

# Transcriptional Profiling of Apoptotic Pathways in Batch and Fed-Batch CHO Cell Cultures

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**Abstract:** Chinese Hamster ovary (CHO) cells are regarded as one of the “work-horses” for complex biotherapeutics production. In these processes, loss in culture viability occurs primarily via apoptosis, a genetically controlled form of cellular suicide. Using our “in-house” developed CHO cDNA array and a mouse oligonucleotide array for time profile expression analysis of batch and fed-batch CHO cell cultures, the genetic circuitry that regulates and executes apoptosis induction were examined. During periods of high viability, most pro-apoptotic genes were down-regulated but upon loss in viability, several early pro-apoptotic signaling genes were up-regulated. At later stages of viability loss, we detected late pro-apoptotic effector genes such as *caspases* and *DNases* being up-regulated. This sequential regulation of apoptotic genes showed that DNA microarrays could be used as a tool to study apoptosis. We found that in batch and fed-batch cultures, apoptosis signaling occurred primarily via death receptor- and mitochondria-mediated signaling pathways rather than endoplasmic reticulum-mediated signaling. These insights provide a greater understanding of the regulatory circuitry of apoptosis during cell culture and allow for subsequent targeting of relevant apoptosis signaling genes to prolong cell culture. © 2006 Wiley Periodicals, Inc.

**Keywords:** CHO cells; fed batch; apoptosis; expression; microarray

## INTRODUCTION

Currently, batch (BC) and fed-batch (FBC) cultures continue to be the main culture modes for a vast majority of industrial bioprocesses due to ease of operation and reliability. The usual practice in BC is to supply all the nutrients needed by the cells for the full duration of a run at the beginning of a culture. However, there is a practical limit as to how much nutrient can be tolerated initially. As soon as nutrient

starvation or depletion occurs, culture viability and productivity start to decrease. Thus FBC were developed whereby feeding at regular intervals is used to prolong culture life and productivity. Although FBC allow for prolonged culture life, viability still decreases but at a later time point compared to BC. In these processes, viability loss due to apoptosis often limits recombinant protein production and increases risk of product degradation (Arden and Betenbaugh, 2004; Fussenegger and Bailey, 1998; Laken and Leonard, 2001; Vives et al., 2003b). Despite being the predominant mode of cell death in bioreactor cultures, apoptosis signaling during bioreactor cultures has not been examined extensively. A better understanding of apoptosis signaling in culture is therefore crucial for understanding cell death in bioprocesses.

Apoptosis activating signals can be mediated either via extrinsic or intrinsic pathways. Extrinsic pathways are usually initiated by the binding of extracellular death ligands to receptors while intrinsic pathways are triggered in response to stimuli generated from within the cell. In the extrinsic death receptor-mediated pathway, receptors on the surface of the cell receive death signals. Upon activation, a group of proteins termed “death inducing signaling complex (DISC)” would assemble at the activated death receptors. As a result of DISC formation, procaspase-8 is autocatalytically cleaved into its active form (Curtin and Cotter, 2003). Active *caspase-8* then cleaves various proteins in the cell including procaspase-3, which results in apoptosis execution. In the intrinsic mitochondria-mediated pathway, apoptosis is initiated by the release of cytochrome *c* into the cytosol. Bcl-2 family members that can either promote survival or apoptosis play a pivotal role in this process (Cory et al., 2003; Gross et al., 1999; van Gurp et al., 2003). Upon activation, cytochrome *c* is released into the cytosol and binds to APAF-1 to form the apoptosome complex that mediates the

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activation of *caspase-9*. Once *caspase-9* is activated, it in turn activates executioner caspases such as *caspase-3* and *-7*. More recently, another intrinsic pathway involving the endoplasmic reticulum (ER) has been implicated in apoptosis regulation (Rao et al., 2004; Szegezdi et al., 2003). ER-mediated apoptosis induction can occur independently of the mitochondria and death receptors through *caspase-12* activation. Upon activation of ER-mediated apoptosis, *caspase-12* is translocated from the ER to the cytosol where it cleaves procaspase-9, which in turn activates the executioner caspase, *caspase-3*. "Cross-signaling" can also occur between these extrinsic and intrinsic pathways through various intermediates (Cory et al., 2003; Curtin and Cotter, 2003). The mechanisms described above illustrate briefly the complexity involved in apoptosis initiation, regulation, and execution.

First described by Schena et al. (1995), DNA microarray technology is based on the simultaneous hybridization of two different DNA populations (each labeled either with red or green fluorescence) onto microarrays containing thousands of distinct gene sequences. The ratio of fluorescence intensity then represents the ratio of expression between the two different populations. Transcriptional profiling using microarrays can be used as a means to track the up- and down-regulation of apoptosis signaling genes in cell culture.

In this article, we examined the apoptosis signaling pathways induced during BC and FBC of a Chinese Hamster ovary (CHO) cell line producing recombinant human interferon gamma ( $\text{IFN-}\gamma$ ) in bioreactors using DNA microarray. The primary aim of this study is to gain a better understanding of apoptosis signaling in BC and FBC in order to develop strategies to delay the onset of apoptosis and concomitantly prolong cell culture.

## MATERIALS AND METHODS

### Cell Line

CHO  $\text{IFN-}\gamma$  is a suspension Chinese Hamster ovary cell line that produces recombinant human interferon gamma (Scahill et al., 1983). CHO  $\text{IFN-}\gamma$  was maintained in commercially available serum-free HyQ CHO MPS media (Hyclone, Logan, UT) supplemented with 4 mM glutamine, 20 mM glucose, and 0.25  $\mu\text{M}$  methotrexate (Sigma, St. Louis, MO).

