Stable transfection of CHO cells with the *c-myc* gene results in increased proliferation rates, reduces serum dependency, and induces anchorage independence

Vasiliki Ifandi and Mohamed Al-Rubeai*

Animal Cell Technology Group, Department of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2 TT, UK; *Author for correspondence (e-mail: m.al-rubeai@bham.ac.uk; phone: +44-121-414-3888)

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Abstract

Induction of the transcription factor Myc promotes cell proliferation and transformation by activating growth-promoting genes and/or by transcriptionally repressing the expression of growth arrest genes. However, a number of studies have shown that c-Myc is a potent inducer of apoptosis in the absence of serum or growth factors. To further examine the role of Myc in cell growth and proliferation, and the advantages of this positive regulator in cell culture we transfected the CHO-K1 cell line with a human *c-myc* gene driven by MMLV 5'-LTR promoter. Over-expression of ectopic c-Myc resulted in a significant increase in growth rate and maximum cell number, in both suspension and attached batch culture accompanied by a similar decrease in specific glucose consumption rate. Interestingly, there was no manifestation of the widely reported apoptotic death by *c-myc* in the absence of serum. Additionally, over-expression of c-Myc appeared to induce morphological transformation and partial anchorage-independence.

Introduction

The *c-myc* gene was first identified as the cellular counterpart of the transforming gene of avian myelocytomatosis virus MC29, and like many proto-oncogenes is found conserved in evolution (reviewed in Cole 1986). It encodes a short-lived nuclear phosphoprotein with sequence specific DNA binding activity. Several studies indicate that c-Myc may act as a transcription factor, and is the main member of a family of proteins which also includes N-Myc, L-Myc, S-Myc and B-Myc. c-Myc is organised in three parts, and the important regions for proliferation, apoptosis and transcriptional activity are present in its terminal domains (reviewed in Spencer and Groudine 1991; Marcu et al. 1992; Meichle et al. 1992; Bouchard et al. 1998). The c-Myc association with Max and the c-Myc binding to DNA are essential for transcriptional activation of target genes as well as its ability to

drive proliferation, malignant cell transformation and apoptosis (Amati et al. 1993).

c-Myc is a member of a set of cellular messengers commonly known as 'immediate early response' genes, because their expression is activated by a variety of mitogenic stimuli independent of *de novo* protein synthesis, early during the Go to G1 transition of cells during the cell cycle (reviewed in Cole 1986). It has been suggested that it plays an important role in the regulation of both entry in the cell cycle and maintenance of cell proliferation because c-Myc expression is maintained throughout the cell cycle. In particular, deregulated c-Myc expression blocks exit from the cell cycle, and ectopic activation of c-Myc is sufficient to drive Go cells into cycle and keep them there (Marcu et al. 1992; Evan et al. 1992, 1996; reviewed in Facchini and Penn 1998).

Although c-Myc appears to play an important role in cell cycle progression, it is also sufficient and

necessary for the induction of apoptosis under certain conditions such as growth limitations (e.g. serum deprivation), transcription and translation inhibitors, hypoxia, glucose deprivation, heat shock, and DNA damage (Evan et al. 1992; Bissonnette et al. 1992; Fanidi et al. 1992; Wahgner et al. 1993). It has been suggested that cell proliferation and apoptosis are coupled, and when *c-myc* is expressed both the proliferation and the apoptotic pathways are induced. Therefore, successful proliferation of cells requires two independent signals: one to activate mitogenesis and another to suppress apoptosis. In serum deprived cells it is thought that *c-Myc* induces apoptosis due to the growth suppressive effects or lack of specific cytokines that are present in the serum, and activate mutagenesis (reviewed in Prendergast 1999).

In the area of mammalian cell engineering, great research interest has been directed towards the development of apoptosis and proliferation controlled cell lines (reviewed in Al-Rubeai 1998; Fussenegger and Bailey 1998). High cell density, apoptosis resistance, controlled proliferation and easy adaptation into suspension culture of serum free medium are desirable characteristics for the cost effective production of biopharmaceuticals, mainly because genetically modified cell lines can afford greater efficiency and control. *c-myc* is a prime candidate from a number of genes that regulate cell proliferation in such a manner as to consider the advantages of its introduction in cell lines. Recently, Sanfeliou and Stephanopoulos (1999) found an increase in *c-Myc* level after insulin stimula-

tion in relation to arrested CHO cells due to glutamine depletion. Therefore, to further study the role of *c-Myc* on cell growth and proliferation in cell culture processes we examined the effect of over-expression of *c-myc* in Chinese hamster ovary cells (CHO-K1). This robust cell line and its derivatives have been widely used for the production of recombinant proteins. The wild type CHO-K1 is an adherent cell line and our data suggest that *c-Myc* over-expression not only enhances growth rate but also adds further transforming characteristics altering the attachment and detachment rates and serum dependency.

