METHODS AND COMPOSITIONS FOR INCREASING LONGEVITY AND PROTEIN YIELD FROM A CELL CULTURE

This application claims priority from U.S. Serial No. 60/590,349 filed on July 23, 2004. This application claims only subject matter disclosed in the aforementioned provisional application and therefore presents no new matter.

BACKGROUND OF THE INVENTION

Culturing cells in vitro, especially in large bioreactors, has been the basis of the production of numerous biotechnology products, and involves the elaboration by these cells of protein products into the support medium, from which these products are isolated and further processed prior to use clinically. The quantity of protein production over time from the cells growing in culture depends on a number of factors, such as, for example, cell density, cell cycle phase, cellular biosynthesis rates of the proteins, condition of the medium used to support cell viability and growth, and the longevity of the cells in culture (i.e., how long before they succumb to programmed cell death, or apoptosis). Various methods of improving the viability and lifespan of the cells in culture have been developed, together with methods of increasing productivity of a desired protein by, for example, controlling nutrients, cell density, oxygen and carbon dioxide content, lactate dehydrogenase, pH, osmolarity, catabolites, etc. For example, increasing cell density can make the process more productive, but can also reduce the lifespan of the cells in culture. Therefore, it may be desirous to reduce the rate of proliferation of such cells in culture when the maximal density is achieved, so as to maintain the cell population in its most productive state as long as possible. This results in increasing or extending the bioreactor cycle at its production peak, elaborating the desired protein products for a longer period, and this results in a higher yield from the bioreactor cycle.

Many different approaches have been pursued to increase the bioreactor cycle time, such as adjusting the medium supporting cell proliferation, addition of certain growth-promoting factors, as well as inhibiting cell proliferation without affecting protein synthesis. One particular approach aims to increase the lifespan of cultured cells via controlling the cell cycle by use of genes or antisense oligonucleotides to affect cell cycle targets, whereby a cell is induced into a pseudo-senescence stage by transfecting, transforming, or infecting with a vector that prevents cell cycle progression and induces a so-called pseudo-senescent state that blocks further cell division and expands the protein synthesis capacity of the cells in culture; in other words, the pseudo-senescent state can be induced by transforming the cells with a vector expressing a cell cycle inhibitor (Bucciarelli *et al.*, US Patent 2002/0160450 A1; idem., WO 02/16590 A2). The latter method, by inhibiting cell duplication, seeks to force cells into a state that may have prolonged cell culture lifetimes, as described by Goldstein and Singal (Exp Cell Res 88, 359-64, 1974; Brenner *et al.*, Oncogene 17:199-205, 1998), and may be resistant to apoptosis (Chang *et al.*, Proc Natl Acad Sci USA 97, 4291-6, 2000; Javeland *et al.*, Oncogene 19, 61-8, 2000).

Still another approach involves establishing primary, diploid human cells or their derivatives with unlimited proliferation following transfection with the adenovirus E1 genes. The new cell lines, one of which is PER.C6 (ECACC deposit number 96022940), which expresses functional Ad5 E1A and E1B gene products, can produce recombinant adenoviruses, as well as other viruses (e.g., influenza, herpes simplex, rotavirus, measles) designed for gene therapy and vaccines, as well as for the production of recombinant therapeutic proteins, such as human growth factors and human antibodies (Vogels *et al.*, WO 02/40665 A2).

Other approaches have focused on the use of caspase inhibitors for preventing or delaying apoptosis in cells. See, for example, US Patent No. 6,586,206. Still other approaches have tried to use apoptosis inhibitors such as members of the Bcl-2 family for preventing or delaying apoptosis in cells. See Arden *et al.*, *Bioprocessing Journal*, 3:23-28 (2004). These approaches have yielded unpredictable results; for example, in one study, expression of Bcl-2 increased cell viability but did not increase protein production. See Tey *et al. Biotechnol. Bioeng.* 68:31-43 (2000). Another example described overexpression of Bcl-2 proteins to delay apoptosis in CHO cells, but Bcl-xL increased protein production whereas Bcl-2 decreased protein production (see WO03/083093). A further example described experiments using expression of Bcl-2 proteins to prolong the survival of Sp2/0-Ag14 (ATCC # CRL-

1581, hereafter referred to as Sp2/0) cells in cultures; however, the cell density of the Bcl-2 expressing clones were 20 to 50% lower than that of their parental cultures, raising concerns for their practical application in biopharmaceutical industry (see WO03/040374).

It is apparent, therefore, that improved host cells for high level expression of recombinant proteins and methods for reliably increasing recombinant protein production, in particular the production of antibodies and antibody fragments, multispecific antibodies, fragments and single-chain constructs, peptides, enzymes, growth factors, hormones, interleukins, interferons, and vaccines, in host cells are greatly to be desired.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide improved host cells and methods to increase the longevity and recombinant protein yields of a cell culture by introducing into the cells agents that inhibit senescence or that promote cell survival, e.g., anti-apoptotic agents. The use of such agents preferentially increases the lifespan and viability of cells in culture used for the production of a desired recombinant protein, concomitantly increasing the productivity of such cells in culture, and thereby the optimal yield of the desired protein. Preferably, the apoptosis inhibitors used in the method of the present invention include but are not limited to Bcl-2 and its family members. Alternately, the longevity and recombinant protein yields of a cell clone can be improved by introducing into the cell agents that down-regulate the level of intracellular pro-apoptotic proteins, such as p53 and Rb, or up-regulate intracellular anti-apoptotic proteins, such as Bcl-2. Preferably, the regulatory agents used in the method of the present invention include, but are not limited to, human papillomavirus type 16 (HPV-16) oncoproteins E6 and E7, and combinations thereof. Additionally, caspase inhibitors, as described herein, may also contribute to blocking or reducing apoptosis, thus increasing cell survival and increasing the production of recombinant proteins by said cells in culture. A further class of anti-apoptotic agents that can be used in these cultures to enhance production of recombinant proteins includes certain members of the cytokine type I superfamily, such as erythropoietin (EPO). EPO, as a prototype molecule of this class, is a major modifier of apoptosis of multiple cell types, not just erythrocytes, and thus has more general cytoprotective functions, such as in endothelial cells, myocardial cells, tubular epithelial cells of the kidney, skin, and neurons [cf. review by P. Ghezzi and M. Brines, Cell Death and Differentiation 11 (suppl. 1), s37-s44, July 2004].

The present invention also teaches cell culture methods incorporating novel combinations of factors including, but not limited to, transfection vectors, screening and selection of cell clones with desired properties, cell culture media, growth conditions, bioreactor configurations, and cell types to create cell culture conditions in which the longevity of the cell culture is increased and/or made optimal and the yield of a desired recombinant protein is increased. These cell culture methods include suspension, perfusion, and fed-batch methods of production. See Tey *et al.*, J. Biotechnol. 79: 147-159 (2000); Zhang *et al.*, J. Chem. Technol. Biotechnol. 79: 171-181 (2004); Zhou *et al.*, Biotechnol. Bioeng. 55: 783-792 (1997).

Unless otherwise defined, all technical and scientific terms used in the invention have the same meaning as commonly understood by one of ordinary skill in the art. In addition, the contents of all patents and other references cited herein are incorporated by reference in their entirety.

BRIEF DESCRIPTION OF DRAWINGS/FIGURES

Figure 1 shows visual images of Sp2/0 and Sp-E26 cells treated with cycloheximide (+ CHX) or untreated (- CHX).

Figure 2 shows the results of screening HPV E6/E7 transduced cells that are more resistant to CHX treatment. A total of 55 clones were screened; in the first experiment, 31 clones were screened (top panel); in the second experiment, 24 clones were screened (bottom panel). Healthy cells of each clone were split into two equal portions. One was treated with CHX for 2 h and the other left untreated. The viable cells in these two cultures were then measured by MTT assay and the ratios of viable cell populations treated (CHX^+) vs. untreated (CHX^-) were plotted. As shown in the top panel, CHX treatment resulted in 30% reduction of viability in Sp2/0 cells, while only 6% reduction in Sp-E26 cells. Seven of the 31 clones screened (indicated by *) performed significantly better (<20% reduction of viability) than Sp2/0 but not as well as Sp-E26. For the 24 clones screened in the second experiment (bottom panel), CHX treatment resulted in ~50% reduction of viability in Sp2/0 cells and <20% reduction of viability in Sp-E26. Ten of the 24 clones (indicated by * or **) screened performed significantly better (<30% reduction) than Sp2/0, and 6 of them (indicated by **) matched or were better than Sp-E26 (<20%). E28 and E36 are two additional control clones that perform better than Sp2/0 but not as well as Sp-E26.

Figure 3 shows the dot plots of Guava Nexin V assay. The percentage of early apoptotic cells (Nexin V-positive and 7-AAD-negative) is indicated in the lower-right quadrant.

Figure 4 shows the DNA fragmentation in Sp2/0 treated by CHX. In contrast, Sp-E26 cells are resistant to the treatment.

Figure 5 shows the growth profiles of Sp2/0 and Sp-E26 cells in T-flasks. Healthy cells (>95% viability) were seeded in T-flasks at an initial cell density of 200,000/ml. Viable and dead cells were counted daily using Guava ViaCount reagent (Guava technologies, Inc.) and PCA instrumentation (Guava Technologies, Inc.). Accumulation of NH_4^+ and lactate also was monitored.

Figure 6 compares the growth profiles of Sp2/0 and Sp-E26 cells as determined for a batch culture in 3-L bioreactors. Healthy cells (>95% viability) were seeded in the bioreactors at an initial cell density of 250,000/ml. Cells were counted daily by trypan blue and microscope.

Figure 7 shows a representative immunoblot stained with Bcl-2 (100) antibody (Santa Cruz Biotech.) and developed with enhanced chemiluminescence for screening of clones for Bcl-2-EEE expression.

Figure 8 shows a graph of flow cytometry results using Guava Express. Cells were fixed and permeabilized before staining with phycoerythrin-conjugated anti-Bcl-2 antibody (Santa Cruz Biotechnology, Inc.) Several sub-clones are compared.

Figure 9 shows a graph of flow cytometry results using Guava Express. Cells were fixed and permeabilized before staining with phycoerythrin conjugated anti-Bcl-2 antibody (Santa Cruz Biotechnology, Inc.). Sp2/0, Raji and Daudi cells were compared to Bcl-2-EEE clones.

Figure 10 shows the results of immunoblot analyses of 665.B4.1C1, Sp2/0, Raji, Daudi, Sp-EEE (87-29 clone) and Sp-EEE (7-16 clone) cell lysates. A. Blots stained with a human Bcl-2 specific antibody (Santa Cruz Biotechnology, Inc). B. Blot stained with an anti-Bcl-2 antibody (Santa Cruz Biotechnology, Inc) that recognizes mouse and human Bcl-2.

Figure 11 shows growth curves (A) and viability (B) of Sp-EEE clones compared to Sp2/0 cells grown in media supplemented with 10% fetal bovine serum.

Figure 12 shows growth curves (A) and viability (B) of Sp-EEE clones compared to Sp2/0 cells grown in media supplemented with 1% fetal bovine serum.

Figure 13 shows growth curves (A) and viability (B) of Sp-EEE clones compared to Sp2/0 cells grown in serum-free media.

Figure 14 shows methotrexate kill curves for Sp-EEE (87-29 clone) cells.

