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Metabolic pathways recruited in the production of a recombinant enveloped virus: Mining targets for process and cell engineering

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ABSTRACT

Biopharmaceuticals derived from enveloped virus comprise an expanding market of vaccines, oncolytic vectors and gene therapy products. Thus, increased attention is given to the development of robust high-titer cell hosts for their manufacture. However, the knowledge on the physiological constraints modulating virus production is still scarce and the use of integrated strategies to improve hosts productivity and upstream bioprocess an under-explored territory.

In this work, we conducted a functional genomics study, including the transcriptional profiling and central carbon metabolism analysis, following the metabolic changes in the transition 'parental-to-producer' of two human cell lines producing recombinant retrovirus. Results were gathered into three comprehensive metabolic maps, providing a broad and integrated overview of gene expression changes for both cell lines. Eight pathways were identified to be recruited in the virus production state: amino acid catabolism, carbohydrate catabolism and integration of the energy metabolism, nucleotide metabolism, glutathione metabolism, pentose phosphate pathway, polyamines biosynthesis and lipid metabolism. Their ability to modulate viral titers was experimentally challenged, leading to improved specific productivities of recombinant retrovirus up to 6-fold. Within recruited pathways in the virus production state, we sought for metabolic engineering gene targets in the low producing phenotypes. A mining strategy was used alternative to the traditional approach 'high *vs.* low producer' clonal comparison. Instead, 'high *vs.* low producer' from different genetic backgrounds (*i.e.* cell origins) were compared. Several genes were identified as limiting in the low-production phenotype, including two enzymes from cholesterol biosynthesis, two enzymes from glutathione biosynthesis and the regulatory machinery of polyamines biosynthesis. This is thus a frontier work, bridging fundamentals to technological research and contributing to enlarge our understanding of enveloped virus production dynamics in mammalian cell hosts.

1. Introduction

Biopharmaceuticals derived from enveloped virus produced in mammalian hosts comprise a myriad of valuable products from vaccines to gene therapy vectors. Such products have been based on different *genus* including gammaretrovirus (Dalba et al., 2007), lentivirus (Cockrell and Kafri, 2007; Di Nunzio et al., 2011), alphavirus (Atkins et al., 2008), orthopoxvirus (Walsh and Dolin, 2011), avulavirus (Schirrmacher and Fournier, 2009), flavivirus (Appaiahgari and Vrati, 2010) and respirovirus (Griesenbach et al., 2005). As this market expands (Kresse and Shah, 2010; Moran, 2012) it increases the interest to develop high-titer production hosts. In this context, metabolic optimization by culture medium and gene manipulation are potential tools to improve both process and cell engineering. But metabolic intervention, particularly at the gene engineering level, requires a broad overview on the physiological constraints modulating the (virus) producing phenotype.

In recombinant protein production, mammalian cell culture has profited from 20 years of functional genomics studies from transcriptomics to proteomics and metabolomics (Griffin et al., 2007; Khoo and Al-Rubeai, 2007). These studies boosted our fundamental understanding on the physiological changes following the development, adaptation, selection and engineering of cell hosts opening the door for a variety of metabolic interventions towards improved productivities (Lim et al., 2010; Seth et al., 2006). In the case of recombinant virus production, however, the translation of gathered knowledge for the rational design of process and cell engineering is still scarce, with some notable exceptions (Hansen et al., 2005; Martinez et al., 2009; Mitta et al., 2005). Although viruses induce

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appreciable changes to cell metabolism as part of their replication strategy, the majority of reported work has been conducted from a basic virology point of view aiming at inhibiting viral replication.

In this work we mine the bases of virus induced metabolic changes and use that knowledge to boost viral titers. A functional genomics study was conducted – including transcriptional profiling and central carbon metabolism analysis – of the metabolic changes occurring in the transition 'parental-to-producer' of two different human cell lines, using recombinant retrovirus as an enveloped virus model. Gathered knowledge was used to increase viral titers by media manipulation and to identify targets for gene metabolic engineering for 293 FLEX cells. 293 FLEX are human embryonic kidney 293 derived retrovirus producer cell lines highly flexible for viral vector exchange thus, a preferential platform for a production process for clinical applications (Coroadinha et al., 2006b; Schucht et al., 2006). Their titers, however, fall in the range of 10^6 infectious particles *per* mL, insufficient for large-scale production after the transition clinical-to-market, demanding improved productivities.

Apart from the biopharmaceutical potential – gene therapy vectors, vaccine candidates and oncolytic agents (Dalba et al., 2007) – retrovirus production is an attractive model for metabolic engineering studies: (i) current producer cells and upstream process are sub-productive offering plenty of room for improvement through metabolic manipulation (Coroadinha et al., 2006a); (ii) they are non-cytotoxic, allowing metabolic manipulation without the constraints of cell growth arrestment and (iii) they represent a complex biopharmaceutical, composed by nucleic acids, lipids and (glycosylated) proteins. Thus, metabolic manipulations resulting in improved retrovirus production are likely to be of interest to other enveloped virus produced in mammalian cells.

