Review

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The impact of process temperature on mammalian cell lines and the implications for the production of recombinant proteins in CHO cells

The use of a temperature-shift to subphysiological temperatures (<37°C) during the culturing of mammalian cell lines engineered to produce a recombinant protein is widely utilized. The effect of such a temperature shift on both the cellular responses and recombinant protein yield is not uniform and is both cell- and product-specific. Despite this, the understanding of the cellular responses to mild subphysiological temperature (27–35°C) culturing has developed markedly over the last decade and, as such, opportunities exist to harness this knowledge to further improve product yields and quality at subphysiological temperatures. In this review we describe the mammalian cell response to mild subphysiological temperature, the effects on recombinant protein production under such conditions, those mechanisms that appear to underpin increases in productivity at such temperatures, and potential future innovations and directions that may further enhance the use of such conditions during bioprocessing of recombinant protein products.

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Mammalian cells & the production of recombinant proteins

Mammalian cell expression systems are routinely used for the generation of recombinant proteins, particularly in the industrial biopharmaceutical manufacturing industry where they are used for the production of therapeutic proteins (rPs). Indeed, many of the currently available recombinant protein biopharmaceuticals (e.g., monoclonal antibodies [mAbs]) are produced in cultured mammalian cells [1], with the most commonly used industrial mammalian cell host being the Chinese hamster ovary (CHO) cell [2] because these are a relatively easy and robust cell line to grow. In addition, from a regulatory prospective, CHO cells have a proven track record of producing safe biotherapeutics. Biotherapeutics now constitute a significant portion of the total pharmaceuticals market and it is predicted that mAb-based products alone will have sales approaching US$89.9 billion by 2017 [3]. Mammalian cells are usually chosen as the expression system of choice over other systems (e.g., yeast, bacteria, plants and transgenic animals) for biotherapeutic production when, first, the recombinant protein product is required to be as similar as possible to that which would be produced in humans, and second, when the correctly assembled and functionally active protein requires specific post-translation modification(s), folding and assembly of the target protein that can only be undertaken within specific cellular compartments/organelles that contain the required cellular machinery (e.g., chaperones and enzymes) to perform these cellular processes [4].

Traditionally, mammalian cells are cultured at the physiological temperature of 37°C to

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Key Term

Subphysiological temperature culturing: The culturing of mammalian cells at temperatures below 37°C, generally covering the range of 25–35°C.

align with conditions that are found in the body of the organism from which the cells were derived [5]. During a typical batch or fed-batch culture of a recombinant cell line the majority of cell proliferation occurs during exponential growth during which time recombinant protein synthesis is suboptimal as cellular resources and energy are diverted towards the essential processes required for doubling of cell mass (and hence protein) and cell proliferation [6]. The synthesis of recombinant protein from mammalian cells is generally greatest when cells are in a stationary growth phase when there is less competition with endogenous protein synthesis and cellular processes [7]. This difference in the production level in relation to the stage of growth and the cellular energy requirements is known as the biphasic production concept.

Over the last 20–30 years the yields of recombinant protein obtained from cultured mammalian

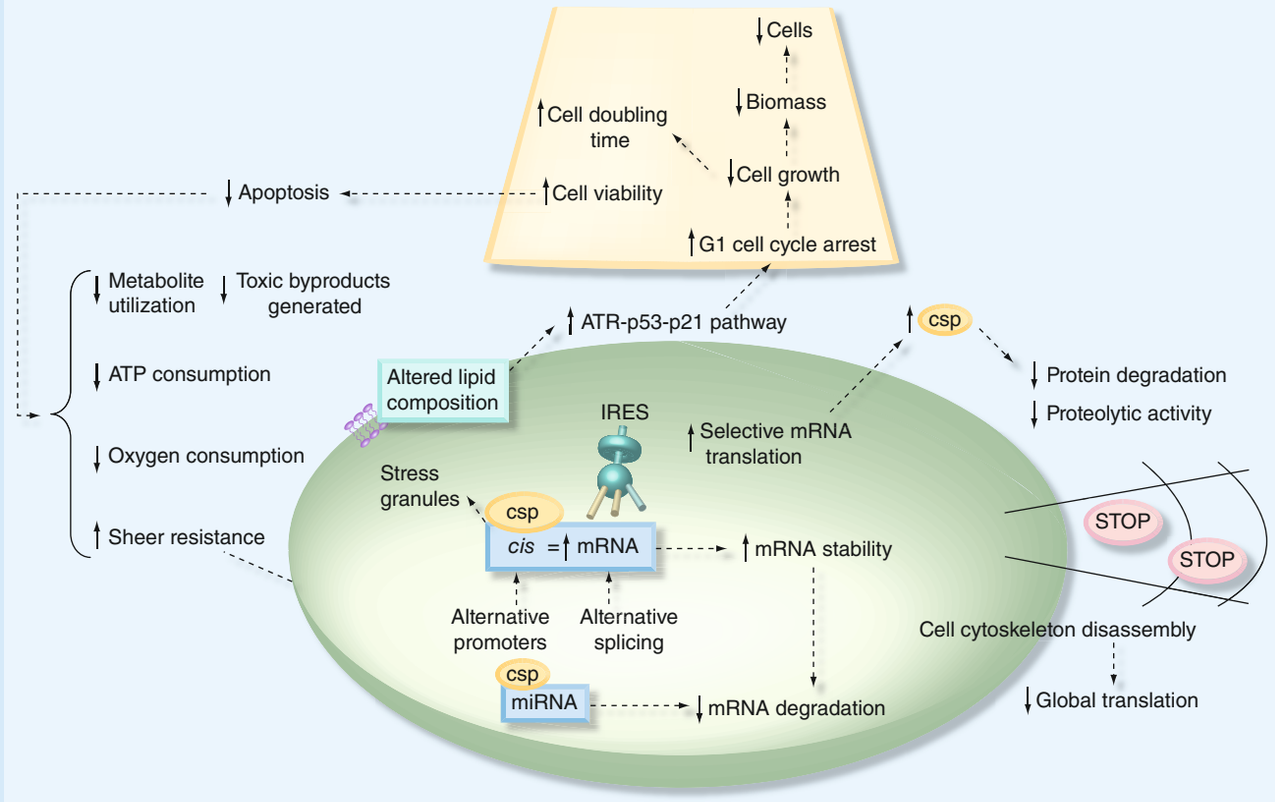

Figure 1. Summary of key mammalian cell cold-shock responses. In response to subphysiological temperature culturing and the exposure of mammalian cells to cold-shock, cells arrest in the G1 phase of the cell cycle, which leads to a reduction in growth rate and as a result decreased biomass and fewer cells. Metabolism is reduced upon cold-shock and therefore less metabolic toxic by-products are generated, there is reduced ATP and oxygen consumption and cells exhibit enhanced sheer resistance and delayed or reduced apoptosis. Consequently, cell viability is enhanced and cell doubling time is lengthened leading to prolonged culture duration. The cell cytoskeleton disassembles disrupting the organization of the translational machinery and results in attenuation of protein biosynthesis, although STOP proteins interact with the microtubule network to stabilize these. The lipid composition of cellular membranes is altered changing the rigidity activating the ataxia telangiectasia mutated and Rad3-realted kinase (ATR)-p53-p21 pathway. p53 activation of p21 in turn causes cell cycle arrest. Global transcription and translation is reduced, although cold shock proteins (CSP) are induced and the synthesis of selective proteins are upregulated. Mechanisms including CSP binding to *cis*-elements of target genes increasing their translation, activation of alternative promoters to generate alternative mRNA transcripts that can be preferentially translated through internal ribosome entry site (IRES) cap-independent translation, alternative splicing leading to preferential cold-specific mRNAs, secondary structure formation in mRNAs such that they are 'stabilized' with reduced turnover, and a reduction in protein turnover. Up arrows indicate a potential increase in the process, down arrows a decrease upon subphysiological temperature culturing. Note, the schematic is a general guide to responses and items are not necessarily in correct locations within the cell, in proportion or all responses always in the same direction across all cells and products (see text for more detail).