Figure 15 shows a graph of flow cytometry results using Guava Express comparing Sp-EEE clones grown in the presence or absence of 1mg/ml zeocin. Cells were fixed and permeabilized before staining with phycoerythrin conjugated anti-Bcl-2 antibody (Santa Cruz Biotechnology, Inc).

Figure 16 shows the map of the pdHL2 vector used to transfect Sp2/0 cells to obtain the 665.2B9 clone with humanized antibody sequences and the SV40 promoter and enhancer sequences.

Figure 17 shows the map of DNA plasmid with incorporated *Bcl-2* gene, used for transfection of clone 665.2B9

Figures 18 and 19 show the growth profiles of Bcl-2 transfected clones 665.2B9#4, Bcl-2 negative clones and untransfected control. Healthy cells (>95% viability) were seeded in 24-well plates at an initial cell density of 400,000/ml. Viable and dead cells were counted daily using Guava ViaCount reagent and PCA instrumentation.

Figures 20 and 21 show growth profiles of Bcl-2 transfected clone 665.259 #4 and Bcl-2 negative clones in different MTX concentration. Healthy cells (>95% viability) were seeded in T-flasks at initial cell density of 100,000/ml. Viable cell density and viability were counted daily using Guava ViaCount reagent and PCA instrumentation.

Figure 22 shows the levels of human Bcl-2 expressed by clone 665.2B9#4 in increasing concentrations of MTX and clone #13 detected by Western blotting.

Figures 23 and 24 show the profiles of cell viability and viable cell density, respectively, of clone 665.2B9#4 cultured in 0.6 and 1 μM of MTX and the Bcl-2-negative clone #13 cultured in 0.3 μM MTX with or without spiking L-glutamine and glucose. Healthy cells (>95% viability) were seeded in roller bottles at an initial cell density of 200,000/ml. On day 2 and 4 (arrows indicated), a nutrient supplement solution containing glucose and L-glutamine was added to the "spiked" culture. Viable and dead cells were counted daily using Guava ViaCount reagent and PCA instrumentation.

Figure 25 shows the process schematics for bioeractor feeding strategies.

Figure 26 shows the growth curves (VCD and the viability) of 665.2B9.1E4 and 665.B4.1C1 cell lines by Process #1, which does not feed recombinant insulin, and Process #2, which is based on Process #1 with a modified linoleic acid and lipid feeding schedule and an additional feeding of insulin.

Figure 27 shows the antibody yields of 665.2B9.1E4 and 665.B4.1C1 cell lines in Processes #1 and #2. The final yield of 665.2B9.1E4 cells was 0.42 g/L in Process #1 and 0.55 g/L in

Process #2. For comparison, 665.B4.1C1 cells delivered a higher final yield of 1.5 g/L in both processes.

Figure 28 shows the daily specific antibody productivities (per cell basis). As shown in the figure, the 665.2B9.1E4 cells had an average daily $Q_{[MAb]}$ of approximately 15 pg/cell/day throughout the course of cultivation for both processes. The additional day of grown at the highest VCD in Process #2 resulted in a higher final antibody concentration.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides improved compositions, including host cell lines, and methods for enhanced production of recombinant proteins in such cell lines. Cell lines have been created that constitutively express one or more anti-apoptotic genes and that can be transfected with an expression construct encoding a protein or peptide of interest, where expression of the anti-apoptotic gene(s) prolongs survival of the transfected cell in culture and provides for enhanced yields of the protein or peptide of interest.

Specifically, the present inventors have created from Sp2/0 myeloma cell line two novel cell lines, referred to as Sp-E26 and Sp-EEE, which show enhanced survival in batch culture. Sp-E26 and Sp-EEE constitutively express the E6 and E7 proteins of HPV-16 and a Bcl-2 mutant, referred to as Bcl-2-EEE, respectively. In addition, recombinant protein production, and particularly production of recombinant antibodies and antibody fragments, can be improved upon transfecting either Sp-E26 or Sp-EEE with an expression vector for the recombinant protein of interest. The E6/E7 or Bcl-2-EEE proteins delay induction of apoptosis in the host cells and permit enhanced recombinant protein production in the host cells. Protein production can be boosted still further by addition of one or more caspase inhibitors (e.g., caspase 1 and/or 3 inhibitors) (Bin Yang et al. *Nephron Experimental Nephrology* 2004;96:e39-e51), and/or by addition of one or more members of the cytokine type I superfamily, such as erythropoietin (EPO), into the growth medium of the cells. A pan-caspase inhibitor is particularly effective in this regard.

The present inventors also have found that production of recombinant proteins, such as antibodies or antibody fragments, can be significantly enhanced in the host cell by co-expression of an apoptosis inhibitor, such as Bcl-2. In particular, protein production is significantly enhanced in a myeloma cell line, such as Sp2/0, that is stably transfected with an expression vector encoding an antibody or antibody fragment and that is co-transfected with an expression vector encoding an apoptosis inhibitor, such as Bcl-2. Increased production of

antibody can also be obtained from a host cell transfected with the E6/E7 gene. Recombinant protein production can be boosted still further by addition of one or more caspase inhibitors into the growth medium of the cells. A pan-caspase inhibitor is particularly effective in this regard. Also, recombinant protein production can be enhanced by feeding EPO, or another anti-apoptotic cytokine, into the medium of the cell culture.

Physiological, or programmed, cell death, referred to as apoptosis (Kerr *et al.*, Br J Cancer., 26:239-257, 1972) is essential for proper tissue development and maintenance and is controlled by an intrinsic genetic program that has been conserved in evolution (Ellis *et al.*, Annu Rev Cell Biol, 7, 663–698, 1991). Hence, when cells grow in artificial environments, such as *ex vivo* cultures, this genetic endowment results in a finite lifespan. Therefore, the utility of such cell cultures for the production of proteins used in medicine and industry, as well as research, is dependent on maintaining such cultures for extended lifespan, or cycles, before they die according to apoptotic mechanisms.

Methods and agents have been discovered that act independently on cell proliferation and cell death events, by differentiating cell cycle from apoptotic effects. Bcl-2, a well-known intracellular regulator of apoptosis (Vaux *et al.*, Nature 335, 440-2, 1988), is a proto-oncogene that has been found to have an anti-apototic effect that is genetically different from its inhibitory influence on cell cycle entry (Huang *et al.*, EMBO J 16, 4628-38, 1997). Two homologues of Bcl-2, Bcl-x_L and Bcl-w, also extend cell survival, but other members of the Bcl-2 family, such as Bax and Bak, are pro-apoptotic (Oltvai *et al.*, Cell 74, 609-19, 1993; Chittenden *et al.*, Nature 374, 733-6, 1995; Farrow *et al.*, Nature 374, 731-3, 1995; Kiefer *et al.*, Nature 374, 736-9, 1995). Other anti-apoptotic genes include *Bcl-6* and *Mcl-1*.

Thus, Bcl-2 and certain of its family members exert protection against apoptosis, and it was therefore hypothesized as a method to increase the lifespan of certain host cells in culture that are used for the production of proteins, thereby enhancing the amount of proteins produced and isolated. Since antibodies are produced by B-lymphocytes, particularly by myeloma cells, over-expression of an anti-apoptotic Bcl-2 family member, such as Bcl-2, Bcl-x_L, Bcl-w or mutant varieties of these proteins, inhibits apoptosis, resulting in increased cell density and longer culture survival. Hence, transfection of anti-apoptotic Bcl-2 family genes avoids the necessity to prolong the cell culture by interfering with the cell cycle per se, as others have proposed (*ibid.*). Similarly, transfection of fibroblasts with genes for Bcl-2 results in over-expression of Bcl-2 in these cells, resulting again in an antagonism of apoptosis and increasing the lifespan of these cells, with a concomitant increase in the production and

isolation of recombinant proteins. It has also been observed that upon cytokine withdrawal, interleukin-6 (IL-6)-dependent murine myeloma cells expire as if they undergo apoptosis. It was also found that IL-6-receptors in such cells could be regulated by Bcl-2 or Bcl-xL in extending apoptosis (Schwarz *et al.*, Cancer Res 55:2262-5, 1995).

Recent literature has also demonstrated that a mutant Bcl-2 possessing three point mutations (T69E, S70E and S87E) exhibited significantly more anti-apoptotic activity compared to wild type or single point mutants (Deng *et al.*, PNAS (101) 153 – 158, 2004). Thus, the invention teaches the construction of an expression vector for a Bcl-2-EEE triple mutant, which was then used to transfect Sp2/0 cells to create Sp-EEE clones and subclones that show improved longevity and recombinant protein production.

Other agents, such as oncogenic viruses, can also oppose apoptosis as part of their eliciting cellular immortalization and ultimately complete malignant transformation, such as high-risk type HPV oncoproteins E6 and E7 (Finzer *et al.*, Cancer Lett 188, 15-24, 2002). For example, the viral E6 protein effectively blocks the epidermal apoptotic response to ultraviolet light (Storey, Trends Mol Med 8, 417-21, 2002). It has also been suggested, from indirect evidence, that the human papillomavirus may cause reduced apoptosis in squamous (but not basal cell) carcinoma (Jackson *et al.*, Br J Cancer 87, 319-23, 2002). However, not all papillomavirus oncoproteins have anti-apoptotic effect. For example, other studies have reported that the papillomavirus E6 protein of bovine species sensitizes cells to apoptosis (Liu *et al.*, Virology 295, 230-7, 2002), which is in contrast to other studies showing that HPV-16 E7 gene protects astrocytes against apoptosis induced by certain stimuli (Lee *et al.*, Yonsei Med J 42, 471-9, 2001). By use of E6-binding peptide aptamers, direct experimental evidence was obtained that HPV E6 oncoprotein has anti-apoptotic activity in HPV-positive tumor cells (Butz *et al.*, Proc Natl Acad Sci USA 97, 6693-7, 2000). However, other HPV oncoproteins can have the opposite effect; the E2 protein induces apoptosis in the absence of other HPV proteins (Webster *et al.*, J Biol Chem 275, 87-94, 2000). Continuous expression of both the E6 and E7 proteins is known to be required for optimal proliferation of cervical cancer cells and that the two viral proteins exert distinct effects on cell survival (DeFilippis *et al.*, J Virol 77, 1551-63, 2003). The primary intracellular target attributed to HPV-16 E6 is p53. E6 forms a ternary complex with p53 and a cellular ubiquitin ligase, E6AP, resulting in the ubiquitination and degradation of p53 through the proteosome pathway and inactivation of p53. On the other hand, HPV-16 E7 protein interacts and destabilizes the tumor suppressor protein Rb. Moreover, levels of a variety of other intracellular proteins involved in apoptosis

and cell cycle pathways were reported to be regulated by E6 and E7 transformation, such as Bcl-2, Bcl-xL, p73, MDM2, p21, cyclins and cdc, cdk proteins, etc. Changes in the expression of these proteins will greatly influence the physiological properties of the cell. The present inventors therefore hypothesized that transfection of cells in culture by HPV-16 E6 and E7 would be very effective in generating genetically modified clones that are resistant to aging-culture-condition induced apoptosis and, therefore, prolong the lifespan of the cell culture. It was also postulated that introduction into a cell of either HPV-16 oncoprotein E7 or E6 alone might be sufficient to generate genetically modified clones with improved resistance to aging-culture-condition induced apoptosis. When the cell is a recombinant protein-producing clone, the improved physiological properties would in turn translate into enhanced overall protein productivity.