Material and methods

c-myc plasmids and cell lines

The plasmids used for the transfection of CHO cells were pDORclG123 (*c-Myc* plasmid) and pDOR cla-Neo (neomycin plasmid) (Figure 1) (kindly donated by Dr T. Littlewood, Imperial Cancer Research Fund, London, UK).

The pDORclG123 and pDOR cla- neo plasmids were amplified in *E. coli* DH5 α and isolated using a QIAGEN[®] plasmid preparation kit and CHO-K1 cells (ECCAC, Porton, UK) were transfected with the plasmids using the calcium mediated transfection of adherent cells in suspension. Briefly, the calcium phosphate –DNA solution was prepared at a ratio of 2.2:2.5:0.3 of DNA solution (plasmid DNA was

Figure 1. The pDORG123 vector and the position of *c-myc* on the vector; LTR, Moloney murine leukaemia virus long terminal repeat; SV40 Simian Virus 40 promoter; pBR Ori, pBR322 origin of replication; *neo^R*, neomycin acetyltransferase resistance gene; *amp^R*, ampicillin resistance gene. Map is not to scale.

dissolved in $0.1 \times \text{TE pH } 8.0$ $1 \text{ Mm Tris-Cl pH } 8.0$, $0.1 \text{ mM EDTA pH } 8.0$ at a concentration of $40 \mu\text{g/ml}$., $2 \times \text{HBS}$ (280 Mm NaCl , 10 mM KCl , $1.5 \text{ mM Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 12 mM dextrose , 50 mM Hepes) and 2 M CaCl_2 respectively. The mixture was then incubated for 30 min and while a precipitate was forming, exponentially growing CHO cells were harvested into aliquots of 10^6 cells. Each aliquot was resuspended in 0.5 ml of the calcium phosphate DNA solution and incubated for 15 min at room temperature followed by the addition of Ham's F12 supplemented with 5% foetal calf serum (FCS; Gibco, Paisley, UK) into the cell suspension. The cultures were incubated for 48 h at 37°C in 5–7% CO_2 before drug selection with 1 mg/ml Geneticin G418 (Gibco, Paisley, UK). The surviving cmc-cho and neo-cho cells were pooled separately and were maintained in Ham's F12 supplemented with 5% FCS. Using dilution cloning, stable cmc-cho and neo-cho clones were selected and used in subsequent experiments. Selection pressure was maintained by incubation with 1 mg/ml Geneticin G418 (Gibco, Paisley, UK) at regular intervals and cell samples were taken before and after incubation to ensure stable over-expression of the c-Myc protein.

Indirect immunofluorescence using flow cytometry

10^6 cells were fixed prior to immunostaining with $1 \text{ ml } 1\%$ paraformaldehyde at 4°C and were washed with PBS twice and then incubated with $100 \mu\text{l}$ of an anti-Myc monoclonal antibody (9E10) for 1 h at room temperature. After incubation the cells were washed with PBS and then incubated for a further 1 h at room temperature in the dark in the presence of $100 \mu\text{l}$ of an anti-mouse FITC antibody (Sigma, UK). Cells were then washed in 1 ml of PBS and were ready for analysis. Data were acquired using a Coulter EPICS Elite Analyser equipped with an argon ion laser set at 488 nm .

