Global mRNA and protein expression analysis

Research applications in cancer and other diseases and in biopharmaceutical production

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Differential protein expression in *Candida albicans* in response to amphotericin B, a crucial antifungal agent for the treatment of fungal infections in cancer patients

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Amphotericin B is a polyene macrolide antibiotic widely used in the treatment of systemic fungal infection in cancer patients and functions by forming pores in the fungal cell membrane which allow the leakage of intracellular constituents and the eventual death of the cell. Although a proven agent in the treatment of fungal infections, amphotericin B has severe side effects and is extremely nephrotoxic. *Candida albicans* is a dimorphic fungus and is a major cause of infection and death in patients immunocompromised as a result of disease (leukaemia) or therapy (chemotherapy or irradiation). This project sought to ascertain whether amphotericin B affected the ability of the fungus to grow in the body and contributed to its virulence. Results will be presented to show that amphotericin B exposure increases the ability of the yeast *C. albicans* to adhere to host tissue and also induces the elevated expression of peptides associated with binding to plasminogen. Several proteins were identified by MALDI-TOF mass spectrometry and the differential expression of these proteins (glyceraldehydes-3-phosphate dehydrogenase, alcohol dehydrogenase, phosphoglycerate mutase, phosphoglycerate kinase, fructose biphosphate aldolase) in response to amphotericin B indicates that this drug has

the potential to increase the ability of *C. albicans* to survive in the body and to adhere to host tissue. Implications of these finding for the treatment of cancer patients with systemic or superficial fungal infections will be discussed.

Adaptation of Adherent Human cell lines to suspension culture

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To meet the demand for pharmaceutically important proteins, the use of mammalian cell lines as working factories for the production of recombinant proteins is increasing. To counteract this looming 'crunch' in production capacity, and to offset rapidly increasing costs, individual productivity of each cell must be improved while maintaining product quality. To study the factors that influence growth, viability and death in bioreactors, five different types of adherent human cell lines were chosen as models including DLKP, DLKP-A10, DLKP-A5F, H1299, and MDA-435S Tax 10p4pSI. These cell lines are very well established and were chosen as bioprocess models, as human cell lines are easier to characterise by conventional PCR and microarray techniques when compared to industrial hamster cell lines, e.g. CHO.

The growth and viability of these cell lines in suspension culture was measured and as a result of initial experiments, H1299 & MDA-435STax10p4SI were selected for the further study. These particular cell lines were then further adapted to suspension growth. The adapted H1299 has shown manifold increase in peak viable cell densities achieved, while MDA-435STax10p4SI has shown similar growth pattern compared to the non-adapted. The inocu-

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lation density and agitation speed were standardised for adapted MDA to achieve better growth. These adapted cell lines offer an exciting opportunity to study the molecular events leading to adaptation of cells to suspension bioreactor culture and hence to use rational design approach to engineer industrial cell lines to achieve greater productivity.

Mode of action of Caspofungin—a novel agent for the control of fungal infections in cancer patients

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Caspofungin is the first approved agent from a new class of antifungal drugs, the echinocandins, and is increasingly being used to treat systemic fungal infections in cancer patients. Caspofungin exhibits a unique mode of action compared to conventional antifungal agents by disrupting the synthesis of the fungal cell wall via the inhibition of β 1, 3 glucan synthetase. The yeast *C. albicans* is an opportunistic fungal pathogen capable of causing serious systemic infections in immuno-compromised individuals (e.g. patients undergoing chemotherapy, neutropenia). *Candida albicans* infection has become the fourth most prevalent infection within the clinical setting and the frequency of candidiasis is increasing worldwide. Current antifungal treatments include use of polyenes (e.g. amphotericin B, nystatin) or triazoles (e.g. ketoconazole, fluconazole, itraconazole); however, these drugs have limited success due to resistance or toxicity. This study examined the effect of caspofungin on *C. albicans* by analysing changes in the adherence to epithelial cells, enzyme secretion, protein production, ergosterol production and membrane integrity. In addition to its effects on the fungal cell wall, caspofungin decreased fungal adherence, induced alterations in the cell membrane and increased trans-membrane leakage. Exposure of fungal cells to caspofungin resulted in altered protein expression. Proteins altered in expression following caspofungin treatment of *C. albicans* have been identified by MALDI ToF analysis and their role in the physiology of the yeast will be discussed.