Generation of New Host Cells Expressing Viral Anti-apoptotic Genes

Host cells, such as myeloma host cells, can be generated that constitutively express viral anti-apoptotic genes, such as HPV-16 E6 and E7 proteins. These host cells can be transfected with an expression vector that encodes a recombinant protein of interest and co-expression of the anti-apoptotic genes results in significantly increased production of the recombinant protein.

The host cell can be essentially any host cell suitable for recombinant protein production that can be stably transformed with the viral anti-apoptosis genes. For many recombinant proteins, host cells such as CHO and COS cells are advantageous, while for other proteins, such as antibodies, host cells such as myeloma cells and CHO cells are the common choices. The viral genes can be introduced into the host cell by any suitable method that results in constitutive or inducible expression of the genes, *i.e.*, any method that permits stable integration of the genes into the host cell chromosome while permitting expression of the genes. Methods for stable transformation of host cells with a gene of interest are well known in the art. A particularly advantageous method is to use a retroviral vector that encodes the viral anti-apoptosis genes. Suitable vectors include the LSXN vector (Miller *et al.* Biotechniques 7, 980-90, 1989).

Advantageously, the vector used to transfect the host cell contains a selectable marker that permits selection of cells containing the vector. Suitable selection markers, such as enzymes that confer antibiotic resistance on transfected cells, are well known in the art. After transfection, cells are maintained in a medium containing the selection agent, such as an

antibiotic, and screened for resistance to the marker. Cells can be selected and cloned by limiting dilution using conventional methods.

The ability of the viral anti-apoptosis genes to increase cell viability can be tested by challenging the cells with an agent that induces apoptosis, such as cycloheximide (CHX). Cells that do not express the viral anti-apoptosis genes tend to demonstrate significant onset of apoptosis, whereas cells expressing the genes exhibit drastically reduced apoptotic activity. Methods of detecting apoptosis are well known in the art and include, for example, cell surface FITC- Annexin V binding assay, DNA laddering assay and TUNEL assay.

Upon selection of suitable cells expressing the viral anti-apoptosis genes, the cells can be transfected with an expression vector encoding the recombinant protein of choice. The expression vector can be a vector suitable for transient expression or, advantageously, can be an episomal vector containing a eukaryotic origin of replication, or an amplifiable vector that permits stable integration and subsequent gene amplification of the expression cassette. Suitable vectors are well known in the art and include, for example, the pdHL2 vector, which is particularly suited for production of antibodies and antibody fragments. When an amplifiable expression cassette is used, it advantageously contains a selectable marker that is different from the selectable marker used in the retroviral vector, to allow selection of transfected cells. Once again, suitably transfected cells can be selected and then cloned by limiting dilution.

Upon selection of suitable clones, the cells can be placed in a suitable medium and cultured to produce the desired protein of interest. The medium can contain serum or, preferably, be serum-free. In addition, cell longevity and protein production also can be increased by adding one or more caspase inhibitors (e.g., caspase 1 or 3) to the culture medium. Preferably the caspase inhibitor acts to inhibit one or more of caspase 3, caspase 9 and/or caspase 12. A cell-penetrating caspase inhibitor advantageously is used, and a pan-caspase inhibitor is particularly advantageous. Suitable inhibitors such as Z-VAD-fmk and Ac-DEVD-cho are well known in the art. Alternatively, the cell line can be further transfected to express a caspase inhibitor, such as Aven or XIAP, to enhance its growth properties by affecting apoptosis. In this regard, certain members of the cytokine type I superfamily, such as EPO, can also increase cell survival by having anti-apoptotic and cytoprotective actions.

The methods described above generate a cell line that can be used for transfection with essentially any desired gene. However, the skilled artisan will recognize that established cell lines that constitutively express a desired protein, and particularly a recombinant protein, can

be subsequently transformed with a suitable vector encoding the viral or Bcl-2 family anti-apoptosis genes. See Example 2 below.

The protein of interest can be essentially any protein that can be produced in detectable quantities in the host cell. Examples include traditional IgG type antibodies, $F(ab')_2$ or Fab fragments, scFv, diabody, IgG-scFv or Fab-scFv fusion antibodies, IgG- or Fab-peptide toxin fusion proteins, or vaccines [e.g., including not limited to, Hepatitis A, B or C; HIV, influenza viruses, respiratory syncytial virus, papilloma viruses, Herpes viruses, Hantaan virus, Ebola viruses, Rota virus, Cytomegalovirus, Leishmania RNA viruses, SARS, malaria, tuberculosis (Mycobacteria), Anthrax, Smallpox, Tularemia, and others listed in www.vaccines.org, incorporated herein by reference in its entirety] . The host cells described herein are particularly suitable for highly efficient production of antibodies and antibody fragments in myeloma cell lines as described in Examples 1 and 2, as well as recombinant growth factors (e.g., EPO, G-CSF, GM-CSF, EGF, VEGF, thrombopoietin), hormones, interleukins (e.g., IL-1 through IL-31), interferons (e.g., alpha, beta, gamma, and consensus), and enzymes. These methods could be applied to any number of cell lines that are used for production of recombinant proteins, including other myeloma cell lines, such as murine NSO or rat YB2/0; epithelial lines, such as CHO and HEK 293; mesenchymal cell lines, such as fibroblast lines COS-1 or COS-7; and neuronal cells, such as retinal cells, as well as glial and glioma cells.

Recombinant Antibody Expression in Cells Expressing Apoptosis Inhibitors

Prior work has described the effects of co-expressing Bcl-2, a naturally occurring apoptosis inhibitor, in recombinant CHO cells producing a chimeric antibody. See Tey *et al.*, *Biotechnol. Bioeng.* 68:31-43 (2000). Although increased cell culture life was observed, antibody production did not increase over equivalent cells that lacked Bcl-2 expression. However, the present inventors have found that production of recombinant antibody from myeloma cells is significantly increased when the cells also express Bcl-2.

Advantageously, the myeloma cell line is stably transfected with an expression cassette encoding the antibody or antibody fragment. A suitable expression cassette contains one or more promoters that controls expression of the antibody heavy and light chains (of single chain in the case of an scFv) together with a selectable marker as described above. A particularly useful vector is pdHL2, which contains a selectable marker gene comprising a promoter operatively linked to a DNA sequence encoding a selectable marker enzyme; a

transcription unit having a promoter operatively linked to a DNA sequence encoding the protein of interest; an enhancer element between the selectable marker gene and the transcription unit, which stimulates transcription of both the selectable marker gene and the first transcription unit compared to the transcription of both the selectable marker gene and the first transcription unit in the absence of the first enhancer. The vector also contains a blocking element having a promoter placed between the first enhancer and the selectable marker gene, which selectively attenuates the stimulation of transcription of the selectable marker gene. V_H and V_L sequences can be ligated into pdHL2, which is an amplifiable vector containing sequences for the human light chain constant region, the heavy chain constant region, and an amplifiable *dhfr* gene, each controlled by separate promoters. See Leung et al., Tumor Targeting 2:184, (1996) and Losman et al., Cancer 80:2660-2667, (1997). This vector can be transfected into cells by, for example, electroporation. Selection can be performed by the addition of 0.1 μM or a suitable concentration of methotrexate (MTX) into the culture media. Amplification can be carried out in a stepwise fashion with increasing concentration of MTX, up to 3 μM or higher. Cells stably transfected with the expression cassette and that constitutively express the antibody of interest can therefore be obtained and characterized using methods that are well known in the art. See also Example 4, below. After selection and cloning, the antibody-expressing cell line can then be transfected with an expression vector that encodes an anti-apoptosis gene, such as Bcl-2. For example, the vector pZeoSV (Invitrogen, Carlsbad, CA) containing the *Bcl-2* gene fused to an SV40 promoter is transfected into the cell using a suitable method such as electroporation, and selection and gene amplification can be carried out if necessary. Antibody production using the resulting cell line can be carried out as above and compared to production in cells that do not express an apoptosis inhibitor.

The methods describe initial preparation of a cell line expressing an antibody or antibody fragment that is subsequently transfected with a vector expressing Bcl-2 or a similar inhibitor. However, the skilled artisan will recognize that cell lines can be established that constitutively express Bcl-2 or another anti-apoptotic protein, which can be subsequently transformed with a suitable vector encoding the antibody or antibody fragment.

Representative examples to illustrate the present invention are given below. Example 1 describes the incorporation of HPV-16 E6/E7 into Sp2/0 cell leads to an improved cell clone, Sp-E26, showing characteristics of reduced/delayed apoptosis. Example 2 describes a method to improve host cell lines by over-expression of the HPV-16 E7 element alone. Example 3

describes using the improved cell, Sp-E26, as a host to develop cell clones producing a recombinant Ab. Example 4 describes the enhanced production of Mab observed for an antibody-producing cell line that co-expresses the E6/E7 element. Example 5 describes the generation and characterization of a modified Sp2/0 cell line that constitutively expresses a mutant Bcl-2 (Bcl-2-EEE) possessing three point mutations, resulting in improved longevity. Example 6 describes the improved growth properties of an antibody-producing cell line that expresses Bcl-2. Example 7 describes the enhanced production of MAb observed for the Bcl-2 expressing cell line of Example 6. Example 8 describes the methods to improve a cell clone producing low-level recombinant protein by introduction of Bcl-2 expression in the cell. Example 9 describes the methods to improve Sp-E26 by introduction of Bcl-2 expression in the cell. Example 10 describes using the improved cell line, Sp-EEE, as a host to develop cell clones producing a recombinant Ab. Example 11 describes the use of fed-batch reactor profiles and feeding schedules to optimize yield.

Example 1. Generation of apoptosis-resistance cell clones by stable expression of HPV-16 E6 and E7 genes.

Selection of cell clones resistant to CHX treatment

Sp2/0 cells were transduced with an LXSN retroviral vector containing the expression cassette of HPV-16 E6 and E7 genes at an MOI (multiple of infection) of 10:1. After recovery for 24 h, the infected cells were selected in G418 (1000 μg/ml) for 10 days. G418-resistant cells were cloned in 96-well cell culture plates by limiting dilution (0.5 cells/well). Stable infectants were screened for resistance to treatment by cycloheximide (CHX), a potent apoptosis-inducing agent. Briefly, healthy cells (viability >95%, Figure 1C and D) were incubated in medium containing 25 μg/ml of CHX and cell morphology was examined under a microscope. While more than 50% of parent Sp2/0 cells underwent morphology change after two to three hours of incubation and became fragmented (Figure 1A), several E6/E7 transfected clones showed less extent of morphology change, indicating resistance to apoptosis. The best clone, designated as Sp-E26, showed no apparent morphology change upon four hours of treatment (Figure 1B).

To avoid tedious visual examination, MTT assay was used to access the changes in viable cell population. After the healthy cells were incubated with or without CHX under normal culture condition for 2-3 h, MTT dye was added to the wells. After further incubation for two

hours, the cells were solubilized by adding a lysis buffer contain SDS and HCl. The plates were incubated overnight at 37°C and OD reading was performed at 590 nm using an ELISA plate reader. As shown in Figure 2, the viable cell population was significantly reduced when Sp2/0 cells were treated with CHX. By comparison, under the same treatment conditions (concentration of CHX and length of time), Sp-E26 cells tolerated better against CHX treatment. With this method, a large number of clones can be screened and selected for further analyses (Figure 2).

