

Active hypothermic growth: a novel means for increasing total interferon- γ production by Chinese hamster ovary cells

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Abstract When grown under hypothermic conditions, Chinese Hamster Ovary (CHO) cells become growth arrested in the G_0/G_1 phase of the cell cycle and also often exhibit increased recombinant protein production. In this study, we have validated this hypothesis by stimulating hypothermic growth using basic fibroblast growth factor and fetal bovine serum supplementation. This method led to 7.7- and 4.9-fold increase in total production compared to the 37 °C and 32 °C control cultures, respectively. This proof-of-concept study will motivate the creation of cell lines capable of growing at low temperatures for use in industrial processes.

Key words: hypothermic growth, CHO cell, Interferon gamma, specific productivity

I. INTRODUCTION

Recombinant human glycoproteins produced by Chinese Hamster Ovary (CHO) cells are an important class of therapeutic molecules and investigating means of improving the production rate of these glycoproteins is therefore of great interest. Culturing CHO cells under mild hypothermia (30-33 °C) leads to growth arrest in the G_0/G_1 phase of the cell cycle [1] and, in some cases, cause an increase in specific productivity of recombinant protein [2-7].

Controlled proliferation, achieved by inducing growth arrest in the G_0/G_1 phase by chemical, environmental or genetic means, is often used to increase CHO specific productivity and thus it is commonly assumed that enhanced hypothermic productivity is due to growth arrest [1]. However, we have shown previously that the positive effect of hypothermia on recombinant protein production is not due to G_0/G_1 growth arrest but rather is due to

elevated recombinant mRNA levels [8]. At both 32 °C and 37 °C, specific productivity was growth-associated, increasing as the percentage of cells in the S phase increased, demonstrating that a cell line can be both a growth-associated producer and have enhanced productivity under hypothermic conditions. In other words, hypothermia enhanced productivity whereas growth arrest diminished productivity for the recombinant CHO line used in the study.

Based on these findings, it was hypothesized that the best conditions for maximizing total recombinant protein production would be cells capable of actively growing at low temperature, as these cultures would maintain the benefits of hypothermia on specific productivity whilst also achieving the high cell densities characteristic of active growth, and consequently total production would be enhanced. The goal of this study was to test this hypothesis by achieving “active” hypothermic growth, an easily achievable culture condition in industrial processes.

Hypothermia is known to lead to growth arrest at least in part by causing increased expression of cell cycle regulatory proteins. When mammalian cells are exposed to cold shock, p53 protein levels increase significantly, followed by an increase in p21 mRNA and then p21 protein [9]. The level of p53 mRNA is unchanged during this sequence of events, indicating that low temperature exerts a post-transcriptional, stabilizing effect on p53 [9]. The mechanism by which cold shock stabilizes p53 remains unknown [9]. For the current study, we hypothesized that in order to stimulate cells to grow at low temperature, various growth factors could be used, providing positive growth signals to counteract the negative effects of hypothermia on the cell cycle engine. Specifically, we used basic fibroblast growth factor (bFGF) and insulin on adherent cultures and fetal

bovine serum (FBS) on suspension cultures, in order to stimulate hypothermic growth. These particular mitogens have previously been shown to have positive effects on CHO proliferation [10-14].

II. MATERIALS AND METHODS

A. Cell Line and Medium

A CHO cell line expressing human IFN- γ (CHO- γ) driven by the SV40 early promoter was obtained from Dr. Walter Fiers [15]. This recombinant cell line was created by cotransfection of dihydrofolate reductase (DHFR) and IFN- γ genes into a DHFR deficient CHO cell line. The basal medium used for adherent cell culture was Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT), 20 U ml⁻¹ penicillin-20 μ g ml⁻¹ streptomycin mix (Invitrogen), and 0.25 μ M methotrexate. The basal medium used for suspension culture was HyQ PF-CHO (HyClone) containing 20 mM glucose and supplemented with 4 mM glutamine, 0.1% Pluronic F-68 (Invitrogen), 20 U ml⁻¹ penicillin-20 μ g ml⁻¹ streptomycin mix (Invitrogen) and 0.25 μ M methotrexate. Reagents were from Sigma Chemical Company (Saint Louis, MO) unless noted otherwise.