cells has improved immensely such that yields in excess of 10 g/l have now been reported (e.g., Huang *et al.* who reported yields of a monoclonal antibody and FC-fusion protein in excess of 10 g/l [8]). The majority of enhancements in yield over the years have resulted from improvements to process conditions (e.g., pH control), enhanced culture media and feeding regimes, manipulated vectors and constructs, development of new host cell lines, and the development of new high-throughput screening strategies that allow the assessment of large numbers (100s–1000s) of cell lines to select those with desirable industrial phenotypes (high growth and productivity) [9]. Many examples of such process improvements are found throughout the literature, including the optimization of culture medium [10–12], cell line development [11] and selection [13,14], improved vector design [15], apoptosis engineering [16], manipulation of mRNA translation initiation factors [17], culture process development [18] and the use of subphysiological temperature culturing to improve recombinant product yields [19]. The prevalence of subphysiological temperature culturing of mammalian cells in industry is difficult to gauge from the literature alone, but it is generally accepted to be widely used, but certainly not in all cases, and is usually applied on a case-by-case basis during the development of a specific bioprocess for a particular molecule and cell line.

When mammalian cells are cultured at temperatures of less than 37°C (subphysiological temperatures) the cell responds to such environmental change. The cellular response to a reduction in culture temperature is discussed in more detail below; however, cells generally respond by initiating a coordinated response (Figure 1) that involves cell cycle arrest and reduced proliferation, modulation of transcription/gene expression and mRNA translation, reduced metabolism, changes in lipid biosynthesis and membrane remodelling, changes to the cell cytoskeleton, and a general reduction in cellular processes [20]. In order to understand and harness the mechanisms by which reduced temperature cultivation can potentially result in enhanced recombinant protein yield (Table 1) it is first necessary to consider the mammalian cell response to subphysiological temperature cultivation.

Cellular response of mammalian cells to subphysiological temperature culturing

Our knowledge of the cellular responses of cultured mammalian cells to subphysiological temperatures and the application of this understanding to the production of recombinant protein as it stood in the middle of the last decade has previously been reviewed [19]. Since this time further advances have been made with respect to understanding the responses of mammalian cells to subphysiological temperatures, although the knowledge in this area compared with that in bacteria and plant systems, where defined cold-shock proteins are expressed and orchestrated waves of modulated gene expression are initiated, remains limited. For example, a reduction in temperature results in the formation of mRNA secondary structure that can result in the inhibition of gene expression as a result of premature transcription termination, altered mRNA degradation and inefficient mRNA translation [21]. *Escherichia coli* respond to this challenge by the expression of a number of cold-shock-induced proteins (CSPs) upon temperature downshift that allow the cell to negate these effects, either through the CspA family of proteins that work as RNA chaperones and influence transcription and potentially translation of specific mRNAs or via RNA helicases and exoribonucleases that together overcome RNA stability to degrade RNA [21]. Such a detailed understanding of the responses in mammalian cells remains

Table 1. Key mammalian cell phenotype responses upon subphysiological temperature culturing (25–35°C) relevant to the production of recombinant proteins.

Mammalian cell phenotype	Response upon subphysiological temperature culturing
Cell proliferation	Reduced
Biomass accumulation	Reduced
Cell viability	Prolonged
Apoptosis	Reduced
Culture duration	Prolonged
Metabolism	Reduced, toxic by-products (e.g., lactate) reduced
Tolerance to shear stress	Increased
Cell-specific and volumetric productivity	Cell-, product- and process-specific effects. May be increased or unchanged. In a few cases actually decreased
Protein folding	May be improved
Product quality	Can be enhanced

to be elucidated, however, a summary of the major cellular processes that are known to be modulated in response to cold-stress in mammalian cells is outlined in Figure 1.

» mRNA translation & protein synthesis at subphysiological temperatures in mammalian cells

In response to subphysiological temperatures, global protein synthesis rates are decreased [22], which would seem at odds with the observation that recombinant protein expression can be increased at such temperatures. The obvious explanation for this is that upon cold-shock the associated reduction in global mRNA translation and metabolism (and hence reduced metabolic burden) coupled with increased mRNA stability/reduced degradation that leads to increased recombinant protein mRNA amounts [5,22–24] together mean that although protein synthesis *per se* is reduced, there is less competition of recombinant mRNAs with endogenous mRNAs for the translational and protein folding/secretory machinery, and hence recombinant protein yields are enhanced. However, the mechanisms that underpin potential increases in recombinant protein yield from mammalian cells are far more complex than this, as discussed below.

In addition to the global decrease in mRNA translation and protein synthesis via the phosphorylation of the initiation factor eIF2α [25,26], cold-shock of mammalian cells can reportedly lead to the formation of cytoplasmic structures termed stress granules (SGs) that contain stalled translation pre-initiation complexes [25]. Recent data suggest that protein synthesis is also attenuated in parallel by activation of AMP-activated protein kinase (AMPK), which results in inhibition of mTOR signaling as a result of impaired mitochondrial function [25]. Furthermore, microRNA (miR) expression is also modulated in CHO cells upon a shift to subphysiological temperatures [7,27]. These small non-coding RNAs mainly act as negative regulators of translation of specific mRNA targets by binding to the 3′-UTR of the target mRNAs [27]. The artificial manipulation of such miRs has been shown to have functional effects in CHO cells and to influence recombinant protein cell-specific productivity [27]. In particular, endogenous miR-7 expression has been shown to decrease to a greater extent in CHO cells shifted to lower temperatures over time in culture compared to those kept at 37°C, however, when miR-7 was transiently overexpressed at 37°C this resulted in an effective block of cell proliferation, while inhibition of miR-7 amounts had no effect on cell growth [27]. The overexpression of miR-7 at 37°C did, however, result in an increase in cell-specific productivity, and a subsequent study of the proteome of CHO cells overexpressing miR-7 showed that the expression of 93 proteins was decreased and of 74 proteins was increased upon miR-7 overexpression. Proteins involved in translational control, RNA and DNA processing were enriched in those proteins whose expression decreased, and those involved in protein folding and secretion were enriched in the upregulated category [28].