### Batch and Fed-Batch Cultures

BC and FBC operations were performed according to methods previously described by Wong et al. (2005). The FBC utilized an online dynamic feeding strategy where at intervals of 1.5 h, an automated measurement of residual glutamine concentration was taken. If the residual glutamine fell below setpoint control concentration of 0.3 mM, feeding was effected with concentrated media to raise glutamine concentration to 0.3 mM.

## Measurement of Cell Viability and Apoptosis

Cell viability was determined by trypan blue exclusion assay. Apoptosis was determined using an Ethidium Bromide/Acridine Orange assay adapted from Mercille and Massie (1994) that allowed for the classification of cells into apoptotic or non-apoptotic populations.

## Total RNA Purification

Total RNA was extracted from cell samples ( $1 \times 10^8$  cells) using Trizol<sup>®</sup> reagent according to the manufacturer's protocol (Invitrogen, CA). RNA samples were examined on a 1% denaturing RNA gel to insure no RNA degradation after extraction. Total RNA concentration and purity were determined using a UV-spectrometer (Amersham Biosciences, NJ).

## Microarray

Mouse microarrays were made from 65mer oligonucleotides (Compugen, NJ). The library consisted of 7,524 oligonucleotides, representing 7,445 unique genes. CHO cDNA microarrays were made from cDNA clones obtained by sequencing of CHO cDNA library as described by Wlaschin et al. (2005). cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) and labeled using Cy3 or Cy5 dyes (Amersham Biosciences) via amino-allyl coupling reaction. Hybridization of the Cy dye labeled probes to microarrays was conducted for a minimum of 16 h in a 42°C water bath in the dark. The microarrays were then washed and scanned on an Axon Genepix<sup>™</sup> 4000B scanner (Molecular Devices Corp, CA).

## Data Processing and Analysis

Comparative microarray analysis was performed according to MIAME guidelines (Brazma et al., 2001). Three technical replicates and two biological replicates were carried out for each time point where samples were collected, giving a total of 12 data sets. The technical replicates included a dye-swap in which the dye labeling was reversed to account for any sample dye-bias. Data normalization, including scale normalization between the slides, was conducted using methods adapted from Yang et al. (2002). Gene expression values were expressed as the  $\log_2$  intensity ratio of each time point with respect to the control. Genes with greater than two-fold change and  $P < 0.05$  were considered to be significantly regulated. Expression data was visualized and presented using TreeView (Eisen et al., 1998).

## Real-Time PCR

Nine genes from the microarray data were validated using quantitative real-time PCR. Primers were designed against conserved regions of the gene based on comparison with its equivalent homolog from mouse, rat, or human (Table I).

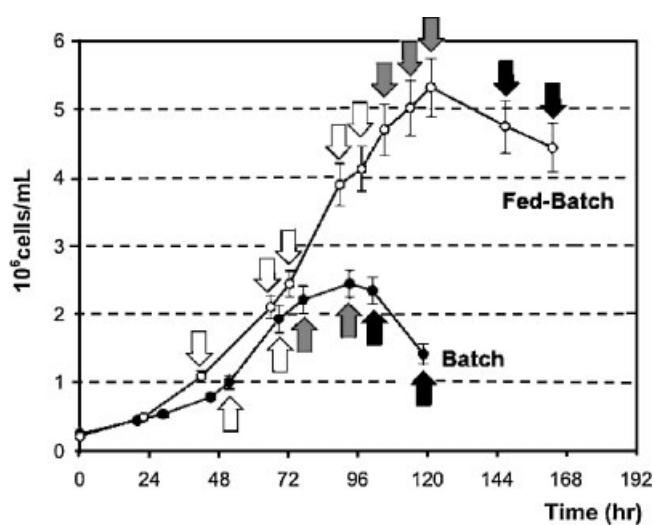
**Table I.** Sequence of primers used for gene specific quantification of *Rip1*, *Fadd*, *Faim*, *Requiem*, *Alg-2*, *Bim*, *Bad*, *Bax*, *Bak*, and  $\beta$ -actin using real time PCR.

Probe	Forward primer	Reverse primer
<i>Rip1</i>	5'-AGCTTTGGCATTGTCCTTGG-3'	5'-GCCTGTTCCCAGATTTTATGC-3'
<i>Fadd</i>	5'-GATATCGGATCCGCCACCATGG-3'	5'-TGCCTCCCTTCCACCAGGTCAG-3'
<i>Faim</i>	5'-TGGAGCTGCGAAAACCAAAG-3'	5'-AAACTCGCCTGCTGTCTCCAT-3'
<i>Requiem</i>	5'-TGGAGTAGCCAGAGCAATTG-3'	5'-TCGACGCTTTTACGCCAG-3'
<i>Alg-2</i>	5'-CAGCGGGTTGATAAAGACAGG-3'	5'-GCCAGCCTGTTTTCTCGG-3'
<i>Bim</i>	5'-TGTGGCAGAGAAGGTGGACAA-3'	5'-CGTCTGGATTACCTTGC GGTT-3'
<i>Bad</i>	5'-TCCGAAGGATGAGCGATGA-3'	5'-ACTGGATAATGCGCGTCCA-3'
<i>Bax</i>	5'-TGGAGCTGCAGAGGATGATTG-3'	5'-CCCAGTTGAAGTTGCCATCAG-3'
<i>Bak</i>	5'-TGATATTAACCGGCGCTACGA-3'	5'-AATAGGCTGGAGGCGATCTTG-3'
$\beta$ -actin	5'-AGCTGAGAGGGAAATTGTGCG-3'	5'-GCAACGG-AACCGCTCATT-3'

