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A platform for context-specific genetic engineering of recombinant protein production by CHO cells

Joseph F. Cartwright[a,1], Claire L. Arnall[a,1], Yash D. Patel[a], Nicholas O.W. Barber[a], Clare S. Lovelady[b], Guglielmo Rosignoli[c], Claire L. Harris[b], Sarah Dunn[b], Ray P. Field[b], Greg Dean[b], Olalekan Daramola[b], Suzanne J. Gibson[b], Andrew A. Peden[d], Adam J. Brown[a], Diane Hatton[b], David C. James[a,*]

[a] Advanced Biomanufacturing Centre, Department of Chemical and Biological Engineering, University of Sheffield, Mappin St., Sheffield S1 3JD, UK
[b] Biopharmaceutical Development, AstraZeneca, Milstein Building, Granta Park, Cambridge, CB21 6GH, UK
[c] Antibody Discovery and Protein Engineering, AstraZeneca, Milstein Building, Granta Park, Cambridge, CB21 6GH, UK
[d] Department of Biomedical Sciences, University of Sheffield, Sheffield, UK

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ABSTRACT

An increasing number of engineered therapeutic recombinant proteins with unpredictable manufacturability are currently filling industrial cell line development pipelines. These proteins can be "difficult-to-express" (DTE) in that production of a sufficient quantity of correctly processed recombinant product by engineered mammalian cells is difficult to achieve. In these circumstances, identification of appropriate cell engineering strategies to increase yield is difficult as constraints are cell line and product-specific. Here we describe and validate the development of a high-throughput microscale platform for multiparallel testing of multiple functional genetic components at varying stoichiometry followed by assessment of their effect on cell functional performance. The platform was used to compare and identify optimal cell engineering solutions for both transient and stable production of a model DTE IgG1 monoclonal antibody. We simultaneously tested the functional effect of 32 genes encoding discrete ER or secretory pathway components, each at varying levels of expression and utilized in different combinations. We show that optimization of functional gene load and relative stoichiometry is critical and optimal cell engineering solutions for stable and transient production contexts are significantly different. Our analysis indicates that cell engineering workflows should be cell line, protein product and production-process specific; and that next-generation cell engineering technology that enables precise control of the relative expression of multiple functional genetic components is necessary to achieve this.

1. Introduction

Chinese hamster ovary (CHO) cells are the predominant cell host used for the production of biopharmaceuticals, largely because of their ability to efficiently produce diverse correctly folded recombinant glycoproteins (Birch and Racher, 2006; Jayapal et al., 2007). Stable production of therapeutic recombinant proteins by CHO cells has been greatly improved over the years and volumetric titers > 10 g/L can now be achieved (Huang et al., 2010; Wurm, 2004). Transient production processes, which enable rapid production of candidate molecules for pre-clinical assessment have also been optimized with volumetric titers > 2 g/L reported (Daramola et al., 2014). However, even using advanced CHO cell expression platforms, some recombinant proteins are relatively difficult-to-express (DTE) due to inherent cellular process constraints – typically protein-specific post-translational folding and assembly processes (e.g. Johari et al., 2015). In these cases, it is not possible to achieve an acceptable volumetric titer of a correctly assembled monomeric product. This is an increasing problem as industrial pipelines of candidate therapeutic proteins begin to fill with more complex, engineered proteins, as these unnatural molecules have unpredictable manufacturability (Aggarwal, 2014; Fischer et al., 2015; Pybus et al., 2014; Lee et al., 2007; Hussain et al., 2018; Tadauchi et al., 2019). As a result, manufacturing cell line development processes can be extended (Browne and Al-Rubeai, 2007). To redress this, many cell engineering strategies have been trialed, with some success, to increase stable (SGE) and transient (TGE) expression of recombinant genes in

* Corresponding author.
E-mail address: d.c.james@sheffield.ac.uk (D.C. James).
[1] These authors contributed equally to this work.

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CHO cells (Fischer et al., 2015) including overexpression of genes to increase secretory pathway capacity (Pybus et al., 2014; Cain et al., 2013; Johari et al., 2015; Davis et al., 2000; Ku et al., 2008; Mohan and Lee, 2010) or improve cell metabolism (Chong et al., 2010; Wlaschin and Hu, 2007). The use of bioactive small molecules to modulate host cell function is also a potentially attractive route to increase cellular productivity (Johari et al., 2015; Cortez and Sim, 2014).

It has been known for some time that increased secretory pathway capacity may lead to increased recombinant protein production, and there is evidence that overexpression of components functioning within these subcellular compartments (i.e. endoplasmic reticulum, Golgi apparatus) or functionally-related transactivators (e.g. associated with the unfolded protein response, UPR) could serve to increase the productivity of CHO cell lines (Rutkowski and Kaufman, 2004; Tigges and Fussenegger, 2006; Kim et al., 2012). However, it is clear that these effects are most often CHO cell line and recombinant protein-specific and so generic solutions that could provide a universal benefit have not been proven. Instead, it is more likely that the specific engineering context (i.e. the synthetic requirements of a specific recombinant protein in a specific host cell background) is a more relevant driver (Pybus et al., 2014; Cain et al., 2013; Borth et al., 2005; Johari et al., 2015). Therefore, effective improvement of cellular synthetic processes will require identification of bespoke cell/product engineering solutions, where a specific combination of genetic effectors may be desirable. To feasibly implement this tailored cell engineering solution, it is essential to use high-throughput (HT) methods for gene delivery and subsequent analysis of cell performance (growth and productivity) to ensure that a product-directed cell engineering strategy is possible within reasonable timescales and at an acceptable cost (Johari et al., 2015; Abbott et al., 2015; Mora et al., 2018; Hansen et al., 2015).

In this study we describe the development and application of a HT microscale transfection platform for simultaneous testing of multiple genetic components at varying stoichiometry, with an assessment of their individual and combined effect on transient and stable production of a recombinant IgG1 monoclonal antibody by CHO cells. Our data reveal that optimal cell engineering solutions are production process specific and discrete combinations of functional genetic effectors operating at an optimal relative stoichiometry are required for maximal efficacy.

2. Materials and methods

2.1. Expression vectors

The MAb-A expression vector contained: *MAb-A* light chain (*LC*) and heavy chain (*HC*) genes driven by CMV promoters; Glutamine synthetase (*GS*) gene driven by a SV40 promoter; OriP element for *Epstein-barr virus nuclear antigen 1* (*EBNA-1*)-based vector expression enhancement (Daramola et al., 2014). Genetic effector cDNA sequences were sourced from the NCBI database and expressed within a universal expression vector containing: Effector gene driven by CMV promoter; OriP element for *EBNA-1*- based vector expression enhancement. Fluorescent protein genes used for stoichiometry analysis were sourced from Evrogen (Moscow, Russia) and Clontech (Mountain View, CA, USA) (for TagBFP and mCherry/eGFP respectively) and were expressed within the universal expression vector. An empty (no cDNA sequence) universal expression vector was used to equalize DNA load across all transfections. All nucleotide sequences expressed within the universal expression vector were optimized for *Cricetulus griseus* expression and synthesised by GeneArt™ (Regensburg, Germany). Vector DNA was purified using a Qiagen maxi prep plus kit (Qiagen, Manchester, UK).

2.2. Cell lines and routine cell culture

A CHO-K1-derived, suspension-adapted transient host (CHO-T2), with stable expression of *EBNA-1* and *GS*, (Daramola et al., 2014) was cultured in CD-CHO medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 25 µM methionine sulfoximine (MSX) (Sigma-Aldrich, St. Louis, MO) and 100 µg mL^{-1} hygromycin-B (Thermo Fisher Scientific). A CHO-K1-derived suspension-adapted host was used to generate a MAb-A producing stable pool (CHO-A) using 50 µM MSX selection. CHO-A cells were cultured in CD-CHO medium (Thermo Fisher Scientific) supplemented with 50 µM MSX (Sigma-Aldrich). Routine culture for both cell lines was at 37 °C in vented, non-baffled Erlenmeyer flasks (Corning, Amsterdam, The Netherlands), in 5% CO_2 (v/v) maintained at 140 rpm. Cells were sub-cultured every 3–4 days at a seeding density of 2×10^5 cells mL^{-1}. Cell density and culture viability were assessed using a Vi-Cell XR automated cell counter and viability analyser (Beckman Coulter, High Wycombe, UK).