To identify gene engineering targets the traditional approach, regardless the bioproduct, has mainly been based on the comparison 'producers *vs.* parental' or 'high *vs.* low producers' for clonal cells with different productivity levels. While this strategy can certainly identify potential targets, their genetic manipulation can often result in small productivity enhancements. A gene already *recruited* in the transition parental-to-producer or in the high producing clone, is likely to be *responsive* to that transition or phenotype; it also indicates that the cells have the intrinsic capability to express it, with no need for further over-expression. Thus in the work herein presented, in addition to analyzing the transition 'parentals-to-producers', we followed a complementary approach by comparing 'high *vs.* low producer' from different genetic backgrounds (two different cell line origins). Comparing different genetic backgrounds for gene engineering has been used, for instance, to evaluate and manipulate glycosylation patterns of recombinant proteins (see for a review Grabenhorst et al. (1999)). To the best of our knowledge, such approach has never been used to mine high productivity transcriptome signatures.

2. Materials and methods

2.1. Cell lines and culture conditions

Te Fly Ga 18 (Cosset et al., 1995; Duisit et al., 1999) and 293 FLEX (Coroadinha et al., 2006b; Schucht et al., 2006) are human derived cell lines producing murine leukemia virus (MLV) based vector harboring a LacZ reporter gene, pseudotyped with gibbon ape leukemia virus (GaLV). Their corresponding parental cell lines are Te 671 (ATCC CCL-136) and HEK 293 (ATCC CRL-1573), respectively. The cells were maintained in Advanced Dulbecco's modified Eagle's medium, DMEM (Gibco, Paisley, UK), with 25 mM of glucose, supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco) and 4 mM of glutamine (Gibco). Te 671 cell line was also used to titrate infectious retroviral particles. All cells were maintained in an incubator with a humidified atmosphere of 92% air and 8% CO_2 at 37 °C.

2.2. Cell growth and viral production studies

Two types of virus production studies were performed: a 7-day batch culture for cell growth/virus production kinetics, transcriptome analysis and metabolite profiling and a 48+24 h production/harvesting process to evaluate media supplementation effect on viral titers.

For the 7-day batch cultures, producer and parental cells were inoculated in t-flasks at 2×10^4 cells/cm^2 for Te 671/Te Fly Ga 18 and 4×10^4 cells/cm^2 for HEK 293/293 FLEX 18. Two samples were collected *per* day: culture supernatant was harvested, filtered through 0.45 μm for clarification, aliquoted and stored at −85 °C until analysis. Cell concentration and viability was determined by the trypan blue exclusion method. At day 3, total RNA was extracted for transcriptome analysis.

For media supplementation studies, cells were inoculated in media containing one or a combination of the following supplements: (i) amino acids (Sigma, Steinheim, Germany); (ii) nucleosides (Millipore, Billerica, MA, USA); (iii) anti-oxidants (Sigma); (iv) polyamines (Sigma); (v) reduced glutathione (Sigma); a non-supplemented control was also prepared. Total RNA was extracted and vector infectivity decay kinetics were assessed as shown in Supplementary Figure SFig. 1. A detailed schematic representation of this experimental setup as well as final concentrations and combinations of supplements used can be found in Supplementary Materials (SM&M1, ST1 and SFig. 1).

For both growth curves as well as for supplemented media studies, three biological replicates were used.

2.3. Measurement of infectious and genome containing retrovirus titer

Infectious procedure and viral titer determination were performed by limiting dilution infection assay, as previously described in Rodrigues et al. (2009). Genome containing particles titer was determined by real-time PCR as described elsewhere (Carmo et al., 2004).

2.4. RNA extraction and RT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The resultant RNA pellet was eluted in 100 μL of nuclease-free water (Qiagen) and stored at −85 °C until further processing. RNA yields were quantified using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) and RNA quality was characterized by the quotient of the 28S to 18S ribosomal RNA electropherogram peak using an Agilent 2100 bioanalyzer and the RNA Nano Chip (Agilent, Santa Clara, CA, USA).

For real time PCR, RPL-22 was chosen as a control gene using as forward primer (F): 5′-CTGCCAATTTTGAGCAGTTT-3′ and as reverse primer (R): 5′-CTTTGCTGTTAGCAACTACGC-3′. Forward (F) and reverse (R) primer sequences for viral genes were the following: envelope (F) 5′-GGACCAAAATAGCGAATGGA-3′/(R) 5′-GGTGAACTGTACGCCTG-GAT-3′; gag-pol (F) 5′-GTCCACTATCGCCAGTTGCT-3′/(R) 5′-CTGGGT-CCTCAGGGTCATAA-3′; transgene (F) 5′-ACTATCCCGACCGCCTTACT-3′/(R) 5′-TAGCGGCTGATGTTGAACTG-3′. The reverse transcription of total RNA and qRT-PCR was performed as described in Rodrigues et al. (2012).