Transcriptomic and Proteomic Analysis of Antibody Producing NS0 Cells cultivated in High Density Perfusion Culture

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The establishment of a gene expression profile in combination with proteomic analysis of industrially important cell lines under various culture conditions is expected to provide a detailed understanding of the complex cellular functions and mechanisms that constrain survival, growth and productivity, thus allowing the rational modification of metabolic pathways to improve cell line performance.

High cell density, achieved by perfusion cultures, is associated with sub-optimal cell physiological state and limited mass transfer resulting in increased apoptotic death and reduction in growth rate, cell cycle duration, productivity and product quality. In this work, we have performed a transcriptomic and proteomic analysis of NS0 cells grown at different densities in 2L spin filter perfusion bioreactor to gain insight into the effect of environmental stress associated with process intensification. The cell line achieved a viable cell density of 1.3×10^7 Cs/mL over a period of 14 days. Transcriptomic and proteomic analysis of cells at three different densities (10^6 , 10^7 and 1.3×10^7 cell/mL) was performed by Affymetrix gene chip and 2D gel electrophoresis. Forty-six proteins with statistically significant changes in expression levels were identified and evaluation by LC-MS/MS mass spectrometry revealed the proteins that are directly associated with changes in physiological and synthetic states at high cell densities. The alteration of transcription at different cell density had a broad spectrum of functions with a wide range of regulated genes that were implicated in the central metabolic and signaling pathways. The contributions of genomic and proteomic analysis to the development of cell engineering strategies to increase productivity and reduce apoptosis will be highlighted and discussed.

Development of targetable Lung cancer cell line for Stable transgene expression

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Stable exogenous gene expression in mammalian cells *in vitro* is a commonly used tool in deciphering the function and importance of the gene in question. In addition it offers a potential tool for gene-therapeutic applications. The biggest challenge in establishing stable transgenic cell lines is genomic instability (loss of transgene expression) and the time involved in generating the stable line. Establishing a targetable lung cancer cell line, DLKP, would be of immense value to researchers at NICB as it is one of the most widely studied cancer cell line here. As well as a considerable amount of *in silico* data generated over the years for different drug variants of this cell line, functionally validating the roles of genes of interest using a targetable system would generate valuable information towards basic cancer research. A targetable cell line was developed by constructing and transfecting a plasmid vector, which had *loxP* sequences flanking a *GFP Neo* fusion gene (Geo) which served as reporter and for stable cell selection. The Geo cassette was subsequently removed by transient *Cre* recombinase expression. A plasmid Shuttle vector, which carries a promoterless *Neo* and targeting *loxP* sequence, was constructed for shuttling genes of interest into the targetable LoxP site in the genome. In this example we demonstrate the utility of the system using a *LacZ* reporter gene.

Proteomic profiling of suspension animal cell cultures

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Animal cells, particularly Chinese Hamster Ovary cells, are the main sources of novel biopharmaceutical molecules. Modern biopharmaceutical molecules are becoming increasingly expensive due to long developmental times and expensive manufacturing conditions. To address this problem, research is underway to better understand the biopharmaceutical process so that the factors that influence productivity can be enhanced at the industrial scale as it is clear that to double the production capacity of a cell could lead to significant decreases in cost burden on health services globally.

Low culture temperature of animal cells is known to enhance protein productivity and to extend the lifespan of production cultures to achieve higher recombinant protein yields; however, the mechanisms of this process are poorly understood.

To address the mechanisms of low culture suspension temperature, both human and CHO-K1 cell lines were adapted for suspension growth in 500 mL spinner cultures and samples were taken throughout the duration of a typical batch culture. Upon selection of optimal conditions of agitation speed, seed density and medium formulation, CHO-K1 cells were cultured at 37 °C for 72 h, and 31 °C for a further 72 h. In a parallel culture, a similar culture was grown at 37 °C for the duration of the culture.

Triplicate protein samples were taken at 72 and 144 h for 2D gel electrophoresis.

It was observed that a number of protein changes were apparent at the later stages of batch suspension culture and work is progressing to identify temperature-specific and temperature-independent protein changes and hence understand the temperature-shift effect and batch culture more clearly.