Anti-apoptosis property of Sp-E26.

CHX-induced apoptosis in Sp-E26 and the parent Sp2/0 cells was evaluated by Annexin V staining and DNA fragmentation assay. After being incubated in the medium containing 25 μg/ml of CHX, the cells were harvested and stained with Guava Nexin reagent (equivalent of Annexin V staining) and analyzed in a Guava Personal Cell Analysis system (Guava Technologies, Inc.). Figure 3 shows that while more than 30% of Sp2/0 cells became Annexin V positive when exposed to CHX treatment for about 1.5 h, indication of apoptosis, Sp-E26 remained healthy, showing no increase in early apoptotic cells.

The induction of apoptosis by CHX can be revealed by analysis of the formation of intracellular oligonucleosomal DNA fragments, a hallmark of apoptosis. The cellular DNA was extracted from CHX-treated and untreated Sp-E26 and Sp2/0 cells and DNA laddering assay was performed. In Sp2/0 cells treated with CHX, extensive DNA fragmentation was detected (Figure 4). In contrast, under identical treatment conditions, the genomic DNA of Sp-E26 was still intact, showing no appearance of DNA fragmentation (Figure 4).

Presence of HPV E6 and E7genes in Sp-E26

To confirm that E6 and E7 genes are stably present in the genome of Sp-E26 cells, oligonucleotide primers specific for E6 and E7 genes were designed and used in a PCR reaction with the genomic DNA extracted from Sp-E26 as the template, resulting in a ~700 bp DNA fragment. The PCR product was cloned and confirmed to be E6 and E7 genes by DNA sequencing. No E6 and E7 genes were detected in the parent Sp2/0 cells.

Improved growth properties of Sp-E26

The growth properties of Sp-E26 were evaluated in T-flask (Figure 5) and 3L-batch bioreactor (Figure 6). Sp-E26 showed improved growth properties over the parent Sp2/0 cell in batch cultures, achieving higher maximum cell density and longer survival time.

Example 2. Generation of apoptosis-resistance cell clones by stable over-expression of HPV16 E7 gene.

The structure of the poly-cistronic HPV16 E6 and E7 genes integrated into the genome of clone Sp-E26 was analyzed by PCR using the primer pair E6-N8$^+$ (5'-ATG TTT CAG GAC CCA CAG GAG CGA-3') and E7-C8$^-$ (5'-TTA TGG TTT CTG AGA ACA GAT GGG-3') and DNA sequencing. Since the sequences of primer E6-N8$^+$ and E7-C8$^-$ match with the coding sequence for the N-terminal 8 amino acid residues of E6 and the complement sequence for the C-terminal 8 codons of E7, respectively, the amplicon of full-length E6 and E7 is expected to be ~850 bp. However, amplification of the genomic DNA prepared from Sp-E26 cell with E6-N8$^+$ and E7-C8$^-$ resulted a PCR fragment of only ~700 bp. DNA sequencing of the 700 bp PCR product revealed a deletion of a 182 poly-nucleotide fragment from the E6 gene. The defective E6 gene is likely resulted from splicing and encodes a truncated E6 peptide with N-terminal 43 amino acid residues. Considering the major physiological activity attributed to E6 is its ability to down-regulate p53 expression, the truncated E6 protein is probably not fully functional because the level of p53 expression in Sp-E26 was found to be more stable than that in Sp2/0.

Thus, to evaluate whether HPV-16 E7 gene alone is sufficient to have anti-apoptotic effect and to improve the growth properties of Sp2/0 cells, transfection of Sp2/0 cell with HPV-16 E7 is performed as follows:

(i) The DNA sequence encoding E7 is cloned from Sp-E26 cell by RT-PCR. Proper restriction sites are introduced to facilitate the ligation of the gene into a mammalian expression vector, pRc/CMV (Invitrogen). Transcription of the viral gene within the vector, designated as E7pRc, is directed from CMV promoter-enhancer sequences. The vector also contains a gene conferring neomycin resistance, which is transcribed from the SV40 promoter.

(ii) Sp2/0 cells are transfected with the expression vector containing the expression cassette of HPV-16 E7 gene. Briefly, 5 ug of E7pRc is linearized by ScaI and transfected into the cell by electroporation.

(iii) After recovery for 24 hours, the transfected cells are selected in G418 (1000 μg/ml) for 10 days.

(iv) G418-resistant cells are then cloned in 96-well cell culture plates by limiting dilution (0.5 cells/well). Stable transfectants are selected and screened for resistance to treatment by cycloheximide (CHX), a potent apoptosis-inducing agent.

(v) Healthy cells (viability >95%) are incubated in medium containing 25 μg/ml of CHX or in the absence of CHX for 3-4 hours under normal culture conditions, followed by the addition of MTT dye into the wells. After further incubation for two hours, the cells are solubilized by adding a lysis buffer contain SDS and HCl. The plates are incubated overnight at 37 °C and an OD reading is performed at 590 nm using an ELISA plate reader. Cell clones showing resistance to CHX treatment are selected and expanded for further analyses.

(vi) The anti-apoptosis property of E7-transfected cells is evaluated by Annexin V staining and DNA fragmentation assays. In the Annexin V assay, after being incubated in the medium containing 25 μg/ml of CHX, the cells are harvested and stained with Guava Nexin reagent (equivalent of Annexin V staining) and analyzed in a Guava Personal Cell Analysis system (Guava Technologies, Inc.). In the DNA fragmentation assay, the cellular DNA is extracted from CHX-treated and untreated E7-transfectants and Sp2/0 cells and analyzed with agarose gel electrophoresis.

(vii) Expression of the viral oncogene in E7-transfectants is evaluated by Southern blot (genomic level), Northern blot (mRNA level), and immunoblot (protein level) analysis. Expression of intracellular proteins that are involved in apoptosis processes and affected by E7 protein are examined by immunoblotting analyses.

(viii) The growth properties of selected E7-transfectants are evaluated in T-flask and in a 3L-batch bioreactor. The transfectants show improved growth properties, i.e. achieving higher maximum cell density and longer survival time, over the parent Sp2/0 cell in batch cultures are considered to be better host cells.

Example 3. High-level expression of hLL2 IgG in Sp-E26.

In this example, Sp-E26 is used as a host to generate cell clones producing hLL2, a humanized anti-CD22 Ab developed for treating patients with NHL and autoimmune diseases. An hLL2-producing clone, 87-2-C9, was previously generated by using Sp2/0 cell as a host (Losman *et al.*, Cancer 80, 2660-2666, 1997), in which case, only one positive clone (a frequency of ~2.5 x 10^{-7}) was identified after transfection, and the maximum productivity

(P_{max}), defined as the concentration of the antibody in conditioned terminal culture medium in T-flask, of the only hLL2-producing clone, before amplification, was 1.4 mg/L. Transfection of Sp-E26 cell with the same hLL2pdHL2 vector and by using similar procedures as described by Losman *et al.* (Cancer 80, 2660-2666, 1997) resulted in more than 200 stable hLL2-producing clones, a frequency of $>10^{-4}$). The P_{max} of 12 randomly selected clones was evaluated and found to be between 13 and 170 mg/L, with a mean of 50 mg/L. The productivities of these clones can be further enhanced by gene amplification with MTX. This example demonstrated the advantage of using Sp-E26 over its parent Sp2/0 cell as a host for the development of cell clones producing recombinant proteins.

Example 4. Improvement of Ab-producing cell lines by stable expression of HPV16 E6 and E7 genes.

607-3u-8 cells were originally generated from Sp2/0 by transfection to produce a humanized monoclonal Ab. The clone was developed by gene amplification (with MTX) and subcloning to enhance the maximum (Ab) productivity up to 150 mg/L, which decreased to ~100 mg/L following weaning off serum supplement in the culture medium.. To obtain higher antibody productivity under serum-free condition, E6/E7 genes of HPV-16 were introduced into 607-3u-8 and the effect of E6/E7 on Ab-productivity was evaluated as follows. 607-3u-8 cells maintained in HSFM supplemented with 10% FBS and 3 uM MTX were transduced with an LXSN retroviral vector containing the expression cassette of HPV-16 E6 and E7 genes at an MOI of 10:1. After recovery for 24 h, stably transfected cells were selected in G418 (400 μg/ml) for 10 days. G418-resistant cells were subcloned in 96-well cell culture plates by limiting dilution (0.5 cells/well). A surviving clone, designated as 607E1C12, was obtained for evaluation. Two subclones, designated as 607-3u-8-7G7 and 607-3u-8-2D10, of 607-3u-8 without E6/E7 transfection were also selected. The P_{max} of these three clones were determined and there were no significant difference (Table 1). These results suggest that introducing E6/E7 genes into the cell does not alter the ability of cells producing Ab. Next, 607E1C12, 607-3u-8-7G7 and 607-3u-8-2D10 were adapted to grow in serum-free medium and the productivities of these clones were determined. All cells were growing well in serum-free medium. The final antibody productivity of clone 607E1C12 was maintained at 150 mg/L, while the two clones without E6/E7 were substantially reduced. In addition, the productivity of 607E1C12 were stable after a freeze (for cryopreservation) and thaw cycle (Table 1)

Table 1. The productivities of Ab-producing clones

Clone	P_{max} (mg/L)[a]	
	With serum	Serum-free
607-3u-8-7G7	127 ± 16 (3)[b]	74 ± 10 (4)
607-3u-8-2D10	140 ± 4 (3)	35 ± 2 (2)
607E1C12	154 (1)	142 ± 13 (6)
607E1C12 (Cryo)[c]		145 ± 17 (5)

a. Determined by protein purification of IgG from terminal culture supernatants.
b. The number in parenthesis indicates the sample size.
c. Cells had been frozen for cryopreservation.

Example 5. Generation and Characterization of a genetically modified Sp2/0 cell line that Constitutively Expresses a Mutant Bcl-2

Evidence suggests that a mutant Bcl-2 possessing three point mutations (T69E, S70E and S87E) exhibits significantly more anti-apoptotic activity compared to wild type or single point mutants (Deng *et al.*, PNAS **101**: 153 – 158, 2004). Thus, an expression vector for this triple mutant (designated as Bcl-2-EEE) was constructed and used to transfect Sp2/0 cells for increased survival and productivity, particularly in bioreactors. Clones were isolated and evaluated for Bcl-2-EEE expression level, growth and apoptotic properties. The nucleic acid sequence for the Bcl-2-EEE is depicted as SEQ. ID. No. 3; the corresponding amino acid sequence for the Bcl-2-EEE protein is depicted as SEQ. ID. No. 4.

A 116 bp synthetic DNA duplex was designed based on the coding sequence for amino acid residues 64 – 101 of human Bcl-2. The codons for residues 69, 70 and 87 were all changed to those for glutamic acid (E). The entire sequence was extraordinarily GC rich and had numerous poly G and poly C runs. Conservative changes were made to several codons to break up the G and C runs and decrease the overall GC content.

Two 80-mer oligonucleotides were synthesized that, combined, span the 116 bp sequence and overlap on their 3' ends with 22 bp (See SEQ. ID. No. 5 & 6). The oligonucleotides were annealed and duplex DNA was generated by primer extension with Taq DNA polymerase. The duplex was amplified using the PCR primers, Bcl-2-EEE PCR Left (5'-TATATGGACCCGGTCGCCAGAGAAG-3'), and Bcl-2-EEE PCR Right (5'-TTAATCGCCGGCCTGGCGGAGGGTC-3').