Although global endogenous protein synthesis is reduced upon subphysiological temperatures in mammalian cells, the synthesis of specific proteins is reportedly upregulated [20,22], presumably these being proteins that are required or essential for the cellular response to, and survival of, cold-shock. There are reportedly two mammalian cold-induced CSPs, Rbm3 (RNA-binding motif protein 3) and CIRBP (cold-inducible RNA-binding protein), these bearing no sequence homology to the bacterial CSPs [20]. There are reports that describe more than 20 additional genes and proteins whose expression changes in mammalian cells at subphysiological temperatures [22,29]. However, care needs to be taken when defining cold-shock-induced protein upregulation as protein degradation is also reduced at subphysiological temperatures, which can result in an apparent increase in protein abundance due to reduced turnover rather than due to an increase in protein expression [22]. The contributions of these two effects (increased protein synthesis vs increased abundance) can be investigated by metabolic radiolabelling of nascent polypeptide chains to determine newly synthesized material and standard protein analysis of total protein lysates. When undertaking such analysis it is seen that the range of proteins synthesized at 37 and 32°C is broadly the same, although global protein synthesis is reduced at 32°C compared with 37°C except in the case of a few polypeptides whose expression does appear to be increased [22,26].

» Effect of subphysiological temperature culturing on the cell cycle

In addition to reduced metabolism and protein synthetic capacity in mammalian cells upon subphysiological temperatures, mammalian cells cell cycle arrest [30], predominantly in the G1 phase of the cell cycle [23]. While cells can proliferate at 32°C [22], at 25°C and below proliferation more-or-less ceases, although this is rapidly restored upon a return of cells to 37°C [30]. The arrest of the cell cycle in mammalian cells upon cold-shock is due to the activation of the p53 signaling pathway [31]. It has now been established that p53 itself is activated as a result of activation of the ataxia telangiectasia mutated and Rad3-related kinase (ATR)–p53–p21 pathway [32]. The mechanism of ATR activation upon reduced temperature

cultivation appears to be due to changes in the lipid composition of cellular membranes at reduced temperature [32]. Subphysiological temperature culturing results in changes to the lipid composition of cellular membranes [33], leading to enhanced rigidity [22] and a reduction in cellular membrane associated roles [20]. Mammalian cells appear to actively respond to maintain the fluidity of the membrane by raising the levels of polyunsaturated fatty acid, activating ATR and the ATR signaling pathway, which ultimately results in p53 and p21 activation and cell cycle arrest [32]. Interestingly, p53 in the CHO-K1 cell line has a point mutation in codon 211 (from ACA to AAA resulting in a change from a Thr to a Lys residue) that manifests in high spontaneous amounts of p53 that do not respond to X-irradiation and mutant function of the protein in terms of a lack of the G1 checkpoint [34]. However, p53 amounts clearly do respond to reduced temperature in CHO-K1 cell lines with reports confirming these are increased [32] and hence it may be the pathway of activation that differs between cold-shock and X-irradiation that activates p53 and subsequent arrest in G1.

» Induction of specific cold-shock proteins at subphysiological temperatures

As described above, two CSPs have been identified in mammalian cells, CIRBP [29,35] and RBM3 [36]. The upregulation of these proteins in response to subphysiological temperatures is maximal at mild hypothermic conditions (e.g., 32°C), while at severe hypothermic conditions (<15°C) there is little to no observed upregulation of these proteins. This suggests that in mammalian cells two distinct responses exist in the adaption to subphysiological temperature culturing; one at mild-hypothermia (32–27°C) and one at severe hypothermia (<15°C). Both CSPs are highly homologous in terms of amino acid sequence and belong to the glycine rich RNA-binding protein family [20].

The exact function of both of these mammalian CSPs is unknown, although like the bacteria CSPs they are suggested to act as RNA chaperones [29,35]. Both have been shown to bind mRNAs, and are thought to be involved in maintaining mRNA stability and assisting in the translation of specific mRNA upon subphysiological temperature culturing [33]. RBM3 is also reported to inhibit miRs, which compromises translation efficiency of target mRNAs by interacting with the miR to prevent it from interacting with the target mRNA [37]. CIRBP, also known as hnRNP A18 [38], was the first of the mammalian CSPs to be identified [33] and is thought to be involved in protecting cells from TNF-induced apoptosis by stimulating the extracellular signal-regulated kinase pathway [39]. CIRBP also binds to ATR mRNA and an increase in CIRBP expression reportedly leads to an increase in the levels of ATR protein [40], a further direct link between the (ATR)–p53–p21 pathway and cold-shock. CIRBP is also thought to bind to the translation initiation factor eIF4G [40]. Via an interaction with the 3′-UTR, such as in the ATR mRNA, and the initiation factor eIF4G, CIRBP may act as a bridge to facilitate translation under cold-stress in a manner similar to that of polyA-binding protein (PABP).

It is interesting to note that the stability of the CIRBP mRNA is not changed upon mild hypothermia even though CIRBP mRNA and protein levels are increased [41]. Thus, mechanisms other than increased CIRBP mRNA stability play a key role during the cold-shock response and CIRBP induction. Indeed, such mechanisms are now beginning to be unraveled, including increased transcription of selected genes due to CSPs binding to *cis* elements in the promoter regions of target genes leading to enhanced transcription and, therefore, in turn leading to higher transcript levels. The regulation of CIRBP itself is now also being unraveled. The CIRBP gene reportedly contains a mild-cold responsive element (MCRE) in its promoter, and specificity protein 1 (Sp1), a transcription factor, binds to the MCRE leading to the induction of CIRBP [38]. The expression and or localization of Sp1 itself also changes under such conditions with Sp1 being localized preferentially to the nucleus at 32°C where it is able to bind to the MCRE of CIRBP [38]. Furthermore, the authors of this study also demonstrated that the MCRE can be added to target genes to specifically enhance recombinant protein expression in mammalian cells at 32°C [38]. The expression of CIRBP is also potentially controlled by the use of alternative promoters, whereby upon mild hypothermia an alternative promoter is activated that results in the production of an alternative CIRBP mRNA to that observed at 37°C [41,42]. This alternative and longer mRNA contains a potential internal ribosome entry site (IRES) that facilitates translation of the mRNA under cold-shock conditions [41]. Interestingly, differential splicing of mRNAs at different subphysiological temperatures has also been reported to generate cold-specific mRNAs, for example alternative splicing of pre-mRNA reportedly occurs in neurofibromatosis type 1 mRNA during exposure to temperatures between 20 and 32°C [29]. Other cold-response promoters and promoter elements have also been identified in CHO cells and shown to increase the transient expression of a luciferase reporter construct at 33°C compared with 37°C over and above the control SV40 promoter [43].

» Additional responses to subphysiological temperatures by mammalian cells

A further response of mammalian cells to subphysiological temperatures is the remodeling of the cell cytoskeleton. An intact cytoskeleton is required for efficient protein synthesis in mammalian cells [44], and the cytoskeleton is disrupted by cold-shock as the microtubules disassemble [45] and the F-actin system is disrupted [44]. Translational components including polyribosomes, mRNAs and initiation factors also co-localize with the cytoskeleton [46]; therefore, if the cytoskeleton integrity is compromised this organization is compromised at a local level. Consequently, disassembly of the cytoskeleton during cold-shock probably leads to the disruption of translation and is a factor in the attenuation of protein biosynthesis. However, mammalian cells can express STOP proteins (stable tubule-only-polypeptides) that at subphysiological temperatures are thought to stabilize the microtubule network [47].

