

# Uncoupling of Cell Growth and Division Results in Enhancement of Productivity in p21<sup>CIP1</sup>-arrested CHO Cells

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## Abstract

Chinese Hamster Ovary cells have been engineered to inducibly over-express the p21<sup>CIP1</sup> cyclin dependent kinase inhibitor, in order to achieve cell cycle arrest and increase cell productivity. In p21<sup>CIP1</sup>-arrested cells production of antibody from a stably integrated IgG4 gene, was enhanced approximately 4-fold. The underlying physiological basis for enhanced productivity was investigated by measuring a range of cellular and metabolic parameters. Interestingly the average size of arrested cells was approximately 1.6-fold greater than that of proliferating cells. This was accompanied by corresponding increases in mitochondrial mass (2-fold), mitochondrial activity (1.6-fold) and ribosomal protein S6 levels (2-fold). Our results suggest that cki-induced cell cycle arrest uncouples cell growth from cell cycle progression, and provides new insight into how improved productivity can be achieved in a cell line commonly used for large scale production of pharmaceutical proteins.

**Keywords:** *cyclin dependent kinase; cell cycle arrest; growth control; metabolic engineering*

## Introduction

Animal cell culture systems have been utilised for secreted pharmaceutical protein production, due to the requirement for post-translational protein modification. To obtain sufficient cells for production, initial high growth rates are essential. However, in certain cell culture processes uncontrolled proliferation beyond a certain desired cell density causes nutrient and oxygen depletion, accumulation of toxic metabolites, cell death and degradation of the product (Al-Rubeai and Singh 1998; Mitchell et al., 1991; Simpson et al., 1998; Zeng et al., 1998 and 1999). The production processes can be improved by regulation of proliferation to allow initial rapid cell growth to achieve a sufficiently high cell density for production, followed by a production period at reduced proliferation rates to prevent excess cell densities (Suzuki and Ollis, 1990; Al-Rubeai et al., 1992; Simpson et al., 1999; Mueller et al., 1999; Kaufmann et al., 2001). Metabolic or cell engineering, involving the introduction of regulatory genes into cells, has become the focus of attention for improving cell productivity (Kirchhoff et al., 1993; Fussenegger et al., 1998, 1999; Fussenegger 2001; Geserick et al., 2000; Carvalhal et al., 2001).

One of the most obvious targets for achieving proliferative control is the cyclin dependent kinases (cdks). This family of protein kinases are key components of the pathways that control cell cycle transitions (Hengst et al., 1994; Fishman et al., 1998; Sugimoto et al., 2002). Cdk activity is regulated by association with cyclin, phosphorylation and the formation of ternary complexes with cyclin dependent kinase inhibitors (ckis) such as p21<sup>CIP1</sup> and p27<sup>KIP1</sup> (Gutierrez et al., 2002; Drissi et al., 1999; Aktas et al., 1997; Bissonnette and Hunting, 1998). The cki p21<sup>CIP1</sup> is known to efficiently inhibit the activity of cdk-cyclin complexes, including cdk2 which has a direct role in the G1/S transition and over-expression of p21<sup>CIP1</sup> in various cell lines results in G1-phase cell cycle arrest (Gartel et al., 1996; Harper et al., 1993 and 1995; Xiong et al., 1993; Sekiguchi and Hunter, 1998; El-Deiry et al., 1993). Recently, it has been also shown that in addition to inducing G1-phase arrest, p21<sup>CIP1</sup> may also mediate G2-phase arrest (Niculescu et al., 1998; Dulic et al., 1998).

In several studies G1-phase arrest of the cell cycle caused by over-expression of p21<sup>CIP1</sup>, p27<sup>KIP1</sup> and the tumour suppresser gene p53 (which induces p21<sup>CIP1</sup>) has been shown to increase cell productivity (Kaufmann et al., 2001; Fussenegger 2001; Mazur et al., 1998; Watanabe et al., 2002; Ibarra et al., 2003). Although the concept of increased productivity arising from proliferative arrest is now well established, the physiological basis for this effect has yet to be investigated thoroughly. An understanding of the physiological basis for increased productivity could inform the design of new strategies to improving cell lines and production processes. In this present report we have used the LacSwitch p21<sup>CIP1</sup> inducible system to modify the CHO-22H11 cell line, which has previously been engineered to contain a stably integrated chimeric IgG4 gene (Bebington et al., 1992). The CHO cell line is one of the most widely used mammalian production cell lines, due to the high stability of chromosomally integrated heterologous transgenes (Schimke 1984). Stable clones of CHO-22H11 harbouring an inducible p21<sup>CIP1</sup> gene were successfully isolated. As predicted from previous studies p21<sup>CIP1</sup>-induced arrest was found to significantly enhance specific antibody production by up to 4-fold in these cells. In order to investigate the basis for this enhancement of antibody productivity a variety of cellular and metabolic parameters have been monitored in the arrested cells. The data suggest that in arrested cells growth is uncoupled from cell cycle

progression, resulting in increased cell size, mitochondrial mass and activity and ribosome biogenesis. Increased cellular productivity most likely arises from these effects on cell growth and metabolism.

## METHODS

### Cell culture and transfection

The parental cell line CHO-22H11, supplied by Lonza Biologics (Slough, UK), has been previously transfected with a human-mouse chimeric B72.3 IgG4 antibody gene, using the glutamine synthetase (GS) expression system (Bebbington et al., 1992). The cells were maintained in high glucose (17mM) DMEM NUT MIXF-12 without glutamine (GIBCO, Paisely, UK) supplemented with 10% foetal calf serum (PAA laboratories GmH), 100  $\mu$ M methylamine sulphoxamine (MSX; Sigma, Poole, UK).