The 126 bp amplimer was then cloned into pGemT PCR cloning vector. The Bcl-2-EEE-pGemT construct was digested with TthI and NgoMI restriction endonucleases and the 105 bp fragment was gel isolated and ligated with hBcl-2-puc19 vector (ATCC 79804) that was digested with TthI and NgoMI to generate hBcl-2(EEE)-puc19. The sequence of this construct was confirmed.

A 948 bp insert fragment was excised from hBcl-2 (EEE)-puc19 with EcoRI and ligated with pZeoSV2+ vector that was digested with EcoRI and treated with alkaline phosphatase. The resulting construct is hBcl-2 (EEE)-pZeoSV2+.

Sp2/0 cells (5.6×10^6) were then transfected by electroporation with 60 µg of hBcl-2 (EEE)-pZeoSV2+ following the standard protocol for Sp2/0 cells. The cells were plated into six 96-well plates that were incubated without selection for 48 hours. After two days, 800 µg/ml of zeocin was added to the media.

Cells from 40 wells were expanded to 24-well plates and analyzed by western blot with anti-hBcl-2 and anti-β actin. All but 5 of the 40 showed medium to high levels of Bcl-2-EEE expression. The results for one of four gels are shown in Figure 7. An Sp2/0 derived hMN14 cell line (Clone 664.B4) that was previously transfected with wild type Bcl-2 was used as a positive control (+). As was demonstrated by Deng *et al.*, the Bcl-2-EEE migrates slightly slower than wild type Bcl-2 in SDS-PAGE.

Three strongly positive wells (#7, #25 and #87) were chosen for further evaluation and sub-cloning. Limiting dilution plating resulted in <20 positive wells per 96-well plate, indicating a very high probability (>99%) that the cells in individual wells are in fact cloned. Initially, 23 subclones from the three original wells were analyzed by Guava Express using anti-hBcl-2-PE (Figure 8). The results confirmed that the original wells contained mixed cell clones. Well #7 yielded clones with the strongest signal and well #25 had those with the lowest. Clones 7-12, 7-16, 87-2 and 87-10 were expanded for further analysis. Subsequently, some initially slower growing subclones were similarly analyzed and one clone, 87-29, gave a signal that was 20% higher than any other clone and was expanded for further analysis. Two high expressing SP-EEE clones (87-29 and 7-16) were compared to the untransfected Sp2/0, Raji and Daudi cells (Figure 9). The Sp-EEE clones expresses about 20-fold higher than Raji and Daudi cells, which are both known to express Bcl-2 at presumably normal cell levels. Sp2/0 cells were negative. This was further verified by anti-Bcl-2 immunoblot (Figure 10). Bcl-2 was not detected with a human Bcl-2 specific antibody in Sp2/0 cells even with high protein loading (50K cells) and long exposure of X-ray film.

Immunoblot analysis with an anti-Bcl-2 MAb (C-2, Santa Cruz Biotech.) that recognizes mouse, rat and human Bcl-2 did not detect any Bcl-2 from untransfected Sp2/0 cells, even with high protein loading (100K cells) and long exposure time (Fig. 10B). If there is any Bcl-2 expressed in Sp2/0 cells, it is at a level that is more than 2 orders of magnitude less than the Bcl-2-EEE in clone 87-29. Growth curves were compared for five Sp-EEE subclones and Sp2/0 cells. Three Sp-EEE subclones displayed a clear advantage over Sp2/0 cells. These three (7-12, 7-16 and 87-29) also express the highest levels of Bcl-2-EEE. As 7-12 and 7-16 are from the same original well and have nearly identical properties (Bcl-2-EEE levels and growth curves), they likely originated from the same initial clone. The best two SP-EEE subclones 7-16 and 87-29 were used for further evaluation.

The clones were plated in media supplemented with 10%, 1% or 0% serum (without weaning) and cell density and viability were monitored. In 10% serum 87-29 grew to a high density and had more than 4 days increased survival compared to Sp2/0 cells (Figure 11). In 1% serum, all cells grew to about 35 – 40% of the density achieved in 10% serum and the Bcl-2-EEE transfectants had a similar survival advantage over Sp2/0 (Figure 12). When transferred directly into serum free media, the Sp2/0 cells only grew to 600K cells/ml while 87-29 cells grew to a two-fold higher density (Figure 13). In each serum concentration 87-29 cells survived 4 – 6 days longer than Sp2/0 cells.

The methotrexate (MTX) sensitivity was determined for 87-29 (Figure 14). The data suggests that a minimum MTX concentration of 0.04 μM is sufficient for initial selection of MTX-resistant clones. Therefore, the same selection and amplification protocols used for Sp2/0 cells can be employed with the SP-EEE cells.

Bcl-2 is a pro-survival/anti-apoptotic protein. It has been demonstrated by several groups that a Bcl-2 deletion mutant missing the flexible loop domain (FLD) has an enhanced ability to inhibit apoptotosis (Figueroa *et al.*, 2001, Biotechnology and Bioengineering, **73**, 211-222; Chang *et al.*, 1997, EMBO J.,**16**, 968-977). More recently, it was demonstrated that mutation of 1 to 3 S/T residues in the FLD of Bcl-2 to glutamic acid, which mimics phosphorylation, significantly enhances its anti-apoptotic ability (Deng *et al.* 2004, PNAS, **101,** 153–158). The triple mutant (T69E, S70E and S87E) provided the most significant survival enhancement. Here, the present invention teaches the generation of a similar Bcl-2 triple mutant construct (Bcl-2-EEE), which is used to stably transfect Sp2/0 cells.

All the aforementioned experiments demonstrate that expression of Bcl-2-EEE reduces apoptosis rates in Sp2/0 cells. This effect was largely dose dependent, in that clones

with higher expression levels survived longer than those with lower levels. The best clone, 87-29, grows to a 15 – 20% higher cell density and survives an additional 4 – 6 days compared to untransfected Sp2/0 cells.

The Bcl-2-EEE level in clone (87-29) is approximately 20-fold higher than normal levels in Daudi or Raji cells. No Bcl-2 expression was detected in untransfected Sp2/0 cells. As described in Example 6, hMN-14-expressing Sp2/0 cells were transfected with a similar construct for expression of wild type Bcl-2 and a clone with exceptional growth properties and enhanced productivity was isolated. When this clone (664.B4) was amplified further with MTX, the Bcl-2 levels increased significantly. Ultimately, the amplified (3 μM MTX) cell line was sub-cloned and the Bcl-2 level of one clone (664.B4.1C1) was two-fold higher than 664.B4. This particular subclone has superior productivity and growth properties. The Bcl-2-EEE level in 87-29 is approximately two-fold higher than the level of Bcl-2 in the amplified 664.B4.1C1. 87-29 cells have a growth rate that is comparable to that of Sp2/0 cells and can apparently continue to grow for one additional day and reach a maximal density that is 15 – 20% higher than Sp2/0. A similar property was found for the E6/E7 expressing Sp-E26 cell line. The Bcl-2-EEE expressing 87-29 clone, which provides an additional 4 – 6 days survival over the parental Sp2/0 cells, is superior to the Sp-E26 clone, which only survives one additional day.

The Sp-EEE cell line as represented by the 87-29 clone is useful as an apoptosis-resistant host for expressing a recombinant protein upon transfection with a suitable vector containing the gene for that recombinant protein. In order for this cell line to be useful it must maintain its Bcl-2-EEE expression and survival advantage following transfection and amplification and during extended culture. It is unlikely that the stably transfected Bcl-2-EEE gene will be lost during subsequent transfection and therefore the survival properties should not diminish. It is possible that MTX amplification could even improve the survival of producing clones via increasing expression of Bcl-2 proteins. Indeed, this was the case with the hMN-14 664.B4 cell line, which was transfected with wild type Bcl-2. Following amplification and sub-cloning, the Bcl-2 level increased several fold and cell survival improved significantly.

Example 6. Improvement of Ab-producing cell survival in stationary batch culture by stable expression of a human *Bcl-2* gene.

Generation of a Bcl-2-transfected cell clone

A cell clone 665.2B9 was originally generated from Sp2/0 by transfection to produce a humanized monoclonal anti-CEA Ab (Qu *et al.*, unpublished results). A vector, designated hMN14pdHL2, was used to transfect Sp2/0 cells to obtain the cell clone 665.2B9. The pdHL2 vector was first described by Gillies *et al.*, and had an amplifiable murine *dhfr* gene that allows subsequent selection and amplification by methotrexate treatment (Gillies *et al.*, J. Immunol. Methods 125:191 (1989)). Generally, the pdHL2 vector provides expression of both IgG heavy and light chain genes that are independently controlled by two metallothionine promoters and IgH enhancers. A diagram of the hMN14pdHL2 vector is shown in Figure 16. SEQ. ID. No. 1 shows the sequence of the vector; SEQ. ID. No. 2 shows the 72 bp sequence defined as the enhancer sequence; the promoter sequence corresponds to nt2908-2979 of hMN14pdHL2.

Sp2/0 cells can be generally transfected by electroporation with linearized pdHL2 vectors such as the hMN14pdHL2 vector used in this instance. Selection can be initiated 48 hours after transfection by incubating cells with medium containing 0.05 to 0.1 μM MTX. Amplification of inserted antibody sequences is achieved by a stepwise increase in MTX concentration up to 5 μM.

The clone was subjected to gene amplification with MTX increased stepwise to 0.3 μM, at which point the maximum productivity (Pmax) of the antibody was increased to about 100 mg/L. To improve cell growth properties, 665.2B9 cells were transfected with a plasmid expression vector (Figure 17) containing the human *Bcl-2* gene by electroporation. *Bcl-2* gene was excised from pB4 plasmid purchased from ATCC (pB4, catalog # 79804) using EcoRI sites and inserted into MCS of mammalian expression vector pZeoSV(+) using the same restriction enzyme. Since zeocin resistance gene is part of the vector, transfected cells were placed into medium containing zeocin ranging from 50 – 300 μg/mL. Stable clones were selected from media containing 300 mg/ml zeocin and subcloned in media without zeocin by plating into 96-well plates at a density of 0.5 cell/100 uL/well. The media without zeocin was used thereafter. Formation of clones in wells was confirmed by visual observation under a microscope. Cells from the wells with only 1 cluster of cells were expanded. Each 96-well plate produced around 30 clones, from which 14 clones were

randomly selected for further studies. The growth characteristics of these clones were evaluated by daily cell counting and viability measurements with ViaCount reagent and Guava PCA. From the 14 clones evaluated in 24-well plates (Figures 18, 19), one Bcl-2-transfected clone showing improved growth characteristics (higher cell densities and prolonged cell survival) was identified and designated as 665.2B9#4 (or clone #4). Comparing to the parent 665.2B9 clone, clone #4 grew to a higher cell density (~1.7-fold) and survived 4 to 6 days longer in T-flasks (Figures 20, 21), and as a consequence of better growth, the P_{max} of clone #4 was increased to about 170 mg/L as determined by ELISA titration and Protein A column purification.