Mammalian cells, therefore, actively respond to the cellular stress incurred due to reduced temperature culturing. It is important to note that cold-stress exposes cells to two types of stress; hypothermia due to reduction in temperature and potentially oxygen-related stress. At reduced temperatures there is a higher dissolved oxygen concentration [19] and decreased oxygen demand from the cells [48]. It is also interesting that the CSPs CIRBP and RBM3 are upregulated in response to hypoxia at both the mRNA and protein levels [49] and cells may experience hypoxia during rewarming to 37°C. Whether the improving understanding of the mammalian cold-shock response can be applied to further design of new systems and approaches that enhance recombinant protein yields at such temperatures remains to be seen and is addressed further in the following section.

Subphysiological temperature culture as an approach to enhance recombinant protein productivity from mammalian cells

Although the concept of subphysiological temperature culturing of mammalian cells is not new [50], knowledge of the processes and mechanisms that determine protein production at low temperatures remains incomplete. As described above, when cells are cultured at subphysiological temperatures growth rate is reduced [23,50] and cell doubling time is increased [23]. As a result culturing of cells at subphysiological temperatures leads to a reduced biomass when compared with cells cultured at 37°C [23]. This inherently seems counterproductive with regard to recombinant protein productivity and, hence, in order for subphysiological temperature culturing to yield greater recombinant protein amounts strategies that either first accumulate biomass before cold-shock, increase growth at reduced temperatures or an increase in cell-specific productivity must be achieved.

» Use of bi- & tri-phasic culture strategies involving temperature down-shifts for recombinant protein production

Partially as a result of the reduction in cell growth at subphysiological temperatures, metabolite depletion or utilization is decreased and in turn the build-up of toxic by-products such as lactate that usually accumulate upon the culturing of mammalian cells is also reduced [23,24,51]. Mild cold-shock also delays or decreases apoptosis, probably due to the reduced metabolism and nutrient use, reduced toxic by-product build up, less ATP and oxygen consumption [20], less sheer stress (or more sheer resistance in cells cultured at lower temperatures [52]) and the fact that the cells mainly arrest at the G1 phase of the cell cycle [23,53]. The reduction in cell death, largely due to reduced apoptosis, leads to prolonged viability of mammalian cells at subphysiological temperatures and, hence, an extension in the duration of the culture [5,23,54]. One factor that can therefore lead to enhanced productivity from mammalian cells at subphysiological temperatures is the prolonged viability to counterbalance the decreased biomass; however, this alone is not sufficient at an industrial scale to warrant the use of culturing at reduced temperatures as having to run bioreactors for extended periods of time to achieve the same or better yields would in the main likely be uneconomical. To overcome such limitations a 'biphasic process' strategy is therefore usually used. This strategy involves two-stage culturing, whereby in the first stage biomass is accumulated by growth of the cells at 37°C until sufficient biomass has been accumulated (often as cells are about to or have entered stationary phase of growth) followed by the second or production phase when the culture temperature is reduced, usually in the range of 27–35°C [55,56]. For example, such a strategy was utilized to enhance the productivity of recombinant human IFN-γ from CHO cells [57]. More recently there have been reports of a three-phase culture strategy involving low-temperature perfusion culture in order to increase productivity and control aggregation of the product [58]. In this process cells were grown at 37°C before transition to 32°C in batch culture mode before the implementation of a third perfusion stage. This approach led to both a cell-specific and volumetric productivity increase over that at 37°C in batch mode, reduced aggregation compared to the batch mode and the sialylation pattern, essential for the target molecules bioactivity, was significantly enhanced in the three-phase perfusion mode compared with batch culture [58].

» Effect of subphysiological temperature culturing on recombinant protein production from mammalian cells is cell & product specific

Reports demonstrate that subphysiological temperature culturing of mammalian cells can positively impact upon both the specific and volumetric productivity with regard to recombinant protein production. However, the effect is variable and may be cell line-, cell type- and target-specific. For example, while reduced temperature culturing was shown to increase the productivity of recombinant human growth hormone from a recombinant CHO cell line, subphysiological temperature culturing actually appeared to decreased cell viability [59]. Other reports have shown that subphysiological temperature culturing of recombinant mammalian cell lines resulted in no change or an actual decrease in cell-specific productivity [57,60–62]. One such example that illustrates this was reported by Yoon *et al.* who showed that reduced temperature cultivation of a CHO cell line expressing an anti-4–1BB antibody had very little effect on productivity at 33°C and a detrimental effect at 30°C (3.9-fold decrease) [5]. On-the-other-hand, there are many reports showing an enhancement in either or both cell-specific and volumetric productivity of recombinant products from subphysiological temperature culture cells compared with that observed at 37°C (e.g., [5,8, 16,23,26,54–59,63–71]) in response to subphysiological temperature culturing. Increases in either cell-specific or volumetric productivity range from modest (1.5–2-fold increases) to much larger increases (7–8-fold increases).

From the reports to date it is clear that even when the same cell type is used (e.g., CHO) there are differences in the effect of subphysiological temperature culturing on productivity. This may be related to differences in how the strategy is applied, feeding regimes, vector design and elements within constructs, specific target protein effects and cell-specific differences. The cell-specific effect was well documented in a study investigating twelve parental clones. In this study 12 parental recombinant clones and their corresponding amplified recombinant clones producing a humanized antibody were investigated with respect to their productivity at subphysiological temperatures. When these CHO cells were cultured at 32°C there was an enhancement in the cell-specific productivity of the parental clones that ranged from 4–25-fold, but only a modest increase in the majority of the amplified clones when compared to the 37°C controls [72]. The differential effect of subphysiological temperature culturing is also variable across different cell types; for example there are reported opposing effects of reduced temperature on productivity in hybridoma and CHO cells. When hybridoma cells expressing a monoclonal antibody were cultured at 33°C there was a 21% drop in cell-specific productivity compared with the 37°C control [60]. Furthermore, there was a 42% drop in the cell-specific productivity of hybridoma cells expressing a cytotoxic monoclonal antibody when cultured at 33°C [61]. On-the-other-hand, in CHO cells subphysiological temperature culturing generally led to either no change or an increase in cell-specific and volumetric productivities. The reason for this discrepancy is unknown.

There have also been reports of a difference in the effect of subphysiological temperature culturing on the cell-specific and volumetric productivity of the same mAb produced in different host cell types. For example, when non-secreting (NS0) murine myeloma cells and CHO cells expressing the same IgG4 monoclonal antibody (cB72.3) were temperature shifted to 32 from 37°C in the CHO cell line, there was a 50% increase in cell-specific (qMab) productivity during the stationary stage of growth when compared with cells cultured at 37°C. Conversely, in the NS0 cells there was a negative effect on productivity upon temperature shift, which was more-or-less halted during stationary phase of growth at 32°C [23]. This difference in qMab was attributed to be due to differences in metabolite utilization, cell growth and recombinant mRNA levels between the two cell lines in response to subphysiological temperature culturing [23]. Finally, the strategy for subphysiological temperature culture also influences the observed effect on yield and cell-specific productivity. This was clearly demonstrated in a study using recombinant erythropoietin and follicle-stimulating hormone producing CHO cell lines that when cultured at 32°C had significantly increased cell-specific productivities, while volumetric activity was not as enhanced due to decreased cell growth. However, in some cases volumetric productivity can be enhanced due to extended culture lifetime allowing the cells more time to produce protein leading to a higher final product yield. In an attempt to further improve yield the erythropoietin and follicle-stimulating hormone-producing CHO cell lines were adapted to grow at 32°C (as opposed to temperature-shifting during culture), and this improved the specific growth rates of both recombinant cell lines at 32°C as opposed to that observed upon temperature-shift, however the cell-specific productivities were actually decreased [71].

