expression platforms, pointing the attention to the bioprocess advantages derived from a stable expression platform and its ease application for volumetric productivity enhancement through an extended culture performance.

Along the way, this work has enabled to generalize the understanding of some biological limitations present within the implementation of RMCE-based strategies in insect cells. Chapter IV focus on the development of a sorting protocol to preclude standing obstacles adjacent to the overall clonal screening which is essential for the establishment of better RMCE-based insect cell lines, thus obviating the translation of similar strategies into this host system.

Finally, Chapter V summarizes and discusses the main results and conclusions of this thesis highlighting future perspectives for applications of the developed cell lines.

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Chapter II

Implementation of Flipase mediated cassette exchange system in Sf9 cells for expression of recombinant proteins

This Chapter is adapted from the manuscript

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and book chapter

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Table of Contents

1. Summary

Site-specific DNA integration allows predictable heterologous gene expression and circumvents extensive clone screening. Herein, the establishment of a Flipase (*Flp*)-mediated cassette exchange system in *Sf9* insect cells for targeted gene integration is described. A tagging cassette harboring a reporter *dsRed* gene was randomly introduced into the cellular genome after screening different promoter activities and transfection protocols. Single-copy integration clones were then co-transfected with both *Flp*-containing plasmid and an *EGFP*-containing targeting cassette. Successful cassette exchange was suggested by emergence of G418-resistant green colonies and confirmed by PCR analysis, showing the absence of the tagging cassette and single integration of the targeting cassette in the same locus. Upon cassette exchange, uniform EGFP expression between clones derived from the same integration site was obtained. Moreover, the resulting cell clones exhibited the expression properties of the parental cell line. EGFP production titers over 40 mg/l were of the same order of magnitude as those achieved through baculovirus infection. This *Sf9* master cell line constitutes a versatile and re-usable platform to produce multiple recombinant proteins for fundamental and applied research.

2. Introduction

The insect cell/baculovirus infection system has been a preferred platform for the production of recombinant proteins due to its eukaryotic protein processing capabilities and relatively short process development timelines (Kost, Condreay et al. 2005). Important applications have included producing therapeutically relevant proteins for structural and functional studies (Massotte 2003; Berger, Fitzgerald et al. 2004), as well as veterinary and human vaccine production (Vieira, Estevao et al. 2005; van Oers 2006; Monie, Hung et al. 2008; Cox and Hollister 2009). Nevertheless, the baculovirus-insect cell expression system (BEVS) has drawbacks related to the lytic nature of virus infection. This makes inevitable the presence of proteases in the reaction bulk at the time of harvest, promoting protein degradation and requiring additional efforts in the purification step (Jarvis and Summers 1989; Murphy, Lennick et al. 1990). Additionally, proteins requiring complex processing are often produced with low quality as the cellular protein processing machinery is less efficient in late stages of infection (Jarvis, Fleming et al. 1990; van Oers, Thomas et al. 2001).

These inefficiencies have motivated the development of stable insect cell lines for heterologous protein expression (Pfeifer 1998; Douris, Swevers et al. 2006). The most common promoters used

for such include the baculovirus immediate early (*IE1* and *IE2*) promoters, insect cell constitutive promoters such as *Actin* and *HSP70*, and inducible promoters such as that of Metallotionein (*Mtn*) (McCarroll and King 1997). However, the establishment of stable cell lines is very laborious and time consuming in order to identify cell clones displaying the best expression properties (Wurm 2004). Furthermore, the random integration of multiple copies of exogenous DNA into chromosomes often gives rise to unpredictable variations in transgene expression, as well as the possibility of inducing mutagenic effects by inhibiting or activating host genes at the integration site.

To overcome these issues, targeted integration strategies have been adopted to express relevant therapeutic proteins (Kito, Itami et al. 2002; Huang, Li et al. 2007). In particular, recombinase-mediated cassette exchange (RMCE) systems make use of site-specific recombinases, such as flipase (Flp) from *Sacharomyces cerevisiae* and Cre from P1 bacteriophage, to mediate the integration of a gene of interest in a pre-characterized chromosome locus flanked by recombinase recognition sites (Turan, Galla et al. 2011). This technology has been successfully used for different purposes in cultured mammalian (Coroadinha, Schucht et al. 2006) and insect (Nakayama, Kawaguchi et al. 2006) cells and in organisms such as *Drosophila* (Horn and Handler 2005) and mice (Cobellis, Nicolaus et al. 2005). Recently, a Flp-mediated cassette exchange system was exploited to generate 25 Chinese Hamster Ovary (CHO) cell lines to produce a human polyclonal anti-RhD antibody mixture composed of equal amounts of 25 monoclonal antibodies (Wiberg, Rasmussen et al. 2006; Frandsen, Naested et al. 2011). This strategy was important to minimize genomic position effects, given that the resulting oligoclonal cell pool displayed highly consistent manufacturing yields of each antibody in the final composition (Frandsen et al. 2011). Despite the recommended single copy integration of the gene of interest, the identification of a potent integration site can enable reach competitive productivity levels in RMCE systems (Wirth, Gama-Norton et al. 2007; Nehlsen, Schucht et al. 2009).

In the present work, the development of a *Sf9* cell line using a Flp-mediated cassette exchange system is reported. Two complementary cassettes (tagging and targeting) were constructed, each flanked by a set of two non-interacting Flp-recombinase recognition target (FRT) sites and encoding different reporter proteins (*dsRed* or *EGFP*) to facilitate the implementation and characterization of the system. Different promoters and transfection protocols were evaluated to define the best conditions which would allow good expression levels from single copy integration of the tagging cassette. Cassette exchange was successfully accomplished in several clones demonstrating the feasibility of FRT/Flp recombination in *Sf9* cells.

3. Materials and Methods

3.1 Plasmid construction

Three vectors with the same backbone were constructed (*pIZT/OpIE2, pIZT/Hsp70 and pIZT/Mtn),* differing only in the promoter *(OpIE2, HSP70 or Mtn)* placed upstream to an enhanced green fluorescence protein (*EGFP*) coding sequence. *EGFP* from *pMDISGFP* (kindly provided by Dr. Dagmar Wirth, HZI Braunschweig, Germany) was amplified by PCR and cloned into an EcoRI/NotI digested *pIZT/V5-His* vector (Invitrogen, Carlsbad, USA), resulting in *pIZT/OpIE2*. The *HSP70 Drosophila* promoter was amplified from *pUAST* (Brand and Perrimon 1993), kindly provided by Dr. Pedro Domingos, ITQB-UNL, Portugal) and cloned into an BspHI/AgeI digested *pIZT/OpIE2* fragment*,* resulting in *pIZT/Hsp70*. The *Mtn* promoter was amplified by PCR from *pRmHa* ((Bunch, Grinblat et al. 1988), kindly provided by Dr. Pedro Domingos, ITQB-UNL, Portugal) and cloned into a BspHI/AgeI digested *pIZT/OpIE2* fragment, resulting in *pIZT/Mtn.*

pTag-OpIE2dsRed cassette (*pTagging*): *OpIE2* and *OpIE1* promoters were amplified from *pIZT/V5-His* (Invitrogen) and cloned into *pCAG-dsRed* (kindly provided by Connie Cepko through Addgene, Cambridge, MA, USA) by digestion with EcoRI/AgeI and HindIII/AvrII, respectively. From this construct a fragment of 2140 bp containing *OpIE2*, dsRed, polyA terminator and *OpIE1* was amplified by PCR. The PCR product was digested with SacI/EcoRI and ligated into *pTagFwF5* (designed in this study and synthesized by GeneArt) that contains a wild-type FRT site (Fw) and a spacer mutant FRT site (F5) with and hygromycin resistance gene followed by an ATG-deleted neomycin (neo) resistance gene.

pTarget-OpIE2eGFP cassette (*pTarget*): The *OpIE2* promoter and *EGFP* were amplified by PCR from *pIZT/OpIE2* and cloned into *pTar(mcs)* by digestion of SphI/XhoI recognition sites. Subsequently, using EcoRI/EcoRV recognition sites, the *OpIE1* promoter was placed upstream to the ATG start codon followed by the F5 site to complement the inactive neo resistance gene after targeting.

pOpIE2FLPe vector: The *OpIE2* promoter was amplified from *pIZT/V5-His* and digested with NheI/SacI. The SV40 promoter was excised from *pSVFLPe* (Coroadinha, Schucht et al. 2006) with NheI/SacI followed by insertion of the *OpIE2* promoter fragment. Primer sequences for all constructions are available upon request.

3.2 Sf9 cell culture maintenance

Spodoptera frugiperda-derived *Sf9* cells (ECACC 89070101) were cultured in Sf900II medium (Gibco Invitrogen Corporation, Paisley, UK) at 27ºC. Routine culture was performed in 100 or 500 ml shake flasks (Corning, USA) with 15 or 50 ml working volume at 110 or 90 rpm, and cells were sub-cultured every 3 to 4 days at 5×10^5 cell/ml. Cell density and viability were determined by cell counting using a Fuchs-Rosenthal chamber after diluting culture bulk samples in 0.4% (v/v) Trypan Blue.

3.3 Baculovirus infection

A recombinant *Autographa californica nucleopolyhedrovirus* (Ac-hisGFP) encoding a GFP gene under the control of the *polyhedrin* promoter (kindly provided by Dr. Monique van Oers, Wageningen University, Netherlands) was used to infect *Sf9* cells. Infection was performed when cell density reached 3×10^6 cell/ml (~72 h after inoculation), using a multiplicity of infection of 0.1. GFP production was quantified in both supernatant and cell extracts at 216 h of culture, when cell viability was about 50%.

3.4 Transfection, selection and limiting dilution

Sf9 cells were transfected at concentrations above 1.5×10^6 cell/ml. For each 1×10^6 cells it was used one unit of Cellfectin II reagent (Invitrogen) and 0.3 µg of DNA were used. To introduce the tagging cassette a lower amount of DNA (0.03 µg) was tested as well. *Sf9* cells were transfected with the tagging cassette also by electroporation (Neon™ Transfection system, Invitrogen, Pailsey, UK). Two protocols were tested: both protocols used 4×10^6 cells and two pulses of 5 ms length each, but different quantities of the tagging construct were added to the cells (either 1 µg or 0.5 µg total, i.e., 0.25 µg or 0.125 µg per 1×10^6 cells), and different voltages applied (250 V or 500 V, respectively).

Culture medium was replaced 24 h post-transfection and selection started 2 days later using medium containing Hygromycin B (200 U/ml) or Zeocin (300 U/ml).

Limiting dilution was applied to obtain cell clones from the four selected populations expressing the tagging cassette. Conditioned medium supplemented with 10% (v/v) of fetal bovine serum (FBS, Gibco) was used to support cell colony growth.

In order to induce the *Mtn* promoter, cells expressing the *pIZT/Mtn* vector were cultured in medium containing different concentrations of $CuSO_4$ (0.2, 0.4 and 0.6 g/l).

3.5 Cassette exchange

For site-specific cassette exchange, cell clones were co-transfected with 0.1 µg of *pTarget* and 0.5 µg of Flpe recombinase-expressing vector (*pOpIE2FLPe)* using Cellfectin® II reagent (Invitrogen) in the same conditions described above. The Flpe variant, which resulted from mutations in the wild-type Flp enzyme to increase its activity at 37ºC, was used in this work (Buchholz, Angrand et al. 1998). The medium was replaced 24 h post-transfection. G418 (300 U/ml) was added two days later to the transfected population, and cells were transferred to 6-well plates. After four to five days in selection the culture medium was replaced by conditioned medium supplemented with 10% FBS and 100 U/ml of G418, and this medium was replaced every five to six days. When colonies started growing faster, the G418 dose was increased back to 300 U/ml. Cell colonies were picked and expanded.

3.6 Reverse transcriptase and standard polymerase chain reaction

EGFP and dsRed mRNA levels were assessed by PCR. mRNA was extracted using the RNeasy kit (Qiagen, Courtaboeuf, France) and cDNA was synthetized using the First Strand cDNA Synthesis kit (Roche Diagnostics, Manheim, Germany). RT-PCR analysis was executed using cDNA diluted (1/10 and 1/20) as template: amplification was performed once at 94^{o}C for 15 min, followed by 35 cycles at 94^{o}C 1 min, 58^{o}C for 30 sec and 72^{o}C for 30 sec, and a final extension step at 72^{o}C for 10 min.

G418-resistant target cell populations were assessed in terms of correct cassette exchange and unspecific *pTarget* integration events by PCR. For both experimental setups a total of 100 ng of genomic DNA was applied. Amplification was performed once at 94^{o}C for 15 min, followed by 35 cycles at 94^{o}C 1 min, 68^{o}C for 30 sec and 72^{o}C for 20 sec, followed by a final extension step at 72^{o}C for 10 min. Primers position can be seen in Figure 2.

The sequences of the primers are summarized in Table 1.

Table 1. Sequence of primers used in PCR analyses.