Bcl-2 expression in 665.2B9#4

To confirm that the improved growth properties of 665.2B9#4 were resulted from transfection of Bcl-2, intracellular level of human Bcl-2 protein was measured by using Guava Express reagent and Guava PCA instrument. Briefly, 4 x 10^5 cells placed in 1.5ml spin-tubes were centrifuged for 5 minutes at 1500 rpm, washed three times with 1x PBS. Supernatants were carefully aspirated. Fixation solution (10x, 60 µL) from Santa Cruz Biotechnology (SCB), Inc. (cat. # sc- 3622) was added to cell pellets for 15 min and incubated on ice. Fixation solution was removed with 4x 1 mL PBS at 4°C, each time spinning as described. Permeabilization buffer (0.5 mL) at –20°C (SCB cat. # sc- 3623) was added dropwise while vortexing, followed by 15 min incubation on ice. Cells were then spun and washed two times with 0.5 mL FCM wash buffer (SCB cat. # sc- 3624). Final cell pellet was resuspended in 100 µL of FCM wash buffer and stained for Bcl-2 intracellular protein with 10 µL of anti-Bcl-2 mouse monoclonal antibody conjugated to PE (obtained from SCB). Incubation was performed at room temperature in dark for one hour. Two washes with 0.5 mL of FCM wash buffer followed. The final cell pellet was resuspended with 0.4 mL FCM wash buffer and the cells analyzed on Guava PC. Mean values of the fluorescence intensity (MFI) for each clone were compared to control staining with non-specific, isotype mouse IgG1 conjugated with PE. The results summarized in Table 2 confirm that clone 665.2B9#4 expresses a higher level of Bcl-2 protein compared to the parental cell line. A zeocin-resistant clone (#13) that showed a similar growth profile as the parent 665.2B9 was negative for Bcl-2 staining, confirming that Bcl-2 expression is necessary for the improvement of growth.

Table 2. Intracellular level of Bcl-2 determined by Guava Express.

Cell	Viability[a] (%)	Mean FI (AU)
665.2B9	84	42
665.2B9#4	97	110
Clone #13	92	14
Non-specific antibody staining		12

a. Determined before the assay to ensure healthy cells were used.
b. 665.2B9 cells stained with an isotype-matched mouse IgG1antibody, PE-conjugated.

With Guava Express analysis it was found that the intensities of fluorescent staining corresponding to Bcl-2 levels are rising with MTX amplification of clone 665.2B9#4, suggesting co-amplification of *Bcl-2* with the dhfr gene. To compare intracellular Bcl-2 levels of amplified cells, Western blotting analysis was performed on cell lysates of clone 665.2B9#4 (Bcl-2 positive) and clone #13 (Bcl-2 negative) using an anti-human Bcl-2 antibody. Densitometric evaluation showed that Bcl-2 signal of clone 665.2B9#4 growing in 1.0 μM MTX is 2x stronger than the cells in 0.6 μM MTX. A lysate of Clone #13 did not reveal the presence of Bcl-2 protein (Figure 22).

Example 7. Improved Ab-production of clone 665.2B9#4 under batch culture condition.

By monitoring nutrients consumption in the cell cultures near the terminal phase, it was found that glucose and L-glutamine are the first components to be consumed. Experiments were carried out to determine whether supplementation of these limiting nutrients would improve the final antibody yields. Two types of cultures were initiated: spiked fed batch - where these limiting components were supplemented upon their consumption; and unfed batch - without nutrients supplementation. Tested were Bcl-2-positive clone 665.2B9#4 growing in medium containing 0.6 and 1 μM of MTX and the Bcl-2-negative clone #13 growing in 0.3 μM MTX. Figures 23 and 24 show the profiles of cell viability and cell density in both culture types until they reached terminal stage. Protein yields, expressed as mg/L, are shown in Table 3. The results of this experiment suggest that nutrient spiking improves total yield of produced antibody about 2-fold for all cultures.

Table 3. Antibody production under batch culture conditions

Cell/MTX (μM)	Unfed batch[a] (mg/L)	Spiked fed batch[a] (mg/L)
665.2B9#4/0.6	117	286
665.2B9#4/1.0	156[b]	296
Clone #13/0.3	74.1	165

a. Determined by Protein A column purification.
b. Average of two purifications.

Example 8. Introduction of *Bcl-2* gene into a cell line producing low-level of recombinant protein.

A cell clone 482.2C4A was originally generated from Sp2/0 by transfection to produce a bispecific Ab in the form of an IgG (anti-CEA) and two scFv (anti-DTPA) (Leung *et al.*, J. Nuc. Med. 41: 270P, 2000; Hayes *et al.*, Proc. Am. Asso. Cancer. Res. 43: 969, 2002), each of which is covalently linked to the C-terminus of the IgG heavy chain. The clone was subjected to gene amplification and had a final productivity of ~20 mg/L. To improve the growth property and eventually the Ab productivity, 482.2C4A cells were transfected with a plasmid expression vector containing the human Bcl-2 gene by electroporation as described in Example 6. The transfectants were selected in medium containing 750 μg/ml of Zeocin after three weeks.

Zeocin-resistant cells were treated with 25 μg/ml of CHX for 5 hours to eliminate apoptosis-sensitive cells. Treated cells were washed twice with fresh culture medium to remove CHX and resuspended in fresh growth medium. After recovering for 24 h, the viable cells were cloned into 96-well cell culture plates by limiting dilution (0.5 cells/well). Clones emerged in the wells in two weeks and were screened for Ab production, resistance to CHX-induced apoptosis, as well as growth profiles. Those clones performed better than the parent 482.2C4A in all aspects are selected and further characterized. The best performer is expected to be more robust when growing under stress condition, resist to aging-culture-condition induced apoptosis, and have a higher maximum Ab productivity (ca.150% or better) comparing to the parent 482.2C4A cell.

Example 9. Introduction of *Bcl-2* gene into Sp-E26 for a further improvement of cell growth properties.

Sp-E26 cells are transfected with a plasmid expression vector containing the human Bcl-2-EEE gene, as described in Example 5, by electroporation. The transfectants are selected in medium containing 500 μg/ml of Zeocin after three weeks. Zeocin-resistant cells are treated with 25 μg/ml of CHX for 5 hours to eliminate apoptosis-sensitive cells. Treated cells are washed twice with fresh culture medium to remove CHX and resuspended in fresh growth medium. After recovering for 24 h, the viable cells are cloned into 96-well cell culture plates by limiting dilution (0.5 cells/well). Clones emerge in the wells in two weeks and are screened for resistance to CHX-induced apoptosis, as well as growth profiles. Those clones perform better than the parent Sp-E26, as well as Sp-EEE, in all aspects are selected and further characterized. The best performer containing HPV-16 E6/E7 and Bcl-2-EEE is expected to be more robust when growing under stress condition and resistant to aging-culture-condition-induced apoptosis than the parent Sp-E26 and Sp/EEE cells; therefore, it is a better host cell for recombinant protein production.

Example 10. Improved production of recombinant proteins with the Sp-EEE cell line.

There are two paths that can be taken when developing a cell line with enhanced survival for production of recombinant proteins. One method, which has been accomplished quite successfully, as described in Example 6, involves stable transfection of an already producing cell line with a pro-survival gene, such as *Bcl-2*. However, this method requires additional transfection, selection and cloning steps, thereby lengthening the cell line development process by at least two months and possibly much more. Further, screening for the "best" clone is rather involved, since a number of parameters need to be determined for each clone, including growth/survival, Bcl-2 expression level and productivity. Thus, only a small number of clones can be evaluated. It is quite possible that clones with the highest productivity may not have superior survival and vice versa. An alternative strategy, employed here, is to develop a parental cell line with superior growth and survival properties, which is subsequently transfected with the expression vector for production of the desired protein.

Compared to Sp2/0 cells, the Sp-EEE cells continue to grow for one additional day, reach a maximal density that is 15 – 20% higher, and survive an additional 4 – 6 days in culture. The cells retain their enhanced growth and survival properties when subsequently

transfected with genes for the production of recombinant proteins, such as IgG, antibody fragments and fusion proteins, growth factors, such as G-CSF, GM-CFS, EPO, EGF, VEGF, cytokines, such as an interleukin family member (IL-1 – IL-31), or interferon family members (such as alpha, beta or gamma interferon), oligonucleotides, peptides, hormones, enzymes, or vaccines (e.g., Hepatitis A, B or C, as well as others described above).

A DNA vector, such as pdHL2, containing one or more expression cassettes for recombinant protein(s), such as an IgG, is used to transfect Sp-EEE cells by standard methods, such as electroporation. The transfectants are plated in 96-well plates and clones are analyzed for protein production by established techniques such as ELISA or Biacore. Productive clones are subjected to increasing concentrations of MTX in the culture media over several months to amplify the genetic copy number. Since the Bcl-2-EEE-expressing clones grow to ~20% higher cell density and survive at least an additional 4 days as compared to clones generated in Bcl-2 negative Sp2/0 cells, the former will produce at least 20% more recombinant protein in standard flask or roller bottle culture. An even greater increase is realized in suspension, perfusion or fed-batch bioreactor cultures.

Example 11. Improved Ab-production of Bcl-2 transfected clone 665.B4.1C1 in bioreactor

Both 665.2B9#4 and the parent clone 665.2B9 of Example 6 were weaned into serum-free media. The cells were adapted to a customized formulation of hybridoma serum-free medium (HSFM) (Immunomedics PN 10070) containing 3 μM MTX by continuous subculture in T-flasks for several months. The adapted cells were scaled up from T-flasks to roller bottles for banking. A master cell bank (MCB) for each cell line was created with 1×10^7 viable cells in each 1-mL vial using an FBS-free cryopreservation solution composed of 45% conditioned medium (medium that is collected as supernatant after centrifugation of a culture in the exponential growth phase), 10% DMSO and 45% HSFM. The MCB cell lines were designated 665.2B9.1E4 (without *Bcl-2* gene) and 665.B4.1C1 (with Bcl-2 gene), respectively. The growth properties and antibody production of these two clones were compared under batch culture conditions.

Experiments were conducted in 3-L bench-scale bioreactors using the above cells expanded from the MCB. The 3-L bioreactor system is the scale-down model of a 2500-L cGMP bioreactor system. Therefore, the evaluation results would support the suitability of these cell lines for large-scale commercial manufacturing.

The same growth HSFM as that used in creating the MCB (Immunomedics PN 10070) was used to maintain the cell line and prepare the inoculum. Basal HSFM, a customized formulation based on the growth HSFM with customized modifications (Immunomedics PN 10194), was used in the 3-L fed-batch bioreactor process. Both media contain insulin and transferrin as the only trace proteins. Additional 0.1% Pluronic F68 was incorporated into the formulation to protect cells from shear caused by agitation and aeration. This media also contained 3 μM MTX.

