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Tuning cell cycle of insect cells for enhanced protein production

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ABSTRACT

The eukaryotic cell cycle consists of many checkpoints during which certain conditions must be met before passing to subsequent stages. These safeguards ensure cells' integrity and survival, but may also limit growth and protein synthesis in protein production processes. In this work, we employ metabolic engineering principles to "tune" the cell cycle to overcome checkpoint processes in order to facilitate faster cell growth, and independently, arrest the cell cycle in gap1 (G1) phase for greater protein productivity. Specifically, we identified the complete *cyclin E* (*cycE*) cDNA sequence from industrially relevant, *Trichoplusia ni* (*T. ni*) derived High Five™ genomes. We then both knocked down (through RNA interference; RNAi) and overexpressed (on an expression plasmid) *cycE* gene expression to tune the cell phenotype. We successfully up- and down-regulated *cycE* transcription, enhancing and hindering cell growth, respectively. We also measured the effects of titrated *cycE* expression on the cell cycle phase distribution. Finally, we investigated the dose-dependent effects of dsCycE on recombinant protein production using the baculovirus expression system and demonstrated a nearly 2-fold increase in expression of model protein (GFPuv).

1. Introduction

With the advent of whole genome engineering, our ability to metabolically engineer cells has undergone a transformational shift. While many of the first generation applications have been directed at small molecule or commodity-based products from microbial hosts, it will not be long before these tools will be turned toward insect and mammalian genomes for enhancing synthesis of designer proteins (Cobb et al., 2012; Cong et al., 2013; Majors et al., 2009; Sun et al., 2012). Genome-wide tools are envisioned to alter global regulatory structures and the overall biosynthetic landscape, while specific bottlenecks are often tackled by transient and local controllers (March and Bentley, 2006, 2007; Ni et al., 2011; Stadlmayr et al., 2010). For example, in many eukaryotic (mammalian, insect, yeast) cell systems, which provide superior glycosylation capabilities, interfering RNA (RNAi) has received attention as a means to target local bottlenecks (Hebert et al., 2008; Kim et al., 2012; Lai et al., 2012; March and Bentley, 2007) as well as global processes (Dietzl et al., 2007; Drinnenberg et al., 2009; Xu et al., 2011).

Owing largely to superior protein quality, eukaryotic cell cultures remain the main sources for pharmaceutical protein production despite their relatively low growth rates and protein yields as compared to prokaryotic expression systems (Kamionka, 2011; Schmidt, 2004; Terpe, 2006). Low mammalian cell growth rates and yields are due to a complicated network of regulatory mechanisms that monitor and check their internal physiological conditions, metabolic activities, as well as the absence of external signals as a precondition to cell proliferation (Boye and Nordstrom, 2003; Goranov et al., 2009; Slavov and Botstein, 2011; Tapon et al., 2001). Cells progress through chromosome duplication and cell division in a process called the "cell cycle". The cell cycle consists of many checkpoints during which certain conditions must be met before passing to the next stages. This strategy which ensures cell integrity and survivability can, however, sometimes compromise growth rate and protein synthesis. Since cell cycle and proliferation status are closely related to protein synthesis activity (Gali-Muhtasib and Bakkar, 2002; Kazi and Lang, 2010; Kumar et al., 2007; Stein and Pardee, 2005; Wurm, 2004) the cell cycle has, for many years, been a target of potential metabolic engineering. That is, metabolic

Abbreviations: *cycE*, *cyclin E*; RNAi, ribonucleic acid interference; GFP, green fluorescent protein; CAT, chloramphenicol acetyl transferase; CHO, Chinese hamster ovary; ds, double stranded; G1, gap-1; G2, gap-2; M, mitosis; MOI, multiplicity of infection; S, DNA synthesis; ss, single stranded; *T. ni*, *Trichoplusia ni*; RT-PCR, reverse transcription polymerase chain reaction.

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engineering approaches to arrest the cell cycle at specific states have been successful (Fussenegger et al., 1998; Harper and Brooks, 2005; Sunley and Butler, 2010; Watanabe et al., 2002), but not currently implemented perhaps due to the means of control. For example, chemical signaling additives are typically not desired (Chen et al., 2011; Jiang and Sharfstein, 2008; Sunley and Butler, 2010).

The cell cycle can be divided into four distinct phases: gap-1(G1), DNA synthesis (S), gap-2 (G2), and mitosis (M) (Cai and Tu, 2012; Harper and Brooks, 2005; van den Heuvel, 2005). Cells arrested at G1 (protein synthesis) phase have been shown to exhibit the highest ribosome biogenesis and protein translation activity of any cell cycle phase, resulting in higher recombinant protein yields (Elledge, 1996; Iba et al., 1978). In G1, cell resources are utilized more efficiently for synthesizing proteins of interests without diverting energy to produce cell components for proliferation (Fussenegger et al., 1998; Mazur et al., 1998; Sunley and Butler, 2010; Watanabe et al., 2002). For example, Bi et al. (2004) showed that over-expression of the cyclin-dependent kinase inhibitor p21 causes G1-phase cell cycle arrest in Chinese hamster ovary (CHO) cells. These G1-arrested cells showed an increase of mitochondrial mass and activity as well as ribosomal protein S6 translation levels, culminating in a 4-fold increase of recombinant protein productivity. Although cell cycle control has been demonstrated in mammalian cell lines for recombinant protein production, few studies have examined the feasibility in insect cell expression systems. March and Bentley (2007) successfully arrested cells in the G1 phase and increased the target GFP production 4-fold in *Drosophila* S2 cellsusing dsRNA technology (RNA interference) to suppress *cyclin E* (positive regulator for G1-to-S phase transition) expression. To date, this study is the only reported use of RNAi approaches to control the cell cycle for the purpose of enhancing recombinant protein production in eukaryotic cells.