Primers Name	Sequence
H	5'-GCACTGCAAAAAAACACGCT-3'
J	5′-CAACGGTGTAGACTATTTATGT-3'
M	5'-GGGAGAGGCGGTTTGCGTAT-3'
L	5′-TACCGCCTTTGAGTGAGCTG-3'
EGFP Forward	5′-CAACAGCCACAACGTCTATATCATG-3'
EGFP Reverse	5'-ATGTTGTGGCGGATCTTGAAG-3'
dsRed Forward	5'-CCCGCCGACATCCCCGACTA-3'
dsRed Reverse	5′-CGGAGGGGAAGTTCACGCCG-3'
18S Forward	5′-AGGGTGTTGGACGCAGATAC-3'
18S Reverse	5'-CTTCTGCCTGTTGAGGAACC-3'

3.7 Flow cytometry

CyFlow® space (Partec GmbH, Münster, Germany) was used to evaluate transfection efficiencies, as well as to characterize tagged and target clones in terms of EGFP or dsRed fluorescence intensity. Exponentially growing cells were harvested and diluted in PBS. EGFP was detected using FL1 (emission filter: 527 ± 13 nm) and dsRed by using the FL2 (emission filter 590 ± 5 nm). Fluorescence intensity from 10000 events per sample was collected and analysed using FlowMax Software (©2009, Quantum Analysis GmbH).

3.8 Southern Blot analysis

Tagged clones were screened by Southern Blot analysis to assess the number of integrated copies. Genomic DNA was extracted from each cell clone using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA). A DIG labeled probe was obtained according to the instructions of the PCR DIG Probe Synthesis Kit (Roche). Primers to amplify the first 500 bp of the neo resistance gene were used. Genomic DNA was digested overnight with SacI and/or EcoRI restriction enzymes. The digested samples were applied onto a 0.7% agarose gel and run for 4 h at 45 V. Nucleic acids were then transferred to a nytran SPC membrane (alkaline transfer method), with the Turboblotter® system (Whatman, Kent, UK) for 1h. Hybridization with the DIG-labeled probe was

performed overnight at 60°C. DIG signal was detected following instructions from the DIG Nucleic Acid Detection Kit (Roche).

3.9 Western Blot Analysis

EGFP was analyzed by western blot in both supernatant and cellular extracts. Proteins were separated under reducing electrophoresis on a 1-mm NuPAGE Novex BisTris gel (Invitrogen) and electrically transferred to a nitrocellulose membrane (HybondTMC extra, Amersham Biosciences, Little Chalfont, UK). Immunochemical staining for recombinant protein detection was carried out with anti-GFP monoclonal antibody (Sigma), at a 1:2000 dilution during 2 h at room temperature. Blots were developed using the enhanced chemiluminescence detection system after 1 h incubation with horseradish peroxidase-labeled anti mouse IgG antibody (Amersham Biosciences), at 1:5000 dilution at room temperature. The product yield was estimated by densitometry analysis of the scanned images (ImageJ software, available at http://rsb.info.nih.gov/ij/) and based on a GFP standard (Sigma).

4. Results

4.1. Promoter selection

This work describes the development of a stable *Sf9* cell line following a Flp-mediated cassette exchange approach. The promoter to drive the expression of the gene-of-interest inside the cassette was selected by comparing the relative activity of three promoters commonly used in insect cell systems, namely the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*OpMNPV*) immediate-early 2 gene (*OpIE2*) promoter, and two *Drosophila melanogaster* promoters from the heat shock protein 70 (*HSP70*), and the metal-inducible metallothionein (*Mtn*) genes. These promoters were placed in the same backbone plasmid, upstream of an enhanced green fluorescence protein (*EGFP*) coding sequence. *Sf9* cells were then transfected independently with each of the three constructs and selected by zeocin resistance over three weeks. EGFP expression was analysed by flow cytometry in the resulting stable cell populations (Figure 3). The *OpIE2* promoter revealed to be significantly stronger than either *HSP70* or *Mtn* promoters. For the *Mtn* promoter, protein expression is dependent on the inducer level (e.g. Cu^{2+}), thus *Sf9* cells were cultured in the presence of different concentrations of $CuSO_4$ (0.2, 0.4 and 0.6 g/l). An increase in fluorescence intensity is observed with increasing $CuSO_4$ concentrations, confirming the inducible pattern of this promoter (Figure 3B). However, even at high induction conditions, EGFP

fluorescence intensity is 8- or 16- fold lower when compared to that from *HSP70* or *OpIE2* promoters, respectively (Figure 3A). In addition, cellular growth is significantly hampered by increasing Cu^{2+} concentrations (Figure 3D). The stronger promoter (OpIE2) was used to drive expression of the gene-of-interest in the scope of the RMCE strategy presented in the next section.

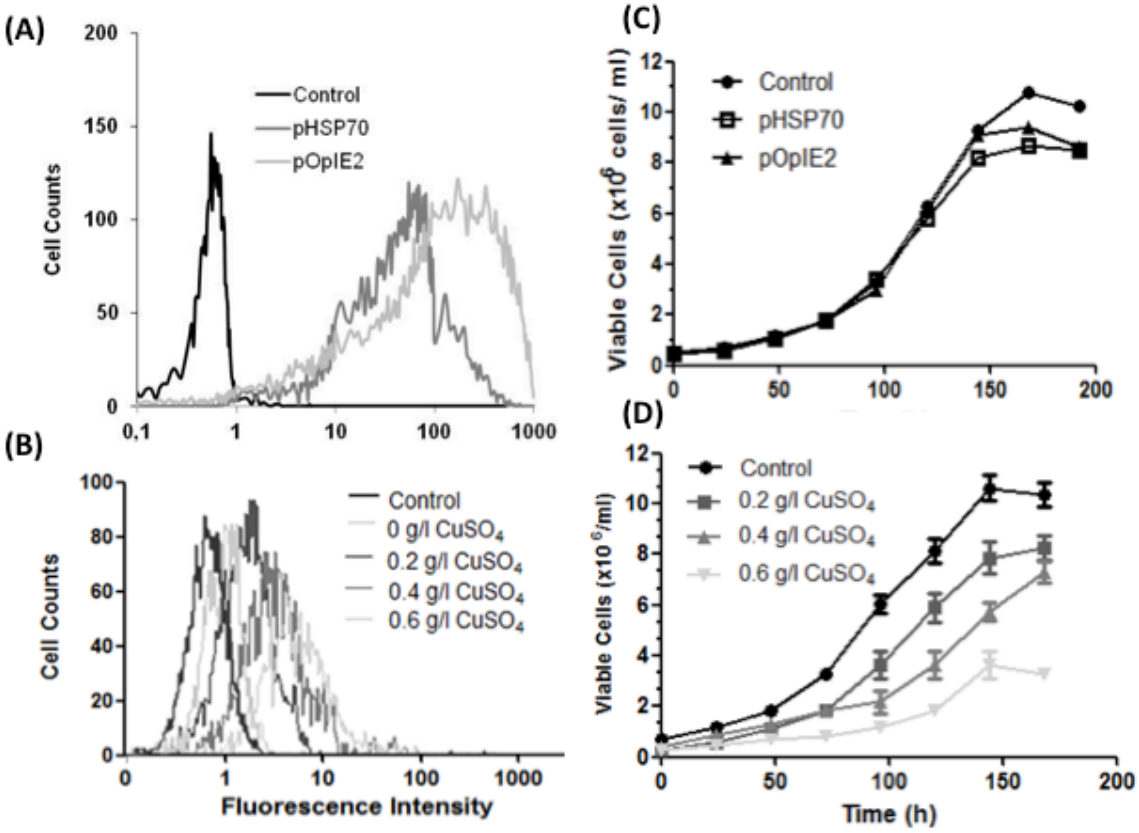

Figure 3. Promoter strength comparison in *Sf9* cells. Flow cytometry analysis of EGFP expression in selected cell populations transfected with **A)** OpIE2 and HSp70 constrcuts and **B)** the *Mtn* construct, 96 h after $CuSO_4$ addition at different concentrations (0, 0.2, 0.4 and 0.6 g/l). At 0.6 g/l $CuSO_4$, the EGFP mean fluorescence intensity from the *Mtn*-promoted construct is 8, whereas for the *OpIE2* and *HSP70* populations it is 123 and 63, respectively. *Sf9* cell density profiles **C)** of *OpIE2* and *HSP70* populations and **D)** *Mtn* populations subjected to different concentrations of $CuSO_4$.

4.2. Tagging and targeting cassettes

The Flp-mediated cassette exchange approach involves two steps: (1) tagging chromosomal sites by transfecting cells with a construct harbouring two non-interacting FRT sites (Fw and F5) and subsequent isolation of parental clones, followed by (2) co-transfection of such cell clones with

both targeting and Flp-harbouring vectors for cassette exchange. To facilitate the establishment and characterization of the RMCE system in *Sf9* cells a different reporter protein was used in each cassette - dsRed in *pTagging* and EGFP in *pTarget* (Figure 4). Both tagging and targeting vectors were assembled with insect cell specific promoters: *OpIE2* drives expression of reporter genes and the *OpIE1*, another *OpMNPV* immediate-early gene promoter, drives expression of the resistance markers. A hygromycin gene assures selection of cells expressing *pTagging.* In the targeting cassette, the *OpIE1* promoter drives the expression of the ATG sequence that complements the ATG-defective neo resistance gene upon successful cassette exchange in the presence of Flp. RMCE is expected to produce the final product as described in Figure 4.

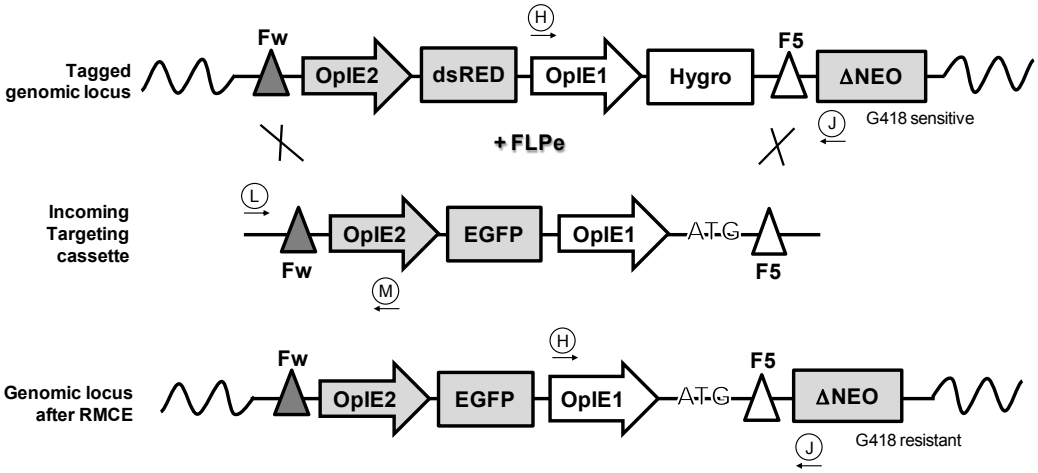

Figure 4. Tagging and targeting strategy. Both *pTagging* and *pTarget* cassettes harbour a wild-type FRT site and a mutant FRT5 site in a parallel orientation. Between Flp recognition sites, the *OpIE2* promoter drives reporter protein expression and the *OpIE1* promoter drives the resistance gene expression. An *ΔATG-neo* is present in the *pTagging* downstream to the F5 site. In the *pTarget* construct the *OpIE1* promoter drives the expression of an ATG sequence, complementary to the *ΔATG-neo* present in the integrated *pTagging* cassette. Co-transfection of the Flpe recombinase-expressing plasmid and the *pTarget* leads to cassette exchange via Fw and F5 and activation of neo resistance marker. As two fluorescent reporter proteins were used, RMCE can be easily monitored by fluorescence microscopy. The letters in circles correspond to the primers in Table 1.

4.3. Stable integration of the tagging cassette

Single copy integration of the tagging cassette into the genome of *Sf9* cells is desirable to achieve reproducible results in RMCE. To cope with the uncertainty and uncontrollability of copy number integration, two transfection methods were used in parallel - lipofection and electroporation; two quantities of tagging cassette DNA were used for each transfection method, and the remaining parameters inherent to electroporation were also tuned to different values (see *Materials and Methods*). As a result, 4 populations were obtained: two using lipofection (0.3 μg or 0.03 μg of

DNA), and two using electroporation (0.125 µg or 0.25 µg of DNA). Different transfection efficiencies were obtained for the different methods as evaluated by flow cytometry analysis at 72 h post-transfection (Table 2), with lipofection allowing a higher percentage of cells receiving at least one copy of the construct. High transfection efficiencies expedited the selection of resistant populations, but also can be associated with a higher probability of multiple copy integration. Hygromycin B selection resulted in 4 populations differing in dsRed expression patterns, with the two populations transfected with higher amounts of DNA having stronger mean fluorescence intensity which can be a result of multiple integration events (Table 2).

Table 2. *Sf9* cells transfection efficiency by lipofection or electroporation and fluorescence intensity of resulting selected cell populations.

	DNA quantity of *pTagging*[a]	Transfection efficiency (%)[b]	Mean fluorescence intensity[c]
Lipofection	0.3 µg	75 ± 4	6.5 ± 0.8
	0.03 µg	3 ± 1.5	3.0 ± 1.3
Electroporation	0.25 µg	0.75 ± 0.3	8.5 ± 2.8
	0.125 µg	1 ± 0.3	2.5 ± 0.3

[a]DNA quantity per 1×10^6 cell; [b]flow cytometry analysis 72 h post-transfection – data shown are the mean and standard deviation from 3 independent transfections; [c]Hygromycin-selected cell populations.

The 4 cell populations were submitted to a limiting dilution procedure in order to obtain cell clones expressing the tagging cassette. The dsRed fluorescence intensity of 14 isolated cell clones, 7 from lipofection and 7 from electroporation can be seen in Figure 5A. To determine the transfected gene copy number in the genome of the different clones, southern blot analysis was performed using a labelled DNA probe to hybridize with the neo resistance marker. Since EcoRI and SacI enzymes cut once within the tagging cassette, in case of single copy integration the neo probe will only detect a single fragment for single copy integration, with size dependent on the genomic integration site of the tagging construct (as depicted in Figure 5B). This was the case for all 14 analysed clones (exemplified in Figure 5B for two clones). Therefore, both transfection protocols resulted in efficient tagging toward the implementation of the RMCE strategy.

