Title: PROCESS FOR PRODUCING A RECOMBINANT PROTEIN

Abstract: The addition of an insulin-like factor and transferrin to serum-free media is synergistic in increasing the expression of recombinant proteins in mammalian cells.
TITLE: PROCESS FOR PRODUCING A RECOMBINANT PROTEIN

Sequence Listing
The present application contains information in the form of a sequence listing, which is appended to the application and also submitted in computer readable form accompanying this application. The contents of sequence listing submitted in computer readable are fully incorporated herein by reference.

FIELD OF THE INVENTION
The invention relates to a process for improving the production of a recombinant protein by cultivation of a transfected mammalian host cell. The invention also relates to a cell culture medium for improving recombinant protein production.

BACKGROUND OF THE INVENTION


SUMMARY OF THE INVENTION

The inventors have found that the addition of an insulin-like factor (e.g. insulin-like growth factor (IGF) or insulin) and transferrin to serum-free media is synergistic in increasing the growth of mammalian cells and the expression of recombinant proteins in mammalian cells.

Accordingly, the invention provides a process for producing a desired protein, comprising:

a) transfecting a mammalian host cell with a DNA sequence encoding the desired protein, and

b) culturing the transfected host cell in a cell culture medium comprising an insulin-like factor (e.g. IGF or insulin) and a transferrin (Tf) under conditions permitting expression of the desired protein.

In a preferred embodiment, the invention further provides a process wherein the combination of the insulin-like factor and the Tf increases the yield of production of the desired protein to a level greater than the sum of the increase in yield from the addition of the insulin-like factor or the Tf separately.

In another preferred embodiment, the invention further provides a process wherein the combination of the insulin-like factor and the Tf increases the growth of the mammalian host cell to a level greater than the sum of the increase in yield from the addition of the insulin-like factor or the Tf separately.

In another preferred embodiment, the invention further provides a process wherein the cell culture medium comprises the insulin-like factor at a concentration of 0.001-200 µg/ml (micro-g/ml), for example 0.001-20 µg/ml (micro-g/ml).

In another preferred embodiment, the invention further provides a process wherein the cell culture medium comprises the Tf at a concentration of 0.1-100 µg/ml (micro-g/ml), for example 0.1-10 µg/ml (micro-g/ml).

The invention also provides a cell culture medium comprising:

(a) basal medium
(b) an insulin-like factor at a concentration of 0.001-200 µg/ml (micro-g/ml); and
(c) a transferrin at a concentration of 0.1-100 µg/ml (micro-g/ml).
The insulin-like factor may be at a concentration of 0.001-20 µg/ml (micro-g/ml). The transferrin may be at a concentration of 0.1-100 µg/ml (micro-g/ml).

For the process or method of the invention, the insulin-like factor may be one or more of insulin and IGF, or a fragment, functional analogue or derivative thereof. The transferrin may be a fragment, functional analogue or derivative thereof.

**DETAILED DESCRIPTION OF THE INVENTION**

**Desired protein**

Various proteins, particularly human proteins and homologues, can be expressed in mammalian cells including therapeutic monoclonal antibodies and antibody fragments and their fusions with other proteins, growth factors, hormones, cytokines, lymphokines, angiogenic factors, enzymes, receptors, extracellular matrix proteins, cell surface markers, etc.


Other examples include agalsidase beta, angiostatin, DWP-404, erythropoietin, etanercept, Factor VIII, Factor VIIIc, herpes simplex vaccine, hepatitis-B vaccine, interferon (alpha), interferon (β1), interferon (gamma), interleukin-1 receptor-1, malaria vaccines, nerve growth factor, PEG-uricase, protein C, somatropin, XR-31 1, mitumprotimut-T, hepatitis-C vaccine, and PHEX peptidase.