In the current work, we report similar *cycE*-targeted efforts, but with an industrially relevant insect cell line the High Five™ cell line derived from the cabbage looper (*Trichoplusia ni*), the gene for which has previously been unknown. During normal cell proliferation, CycE is regulated so that a natural increase and subsequent decrease guides the G1 to S transition (Cai and Tu, 2012; Stein and Pardee, 2005). Hence, our hypothesis is that altered CycE level can lead to altered cell cycle decision-making and subsequently, altered phenotype. Specifically the sustained up-regulation of CycE is hypothesized to yield over-proliferation. Also CycE down-regulation would stall the transition to S from G1. Thus, we investigated the effects of *cycE*-targeted RNAi molecules that are added to *T. ni* cells for their impact on regulation of the cell cycle, on cell physiology, and on production of recombinant protein following baculovirus infection. To do this, we identified the complete *T. ni cycE* cDNA sequence, then up-regulated *cycE* gene expression by transfecting High Five™ cultures with an expression vector carrying *cycE*. Next, *cycE* gene expression was down-regulated via dsRNA against *cycE* (denoted dsTnCycE). For both experiments, *cycE* transcription level, cell growth, as well as the cell cycle distribution (via FACS) were examined. Finally, a dosage-dependent effect of dsTnCycE on recombinant protein production using the baculovirus-mediated expression was revealed. In this study and in our previous work ((Cha et al., 1999b, 1997; Kim et al., 2007; Kramer and Bentley, 2003; Kramer et al., 2003; Wu et al., 2000), GFP is used as a model product in that the results might be transferrable to other more commercially relevant proteins. GFP is a robust protein known to fold properly in a variety of eukaryotic cell lines, hence its use here is reflects a base case for expression without confounding issues relative to posttranslational processing.

2. Materials and methods

2.1. Cell culture

T. ni BTI-TN-5B1-4 (High Five™, Invitrogen) cells were cultivated in EX-CELL™-405 media (SAFC Biosciences) at 27 °C in T75 flasks (Corning) for adhesion culture. Cells were also adapted to suspension culture by cultivating in 125 ml shaker flasks (Corning) at ~120 rpm and at room temperature. Cell passage was conducted every two to three days, or whenever cell density reached confluency (2.5–3 million cells/ml) and then split into 0.6–0.8 million cells/ml.

2.2. Baculovirus amplification and infection

A recombinant *A. californica* multiple nucleopolyhedrovirus (AcMNPV) that expresses GFPuv under the control of the polyhedrin promoter was created previously (Cha et al., 1999a). The baculovirus was amplified in *S. frugiperda* (Sf9) cells (Invitrogen) according to standard protocols with MOI (=0.1) and supernatants harvested after centrifugation. Baculovirus titer was determined by endpoint dilution with GFPuv fluorescence measurement using fluorescence microscopy (BX60; Olympus), a plate reader (SpectraMax M2; Molecular Devices), or flow cytometry (FACSCanto II; BD Biosciences).

2.3. Identification of T. ni cycE sequence

In order to determine the initial putative primers sequences for RT-PCR, *cycE* sequences among inter-interphylum species were collected from NCBI GenBank and multiple sequence alignment was performed by clustalw2 software (EMBL-EBI) to obtain the consensus regions. Subsequently, degenerate primers for PCR were designed: 5′-GARGARATYTAYCCNCCHAAR-3′ (R: A/G; Y: C/T; N: A/T/C/G; H: A/T/C). Next, RACE RT-PCR was performed with the degenerate primers. Briefly, total RNA of High Five™ cells was extracted using RNAquous (Ambion) followed by reverse-transcription via the SMART-RACE™ cDNA amplification kit (Clontech). The resultant PCR products were ligated into a TOPO TA vector (Invitrogen) and subsequent DNA sequencing was carried out by the IBBR sequencing facility (DNA sequencer 3730; Applied Biosystems) to obtain the *T. ni cycE* cDNA sequence.

2.4. pIB-TncycE vector construction

The *cycE* gene was first PCR amplified using accuprime Hi Fi polymerase (Invitrogen) and TOPO-ligated into a pIB/V5-His vector (Invitrogen) to generate the pIB-*TncycE* vector. The ligation products were transformed into *E. coli* TOP 10 competent cells (Invitrogen) and then plated to LB/agar (Fisher Scientific) with Kanamycin 50 μg/ml at 37 °C overnight. Single colonies were picked and inoculated into LB broth (Fisher Scientific) with Kanamycin 50 μg/ml at 37 °C 250 rpm for overnight. Plasmids were purified with a miniprep kit (Qiagen) and DNA concentrations were quantified by NanoDrop (Thermo Scientific). DNA sequencing of *cycE* insertion was carried out to confirm cloning success.

2.5. In vitro double stranded RNA (dsRNA) synthesis

In vitro doubled stranded CycE (dsTnCycE) RNA was synthesized using the Megascript Kit (Ambion). T7 promoter sequences were incorporated into both the 3′ and 5′ ends of partial *cycE* fragments by PCR with primers having extra T7 promoter overhangs. In vitro transcription of dsTnCycE (800 bp) was then generated according to the manufacturer's instructions. Briefly, single stranded TnCycE was synthesized and then extracted using phenol/chloroform