The Lacswitch system was chosen to obtain inducible expression of p21<sup>CIP1</sup>. Briefly, this system involves introducing the gene of interest, under the control of hybrid RSV promoter containing *lac* repressor binding sites, into cells that are constitutively expressing the *lac* repressor protein. Firstly, *lac* repressor expressing cells were constructed using CMVlacI plasmid DNA (Stratagene). DNA was introduced into CHO-22H11 cells by liposome-mediated transfection using Lipofectin<sup>®</sup> reagent (Life technology, UK) according to manufacturer's instructions. Transfected cells were selected in medium containing 400 $\mu$ g/ml Hygromycin B (Sigma). After two weeks selection, the whole population of stable transfectants was then transfected with pOPRSV1/p21<sup>CIP1</sup> plasmid DNA using Lipofectin<sup>®</sup> reagent (Life technology, UK). Plasmid pOPRSV1/p21<sup>CIP1</sup>, which contains the mouse p21<sup>CIP1</sup> cDNA (Huppi et al., 1994; Erhardt and Pittman, 1998) was kindly provided by Dr. R. Pittman (University of Pennsylvania, Philadelphia, PA). Stable clones were selected in 400 $\mu$ g/ml Geneticin 418 (GIBCO), and isolated by limiting dilution. Induction of p21<sup>CIP1</sup> expression was achieved by adding 5mM isopropyl- $\beta$ -D-thiogalactoside (IPTG, GIBCO) into culture medium.

Cell concentration was estimated by haemocytometer counting, using trypan blue dye exclusion to distinguish viable cells.

### Western Blot Analysis

For Western blot analysis  $2 \times 10^5$  cells were lysed in SDS sample buffer, and proteins were separated by 12% SDS-PAGE, and then transferred to nitrocellulose membrane as described by Watanabe et al. (2002). Membranes were probed with either anti-p21<sup>CIP1</sup> antibody (1:1,000 dilution of mouse monoclonal antibody F5, (Santa Cruz Biotechnology Inc, UK) for p21 protein analysis or anti-ribosomal protein S6, (1:1000 dilution of rabbit polyclonal antibody, Santa

Cruz Biotechnology Inc, UK, sc-20085) for evaluating ribosomal protein in TBST with 4% dried skimmed milk. Blots were developed using horse radish peroxidase-conjugated second antibody (1:2000 dilution for p21<sup>CIP1</sup> and 1:2500 dilution for ribosomal protein S6) and ECL detection reagents (Amersham, UK), according to manufacturer's instruction.

### Determination of Monoclonal Antibody Concentration

The human-mouse B72.3 IgG4 chimeric MAb concentration was determined by enzyme-linked immunosorbent assay (ELISA). Chimeric MAb produced by cells was sandwiched by monoclonal anti-human IgG (Fc specific, Sigma) and peroxidase-conjugated anti-human kappa light chain antibody (Sigma). The amount of chimeric MAb was determined by measuring absorbance using o-phenyedianine dihydrochloride (OPD, Sigma) as substrate for peroxide.

### DNA content and cell size analysis

To assess the changes in cell cycle distribution cellular DNA content were analysed by propidium iodide staining and flow cytometry. Briefly, about  $1 \times 10^6$  cells from batch cultures were fixed with 70 % ice-cold ethanol and stored at  $-20^\circ\text{C}$  until use. To stain DNA, fixed cells were wash twice with 1xPBS buffer, resuspended in 100  $\mu$ l of 1x PBS containing 50  $\mu$ g/ml Ribonuclease A (Sigma), and incubated at  $37^\circ\text{C}$  for 60 minutes. Fixed cell were then stained with 300  $\mu$ l of 200  $\mu$ g/ml Propidium Iodide (PI, Sigma). Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, USA) and Modfit LT 2. software program was used for data analysis.

Cell diameter was measured using a Mastersizer S (Malvern Instrument Ltd, UK), with Malvern Mastersizer software version 2.18 to acquire the data.

### Measurement of mitochondrial mass and dehydrogenase activity

The mitochondria mass per cell was measured by flow cytometry using Mito Tracker Green FM (Green FM, Molecular Probes), as described by Metivier et al., 1998.  $1 \times 10^6$  viable cells were collected by trypsinization, suspended in 0.5 ml fresh medium with 150 nM Mito Tracker Green FM for 45 minutes in the dark. After two times of washes with PBS, cells were immediately analysed using a FACSCalibur flow cytometer (Becton Dickinson, USA) and green fluorescent intensity was detected. Approximately  $1.0 - 2.0 \times 10^4$  cells were analyzed at the rate of 200-600 cells/sec, and CellQuest3.3 software program was used for data analysis.

The mitochondrial dehydrogenase enzymes in living cells was determined by colorimetric measurement using substrate 3-(4, 5-dimethyliazol-2-yl)-2, 5-