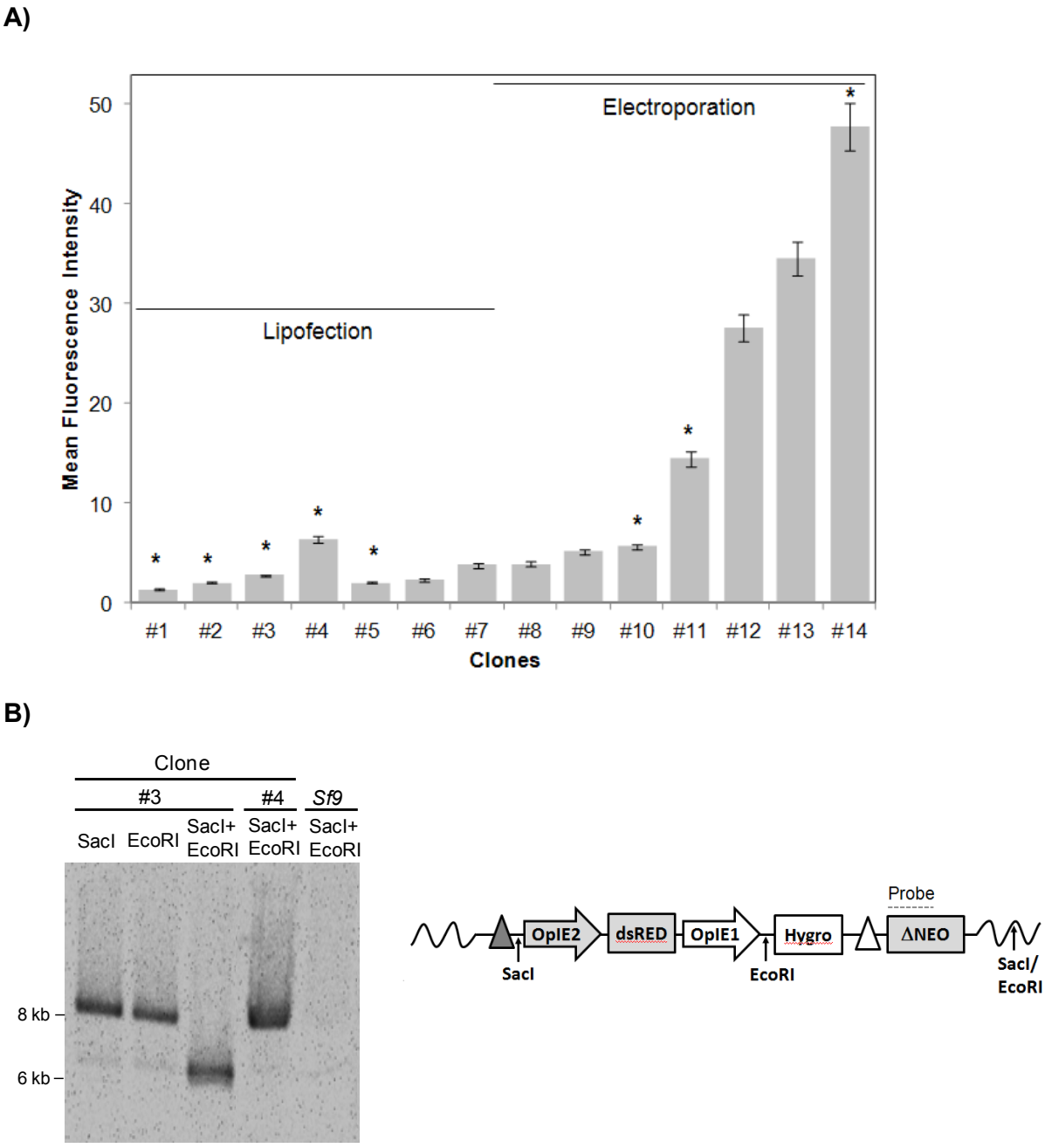

Figure 5. Screening of tagged clones. A) Mean fluorescence intensity of randomly picked clones derived from different transfection methods. Clones #1 through #7 were derived from lipofection whereas clones #8 through #14 were obtained from electroporation. Cassette exchange studies were performed in clones marked with (*). **B)** Southern blot analysis of two tagged clones (#3 and #4). EcoRI and SacI enzymes cut once within the tagging cassette. Upon digestion with each enzyme independently, the Dig-labeled probe that hybridizes with the neo resistance marker will produce only one fragment for single copy integration clones, with size dependent on the genomic integration site of the tagging construct. Simultaneous digestion with both enzymes produces a smaller fragment than digestion with each enzyme individually.

4.4. Flp-mediated cassette exchange

Cassette exchange studies were performed in 8 tagged clones (marked with an asterisk in Figure 5A), by co-transfection with both Flpe and *pTarget* constructs. To minimize multiple copy integration events a small quantity (0.1 µg) of targeting cassette DNA was used while a fivefold excess of Flp-plasmid (0.5 µg) was employed to favour cassette replacement (Baer and Bode 2001; Sorrell, Robinson et al. 2010). Upon cassette exchange, resistance to G418 is activated by restoring the ATG start codon in the neo resistance gene. Although transfection efficiencies over 50% were obtained by flow cytometry analysis at 72 h post-transfection, less than 10% of the cells survived during the first week in G418 selection. The performance of Flp-mediated cassette exchange was different within targeted clones. For clones #1, #2 and #3, dsRed positive cells decreased over time until the third week in selection when they were no longer visible by fluorescence microscopy (Figure 6A). Resistant colonies started growing faster at the third week in selection and were all green, suggesting successful cassette exchange. This was confirmed by PCR analysis which showed the absence of dsRed and the presence of GFP transcripts (Figure 6B). For these clones, about 80-100 green colonies were obtained per 10^6 transfected cells. Cassette exchange was less efficient for the remaining clones, resulting in about 5-10 green colonies per 10^6 transfected cells. Surprisingly, for clones #4 and #11 a major portion of the colonies was red. To exclude the hypothesis of read-through transcription of the neo marker, these tagged clones were confirmed to be sensitive to G418. The number of red colonies was significantly reduced when initial selection was performed in suspension cultures before plating to form isolated colonies.

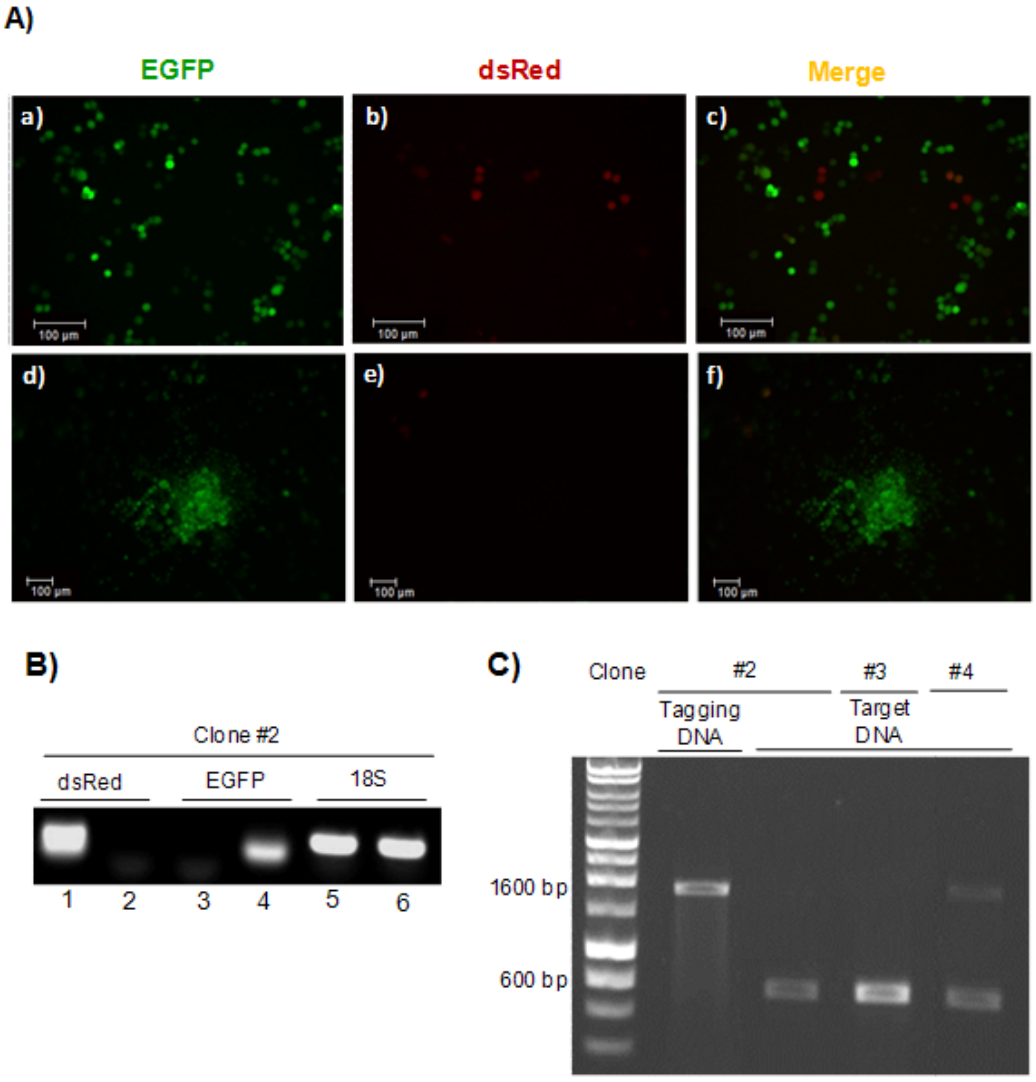

Figure 6. Flipase-mediated cassette exchange. A) Fluorescence microscopy images of clone #2, 72 h (a - c) or 3 weeks (d – f) after targeting. **B)** mRNA levels of dsRed and EGFP in clone #2 before (lanes 1, 3 and 5) and after 3 weeks (lanes 2, 4 and 6) of cassette exchange. **C)** PCR of genomic DNA to confirm insertion of the targeting cassette in the tagged locus of clones #2, #3 and #4. Using primers located at OpIE1 promoter and neo resistance gene, the amplification of a 600 bp fragment from the population selected after targeting confirms correct cassette exchange. For clone #4 the analysis of the pooled cells after 3 weeks in G418-selection still detects tagging cassette (1600 bp band).

Cassette replacement was further confirmed by genomic PCR. The same primer pair (H and J, see Figure 4 for position) was used in DNA from tagged clones and G418-resistant pooled cells after targeting. A difference of 1000 bp in size between the products of the two PCR products confirmed cassette exchange, since the target cassette lacks the hygromycin gene between the amplified

regions (Figure 6C). For clones #4 and #11, PCR analysis of the pooled cells after 3 weeks in selection still detected the presence of tagging cassette (Figure 6C), confirming the fluorescence microscopy results. Additional random integrations of the targeting cassette in the cells genome could not be confirmed by PCR as no amplification occurred from primers flanking the Fw site in *pTarget* (L and M, see Figure 4) (data not shown).

Green colonies from three targeted master cell lines were randomly picked and compared in terms of EGFP fluorescence intensity (Figure 7). Colonies of the same origin presented similar EGFP expression patterns, highlighting the advantage of using RMCE to develop producer cell lines, as the time-consuming screening of best producer clones is not required. Moreover, a high degree of correlation is observed between dsRed and EGFP fluorescence intensity from the same chromosomal locus, i.e., clones with higher dsRed expression have higher EGFP expression (Figure 7). This strong positive correlation suggests that the characteristic expression levels of chromosomal *loci* in each tagged clone were not altered after Flp-mediated cassette exchange, thus in relative terms target gene expression can be reasonably predicted from the tagged clones.

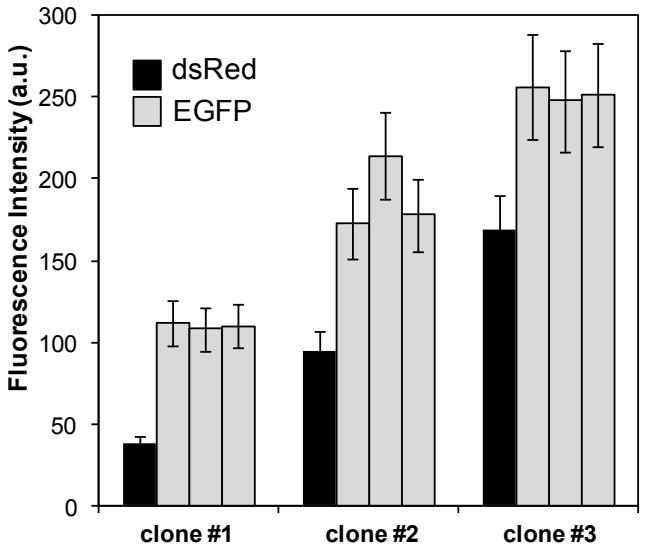

Figure 7. Comparison of reporter expression levels in tagged and target clones. Clones derived from three tagged parental clones (#1 through #3) were analysed in terms of EGFP expression. dsRed fluorescence intensity was multiplied by 50 to allow clear visualization of both dsRed and EGFP in the same graph. dsRed and EGFP fluorescence intensities correlate well for these 3 clones. Error bars correspond to standard deviations from triplicate cultures.