2.3. Transient transfection using electroporation

Electroporation (Nucleofection) was carried out on mid-exponential phase cells using the Amaxa™ Nucleofector™ system (Lonza, Basel, Switzerland) with the Nucleofector™ 96-well Shuttle™ add-on device (Lonza) as per manufacturer's instructions. For each transfection: 1 µg of DNA (resuspended in 2.5 µL H_2O + 7.5 µL SG cell-line Nucleofection solution) was combined with 2.33×10^6 cells (resuspended in 15 µL SG cell-line Nucleofection solution); 20 µL (1.86×10^6 cells and 800 ng DNA) of the cell-DNA mix was nucleofected within the 96-well Nucleocuvette™ Plate (Lonza) using programme FF-158. Then, 80 µL of pre-warmed CD-CHO was added to each transfected well. Transfected cells were seeded into 96-well deep well plates (DWP) at 4×10^5 cells mL^{-1} in 475 µL of pre-warmed CD-CHO and cultured for five days at 37 °C, 320 rpm (25 mm throw), 85 % humidification, 5% CO_2, with Duetz sandwich covers for reduced evaporation. Nutrient supplementation took place at 72 h using CHO CD EfficientFeed™ (5% v/v CHO CD EfficientFeed™ A, 5% v/v CHO CD EfficientFeed™ B; Thermo Fisher Scientific). To equalize DNA load (at 800 ng) CHO-T2 cells were transfected with 533 ng (66 %) MAb-A expression vector, alongside effector gene vectors and empty vector (totaling 267 ng – 33 %). CHO-A cells were transfected with the same DNA quantities, but with 533 ng of empty vector in place of MAb-A expression vector. Transfection efficiency was determined by pmaxGFP vector vector (Lonza) co-transfection and flow cytometry analysis using an Attune Acoustic Focusing Cytometer (Thermo Fisher Scientific) 24 h post-transfection.

2.4. Determination of integral of viable cell density (IVCD), protein (IgG) titer and cell specific production rate (qP)

PrestoBlue® cell viability assay (Thermo Fisher Scientific) was used as per manufacturer instructions to measure cell density on day 0 and day 5 post-transfection to calculate the integral of viable cell density (IVCD) using a Pherastar Plus microplate reader (BMG Labtech, Ortenberg, Germany). Culture supernatants were isolated by centrifugation at 1000 x *g* for 5 min. Due to differing MAb production levels of transient and stable cells, MAb-A titer was measured by FastELISA® Human IgG Quantification Kit (RD Biotech, Besancon, France) and ValitaTITER IgG assay (Valitacell, Dublin, Ireland; (Thompson et al., 2017) for CHO-T2 and CHO-A cells respectively. The following formulae were used to calculate (1) IVCD (10^6 cell/day/mL $^{-1}$ or RFU/day and (2) qP (pg/cell/day or mg/L/RFU/day):

$$IVCD = \left(\frac{N_1 + N_2}{2} \times \Delta t\right) \quad (1)$$

$$qP = \left(\frac{T_2 - T_1}{IVCD}\right) \quad (2)$$

Where N_1 and N_2 are the viable cell concentration (cells mL^{-1} or normalized RFU) at the first and second time points, respectively; t represents time (days); T_1 and T_2 are the titers (mg L^{-1}) at the first and second time points, respectively.

2.5. Flow cytometry

CHO-K1 derived, suspension adapted cells were transfected with three fluorescent protein-expressing vectors (TagBFP, eGFP, mCherry) at varying levels using the nucleofection protocol described above. Cells were seeded at 1×10^6 cells mL^{-1} and gene expression was analyzed 24 h post-transfection using a BD LSR Fortessa™ flow cytometer (BD Biosciences). Prior to analysis DAPI (4′,6-diamidino-2-phenylindole) viability dye (at 10 μg mL^{-1}; BioLegend®, San Diego, USA) was added (1 in 2 dilution) to samples to select 10,000 viable cells for analysis. The excitation lasers and emission bandpass filters used for multiparametric analysis of DAPI, TagBFP, eGFP and mCherry were, respectively: 355 nm, 405 nm, 488 nm and 532 nm lasers; 450/40, 450/50, 525/50 and 610/20 bandpass filters. FlowJo® Software (FlowJo LLC, USA) was used to carry out minimal amounts of fluorescence compensation and analysis of raw data. Raw data was extracted to enable a single-cell analysis of gene expression.

3. Results

3.1. Establishment of a HT transient transfection platform for standardized screening of genetic components at varying relative stoichiometry

A HT transient gene expression platform was established to facilitate simultaneous screening of multiple genetic components in parallel, enabling rapid elucidation of bespoke engineering solutions for any given recombinant protein or host cell line. The platform is capable of identifying functional interactions between different genetic components as well as their optimal relative stoichiometry, resulting in the identification of a carefully titrated combination of potential effectors of cell performance.

HT gene delivery was achieved by microscale electroporation (nucleofection) using the 96-well Nucleofection system (Lonza). Cells were then maintained within 96 DWPs for 5 days (exponential growth phase) followed by measurement of cell growth and recombinant protein production. The nucleofection protocol was optimized using a GFP expression vector with respect to electroporation transfection program, cell number and DNA load. The optimized method produced a high and consistent transfection efficiency (> 94 %, Fig. 1A), high level gene expression (median fluorescence intensity – MFI, Fig. 1A) and a high cell viability post-transfection (> 85 % at 72 h post-transfection, data not shown). The platform enabled precise control of effector gene expression over a wide linear dynamic range (> 10-fold) by titration of vector DNA load during transfection, as demonstrated using vectors encoding GFP (Fig. 1B) or more complex proteins such as MAb-A (Fig. 1C). With respect to the latter, cell specific growth rate and MAb-A transient production titer were inversely correlated, indicative of either a progressive cellular stress response or metabolic/expression burden associated with recombinant protein production Gutierrez et al., 2018).

We considered that microscale transfections may exhibit inter-assay or inter-plate variation due to accumulated technical error within our protocol. Therefore, we measured assay variation associated with both cell growth and productivity to determine the optimal number of replicate wells required to compare discrete genetic effector treatments whilst maintaining maximum throughput. Nine microplates (864 wells) were identically transfected with 533 ng MAb-A and 267 ng empty vector. The coefficient of variation (CV, %) for cell growth and productivity measurements was calculated for differing numbers of replicates (Fig. 1D). CV (%) decreases as the number of replicates increases. However, this trend diminishes as replicates increase and so it was determined that 3 replicates yielded a substantial reduction in technical variation, whilst retaining a reasonably HT per microplate (> 30). Also, it is common practice to use 3 replicates when measuring a response variable (Singer et al., 2007) and so we decided that this was the appropriate number of replicates. To account for biological variation (e.g. between cell populations), each subsequent experiment was conducted in biological triplicate, i.e. each experimental permutation was repeated a total of 9 times.

3.2. The relative stoichiometry at which multiple recombinant genes are expressed can be accurately controlled using HT microscale co-transfection of single vectors

We considered that it would be necessary to simultaneously transfect cells with multiple genes at varying relative stoichiometry in order to measure their combined effect on cell functional performance. Due to the impracticality of constructing multigene vectors for each testable stoichiometric and combinatorial permutation of effector genes and for absolute flexibility in gene expression stoichiometry and experimental design, we screened effector genes by simultaneously co-transfecting multiple single-gene vectors at varying relative ratios. To quantitatively validate whether this approach yielded the expected relative expression of encoded genes, three spectrally distinct fluorescent reporter proteins (mCherry, eGFP and TagBFP), each encoded within discrete vectors, were co-transfected at varying relative ratios and reporter protein production was measured after 24 h using flow cytometry. In each case an identical vector backbone was employed, and the similarity in reporter gene sequence length (total vector length: mCherry, 6029 bp; eGFP, 6038 bp; TagBFP, 6020 bp) meant that minimal adjustment of relative DNA mass per transfection was necessary to achieve a desired gene ratio. Total DNA load (800 ng) was equalized using an empty vector. Using this standardized approach, the relative stoichiometry of different recombinant genes was user-controllable with minimal technical background variation in transfection conditions. To control for reporter protein-specific variation in post-transcriptional processing (e.g. mRNA dynamics, protein synthesis etc.) and the discrete spectral properties of a fluorescent protein (governed by fluorescence quantum yield and extinction coefficient), data were normalized with respect to the differing inherent fluorescent intensity of each fluorescent protein by separate calibration of reporter gene load against fluorescence intensity in each case (data not shown).

As total reporter protein expression in individual cells may vary (due to cell-specific variation in transfection efficiency), it was important to establish whether precise control over reporter protein expression was achieved at the single-cell level. Therefore, the individual fluorescence intensity of reporter proteins within each cell were further normalized with respect to total cell fluorescence to enable direct comparison of relative reporter protein expression within individual cells. However, individual cell data were weighted according to total reporter protein content as highly-transfected cells contribute proportionately more to the overall rDNA output (i.e. recombinant protein production) of the population.