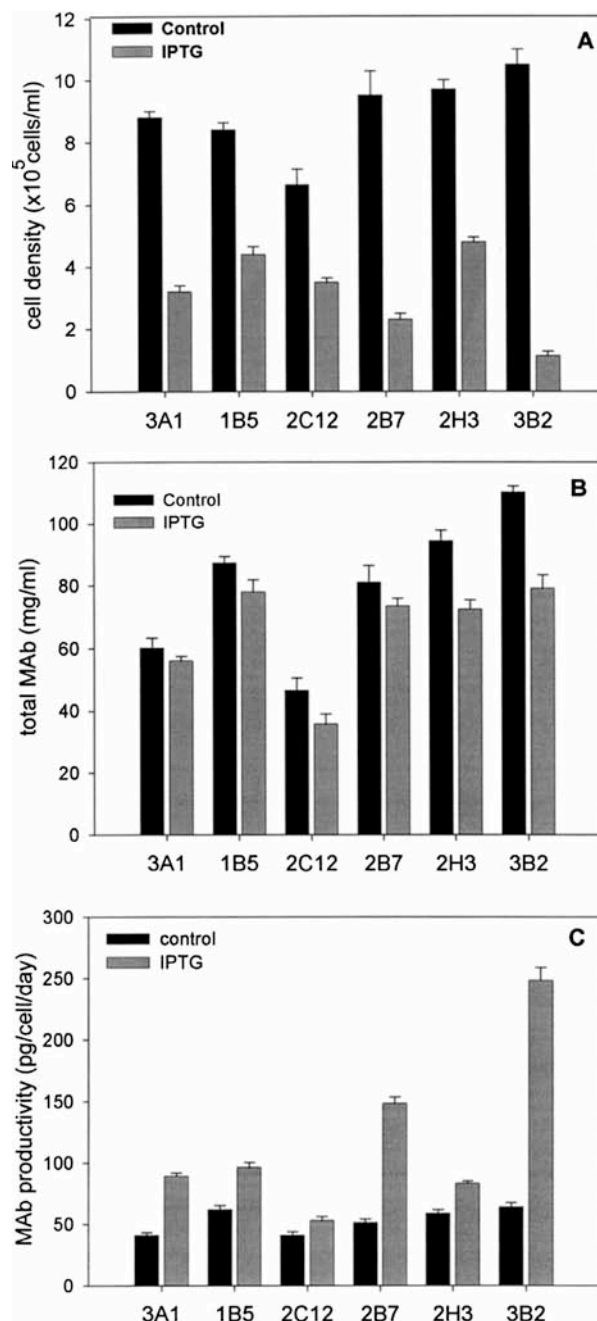
diphenyl tetrazolium bromide (MTT, Sigma) (Mosmann 1983). Briefly,  $2 \times 10^6$  living cells were harvested and resuspended in 1 ml medium plus 100  $\mu$ l 5 mg/ml MTT. The samples were incubated at 37 °C for 2 hr to allow the MTT to diffuse into and react with the cells. After centrifugation, supernatant was replaced by 1 ml iso-propanol and shaken at 1000 rpm for 20 minutes to release the formazan products. The absorbance of formazan product at 540 nm was measured using an SLT Spectra Elisa plate reader.

## RESULTS & DISCUSSION

### Establishment and characterization CHO cell lines that inducibly express the p21<sup>CIP1</sup> cki

To investigate the effects of p21<sup>CIP1</sup>-induced proliferation control on cell productivity, CHO-22H11 cells were first transfected with the plasmid CMV-LacI encoding *lac* repressor protein, and selected in medium containing 400 $\mu$ g/ml Hygromycin B. Cells expressing *lac* repressor protein were then transfected with the plasmid pOPRSV1/p21, which contains the mouse p21<sup>CIP1</sup> cDNA under the control of a hybrid RSV-LTR-*lac* promoter (Huppi et al., 1994; Erhardt and Pitmann, 1998). Cells were selected in Geneticin G418, and 20 clones displaying IPTG-inducible effects on proliferation were obtained (data not shown). Six of the clones showing the greatest effect (3A1, 1B5, 2C12, 2B7, 2H3 and 3B2) were chosen for further study.

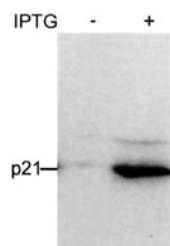
In order to characterise the effect of inducible p21<sup>CIP1</sup> over-expression on cell proliferation and antibody productivity, the six selected clones were incubated in the absence and presence of IPTG. Cells and supernatants were harvested at 96 hr, viable cells were counted and the amount of B72.3 IgG4 antibody secreted into the supernatant was assayed by ELISA. Fig.1 shows that in the absence of IPTG all tested clones had a similar basal proliferation rate except clone CHO-C12, which had a lower proliferation rate. Transfection and cloning have therefore not selected cells with grossly different proliferation rates. In the presence of IPTG the effect of p21<sup>CIP1</sup> over-expression on proliferation varied in each of the clones, clone CHO-3B2 with effectively no increase in cell numbers showed the most pronounced cessation of cell proliferation. The differential effect on cell proliferation is most likely due to variation in the level of p21<sup>CIP1</sup> over-expression in each clone. This has already been demonstrated in previous studies using NSO myeloma cells (Wanatabe et al., 2002), where it was clear that lower levels of exogenous p21<sup>CIP1</sup> expression failed to completely arrest cell cycle progression. Sufficiently high levels of p21<sup>CIP1</sup> need to be expressed in order to inhibit cdk activity and completely block cell cycle progress. Fig.1 also shows that although total yield of IgG4 antibody might be slightly higher in non-arrested cells, specific productivity in each of the cloned cell lines was enhanced by p21<sup>CIP1</sup> over-expression.



**Fig. 1.** The effect of p21<sup>CIP1</sup> expression on cell proliferation and cell productivity in different transfected CHO sub-clones. Cultures were set up at approximately  $1 \times 10^5$  cells /ml, and incubated in the absence (Control) or presence (IPTG) of 5mM IPTG. After 72 hours viable cells were counted (A) and supernatants were harvested and assayed for chimeric 72.3 IgG4 antibody (MAb) concentration (B). From these data cell productivity was calculated (C). Bars indicate standard deviation.