Some further examples are A-74187, pexelizumab, aflimeprase, darbepoetin alfa, ersofermin, MVA-BN DF (Bavarian Nordic), MVA-BN HIV nef (Bavarian Nordic), BAY-50-4798, repinotan, desmoteplase, rolipram, galsulfase, BIT-225, belatacept, Leucotropin, Endostatin, SARS coronavirus vaccine, rusalatide, rhCCIO, Claragen, velafermin, eptotermin alfa, anti-endotheliase antibodies), Dendreon/Dyax, alglucosidase alfa, monteplase, drotrecogin alfa, eglumatad, pemetrexed, mitumprotimut-t, pexiganan acetate, dornase alfa, tenecteplase, personalized recombinant protein vaccines, Genitope, chitinase, Genzyme Corp, recombinant prolactin, sertindole, CD4 red blood cell electroinsertion, cetuximab,

DNA sequence

Any DNA sequence encoding the desired protein can be used, e.g. the sequence described in the indicated literature or a sequence obtained by codon optimization. The coding DNA is coupled to a promoter sequence that will enable the host cell proteins to transcribe the information to mRNA and then translate mRNA to the target protein. Persons skilled in the art would readily and with a reasonable expectation of success be able to follow a protocol such as that described in Curr. Protocols in Mol. Biol. (2001) (John Wiley and Sons.), to design the DNA sequence required.

Mammalian host cell

Examples of mammalian cells are CHO and its variants, hybridomas (NSO and sp2/0), Per.C6, HEK293, Vero, MDCK, and BHK cell lines, e.g. MK2.7 cells, BHK-21, PER.C6® cells, NSO, GS-NSO, CHO K1, CHO-S, CHO-GS, CHO-DukxB1 1, CHO DG44 cells, HEK 293 cells, COS cells and Sp2/0 cells.

MK2.7 (ATCC Catalogue Number CRL 1909) is an anti-murine VCAM IgGl expressing hybridoma cell line derived from the fusion of a rat splenocyte and a mouse Sp2/0 myeloma. MK2.7 is a non-adherent cell line that can be grown in serum-free media. Other types of cells can be selected from the group consisting of 5L8 hybridoma cells, Daudi cells, EL4 cells, HeLa cells, HL-60 cells, K562 cells, Jurkat cells, THP-1 cells, Sp2/0 cells; and/or the hybridoma cells listed in table 2, WO 2005/070120 which is hereby incorporated by reference or any other cell type disclosed herein or known to one skilled in the art.

Additional mammalian cell types can include primary cells derived from endodermal,
mesodermal and ectodermal origins, including epithelial cells (e.g., keratinocytes, cervical epithelial cells, bronchial epithelial cells, tracheal epithelial cells, kidney epithelial cells and retinal epithelial cells) and established cell lines and their strains (e.g., 293 embryonic kidney cells, BHK cells, HeLa cervical epithelial cells and PER-C6 retinal cells, MDBK (NBL-1) cells, 911 cells, CRFK cells, MDCK cells, CHO cells, BeWo cells, Chang cells, Detroit 562 cells, HeLa 229 cells, HeLa S3 cells, Hep-2 cells, KB cells, LS 180 cells, LS 174T cells, NCI-H-548 cells, RPMI2650 cells, SW-13 cells, T24 cells, WI-28 VA13, 2RA cells, WISH cells, BS-C-I cells, LLC-PK.sub.2 cells, Clone M-3 cells, 1-10 cells, RAG cells, TCMK-1 cells, Y-1 cells, LLC-PK.sub.1 cells, PK(15) cells, GH.1 cells, GH3 cells, L2 cells, LLC-RC 256 cells, MH.sub.IC1 cells, XC cells, MDOK cells, VSW cells, and TH-I, B1 cells, or derivatives thereof), fibroblast cells from any tissue or organ (including but not limited to heart, liver, kidney, colon, intestines, esophagus, stomach, neural tissue (brain, spinal cord), lung, vascular tissue (artery, vein, capillary), lymphoid tissue (lymph gland, adenoid, tonsil, bone marrow, and blood), spleen, and fibroblast and fibroblast-like cell lines (e.g., CHO cells, TRG-2 cells, IMR-33 cells, Don cells, GHK-21 cells, citrullinemia cells, Dempsey cells, Detroit 551 cells, Detroit 510 cells, Detroit 525 cells, Detroit 529 cells, Detroit 532 cells, Detroit 539 cells, Detroit 548 cells, Detroit 573 cells, HEL 299 cells, IMR-90 cells, MRC-5 cells, WI-38 cells, WI-26 cells, MiCl.sub.1 cells, CHO cells, CV-1 cells, COS-1 cells, COS-3 cells, COS-7 cells, Vero cells, DBS-FrhL-2 cells, BALB/3T3 cells, F9 cells, SV-T2 cells, M-MSV-BALB/3T3 cells, K-BALB cells, BLO-11 cells, NOR-10 cells, C3H/10T1/2 cells, HSDM.sub.IC3 cells, KLN205 cells, McCoy cells, Mouse L cells, Strain 2071 (Mouse L) cells, L-M strain (Mouse L) cells, L-MTK (Mouse L) cells, NCTC clones 2472 and 2555, SCC-PSA1 cells, Swiss/3T3 cells, Indian muntjac cells, SIRC cells, CII cells, and Jensen cells, or derivatives thereof).