Proteomic analysis of differential protein expression induced by c-Myc over-expression in CHO cells

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Induction of the transcription factor Myc promotes cell proliferation and transformation by activating growth promoting genes or by repressing the expression of growth arrest genes. To examine the role of this gene in cell culture processes, an adherent CHO cell line was co-transfected with the human c-Myc and the secreted alkaline phosphatase (hSEAPFc) fusion antibody genes and a new stable cell line was generated. A significant increase in proliferation rate, maximum cell number and productivity, as well as a reduced serum dependency was observed in batch cultures. To understand the broad range of proteins involved in this stimulation of cellular activity, we carried out a systematic study to identify specific proteins involved in c-Myc induced proliferation using two-dimen-

sional gel electrophoresis and mass spectrometry. Unique changes in protein expression level for 32 proteins were observed at day 5 of batch culture, including Hsp70 and annexin A2 proteins which have been implicated in cell proliferation and cell growth, ATP synthetase, actin-like protein, β -actin, phosphoglycerate mutase-1 and triphosphate isomerase. This proteomic approach provides opportunities to gain insights into the mechanism by which c-Myc modulates cell growth, apoptosis and differentiation.

Amplification of mRNAs from Medium Conditioned by Insulin-producing Cultured Cells

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Background Type 1 diabetes results from lack of beta cells, while reduced beta cell mass may contribute to type 2 diabetes. A panel of circulating biomarkers has yet to be identified that is indicative of beta cell mass and function. We have recently developed methods enabling detection of mRNAs apparently secreted from cancer cells (1). Applying these techniques, this study aimed to establish if: (i) extracellular mRNAs are detectable in medium conditioned by beta cells; (ii) levels of such mRNAs are indicative of beta cell number and/or function.

Methods MIN-6(L) and MIN-6(H) (glucose-responsive and non-responsive, respectively, variants of MIN-6 murine beta cell line), MIN6-B1 (clonal population), and Vero cells engineered to produce human insulin (Vero-PPI) (2) were included in this study. Cells were seeded at a range of densities and were cultured for 48 h. Medium conditioned by the cells (conditioned medium; CM) was filtered and our protocol for RNA isolation was applied. RT-PCR was performed for 9 mRNAs.

Result Amplifiable extracellular mRNAs were detected in CM, suggesting that beta cells secrete gene transcripts. *Preproinsulin* mRNA was detected in Vero-PPI CM, the levels of which approximately correlated with the cell number conditioning the medium. Similarly, levels of *pdx1*, *npv*, *egr1*, *pld1*, *chgb*, *gad1*, *INSI*, and *beta-actin* reflected the density of MIN6-B1 cells conditioning medium. Although *Pax4* and *INSII* were amplified using MIN6-B1 cell RNA, these mRNAs were not detected in corresponding CM, suggesting a selective secretion mechanism. Higher levels of *egr1*, *pld1* and *chgb* mRNAs were found in CM from glucose-responsive MIN6 (L), compared to non-responsive MIN6 (H), cells.

Conclusion Results from this study suggest that mRNAs detected in CM may reflect beta cell numbers and function.

References

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Transcriptome Analysis of Antibody Producing NS0 Cell Line Cultivated at Sub-Physiological Temperatures

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Lowering culture temperature has been reported as a significant factor in improvement of mammalian cell productivity. To determine the physiological changes which take place at different temperature cultivations, an NS0 cell line producing human-mouse chimeric antibody was cultured at 22, 34 and 37 °C. Various cellular parameters such as viability, productivity, metabolism, apoptosis and cell cycle were studied and notable changes were shown to be accompanied by changes in metabolic rates. Reduction of the temperature to 22 °C resulted in a decrease in the growth rate, increase in viability, inhibition of antibody production, arrest of cell cycle in G2 phase and delay in apoptosis. Slight increase in antibody production was observed at 34 °C due to the increase of growth rate and prolonged stationary phase. To better understand and

explore the mechanisms underpinning the biological alterations and to identify the genes involved in the genetic reprogramming, genome-wide analyses were performed using GeneChip Mouse Genome arrays. The examination of differential gene expression induced by temperature reduction demonstrated a specific pattern of gene expression of NS0 cells in response to temperature stress. The alteration of transcription at different temperatures had a broad spectrum of functions with a wide range of regulated genes that were implicated in the central metabolic and signaling pathways. Most deregulated genes involved in stress (death receptor and endoplasmic reticulum stress pathway) and apoptosis response were induced in cells cultured at 22 °C, while genes involved in metabolic pathways (glycolysis/gluconeogenesis, pentose phosphate pathway and inositol metabolism) were mostly repressed at this temperature. By combining gene expression and physiological changes at different temperatures, it was possible to provide greater understanding of the cellular response to hypothermic conditions.