These data (Fig. 2) reveal that at the cell population level, median reporter protein expression closely corresponded to the pre-defined ratio of transfected reporter genes (at up to a 4-fold excess of a single gene over another). Across all transfections the mean percent difference between any transfected gene ratio (i.e. either 1, 2, 3, or 4:1) and the observed population median relative ratio of the corresponding reporter protein was 3.6 % (SD = 3%). At the single-cell level, variation in reporter protein expression (at any given gene load) was evident, yielding a mean CV of 30.2 % (SD = 4.4 %) across all transfections. The mean percentage difference between the expression ratio of a given reporter protein and its transfected gene ratio within single cells was 22.8 % (SD = 2.5 %). It should be noted that the absolute range of variation increases with transfected gene load, which is illustrated by the differential breadth of the histograms (Fig. 2). However, the relative range of variation is consistent. We conclude that the relative stoichiometry of multiple co-transfected vectors encoding different recombinant proteins reasonably accurately represents their relative level of transcription across the cell population.

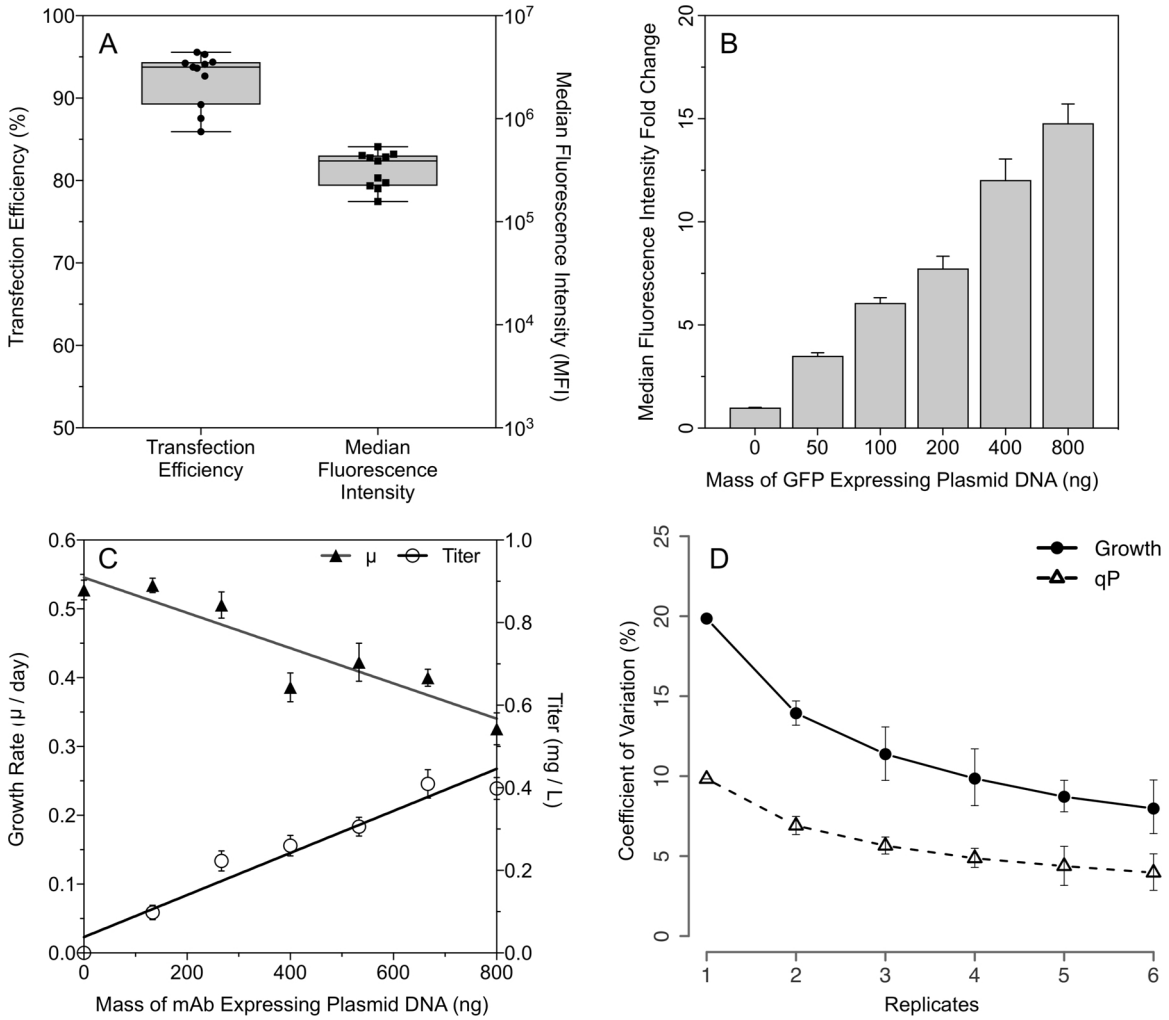

Fig. 1. The HT transient transfection platform can reliably titrate genetic component delivery to alter measurable responses in cell performance.
Here, using our standard HT nucleofection conditions we demonstrate that we achieve: (A) High and consistent levels of gene expression - Box and whisker plots representing *GFP* transfection efficiency (~93 %) and median fluorescence intensity (~6×10^5; MFI) 24 h post-transfection, measured from 11 transfections with expression-vector encoding *eGFP*; (B) Precise and titratable control of gene expression level through transfection of *eGFP*, measured here by MFI 24 h post-transfection; (C) Precise and titratable control of cell performance in response to transfection of MAb-A - shown via the impact upon growth rate (μ) and titer, measured at DNA 72 h post-transfection. (D) Minimized error through sample replication – measured using the CV (%) for growth and qP in response to transfection of MAb-A.

3.3. Transient transfection of constitutive secretory pathway genetic effectors can increase transient and stable MAb-A production in a context-specific manner

Genes to be co-expressed with MAb-A were selected based upon the hypothetical ability of the encoded protein to enhance MAb-A processing, folding, assembly or secretion. The proteins are active within the constitutive secretory pathway, predominantly within the ER, Golgi complex or vesicular transport machinery. The 32-gene library included a diverse array of molecular chaperones, redox/foldases, transactivators, ER to Golgi trafficking components, vesicular transport effectors, lipid synthesis modulators and the MAb-A light chain (*LC*) gene. An annotated list of the specific "effector genes" employed is given in Supplementary Table 1. The criteria used to select effector genes included: (i) a previously reported effect of over-expression resulting in increased productivity of CHO cells; (ii) association with an increased secretion phenotype, derived from differences in transcript analysis between B cells and plasma cells (data not shown); (iii) association with increased CHO cell production capacity from published 'omics studies (Carlage et al., 2012; Doolan et al., 2008; Harreither et al., 2015; Nissom et al., 2006; Yee et al., 2009) or (iv) in the absence of the above, a known function related to protein processing and secretion.

Initially, effector genes were assessed for their individual ability (one factor at a time, OFAT) to impact cell functional performance (cell growth and transient or stable MAb-A production). Effector gene vectors were both (i) co-transfected with a vector encoding MAb-A into a non-producing host (CHO-T2) and (ii) transfected into CHO cells engineered stably expressing MAb-A using glutamine synthetase selection/amplification (CHO-A, qP = 14 pg/cell/day at day 5 of a batch overgrow). In both cases, using the HT platform described above, individual gene dose was titrated across a defined range with empty vector used to equalize DNA load to 800 ng per well. At 5 days post-transfection MAb-A volumetric titer and viable cell biomass (expressed as IVCD) were measured and used to derive a quantitative estimate of qP. CHO-T2 cell performance in response to transfection, with effector genes tested at four concentrations (1.25 %, 2.5 %, 5% and 10 % of total DNA load), is shown in Fig. 3 relative to that of control cells transfected with only MAb-A vector and empty vector. CHO-A cell performance in response to transfection, with effector genes tested at three concentrations relative to that of control cells transfected with empty vector (3.3 %, 11.7 % and 20 % of total DNA load) is shown in Fig. 4. Effector genes were ranked according to their effect on overall MAb-A titer (Figs. 3A and 4 A). Effector genes were employed over a broader range of expression for stably transfected CHO-A cells as these