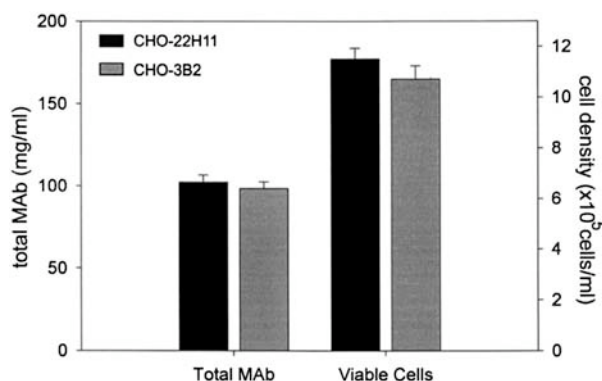
The scale of effect directly reflected the degree of cytostasis, and again clone CHO-3B2 shows the most significant effect with an increase in cell productivity of approximately 4-fold at 72hr. This compares closely with our previous findings in NSO cells, where p21<sup>CIP1</sup> over-expression caused effective proliferative arrest,

and a similar 4-fold increase in productivity (Wanatabe et al., 2002). Clone CHO-3B2 was selected for further study because in these cells p21<sup>CIP1</sup> expression had the most pronounced effect on proliferation and productivity.



**Fig. 2.** Western blot analysis of p21<sup>CIP1</sup> expression in CHO-3B2 cells. Equal numbers cells were analysed following 24 hr incubation in the absence and presence of 5 mM IPTG for 24 hr. Protein in cell lysates was analysed by immunoblotting with anti-p21<sup>CIP1</sup> monoclonal antibody.

In order to confirm that IPTG treatment is indeed affecting p21<sup>CIP1</sup> expression, CHO-3B2 cells were incubated in the absence and presence of IPTG for 24 hours, then analysed by western blotting. Fig.2, lane 1 shows that very little endogenous or exogenous p21<sup>CIP1</sup> protein can be detected in untreated, proliferating cells. The steady-state level of p21<sup>CIP1</sup> protein was comparable to that found in non-transfected CHO-22H11 cells (data not shown), confirming that the Lacswitch system is tightly regulating expression in these cells. Fig.2, lane 2 shows that 24 hours after addition of IPTG there is a considerable increase in the amount of p21<sup>CIP1</sup> protein present in cells, confirming that induction is rapid and effective. Steady-state levels of p21<sup>CIP1</sup> protein did not increase further at later time points (data not shown).



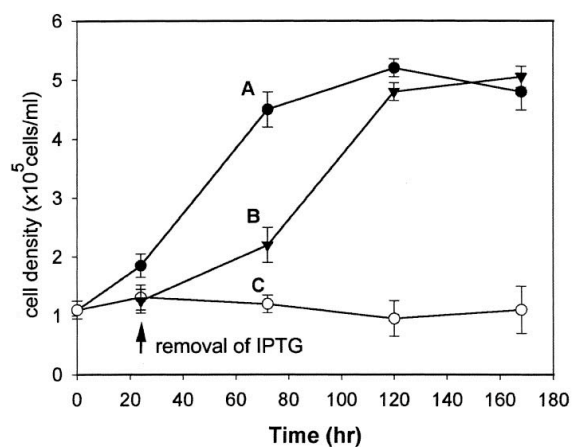
**Fig. 3.** Comparison of cell number and productivity in parental and transfected, cloned cells. Parental CHO-22H11 and transfected CHO-3B2 cells were incubated in the absence of IPTG for 96 hr, after which viable cells were counted and yield of IgG4 antibody was assayed.

To eliminate the possibility that any of the observed effects on proliferation and productivity have arisen through clonal selection, cell proliferation and productivity was assessed in the parental CHO22H11

cell line and the transfected CHO-3B2 cell line harbouring p21<sup>CIP1</sup>, in the absence of IPTG. Fig.3 confirms that after 96 hr of culture in the non-induced state, viable cell number and productivity were similar in both cell lines.