Multiple sequence alignments to determine conserved regions were performed using ClustalX (1.81) using default alignment parameters (Thompson et al., 1997). Real time PCR was carried out in duplicates using the ABI PRISM<sup>®</sup> 7000 Sequence Detection System using SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystem, CA). Sequence specificities were confirmed by DNA sequencing. Fold change in gene expression was calculated using the delta–delta threshold cycle ( $\Delta C_T$ ) method (Livak and Schmittgen, 2001) and normalized against *Cricetulus griseus*  $\beta$ -actin (U20114).

## RESULTS

As shown in Figure 1, based on the dynamic online fed-batch strategy developed previously (Wong et al., 2005), significant improvement in cell densities and viability were achieved for the FBC compared to the BC. However, in spite of the feeding strategy, CHO cells were still susceptible to loss in culture viability albeit at a later time. Based on the Ethidium Bromide/Acridine Orange assay (Merville and Massie, 1994), cell death in both BC and FBC was due mainly to



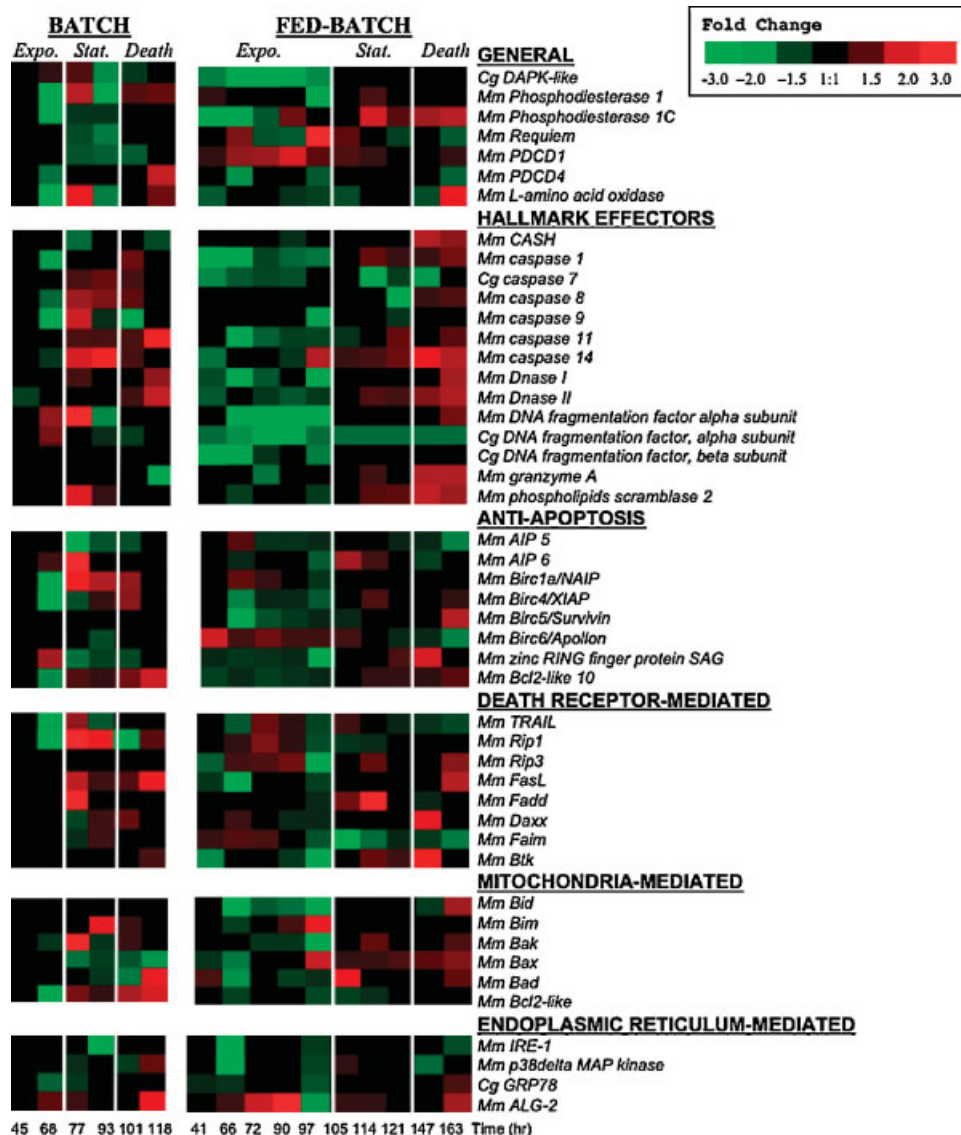
**Figure 1.** Cell growth during BC and FBC of CHO cells. Samples are taken during the exponential  $\uparrow$ , stationary  $\uparrow$  and death  $\uparrow$  phase of BC ( $\bullet$ ) and FBC ( $\circ$ ) for transcription analysis using microarray technology.

apoptosis (data not shown). Transcriptional analysis of samples collected across multiple time points during the exponential, stationary and death phases of BC and FBC were undertaken and the results summarized in Figure 2.