4.5. EGFP production

Shake flask cultures of two target clones (derived from parental clones #2 and #3) were sampled daily for cell counting and further EGFP quantification through western blot (Figure 8). The population doubling time was similar for both clones but the maximum cell density (X_{max}) achieved for clone #2 was slightly higher than that for clone #3 (Table 3). EGFP production was analyzed at the end of the stationary phase (164 h of culture) in supernatant and cell extracts (Figure 8B). Although higher EGFP fluorescence intensity was obtained for clone #3 by flow cytometry analysis, the western blot results show similar cell specific productivities (q_{EGFP}) resulting in similar total protein titers for both clones (45.0 and 44.2 mg/l). These EGFP production levels are comparable to those achieved through recombinant baculovirus infection carrying GFP under the *polyhedrin* promoter. By infecting *Sf9* cells at 3×10^6 cell/ml, the total GFP titer after 144 h from infection was 67 mg/l (Table 3).

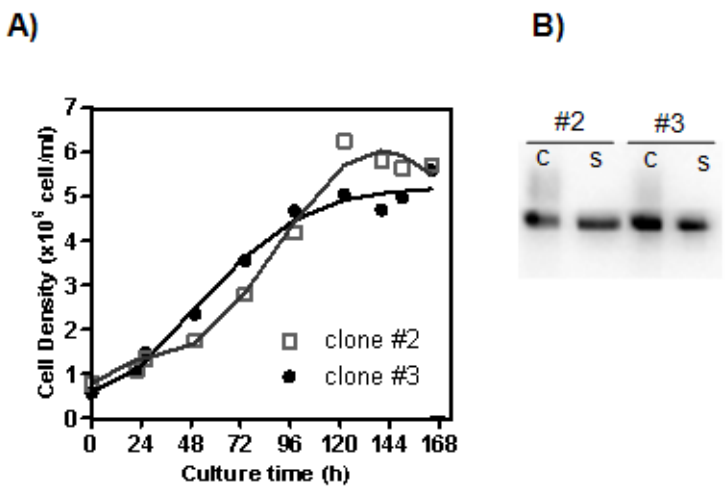

Figure 8. Cell growth and EGFP production of target clones. A) Cell density profiles of sub-clones derived from parental clones #2 and #3. **B)** Western Blot analysis of EGFP accumulation at the end of culture (164 h), both intracellularly (c) and in the supernatant (s).

Table 3. Comparison of cellular growth and EGFP production of master cell clones with baculovirus infected-*Sf9* cells.

	doubling time (h)	X_{max} (10^6cell/ml)	q_{EGFP} (µg/10^6cell/h)	EGFP titer (mg/l)[b]
Clone 2	41.9 ± 4.2	6.2 ± 0.6	0.079 ± 0.004	45.0 ± 4.07
Clone 3	41.7 ± 5.8	4.8 ± 0.5	0.078 ± 0.004	44.2 ± 3.69
Baculovirus infection (in *Sf9* cells)	[a]	3.9 ± 0.4	0.140 ± 0.008	67.9 ± 5.67

[a]Cell growth was impaired by baculovirus infection (MOI=0.1); [b] Final titer based on the sum of protein in the supernatant and intracellularly. Data shown are the mean and standard deviation from three independent cultures.

5. Discussion

In the current study, we have combined the advantageous culture characteristics of *Sf9* insect cells with the powerful RMCE technology to develop a stable protein expression platform while shortening cell line establishment timelines through targeted gene integration. Although the Flp/FRT recombination system has previously been applied to several biological systems (Verhoeyen, Hauser et al. 2001; Cobellis, Nicolaus et al. 2005; Schebelle, Wolf et al. 2010; Turan, Kuehle et al. 2010), its performance was here evaluated in *Sf9* cells for the first time.

To choose a suitable promoter for tagging/targeting vector design, the performance of three promoters was evaluated for stable expression in *Sf9* cells. The capacity for baculovirus early promoters to induce high transcriptional rates in most lepidopteran cell lines have been described previously (Pfeifer, Hegedus et al. 1997). From our results, the *OpIE2* promoter allowed a two-fold increase in gene expression when compared to the *Drosophila*-derived *HSP70* promoter. The *Drosophila Mtn* promoter was also tested in this study, but was revealed to be unsuitable for inducible protein expression in *Sf9* cells as it required cytotoxic concentration of heavy-metals to reach measurable protein expression levels. A similar result had been reported by (Hegedus, Pfeifer et al. 1998) who failed to observe β-galactosidase expression from the *Mtn* promoter in two lepidopteran cell lines.

Different transfection protocols, either based on lipofection or electroporation, were adopted in parallel for stable integration of the tagging cassette in order to address the uncertainty of copy number integration. Lipofection allowed higher transfection efficiencies and shorter antibiotic selection periods when compared to electroporation-based transfections. Still, all transfection methods were able to generate single copy integration of the tagging cassette. This is in part due to the small quantities of tagging DNA used in both transfection methods, at least 10-fold lower when compared to the amounts which have been used for RMCE applications in mammalian cells (Lauth, Spreafico et al. 2002; Obayashi, Kawabe et al. 2011).

The relative and absolute quantities of the targeting and recombinase plasmids have been reported to influence cassette exchange efficiency (Sorrell, Robinson et al. 2010). In this study, a small amount of the targeting cassette was also used to minimize multiple copy integration events. Furthermore, an excess of Flp plasmid in relation to the targeting cassette was used as recommended in other RMCE studies (Lauth, Spreafico et al. 2002; Schebelle, Wolf et al. 2010). The trap system based on a defective neo resistance gene that becomes active by ATG complementation upon cassette exchange enabled the screening of recombinant clones which have been correctly targeted. High efficiencies of these trap systems are usually reported based on the percentage of correctly targeted clones obtained after selection (Coroadinha, Schucht et al. 2006; Nehlsen, Schucht et al. 2009), but few studies report on the efficiency of Flp-RMCE upon transfection. In this study, as previously observed for Flp-RMCE in CHO cells (Wilke et al. 2011), cassette exchange efficiency was rather low considering the small number of green colonies resulting from transfectants. Furthermore, these results corroborate the hypothesis that not all chromosomal *loci* are equally amenable to Flp-RMCE; the accessibility of the recombinase to the FRT sites is likely to be dependent on local structure of the genome (Turan et al. 2010). In our particular case, one way to improve recombination efficiency could be to use the wild-type Flp instead of the Flpe variant, as its optimum activity temperature (30ºC) is closer to the cells maintenance temperature (27ºC). Further improvements in RMCE efficiency have been achieved by the addition of a nuclear localisation sequence signal to Flp for enhanced nuclear uptake (Schaft, Ashery-Padan et al. 2001; Patsch, Peitz et al. 2010). Targeted clones derived from the same tagged master cell line showed similar EGFP expression patterns, confirming that site-specific integration significantly reduces the variability in expression level as reported before (Coroadinha, Schucht et al. 2006; Huang, Li et al. 2007; Nehlsen, Schucht et al. 2009; Turan, Galla et al. 2011). Furthermore, consistent expression levels of dsRed and EGFP were obtained for corresponding tagged and target cell clones. Therefore, the inherent advantages of the RMCE

system for cell line development were demonstrated in *Sf9* cells. By using the same locus and the same promoter, predictable expression levels were obtained for reporter proteins, thus obviating the need for extensive clone screening after transfection with a gene-of-interest.

EGFP production titers in small-scale batch cultures of two clones were similar to those achieved using the BEVS. Although the cell specific productivity in the stable expression system is still lower than that driven by the strong baculovirus *polyhedrin* promoter, it allows wider room for improvement of cellular growth and culture duration through medium and/or process engineering, which should be conductive to higher volumetric titers.

In conclusion, the developed cell line can be rapidly converted into a producer of different recombinant proteins, avoiding the obstacles caused by random gene integration (Siegal and Hartl 1998). Furthermore, it can be advantageous over the BEVS by de-bottlenecking the expression of proteins requiring complex processing and allowing the optimization of culture conditions to increase producer cell concentrations. Lastly, the developed cell line may also improve the performance of the BEVS by overexpressing accessory genes during baculovirus infection with impact on final recombinant protein quality and quantity (Lin, Li et al. 2001).

6. Acknowledgements

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Chapter III

Insect cell expression of complex proteins through stable targeted integration

This Chapter is adapted from the manuscript

Fernandes F, Dias MM, Vidigal J, Patrone M, Teixeira AP, Alves PM. Production of rotavirus core-like particles in Sf9 cells using recombinase-mediated cassette exchange. Journal of Biotechnology 172, 34-38 (2014)

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1. Summary

A flexible *Sf9* insect cell line was recently developed leveraging the recombinase mediated cassette exchange (RMCE) technology, which competes with the popular baculovirus expression vector system (BEVS) in terms of speed to produce new proteins. Herein, the ability of this stable cell platform to produce complex proteins, such as rotavirus core-like particles, was evaluated. A gene construct coding for a VP2-GFP fusion protein was targeted to a previously characterized high recombination efficiency *locus* flanked by flipase (Flp) recognition target sites and, after three weeks in selection, an isogenic cell population was obtained. Despite the lower cell specific productivities with respect to those obtained by baculovirus infection, the titers of VP2-GFP reached in shake flask batch cultures were comparable as a result of higher cell densities. To further improve the VP2-GFP levels from stable expression, analysis of exhausted medium over culture time was undertaken to design feeding strategies enabling higher cell densities as well as increased duration of cultures. The implementation of the best strategy allowed reaching 20 million cells per ml in bioreactor cultures; the integrity of the rotavirus core-like particles could be confirmed by electron microscopy. Overall, we show that this *Sf9* -Flp cell platform represents a valuable alternative to BEVS for producing complex recombinant proteins, namely rotavirus core-like particles.

2. Introduction

The baculovirus-insect cell system represents a well established tool for the production of recombinant proteins, due to its eukaryotic protein processing capabilities and relatively short development timelines (Kost, Condreay et al. 2005). Several vaccines under development or already licensed (including the recently approved HA subunit influenza vaccine), are produced using this platform (Fernandes, Teixeira et al. 2013). However, inherent drawbacks include the effort to maintain the virus stock and, more importantly, the lytic infection cycle: proteins requiring complex processing are often produced in low quality and/or quantity because the cellular protein processing machinery is compromised during late stages of infection (Jarvis, Fleming et al. 1990; van Oers, Thomas et al. 2001).

Such inefficiencies have motivated the development of stable insect cell lines that are able to continuously express the desired protein in a virus-free process (Jarvis, Fleming et al. 1990; Harrison and Jarvis 2007). In particular, clonal isolates from *Spodoptera frugiperda* (*Sf9* or *Sf21*) present advantageous growth and metabolic characteristics, reaching high cell densities in batch

cultures, as opposed to the majority of mammalian cell lines where excessive by-product excretion, such as lactate and ammonia limits culture performance (Ikonomou, Schneider et al. 2003). However, the establishment of cell lines is often a tedious and time-consuming process, requiring extensive screening to identify the cell clones that display the best expression properties due to random integration of the heterologous gene. To overcome these issues, our group has recently developed a *Sf9* insect cell line using targeted integration by recombinase-mediated cassette exchange (RMCE) (Fernandes, Vidigal et al. 2012). This system allows for rapid generation of cell lines to produce different recombinant proteins making use of flipase (Flp) to mediate the integration of the gene-of-interest in a pre-characterized chromosome *locus* flanked by Flp recognition target (FRT) sites (Qiao, Oumard et al. 2009; Turan, Galla et al. 2011). The feasibility of FRT/Flp recombination in *Sf9* cells was demonstrated with reporter proteins: a cassette encoding DsRed in a specific *locus* could be exchanged to an EGFP-encoding cassette in several tagged clones. Furthermore, EGFP-expressing cell clones derived from the same tagged clone displayed similar EGFP fluorescence patterns. This suggests the isogenic nature of the resulting targeted cells thus avoiding cloning and shortening the cell line development time-frame to 3 weeks (Fernandes, Vidigal et al. 2012).

In the present work, we evaluated the performance of *Sf9* -Flp cell line to produce complex proteins with biomedical interest, such as the rotavirus inner capsid protein VP2. This protein is the most abundant polypeptide of the rotavirus core (accounting for 90% of total protein mass). When expressed alone in recombinant form, VP2 assembles in core-like particles with 50 nm in diameter, consisting of 120 monomers of 102.5 kDa each, organized in 60 dimers (Lawton, Zeng et al. 1997). VP2 core particles as well as more complex multi-layered rotavirus-like particles (RLPs) have been produced using the baculovirus expression vector system (BEVS) (Labbe, Charpilienne et al. 1991; Lawton, Zeng et al. 1997; Vieira, Estevao et al. 2005; Roldao, Vieira et al. 2007), mostly due to its ability to simultaneously express various proteins driven by strong very-late baculovirus promoters (van Oers 2011; Fernandes, Teixeira et al. 2013). Here, we compare both expression systems, *Sf9* -Flp and BEVS, using a VP2-GFP fusion protein developed by Charpilienne *et al.* (2001), which retains the ability to self-assemble into core-like particles and can be easily followed by fluorescence (Charpilienne, Nejmeddine et al. 2001).

3. Materials and Methods

3.1 Construction of the target cassette encoding VP2-GFP

The ORF coding VP2-GFP was amplified by PCR from the genome of a recombinant *Autographa californica nucleopolyhedrovirus* vector (Ac-VP2GFP, kindly provided by Dr. Monique van Oers, Wageningen University, Netherlands). The genome was extracted and purified by High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Penzberg, Germany). The VP2-GFP amplimer was then inserted at AgeI-NotI sites within pTarget-OpIE2eGFP (Fernandes, Vidigal et al. 2012), replacing GFP ORF. The resulting plasmid vector (pTarget-VP2GFP) was propagated in *E. coli* Library Efficiency® DH5a (Life Technologies) and purified using a silica-based anion-exchanGenElute plasmid MiniPrep kit (Sigma), according to the manufacturer protocol.