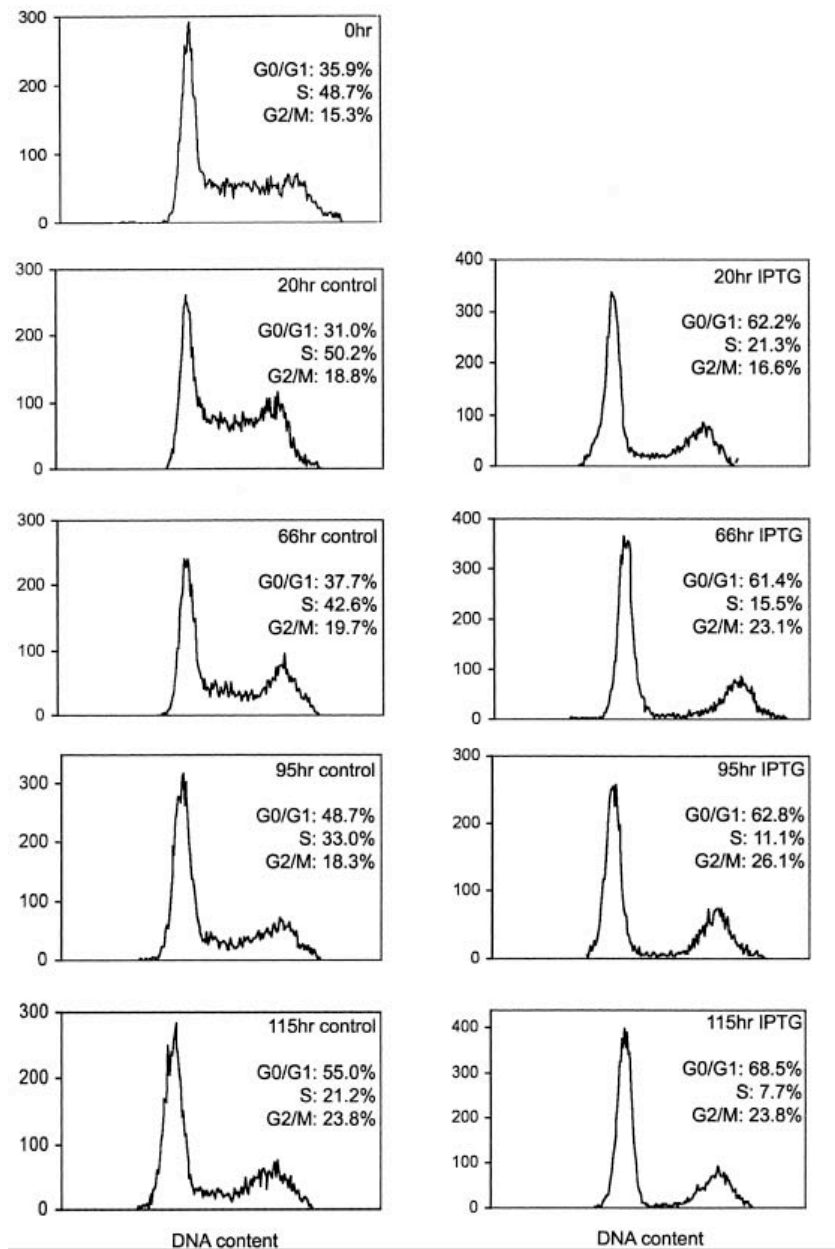
### p21<sup>CIP1</sup>-induced proliferative control is complete and reversible

The use of controlled proliferation technology requires an efficiently regulated gene expression system which allows expression of the cytostatic gene precisely at a desired cell culture state. In order to confirm that the Lacswitch system fulfils these requirements in CHO cells, the effectiveness and reversibility of the proliferative arrest caused by p21<sup>CIP1</sup> over-expression was tested in CHO-3B2.



**Fig. 4.** p21<sup>CIP1</sup>-induced proliferative arrest of CHO-3B2 cells is complete and fully reversible. Cultures were set up at approximately 1x10<sup>5</sup> cells /ml, and incubated for 168 hr in the absence (A) or presence (C) of 5 mM IPTG. IPTG was removed from some of the cells at 1 day, and incubation continued for a further 6 days in the absence of IPTG (B). Viable cells were counted at the intervals shown.

Cells were cultured for 168 hr in the absence or presence of IPTG, and counted at 48 hr intervals. Some of the cells were removed from IPTG after 24 hr and placed in IPTG-free medium then cultured for a further 144 hr. Fig.4 shows that in the presence of IPTG there is no increase in cell numbers, confirming that p21<sup>CIP1</sup> induces a complete and stable arrest of cell proliferation. In cells that were removed from IPTG after 24 hr cell proliferation resumed at a rate equivalent to non-induced cells within 48 hr, and the culture achieved the same final cell density as non-induced cells by 168 hr. The lag presumably reflects the time taken for p21<sup>CIP1</sup> to be degraded and removed from the cells, and for functional *lac* repressor to accumulate to a sufficient level to repress further p21<sup>CIP1</sup> expression. The effects of p21<sup>CIP1</sup> over-expression are therefore complete and fully reversible, confirming the physiological nature of the arrest that is achieved.



**Fig. 5.** The cell cycle distribution of  $p21^{CIP1}$ -arrested CHO cells. CHO-3B2 cells were incubated in the absence or presence of 5mM IPTG for 115 hours. At the intervals shown cells were removed and stained with propidium iodide, then analysed by flow cytometry. The profiles of DNA content for non-induced (control) and induced (IPTG) cells are shown, together with the cell cycle distribution, calculated using Modfit LT 2.0 software programme.

### Over-expression of $p21^{CIP1}$ causes cell cycle arrest in CHO-3B2 cells

Figs. 4 clearly shows that  $p21^{CIP1}$  over-expression inhibits proliferation of CHO-3B2 cells. In order to determine the effect of  $p21^{CIP1}$  over-expression on cell cycle progression, CHO-3B2 were analysed by propidium iodide (PI) staining and flow-cytometry, after incubation in the absence and presence of IPTG. Fig. 5 shows that after 20 hr in the absence of IPTG 31.0 % of cells were in G0/G1-, 50.2 % in S- and 18.8% in G2-phase. In the absence of IPTG there is a progressive decline in S-phase cells, as stationary

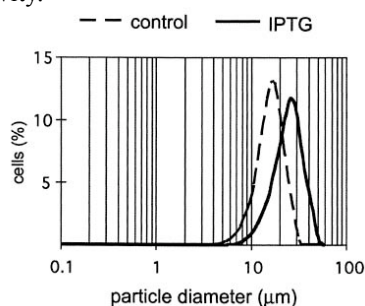
phase is established, around 115 hr. By comparison, after 20 hr in the presence of IPTG 62.2 % of CHO-

3B2 cells were found to be in G0/G1, 21.3% in S, 16.6% in G2/M. Over-expression of  $p21^{CIP1}$  therefore rapidly induces G0/G1-phase arrest in CHO-3B2 cells. In the continued presence of IPTG there is a further gradual decline in S-phase cells and increase in G2/M-phase cells, until at 115 hr 68.51% of CHO-3B2 cells were found to be in G0/G1, 7.68% in S, 23.80% in G2/M. These results indicate that a majority of the cells were rapidly blocked in G0/G1-phase after only 20 hr induction with IPTG, while a small percentage of cells appear to be blocked in G2/M-phase at later stages of culture.