Figure 2 shows that for both BC and FBC, 47 out of a total of 170 apoptosis signaling genes present on the mouse and CHO microarrays were greater than two-fold up- or down-regulated in transcript levels, at a statistical confidence level of 95% ( $P < 0.05$ ). It should be noted that apoptosis signaling genes, which were not significantly differentially expressed, could still play important roles in apoptosis. During the exponential phase, the majority of apoptosis-related genes, such as the hallmark effectors, were either unchanged or down-regulated. Upon transition to the stationary phase, there was a noticeable increase in up-regulation of early (initiator) apoptosis genes involved in the death receptor- and mitochondria-mediated apoptosis signaling pathways. And, in the death phase, there were up-regulation of late (executioner) apoptosis genes namely *caspases*, *DNases*, and *DNA fragmentation factors*. These genes mediate events such as nucleus condensation, DNA fragmentation and phosphatidylserine exposure on the cell surface, which are physiological hallmarks of apoptosis (Evans and Aguilera, 2003). These results show that transcriptional profiling can be used to follow the sequential regulation of apoptosis in cell culture.

The validation of microarray data carried out using real time PCR is shown in Figure 3. The panel of apoptosis genes examined comprised of death receptor-mediated signaling genes (*Rip1*, *Fadd*); mitochondria-mediated signaling genes (*Bim*, *Bad*, *Bax*, and *Bak*); and other important apoptosis signaling genes (*Alg-2*, *Requiem*, and *Faim*). The real time PCR data showed that the trends in the differentially expressed genes were similar to the microarray data, indicating that expression profiling can be used reliably to follow apoptosis events.

Figure 4 shows a network comprising of the three major apoptosis signaling pathways which transduce death and survival signals and the interactions between these pathways for BC (Fig. 4A) and FBC (Fig. 4B) (Cory et al., 2003; Curtin and Cotter, 2003; Gross et al., 1999; Liston et al., 2003; Rao et al., 2004). This network integrates the death receptor-mediated (I), mitochondria-mediated (II), and ER-mediated



**Figure 2.** Apoptosis-related genes regulated during BC and FBC of CHO cells. Genes from CHO cDNA array are denoted by *Cg* (*Cricetulus griseus*) while those from mouse oligomers array are denoted by *Mm* (*Mus musculus*).

(III) apoptosis signaling pathways. Transcriptional profiling data as indicated in the legend are represented as blocks for the different culture phases (exponential, stationary, and death) and up- or down-regulated genes are color-coded.

From this complex signaling network, the following major observations were made:

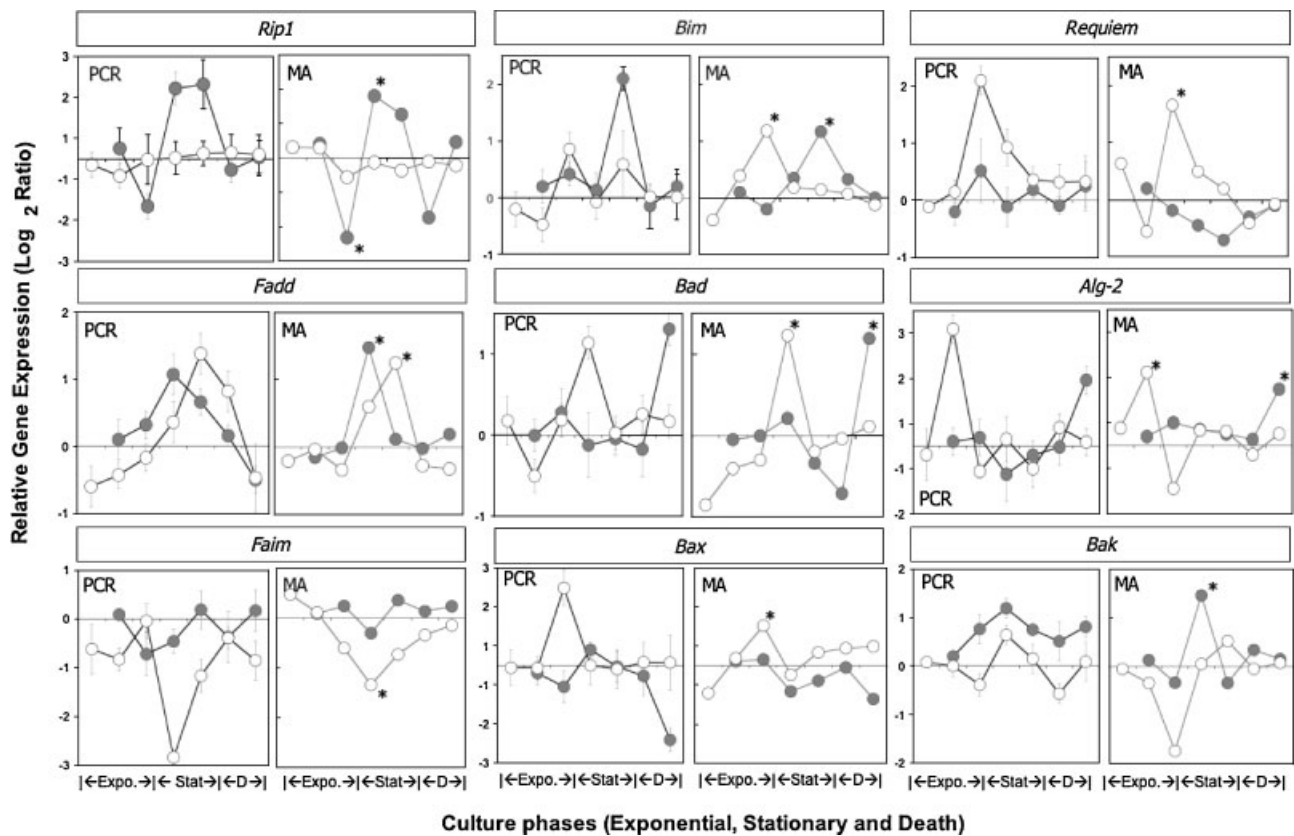
- (1) Up-regulation of *FasL* and *Fadd* in the death receptor-mediated apoptosis signaling pathway during BC and FBC.