c-Myc immunoprecipitation and western blot analysis

For the combined immunoprecipitation/western blot 1×10^6 cells from both the control (neo-cho) and the c-myc (cmc-cho) cell lines were lysed and an anti-myc rabbit serum (a murine c-Myc specific rabbit polyclonal immunoglobulin G (IgG)) was added and the mixture was incubated for 1 h. After incubation $100 \mu\text{l}$ of protein A sepharose 4B were added to

capture the c-Myc-antibody complexes, and the proteins were released from the beads by boiling the samples in loading buffer ($20 \text{ mM Tris-Cl pH } 7.5$, 50 mM NaCl , 2% bromophenol blue and 4% 2-mercaptoethanol 10% glycerol –Sigma, UK) and loaded on a SDS-10% polyacrylamide gel. After electrophoresis the proteins were transferred on nitrocellulose membranes and the bands were revealed by an anti-Myc monoclonal antibody (9E10 kindly provided by Dr T. Littlewood, Imperial Cancer Research Fund, London, UK) and a horseradish-peroxidase-conjugated sheep anti-mouse IgG (Amersham, UK) as the secondary antibody. The reaction was developed by chemiluminescence ECL detection (Amersham, UK).

Static and suspension batch and fed batch cultures of cmc-cho and neo-cho

Static cultures from each cell line were plated in 25 cm^2 T-flasks with a density of $2 \times 10^5/\text{ml}$ allowing duplicate flasks to be sacrificed at regular intervals for each cell line, in Ham's F12 in both the presence and absence of 5% FCS (Gibco, Paisley, UK). To ensure that the cells used in the cultures grown in the absence of FCS were FCS free, the inoculum was washed twice with Ham's F12 prior to plating. Cell viability was determined by taking frequent samples; floating cells were collected by centrifugation of the aspirated medium, and adherent cells were trypsinised and collected by centrifugation. Numbers of viable and non-viable cells were determined by trypan-blue exclusion counts of the pooled cell samples. Apoptosis and necrosis were scored using propidium iodide and acridine orange staining and fluorescent microscopy (Simpson et al. 1997), while glucose concentration was measured (in duplicate) using the REFLOLUX 2 (Boehringer-Mannheim) kit. Similarly, fed batch cultures were established for each cell line, in duplicate; on day 3 and every 2 days after initial feeding 50% of the medium was changed, with cell and media samples taken as before.

For the suspension cultures stock cultures of cmc-cho and control cell line were adapted in a 100 ml spinner flask (Bellco) at an initial concentration of $2 \times 10^5/\text{ml}$. The time required to obtain a single cell suspension for the cmc-cho and neo-cho cell lines was 3 and 6 wk respectively. Following adaptation to a single cell suspension the cells were harvested at mid-exponential phase by centrifugation and used to inoculate a 100 ml culture in spinner flasks (Bellco) in duplicate using Ham's F12 supplemented with 5%

FCS (Gibco, Paisley, UK). Samples of cells and media were taken as before for cell viability studies and to determine the glucose concentration.

Cell attachment and detachment rates

Duplicate cultures from each cell line were plated on 9 mm diameter 6-well plates with a density of 8×10^4 /ml in Ham's F12 in both the presence and absence of 5% FCS (Gibco, Paisley, UK). For the attachment study, cells were added into a culture dish and media samples were removed at regular intervals to determine the number of unattached cells by trypan-blue exclusion counts until no cells were recovered from the samples. For the detachment study, cells were added into a culture dish and allowed to attach for 4 h; following attachment of the plated cells, trypsin-EDTA (Sigma, UK) at a $\times 10$ concentration of was used to detach the cells from the substratum. Cell samples were removed at regular intervals to determine the time required for the cells to detach.

Results

Over-expression of c-Myc in CHO-K1 cells

To assay the effect of expression of c-Myc we used the pDORclaG123 plasmid (Figure 1) to transfect CHO-K1 so as to generate cells over-expressing c-Myc. Geneticin resistant colonies were pooled together and limiting dilution cloning was used to establish cmyc-cho clones that over-express c-Myc. Evidence of the over-expression of c-Myc in cmyc-cho cells was obtained using indirect immunofluorescence and flow cytometry (Figure 2). This method clearly showed that cmyc-cho cells over-express exogenous c-Myc 30 times more than the control cell line. Initial experiments showed that the selected cmyc-cho clones were not exhibiting any significant differences between them regarding c-Myc over-expression and further studies also indicated that they followed similar growth and proliferation patterns (data not shown); for the purposes of this study one clone was chosen randomly for subsequent experiments.

In addition, the combination of the immunoprecipitation/western blot analysis of the transfected cells confirmed that over-expression of the c-Myc was constant and stable during a batch culture with sam-

Figure 2. Indirect immunofluorescence analysis using flow cytometry of c-Myc expression in the *neo* (control) and *c-myc* transfected cell lines. Dead cells and debris were removed from the analysis of the total population in a forward scatter against side scatter plot. The x-axis represents the c-Myc-FITC fluorescence.

ples taken randomly at different days of the culture. (Figure 3) c-Myc expression in neo-cho cells was below the limits of detection for this particular method.

