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Research paper

Engineering mammalian cell factories with SINEUP noncoding RNAs to improve translation of secreted proteins

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ABSTRACT

Whenever the function of a recombinant protein depends on post-translational processing, mammalian cells become an indispensable tool for their production. This is particularly true for biologics and therapeutic monoclonal antibodies (MAbs). Despite some drawbacks, Chinese Hamster Ovary (CHO) cells are the workhorse for MAbs production in academia and industry. Several methodologies have been adopted to improve expression and stability, including methods based on selective pressure or cell engineering.

We have previously identified SINEUPs as a new functional class of natural and synthetic long non-coding RNAs that through the activity of an inverted SINEB2 element are able to promote translation of partially overlapping sense coding mRNAs.

Here we show that by taking advantage of their modular structure, synthetic SINEUPs can be designed to increase production of secreted proteins. Furthermore, by experimentally validating antisense to elastin (AS-eln) RNA as a natural SINEUP, we show that SINEUP-mediated control may target extracellular proteins.

These results lead us to propose synthetic SINEUPs as new versatile tools to optimize production of secreted proteins in manufacturing pipelines and natural SINEUPs as new regulatory RNAs in the secretory pathways.

1. Introduction

Mammalian recombinant proteins are fundamental resources for basic and applied research, as well as for biotechnological applications. They can be produced in a variety of expression systems although mammalian cells are the first choice when post-translational processing is required for function. This is particularly true for proteins with therapeutic potential, the so-called biologics. In the last decade over hundred biologics have been introduced in the market with applications ranging from diagnostics to in vivo therapeutics. Among these proteins, monoclonal antibodies (MAbs) constitute the fastest growing sector (Leader et al., 2008; O'Callaghan and James, 2008).

Chinese Hamster Ovary (CHO) cells are the primary factories for the production of recombinant MAbs in academia and industry (Lai et al., 2013). They are safe hosts in which highly specific productivity can be achieved via gene amplification (e.g.: DHFR- or GS-based systems) (Baldi et al., 2005). In addition, CHO-derived clones have been adapted to grow in suspension, at high-cell densities and in serum-free conditions (Kim et al., 2012). However, CHO cells present some undesired features, such as a significant genome instability and the propensity to epigenetic transgene silencing (Kim et al., 2011). Moreover, recent-omics and modeling analyses have showed that genomic and phenotypic differences are not distributed uniformly among clonally-derived cells. These findings support the concept that cells use different road maps to adapt to selective pressure, favoring viability rather than recombinant protein synthesis (O'Callaghan and James, 2008). Since this affects the productivity of clones quantitatively and/or qualitatively, different strategies have been proposed to attenuate these drawbacks. Some of them concern vector design, featuring inducible promoters and/or epigenetic regulators to increase and extend transgene expression (Lai et al., 2013). Others aim at improving cell viability and stress resistance throughout cell engineering or RNA interference (Lai et al., 2013). Whatever strategy is adopted, clonal variability and a substantial proportion of cells incapable to produce high levels of recombinant MAbs remain a burden, particularly for the industry sector.

Abbreviations: CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; GFP, green fluorescent protein; GS, glutamine synthetase; lncRNA, long noncoding RNA; MAb, monoclonal antibody; PBS, phosphate buffered saline.

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We have recently discovered a new class of natural and synthetic antisense long noncoding RNAs – named SINEUPs – whose effect is to promote translation of partially overlapping sense coding mRNAs with no consequence on RNA levels (Carrieri et al., 2012). SINEUP activity depends on two functional domains: a 5′ overlap with the protein-coding target (Binding Domain or BD) and an embedded inverted SINEB2 element that functions as activator of translation (Effector Domain or ED) (Zucchelli et al., 2015). These features are shared by a group of sense/antisense pairs in the mouse genome that identifies 31 candidates for natural SINEUPs.

Synthetic SINEUPs may thus be designed by targeting the BD to the mRNA of choice representing the first scalable tool to increase protein synthesis of potentially any gene of interest. Although the level of protein increase may differ, they have been shown to work in different cell lines of mouse, hamster, monkey and human origin including HEK293, HeLa and HepG2 cells (Yao et al., 2015; Zucchelli et al, 2015). SINEUPs have been successfully designed to target commonly used tags to increase expression of tag-fused proteins. Importantly, they have been able to increase translation of endogenous cellular mRNAs (Yao et al., 2015; Zucchelli et al., 2015). So far all the experimentally-validated natural and synthetic SINEUPs act on transcripts encoding for cytosolic or nuclear proteins.

Here we show that synthetic SINEUPs are active in CHO cells grown in suspension, a suitable model for large-scale production of recombinant proteins. SINEUP modular structure is then exploited to successfully design leader-sequence specific BD to up-regulate secreted proteins, including monoclonal antibodies (MAbs). Finally, we find that a natural lncRNA antisense to elastin gene (AS eln) acts as a natural SINEUP for this secreted protein and it can be employed to build a new formulation of synthetic SINEUPs.