*FasL* was up-regulated at the start of stationary phase for BC (Fig. 4A) but at the end of death phase for FBC (Fig. 4B). However, *Fas associated death domain (Fadd)* was up-regulated at the stationary phase for both BC and FBC. Both of these apoptosis-associated genes are involved in CD95 death receptor signaling.

In the case of BC, up-regulation of *Rip1*, which encodes for a DISC adaptor protein recruited by

TNF-R1 and DR3 death receptors (Curtin and Cotter, 2003; Peter et al., 1996), during the stationary phase in BC, indicated that in addition to CD95 death signaling, TNF-R1 and DR3 death receptors may also be involved in apoptosis. *Caspase-8*, a key initiator caspase, which is downstream of *Fadd/Rip* activation and forms part of the DISC complex, was also observed to be up-regulated at the stationary phase of BC.

In contrast, the lack of *Rip1* up-regulation in FBC seems to suggest that death receptor-mediated signaling occurred primarily via CD95 rather than TNF-R1 or DR3 death receptors. Evidence of the importance of CD95 death receptor signaling during FBC was further supported by the involvement of *Btk*, *Faim*, and *Cash* which have been shown to modulate CD95 apoptosis signaling (Goltsev et al., 1997; Schneider et al., 1999; Vassilev et al., 1999).



**Figure 3.** Validation of microarray expression profiles of apoptosis signaling genes across exponential (Expo), stationary (Stat) and death (D) phases of BC (●) and FBC (○) of CHO cells using real time PCR (real time PCR data (PCR) points represent averages of duplicates and error bars represent standard deviations; microarray data (MA) with \*indicating  $P < 0.05$ ).

- (2) Up-regulation of *Bim* and *Bad* in the mitochondria-mediated apoptosis signaling pathways during BC and FBC.

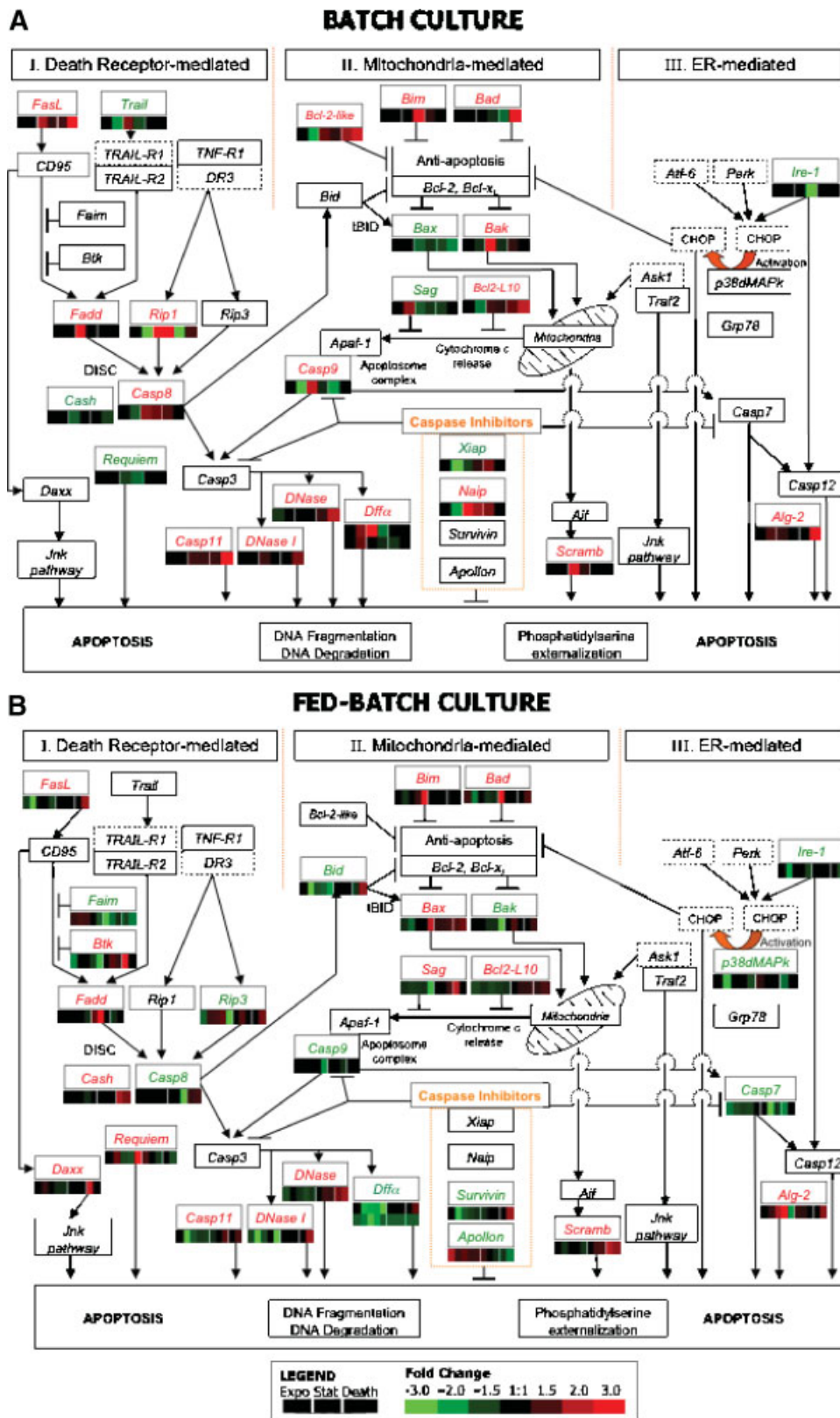
It was found that *Bim* was up-regulated during the stationary phase for BC (Fig. 4A) and just prior to stationary phase in FBC (Fig. 4B). However, *Bad* was up-regulated at the end of death phase for BC but at the start of stationary phase for FBC. *Bim* and *Bad* encode for BH3-only Bcl-2 family proteins that function as upstream sensors of death cues and intracellular damage in the mitochondria-mediated apoptosis signaling pathway.