» Subphysiological temperature culturing can influence recombinant protein quality

In addition to potential benefits on product yield, subphysiological temperature culturing has also been shown to improve product quality [26,73] and protein

glycosylation [74,75]. These improvements have been attributed to improved and more accurate protein folding and post-translational modifications in cells cultured at lower temperatures [26], and therefore reduced product aggregation [55,68]. However, once again this may be a product- and cell-specific effect. In contrast to those reports that show improved folding and reduced aggregation at subphysiological temperatures, others have shown increased aggregation. In one such study subphysiological temperature culturing led to amplification of recombinant antibody aggregates. This was attributed to enhanced levels of light chain (LC) and heavy chain (HC) mRNA generating an unfavorable LC/HC polypeptide/protein ratio combined with restricted ER machinery, limiting the folding and assembly of antibody, hence initiating the formation of aggregates [76]. Increased aggregation was also observed of a flag-tagged COMP angiopoietin-1 (FCA1) molecule from CHO cells at 33°C and pH 7.2 compared with that at 37°C, although aggregation was dramatically decreased at both temperatures at pH 7.5, suggesting that temperature alone was not responsible for the observed aggregation [54].

Cellular mechanisms by which subphysiological temperature culturing influences recombinant protein production & quality

Subphysiological temperature can have a positive effect on recombinant protein productivity from mammalian cells, although this is not universally the case. But what is/are the mechanism(s) by which an improvement in productivity is attained, or not, upon subphysiological temperature culturing? As described previously, subphysiological temperature culturing leads to extended culture longevity and cell cycle arrest and many of the original investigations attributed increases in cell-specific and volumetric productivity to these responses as there had been reports of a correlation between G1 cycle arrest and higher specific cellular productivity in mammalian cells [53,75]. However, even though cells residing in the G1 phase of the cell cycle has been linked with enhanced productivity, other phases of the cell cycle have also been reported as preferable for recombinant protein production. For example, Fox *et al.* reported that the S-phase of the cell cycle is the most productive in terms of recombinant protein yield in CHO cells [77]. During the S-phase, DNA synthesis occurs and it is likely that increased cell-specific productivity during G1 or S-phase cell cycle arrest is due to reduced competition between endogenous and recombinant mRNA and polypeptides for the cellular machinery required to synthesize, fold and assemble recombinant protein.

A number of more recent studies have suggested that changes in recombinant mRNA amounts in mammalian cells upon mild subphysiological temperature culturing is a key factor in enhanced productivity at such temperatures [5,23,26,70,77] and that growth arrest is not the key factor in enhanced productivity under such conditions [77]. During subphysiological temperature culturing the amounts of recombinant mRNA present have been shown to be elevated compared with those observed at 37°C [23,70,77] due to increased mRNA stability [70] through the formation of secondary structures and a reduction in the rate of degradation [22,77]. Several studies have now shown this effect; for example, one such study in CHO cells reported that the levels of recombinant IFN-γ mRNA increase at subphysiological temperatures and are responsible for the associated increase in IFN-γ production at such temperatures [67,77]. Another similar study reported that the specific productivity of a TNF receptor-immunoglobulin G1 Fc fusion protein (TNFR-Fc) was increased fivefold at 30°C above that at 37°C when expressed in CHO cells, and that the increase in specific productivity was related to an increase in TNFR-Fc mRNA levels [70]. These authors also showed that the increase in mRNA levels was due to a change in the half-life of the mRNA at 32°C (5.55 h at 32°C vs 3.69 h at 37°C) as a result of enhanced mRNA stability at the reduced temperature and not due to transcriptional activity [70].

As described previously, upon subphysiological temperature culturing of mammalian cells the expression of the CSP CIRBP is induced. Hence, investigators have determined the effect of overexpressing CIRBP at physiological temperature on recombinant protein expression. Overexpression of CIRBP in CHO cells stably expressing IFN-γ cultured at 37°C increased cell-specific IFN-γ productivity and led to a 40% increase in final IFN-γ yields. This approach generated higher yields than in cells not stably overexpressing CIRBP cultured using a biphasic temperature-shift approach or by culturing at one temperature – 37°C or 32°C [78]. This improvement was once again reported to be due to higher IFN-γ mRNA levels [78]. Whether this was due to CIRBP, an RNA-binding protein, directly stabilizing the recombinant mRNA levels, due to an indirect effect or just a cell line-specific effect was not established.

Increased mRNA amounts at mild subphysiological temperatures would seem to be a key factor in enhanced protein yield from mammalian cells at such temperatures. Global translation is attenuated at mild-hypothermic temperatures, but not halted [22,26], and hence the increased mRNA levels must more than compensate for this decrease such that additional mRNA molecules are actually being translated at the

reduced temperature compared with that at 37°C in order for enhanced protein synthesis to occur. This may also be aided by the global reduction in mRNA translation resulting in less 'competition' with endogenous mRNAs for the cellular translational machinery leading to more recombinant mRNAs being located on ribosomes. Indeed, it has been suggested that via manipulation of the translational machinery it may be possible to further enhance recombinant protein yields from mammalian cells at subphysiological temperatures [17]. The effect of miRs in modulating mRNA translation and protein synthesis is also likely to be a key determinant underpinning recombinant protein yields and quality at subphysiological temperatures [7,27,28]. Once temperatures drop below approximately 27°C translation is more-or-less completely halted [22] and, hence, even though mRNA levels may increase, protein production levels will not be.

The majority of studies described herein refer to observations and mechanisms in stably expressing recombinant cell lines. However, the application of subphysiological temperature culturing to enhance recombinant protein yields from mammalian cells has now been applied to transient systems to enhance yields [69]. A transient approach is often desirable when recombinant protein material is rapidly required for candidate screening, research purposes and preclinical studies [79]. For example, when using a double-knockout CHO-K1 cell line, whereby the two pro-apoptotic factors Bax and Bak had been knocked out using zinc finger nuclease technology, these cells produced high levels of lactate; however, this effect was mitigated against, and the transient productivity enhanced, by shifting the culture temperature to 31°C [79]. Others have used mild-hypothermic culturing in combination with additional treatments to successfully enhance the yields of various recombinant protein products from cultured mammalian cells [79–81]. The exact mechanism(s) by which subphysiological temperature enhances transient recombinant protein yields are yet to be elucidated, however, they are likely to include those defined in stable systems.

Conclusion

The subphysiological (<37°C) temperature culturing of mammalian cells clearly results in a coordinated cellular response invoking reduced growth rate, increased cell viability, a reduction in metabolism, reduced oxygen and ATP utilization, decreased toxic by-product generation, delayed apoptosis, cell cycle arrest, attenuation of global transcription and translation, and in turn protein synthesis, preferential translation of specific mRNAs, formation of mRNA secondary structure and mRNA stabilization, induction of CSPs, reduced protein turnover, improved tolerance against shear stress, alterations of the cell cytoskeleton and cell membranes, and in some situations enhanced cell-specific recombinant protein productivity and improved protein quality. The actual response is also temperature-dependent with different responses at mild hypothermic conditions to those activated at more severe temperatures. The exact cellular mechanisms that are activated and how these are interconnected is still not well understood, but the cell does actively respond to reduced temperature cultivation through a range of mechanisms and processes.