3.2 Cassette Exchange

For site-specific cassette exchange, the *Sf9* -Flp cell line was co-transfected at a cell density of 1.0×10^6 cell/ml with 0.1 µg of *pTarget-VP2GFP* and 0.5 µg of Flpe recombinase-expressing vector (*pOpIE2FLPe,* Fernandes *et al.* (2012)) using one unit of Cellfectin® II reagent (Invitrogen) per 1×10^6 cells. G418 (300 U/ml) was added three days later to the transfected population, and cells were transferred to 6-well plates. The culture medium was replaced every five to six days by conditioned medium supplemented with 10% FBS and 300 U/ml of G418. After three weeks in selection, growing colonies were pooled and expanded. This population (*Sf9* -VP2GFP) was assessed for correct cassette exchange by PCR.

3.3 Cell culture maintenance

Parental Sf9 cells (ECACC 89070101), as well as *Sf9* -Flp and *Sf9* -VP2GFP cells were all cultured in Sf-900 II medium (Gibco Invitrogen Corporation, Paisley, UK) at 27ºC. Routine culture was performed in 125/500 ml shake flasks (Corning, USA) with 20/50 ml working volume, agitated at 115/90 rpm; cells were sub-cultured every 3 to 4 days at 5×10^5 cell/ml. Cell density and viability were determined by cell counting using a Fuchs-Rosenthal (Brandt, Wertheim/Main, Germany) chamber after diluting culture bulk samples in 0.4% (v/v) Trypan Blue.

3.4 Baculovirus infection

For VP2-GFP protein production with the BEVS, the baculovirus vector Ac-VP2GFP (encoding VP2-GFP under the polyhedrin promoter) was used to infect *Sf9* cells. The cell concentration at infection (CCI) was 4×10^6 cell/ml, using a multiplicity of infection (MOI) of 3 pfu/cell, with 50% medium exchange 24h before infection. Both supernatant and cell extracts were collected daily until 96 hpi, when cell viability was about 70%. For virus propagation, infection was performed at CCI of 1×10^6 cell/ml, using a MOI of 0.1. Virus titers were determined as described elsewhere (Carinhas, Bernal et al. 2010).

3.5. Exometabolome analysis

^{1}H-NMR was performed in a 500 MHz Avance spectrometer (Bruker, USA) equipped with a 5 mm QXI inversed probe. Spectra were acquired using a NOESY-based pulse sequence with water presaturation, performing 256 scans with 4 s acquisition time, 1 s relaxation delay and 100 ms mixing time at 25°C. DSS-d6 (Cat. No. 613150, Isotec, USA) was used as internal standard for metabolite quantification in all samples. Sample preparation was performed accordingly to Carinhas *et al.* (2013) (Carinhas, Duarte et al. 2013). Each spectrum was phased, baseline corrected and integrated using the Chenomx NMR Suite 7.1 (Chenomx Inc., Canada).

3.6. Process optimization

Consecutive feedings, each corresponding to approximately 10% of the working volume, were implemented in shake flask and bioreactor cultures in order to improve cell density and viability over time. The applied feeding formulations (Table I) comprised glucose, amino acids, peptones (wheat peptone E1, Organotechinie S.A.S), lipids (lipid supplement 100×, Gibco), vitamins (RPMI 1640 Vitamins solution 100×, Sigma-Aldrich) and extra supplements (Insect Medium Supplement 10×, Sigma-Aldrich). Bioreactor cultures were performed in BIOSTAT® B-DCU 1L vessels (Sartorius, Goettingen, DE), with 800 mL working volume. Dissolved oxygen was kept at 30% of air saturation through sparging (aeration rate of 0.01 vvm) by using a sequential *cascade* of agitation between 80 and 290 rpm, followed by an increasing percentage of O_2 in the airflow over culture time. The pH was monitored online and remained stable during culture time (within 6.0-6.2 pH unit interval).

3.7. Flow cytometry

CyFlow® space (Partec GmbH, Münster, Germany*) flow cytometer* was used to evaluate VP2-GFP protein fluorescence intensity in both production systems. Cells were harvested and diluted in PBS. GFP was detected using FL1 (emission filter: 527 ± 13 nm). Fluorescence intensity from 10 000 events per sample was collected and analyzed using FlowMax Software (©2009, Quantum Analysis GmbH).

3.8 Confocal microscopy

Cells expressing VP2-GFP were seeded on a 22 × 22-mm coverslips and washed with PBS, fixed with 4% (w/v) paraformaldehyde (PFA) in PBS for 15 min, washed twice with PBS and stored at 4ºC. The samples were mounted in Prolong® gold anti-fade containing 4,6-diamidino-2-phenylindole (DAPI). Specimens were observed at a spinning disk confocal microscope (Andor Technology, Belfast, Northern Ireland), using a 100× planapochromatic oil objective, with excitation and detection wavelength parameters set at 488 nm and >505 nm, respectively.

3.9 Solubilization and purification of VP2-GFP particles

Cells expressiongVP2-GFP were harvested, pelleted by centrifugation (200 × *g* 10 min at 4ºC) and resuspended in TED buffer (10 mM Tris-HCl [pH 7.4], 0.1 mM EDTA, cOmplete EDTA-free protease inhibitor cocktail (Roche). Cell suspensions were vortexed for 1 min, followed by sonication on ice (3 min, 20 kHz, 6 pulses with 30s/pulse, ON/OFF mode alternatively 250-450 Sonifier Analog Cell Disruptor, Branson.), then supplemented with in 1% sodium deoxycholate (DOC) – 0.1% SDS and clarified by centrifugation (10000 *g* for 10 min at 4ºC). The resulting supernatants were either analyzed by Western Blot or loaded onto a 5 to 54.85 % (w/v) sucrose gradient in 0.1% SDS, 1% DOC –TED buffer and centrifuged for 120 min in a SW41ti rotor (Beckman) at 111000 *g* (4ºC). Fractions were collected, analyzed by SDS-PAGE, dialyzed and subsequently examined by electron microscopy.

3*.10 Western Blot*

The samples were run in reducing and denaturing conditions and separated by electrophoresis on a 1-mm Novex® NuPAGE® Bis-Tris gel (Life Technologies) and blotted to a nitrocellulose membrane (HybondTMC extra; Amersham Biosciences, Little Chalfont, UK). VP2-GFP immunochemical staining was carried out with anti-GFP monoclonal antibody (GFP-20 clone,

Sigma) at a 1:2000 dilution during 2h at room temperature. Blots were developed using the enhanced chemiluminescence detection system after 1 h incubation with horseradish peroxidase-labeled anti mouse IgG antibody (GE Healthcare) against GFP antibody.

3.11 Electron Microscopy

The morphology and integrity of VP2-GFP VLPs were evaluated by electron microscopy after negative staining. Three microlitres of sample were fixed for 1 min in a 400 mesh copper grid coated with Formvar-carbon (Electron Microscopy Sciences, Ft. Washington, PA, USA). The grids were then washed, soaked in 2% uranyl acetate for 30 sec left to dry after rinsing and observed at a Hitachi H-7650 Transmission Electron Microscope (JEOL, Tokyo, Japan).

4. Results

4.1 Site-specific integration of VP2-GFP into the *Sf9* -Flp cell line

A previously developed master cell line (*Sf9* -Flp) tagged with DsRed expression cassette flanked by two non cross-interacting FRT sites in a high recombination efficiency *locus* (Fernandes *et al.* 2012) was exploited to produce rotavirus core-like particles. *Sf9* -Flp cells were co-transfected with a Flp-encoding plasmid and a target cassette encoding VP2-GFP flanked by an identical FRT pair. Cells which exchanged cassettes became resistant to G418 by ATG complementation of the neomycin gene in the tagged *locus* (Figure 1a). After three weeks in selection as adherent cultures, the growing green colonies were pooled together and the resulting cell population (*Sf9* -VP2GFP) was analyzed by genomic PCR; a 600 bp length product (H and J primers from Figure 9 A) confirmed the loss of the hygromycin resistance gene and that correct cassette exchange occurred (Figure 9 B). At the end of the three-week selection process, a small percentage of DsRed positive cells (5%) is still present, in part justified by the long half-life of the DsRed protein (~5 d), which progressively disappears by maintaining cells under G418-selective pressure.

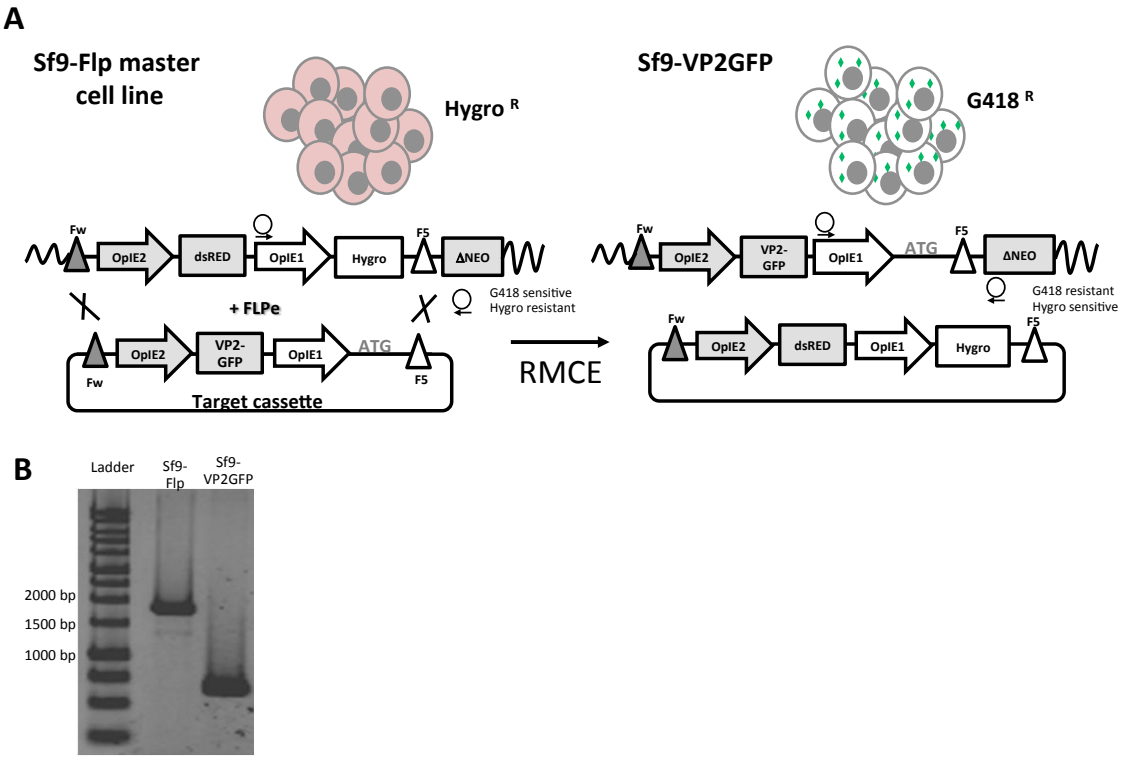

Figure 9. Integration of a VP2-GFP transgene in a pre-tagged locus. A) The *Sf9* -Flp master cell line is tagged in a locus with a DsRed encoding cassette flanked by a pair of incompatible Flp recognition target (FRT) sites (Fw and F5). The target cassette is flanked by the same FRT pair and contains the VP2-GFP transgene and the ATG sequence complementary to the defective neomycin resistance gene downstream to F5 in the tagged locus. **B)** Genomic PCR analyses (using H and J primers) resulted in a 600 bp amplicon for the VP2-GFP target population in contrast to a 1600 bp amplicon for the tagging master cell line (*Sf9* -Flp), thus confirming the integration of the target cassette into the tagged locus.

4.2 VP2-GFP production in stable *Sf9* -VP2GFP cells vs BEVS

Cell specific and volumetric VP2-GFP expression levels from single gene copy integrated in the *Sf9* -Flp cell line were compared to that obtained with baculovirus infection in shake flask cultures. Parental *Sf9* cells infected with Ac-VP2GFP (as described in Materials and Methods) underwent the infection-induced growth arrest by 24 hpi and the cell density progressively declined until 96 hpi when the culture was harvested (Figure 10 A). In the same time frame, *Sf9* -VP2GFP cells

continuously proliferated, reaching a maximum cell density of 12×10^6 cells/mL at 144 h after inoculum (Figure 10 A). Flow cytometry analysis showed the expected higher fluorescence intensity at population level by the transient lytic infection as a consequence of the high transgene copy number resulting from the viral DNA amplification (Figure 10 B). Nevertheless, confocal microscopy highlighted that VP2-GFP is mainly present as large amorphous aggregates within infected cells, while *Sf9* -VP2GFP expressed mainly regular dotted structures, suggestive of correctly assembled core-like particles (Figure 10 C). Moreover, immunoblot performed by loading protein extracts corresponding to equal culture volumes, proved that single copy VP2-GFP gene is well compensated by the higher cell densities achieved by *Sf9* -VP2GFP, leading to volumetric yields similar to those obtained with BEVS (Figure 10 C).