Upon activation, *BIM* and *BAD* proteins can bind to *BCL-2* and *BCL-X<sub>L</sub>* proteins and neutralize their anti-apoptotic activities (Cory et al., 2003; Gross et al., 1999; O'Connor et al., 1998). Interestingly, both *Bcl-2* and *Bcl-x<sub>L</sub>*, were not differentially expressed during BC and FBC in this study. Instead, *Bcl2-L-10*, another Bcl-2 member, which can also prevent cytochrome *c* release from the mitochondria (Zhang et al., 2001), was up-regulated from the stationary phase until the death phase of both BC and FBC.

It has been reported that *Bim* and *Bad* do not induce apoptosis in the absence of *Bax* and *Bak* (Wei et al., 2001). In the case of BC, up-regulation of *Bak* but not *Bax* was observed during stationary phase. In contrast, during FBC, *Bax* but not *Bak* was up-regulated. Upon

activation, *BAX* and/or *BAK* proteins have been reported to undergo conformational changes which alter the mitochondrial membrane permeability leading to cytochrome *c* release and apoptosome complex formation. *Caspase-9*, a key initiator caspase of mitochondria-mediated apoptosis signaling is downstream of cytochrome *c* release. In this study, *Caspase-9* and *-8* were up-regulated in BC but in contrast, both caspases were down-regulated in FBC. The difference in the regulation of these two key caspases can be due to the differential expression of inhibitor of apoptosis protein (IAP) family members that can modulate caspases activities (Liston et al., 2003). This will be discussed in "Point (4)." (3) Down-regulation of *Ire-1* and up-regulation of *Alg-2* in the endoplasmic reticulum (ER)-mediated Apoptosis Signaling during BC and FBC.

The ER-mediated apoptosis signaling pathway is still poorly understood but proteins such as *IRE-1*, *PERK*, and *ATF-6*, which respond to unfolded/misfolded protein accumulation are known to play roles as ER stress sensors (Breckenridge et al., 2003; Rao et al., 2004). As shown in Figure 4A and B, *Ire-1* was down-regulated for both BC and FBC suggesting that protein misfolding may not be a major issue. Unfortunately, *Atf-6* and *Perk* were not represented on our microarrays. In addition, *Grp78*, which encodes for an ER chaperone protein that responds to ER stress (Rao et al., 2004) and



**Figure 4.** Apoptosis Signaling in BC (A) and FBC (B) of CHO cells (solid-lined box: genes represented in microarray; semi-solid box: genes not represented in microarray; genes labeled in red indicate significant upregulation while genes labeled in green indicate significant down-regulation in culture. Abbreviations as in Glossary).

both *Caspase-12* and *-7*, which encodes for caspases critical to ER-mediated apoptosis signaling (Rao et al., 2001; Szegezdi et al., 2003), were also not differentially expressed. Furthermore, *p38MAPk*, which encodes for a protein responsible for CHOP activation, was found to be down-regulated during FBC. It has been reported that CHOP activation occurs in response to ER stress (Rao et al., 2004). The down-regulation of *Ire-1* and *p38MAPk* coupled with the lack of differential gene expression in *Grp78*, *Caspase-7*, and *-12* seems to imply that apoptosis signaling via this ER-mediated pathway was not significant.

The up-regulation of *apoptosis linked gene 2 (Alg-2)* was observed in both BC (late death phase) and FBC (exponential phase). However, the exact role of *Alg-2* is not very clear. Rao et al. (2004) had suggested that *Alg-2* is part of an ER-stress induced caspase-activating complex but *Alg-2* has also been found to be involved in CD95 death receptor-mediated apoptosis (Jung et al., 2001).

- (4) Differential expression of inhibitors of apoptosis proteins (IAP).

Inhibitor of apoptosis protein (IAP) family members can associate with and inhibit caspase activities (Liston et al., 2003). It has been reported that the IAPs themselves are subject to negative regulation by *DIABLO* and *Omi* (Liston et al., 2003; van Gurp et al., 2003). As *DIABLO* and *Omi* were not differentially expressed in this study (data not shown), negative regulation of the IAPs was probably minimal. Four of these IAPs, *Birc1*, *Birc4*, *Birc5*, and *Birc6*, which are more commonly known as *NAIP*, *XIAP*, *Survivin*, and *Apollon* respectively, were differentially expressed during either BC (Fig. 4A) and FBC (Fig. 4B).