## The effect of cell cycle arrest on cell size

Observation of p21<sup>CIP1</sup>-arrested cells revealed obvious morphological changes in the cells (data not shown). Following p21<sup>CIP1</sup> induction the arrested cells appeared to become larger, by comparison with proliferating cells. This observation was confirmed by measuring cell size in proliferating and arrested cultures. Fig. 6 shows that average cell size is significantly larger in arrested cells. After 72 hr of culture in the absence of IPTG average cell diameter was 17.6  $\mu$ m, compared to 27.8  $\mu$ m in the presence of IPTG. This corresponds to an increase in cell size of 1.6-fold.

These data therefore clearly show that CHO-3B2 cells arrested by p21<sup>CIP1</sup> over-expression continue to grow and increase in size, even though cell cycle progression and cell division are inhibited, at the G1/S and G2/M transition. The increase in average size is equivalent to slightly less than the increase required to support 2 cell doublings. By comparison, proliferating CHO-3B2 cells have undergone just over 2 cell doublings during a 72 hr period (data not shown). This uncoupling of cell growth from cell proliferation has obvious implications for understanding how the processes of cell growth and cell division are normally controlled, as well as how induced cytostasis might increase cell productivity.



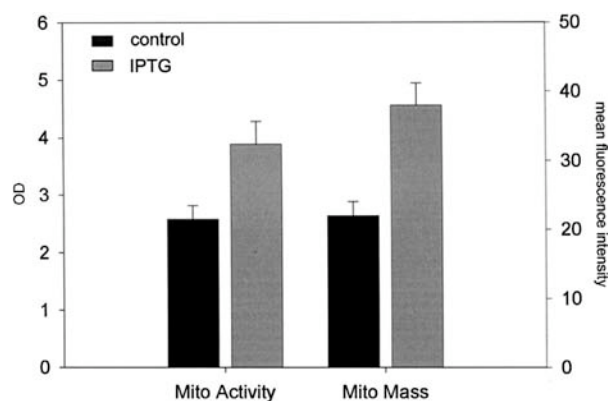
**Fig. 6.** The size of proliferating and arrested CHO-3B2 cells. Approximately  $4 \times 10^6$  cells were harvested from cultures that had been incubated for 72 hr in the absence (dash line) and presence (solid line) of 5mM IPTG, and cell size was measured by using a Malvern Mastersizer S. The mean diameter of non-induced and induced CHO-3B2 cells was  $17.6 \pm 2.3 \mu$ m,  $27.8 \pm 1.8 \mu$ m respectively.

The mechanisms that coordinate cell growth with proliferation remain poorly understood, despite the significant progress in identifying the molecular basis of cell cycle control. Recent studies have established a close link between growth factor signalling and cell growth (Tapon et al., 2001). Mitogenic and growth signalling pathways activate regulatory proteins such as Ras, phosphatidylinositol-3-OH kinase (PI3K) and c-myc, resulting in well established effects on the biosynthetic capabilities of cells.

Notably, ribosome biogenesis together with accumulation and activation of translation initiation factors upregulates rates of protein synthesis during G1-phase (Tapon et al., 2001; Thomas, 2002). Similarly, biogenesis of mitochondria is also tightly linked to cell cycle progress during G1-phase (Margeintu et al., 2002).

## Comparison of biosynthetic activities in proliferating and arrested cells

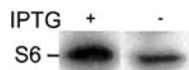
In order to investigate further the physiological mechanisms operating in p21<sup>CIP1</sup>-arrested cells, mitochondrial dehydrogenase enzyme activity and mitochondrial mass were investigated in proliferating and arrested CHO-3B2 cells. Mitochondrial activity was analyzed by a colorimetric MTT assay for mitochondrial dehydrogenase enzymes in living cells which were incubated in the absence and presence of IPTG for 48 hr.



**Fig. 7:** Mitochondrial mass and mitochondrial dehydrogenase activity in proliferating and arrested CHO-3B2 cells. Cells were incubated for 48 hr in the absence (black column) or presence (grey column) of 5mM IPTG. Mitochondrial mass was determined by staining cells with Mito Tracker Green, and flow cytometric analysis. Mitochondrial dehydrogenase activity was measured in  $1 \times 10^6$  cells, using a colorimetric assay based on MTT.

Fig.7 shows that mitochondrial dehydrogenase activity is increased by approximately 1.6-fold in IPTG treated, arrested cells compared to proliferating cells and this increase in activity was accompanied by a similar approximately 2-fold increase in mitochondrial mass IPTG induced, arrested cells, as assessed by Mito Tracker Green FM staining and flow cytometric analysis (Metivier et al., 1998). Together these data confirm that the increased size of arrested cells is accompanied by a comparable increase in mitochondrial activity, and that this is due to biogenesis of new mitochondria, not activation of existing organelles.

Finally, ribosome biogenesis was evaluated by assaying the amounts of ribosomal protein S6 in lysates from cells which were incubated in the presence and absence of IPTG for 48 hr using immunoblotting.



**Fig. 8.** Western blot analysis of ribosomal protein S6 levels in proliferating and arrested CHO-3B2 cells. Equal numbers cells were analysed following 48 hr incubation in the presence and absence of 5 mM IPTG. Protein in cell lysates was analysed by immunoblotting with anti-ribosomal protein S6.

Fig. 8 shows that there is about approximately a 2-fold increase in S6 protein levels in arrested cells. This result is consistent with increased ribosome biogenesis and fits with the increased mitochondrial mass shown by Fig. 7.