Future perspective

Understanding how the cellular mechanisms initiated upon the perception of cold-shock are coordinated, and why recombinant protein production is enhanced in some but not all cases under such conditions, will be crucial in developing new processes, cell lines and vector systems that utilize these responses to further enhance product yield and quality at reduced temperatures. Mechanistic modelling approaches that look across cellular processes are likely to be useful in predicting (and therefore helping to select) the effects of varying cellular responses under varying temperature (and indeed other) conditions, and such approaches are now being developed [82]. Future developments are likely to harness elements taken from the cellular cold-shock response to enhance recombinant protein synthesis or require the engineering of cells to combat negative regulation of processes that limit recombinant protein expression at reduced culture temperatures. New approaches are likely to involve manipulation of global regulators, which, while more challenging in predicting the outcome or in their manipulation, are likely to yield larger improvements. This approach, coupled with a bi-phasic temperature culture strategy, is likely to be utilized in the future not necessarily to further improve protein yields beyond those currently achievable, but as a method to help in the production of difficult-to-express proteins and as a means to more closely control recombinant protein product quality.

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Executive summary

Mammalian cells & the production of recombinant proteins

- Mammalian cells are often the expression system of choice for the production of industrial recombinant biopharmaceuticals, and these products are having a large impact of the pharmaceutical market.
- The most commonly used mammalian host cell line in industry is the Chinese hamster ovary (CHO) cell line.
- Traditionally mammalian cells are cultured at 37°C, however subphysiological temperature culturing (<37°C) is an approach used to try to increase recombinant protein yield and product quality.

The cellular response of mammalian cells to subphysiological temperature culturing

- mRNA translation is a key control point of gene expression regulated at both the global and mRNA specific level upon perception of cold stress in mammalian cells.
- Cell cycle arrest occurs upon perception of cold-stress in mammalian cells that reduces cell proliferation and accumulation of biomass.
- Two cold-shock proteins have been identified in mammalian cells, Rbm3 (RNA-binding motif protein 3) and CIRBP (cold-inducible RNA-binding protein).
- Cold-shock actually exposes cells to two types of stress; hypothermia due to reduction in temperature and oxygen related stress due to a higher dissolved oxygen concentration and decreased oxygen demand from the cells.

Subphysiological temperature culture as an approach to enhance recombinant protein productivity from mammalian cells

- Subphysiological temperature culturing can be used as an approach to enhance recombinant protein yields in terms of volumetric and cell-specific productivity.
- The effect is cell line, product and process specific.

The cellular mechanisms by which subphysiological temperature culturing influences recombinant protein production & quality

- Changes in recombinant mRNA amounts and turnover upon cold-stress is a key factor in enhanced productivity at subphysiological temperatures.

Conclusion

- Mammalian cells actively respond to subphysiological temperature culturing in a cell line, product and temperature specific manner.

Future perspective

- As our understanding of the mechanisms involved in the cold-shock response continues to be refined this will facilitate further manipulation of the cell and bioprocesses to improve product quality and to enhance protein yields further.
- Such future approaches include:
  - Mechanistic modelling of cellular processes.
  - Manipulation of global cellular regulators that effect recombinant protein production.
  - Use of a bi-phasic temperature shift approach coupled with engineering of cellular regulators.
  - Manipulation of cellular processes that elicit the same response as cold-stress at 37°C.

References

Papers of special note have been highlighted as:
▪ of interest

1 Walsh G. Biopharmaceutical benchmarks 2010. *Nat. Biotechnol.* 28(9), 917–924 (2010).

2 Kim JY, Kim YG, Lee GM. CHO cells in biotechnology for production of recombinant proteins: current state and further potential. *Appl. Microbiol. Biotechnol.* 93(3), 917–930 (2012).

3 Highsmith J. Biologic therapeutic drugs: technologies and global markets. BIO079B (2013).

4 Zhu J. Mammalian cell protein expression for biopharmaceutical production. *Biotechnol. Adv.* 30(5), 1158–1170 (2012).

5 Yoon SK, Song JY, Lee GM. Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. *Biotechnol. Bioeng.* 82(3), 289–298 (2003).

▪ **One of the first reports to relate cell-specific productivity to increases in mRNA at subphysiological temperatures.**

6 Smales CM, Masterton RJ. *5.39 - Biopharmaceutical Development*. In: *Comprehensive Biotechnology (Second Edition)*. Murray M-Y (Ed.). Academic Press, MA, USA, 489–498 (2011).

7 Gammell P, Barron N, Kumar N, Clynes M. Initial identification of low temperature and culture stage induction of miRNANA expression in suspension CHO-K1 cells. *J. Biotechnol.*130(3), 213–218 (2007).

▪ **The first report of microRNAs and their regulation in CHO cells, specifically the regulation of the expression of specific microRNAs upon subphysiological temperature culturing of recombinant CHO cell lines.**

8 Huang YM, Hu W, Rustandi E, Chang K, Yusuf-Makagiansar H, Ryll T. Maximizing productivity of

CHO cell-based fed-batch culture using chemically defined media conditions and typical manufacturing equipment. *Biotechnol. Prog.* 26(5), 1400–1410 (2010).

9 Mead EJ, Chiverton LM, Spurgeon SK *et al.* Experimental and *in silico* modelling analyses of the gene expression pathway for recombinant antibody and by-product production in NS0 cell lines. *PLoS ONE* 7(10), e47422 (2012).

10 Sellick CA, Croxford AS, Maqsood AR *et al.* Metabolite profiling of recombinant CHO cells: designing tailored feeding regimes that enhance recombinant antibody production. *Biotechnol. Bioeng.* 108(12), 3025–3031 (2011).

11 Hacker DL, De Jesus M, Wurm FM. 25 years of recombinant proteins from reactor-grown cells – where do we go from here? *Biotechnol. Adv.* 27(6), 1023–1027 (2009).

12 Hunt L, Hacker DL, Grosjean F *et al.* Low-temperature pausing of cultivated mammalian cells. *Biotechnol. Bioeng.* 89(2), 157–163 (2005).

13 Porter AJ, Racher AJ, Preziosi R, Dickson AJ: Strategies for selecting recombinant CHO cell lines for cGMP manufacturing: improving the efficiency of cell line generation. *Biotechnol. Prog.* 26(5), 1455–1464 (2010).

14 Pichler J, Galosy S, Mott J, Borth N. Selection of CHO host cell subclones with increased specific antibody production rates by repeated cycles of transient transfection and cell sorting. *Biotechnol. Bioeng.* 108(2), 386–394 (2011).

15 Jalah R, Rosati M, Kulkarni V *et al.* Efficient systemic expression of bioactive IL-15 in mice upon delivery of optimized DNA expression plasmids. *DNA Cell Biol.* 26(12), 827–840 (2007).

16 Sunley K, Butler M. Strategies for the enhancement of recombinant protein production from mammalian cells by growth arrest. *Biotechnol. Adv.* 28(3), 385–394 (2010).

17 Underhill MF, Marchant RJ, Carden MJ, James DC, Smales CM. On the effect of transient expression of mutated eIF2alpha and eIF4E eukaryotic translation initiation factors on reporter gene expression in mammalian cells upon cold-shock. *Mol. Biotechnol.* 34(2), 141–149 (2006).