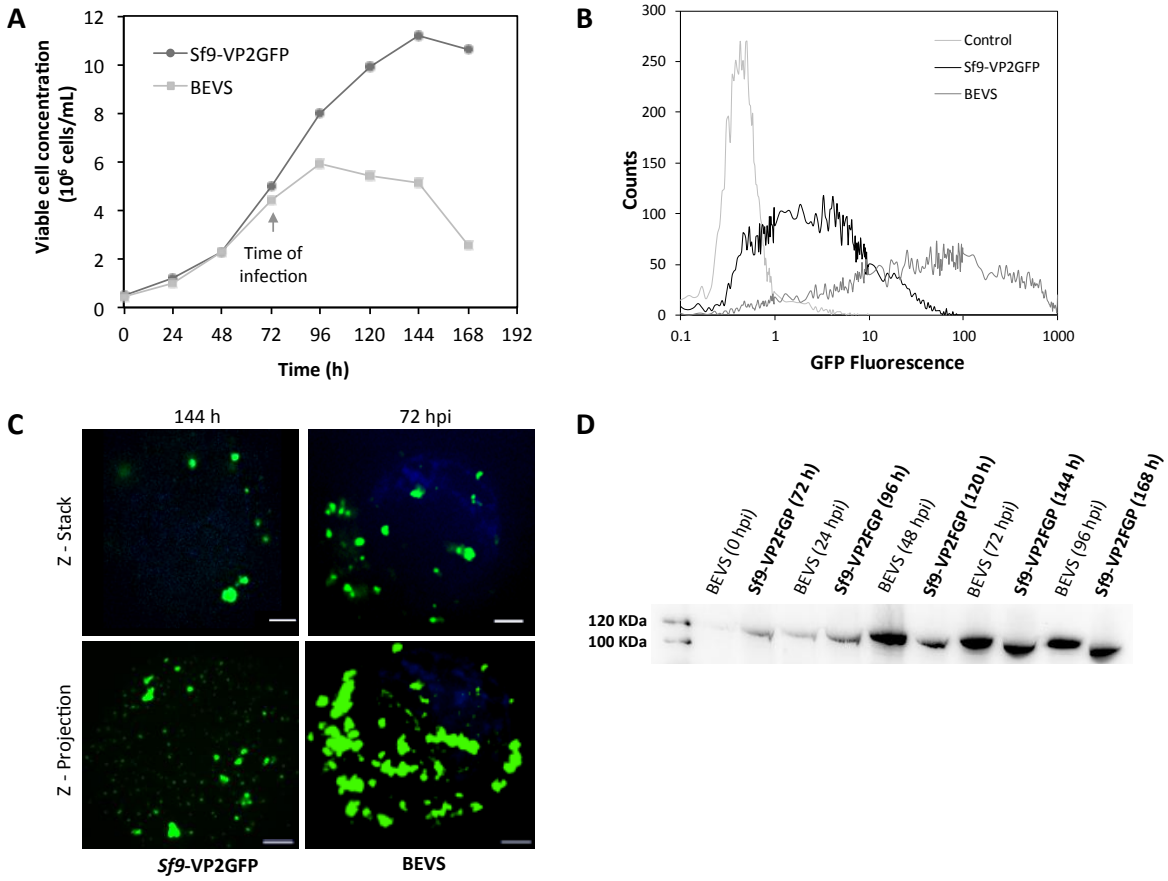

Figure 10. Comparison of VP2-GFP production between the *Sf9* -VP2-GFP cell population and the BEVS. A) Viable cell concentrations for both expression systems (the arrow points to the time of infection (CCI 4, MOI 3)). **B)** Flow cytometry analysis of VP2-GFP fluorescence from constitutive expression (at 144 h of culture) and baculovirus-infected cells (at 96 hpi). **C)** Confocal microscopy images of a representative

cell from both expression systems (upper panels are z-stacks and lower panels are projections of 0.5 µm z-stacks collected through the 100× objective; scale bar corresponds to 3 µm). **D)** Western blot analysis of VP2-GFP from both stable and infected cells over culture time; all lanes were loaded with protein extracts from equal culture volumes.

4.3 Optimization of *Sf9* -VP2GFP culture growth through nutrient feeding

To explore the advantages offered by a stable *Sf9* platform over the lytic baculovirus infection protocol, a small scale optimization study was performed based on the analysis of nutrient consumption trends. As *Sf9* cells are not affected by accumulation of inhibitory metabolites (Ikonomou, Schneider et al. 2003), fed-batch operation is a feasible culture mode to obtain high cell densities and increase the length of the cell productive/proliferative phase by selected nutrient addition (Bédard, Perret et al. 1997). The concentrations of glucose and of all amino acids were analyzed in the supernatant over culture time. From these, only serine (Ser) and cystine (Cys) were depleted (Figure 11 A); in fact, Ser exhaustion occurred at the onset of the stationary phase (96 h after inoculation). Interestingly, after Ser became undetectable the concentrations of the remaining amino acids either prevailed constant or increased in the supernatant, suggesting that cells start to degrade other amino acid sources (yestolates) present in the culture medium.

Based on these results, a simple fed-batch optimization study was performed to evaluate if the cell density from batch cultures could be further improved. Table 4 describes the regimen and composition of the two feeding strategies tested, both composed by Ser, Cys, glucose (Gluc), glutamine (Gln) and differing only in the source of lipids, vitamins and amino acids. Both feeding strategies allowed increasing the maximum cell densities (up to 16×10^6 cell/ml) and to extend culture viability in comparison to the control batch culture (Figure 11 B). The feeding strategy A was then implemented in bioreactor cultures, further increasing the maximum cell density to 20×10^6 cells/ml which was sustained for three days (Figure 11 C). VP2-GFP produced from fed-batch cultures was purified by sucrose density gradient centrifugation followed by Western blot analysis (Figure 11 D). Four fractions were observed to be the most enriched in VP2-GFP particles (lanes 4-7). Fraction 6 was selected for electron microscopy analysis confirming the presence of well assembled ~50 nm core-like particles (Figure 11 E) (Zeng, Labbe et al. 1994).

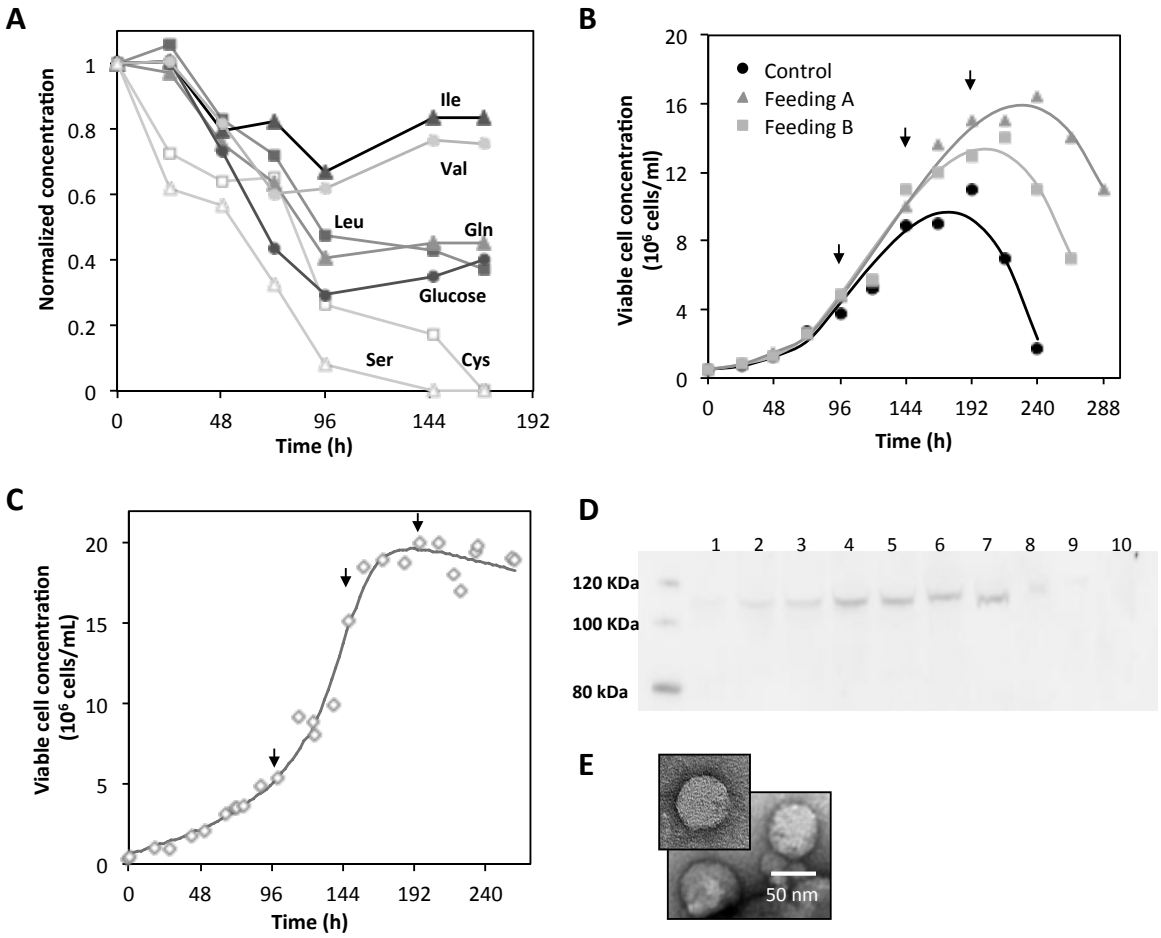

Figure 11. Bioprocess optimization of stable VP2-GFP production. A) Concentration profiles of glucose and some amino acids, each normalized to its initial concentration. **B)** Growth curves of *Sf9* -VP2GFP cells resulting from implementation of feeding strategies. Arrows indicate feeding times; each feeding formulation is listed in Table I. **C)** Growth curve of *Sf9* -VP2GFP cells in bioreactor cultures resulting from feeding strategy A. **D)** Western blot analysis of sucrose gradient ultracentrifugation fractions from bioreactor culture extracts. VP2-GFP particles were detected in 4 (lanes 4-7) of the 9 fractions collected (lanes 1-9). Lane 10 corresponds to the negative control. **E)** Electron microscopy images of VP2-GFP particles from the fraction corresponding to lane 6.

Table 4. Feeding strategies implemented in *Sf9* cultures.

	Feeding Strategy A	Feeding Strategy B
Feed 1 (96h)	10 mM Ser, 1 mM Cys prepared in insect medium supplement (1x)	10 mM Ser, 1 mM Cys prepared in Sf-900II medium
Feed 2 (144h)	20 mM Glucose, 2 mM Gln and insect medium supplement (1x)	20 mM Glucose, 2 mM Gln, 4g/L peptones, lipidic cocktail (1x) and vitamin solution (1x)
Feed 3 (192h)	15 mM Glucose, 2.5 mM Gln and insect medium supplement (1x)	15 mM Glucose, 2.5 mM Gln, lipidic cocktail (1x) and vitamin solution (1x)

5. Discussion

We have previously established multiple *Sf9* -Flp master cell lines, each randomly tagged in a single genomic locus, that can be used to produce different target proteins by Flp-mediated site specific integration of genes of interest (Fernandes, Vidigal et al. 2012). In this work, we used the cell line with the highest recombination efficiency among those previously generated, which displays average transgene expression levels, and turned it into a producer of rotavirus core-like particles. Due to the high recombination efficiency, in less than four weeks we could obtain a cell population stably expressing the target protein over several passages. RMCE excludes the need for a time-consuming clone screening protocol allowing short development timelines comparable to those possible with the baculovirus expression system. This time frame can likely be further reduced using a fluorescence activated cell sorting protocol recently developed at our lab for insect cell lines (submitted manuscript), exploiting the loss of red fluorescence by cells which undergo cassette exchanged for recombinant protein expression.

Even though infected cells displayed higher specific productivities, overall volumetric yields were similar to those obtained through *Sf9* -VP2GFP constitutive expression as higher cell concentrations were reached. Noteworthy, constitutive expression of VP2-GFP is driven by an immediate early baculovirus promoter which is much weaker than the polyhedrin promoter controlling protein expression in the BEVS (Douris, Swevers et al. 2006). By designing feeding strategies to satisfy insect cell nutritional needs, significant improvements in cell densities and

culture time extension were obtained to drive further increments in protein production. Upon sucrose gradient ultracentrifugation of the cell extracts, particles with the expected diameter (approximately 50 nm) (Labbe, Charpilienne et al. 1991; Zeng, Labbe et al. 1994) were observed by electron microscopy, indicating that this stable expression system efficiently delivers assembled rotavirus core-like particles. A similar bioprocess intensification strategy was followed by Jardin *et al.* (2007), demonstrating that significantly higher titers can be achieved from high cell density fed-batch and perfusion cultures of a cell line constitutively expressing a secreted reporter protein when compared to those from infection batch cultures (Jardin, Montes et al. 2007).

In conclusion, this work validates the use of a previously developed master cell line that exploits the RMCE strategy to rapidly produce complex proteins, demonstrating it as a valuable alternative to the BEVS. The added value of our cell platform is expected to be more prominent for expression of secreted proteins, not only in terms of quantity, as high cell densities and an extended production phase can be easily obtained, but also in terms of quality, since the protein processing and secretion machinery is not compromised as in late stages of infection.