B. Adherent Cell Culture

Following 2-4 passages of stock cells, mid exponential cells with viability greater than 95% were centrifuged at 950 rpm for 5-10 minutes and resuspended in fresh medium. One quarter million cells were resuspended in 3 ml of medium and added to each well of a group of 6-well plates. Cells were incubated at 32 °C, or 37 °C in humidified incubators with 7.5% CO₂ overlay. A temperature of 32 °C was used because this temperature has been shown previously to increase CHO- γ specific productivity [7]. However, 32 °C is not necessarily the optimal temperature for hypothermic culture. Every day, 2 wells per condition were sacrificed for cell counting. The experiments were terminated once the percent viability was less than 80%.

Various amounts of stock growth factor solutions were added to the medium at the beginning of the experiments. In all cases, the growth factors were dissolved in the standard basal medium containing the same supplements as the control cultures. Thus, all conditions were identical except for the presence or absence of growth factors. The insulin stock solution contained 100 μ g ml⁻¹ insulin and the bFGF stock solution contained 25 μ g ml⁻¹ bFGF. Insulin concentrations of 5, 15 and 25 μ g ml⁻¹, bFGF concentrations of 13, 21, 42 and 45 ng ml⁻¹, and a

mixed culture containing 15 μ g ml⁻¹ insulin and 21 ng ml⁻¹ bFGF were tested.

C. Suspension Cell Culture

Following 2-4 passages of stock cells, mid exponential cells with viability greater than 95% were centrifuged at 950 rpm for 5-10 minutes and resuspended in fresh medium at a density of 2.5x10⁵ viable cells ml⁻¹. The experiments were conducted in 250-ml Erlenmeyer flasks with a starting volume of 50 ml. Cells were incubated at 32 °C, or 37 °C on shaker platforms set at 100 rpm in humidified incubators with 7.5% CO₂ overlay. All cell culture experiments were conducted in duplicate. Samples were taken at least daily for cell counting and viability determination. The experiments were terminated once the percent viability was less than 80%.

For cultures aimed at achieving active hypothermic growth, the procedure was the same as outlined above except that 10 or 25% FBS was added to the initial culture. Volume was maintained the same as the other cultures (50 ml) by adding less medium to the FBS-containing cultures.

D. Cell Enumeration and Viability

The procedure used for counting adherent cell cultures was designed to ensure that both the adherent cells and any dead or viable cells that had become detached during the course of the experiment were included in the total cell count. Medium was removed from the cells and centrifuged at 950 rpm for 5 minutes to retain detached cells for counting. After centrifugation, the supernatant was removed and stored at -80 °C for future analyses. The adherent cells were treated with a 0.05% Trypsin / EDTA solution (Invitrogen) for 5-10 minutes at 37 °C. The trypsin was removed and the cells were pooled with the previously centrifuged, detached cells. The well was rinsed with fresh medium to remove any remaining cells and the rinse solution was combined with the trypsin pool. The cell number per well was then determined by counting the cells using a hemacytometer and multiplying the concentration by the known volume of the cell solution. Each sample was counted in duplicate and each count consisted of at least 200 total cells. If the results of the duplicate counts did not agree within 10% of each other, additional counts were conducted. The cell viability was determined using the trypan blue exclusion assay in conjunction with cell counting.

Approximately 0.5 – 1 ml samples were removed from well-mixed suspension cultures. The cell

concentration was determined by counting the cells using a hemacytometer. Each sample was counted in duplicate and each count consisted of at least 200 total cells. If the results of the duplicate counts did not agree within 10% of each other, additional counts were conducted. The cell viability was determined using the trypan blue exclusion assay in conjunction with the cell counting. After counting, samples were centrifuged at 950 rpm for 5 minutes and the supernatant was removed from the cell pellet and stored at -80 °C for future analyses.

E. IFN- γ Concentration

IFN- γ concentration was measured using an ELISA kit (HyCult biotechnology b.v., Uden, the Netherlands). To reduce the uncertainty of ELISA measurements, samples were measured in triplicate.