*NAIP* and *XIAP* were up-regulated during the stationary phase of BC but not FBC. When over-expressed, *NAIP* can inhibit *caspase-3* and *-7* while *XIAP* can inhibit *caspase-9* in addition to *caspase-3* and *-7* (Liston et al., 2003; Shin et al., 2001). In contrast, *Survivin* and *Apollon* were only differentially expressed during FBC and not BC. In FBC, *Survivin*, a potent inhibitor of *caspase-3* and *-7* (Liston et al., 2003), was only up-regulated very late in the death phase indicating that *Survivin* was a late survival response. *Apollon* on the other hand was up-regulated during the growth phase of FBC although the level of up-regulation decreased with time and ultimately, it was down-regulated from 160 h onwards. Cells that overexpress *Apollon* has been shown to be resistant to cell death induced by transient expression of *Caspase-8* and *-9* (Hao et al., 2004). The specific early up-regulation of *Apollon* in FBC could have accounted for the down-regulation of *Caspase-8* and *-9* in FBC but not BC as discussed in "Point (3)." However, despite its initial up-regulation, the decreasing expression of *Apollon* showed that this survival pathway was compromised with time in FBC.

## DISCUSSIONS

### Apoptosis-Related Cell Death in CHO Cell BC and FBC

Nutrient deprivation, hypoxia, toxic metabolite accumulation, growth factor withdrawal, and other factors can induce apoptosis (Arden and Betenbaugh, 2004; Laken and Leonard, 2001; Mercille and Massie, 1994; Vives et al., 2003a). It is interesting to note that for BC, the up-regulation of apoptosis genes such as *FasL*, *Rip1*, *Bak*, *Caspase-8*, and *-9* (Fig. 4A) occurred at the stationary phase, which coincided with the depletion of glucose and/or glutamine in the culture medium and loss in culture viability as reported by Wong et al. (2005). However, cell death in FBC was not due to nutrient limitation because of the feeding strategy employed. Since critical parameters such as dissolved oxygen and pH were tightly controlled and lactate and ammonia concentrations were below reported toxic levels (Lao and Toth, 1997), we concluded that these factors also did not induce apoptosis in FBC.

As shown in Figure 4B, the apoptosis genes *Fadd*, *Bim*, *Bad*, *Requiem*, and *Alg-2* were up-regulated in the exponential and stationary phases of FBC. The up-regulation of *Fadd*, which involves CD95 death receptor signaling, is known to be activated by IFN- $\gamma$  or other extracellular death ligands (Curtin and Cotter, 2003; Ossina et al., 1997; Spanaus et al., 1998; Wang et al., 2004). Although there could have been cross-reactivity between human IFN- $\gamma$ , which is the recombinant product with the CHO cell CD95 death receptors, it seems unlikely due to the following reasons. Firstly, Ossina et al. (1997) and Wang et al. (2004) reported that IFN- $\gamma$  induces *Bak* expression, but *Bak* was not up-regulated in FBC. Furthermore, CD95, *Bcl-2*, and *Bcl-x<sub>L</sub>* were not differentially expressed in this study, contrary to findings by Spanaus et al. (1998), which found that IFN- $\gamma$  induced *CD95* expression and down-regulated *Bcl-2* and *Bcl-x<sub>L</sub>*. As such, the up-regulation of *Fadd* during FBC cannot be attributed to the presence of recombinant human IFN- $\gamma$  in the culture medium activating the CHO cell CD95 death receptor. Thus, the up-regulation of *Fadd* is attributed to the binding of extracellular death ligands secreted either by the cell itself or by other surrounding cells (Curtin and Cotter, 2003).

We propose that the observed up-regulation of *Bim*, *Bad*, and *Requiem* may have resulted from a decreased production of autocrine growth factors during prolonged FBC triggering apoptosis and loss in culture viability. The up-regulation of *Bim* has been attributed to cytokine withdrawal by Dijkers et al. (2000) and *Bad* up-regulation has also been associated with growth factor deprivation (Gross et al., 1999; van Gurp et al., 2003). Glucose starvation is known to induce *Bad* up-regulation as well (Danial et al., 2003) but it is unlikely to be the cause of *Bad* up-regulation in our FBC since glucose was not limiting. *Requiem*, a zinc finger protein transcription factor, has also been shown to trigger apoptosis upon cytokine withdrawal (Gabig et al., 1994).

As discussed in “Point (3)” of the Section “Results,” the up-regulation of *Alg-2*, which has been implicated in both ER- and death receptor mediated apoptosis signaling was observed in both BC and FBC. However, other ER-mediated apoptosis signaling genes such as *Ire1*, *p38MAPk*, *Grp78*, *Caspase-7*, and *-12* were either down-regulated or not differentially expressed during FBC. As such, *Alg-2* up-regulation in this study was most likely due to CD95 death receptor-mediated apoptosis signaling rather than ER-mediated apoptosis signaling. In addition, it is speculated that ER stress due to protein misfolding was not an issue since the cell line has a relatively low specific productivity of recombinant protein (~1 pg/cell/day).