Effect of c-Myc over-expression on cell growth and death rate

Static batch and fed batch cultures

Figure 4a shows the effect of c-Myc over-expression on viable cell number and viability in a static culture in the presence of serum. Maximum cell number was 1.7×10^6 /ml in the cmyc-cho cultures with the neo-cho control cultures reaching 1.1×10^6 /ml. Viability on the other hand remained above 80% for both the cmyc-cho and the neo-cho cultures over the first 6

Figure 3. Western blot analysis of c-Myc over-expression in cmyc-cho cell line. Samples were taken randomly at different days of the batch culture; day 0 indicates over-expression at the time of inoculation and days 1, 3, 6, 7, and 9, indicate over-expression of random samples taken at various points during the batch culture. The results show that c-Myc over-expression remains constant throughout the course of the batch culture.

days. In the absence of serum (Figure 4b), the effect of c-Myc on cell proliferation was more pronounced with cmyc-cho cultures reaching maximum cell number of $5.1 \times 10^5/\text{ml}$ whilst the control cultures reached only $2.2 \times 10^5/\text{ml}$, which corresponds to a 130% increase in cell number. Moreover, the difference in viability between cmyc-cho and neo-cho cultures was higher in the absence than in the presence of serum, with the neo-cho cell viability declining, approximately, 20% faster than the cmyc-cho cells.

The pattern of cell death was established using fluorescence microscopy. Figure 5a shows the percentage of apoptotic death in the cultures of cmyc-cho and neo-cho in the presence of serum. Apoptosis in both cultures increased with time at a constant rate and remained very low even on day 9 when the viability had decreased below 50% indicating the transient nature of the process with progression from viable to apoptosis and finally to secondary necrosis.

In the absence of serum (Figure 5b), the difference in the percentage of apoptosis between cmyc-cho and

Figure 4. Static batch cultures of cmyc-cho and neo-cho cells in a) presence and b) absence of serum.

Figure 5. Apoptosis in static batch cultures of cmyc-cho and neo-cho cells in a) presence and b) absence of serum.

neo-cho cells is apparent from day 3 of the culture, with cmyc-cho showing relatively higher apoptotic rates than neo-cho. Necrosis was apparently the main morphological form of cell death in all cultures studied. However, in the absence of serum necrosis in the cmyc-cho cultures was less predominant than in the other cultures and DNA gel electrophoresis carried out on samples taken during the duration of the cultures (data not shown) revealed a clear ladder-pattern which confirmed that apoptosis was transient but nevertheless a significant mechanism of cell death, i.e. cells died by apoptosis but they became necrotic within a short period of time.

A more apparent difference in cell number between c-Myc transfectants and control cells is shown in fed-batch cultures (fed with Ham's F12, 5% FCS) with cmyc-cho reaching maximum cell number of $2.4 \times 10^6/\text{ml}$ on day 11 of the culture whilst the control

Figure 6. Static fed cultures of cmyc-cho and neo-cho cells in the presence of serum.

only reached a maximum of 9.31×10^5 /ml on day 7 (Figure 6). Average viability for cmyc-cho cultures remained high up until day 11, and as cell number started to decline viability decreased but at a lower rate than that in the neo-cho cultures. The difference was most apparent on day 13 when viability of cmyc-cho and neo-cho was approximately 90% and 60% respectively. Apoptosis in both cultures, as measured by fluorescence microscopy, was also at similar low levels with cmyc-cho continuing showing slightly higher levels than neo-cho (Figure 7).

During batch and fed-batch cultures the glucose consumption rate for cmyc-cho was much lower than that of the neo-cho cells with a significantly higher glucose consumption rate in the control cultures than that in cmyc-cho cultures (data not shown).

Figure 7. Apoptosis in static fed culture of cmyc-cho and neo-cho cells in the presence of serum.

Figure 8. Suspension batch culture the presence of serum.