Transfection of DNA into Mammalian Cells

The transfection may be carried out as described in Curr. Protocols in Mol. Biol. Chap 9, 2001 (John Wiley and Sons.).

The mammalian cell may be transfected with a product gene and a dhfr gene which enables selection of transfected host cells of the dhfr+ phenotype. Selection is carried out by culturing the colonies in media devoid of thymidine and hypoxanthine, the absence of which prevents cells from growing. The stable transfectants usually express low levels of the product gene by virtue of co-integration of both transfected genes. The expression levels for the product gene may be increased by amplification using methotrexate. This drug is a direct inhibitor of
the dhfr enzyme and allows isolation of resistant colonies which have amplified their dhfr gene copy number sufficiently to survive under these conditions. Since the dhfr and product genes are usually closely linked in the original transfected cells, there is normally concomitant amplification resulting in increased expression of the desired product gene.

**Insulin-like factor (IGF or insulin)**

Preferably the insulin-like factor is selected from the group consisting of insulin, IGF-I, IGF-II and homologues thereof, particularly homologues of the human sequences. Preferably the homologues, e.g. sequence variants, retain some affinity for the IGF-I receptor. The affinity for the IGF-I receptor may be diminished but preferably retains sufficient potency, for example sufficient ability to enhance cell growth. Preferably the homologue shows at least the same potency as insulin, more preferably a potency higher than that of insulin and most preferably a potency which is the same as or higher than that of IGF-I. A homologue might have other desirable sequence changes that improve its potency in enhancing the growth or productivity of cultured mammalian cells, e.g. reduced binding to inhibitory IGF-binding proteins (IGFBPs). Examples are [Arg]3IGF-I, [Arg]6IGF-II, [Gly]3IGF-I des(1-3)IGF-I and des(1-6)IGF-II and indeed any IGF with the first 3 to 6 amino acids in its sequence altered to remove or replace the most N-terminal glutamic acid residue. For the purposes of this specification, insulin, IGF-I, IGF-II and biologically active fragments, functional analogues or derivatives are considered to be equivalent, and are referred to collectively as "insulin-like factors".

In one preferred embodiment the insulin-like factors has 100% amino acid sequence identity to the amino acid sequence of human IGF-I. The amino acid sequence for human IGF-I is as follows:

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GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSRRAPQTGIVDECCFRSCDLRRLEMY
CAPLKPAKSA [SEQ ID NO. 1]
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Alternatively the insulin-like factor has 100% amino acid sequence identity to the amino acid sequence of human IGF-II. The amino acid sequence for human IGF-II is as follows:

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AYRPSETLCGGELVDTLQFVCGDRGFYSRPASRVSRRGIVEECCFRSCDLALLETYCA
TPAKSE [SEQ ID No. 2]
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Alternatively the insulin-like factor has 100% amino acid sequence homology to the amino acid sequence of human insulin. The amino acid sequence for human insulin is as follows:

B Chain: FVNQH LCGSH LVEALYLVCGERGFFYTPKT [SEQ ID No. 3]

A Chain: GIVEQCCTSICSLYQLENYC [SEQ ID No. 4]

More preferably the insulin-like factor is selected from the group consisting of:

MFPAMLSSLF-[Arg]3IGF-I, [SEQ ID No. 5]

MFPAMLSSLFVN-[Arg]3IGF-I, [SEQ ID No. 6]