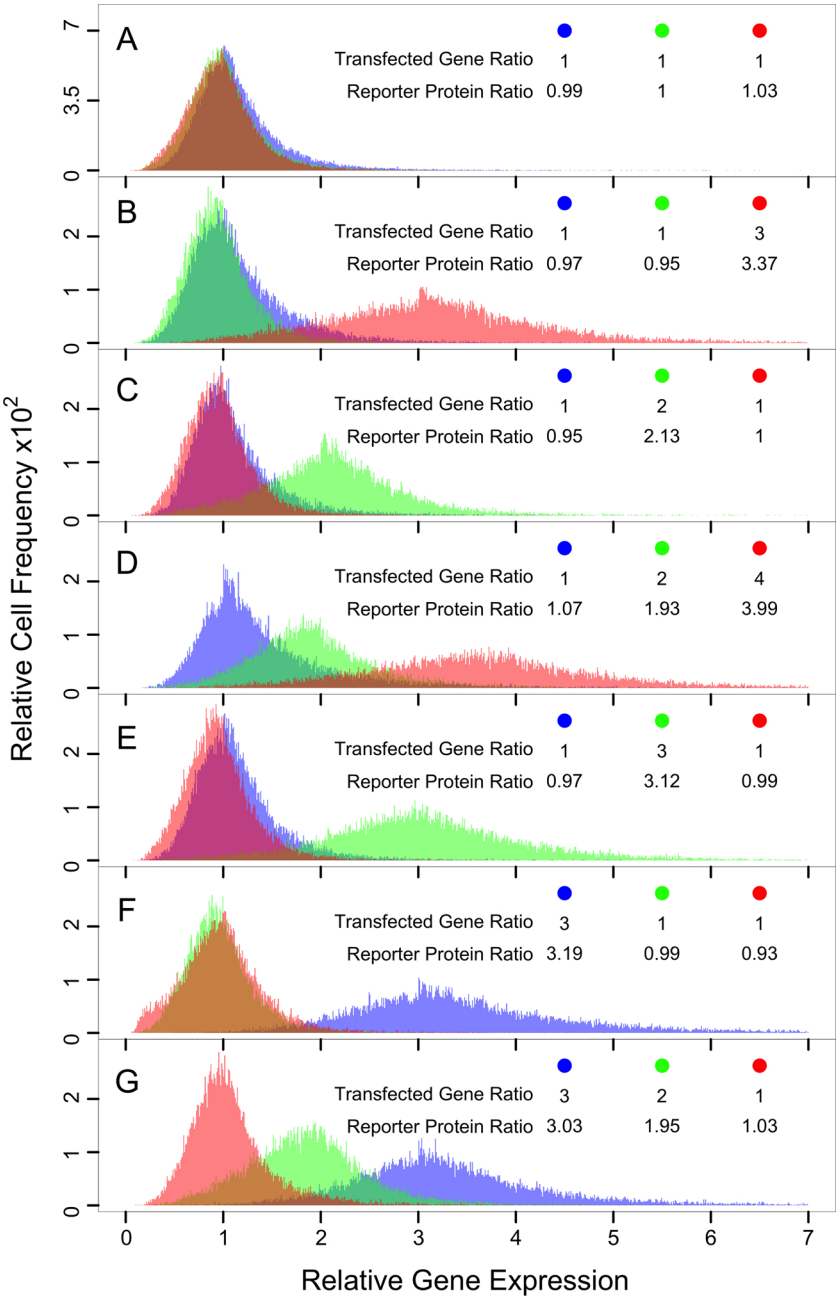

Fig. 2. The HT platform is capable of delivering multiple single-gene vectors for expression at varying relative stoichiometries.
TagBFP (blue), *eGFP* (green) and *mCherry* (red) single-gene vectors were co-transfected at varying relative stoichiometries by titration of DNA load: 1:1:1 (A), 1:1:3 (B), 1:2:1 (C), 1:2:4 (D), 1:3:1 (E), 3:1:1 (F) and 3:2:1 (G). Fluorescence values were normalized for reporter protein brightness by recalibration to a reporter protein titration. Reporter protein expression was then standardized using total cell fluorescence to generate a relative ratio for gene expression within each cell. Analysis was weighted towards those cells with higher overall gene expression. The histograms illustrate the comparison of reporter protein expression from the transfected gene load using a composite of single-cell ratio values. The transfected DNA load and corresponding mean reporter protein expression are outlined in the plot legends. Across all the transfections the mean percentage difference between transfected gene load and median reporter protein expression was 3.6 % (SD = 3%). The CV of reporter protein expression 30.2 % (SD = 4.4 %). The mean percentage difference between the expression ratio of a given reporter protein and its transfected gene ratio within single cells was 22.8 % (SD = 2.5 %).

Table 1
The number of effector genes that had either a positive (+), negative (-), or no (None) impact upon the three cell performance metrics titer, IVCD and qP for at least one of the concentrations used ($p < 0.05$).

	Titer			IVCD			qP		
	+	None	–	+	None	–	+	None	–
CHO-T2	7	8	17	3	26	3	3	14	15
CHO-A	13	16	4	2	17	13	19	11	2

*Genes were counted twice where both a positive and negative impact was seen at different concentrations for a given metric.

cells exhibited higher productivity and we decided to reduce the number of concentrations to increase platform throughput. Table I summarizes the number of effector genes that had either a positive, negative or no impact on titer, IVCD and qP.

Generally, across all transfections the HT platform exhibited reasonably precise measurements of both MAb-A titer (mean CV = 20.3 %) and IVCD (mean CV = 16.1 %), validating previous observations (Fig. 1). With respect to both sets of (transient and stable MAb-A production) data it was evident that the impact of many effector genes was dose dependent. This is exemplified by the impact of *ATF6c* upon MAb-A titer in stably-transfected CHO-A cells, where there was a significant increase and decrease in titer at different concentrations (20 % and 11.7 % respectively). Overall, effector genes had a greater impact on cell-specific production of MAb-A (qP, Figs. 3C and 4 C) than on cell growth (IVCD, Figs. 3B and 4 B), with gene-specific effects on qP tending to have the largest impact upon observed MAb-A titers. However, there was a clear difference in the respective responses to genetic effectors on comparison of cells transiently (CHO-T2) or stably (CHO-A) producing MAb-A. Indeed, co-expression of only 7 effector genes (at any dose) yielded a significant increase in MAb-A titer during transient co-production using CHO-T2 cells, whereas expression of 17 effector genes caused a significant decrease (Fig. 3A, C and Table 1). For stable

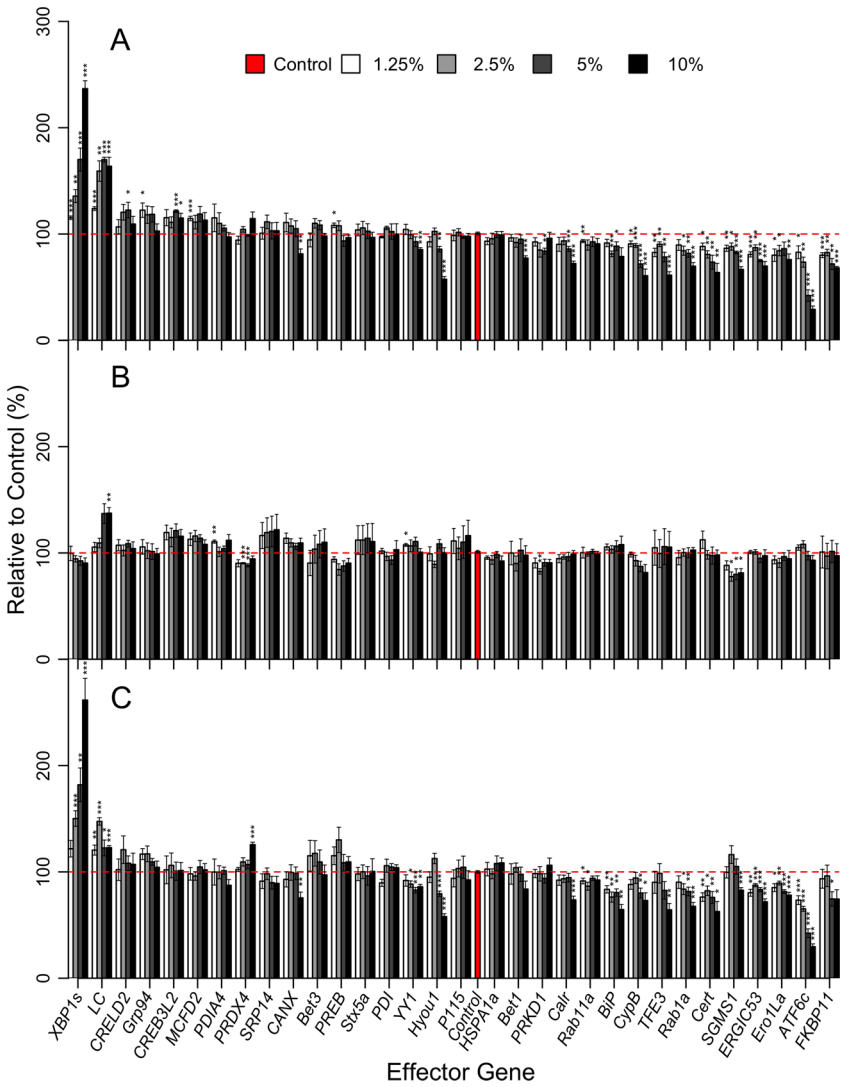

Fig. 3. Transient co-transfection of MAb-A alongside 32 genetic effectors of the constitutive secretory pathway can increase transient MAb-A production in a context-specific manner.