Suspension batch cultures

Prior to adaptation to suspension, it was observed that in the absence of serum, neither the control nor cmyc-cho cells could survive for prolonged period in contrast to the results obtained in static cultures (Figure 4a and b). It was observed that cmyc-cho adapted to grow in suspension cultures faster than the control cells. In fact, the time required to obtain a neo-cho single cell suspension culture was double the time required by cmyc-cho (data not shown).

In batch suspension cultures with serum supplementation both cmyc-cho and control cells reached on day 10 the maximum cell number of 6.1×10^5 /ml and 4.9×10^5 /ml respectively with viability following the same pattern as that seen in static cultures (Figure 8).

Effect of c-myc over-expression on morphology and anchorage dependance

Effect on morphology

An example of effect of c-Myc over-expression on the cell morphology is shown in Figure 9a and b. CHO-K1 cells that over-express c-Myc were observed to form small colonies or aggregates that form clusters (focci) of viable cells (Figure 9a); a characteristic loss of growth regulation by fully transformed cell lines. In addition to these colonies a number of cells also appear rounded in contrast to the flat appearance of neo-cho cells (Figure 9b); those clusters and rounded cells when trypsinised and re-seeded can easily attach and proliferate as viable cells.

Effect on anchorage dependency

The effect of c-Myc over-expression on attachment

Figure 9. Photographs of cmyp-cho (a) and neo-cho (b) cells ($\times 100$ magnification).

rates is shown in Figure 10a and b. In both conditions of presence and absence of serum the control cell line required less time to attach to the substratum surface as a monolayer than the cmyp-cho cell line. In the presence of serum all of the control cells attached within 90 min, while cmyp-cho remained at 85% even

after 200 min from inoculation. In the absence of serum cmyp-cho cells could only fully attach 90 min after the time for full attachment of the control cells (120 min). On the other hand, when the rate of detachment was examined it was observed that cmyp-cho detached at much faster rates than the control cell

Figure 10. Attachment rates of cmyc-cho and neo-cho cells in a) presence and b) absence of serum.

line (Figure 11a and b) in both conditions. The time required for the cmyc-cho cells to detach was 50% and 30% less than the time required for neo-cho cells in the presence of serum and absence of serum, respectively.

Discussion

Cell proliferation is a process that is highly regulated by intracellular factors and depends on appropriate and specific extracellular signals. A number of studies have shown that *c-myc* is such an intracellular factor that affects cell proliferation in normal, non tumorigenic cell lines, with unregulated expression of the protein resulting in tumorigenic phenotypes (reviewed in Hoffman and Liebermann 1998). Furthermore, several cell systems have been developed where c-Myc is over-expressed either by transfection,

Figure 11. Detachment rates of cmyc-cho and neo-cho cells in a) presence and b) absence of serum.

infection or microinjection that affected proliferation rates. However, evidence up to date suggested that *c-myc* although itself an important regulator of cell proliferation is still regulated by extracellular signals in normal cells (Hoffman and Liebermann 1998; Prendergast 1999).

Our results show that constitutive over-expression of c-Myc results in an increase of proliferation rate with an additional, but relative, independence to external factors affecting and regulating proliferation, such as cell to cell contact and growth factors present in serum. Table 1, shows a summary of the results in static cultures; cmc-cho cells reached higher cell numbers (Figure 4) and exhibited higher growth rates with less glucose utilisation when compared to the control cell line, even under conditions of absent growth factors (serum). Although the cell numbers for the cmc-cho cultures were significantly higher in the

presence of serum, the positive effect of c-Myc on cell proliferation was more obvious in the cultures grown in the absence of serum. Indeed, it can be suggested that, although the cmc-cho proliferation rate is governed and affected by the same factors as the control cell line, over-expression of the c-Myc protein has provided the cell line with a specific advantage under growth-limiting conditions. However, growth of the cell line was not totally independent of cell-cell contact and growth factors present in serum as observed in static batch and fed batch cultures. In fact, the static fed batch results show that feeding affects the cell number and the duration of the culture as it would have been expected; but again, c-Myc over-expression had a positive effect on proliferation rate, which was further amplified by the addition of nutrients and growth factors. Interestingly, feeding had no significant effect on neo-cho cultures.