### Strategies to Delay Onset of Apoptosis in Culture

Genetic modification to confer apoptosis resistance is a promising strategy for apoptosis inhibition in culture systems (Arden and Betenbaugh, 2004; Laken and Leonard, 2001; Vives et al., 2003a). The most common genetic modification reported in literature involves overexpression of either *Bcl-2* or *Bcl-x<sub>L</sub>* to prevent mitochondrial release of cytochrome *c* (Arden and Betenbaugh, 2004; Laken and Leonard, 2001; Vives et al., 2003a). Alternatively, the viral homologs of *Bcl-2* such as *Bhrf-1* and *E1B19K* can also be used to confer apoptosis resistance. Other strategies include approaches that interfere with caspase activation such as anti-sense RNA against caspases or overexpression of caspases inhibitors such as *XIAP* and *Crma* (Kim and Lee, 2002; Sauerwald et al., 2002, 2003).

The strategy we propose to adopt to delay the onset of apoptosis is to target the apoptosis genes, which were up-regulated going from the exponential to the stationary phase. We postulated that these genes are involved in early apoptosis signaling. From BC (Fig. 4A), these potential targets include genes such as *FasL*, *Fadd*, *Bim*, and *Bak* and from FBC (Fig. 4B) the promising targets include genes such as *Fadd*, *Bim*, *Bad*, *Bax*, *Alg-2*, and *Requiem*.

Since *FasL* and *Fadd* are genes involved in CD95 death receptor apoptosis signaling, attenuation of CD95 death receptor signaling could be a promising strategy to delay the onset of apoptosis in culture. The construction of a dominant negative form of *Fadd* has been demonstrated by Chinnaiyan et al. (1996) to effectively suppress CD95 signaling and prevent the activation of downstream *caspase-8*. An alternatively strategy is the overexpression of *Faim* to suppress CD95 death receptor signaling, restoring this pro-survival pathway which was observed to be down-regulated in FBC (Fig. 4B).

To target *Bim*, *Bad*, *Bak*, and *Bax*, which are involved in mitochondria-mediated apoptosis signaling, the overexpression of *Bcl-2* or *Bcl-x<sub>L</sub>* has been reported to block the apoptosis signals induced by all four genes (Cory et al., 2003; Espoti and Dive, 2003; Gross et al., 1999). Specifically, *Bim* and *Bax* signaling can be suppressed by either *Bcl-2* or *Bcl-x<sub>L</sub>* (O'Connor et al., 1998) whereas

*Bad* and *Bak* signaling are more effectively suppressed by *Bcl-x<sub>L</sub>* (Espoti and Dive, 2003; Yang et al., 1995).

The two other promising apoptosis gene targets are *Alg-2* and *Requiem*. *Alg-2* is involved in both death receptor- and ER-mediated apoptosis signaling while the role played by *Requiem* in apoptosis signaling is still unclear. Although the exact signaling roles of these two genes are not well defined, targeting can be achieved by directly “silencing” these pro-apoptotic genes through the use of small interfering RNA (Hammond et al., 2001).

The gene targets discussed above comprised of genes involved in both death receptor- and mitochondria-mediated apoptosis signaling (Fig. 4A and B). The important implication is that to be effective in delaying the onset of apoptosis, it may be necessary to suppress both death receptor- and mitochondria-mediated signaling concurrently. A possible approach would be the overexpression of either *Bcl-2* or *Bcl-x<sub>L</sub>* to block mitochondria-mediated signaling while overexpressing *Fadd dominant negative* or *Faim* pro-survival genes to block receptor-mediated signaling concomitantly.

The apoptosis transcriptome analysis described here has enabled our group to develop CHO cell lines, which are apoptosis resistant (Wong et al., 2006). A similar strategy can be applied to other cell lines and conditions to identify key apoptosis signaling genes to prolong culture.

### GLOSSARY

<i>Aif</i>	Apoptosis inducing factor
<i>Alg-2</i>	Apoptosis linked gene 2
<i>Apaf-1</i>	Apoptotic protease-activating factor 1
<i>Ask1</i>	Apoptosis signal-regulating kinase
<i>Atf-6</i>	Activating transcription factor 6
<i>Birc1/Naip</i>	Baculoviral IAP repeat-containing protein 1/neuronal apoptosis inhibitory protein
<i>Birc4/Xiap</i>	Baculoviral IAP repeat-containing protein 4/X-linked inhibitor of apoptosis
<i>Birc5/Survivin</i>	Baculoviral IAP repeat-containing protein 4/SURVIVIN
<i>Btk</i>	Bruton agammaglobulinemia tyrosine kinase
<i>Cash</i>	Caspase 8 and <i>FADD</i> -like apoptosis regulator, Cflar
<i>Casp</i>	Caspase
<i>CHOP</i>	Gadd153
<i>DIABLO</i>	Direct IAP binding protein with low PI mRNA
<i>DISC</i>	Death inducing signaling complex
<i>Diffα</i>	DNA fragmentation factor alpha subunit
<i>Fadd</i>	Fas associated death domain
<i>Faim</i>	Fas apoptosis inhibitory molecule
<i>FasL</i>	Fas Ligand
<i>Grp78</i>	Glucose regulated protein of 78 kDa
<i>Perk</i>	PKR-like ER kinase
<i>Rip</i>	Receptor (TNFRSF)-interacting protein
<i>Sag</i>	zinc RING finger sensitive to apoptosis gene
<i>Scramb.</i>	Scramblase 2
<i>TNF-R1</i>	Tumor necrosis factor receptor 1
<i>Trail</i>	Tumor necrosis factor inducing ligand
<i>TRAIL-R1</i>	Tumor necrosis factor inducing ligand receptor
<i>TRAF2</i>	TNF receptor associated factor 2

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