MAb-A vector (66 % w/w – 533 ng) was co-transfected into CHO-T2 cells alongside a total of 32 genetic effectors at four different concentrations (OFAT): 1.25, 2.5, 5, 10 % (w/w) of total mass vector DNA. Empty vector was used to equalize total DNA mass transfected (800 ng). Titer (A), IVCD (B) and qP (C) were calculated after 5 days of culture. Data is shown as a percentage change relative to control transfections (red bar/dashed line) containing no genetic effector. Genes are ordered by their impact upon titer. The data points and error bars, respectively, represent the average and SEM of three separate experiments, each with three technical replicates. Significance, represented by *(< 0.05), **(< 0.01), or ***(< 0.001), was calculated by *t*-test followed by a Benjamini Hochberg adjustment to allow for multiple comparisons.

production of MAb-A in CHO-A cells, expression of 13 effector genes yielded a significant titer improvement and only 4 effector genes caused a significant decrease in titer (Fig. 4A, C and Table 1). Further, comparing the relative efficacy of specific functional genes it was clear that different genes were active in both cases. *XBP1s* and *LC* genes led to substantial MAb-A titer increases (max. 237 % and 170 % respectively) when transiently co-expressed with MAb-A in CHO-T2 cells. However, *XBP1s* expression in stably transfected CHO-A cells had a much less impact on MAb-A titer and qP (max. 126 % and 142 % respectively), and *LC* expression actually inhibited MAb-A production (max. 83 %) and IVCD. Moreover, there was no overlap between the "top 5" ranked most effective genes (with respect to titer) for each MAb-A production system (**CHO-T2**: *XBP1s*, *LC*, *CREDL2*, *GRP94*, *CREB3L2*; **CHO-A**: *PDI*, *ERO1La*, *SRP14*, *FKBP11*, *PREB*), with only 2/10 effector genes (*XBP1s*, *GRP94*) yielding an improvement in cell performance in both MAb-A production systems. In both cases, even though all "top 5" most effective genes function or impact upon function within the ER (Supplementary Table 1), their specific mechanisms of actions differ when comparing transiently transfected CHO-T2 and stably transfected CHO-A cells. For example, 2 of the top 5 cell performance enhancers of CHO-T2 cells are transcription factors (*XBP1s* and *CREB3L2*), which may indicate the need for a more global modification to ER function and capacity for efficient MAb-A production in comparison to CHO-A cells, which may require more specific mechanistic alterations to ER processes. Indeed, an example of this functional specificity is revealed by the relative success of *PDI* and *ERO1La* in both improving performance in CHO-A cells, where both genes are involved in disulfide bond formation within the ER.

3.4. Combinations of efficacious genetic effectors identified within OFAT screening can provide further increases to transient and stable MAb-A production

Many of the genes selected for evaluation in this study function within the same cellular organelle, process or pathway. It is therefore reasonable to hypothesize that they could act in synergy. Even genes that do not functionally interact directly could provide additive benefits if combined. The number of possible combinations (of effector genes and their relative stoichiometry) is theoretically impossible to implement practically so we employed a simple data-driven approach to select genes based on their ranked OFAT performance to determine if further improvement in transient or stable production of MAb-A was achievable. Of course, we note that it would be possible to combine genes using a hypothesis-driven approach based on related function (e.g. *BIP*, *PDI* and *GRP94* are closely associated within antibody folding and assembly; (Walter and Ron, 2011). However, for the purpose of this study we sought to demonstrate ability of the HT platform to ascertain the impact of multiple effector genes acting in concert by using effector genes known to yield measurable increases in MAb-A production when employed OFAT.

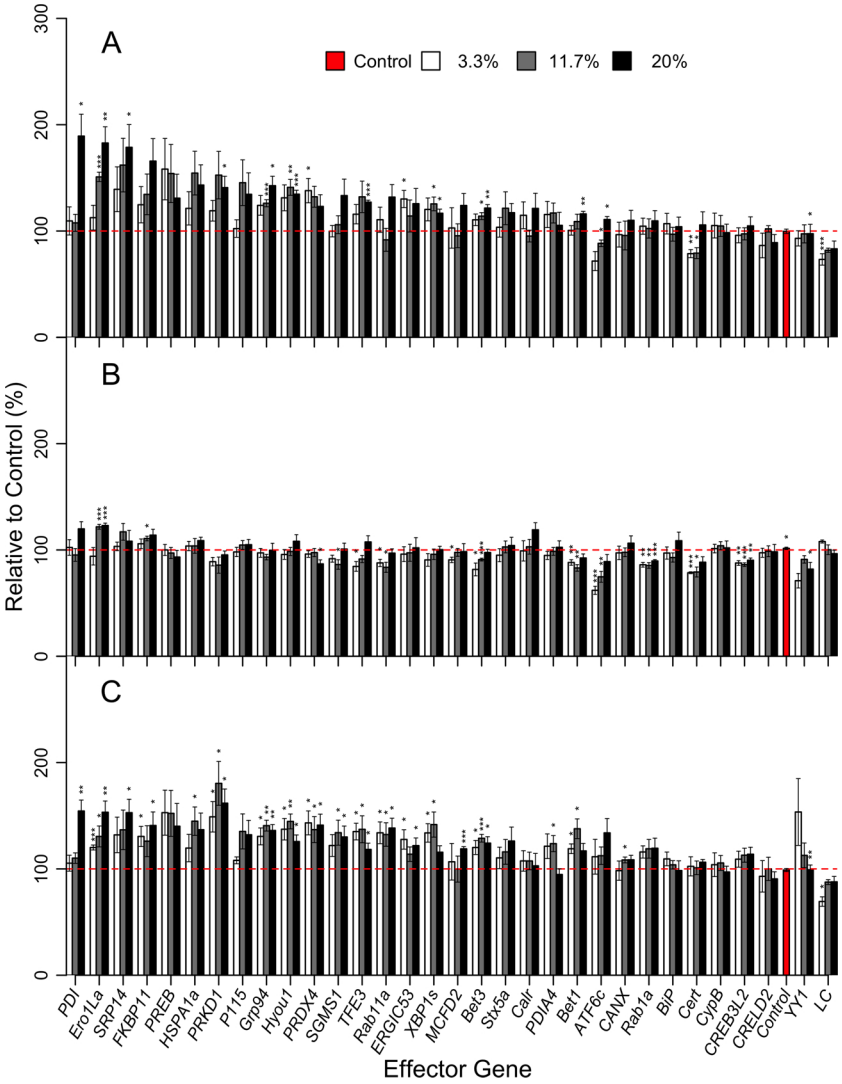

Fig. 4. Transient transfection of 32 genetic effectors of the constitutive secretory pathway can increase stable MAb-A production in a context-specific manner.
A total of 32 genetic effectors were transfected into CHO-A cells (stably producing MAb-A) at three different concentrations OFAT, 3.3, 11.7, 20 % (w/w) of total mass vector DNA. Empty vector was used to equalize total DNA mass transfected (800 ng). Culture titer (A), IVCD (B) and qP (C) were calculated after 5 days of culture. Data is shown as a percentage change relative to control transfections (red bar/dashed line) containing no genetic effector. Genes are ordered by their impact upon titer. The data points and error bars, respectively, represent the average and SEM of three separate experiments, each with three technical replicates. Significance, represented by *(< 0.05), **(< 0.01), or ***(< 0.001), was calculated by *t*-test followed by a Benjamini Hochberg adjustment to allow for multiple comparisons.