6. Acknowledgements

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Chapter IV

Streamlining insect-based cell line development that leverages RMCE technology

This Chapter is adapted from the manuscript

Vidigal J, Dias MM, Fernandes F, Patrone M, Bispo C, Andrade C, Gardner R, Carrondo MJ, Alves PM, Teixeira AP. A cell sorting protocol for selecting high-producing sub-populations of Sf9 and High Five cells. Journal of Biotechnology 168 (4), 436-439 (2013)

Table of Contents

1.Summary

Insect cell lines such as *Sf9* and High FiveTM have been widely used to produce recombinant proteins mostly by the lytic baculovirus vector system. We have recently established an expression platform in *Sf9* cells using a fluorescence-based recombinase mediated cassette exchange (RMCE) strategy that has similar development timelines but avoids baculovirus infection. To expedite cell engineering efforts, a robust fluorescence-activated cell sorting (FACS) protocol optimized for insect cells was here developed. The standard sorting conditions used for mammalian cells proved to be unsuitable, resulting in post-sorting viabilities below 10% for both cell lines. We found that the extreme sensitivity to the shear stress displayed by *Sf9* and High Five^{TM} cells was the limiting factor, and using a biocompatible surfactant in the cell suspension could increase post-sorting viabilities in a dose dependent manner. The newly developed protocol was then used to sort stable populations of both cell lines tagged with a dsRed-expressing cassette. Before sorting, the average fluorescence intensity of the *Sf9* cell population was 3- fold higher than that of the High FiveTM cell population. By enriching with the 10% strongest dsRed-fluorescent cells, the productivity of both cell populations could be successfully improved. The established sorting protocol potentiates the use of RMCE technology for recombinant protein production in insect cells.

2. Introduction

Insect cell lines have been widely used to produce recombinant proteins using the baculovirus expression vector system (BEVS) (Kost et al, 2005). To bypass baculovirus infection and still take advantage of insect cells as production hosts, stable cell lines have also been developed (McCarroll et al, 1997, Harrison and Jarvis, 2007). Traditionally, this process is very time-consuming as extensive screening is needed to find those clones which integrated the gene of interest into superior *loci* (Wurm, 2004). To circumvent the positional effect, site-specific recombinases have been applied to Sf9 cells (Fernandes et al., 2012), allowing the re-use of a previously tagged and characterized locus and reducing the time needed for the implementation of cell lines stably expressing the target of interest. To further potentiate the use of site-specific integration strategies in insect cells, a fast high- throughput cell screening approach based on fluorescence-activated cell sorting (FACS) is needed to reduce the work-oad associated with standard limiting dilution cloning methods. This technology has been widely applied to develop recombinant mammalian cell lines (Weaver et al., 1997; Mattanovich and Borth, 2006; Brezinsky et al., 2003; Turner et al, 2004), including mammalian RMCE applications (Qiao et al, 2009; Coroadinha et al, 2006; Turan et al, 2010; Mata et al. 2012). However, FACS methodologies have been poorly explored in insect cell

line development.

In this study, we present a FACS-based methodology to swiftly select high-producing sub-populations of fluorescence-tagged *Spodoptera frugiperda* and *Trichoplusia ni* cell lines (*Sf9* and High Five™, respectively) to be later used for targeted recombination with a gene of interest. Both cell lines have been widely used to produce recombinant proteins through baculovirus infection. Although Sf9 cells have improved growth and metabolic efficiency, making them better hosts for baculovirus replication, High Five™ cells have been described as better recombinant protein producers (Krammer et al., 2010; Rhiel et al., 1997; Monteiro et al. 2014). Herein, the first comparison of the two cell lines under virus-free constitutive expression of reporter genes is provided.

3. Material and Methods

3.1 Cell lines, cultivation and transfection

Spodoptera frugiperda Sf9 cells (ECACC 89070101) and *Trichoplusia ni* BTI-TN5B1-4 (High FiveTM, Hi5) cells (ATCC CRL-10859) were cultured in Sf-900II SFM (Gibco Invitrogen Corporation, Paisley, UK) and Insect-XPRESSTM (Lonza, Basel, Switzerland) media, respectively. Routine culture was performed in 125 ml shake flasks (Corning, NY) with 15 ml working volume at 110 rpm. Hi5 and *Sf9* cells were sub-cultured every 3–4 days at 0.3×106 and 0.5×106 cell/mL, respectively. The same cell densities were applied for transfection; for each 1×106 cells, it was used 8 µl of Cellfectin II reagent (Invitrogen) and 0.3 µg of the tagging plasmid containing a dsRed reporter gene and hygromycin as selective marker (Fernandes et al. 2012). Selection started 72h post-transfection using medium containing Hygromycin B (200 U/ml).

3.2 Flow Cytometry

Cells were sorted in a MoFlo High Speed cell sorter (Beckman Coulter, Fort Collins, USA) equipped with a 488 nm laser (200 mW air-cooled Sapphire, Coherent) for scatter measurements and a 561 nm laser (50 mW DPSS, CrystaLaser) for dsRed excitation. DsRed was detected using a 630/75 nm bandpass emission filter. As a special requirement for insect cells, cells were resuspended in Phosphate Buffer Saline (PBS) supplemented with Pluronic acid F68 (Sigma, St. Louis, USA). PBS was used as sheath fluid, and run at a constant pressure of 207 kPa with a 100 µm nozzle and a frequency of drop formation of approximately 30 kHz. Sorting rates were typically 2×106 cells per hour. Viability tests were performed by Propidium iodide staining. Cells were

collected into ~1 ml of PBS also supplemented with Pluronic maintained at 4 ^{o}C. After sorting, cells were pelleted (300 × *g* for 10 min) and kept in culture medium with Antibiotic–Antimycotic (Invitrogen).

CyFlow R space (Partec GmbH, Münster, Germany) was used to evaluate transfection efficiencies as well as to measure dsRed intensity. Exponentially growing cells were harvested and diluted in PBS. DsRed was detected using a 590/10 nm emission filter. Fluorescence intensity from 10 000 events per sample was collected and analyzed using the FlowMax Software (2009, Quantum Analysis GmbH).

4. Results and Discussion

4.1 Optimization of insect cells sorting conditions

The insect cell lines *Sf9* and Hi5 revealed to be extremely sensitive to the cell sorting process. The standard sorting conditions (2% FBS in PBS), which allow over 90% post-sorting viabilities for CHO cells, were not suitable for either of the above insect cell lines, collected with viabilities below 10% (Figure 12). Cells are exposed to high hydrodynamic forces when flowing through the sorter's microfluidic system and subjected to relatively high electrical charges. However, reducing sheath fluid pressure to minimal levels (<85 kPa) did not significantly improve the post sorting viability and reducing the applied charge did not improve beyond 50% post sort viability (data not shown). On theother hand, adding the surfactant Pluronic acid F68 (PA), which is normally present in culture media to protect cells from shear stress in stirred and gas-sparged bioreactors, could restore cell viabilities. The effective PA concentration was proportional to the sensitivity of each cell line to the shearing insult: while 0.5% PA (v/v) was sufficient to collect Hi5 cells with viabilities over 90%, *Sf9* cells required 3% PA to reach the same post-sorting survivability (Figure 12). Despite the high PA concentrations, cells resuspended in fresh medium displayed normal growth behavior. As an alternative if PA sensitive cells are used, we have obtained similar high viabilities using 0.1% PA in both the cell suspension solution and the sheath fluid (Figure 12). A similar result had been obtained for *Drosophila* S2 cells (Bryantsev and Cripps 2012).

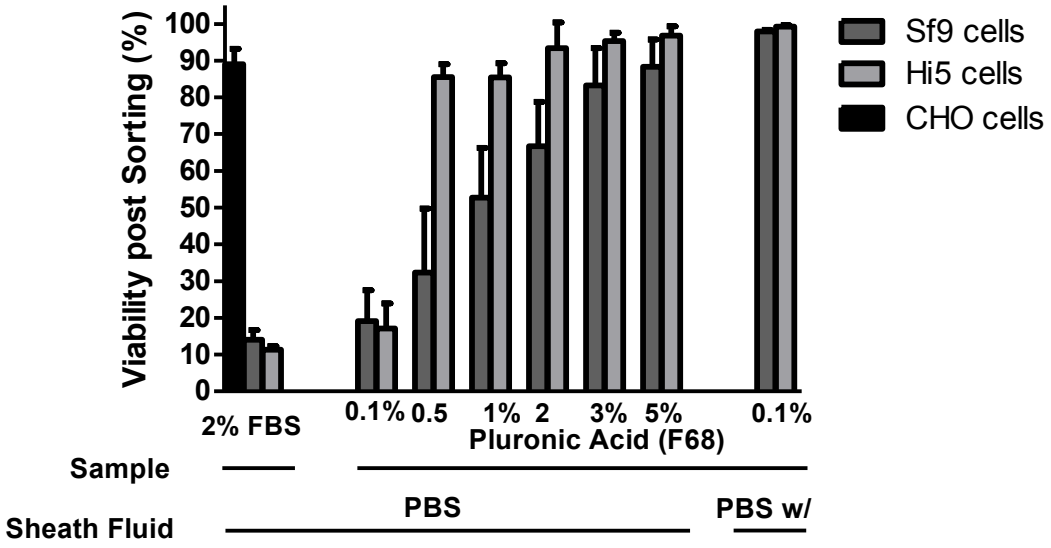

Figure 12. Post sorting viabilities of mammalian and insect cell lines. The standard mammalian cell sorting conditions, in which cells to be sorted are resuspended in PBS containing 2% of FBS, resulted in very low recovering viabilities for both *Sf9* and Hi5 cells. The addition of pluronic acid F68 to the cell preparation significantly improved post sorting viabilities for both insect cell lines; Hi5 cells required a lower concentration (0.5%) to reach viability over 90%. Alternatively, high viabilities were also obtained when using 0.1% PA in both the cell suspension solution and in the sheath fluid. Pre-sorting viabilities were always higher than 94% for all cells.

4.2 Selecting sub-populations tagged in high expressing *loci*

Hi5 and *Sf9* cells were transfected with the same plasmid harboring dsRed under the control of the immediate early baculovirus promoter OpIE2 (Fernandes et al. 2012). This expression cassette, flanked by FRT sites, allows for subsequent exchange with a cassette containing a gene-of-interest. As the aim is to re-use an integration *locus* with high expression properties, FACS can be exploited to select the corresponding sub-population, reducing the number of clones to evaluate for establishing a master cell line. The fluorescence distribution of both Sf9 and Hi5 cell populations evaluated 48h after transfection was similar as analyzed by flow cytometry (Figure 13 A); analogous results were obtained when EGFP was used as reporter protein (data not shown). Noteworthy, upon two weeks of antibiotic selection, the mean fluorescence of the *Sf9* cell pool was almost 3 times higher than that of the Hi5 pool (Figure 13 B). These results suggest *Sf9* cells are more prone to

integrate exogenous DNA, possibly resulting in multiple integrations and/or insertion in higher-expressing *loci.* A difference of 3 fold in recombinant expression of two similar fucosyltransferases had been previously obtained in two independent studies, both using the OpIE2 promoter; the titers reached were 13.4 mg/L in *Sf9* cells (Morais and Costa, 2003) and 3.92 mg/L in Hi5 cells (Münster et al, 2006). Following enrichment by FACS of the top 10% dsRed-fluorescent cells of each population and two additional weeks in culture, the mean fluorescence intensities of the sorted cell populations increased significantly (5.4 and 9.7-fold for *Sf9* and Hi5, respectively), bringing closer the expression levels obtained from each cell type (Figure 13 C, D and Table 5). Overall, repeated rounds of FACS can be used to select for the best performing clones from polyclonal cell populations, speeding up the development process of insect cell lines using site-specific integration approaches.

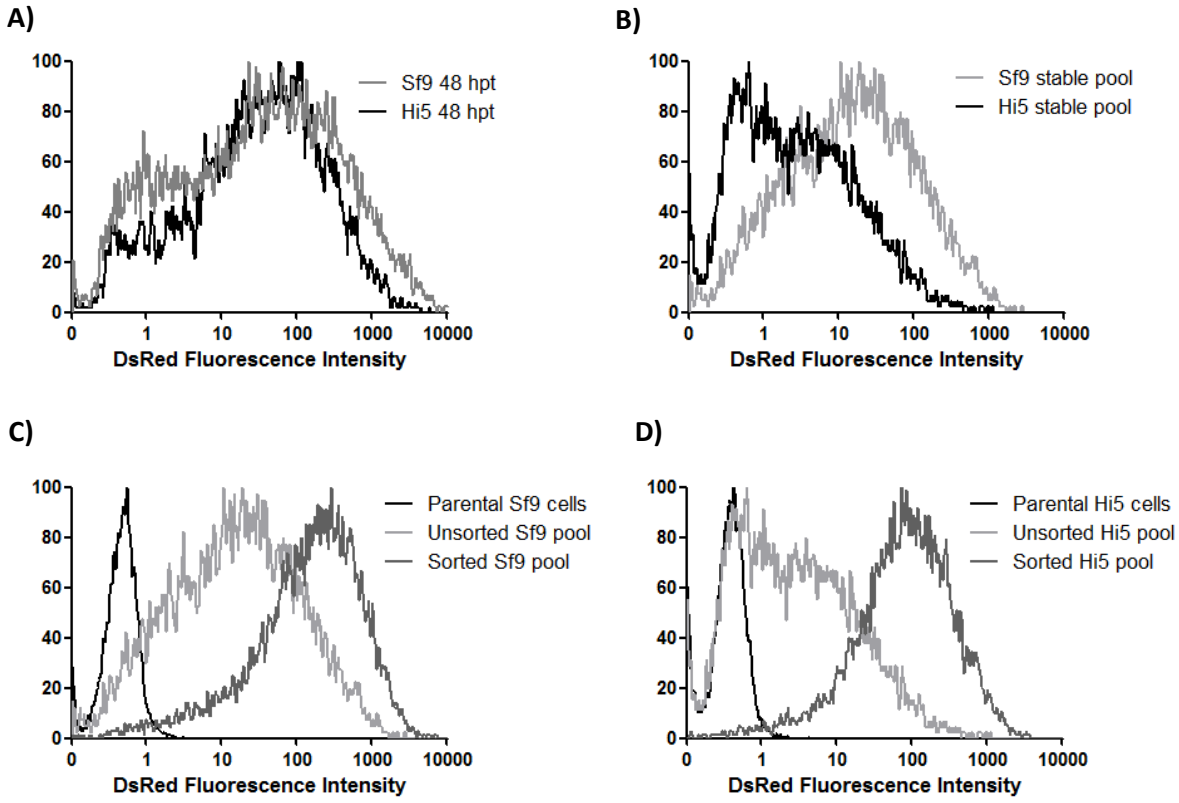

Figure 13. Comparison of dsRed fluorescence distribution from *Sf9* and Hi5 cells transfected with the same plasmid. A) 48h after transfection, **B)** upon two weeks in selection. Comparison of unsorted and sorted **C)** *Sf9* and **D)** Hi5 cell pools; the sorted pools were obtained from the top 10 % dsRed-fluorescent cells in the unsorted pools.