F. Cell Cycle Analysis

At least one million cells per sample were collected by centrifugation for 5 minutes at 950 rpm and washed once with 5 ml of PBS. After a second centrifugation step, cells were resuspended in 0.5 ml PBS, followed by addition of 4.5 ml of ice-cold 70% ethanol and stored at -20 °C for 1-4 weeks. The cells were centrifuged and the ethanol was aspirated. Then, the cells were resuspended in 5 ml PBS, held at room temperature for 60 seconds and centrifuged again. Finally, the cells were resuspended in 1 ml of the propidium iodide (PI)/Triton X-100 staining solution [0.1% (v/v) Triton X-100; 10 $\mu\text{g ml}^{-1}$ PI; and 0.2 mg ml^{-1} RNase A in PBS] and incubated at room temperature for 30 minutes prior to the flow cytometry analysis. The flow cytometry measurements were carried out on EPICS[®] Elite flow cytometer (Coulter Corporation, Miami, FL) equipped with a single argon ion laser with the excitation wavelength at 488 nm and emission set at 610 nm. DNA distribution histograms were analyzed with the WinMDI Multi-Cycler 2.7 software

III. RESULTS

A. Insulin and bFGF stimulate CHO growth at low temperature

Given that hypothermia serves as a negative growth signal, it was hypothesized that hypothermic active growth could be achieved by providing high concentrations of growth factors to serve as positive growth signals, thereby overcoming the hypothermic arrest. To test the feasibility of this approach, the effect of different concentrations of the growth factors insulin and bFGF on cell density was

determined. The concentrations tested were similar to those shown by other researchers to have a positive effect on CHO cells [5 $\mu\text{g ml}^{-1}$ insulin [13] and 20 ng ml^{-1} bFGF [10]. Figure 1 shows the peak cell density obtained at 32 °C for the different growth factor conditions tested. Clearly, both insulin and bFGF treatment cause higher cell densities relative to the control and some level of hypothermic active growth is therefore achieved.

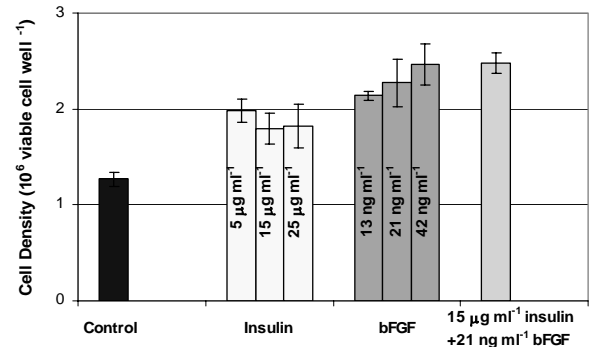


Figure 1: Effect of Growth Factors on Peak Cell Density: Adherence Cells

The combination of insulin and bFGF gave a slightly higher cell density than either of the two growth factors alone, but the additive effect was not significant. The viability for the cultures containing insulin was consistently lower than the bFGF cultures. Insulin has previously been observed to cause cells growth-arrested by serum withdrawal to proliferate but also to undergo apoptosis [13] and it is likely that a similar event is taking place here, resulting in lower cell viability. Because of this detrimental effect and the fact that insulin did not give higher cell density than bFGF and therefore only bFGF was tested in the subsequent experiments.

B. bFGF supplementation leads to a higher fraction of S phase cells during hypothermic culture

The cell growth curves of control and bFGF-treated cultures at 32 °C and 37 °C are shown in Figure 2. The growth factor causes the peak cell density to decrease slightly at 37 °C whereas it causes a significant increase in cell density at 32 °C, with peak cell density doubling relative to the untreated control.

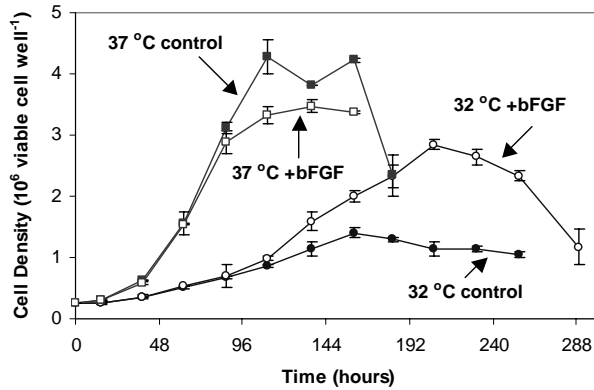


Figure 2: bFGF Stimulation of Cell Growth

The effect of bFGF at 32 °C is evident at the cell cycle level, as shown in Figure 3, which shows the percentage of S phase cells versus time. As early as day 2, the bFGF-treated hypothermic culture has a significantly higher percent of cells in S phase, and this trend continues for the remainder of the culture.