In summary, we present proof-of-principle experiments to propose SINEUPs as a new versatile tool for implementing manufacturing of recombinant secreted proteins in mammalian cells.

2. Materials and methods

2.1. Plasmids and clonings

The following commercially available vectors were used: pEGFP-C2 (green fluorescent protein) from Clontech; pcDNA3.1(−) from Life Technologies; pNL1.1 (cytosolic NanoLuc) and pNL1.3 (secreted NanoLuc) from Promega; clone ID: A630042I19 (mouse elastin cDNA) and B230220P09 (natural SINEUP antisense to elastin) from RIKEN (Yokohama, Japan); IMAGE clone 40008000 (human periostin cDNA) from Source Bioscience (Nottingham, UK). The pEGFP-C2 vector was used as a backbone to build the luciferase reporters, in which the GFP was replaced with the coding sequences of the appropriate NanoLuc. The Eln-NLuc reporter vector was constructed replacing the IL-6 secretory peptide with a sequence containing the elastin leader peptide and the 5′UTR, obtained from the RIKEN clone A630042I19. SINEUP targeting EGFP (AS-GFP, here named SINEUP-GFP) has been described previously (Carrieri et al., 2012). SINEUP-Luc and SINEUP-MB were generated by annealing and ligation of phosphorylated oligonucleotides into a SINEUP-backbone as described (Zucchelli et al., 2015). SINEUP antisense to mouse elastin (SINEUP-Eln) was obtained by subcloning the corresponding cDNA from the original RIKEN clone into pcDNA3.1(−).

The leaderless, full-length coding sequence of human periostin (aa 23–836) was cloned into the pMB vector (Di Niro et al., 2007).

2.2. Cell culture and transfections

CHO-S cells (Life Technologies cat. R800-07) were seeded on 24 well plates in CHO-S-SFM II medium (Life Technologies cat. 12052) at a density of 200,000 cells per well and transfected with the FreeStyle™ MAX reagent (Life Technologies). HEK293T cells were seeded on 24 well plates in DMEM containing 10% FBS at a density of 125,000 cells per well and transfected with Lipofectamine 2000 (Life Technologies). All cells were transfected with a 1:6 ratio between sense and SINEUP encoding plasmids. Medium was changed 5 h after transfection to reduce toxicity.

To generate a CHO-S cell line stably expressing periostin (POSTN), cells were prepared as above and transfected with the corresponding POSTN expression vector. Transfected cells were cultured under selective conditions in ProCHO5 Medium (Lonza cat. BE12-766Q) containing 0.2 mg/ml hygromicin B and serially diluted to 2–3 cells/well in a 96 well plate. After 3 weeks the presence of the recombinant protein in the medium was tested by ELISA assay and the higher productive clone was chosen.

2.3. Immunoblotting

Transfected cells were lysed in Laemmli buffer and proteins were separated by SDS-PAGE. After separation and transfer, the nitrocellulose membranes were incubated with an anti-GFP antibody (clone JL-8, Clontech) and revealed by chemiluminescence. The band density was quantified by ImageJ (http://imagej.nih.gov) and normalized to β-actin.

2.4. Flow cytometry

Transfected CHO-S cells were collected in PBS containing 0.5% FBS. GFP fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, Erembodegem, Belgium) using the 488 nm laser. 20,000 events for each condition were acquired.

2.5. Luciferase assays

Luciferase activity was measured at different time points after transfection with the respective NanoLuc reporters. The secreted NanoLuc (secNLuc) was measured in the conditioned media by mixing with an equal volume of Nano-Glo Luciferase assay reagents. To detect the cytosolic NanoLuc (cytNLuc), cells were lysed in PBS Triton 1% and cleared surnatants mixed with an equal volume Nano-Glo Luciferase assay reagent. Luminescence was measured with a Victor™ X4 multilabel plate reader (Perkin Elmer, Waltham, MT).

2.6. ELISA

Supernatants of cells expressing the mAb and Periostin were collected at different time points, diluted 1:50 in PBS and used to coat a 96 wells plate (Greiner Bio-one). ELISA was performed as reported (Boscolo et al., 2012).

2.7. qRT-PCR

Total RNA was extracted using the PureLink RNA microscale kit (Life Technologies). RNA (1 μg) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was diluted 1:10 and analyzed by qPCR using Platinum SYBR Green qPCR Super Mix-UDG (Life Technologies) on a CFX96 Real-Time PCR detection system (Bio-Rad). Expression was normalized to GAPDH.

2.8. Statistical analysis

All data are presented as mean ± s.e. ($n \geq 3$). Data were analyzed by Student's *t*-test and considered significant at $p < 0.05$ (*) or extremely significant at $p < 0.01$ (**).