Table 5. Comparison of fluorescence distributions of *Sf9* and Hi5 pools before and after sorting.

	Median Fluorescence		Mean Fluorescence		Mean Fluorescence ratio
	Sf9	High Five™	Sf9	High Five™	Sf9 / High Five™
Unsorted Pools	22.6 ± 7.8	4.9 ± 1.1	97.8 ± 24.8	34.1 ± 1.0	2.9
Sorted Pools	319.1 ± 34.0	191 ± 13.9	524.5± 52.2	329.9 ± 21.8	1.6
Fold increase	14.1	39.0	5.4	9.7	-

5. Conclusions

The established FACS protocol fills an important gap in applications of insect cell line engineering, in particular strengthening the potential of RMCE for fast recombinant protein expression. Besides aiding to establish tagged master cell clones, the described method can accelerate the isolation process upon cassette exchange of final cell lines by counter-selection of fluorescent markers. This report also provides a comparison of stable expression between *Sf9* and Hi5 cells. To our knowledge, this is the first time recombinant protein production has been benchmarked in these two important cell lines independently of baculovirus infection. Although transient expression is comparable, the stable populations differ significantly, suggesting that *Sf9* cells have a higher propensity to incorporate plasmid DNA in productive genomic hotspots. Thus, the choice of Hi5 cells as the preferred host for recombinant protein production is likely restricted to the transient lytic infection system. This apparent discrepancy is an issue of interest to be further explored in the future.

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Chapter V

Discussion and Conclusions

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1. Discussion

The work presented on this thesis focuses on the development and optimization of *Sf9*-based cell lines for stable expression of proteins as an alternative to the well-established baculovirus-mediated insect cell expression system. Site-directed engineering of defined chromosomal sites was evaluated to develop protein producing systems in an expedite way. This technology allows to reuse a previously identified hot spot to express different target protein, reducing the time and effort invested in the development of high producer cell lines (Figure 14) (Vidigal, Fernandes et al.). The timelines are significantly cut compared to conventional cell line development based on random transgene integration.

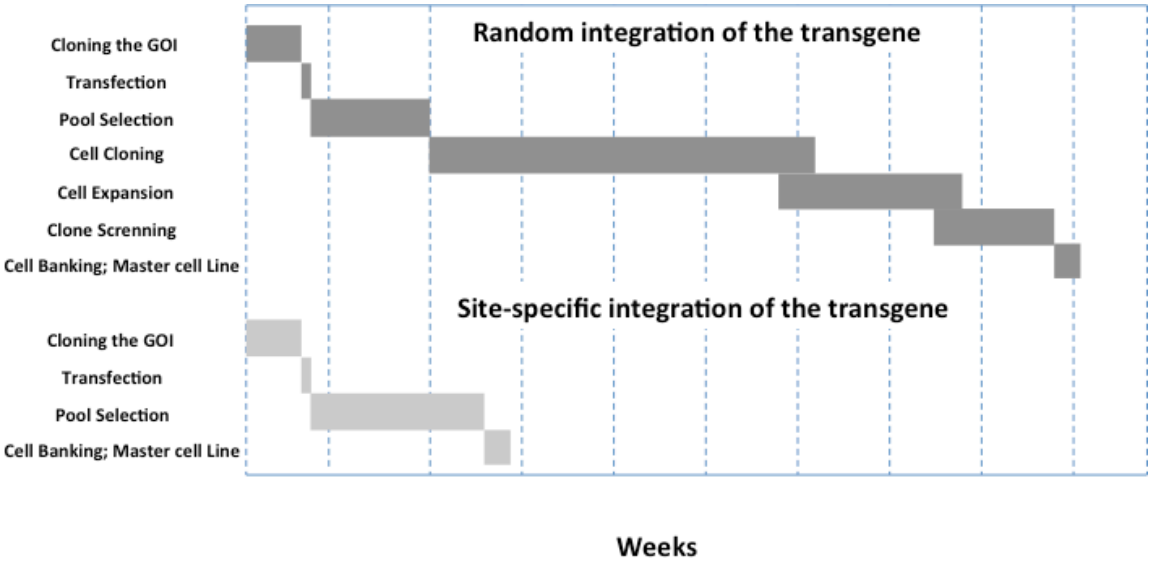

Figure 14. Timelines for cell line development through random or site-specific integration of the transgene. The process that employs site-specific integration of transgenes is much faster due to the re-use of pre-tagged high-level expression locus.

At the onset of this work, stable insect cell lines for protein production were routinely established through conventional transfection and selection strategies, where several copies of the GOI are randomly inserted into the cell genome. Even though RMCE technology has been widely exploited during the last ten years for mammalian cell line development, its use in the development of insect-based producer cell lines is still in its infancy. Nakayama *et al.* reported a RMCE-like method using phiC31 attP and attB sites in direct orientation for site specific integration recombination system; although cassettes were not exchanged this method permitted the integration of a gene into the cell genome (Nakayama *et al.* 2006). Similar methodologies for RMCE using the FRT/FLP (Horn and Handler 2005) and loxP/Cre (Oberstein *et al.* 2005) recombinase systems have only

been described for the organism *Drosophila melanogaster*, not in transformed cell lines. Flipase site-specific recombination (FLP/FRT) strategies have been used for mammalian-based production of several proteins, including monoclonal antibodies, as well as multiprotein complexes and viruses, e.g such as retroviral vectors (Coroadinha, Schucht et al. 2006; Schucht, Coroadinha et al. 2006; Wiberg, Rasmussen et al. 2006; Huang, Li et al. 2007).

In Chapter II of this thesis, the establishment of a flipase-based recombination system in *Sf9* cells has been undertaken, aiming at flexible and fast development of cell lines expressing target proteins. Initial efforts were put in vector design, mainly to select suitable promoters that could drive satisfying and consistent levels of protein expression in these cells. The BEVS prominence in the biotechnology field has limited the use of stable insect cell expression platforms; thus the number of promoters available to drive stable expression in insect cells is low. Moreover, the lack of *Sf9* genomic data compromises the implementation of refined cell line engineering strategies. Promoters from *Drosophila melanogaster* and immediate early promoters from baculoviruses were considered, with the later ones clearly allowing higher transcriptional rates.

To take full advantage of RMCE systems, some critical conditions must be met: a) an efficient tagging of a hotspot allowing high and stable expression, b) integration of single copy of the tagging vector and c) the tagging hotspot should allow an efficient targeting process each time a transgene needs to be integrated. Having this in mind, the proof of concept of cassette exchangeability has been illustrated by the expression of reporter proteins - dsRed and EGFP were encoded in the tagging and target cassettes, respectively; this is a remarkable advantage for the pre-selection of high producer cell lines that should also sustain flipase-driven recombination. As reported in RMCE-based mammalian cell line development, we observed that effective cassette exchange is highly dependable on the chromosomal site in which the tagging cassette is inserted. From the number of isolated clones with single copy integration of the tagging cassette, only less than 20% of them could support an efficient cassette exchange process; unfortunately, the highest dsRed-expressing clones turn out to be tagged in non-targetable loci. As expected, expression patterns of dsRed and EGFP from the same tagged locus are correlated. Furthermore, EGFP expression levels from single copy integration in Sf9 cells were comparable to those obtained when parental *Sf9* cells are infected with baculovirus vectors expressing the same reporter protein under the control of the stronger very late *polh* promoter.

The use of these producer cell lines for the expression of complex proteins with biomedical relevance was evaluated in Chapter III, where flipase-mediated cassette exchange promoted the

integration of the rotavirus VP2 gene into a pre-tagged locus of the *Sf9* cell genome. Additionally, we took advantage of this virus-free process by looking into culture conditions allowing higher cell densities and extended production phases. High volumetric productivity without compromising protein quality is the ultimate goal during cell culture process development. The influence of different media supplements has shown to improve cell densities and as a consequence the production levels of VP2 rotavirus core-like particles at the end of the process. *Sf9*-RMCE cell lines constitute a viable alternative to the BEVS as a platform for fast production of complex proteins. Besides the application of the RMCE concept for the production of the rotavirus VP2 protein, we have applied it to express other rotavirus proteins (VP6 and VP7), influenza proteins (Na, HA and M1) and the human cytomegalovirus gB glycoprotein.

In parallel, some bottlenecks faced during the development of the RMCE-based insect cell lines were addressed to further streamline the cell line development process (Chapter IV). A FACS protocol has been established for insect cells, ensuring high post-sorting viabilities, thus representing a vital tool to accelerate cell line development by targeted integration.

In summary, site-directed engineering of defined chromosomal sites has shown to be a valuable approach for the generation of reliable and flexible insect cell factories aiming at stable and consistent production of proteins. The established protein expression platforms constitute a suitable alternative to the BEVS, presenting similar fast development timelines and protein productivities, but with the added advantages associated to stable expression systems.

2. Future work

The RMCE concept has been recently extended to a simultaneous cassette-exchange process of two pre-characterized loci (Turan, Kuehle et al. 2010). The combination of different non-interacting recombinase sites (multiplexing) or the combination of recombinase systems (e.g., Cre/LoxP and FLP/FRT) permits a distinct introduction of two or more expression cassettes within independent chromosomal integration sites. In this respect, several applications would benefit from such expression approach in insect cells, namely the production of VLPs composed of different protein subunits, since multiple integration sites are preferred over a single-site, as repeated insertion of DNA sequences in the same locus is a main cause of genetic instability (Bzymek and Lovett 2001; Schucht, Coroadinha et al. 2006). Furthermore, predominant obstacles associated to assembly efficiency of protein complexes can be addressed by taking advantage of synthetic biology tools to govern a timely and stoichiometrically coordinated expression of all their components (Vieira,

Estevao et al. 2005). Specifically, transcriptional circuits and/or post-transcriptional regulation elements can be incorporated in the target cassettes enabling independent control of expression of each component (Bhalerao 2009; Weber and Fussenegger 2009), thus promoting higher yields of correctly assembled complexes.

The work developed within this thesis can be straightforwardly translated to other transformed insect cell lines (e.g. High Five^{TM}, also widely used for rBVs infection), enabling a broader portfolio of stable insect-based expression platforms, to cope with the production of challenging proteins. It is anticipated that a new generation of insect-based producer cell lines can be pursued eying future commercialization of protein manufacturing services within a vast number of applications.

In another perspective, RMCE technology also represents a valuable tool for the development of optimized expression vectors/cassettes; the presence of specific regulatory elements to take maximal expression profit from a specific tagged chromosomal site in which they are integrated can be tuned according to expression needs. Therefore, a systematic approach to study the performance of different regulatory elements concerning expression level optimization in a given integration site could be performed through this established cell platform. In this perspective, RMCE can be a technology of excellence in the re-engineering of genomic loci towards their optimal expression potential. Furthermore, the same approach can be used to study inducible expression systems, benefiting from a controlled and defined chromosomal environment, in which different elements can be tested to achieve the desired level of regulation (Wong, Kolman et al. 2005; Iacovino, Bosnakovski et al. 2011).

We have shown how simple bioprocess engineering can benefit stable expression titers of our master cell line, comparable to those obtained by the BEVS without compromise final protein quality (Fernandes, Dias et al. 2014), but there is still much room for improvements at the bioprocess level to achieve higher protein levels. In respect to mammalian producer cell lines, great improvements have been made in protein titers as a consequence of better media formulations and feeding strategies to sustain higher cell densities (Hacker, De Jesus et al. 2009), however the underlying molecular and physiological factors are not well understood, particularly on the systems-level. The need to understand and manipulate cellular systems for increased biosynthesis of recombinant products has been a consistent milestone, supported by data generation at different cellular levels. Still, deciphering these data to rationally develop cell culture optimization strategies is not yet a straightforward proceeding. The link between molecular traits and the desired

phenotype is still a black box. Ideally, we would be able to incorporate these data into mechanistic models enabling accurate prediction of cell behavior in response to cell culture perturbations, which could be further exploited for forward cell engineering and/or bioprocess optimization.

Despite significant contributions on rational cell line development and engineering are herein presented, the systematic exploitation of such platforms and its broader applicability are yet in its beginning. We have taken the first steps towards the endorsement of targeted integration based-technologies for stable insect cell line development by developing further refinements to these expression platforms. Future work will benefit from a complete cellular understanding acquired from holistically-derived data that will enable the development of better insect cell factories and bioprocess methodologies.

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