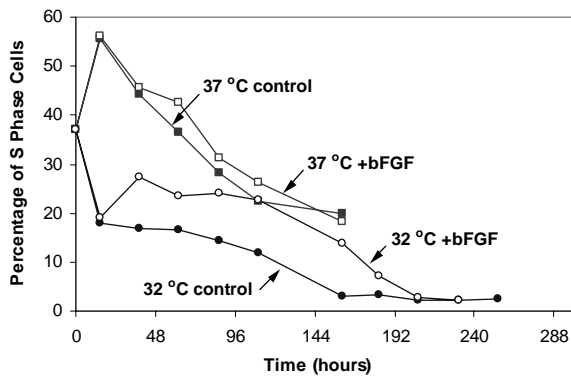


Figure 3: bFGF On Percent of S Phase Cells

C. Hypothermic active growth increases the total IFN- γ production

At the end of the cultivation, the IFN- γ concentrations were determined and it can be seen that the IFN- γ is significantly higher for the bFGF-treated, hypothermic culture (Figure 4) compared to any of the other three conditions. The total IFN- γ production was 110% compared to the 37 °C control and by 50% compared to the 32 °C control. Thus, this culture concept, active hypothermic growth via growth factor addition, is validated as a means for improving total production of recombinant glycoproteins.

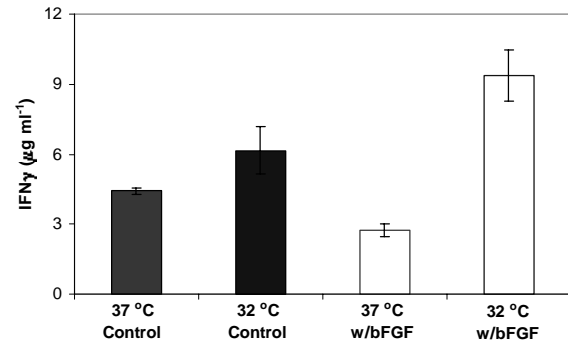


Figure 4: Active Hypothermic Growth Effect on Total IFN- γ Production

The bFGF itself appears to have a negative effect on specific productivity, as can be seen by the 20-25% decrease relative to the non-treated control (Figure 5), regardless of culture temperature. The molecular mechanism for this detrimental effect is unknown. However, the critical point for validating *active hypothermic growth* is that the ratio of 32 °C to 37 °C specific productivity remains essentially unchanged for the non-treated and bFGF-treated samples (Figure 6) showing that the positive effect of hypothermia on specific productivity can be achieved simultaneously with active growth.

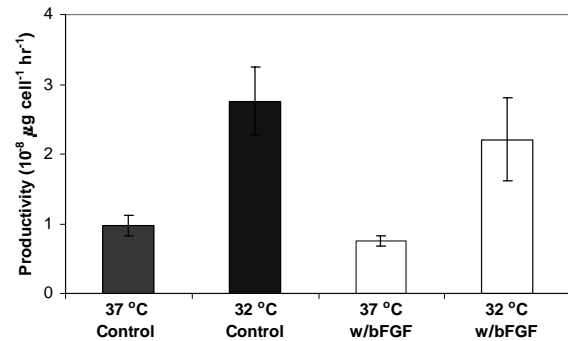


Figure 5: bFGF Reducing Average Specific Productivity at Different Temperatures

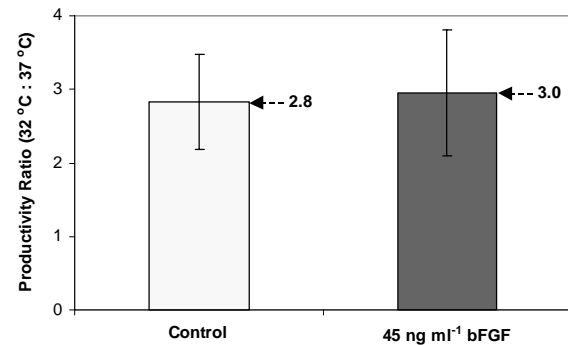


Figure 6: Positive Effect of Hypothermic Growth

D. Hypothermic active growth in suspension culture

For an industrial production process, cells are usually grown in suspension culture. To verify that hypothermic active growth is also feasible in suspension culture, suspension cultures were subjected to mitogen stimulation using high doses of FBS. The basal medium used was a commercially available protein-free medium. The effect of 0, 10 and 25% (v/v) FBS on cell growth at 37 °C and 32 °C is shown in Figure 7. Clearly, hypothermic active growth is feasible in suspension culture, as seen from the data. In fact, the hypothermic growth is far more impressive than what had been obtained for the bFGF-stimulated adherent cells, with hypothermic culture in the presence of FBS reaching the same peak density as 37 °C control.