18 Wurm FM. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat. Biotechnol.* 22(11), 1393–1398 (2004).

19 Al-Fageeh MB, Marchant RJ, Carden MJ, Smales CM. The cold-shock response in cultured mammalian cells: harnessing the response for the improvement of recombinant protein production. *Biotechnol. Bioeng.* 93(5), 829–835 (2006).

▪ **Published in 2006, this review outlines in detail the impact of the cold-shock response on mammalian cells and the current understanding at the time of how this related to recombinant protein production.**

20 Al-Fageeh MB, Smales CM. Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. *Biochem. J.* 397(2), 247–259 (2006).

21 Phadtare S, Severinov K. RNA remodeling and gene regulation by cold shock proteins. *RNA Biol.* 7(6), 788–795 (2010).

22 Roobol A, Carden MJ, Newsam RJ, Smales CM. Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming. *FEBS J.* 276(1), 286–302 (2009).

23 Marchant RJ, Al-Fageeh MB, Underhill MF, Racher AJ, Smales CM. Metabolic rates, growth phase, and mRNA levels influence cell-specific antibody production levels from *in vitro*-cultured mammalian cells at subphysiological temperatures. *Mol. Biotechnol.* 39(1), 69–77 (2008).

24 Wagstaff JL, Masterton RJ, Povey JF, Smales CM, Howard MJ. (1)H NMR spectroscopy profiling of metabolic reprogramming of Chinese hamster ovary cells upon a temperature shift during culture. *PLoS ONE* 8(10), e77195 (2013).

25 Hofmann S, Cherkasova V, Bankhead P, Bukau B, Stoecklin G. Translation suppression promotes stress granule formation and cell survival in response to cold shock. *Mol. Biol. Cell.* 23(19), 3786–3800 (2012).

26 Masterton RJ, Roobol A, Al-Fageeh MB, Carden MJ, Smales CM. Post-translational events of a model reporter protein proceed with higher fidelity and accuracy upon mild hypothermic culturing of Chinese hamster ovary cells. *Biotechnol. Bioeng.* 105(1), 215–220 (2010).

27 Barron N, Kumar N, Sanchez N *et al.* Engineering CHO cell growth and recombinant protein productivity by overexpression of miRNA-7. *J. Biotechnol.*151(2), 204–211 (2011).

28 Meleady P, Gallagher M, Clarke C *et al.* Impact of miRNA-7 over-expression on the proteome of Chinese hamster ovary cells. *J. Biotechnol.*160(3–4), 251–262 (2012).

29 Sonna LA, Fujita J, Gaffin SL, Lilly CM. Effects of heat and cold stress on mammalian gene expression. *J. Appl. Physiol.* 92(4), 1725–1742 (2002).

30 Neutelings T, Lambert CA, Nusgens BV, Colige AC. Effects of mild cold shock (25 degrees c) followed by warming up at 37°C on the cellular stress response. *PLoS ONE* 8(7), e69687 (2013).

31 Ohnishi T, Wang X, Ohnishi K, Takahashi A. p53-dependent induction of WAF1 by cold shock in human glioblastoma cells. *Oncogene* 16(11), 1507–1511 (1998).

32 Roobol A, Roobol J, Carden MJ *et al.* ATR (ataxia telangiectasia mutated- and Rad3-related kinase) is activated by mild hypothermia in mammalian cells and subsequently activates p53. *Biochem. J.* 435(2), 499–508 (2011).

▪ **First report to define the cellular mechanim by which reduced temperature results in activation of p53 and, thus, leads to cell cycle arrest.**

33 Fujita J. Cold shock response in mammalian cells. *J. Mol. Microbiol. Biotechnol.* 1(2), 243–255 (1999).

34 Hu T, Miller CM, Ridder GM, Aardema MJ. Characterization of p53 in Chinese hamster cell lines CHO-K1, CHO-WBL, and CHL: implications for genotoxicity testing. *Mutat. Res.* 426(1), 51–62 (1999).

35 Nishiyama H, Higashitsuji H, Yokoi H *et al.* Cloning and characterization of human CIRP (cold-inducible RNA-binding protein) cDNA and chromosomal assignment of the gene. *Gene* 204(1–2), 115–120 (1997).

36 Danno S, Itoh K, Matsuda T, Fujita J. Decreased expression of mouse Rbm3, a cold-shock protein, in Sertoli cells of cryptorchid testis. *Am. J. Pathol.* 156(5), 1685–1692 (2000).

37 Dresios J, Aschrafi A, Owens GC, Vanderklish PW, Edelman GM, Mauro VP. Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. *Proc. Natl Acad. Sci. USA* 102(6), 1865–1870 (2005).

38 Sumitomo Y, Higashitsuji H, Liu Y *et al.* Identification of a novel enhancer that binds Sp1 and contributes to induction of cold-inducible RNA-binding protein (cirp) expression in mammalian cells. *BMC Biotechnol.* 12, 72 (2012).

39 Sakurai T, Itoh K, Higashitsuji H *et al.* Cirp protects against tumor necrosis factor-alpha-induced apoptosis via activation of extracellular signal-regulated kinase. *Biochim. Biophys. Acta* 1763(3), 290–295 (2006).

40 Yang R, Zhan M, Nalabothula NR, Yang Q, Indig FE, Carrier F. Functional significance for a heterogenous ribonucleoprotein A18 signature RNA motif in the 3′-untranslated region of ataxia telangiectasia mutated and Rad3-related (ATR) transcript. *J. Biol. Chem.* 285(12), 8887–8893 (2010).

41 Al-Fageeh MB, Smales CM. Cold-inducible RNA binding protein (CIRP) expression is modulated by alternative mRNAs. *RNA* 15(6), 1164–1176 (2009).

42 Al-Fageeh MB, Smales CM. Alternative promoters regulate cold inducible RNA-binding (CIRP) gene expression and enhance transgene expression in mammalian cells. *Mol. Biotechnol.* 54(2), 238–249 (2013).

43 Thaisuchat H, Baumann M, Pontiller J, Hesse F, Ernst W. Identification of a novel temperature sensitive promoter in CHO cells. *BMC Biotechnol.* 11, 51 (2011).

▪ **Report on the identification of a highly active endogenous Chines hamster ovary gene promoter that displays conditionally temperature-inducible gene expression.**

44 Sieck GC. Molecular biology of thermoregulation. *J. Appl. Physiol.* 92(4), 1365–1366 (2002).

45 Thieringer HA, Jones PG, Inouye M. Cold shock and adaptation. *Bioessays* 20(1), 49–57 (1998).

46 Stapulionis R, Kolli S, Deutscher MP. Efficient mammalian protein synthesis requires an intact F-actin system. *J. Biol. Chem.* 272(40), 24980–24986 (1997).

47 Bosc C, Andrieux A, Job D. STOP proteins. *Biochemistry* 42(42), 12125–12132 (2003).

48 Jorjani P, Ozturk SS. Effects of cell density and temperature on oxygen consumption rate for different mammalian cell lines. *Biotechnol. Bioeng.* 64(3), 349–356 (1999).

49 Wellmann S, Buhrer C, Moderegger E *et al.* Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism. *J. Cell Sci.* 117(Pt 9), 1785–1794 (2004).

50 Rao PN, Engelberg J. Hela cells: effects of temperature on the life cycle. *Science* 148, 1092–1094 (1965).

51 Szperalski B, Jung C, Shao Z, Kantardjieff A, Hu WS. LDH-C can be differentially expressed during fermentation of CHO cells. *BMC Proc.* 5(Suppl. 8), P107 (2011).