MFPAMLSSLFVNFAHY-[Arg]3IGF-I [SEQ ID No. 7],

MFPAMLSSLFVNGFAHY-[Arg]3IGF-I [SEQ ID No. 8],

MFPAMLSSLFANAVLRAQHQLAADTYYKEFERAYIPEGQRYSIQ-[ATg]3IGF-I [SEQ ID No. 9],

MFPAMLSSLFANAVLRAQHQLAADTYYKEFERAYIPEGQRYSIQVN-[Arg]3IGF-I [SEQ ID No. 10],

MFPAMLSSLFANAVLRAQHQLAADTYYKEFERAYIPEGQRYSIQVNFAHY-[Arg]3IGF-I [SEQ ID No. 11],

MFPAMLSSLFANAVLRAQHQLAADTYYKEFERAYIPEGQRYSIQVNGFAHY-[Arg]3IGF-I [SEQ ID No. 12],

MFPAMLSSLFLSTQ-[Arg]3IGF-I [SEQ ID No. 13],

MFPAMLSSLFVN LSTQ-[Arg]3IGF-I [SEQ ID No. 14],

MFPAMLSSLFVNLSTQ-[Arg]3IGF-I [SEQ ID No. 15],

MFPAMLSSLFANAVLRAQHQLAADTYYKEFERAYIPEGQRYSIQLSTQ-[Arg]3IGF-I [SEQ ID No. 16],

MFPAMLSSLFANAVLRAQHQLAADTYYKEFERAYIPEGQRYSIQVNFAHY-[Arg]3IGF-I [SEQ ID No. 17],

MFPAMLSSLFANAVLRAQHQLAADTYYKEFERAYIPEGQRYSIQVNGFAHY-[Arg]3IGF-I [SEQ ID No. 18].
MFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYSIQVNLSTQ-[Arg]3IGF-I
[SEQ ID No. 17], or

MFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYSIQVNGLSTQ-[Arg]3IGF-I
[SEQ ID No. 18],

MFPAMPLSSLF-des(1-3)IGF-I [SEQ ID No. 19],

MFPAMPLSSLFVN-des(1-3)IGF-I [SEQ ID No. 20],

MFPAMPLSSLFVNFAHY-des(1-3)IGF-I [SEQ ID No. 21],

MFPAMPLSSLFVNGFAHY-des(1-3)IGF-I [SEQ ID No. 22],

MFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYSIQ-desO^IGF-I
[SEQ ID No. 23],

MFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYSIQVN-des(1-3)IGF-I
[SEQ ID No. 24],

MFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYSIQVNFAHY-des(1-3)IGF-I
[SEQ ID No. 25],

MFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYSIQVNGFAHY-des(1-3)IGF-I
[SEQ ID No. 26],

MFPAMPLSSLFLSTQ-des(1-3)IGF-I [SEQ ID No. 27],

MFPAMPLSSLFVNLTQ-des(1-3)IGF-I [SEQ ID No. 28],

MFPAMPLSSLFVNLSTQ-des(1-3)IGF-I [SEQ ID No. 29],

MFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYSIQLSTQ-des(1-3)IGF-I
[SEQ ID No. 30],

MFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYSIQVNLSTQ-des(1-3)IGF-
In this specification, abbreviations such as: 'MFPAMPLSSLF-[Arg]3IGF-I' mean that the sequence preceding '[Arg3IGF-I]' is fused to a protein analogue of IGF-I in which the third amino acid of the sequence of native IGF-I is substituted for an Arg. For example, 'MFPAMPLSSLF-[Arg]3IGF-I, [SEQ ID No. 5]' is an abbreviation for MFPAMPLSSLF GPRTLCGAELVDALQFVCGRDFYFNKPTGYGSSSRAPPQTGIVDECCFRSCDLRRLEMY CAPLKPAKSA.

Likewise, abbreviations such as:
'MFPAMPLSSLFANAVLRAQHLHQLAATYYKEFERAYIPEGQRYSIQVNFAHY-des(1-3)IGF-I', mean that the sequence preceding 'des(1-3)IGF-I' is fused to a protein analogue of IGF-I in which the first three amino acids of the sequence of native IGF-I are deleted. For example, 'MFPAMPLSSLFANAVLRAQHLHQLAATYYKEFERAYIPEGQRYSIQVNFAHY-des(1-3)IGF-I' is an abbreviation for:

MFPAMPLSSLFANAVLRAQHLHQLAATYYKEFERAYIPEGQRYSIQVNFAHYTLCGAELVD ALQFVCGRDFYFNKPTGYGSSSRAPPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA.

Even more preferably the insulin-like factor is MFPAMPLSSLFVNLSTQ-des(1-3)IGF-I [SEQ ID No. 6]. Alternatively the insulin-like factor is MFPAMLSSLFV-[Arg]3IGF-I [SEQ ID No. 28] (also referred to as LONG®R3IGF-I). LONG®R3IGF-I is sometimes referred to as LR3 or L®R3.