E. Hypothermic active growth greatly enhances total IFN- γ production in suspension culture

As hypothesized, the hypothermic active growth cultures were able to simultaneously maintain high productivity and achieve high cell density. The combined factors yielded a significant enhancement in the total IFN- γ production (Figure 8). The 25% FBS culture at low temperature had 7.7-fold higher total production than the 37 °C control and 4.9-fold higher than the 32 °C control. The FBS had an insignificant effect on 37 °C total production, and thus the enhancements under hypothermic conditions can be attributed directly to the improved growth rather than to a secondary effect of the FBS on productivity.

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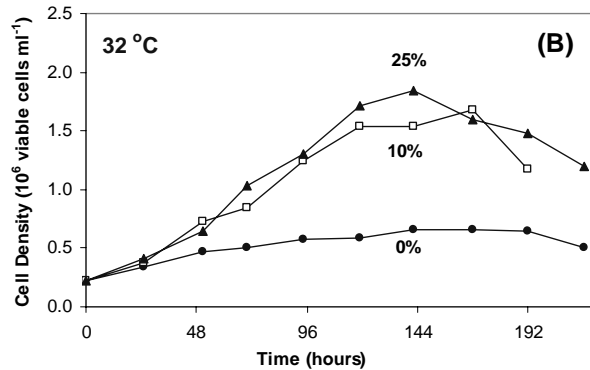
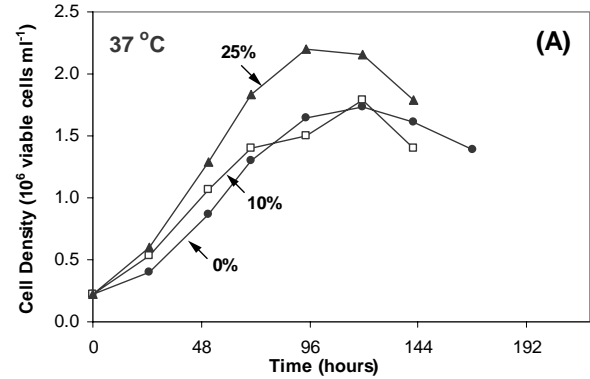


Figure 7: Serum Supplementation on Cell Densities

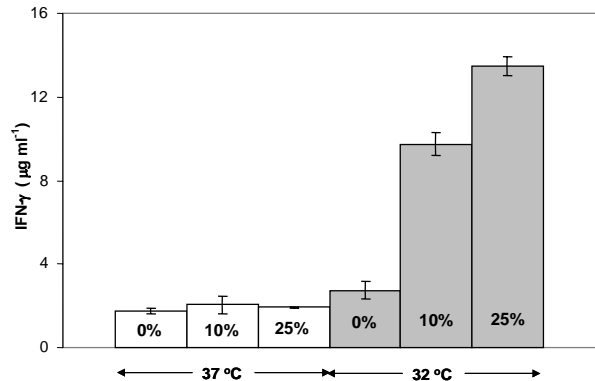


Figure 8: Serum Supplementation on IFN- γ Production

IV. CONCLUSIONS AND DISCUSSION

It was hypothesized that cells capable of maintaining active growth at low temperature would prove to be the optimal production platform for maximizing IFN- γ production. This is mainly attributed to the fact that CHO- γ was a growth-associated producer that also had enhanced productivity at low temperature [8]. The hypothesis was proven to be correct by stimulating hypothermic growth using mitogens. The growth factors maintained the cells in its cell division cycles instead of arresting in G₀/G₁, resulting in much higher cell

densities at low temperature without sacrificing the enhanced specific productivity. This, consequently, resulted in higher IFN- γ concentrations.

The greatly improved cell densities are due somewhat to improved growth rates but are also due to exponential growth being maintained for a longer period of time. The growth rate was improved by about 10-35% at the low temperature. More importantly, the mitogen-stimulated cells maintained exponential growth for about 2 days longer than the control for all the hypothermic conditions tested. As a result, the integrated viable cell densities (IVCD) of the active hypothermic growth cultures were comparable to, or greater than, the 37 °C controls.