52 Weidemann R, Ludwig A, Kretzmer G. Low temperature cultivation--a step towards process optimisation. *Cytotechnology* 15(1–3), 111–116 (1994).

53 Kaufmann H, Mazur X, Fussenegger M, Bailey JE. Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells. *Biotechnol. Bioeng.* 63(5), 573–582 (1999).

54 Hwang SJ, Yoon SK, Koh GY, Lee GM. Effects of culture temperature and pH on flag-tagged COMP angiopoietin-1 (FCA1) production from recombinant CHO cells: FCA1 aggregation. *Appl. Microbiol. Biotechnol.* 91(2), 305–315 (2011).

55 Sunley K, Tharmalingam T, Butler M. CHO cells adapted to hypothermic growth produce high yields of recombinant beta-interferon. *Biotechnol. Prog.* 24(4), 898–906 (2008).

56 Yoon SK, Kim SH, Song JY, Min. LG. Biphasic culture strategy for enhancing volumetric erythropoietin productivity of chinese hamster ovary cells. *Enzyme and Microbial. Technol.* 39, 362–365 (2006).

57 Fox SR, Patel UA, Yap MG, Wang DI. Maximizing interferon-gamma production by Chinese hamster ovary cells through temperature shift optimization: experimental and modeling. *Biotechnol. Bioeng.* 85(2), 177–184 (2004).

58 Rodriguez J, Spearman M, Tharmalingam T *et al.* High productivity of human recombinant beta-interferon from a low-temperature perfusion culture. *J. Biotechnol.*150(4), 509–518 (2010).

59 Rezaei M, Zarkesh-Esfahani SH, Gharagozloo M. The effect of different media composition and temperatures on the production of recombinant human growth hormone by CHO cells. *Res. Pharm. Sci.* 8(3), 211–217 (2013).

60 Barnabe N, Butler M. Effect of temperature on nucleotide pools and monoclonal- antibody production in a mouse hybridoma. *Biotechnol. Bioeng.* 44(10), 1235–1245 (1994).

61 Sureshkumar GK, Mutharasan R. The influence of temperature on a mouse hybridoma growth and monoclonal-antibody production. *Biotechnol. Bioeng.* 37(3), 292–295 (1991).

62 Reuveny S, Velez D, Macmillan JD, Miller L. Factors affecting cell growth and monoclonal antibody production in stirred reactors. *J. Immunol. Methods* 86(1), 53–59 (1986).

63 Beer C, Buhr P, Hahn H, Laubner D, Wirth M. Gene expression analysis of murine cells producing amphotropic mouse leukaemia virus at a cultivation temperature of 32 and 37 degrees C. *J. Gen. Virol.* 84(Pt 7), 1677–1686 (2003).

64 Ducommun P, Ruffieux PA, Kadouri A, Von Stockar U, Marison IW. Monitoring of temperature effects on animal cell metabolism in a packed bed process. *Biotechnol. Bioeng.* 77(7), 838–842 (2002).

65 Fogolin MB, Wagner R, Etcheverrigaray M, Kratje R. Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. *J. Biotechnol.* 109(1–2), 179–191 (2004).

66 Schatz SM, Kerschbaumer RJ, Gerstenbauer G, Kral M, Dorner F, Scheiflinger F. Higher expression of Fab antibody fragments in a CHO cell line at reduced temperature. *Biotechnol. Bioeng.* 84(4), 433–438 (2003).

67 Fox SR, Yap MX, Yap MG, Wang DI. Active hypothermic growth: a novel means for increasing total interferon-gamma production by Chinese-hamster ovary cells. *Biotechnol. Appl. Biochem.* 41(Pt 3), 265–272 (2005).

68 Rodriguez J, Spearman M, Huzel N, Butler M. Enhanced production of monomeric interferon-beta by CHO cells through the control of culture conditions. *Biotechnol. Prog.* 21(1), 22–30 (2005).

69 Wulhfard S, Tissot S, Bouchet S *et al.* Mild hypothermia improves transient gene expression yields several fold in Chinese hamster ovary cells. *Biotechnol. Prog.* 24(2), 458–465 (2008).

70 Kou TC, Fan L, Zhou Y *et al.* Detailed understanding of enhanced specific productivity in Chinese hamster ovary cells at low culture temperature. *J. Biosci. Bioeng.* 111(3), 365–369 (2011).

71 Yoon SK, Hong JK, Choo SH, Song JY, Park HW, Lee GM. Adaptation of Chinese hamster ovary cells to low culture temperature: cell growth and recombinant protein production. *J. Biotechnol.*122(4), 463–472 (2006).

72 Yoon SK, Hwang SO, Lee GM. Enhancing effect of low culture temperature on specific antibody productivity of recombinant Chinese hamster ovary cells: clonal variation. *Biotechnol. Prog.* 20(6), 1683–1688 (2004).

73 Chuppa S, Tsai YS, Yoon S *et al.* Fermentor temperature as a tool for control of high-density perfusion cultures of mammalian cells. *Biotechnol. Bioeng.* 55(2), 328–338 (1997).

74 Borys MC, Dalal NG, Abu-Absi NR *et al.* Effects of culture conditions on N-glycolylneuraminic acid (Neu5Gc) content of a recombinant fusion protein produced in CHO cells. *Biotechnol. Bioeng.* 105(6), 1048–1057 (2010).

75 Fussenegger M. The impact of mammalian gene regulation concepts on functional genomic research, metabolic engineering, and advanced gene therapies. *Biotechnol. Prog.* 17(1), 1–51 (2001).

76 Gomez N, Subramanian J, Ouyang J *et al.* Culture temperature modulates aggregation of recombinant antibody in cho cells. *Biotechnol. Bioeng.* 109(1), 125–136 (2012).

77 Fox SR, Tan HK, Tan MC, Wong SC, Yap MG, Wang DI. A detailed understanding of the enhanced hypothermic productivity of interferon-gamma by Chinese-hamster ovary cells. *Biotechnol. Appl. Biochem.* 41(Pt 3), 255–264 (2005).

78 Tan HK, Lee MM, Yap MG, Wang DI. Overexpression of cold-inducible RNA-binding protein increases interferon-gamma production in Chinese-hamster ovary cells. *Biotechnol. Appl. Biochem.* 49(Pt 4), 247–257 (2008).

79 Macaraeg NF, Reilly DE, Wong AW. Use of an anti-apoptotic CHO cell line for transient gene expression. *Biotechnol. Prog.* 29(4), 1050–1058 (2013).

80 Wulhfard S, Baldi L, Hacker DL, Wurm F. Valproic acid enhances recombinant mRNA and protein levels in transiently transfected Chinese hamster ovary cells. *J. Biotechnol.* 148(2–3), 128–132 (2010).

81 Galbraith DJ, Tait AS, Racher AJ, Birch JR, James DC. Control of culture environment for improved polyethylenimine-mediated transient production of recombinant monoclonal antibodies by CHO cells. *Biotechnol. Prog.* 22(3), 753–762 (2006).

82 Clarke C, Doolan P, Barron N *et al.* Predicting cell-specific productivity from CHO gene expression. *J. Biotechnol.* 151(2), 159–165 (2011).