The specific characteristics of the continuous feeding solutions and the pulse feeding solutions are shown in tables 4 and 5 as follows:

Table 4: Continuous feeding solutions

Solutions	**Formulation (Dissolve in WFI unless specified)**
Glucose and glutamine solution (G/G)	Glucose, 13.3g/L; Glutamine, 20mM
ImmuC2 solution	Glucose, 13.3g/L; Glutamine, 20mM; PNS A, 50ml/L; NaOH, 50mM
ImmuC5 solution	Glucose, 26.6g/L; Glutamine, 40mM; PNS A, 100ml/L; NaOH, 100mM

Table 5: Pulse feeding Solutions

Solutions	**Formulation (Dissolve in WFI unless specified)**
TC Soy Plus	120g/L
Linoleic acid/cyclodextrin	1.5mg/ml
HD lipid	500X
β-mercaptoethanol/EDTA	BME, 0.01M; EDTA, 0.1mM.
ImmuVitamin	MEM Vitamin Solution (100x), as solvent; Choline Chloride, 500mg/L; Myo-inositol, 600mg/L.
TEC solution	Transferrin solution (4mg/mL), as solvent; CaCl2, 125mM; Ethanolamine-HCl 1g/L.
Insulin	4mg/ml

The fed-batch experiments were conducted in 3L Bellco spinner-flask bioreactor systems (Bellco glasses, Vineland, NJ) with 2 L of working volume. The bioreactor temperature, pH and dissolved oxygen (DO) were monitored and controlled by single loop controllers. The reactor temperature was controlled at 37°C by a heating blanket. The culture pH was controlled at 7.3 by the addition of CO_2 or 6% Na_2CO_3. Aeration was performed through a cylindrical sintered sparger at 10ml/min. DO was controlled above 40% of air

saturation by intermittent sparging of O_2 into the medium. A constant agitation rate of 50 ~ 60rpm was used throughout the cultivation.

A frozen vial from MCB was thawed and recovered in T-flasks in approximately 1 to 2 weeks. The cells were then expanded from T-flasks to roller bottles prior to inoculation into the bioreactors. Cells were cultured at 37°C in a 5% CO_2 atmosphere and maintained in the exponential growth phase throughout the expansion process.

Prior to the inoculation, 1.2 liters of Basal HSFM was pump-transferred into the bioreactor aseptically. The medium was air saturated to calibrate the dissolved oxygen (DO) probe. A medium sample was also taken to calibrate the pH probe. Once pH probes and DO probes were calibrated, both controllers were set to AUTO modes. Once the system reached set points of pH (7.3) and temperature (37°C), calculated amount of inoculum from roller bottle was pump transferred into the bioreactor. The post-inoculation viable cell density (VCD) was around 2×10^5 vial cells/ml.

The feeding strategy is as follows. During the cultivation, concentrated nutrient solutions were fed into the bioreactor to provide the cells with necessary and non-excessive nutrients (See figure 25 for the overall process schematics). Concentrated nutrient solutions were delivered to the culture via continuous feeding and pulse feeding. The continuous feeding solutions were pump transferred into the reactor continuously using peristaltic pumps (Watson-Marlow 101U/R). The pulse feeding solutions were pulse fed once a day into the culture.

Two fed-batch feeding strategies were developed and applied to both cell lines. Process #1 does not feed recombinant insulin during the cultivation. Process #2 is designed based on Process #1 with a modified linoleic acid and lipid feeding schedule and an additional feeding of insulin.

The following tables summarize the feedings of both processes for both cell lines.

Table 6: Process #1 for cell line 665.2B9.1E4

Continuous feeding

Day	Expected Viable Cell Density (cells/mL)	Continuous Feeding Rate (ml/day)		
		Glucose and Glutamine	ImmuC2	ImmuC5
Day2	0.4 ~ 0.7 E6	60	0	0
Day3am	1.0 ~ 1.7E6	0	60	0
Day3pm	1.01 ~ 2.5E6	0	90	0
Day4am	2.51~3.5E6	0	90	0
Day4pm	2.51~4.5E6	0	150	0
Day5am	4.51~6.5E6	0	0	90
Day5pm	4.51~7.5E6	0	0	120
Day6	7.51 ~ 12E6	0	0	120
Day7	if <13E6	0	0	120
	if >13.1E6	0	0	150

Pulse feeding

Day	Pulse Feeding (mL)					
	TC Soy Plus (120g/L)	LA/CD (1.5mg/ml)	Lipid (500X)	TEC Solution	Immu Vitamin	BME/EDTA
Day3	12.5	4	3	-	-	15
Day4	25	8	-	-	-	-
Day5	50	12	-	4	15	15
Day6	60	8	2	8	-	-
Day7	60	-	1	-	-	-
Day8	25					

Table 7: Process #2 for cell line 665.2B9.1E4

Continuous feeding				
Day	**Expected Viable Cell Density (cells/mL)**	**Continuous Feeding Rate (ml/day)**		
		Glucose and Glutamine	ImmuC2	ImmuC5
Day2	0.4 ~ 0.7 E6	60	0	0
Day3am	1.0 ~ 1.7E6	0	60	0
Day3pm	1.01 ~ 2.5E6	0	90	0
Day4am	2.51~3.5E6	0	90	0
Day4pm	2.51~4.5E6	0	150	0
Day5am	4.51~6.5E6	0	0	90
Day5pm	4.51~7.5E6	0	0	120
Day6	7.51 ~ 12E6	0	0	120
Day7	if <13E6	0	0	120
	if >13.1E6	0	0	150
Day8	if < 10E6	0	0	90
	if 10.1 ~ 13E6	0	0	120
	if >13.1E6	0	0	150

Pulse feeding

Day	**Pulse Feeding (mL)**						
	TC Soy Plus (120g/L)	LA/CD (1.5mg/ml)	Lipid (500X)	TEC Solution	Immu Vitamin	BME /EDTA	Insulin (4mg/ml)
Day3	12.5	2	3	-	-	15	-
Day4	25	4	-	-	-	-	-
Day5	50	6	-	4	15	15	4
Day6	60	4	-	8	-	-	8
Day7	60	4	-	-	-	15	8
Day8	50	-	-	-	-	-	8

Table 8: Process #1 for cell line 665.B4.1C1

Continuous feeding				
Day	**Expected Viable Cell Density (cells/mL)**	**Continuous Feeding Rate (ml/day)**		
		Glucose and Glutamine	ImmuC2	ImmuC5
Day2	0.4 ~ 0.7 E6	60	0	0
Day3am	1.0 ~ 1.7E6	0	90	0
Day3pm	1.01 ~ 2.5E6	0	120	0
Day4am	2.51~3.5E6	0	0	60
Day4pm	2.51~4.5E6	0	0	90
Day5am	4.51~6.5E6	0	0	120
Day5pm	4.51~7.5E6	0	0	150
Day6	7.51 ~ 12E6	0	0	180
Day7, 8, 9	if <15E6	0	0	180
	if >15.1E6	0	0	240
Day10	if < 10E6	0	0	120
	if 10.1 ~ 13E6	0	0	150
	if >13.1E6	0	0	180

Pulse feeding						
Day	**Pulse Feeding (mL)**					
	TC Soy Plus (120g/L)	LA/CD (1.5mg/ml)	Lipid (500X)	TEC Solution	Immu Vitamin	BME/EDTA
Day3	12.5	4	3	-	-	15
Day4	25	8	-	-	-	-
Day5	50	12	-	4	15	15
Day6	60	8	2	8	-	-
Day7	60	-	1	-	-	15
Day8	60	-	-	-	-	-
Day9	60	-	-	-	-	15
Day10	50	-	-	-	-	-

Table 9: Process #2 for cell line 665.B4.1C1

Continuous feeding				
Day	**Expected Viable Cell Density (cells/mL)**	**Continuous Feeding Rate (ml/day)**		
		Glucose and Glutamine	ImmuC2	ImmuC5
Day2	0.4 ~ 0.7 E6	60	0	0
Day3am	1.0 ~ 1.7E6	0	90	0
Day3pm	1.01 ~ 2.5E6	0	120	0
Day4am	2.51~3.5E6	0	0	60
Day4pm	2.51~4.5E6	0	0	90
Day5am	4.51~6.5E6	0	0	120
Day5pm	4.51~7.5E6	0	0	150
Day6	7.51 ~ 12E6	0	0	180
Day7, 8, 9	if <15E6	0	0	180
	if >15.1E6	0	0	240
Day10	if < 10E6	0	0	120
	if 10.1 ~ 13E6	0	0	150
	if >13.1E6	0	0	180

Pulse feeding							
Day	**Pulse Feeding (mL)**						
	TC Soy Plus (120g/L)	LA/CD (1.5mg/ml)	Lipid (500X)	TEC Solution	Immu Vitamin	BME /EDTA	Insulin (4mg/ml)
Day3	12.5	2	3	-	-	15	-
Day4	25	4	-	-	-	-	-
Day5	50	6	-	4	15	15	4
Day6	60	4	-	8	-	-	8
Day7	60	4	-	-	-	15	8
Day8	60	4	-	-	-	-	8
Day9	60	4	-	4	15	15	8
Day10	60	-	-	-	-	1-	8

During the cultivation, bioreactor samples were taken periodically for off-line analysis. The viable cell density (VCD) and the cell viability were measured by microscopic counting using a hemocytometer after staining with 0.4% trypan blue dye. The glucose, lactate, glutamine, ammonia concentrations were measured using a Nova Bioprofile 200. The antibody concentration was determined by HPLC using a protein A affinity chromatography column (Applied Biosystems, P/N 2-1001-00).

The specific antibody productivity was calculated by dividing the cumulative antibody produced by the time integral of the total viable cell in the culture:

$$Q_{[MAb]} = \frac{([Mab]_{t1} \bullet V_{t1} - [Mab]_{t0} \bullet V_{t0}}{\int_{t0}^{t1} VCD \bullet Vdt}$$, in which

$\int_{t0}^{t1} VCD \bullet Vdt$ is approximated by the Trapezium Rule: $\frac{(VCD_{t0} \bullet V_{t0} + VCD_{t1} \bullet V_{t1})(t1 - t0)}{2}$

Figure 26 shows the growth curves (VCD and the viability) of both cell lines by Process #1 and Process #2. By Process #1, 665.2B9.1E4 cells grew to attain a maximal VCD of 1×10^7 viable cells/ml on day 6 with 86% of viability. After day 6, VCD and V% decreased quickly and the culture was harvested on day 8. Process #2 helped the culture reach a higher VCD of 1.2×10^7 viable cells/ml and sustain one day longer.

As compared to 665.2B9.1E4 cells, 665.B4.1C1 cells exhibited much better growth in both processes. In Process #1, its VCD reached 2×10^7 viable cells/ml on day 7 with 97% viability. The culture also maintained this VCD and V% for two more days before it started to decline. The culture was harvested on day 11. In Process #2, 665.B4.1C1 cells showed a similar growth profile as in Processes #1. More specifically, the cells reached the highest VCD of 2.3×10^7 viable cells/ml and the viability declined a little slower with the harvest occurring on day 11. This observation was somewhat different from the 665.2B9.1E4 cell line, which demonstrated a growth advantage in Process #2.

The antibody yields of two cell lines in Processes #1 and #2 were compared in figure 27. The final yield of 665.2B9.1E4 cells was 0.42 g/L in Process #1 and 0.55 g/L in Process #2. For comparison, 665.B4.1C1 cells delivered a higher final yield of 1.5 g/L in both processes.

The daily specific antibody productivities (per cell basis) were calculated and are shown in figure 28. As shown in the figure, the 665.2B9.1E4 cells had an average daily $Q_{[MAb]}$ of approximately 15 pg/cell/day throughout the course of cultivation for both processes. The additional day of growth at the highest VCD in Process #2 resulted in a higher final antibody concentration.

The 665.B4.1C1 cells showed a similar daily specific antibody productivity profile in both processes with Process #1 yielding slightly higher productivity. The daily $Q_{[MAb]}$ were maintained between 20~25 pg/cell/day until day 9. Thereafter the productivity declined.