The effect of c-Myc on cell growth rate can be considered as both synergistic and antagonistic, as it is widely accepted and shown in numerous studies. Indeed it plays both a positive and negative role in growth control by influencing cell proliferation and apoptosis. Several investigators have suggested three general models for the induction of apoptosis, namely the conflict, dual signal and the modified dual signal models (reviewed in Prendergast 1999). The conflict model suggests that deregulated c-Myc expression may lead to inappropriate growth signals that result in apoptosis, without, however, c-Myc being directly responsible for the apoptosis. This comes to an antithesis with the dual signal model that suggests a direct role for c-Myc in the induction of apoptosis, along with proliferation, by regulating death effector systems. Lastly, the modified dual signal model suggests that while c-Myc does indeed co-ordinate cell proliferation and apoptosis there are two distinct pathways that might lead to apoptosis, one being the pathway that primes apoptosis following proliferation, and then a second being the triggering pathway which can be suppressed by survival signals. Although our studies on c-Myc expression are focused mainly on the effect on cell culture kinetics rather than the molecular and biochemical mechanisms that

lead to that effect, our results show that the observed apoptosis seems to follow a combination of the above models. It has to be noted that these models explain the effect of c-Myc based on complex studies that include structure-function and genetic analysis of the gene/protein. However they can be used to draw a general hypothesis based on the observed results. c-Myc enhanced proliferation in a manner partially independent of the serum effect. Although c-Myc appeared to increase the rate of apoptosis in the growth limiting conditions as seen in the serum-free cultures; nevertheless, c-Myc may have to overridden to a certain extent, the effect of those growth limiting factors, since there was an increase in cell proliferation rate and cell density. Additionally, the triggered apoptosis might have been 'postponed' by the addition of survival factors normally present in the serum, as it can be seen in the fed batch cultures, yet cell death is inevitable due to exhaustion of nutrients and accumulation of waste products.

Several studies have shown that cell adhesion and cell contact might also influence cell proliferation and susceptibility to apoptosis. In particular, it has been shown that cell to cell contact might affect the proliferation of anchorage dependent cells as a limiting factor (as reported in Prendergast 1999); however, our results show that cell contact did not play such an important role when considering the proliferation of cmc-cho. When confluent and covering the whole of the adhesion area cmc-cho cells continue to divide and form clusters of viable cells, which seem to adhere on top of the monolayer without affecting viability, unlike the control cell line which grow uniformly and at a slower growth rate. The morphology and growth characteristics of the transfected cmc cell line indicated that over-expression indeed resulted in morphological transformation and partial anchorage independent growth as reported previously on other cell lines (Small et al. 1987; reviewed in Claasen and Hann 1999). Additionally the cmc-transfected cells appeared to show partial anchorage independence characteristics in both the presence and absence of serum. These results led to the proposition and later the demonstration that it is easier to adapt

Table 1. Summary of the results in static cultures of cmc-cho and neo-cho cells.

Cell line	Growth rate (day ⁻¹)		Glucose consumption rate ($\mu\text{mol}/10^5$ cells/day)	
	In serum	No serum	In serum	No serum
cmc-cho	0.38	0.27	0.06	0.14
neo-cho	0.18	0.08	0.1	0.5

cmcy-cho cells into suspension than the control cells since anchorage did not seem to play an important role for their survival and proliferation. Furthermore, studies have shown that cell adhesion did alter the susceptibility to apoptosis in serum deprived cultures of CHO cells (Prendergast 1999) suggesting that cell-cell interactions might limit death in cells cultured at higher density. Additionally, it was also shown in different cell systems, that cells would undergo apoptosis when deprived of a cell adhesion matrix. When cells in this study were adapted to suspension, the batch and fed batch results showed that c-Myc had a positive effect on cell proliferation, with reduced glucose utilisation rate. As expected apoptosis in suspension cultures was generally higher than in static culture for both cell lines, with cmcy-cho exhibiting slightly higher apoptosis rates which may be due to shear stress induced by agitation (Al-Rubeai et al. 1995).

Conclusion

Using a cell (metabolic) engineering approach we have developed a new CHO cell line with increased proliferation rate, reduced serum dependency and increased anchorage independence. Additionally, it seems that the positive effect of cmcy on cell proliferation was not affected to a large extent by its widely reported effect on apoptosis. The % of apoptosis observed in our study was not as high as described in different systems that required the introduction of an anti-apoptotic gene such as *bcl-2* for developing a successful and viable cell culture process. Furthermore, we have obtained a cell line that comprises the positive effect of c-Myc with the robust nature of CHO-K1, in that it is highly proliferative and easy to grow in suspension.

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