The three effector genes that yielded the largest increases in MAb-A titer for each production system during the OFAT screen, with no significant decrease in cell growth, (**CHO-T2/transient**: *XBP1s*, *LC*, *CRELD2*; **CHO-A/stable**: *PDI*, *ERO1Lα*, *SRP14*) were selected to assess whether any of their combinations could further improve MAb-A production in each case (Fig. 5). A 3 factor, 2 level factorial design was used to test all combinations within each 3-gene selection, where the DNA loads (levels) were set at 0 and the percentage of DNA load that yielded the highest titer during the OFAT gene screen (**CHO-T2/transient**: *XBP1s* – 10 %, *LC* – 5%, *CRELD2* – 5%; **CHO-A/stable**: *PDI* – 20 %, *ERO1Lα* – 20 %, *SRP14* – 20 %). The relative stoichiometry of these genes was 2:1:1 (*XBP1s:LC:CRELD2*) and 1:1:1 (*PDI: ERO1Lα:SRP14*) for the transient and stable screens respectively. A pairwise combination that significantly exceeded the impact of its composite genes was considered successful. Similarly, a triple combination that exceeded the impact of its composite individual components and pairwise combinations was considered as successful. A successful combination was characterized as either greater than the sum of the impact of its components (synergistic – S), equal to the sum of the impact of its components (additive – A) or less than the sum of the impact of its components (enhancing – E). For transient MAb-A production by CHO-T2 cells (Fig. 5A-C) the *LC/XBP1s* combination was enhancing in its improvement of titer (312 %) and qP (355 %), but caused a minor but significant decrease in IVCD (84 %). *CRELD2* was additive in its pairwise combinations with *LC* and *XBP1s* for titer (261 % and 283 % respectively), with the latter having a small significant decrease in IVCD (88 %). Neither of these combinations yielded a significant increase in qP. The triple combination of *LC/XBP1s/CRELD2* did not improve cell performance beyond that achieved either by single genes or pairwise combinations with respect to titer, IVCD or qP. For CHO-A cells stably producing MAb-A (Fig. 5D-F) the *PDI/ ERO1Lα* combination was additive in its improvement of titer (159 %) and qP (161 %), without having a significant impact upon IVCD (98 %). The *ERO1Lα/ SRP14* combination was enhancing in its improvement of titer (162 %) but did not outperform *SRP14* in terms of qP (153 %) and did not have a significant impact upon IVCD (105 %). Neither the *PDI/SRP14* pairwise combination or the *PDI/ERO1Lα/SRP14* triple combination led to significant improvements over their composite pairwise combinations or single genes considering titer, IVCD or qP.

4. Discussion

A core premise of this work was that despite the fact that there have been many published examples of CHO cell engineering to improve complex multigenic functions such as cell growth and productivity (Pybus et al., 2014; Cain et al., 2013; Johari et al., 2015; Davis et al., 2000; Ku et al., 2008; Mohan and Lee, 2010), very few, if any, are demonstrably generally applicable. Indeed, there were several genes reported to enhance productivity, such as *CYPB*, *CALR* and *CERT*, which had a negative impact on productivity in this study (Johari et al., 2015;

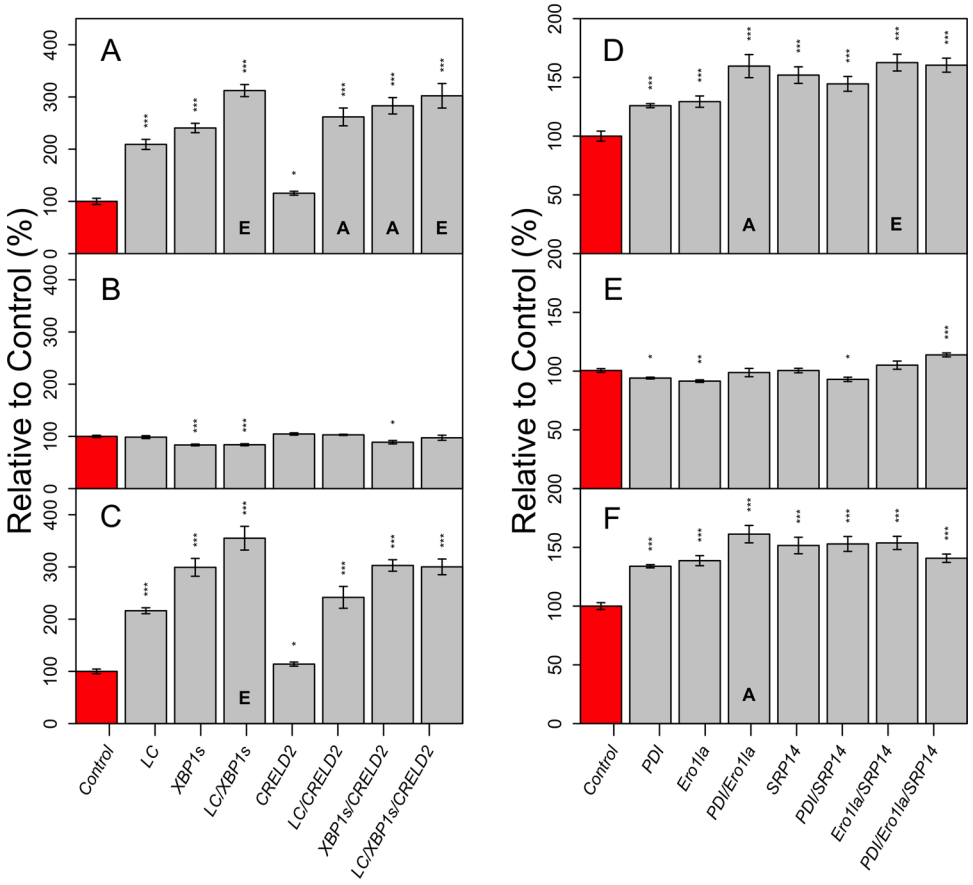

Fig. 5. Combinations of efficacious genetic effectors identified within OFAT screening can provide further increases to transient and stable MAb-A production.
Combinations of genetic effectors were assessed for their impact on cell performance within 2-level, 3 factor factorial designs for CHO-T2 (A – titer, B – IVCD, C – qP) and CHO-A (D – titer, E – IVCD, F – qP) production of MAb-A. DNA load (levels) was set at 0 and the most successful gene dose from the single-gene screen (CHO-T2: *LC* – 10 %, *XBP1s* – 10 %, *CRELD2* – 5%; CHO-A: *PDI* – 20 %, *ERO1LA* – 20 %, *SRP14* – 20 %). The data points and error bars, respectively, represent the average and SEM of three separate experiments, each with three technical replicates. Data is shown as a percentage change relative to control transfections (red bar) containing no genetic effector. Significance, represented by *(< 0.05), **(< 0.01), or ***(< 0.001), was calculated by *t*-test followed by a Benjamini Hochberg adjustment to allow for multiple comparisons. A successful combination was characterized as either greater than the sum of the impact of its components (synergistic – S), equal to the sum of the impact of its components (additive – A) or less than the sum of the impact of its components (enhancing – E).

Chung et al., 2004; Florin et al., 2009; Rahimpour et al., 2013). This is likely explained by the genetic and phenotypic diversity of CHO cell families / cell lines (Wurm and Hacker, 2011; Lewis et al., 2013) as well as the molecule-specific demands that a given recombinant protein places on a particular host CHO cell (e.g. at critical cell/product-specific interfaces such as folding/assembly). Essentially, everything is variable; host cell, protein product, genetic vector/component design, cell growth medium, production process and so despite all the directed cell engineering and comparative 'omic studies conducted to date, literature mining to select a genetic engineering strategy to improve the growth or productivity of a particular cell line/product combination is rather unreliable (Kuo et al., 2018; Lund et al., 2017; Hansen et al., 2017, Gutierrez, 2018, Prashad and Mehra, 2015). Therefore, engineering of functional genetic components is not generally employed within industrial CHO cell line development, and instead screening of "natural" genetic/functional variation within stable pools is still relied upon to enhance functional performance (Davies et al., 2012; O'callaghan et al., 2015; Fernandez-Martell et al., 2018). Advances in new technologies for clone screening and isolation have made this approach generally successful (Priola et al., 2016). However, for some DTE proteins, manufacturability can be an issue (Aggarwal, 2014; Fischer et al., 2015; Pybus et al., 2014; Lee et al., 2007; Hussain et al., 2018; Tadauchi et al., 2019) without the cell engineer understanding the underlying mechanisms. It is often assumed that a bottleneck in ER-resident folding and assembly exists, although even if this is the case it is certainly not obvious which ER processes to target (Johari et al., 2015; Pybus et al., 2014; Kaneyoshi et al., 2019). Where the ability of natural variation (Davies et al., 2012) is exhausted it has become increasingly necessary to design targeted, non-natural solutions to redress the situation and so it is imperative to overcome the context-specificity that is currently limiting cell factory engineering.