## CONCLUSION

This present study has confirmed that p21<sup>CIP1</sup> over-expression arrests CHO cells predominantly in G1-phase of the cell cycle, where growth and organelle biogenesis continue in the absence of cell cycle progression and cell division, consistent with previous studies. With the aims of the present study in mind, p21<sup>CIP1</sup>-arrested cells are metabolically more active, and will most certainly have a correspondingly increased capacity for protein synthesis, compared with smaller, proliferating cells. This therefore forms the physiological basis for enhanced productivity in arrested cells. At the start of the cell cycle, when cells are preparing for DNA replication and cell division, there is a requirement to replenish energy pools and metabolic precursors as well as increase cell mass (Ko and Prives, 1996; Linke et al., 1996). Consequently, by arresting cell cycle progress at the end of G1-phase, in the continued presence of growth signals, growth is allowed to continue and physiological activity is maintained. Furthermore, the extent of cell “overgrowth” observed in this study suggests that mammalian cells do not possess a mechanism to prevent continued growth in the absence of cell division. This reinforces the view that cell size is ultimately determined by mechanisms that trigger entry into mitosis and cytokinesis when an appropriate cell size has been achieved.

## REFERENCES

Aktas H, Cai H, Cooper, GM. 1997. Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27<sup>KIP1</sup>. *Mol Cell Biol* **17**: 3850-3857.

Al-Rubeai M, Emery AN. 1990. Mechanisms and kinetics of monoclonal antibody synthesis and

secretion in synchronous and asynchronous hybridoma cell cultures. *J Biotechnol* **16**: 67-85.

Al-Rubeai M, Chalder S, Bird R, Emery AN. 1991. Cell cycle, cell size and mitochondrial activity of hybridoma cells during batch cultivation. *Cytotechnology* **7**: 179-186.

Al-Rubeai M, Emery AN, Chalder S, Jan D. 1992. Specific monoclonal antibody productivity and the cell cycle-comparisons of batch, continuous and perfusion cultures. *Cytotechnolog* **9**: 85-97.

Al-Rubeai M, Singh RP. 1998. Apoptosis in cell culture, *Curr Opin Biotechnol* **9**:152-156.

Bebbington CR, Renner G, Thomson S, King D, Abrams D, Yarranton GT. 1992. High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. *Bio/Technology* **10**:169-175.

Bissonnette N, Hunting DJ. 1998. p21-induced cycle arrest in G1 protect cells from apoptosis induced by UV-irradiation of RNA polymerase II blockage. *Oncogene* **16**:3461-3469.

Carvalho AV, Moreira JL, Carrondo MJ. 2001. Strategies to modulate BHK cell proliferation by the regulation of IRF-1 expression. *J Biotechnol* **92**: 47-59.

Drissi H, Hushka D, Aslam F, Nguyen Q, Buffone E, Koff A, van Wijnen A, Lian JB, Stein JL, Stein GS. 1999. The cell cycle regulator p27<sup>KIP1</sup> contributes to growth and differentiation of osteoblasts. *Cancer Res* **59**: 3705-3711.

Dulic V, Stein GH, Fa, DF, Reed SI. 1998. Nuclear accumulation of p21Cip1 at the onset of mitosis: a role at the G2/M-phase transition. *Mol Cell Biol* **18**:546-557.

El-Deiry W, Tokino T, Velculescu V, Levy D, Parsons R, Trent J, Lin D, Mercer W, Kinzler K, Vogelstein B. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**:817-825.

Erhardt JA, Pittman RN. 1998. p21WAF1 induces permanent growth arrest and enhances differentiation, but does not alter apoptosis in PC12 cells. *Oncogene* **16**:443-451.

Fishman DD, Segal S, Livneh E. 1998. The role of protein kinase C in G1 and G2/M phases of the cell cycle (review). *Int J Oncol* **12**:181-186.

Fussenegger M, Schlatter S, Datwyler D, Mazur X, Bailey JE. 1998. Controlled proliferation by multigene metabolic engineering enhances the