The molecular mechanism by which the mitogens maintain the cells in active growth is likely by exerting a positive effect on cell cycle progression. Growth factors, such as bFGF, bind specific cell surface receptors. This binding event in turn increases the kinase activity of the receptor, which then leads to a cascade of signals promoting growth [16]. It is known that hypothermia serves as a negative signal to the cell cycle control system, for example activating p53 and thereby triggering a further cascade that ultimately prevents the cyclin-dependent kinase (CDK) molecules from activating cell passage to the S phase [9]. Growth factors, on the other hand, will have the opposite effect, promoting CDK activity or inhibiting cyclin-dependent kinase inhibitors (CKI) as a result of their initial binding and activation of the cell surface receptors and thereby stimulating cells to progress into the S phase. Thus, there appears to be a “tug-of-war” effect on the cell cycle, with hypothermia promoting arrest and mitogens promoting growth and the outcome depending on mitogen level. For example, increasing the bFGF concentration has a positive effect on improving the peak cell density achievable in a batch culture (Figure 1). Whether this trend would continue beyond the 45 ng ml⁻¹ bFGF concentration tested is unknown. Insulin, on the other hand, did not have such an effect, with 5-25 mg ml⁻¹ causing essentially the same peak density. In this case, this may be due to the previously mentioned increasing levels of apoptosis with increasing levels of insulin, and thus the positive effect on hypothermic growth being masked by the cell death.

The hypothermic growth-promoting effect of FBS in suspension culture was more impressive than the effect of bFGF in adherent culture. Whereas bFGF treatment at 32 °C caused cells to reach 44% and 67% of 37 °C growth rate and peak density, respectively, FBS treatment at 32 °C allowed cells to reach 80% and 103% of the 37 °C values of these two parameters. The end result was a spectacular, multi-

fold increase in total IFN- γ production (Figure 8). Unfortunately, FBS cannot be used for new bioprocesses producing human therapeutics. Nevertheless, these results should motivate the search for a well-defined, FDA-approved growth factor or chemical formulation capable of achieving the level of hypothermic growth in suspension culture caused by FBS. The effect of FBS on promoting hypothermic growth in adherent cultures was not tested here, because the CHO- γ cell line is FBS-dependent when grown in adherent culture using DMEM medium, and thus a serum-free control was not practical.

One of the major drawbacks of controlled proliferation strategies in general, including hypothermic culture, is that although specific productivity is higher, total production is not always significantly improved, due to the detrimental effect on cell density caused by controlled proliferation. This is evident in the hypothermic culture literature. For example, Furukawa and Ohsuye [2] found that *specific* productivity was highest at 32 °C, within the range 30 – 37 °C, for their recombinant CHO cell line. However, culturing at 35 °C maximized *total* production, because this temperature achieved the optimum in the trade-off between higher productivity at low temperature and higher growth rates at elevated temperatures. This was despite the fact that 32 °C had a 50% higher specific productivity than 35 °C. A similar finding can be found in another hypothermic study, wherein specific productivity at 30 °C was 40% higher than that at 33 °C, yet total production at the slightly higher temperature was 3 times higher than at 30 °C [6]. By promoting active growth at low temperature, one no longer needs to compromise as significantly on the reduced growth rate and can operate closer to the temperature that gives highest specific productivity. From the findings in this study, it is predicted that the above mentioned researchers would achieve maximum total production by stimulating growth at the lower temperatures, thereby achieving the high cell density whilst simultaneously maintaining the high specific productivity.

Adding growth factors to medium to obtain active hypothermic growth may not be cost effective in an industrial process. It may be far more practical to produce cell lines capable of growing under hypothermic conditions, while maintaining increased specific productivity. We are currently in the process of isolating such CHO cells, which would result in a far more cost effective means of achieving active hypothermic growth.

The general concept shown here, namely promoting growth under otherwise unfavorable conditions, may also be applicable and beneficial to

other controlled proliferation methods. For example, it may be useful to promote active growth in the presence of sodium butyrate, the enhanced specific productivity of which does not appear to depend on growth arrest [8].

In conclusion, this study introduced and validated a novel cell culture strategy, namely active hypothermic growth. This finding is envisioned to be applicable to any cell line that has growth-associated productivity and also has enhanced productivity under hypothermic conditions. The finding also applies to suspension culture and therefore is relevant for improving production in industrial processes.

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