To address these problems of (cell line/product) specificity we developed a HT platform that allows a user to functionally trial many potential genetic effectors (e.g. genes, miRNAs, esiRNAs etc) simultaneously, either OFAT or in combination. We demonstrated the platform's capability to assess the efficacy of a wide array of secretory pathway components OFAT or in combination using both transient and stable production systems with differing CHO cell backgrounds. This clearly exemplified the necessary context-specificity that future cell engineering strategies should employ (Johari et al., 2015; Chung et al., 2004; Florin et al., 2009, Rahimpour et al., 2013). Of course, it would be possible to significantly increase the number of combinations tested using data-driven or hypothesis-led strategies, varying gene stoichiometry within, for example, a Design of Experiments experimental framework. For both transient or stable production scenarios effector gene combinations could be identified that address a specific user-defined objective, such as increased qP, increased cell growth or improvement of a product quality parameter such as reduced product aggregation. Ideally, all of these metrics should be considered simultaneously, so that one is not improved at the expense of another. For example, an improvement in qP is immaterial if the quality of the product, cell growth or cell viability is insufficient. Our study also highlights the importance of controlling effector gene expression level and relative stoichiometry for tunable control of cell function. In all the examples given, effector genes were expressed at a much lower level than MAb-A genes, and frequently functional responses were titratable with respect to effector gene dose. This is in accord with the general observation that for most highly productive stable cell lines, expression of recombinant genes is far in excess of the vast majority of host cell genes. Stoichiometric control of transient expression of genetic effectors, as shown here, can be implemented via titration of gene loads during transfection. Implementation of stoichiometric control within a stable expression context will need to utilize mammalian expression technology that enables precise transcriptional control. To achieve this, novel synthetic

promoter technology has been developed in our laboratory (Brown et al., 2017) and recently exemplified for whole synthetic pathway engineering of recombinant protein production in CHO cells (Brown et al., 2019). We envisage that multigene expression constructs could easily be created (e.g. using Gibson or Golden Gate cloning and vector assembly methods; Torella et al., 2014; Duportet et al., 2014), that would encode both product and effectors (or effectors alone), in which the optimal relative recombinant gene expression ratio is controlled using synthetic promoters with pre-defined transcriptional activity. Assuming that the transient and stable impacts of genetic effectors are approximately synonymous then this approach could yield more productive stable cell lines. Therefore, the HT genetic effector testing system described in this study would be an essential part of a synthetic biology-based CHO cell engineering and design platform technology that utilizes repositories of genetic parts ("biobricks"; effector genes, miRNAs, promoters, etc.) that are used in unique combinations to achieve cell line and product-specific manufacturing objectives.

The data presented in Figs. 3 and 4 can also be interpreted in an analytical context as we effectively examine the effect of perturbing the rate of multiple cellular processes simultaneously. In this regard, the positive or negative effect of a particular functional gene implies that the related cellular process is either a constraint or is functioning optimally respectively. The specific genes that cause a favorable cell response may provide evidence of synthetic bottlenecks. For example, with respect to stable production, the efficacy of *PDI*, *ERO1Lα* and *FKBP11* may indicate a general MAb-A specific constraint (Mason et al., 2012), whereas *SRP14* and *PREB* may indicate a general secretory pathway constraint. Context specificity was clearly evident in the comparison of effector gene functionality for transient and stable MAb-A production systems. It is probable that differences in recombinant MAb-A expression level in combination with cell background differences related to MAb-A production between the two systems influenced cell engineering outcomes. This would directly affect the responsiveness of the MAb-producing cell to co-expression of specific effector genes. For example, transient production was significantly improved by co-expression of *XBP1s*, which likely increases productivity via global upregulation of ER-resident secretory pathway machinery (Tigges and Fussenegger, 2006; Cain et al., 2013; Tirosh et al., 2005; Becker et al., 2008, 2010). This implies that a comprehensive, multifaceted increase in secretory pathway capacity (or an undefined target of *XBP1s*) is necessary to improve the relatively low qP of transiently-transfected cells – i.e. everything has to improve for a benefit. In this transient MAb-A production context the effect of overexpression of individual ER components is either limited (*GRP94, PDIA4*), negligible (*HYOU1, HSPA1A*) or even detrimental (*BIP, FKBP11*). Therefore, in transiently transfected host cells ER chaperone and vesicular transport processes (*P115, RAB11A, PREB, BET1, BET3*) may be collectively unsaturated or more likely, collectively limiting such that improvement of only one process merely shifts the limitation onto the next with limited benefit. The beneficial effect of *LC* expression on transient production and cell growth indicates a MAb-A specific constraint – that the *LC:HC* expression ratio specified by the genetic vector is sub-optimal and may even negatively affect cell growth via an unfolded protein response (UPR)-induced suppression (Fig. 1C; (Pybus et al., 2014; Davies et al., 2011; O'callaghan et al., 2010; Schlatter et al., 2005). In contrast, for CHO-A cells selected for stable production of MAb-A, *XBP1s* had minimal effect on the productivity of stably-transfected cells and *LC* inhibited MAb-A production. Instead, the stable production performance of CHO-A cells benefitted from engineering discrete secretory pathway components to boost qP, such as *PDI* and *ERO1Lα* (disulfide bond formation; (Borth et al., 2005; Cain et al., 2013). We infer that it is likely that stably transfected CHO-A cells already had constitutively enhanced secretory pathway capacity attributes and an altered *LC:HC* ratio to favor folding/assembly kinetics. Thus, further general capacity enhancement via increased *XBP1s* or *LC* expression would not further enhance qP.

The stable transfectant context for cell engineering is fundamentally different to the parental transient context. In the former, an acute UPR associated with high recombinant mRNA/nascent protein production would be incompatible with cell growth (Rutkowski and Kaufman, 2004; Rutkowski et al., 2006; DuRose et al., 2006; Merquiol et al., 2011; Prashad and Mehra, 2015). Therefore, we speculate that it is necessary for stably transfected cells to essentially avoid (or raise the threshold for) UPR induction via maintenance of a constitutively higher secretory pathway capacity or to adapt to the persistent, chronic UPR stress associated with recombinant protein production via altered regulation (de-sensitization) of UPR signaling pathways (Harreither et al., 2015; Smales et al., 2004; Dinnis et al., 2006). Associated with this, in the case of MAbs, as optimal *LC:HC* gene expression ratio is likely to be MAb-specific (Pybus et al., 2014; Davies et al., 2011; O'callaghan et al., 2010; Schlatter et al., 2005) then altered regulation of *HC* and *LC* gene transcription towards the MAb-specific optimum may also be a necessary pre-requisite for cells to achieve both high level cell growth and productivity, again avoiding UPR induction.

Whilst different recombinant proteins may impose a different biosynthetic/metabolic burden on host cells, contributing to reduced cell growth rate in proportion to their cellular resource (e.g. ATP equivalents) utilization (Gutierrez et al., 2018), DTE recombinant proteins represent an additional threat to cell growth as they will induce cell signaling/stress response pathways that slow or halt cell growth irrespective of resource utilization (Pybus et al., 2014; Johari et al., 2015; Hansen et al., 2017; Sommeregger et al., 2016; Le Fourn et al., 2014). DTE proteins with slow folding and assembly kinetics may induce a UPR at relatively lower (compared to ETE recombinant proteins) levels of recombinant mRNA translation as the unfolded proteins accumulate in the ER, thus only stable transfectants with commensurately low, sub-UPR threshold transcriptional activity can proliferate post-transfection, leading to a less productive cell population (Moore and Hollien, 2012; Walter and Ron, 2011). Indeed, it has been found that molecules with secretory bottlenecks in TGE lead to the generation of less productive stable cells due to a diminished recombinant mRNA abundance (Mason et al., 2012). Therefore, a given clone's response to a molecule-specific ER stress (Rutkowski and Kaufman, 2004; Rutkowski et al., 2006; DuRose et al., 2006; Merquiol et al., 2011; Prashad and Mehra, 2015) likely functions as a population dynamic driver during selection and outgrowth of stable transfectants. Therefore, the general transcriptional and protein synthetic landscape (the cellular context) into which effector genes are transfected is a crucial determinant of efficacy and stable transfectants (especially expressing a DTE protein) are clonal variants with constitutive or adaptive changes to the landscape that diminish the effect of recombinant protein production on cell growth.

Given the above, how do we best design tailored host cell and product-specific cell engineering solutions to improve transient or stable production of specific DTE proteins? Clearly, in a transient context an obvious extension of our platform would be to include an expression cassette within the product expression vector (or co-transfect a separate vector) that encodes a stoichiometrically-balanced, product-specific set of effector genes. Alternatively, if generic effectors applicable to different recombinant products are identified (obtained by HT testing of the effect of multiple genetic effectors across different recombinant products simultaneously), these could be stably-transfected into the transient host cell line in advance (Cain et al., 2013).