- productivity of Chinese hamster ovary cells. *Nat Biotechnol* **16**:468-472.
- Fussenegger M, Baily J E, Hauser H, Mueller P. 1999. Genetic optimization of recombinant glycoprotein production by mammalian cells. *TIBTECH* **17**:35-42.
- Fussenegger M. 2001. The impact of mammalian gene regulation concepts on functional genomic research, metabolic engineering, and advanced gene therapies. *Biotechnol Prog* **17**:1-51.
- Gartel AL, Serfas MS, Tynerm AL. 1996. p21-negative regulator of the cell cycle. *Proc Soc Exp Bio Med* **213**:138-149.
- Geserick C, Bonarius HP, Kongerslev L, Hauser H, Mueller PP. 2000. Enhanced productivity during controlled proliferation of BHK cells in continuously perfused bioreactors. *Biotechnol Bioeng* **69**:266-274.
- Gutierrez C, Ramirez-Parra E, Castellano MM, del Pozo JC. 2002. G (1) to S transition: more than a cell cycle engine switch. *Curr Opin Plant Biol* **5**:480-486.
- Harper JW, Adami G, Wei N, Keyomarsi K, Elledge S. 1993. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**:805-816.
- Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai L, Zhang P, Dobtowski S, Bai C, Connell-Crowley L, Swindell E, Fox PMB, Wei N. 1995. Inhibition of cyclin dependent kinases by p21. *Mol Biol Cell* **6**:387-400.
- Hengst L, Dulic V, Slingerland JM, Lees E, Reed SI. 1994. A cell cycle-regulated inhibitor of cyclin-dependent kinases. *Proc Natl Acad Sci USA* **91**:5291-5295.
- Huppi K, Siwarski D, Dosik J, Michieli P, Chedid M, Reed S, Mock B, Givol D, Mushinski JF. 1994. Molecular cloning, sequencing, chromosomal localization and expression of mouse p21 (Waf1). *Oncogene* **9**:3017-3020.
- Ibarra N, Watanabe S, Bi J-X, Shuttleworth J, Al-Rubeai M. 2003. Modulation of cell cycle for enhancement of antibody productivity in perfusion culture of NS0 cells. *Biotech Prog* **19** ( in press)
- Kaufmann H, Mazur X, Marone R, Bailey JE, Fussenegger M. 2001. Comparative analysis of two controlled proliferation strategies regarding product quality, influence on tetracycline-regulated gene expression, and productivity. *Biotechnol Bioeng* **72**:592-602.
- Kirchhoff S, Schaper F, Hauser H. 1993. Interferon regulatory factor 1 (IRF-1) mediate cell growth inhibition by transactivation of downstream target genes. *Nucleic Acid Res* **21**:2881-2889.
- Ko LJ, Prives C. 1996. p53: puzzle and paradigm. *Gene Dev* **10**:1054-1072.
- Linke SP, Clarkin KC, Leonardo AD, Tsou A, Wahl GM. 1996. A reversible, p53-dependent G0/G1 cell-cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. *Gene Dev*:**10**, 934-947.
- Lloyd DR, Holmes P, Jackson LP, Emery AN, Al-Rubeai M. 2000. Relationship between cell size, cell cycle and specific recombinant protein productivity. *Cytotechnology* **34**:59-70.
- Margineantu DH, Cox WG, Sundell L, Sherwood SW, Beecham JM, Capaldi R. A. 2002. Cell cycle dependent morphology changes and associated mitochondrial DNA redistribution in mitochondria of human cell lines. *Mitochondrion* **1**:425-435
- Mazur X, Fussenegger M, Renner WA, Bailey JE. 1998. Higher productivity of growth-arrested Chinese hamster ovary cells expressing the cyclin-dependent kinase inhibitor p27. *Biotechnol Prog* **14**:705-713.
- Metivier D, Dallaporta B, Zamzami N, Larochette N, Susin SA, Marzo I, Kroemer G. (1998) Cytofluorometric detection of mitochondrial alterations in early CD95/Fas/APO-1-triggered apoptosis of Jurkat T lymphoma cells. Comparison of seven mitochondrion-specific fluorochromes. *Immunol Lett* **61**:157-163
- Mitchell CA, Beall JA, Wells JRE, Gray PP. 1991. Growth and protein production kinetics of a murine myeloma cell line transfected with the human growth hormone gene. *Cytotechnology* **5**:223-231.
- Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**:55-63.
- Mueller PP, Schlenke P, Nimtz M, Conradt HS, Hauser H. 1999. Recombinant glycoprotein product quality in proliferation-controlled BHK-21 cells. *Biotechnol Bioeng* **65**:529-536.
- Niculescu AB, Chen X, Smeets M, Hengst L, Prives C, Reed SI. 1998. Effects of p21 (Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. *Mol Cell Biol* **18**:629-643.
- Schimke RT. 1984. Gene amplification in cultured animal cells. *Cell* **37**:705-713.

Sekiguchi T, Hunter T. 1998. Induction of growth arrest and cell death by overexpression of the cyclin-CDK inhibitor p21 in BHK cells. *Oncogene* **16**:369-380.

Simpson NH, Singh RP, Perani A, Goldenzon C, Al-Rubeai M. 1998. In hybridoma cultures, deprivation of any single amino acid leads to apoptotic death, which is suppressed by the expression of the bcl-2 gene. *Biotechnol Bioeng* **59**:90-98.

Simpson NH, Singh RP, Emery AN, Al-Rubeai M. 1999. Bcl-2 over-expression reduces growth rate and prolongs G1 phase in continuous chemostat cultures of hybridoma cells. *Biotechnol Bioeng* **64**:174-186.

Sugimoto M, Martin N, Wilks DP, Tamai K, Huot TJ, Pantoja C, Okumura K, Serrano M, Hara E. 2002. Activation of cyclin D1-kinase in murine fibroblasts lacking both p21 (Cip1) and p27 (Kip1). *Oncogene* **21**:8067-8074.

Suzuki E, Ollis DF. 1990. Enhanced antibody production at slowed growth rates: Experimental demonstration and a simple structured model. *Biotechnol Prog* **6**:231-236.

Tapon N, Moberg KH, Hariharan IS. 2001. The coupling of cell growth to the cell cycle. *Curr Opin Cell Biol* **13**:731-737.

Thomas G. 2000. An encore for ribosome biogenesis in the control of cell proliferation. *Nat Cell Biol* **2**:E71-72.

Watanabe S, Shuttleworth J, Al-Rubeai M. 2002. Regulation of cell cycle and productivity in NS0 cells by the over-expression of p21<sup>CIP1</sup>. *Biotechnol Bioeng* **77**:1-7.

Xiong Y, Hannon G, Zhang H, Casso D, Kobayashi R, Beach D. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* **366**:701-704.

Zeng AP, Deckwer WD, Hu WS. 1998. Determinants and rate laws of growth and death of hybridoma cells in continuous culture. *Biotechnol Bioeng* **57**:642-654.

Zeng AP, Deckwer WD. 1999. Model simulation and analysis of perfusion culture of mammalian cells at high cell density. *Biotechnol Prog* **15**:373-382.