Comparing with the 665.2B9.1E4 cell line, the 665.B4.1C1 cell line exhibited a higher specific antibody productivity of 20~25 pg/cell/day as compared to 15 pg/cell/day. Combining with its better growth, the 665.B4.1C1 cell line tripled the final antibody yield to 1.5 g/L as compared to 0.55 g/L achieved by the 665.2B9.1E4 cell line. These results demonstrate the benefit of incorporating *Bcl-2* gene into the host cell line to enhance its growth and antibody yield in serum-free media in a bioreactor modeled for large-scale commercial preparation of a recombinant protein, in this case an antibody for clinical use. The methods and processes disclosed herein can be modified, as appropriate, by one skilled in the art. All publications, patents and patent applications, and references contained therein, are incorporated herein by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method of making a recombinant protein, comprising culturing a host cell in a suitable medium under conditions suitable for expression of said recombinant protein, wherein said host cell comprises a nucleic acid sequence encoding said recombinant protein and wherein said host cell has been modified by an agent that inhibits apoptosis and wherein said host cell is a lymphocyte, an epithelial cell, or a mesenchymal cell or a malignant form thereof.

2. The method according to claim 1 wherein said host cell is a myeloma cell.

3. The method according to claim 2 wherein said myeloma cell is an Sp2/0 cell or derivative thereof, a murine NSO cell or rat YB2/0 cell.

4. The method according to claim 1 wherein said host cell is an epithelial cell, or a malignant form thereof.

5. The method according to claim 3 wherein said epithelial cell is a CHO or HEK 293 cell.

6. The method according to claim 1 wherein said host cell is a mesenchymal cell, or a malignant form thereof.

7. The method according to claim 6 wherein said mesenchymal cell is a fibroblast.

8. The method according to claim 7 wherein said fibroblast is a COS-1 or COS-7 cell.

9. The method according to claim 1 wherein the host cell is first modified with the agent that inhibits apoptosis.

10. The method according to claim 1 wherein said agent that inhibits apoptosis is a heterologous protein expressed by said host cell.

11. The method according to claim 10 wherein said heterologous protein is encoded by a nucleic acid sequence integrated in the chromosomal DNA of said host cell.

12. The method according to claim 10 wherein said agent that inhibits apoptosis is a papillomavirus E6 protein.

13. The method according to claim 10 wherein said agent that inhibits apoptosis is a papillomavirus E7 protein.

14. The method according to claim 10 wherein said agent that inhibits apoptosis is a papillomavirus E6 protein and a papillomavirus E7 protein.

15. The method according to claim 10 wherein said agent that inhibits apoptosis is an apoptosis inhibitor selected from the Bcl-2 family of apoptosis inhibitors.

16. The method according to claim 15 wherein said apoptosis inhibitor is selected from the group consisting of Bcl-2, Bcl-xL, Bcl-w, Bcl-EEE, Bhrf1, KS-Bcl-2, E1B-19K, Bcl-6 and Mcl-1.

17. The method according to claim 1 wherein said medium further comprises at least one caspase inhibitor.

18. The method according to claim 17 wherein said caspase inhibitor is selected from the group consisting of caspase-1, caspase-3, caspase-9, caspase-12 and pan-caspase inhibitors.

19. The method according to claim 17 wherein said inhibitor is selected from the group consisting of Z-VAD-fmk, Ac-DEVD-cho, Aven and XIAP.

20. The method according to claim 1 wherein said medium further comprises an exogenously added agent that inhibits apoptosis and/or functions as a cytoprotective agent.

21. The method according to claim 20 wherein said exogenous agent is a member of the cytokine type I superfamily.

22. The method according to claim 21 wherein said member of the cytokine type I superfamily is erythropoietin.

23. The method according to claim 1, wherein said recombinant protein is selected from the group consisting of immunoglobulins, peptides, enzymes, growth factors, hormones, vaccines, lymphokines and cytokines.

24. The method according to claim 23 wherein said recombinant protein is an immunoglobulin selected from the group consisting of antibodies, antibody fragments, multispecific antibodies, and single chain antibodies.

25. The method according to claim 23 wherein said protein is a growth factor selected from the group consisting of Erythropoietin, G-CSF, GM-CSF, EGF, VEGF, and thrombopoietin.

26. The method according to claim 23 wherein said protein is a lymphokine selected from the group consisting of any of IL-1 to IL-31, alpha interferon, beta interferon, gamma interferon and consensus interferon.

27. The method according to any preceding claim wherein the cell is placed in a serum free medium and cultured to produce the protein.

28. A host cell comprising an exogenous nucleic acid sequence encoding a recombinant protein that inhibits apoptosis, wherein the host cell is Sp-E26 or Sp-EEE.

29. The method of claim 1, resulting in an increase in cell culture longevity of at least 2 days.

30. The method of claim 1, resulting in an increase in cell culture longevity of at least 4 days.

31. The method of claim 1, resulting in an increase in cell culture longevity of at least 6 days.

SEQUENCE LISTINGS

SEQ.: ID. No. 1: Complete sequence of the hMN14pdHL2 vector.

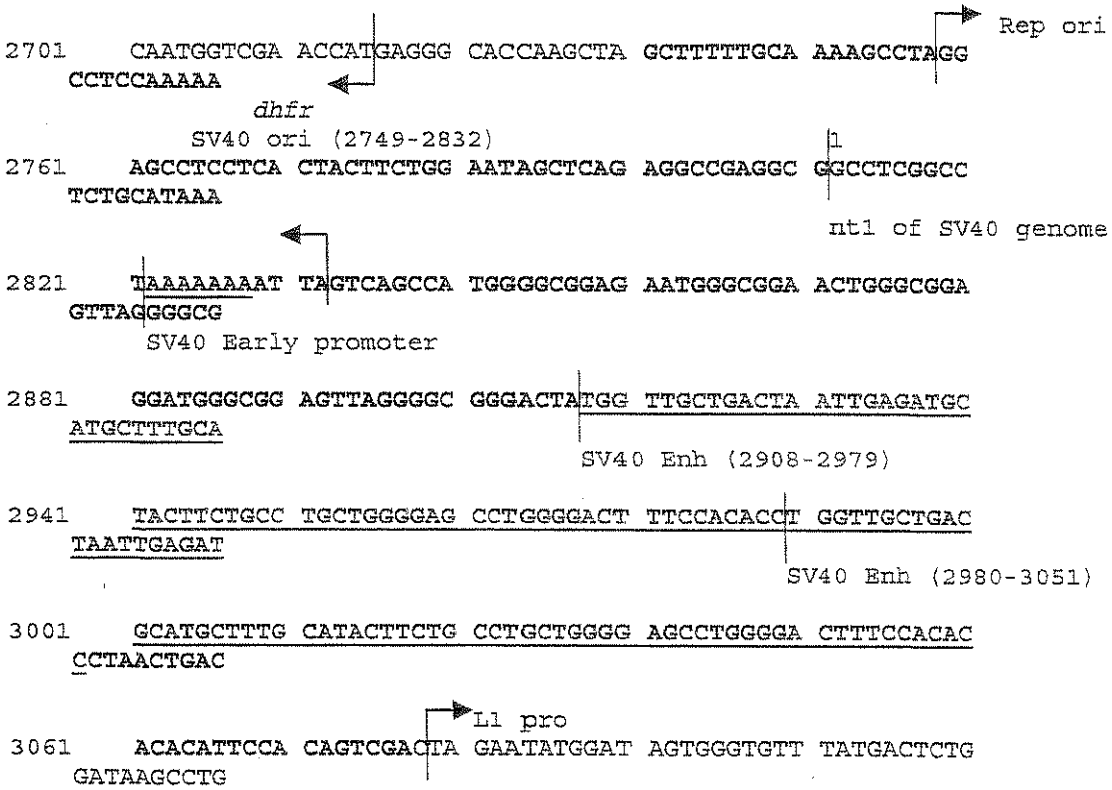
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DEFINITION  hMN14pdHL2 vector.
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9661 gctcactcaa aggcggtaat acggttatcc acagaatcag gggataacgc aggaaagaac
9721 atgtgagcaa aaggccagca aaaggccagg aaccgtaaaa aggccgcgtt gctggcgttt

SEQ: ID. No. 2: Enhancer and promoter sequences of the SV40 enhancer and promoter respectively, that are present in the hMN-14pdHL2 vector used to transfect the SP2/0 cells to obtain the 665.2B9 clone.

hLL2pdHL2



SEQ. ID. NO. 3: Nucleic acid sequence of Bcl-2-EEE gene

5'-

ATGGCGCACG CTGGGAGAAC GGGGTACGAT AACCGGGAGA TAGTGATGAA GTACATCCAT

TATAAGCTGT CGCAGAGGGG CTACGAGTGG GATGCGGGAG ATGTGGGCGC CGCGCCCCCG

GGGGCCGCCC CCGCACCGGG CATCTTCTCC TCCCAGCCCG GGCACACGCC CCATCCAGCC

GCATCCCGCG ACCCGGTCGC CAGAGAAGAA CCGCTGCAGA CTCCGGCTGC TCCTGGAGCA

GCTGCAGGAC CTGCGCTCGA ACCGGTGCCA CCTGTGGTCC ACCTGACCCT CCGCCAGGCC

GGCGACGACT TCTCCCGCCG CTACCGCCGC GACTTCGCCG AGATGTCCAG CCAGCTGCAC

CTGACGCCCT TCACCGCGCG GGGACGCTTT GCCACGGTGG TGGAGGAGCT CTTCAGGGAC

GGGGTGAACT GGGGGAGGAT TGTGGCCTTC TTTGAGTTCG GTGGGGTCAT GTGTGTGGAG

AGCGTCAACC GGGAGATGTC GCCCCTGGTG GACAACATCG CCCTGTGGAT GACTGAGTAC

CTGAACCGGC ACCTGCACAC CTGGATCCAG GATAACGGAG GCTGGGATGC CTTTGTGGAA

CTGTACGGCC CCAGCATGCG GCCTCTGTTT GATTTCTCCT GGCTGTCTCT GAAGACTCTG

CTCAGTTTGG CCCTGGTGGG AGCTTGCATC ACCCTGGGTG CCTATCTGGG CCACAAGTGA-3'

SEQ. ID. NO. 4: Amino acid sequence of Bcl-2-EEE protein

1 MAHAGRTGYD NREIVMKYIH YKLSQRGYEW DAGDVGAAPP GAAPAPGIFS SQPGHTPHPA

61 ASRDPVAREE PLQTPAAPGA AAGPALEPVP PVVHLTLRQA GDDFSRRYRR DFAEMSSQLH

121 LTPFTARGRF ATVVEELFRD GVNWGRIVAF FEFGGVMCVE SVNREMSPLV DNIALWMTEY

181 LNRHLHTWIQ DNGGWDAFVE LYGPSMRPLF DFSWLSLKTL LSLALVGACI TLGAYLGHK

SEQ. ID. NO. 5: Bcl-2-EEE Left (80)

5'GGACCCGGTCGCCAGAGAAGAACCGCTGCAGACTCCGGCTGCTCCTGGAGCAG
CTGCAGGACCTGCGCTCGAACCGGTGC-3'

SEQ. ID. NO. 6: Bcl-2-EEE Right (80)

5'CGCCGGCCTGGCGGAGGGTCAGGTGGACCACAGGTGGCACCGGTTCGAGCGCA
GGTCCTGCAGCTGCTCCAGGAGCAGCC-3'