In a stable production context, we can consider both forward and reverse engineering strategies. In both cases we want transfected cells to be able to harness the full extent of natural (clonal) variation possible, but then to extend beyond this in a product-specific manner via directed cell engineering. The optimal engineering objective is to increase product titer (or quality) at equivalent or lower cell biomass concentrations. The forward engineering approach would be to pre-equip parental cells with the necessary product-specific or generic capacity-enhancing components followed by stable transfection of product gene(s), or to co-transfect these with product gene(s) directly, as mentioned above. This has the theoretical advantage that transfectants

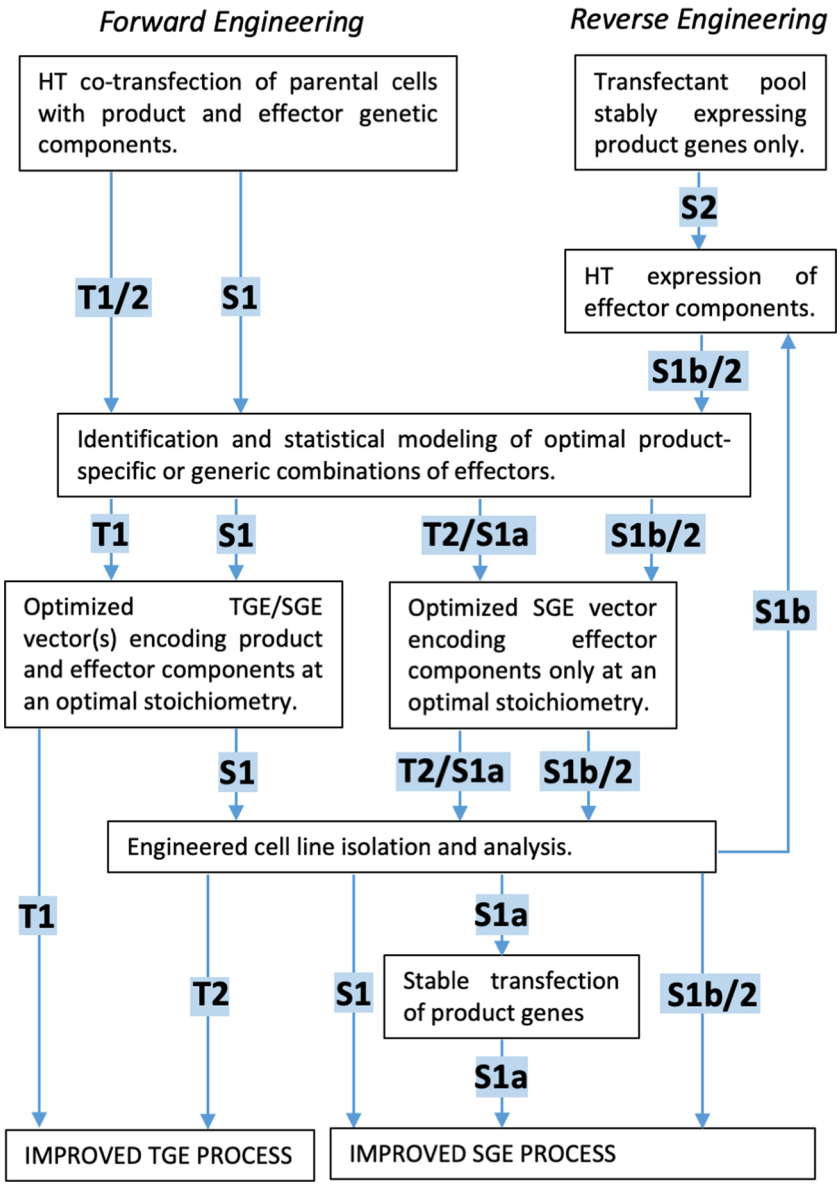

Fig. 6. Workflow strategies for host cell and product-specific engineering of transient and stable recombinant protein production using HT genetic effector testing.
Forward engineering strategies begin with HT co-transfection of parental cells with product and effector genes, followed by identification and statistical modeling of optimal product-specific or generic combinations of effectors. Using optimized TGE/SGE vectors encoding product and effector components at optimal stoichiometry parental cells can either be transfected for improved TGE (T1) or transfected to create an engineered recombinant cell line for improved SGE (S1). Alternatively, an optimized SGE vector encoding only effector components can be used to generate an enhanced parental host via stable transfection. This enhanced host can either be used for improved TGE via transient transfection of product genes (T2) or a subsequent stable transfection of product genes could be utilized to improve SGE (S1a). The reverse engineering strategy begins with HT transfection of a transfectant pool stably expressing product genes with effector components, followed by identification and statistical modeling of optimal product-specific or generic combinations of effectors. Using an optimized SGE vector containing effector components the stable transfectant pool can be transfected to generate an engineered cell line with improved SGE (S2). A combination of forward and reverse engineering strategies could be utilized, whereby the resulting stable pool of route S1 then goes on to follow route S2, yielding a further improvement to SGE (S1b).

would then be able to achieve a significantly higher qP before reaching the threshold for a signaling-pathway mediated reduction in cell growth. However, how do we determine what the optimal combination of genetic effectors is in advance? Clearly, in our example, if we had relied just upon screening transiently transfected CHO-T2 cells (co-expression of product and genetic effectors; Fig. 3) then we would have concluded that (i) titrated, low level *XBP1s* expression would be advantageous to increase cellular secretory pathway capacity and (ii) vector engineering to increase *LC* expression relative to that of the *HC* gene would be advisable. However, this would not, *per se,* have anticipated the further potential utility of titrated *PDI*, *ERO1Lα* and *SRP14* expression in specifically tuning stable transfectants for improved MAb-A production (Figs. 4 and 5). In this circumstance it would be possible to (i) construct an augmented stable transfectant pool, (ii) conduct a second round of HT genetic effector screening, (iii) re-transfect with an optimal effector gene combination and (iv) screen and isolate clones. Alternatively, using a pure reverse-engineering strategy, it would be possible to isolate a specific clonal derivative expressing product gene (s) only (to reach the limits of natural variation) and then apply HT genetic effector screening to identify cell line/product-specific bottlenecks that could be alleviated by retro-engineering of a bespoke multigene construct. However, this strategy could be limited due to the adaptive constraints imposed upon the cell population during selection, such as reduced transcript abundance (Mason et al., 2012). Practically, in both cases, the use of both random recombinant gene integration (e.g. with a metabolic selection marker, to maximize production and clonal variation) combined with site-specific integration (SSI) technology (to insert titrated effector genes at a defined locus for controlled expression) would together provide a solution. A summary of cell line development strategies is provided in Fig. 6. Clearly, it would be of great benefit to the biopharmaceutical community to establish which of these strategies, or combination of strategies, is most useful.

Lastly, although we have concentrated upon secretory pathway engineering in this study, it is clearly evident that this cell engineering design platform could be applied to almost any aspect of cell factory performance to control product molecular heterogeneity, cell growth and metabolic characteristics or genetic vector design. Moreover, we recognize that dynamic process-specific solutions may be required, i.e. effector gene functionality during late-stage culture may be particularly advantageous or the response of the host cell to effector genes may change during a production process. Indeed, it might be the case that a 5-day batch culture (used in this study) does not fully assess the potential impact of a given genetic effector. Here, the use of inducible promoter technology may be employed to titrate expression of

combinations of genetic effectors as required (e.g. Poulain et al., 2017). It might also be useful to determine the impact of genetic effectors at the single-cell level (e.g. via flow cytometry), i.e. how does a given genetic effector impact upon the productivity distribution within a population of cells? This is particularly important when considering how genetic engineering will impact clonal selection. In summary, CHO cells, recombinant protein products and production processes are inherently variable and therefore cell engineering technology that embraces uncertainty to derive tailored outcomes is necessary. The platform we describe can be applied to both compare novel synthetic assemblies or components with unpredictable performance (e.g. signal peptides), or test hypothetical predictions derived from advances in core molecular cell biology, comparative genome-scale informatics or computational modelling of cell function. As these approaches mature, we anticipate that rapid HT testing of *in silico*-predicted cell engineering strategies will become critically important to guide engineering design.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Joseph F. Cartwright: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Claire L. Arnall:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Project administration. **Yash D. Patel:** Investigation. **Nicholas O.W. Barber:** Investigation. **Clare S. Lovelady:** Supervision, Resources. **Guglielmo Rosignoli:** Supervision, Methodology, Resources. **Claire L. Harris:** Supervision, Resources. **Sarah Dunn:** Supervision, Resources. **Ray P. Field:** Supervision, Conceptualization, Resources. **Greg Dean:** Supervision, Resources. **Olalekan Daramola:** Supervision, Writing - review & editing, Resources, Project administration. **Suzanne J. Gibson:** Supervision, Conceptualization, Writing - review & editing, Resources, Project administration. **Andrew A. Peden:** Supervision, Conceptualization. **Adam J. Brown:** Supervision, Conceptualization, Funding acquisition. **Diane Hatton:** Supervision, Conceptualization, Writing - review & editing, Resources, Project administration. **David C. James:** Supervision, Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition, Project administration.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2020.02.012.

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