Preferably the insulin-like factor has a molecular weight of 9.1 KDa as determined by mass spectrometry. Alternatively the insulin-like factor has a molecular weight of 9.2 KDa as determined by mass spectrometry.

In another preferred embodiment the insulin-like factor has biological activity, determined by the L6 rat myoblast protein synthesis assay. Preferably the insulin-like factor has biological activity with an ED50 below 10ng/ml as determined by protein synthesis assay in rat myoblasts cells.

In another preferred embodiment the insulin-like factor maintains at least 90% of its original
bioactivity as measured using the L6 rat myoblast protein synthesis assay following storage for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 months at temperatures between 2 and 8°C, 2 and 27°C or 2 and 37°C.

The insulin-like factor may be recombinant and manufactured using recombinant DNA technology. For example, the insulin-like factor is recombinant and manufactured using techniques described in T. Maniatis et al., in Molecular Cloning. A. Laboratory Manual. CSH Lab. N.Y. (1989). Preferably the insulin-like factor is substantially isolated. For example, it may be substantially isolated from a culture medium following recombinant production of the insulin-like factor.

Suitable methods for the expression and purification of an insulin-like factor are discussed in United States patent No. 5,330,971. It is to be clearly understood, that the present invention extends to biologically active fragments or functional analogues of any human insulin-like factor, i.e. analogues or derivatives of human insulin-like factor in which the wild-type insulin-like factor sequence contains additions, deletions or substitutions by other amino acids or amino acid analogues, in which the biological activity of the insulin-like factor is retained. Methods for identification, manufacture and biological characterisation of active fragments, functional analogues or derivatives of the insulin-like factor are well known to those of ordinary skill in the art, and can be addressed with no more than routine experimentation. In a preferred form, the biologically active fragment, functional analogue or derivative of the insulin-like factor demonstrates biological activity. Insulin-like factor analogues suitable for use in the invention include those described in United States patents U.S. 5,077,276, U.S. 5,164,370, U.S. 5,470,828, and U.S. 5,330,971, and in International Patent Application No. PCT/AU99/00292, which include des(1-3)IGF-I, des(1-6)IGF-II, analogues of IGF-I with amino acid substitutions at amino acid position 1 to 3, analogues of IGF-II with amino acid substitutions at amino acid position 1 to 6, porcine growth hormone IGF fusion proteins and IGF analogues incorporating heparin-binding motifs which enable the IGF analogue to bind to fixed surfaces. In a preferred form the insulin-like factor fragment, functional analogue or derivative has at least 70% amino acid sequence identity with the native insulin-like factor amino acid sequence in question, or preferably at least 90%, more preferably 95%. Methods for assessing the amino acid sequence identity are well known in the art. For example, a suitable program for determining percentage sequence identity is BLAST 2.0 Sequence Comparison (NIH) (http://www.ncbi.nlm.nih.gov/blast/b12seq/b12.html). Preferably, the limiting parameters imposed for such a task are the default setting for the program as displayed on this web site. Thus persons skilled in the art would, readily and with a
reasonable expectation of success, know which alterations to the protein sequence would affect the structure of the IGF polypeptide, and affect the biological activity of the protein. Preferably the alteration does not alter the insulin-like factor polypeptide domain responsible for binding affinity to the insulin or type 1 IGF receptors.

Thus based on the teachings in this specification, the skilled person would readily anticipate the effect of a change within the sequence and would be able to identify biologically active fragments or functional analogues with a high expectation of success. While experimentation may be required to identify biologically active fragments of functional analogues this experimentation is not unduly complex but merely routine. For example, skilled persons will readily be able to use the rat L6 myoblast protein synthesis assay (G. L. Francis et al. Biochem. J. 233, 207, 1986.) to identify biological active fragments or functional analogues of IGFs. The terms "fragment", "analogue", and "derivative" of the insulin-like factor, mean a molecule that retains essentially the same biological function or activity as insulin or IGF.
**Transferrin (Tf)**

The transferrin may be any serum transferrin, such as a mammalian serum transferrin, particularly human serum transferrin (HST) having the amino acid sequence with 679 amino acids ([SEQ ID No. 33], *i.e.* without a 19 amino acid leader sequence: MRLAVGALLVCAVLGLCLA) or 698 amino acids (Ae. with the 19 amino acid leader sequence [SEQ ID No. 34], Accession numbers NP_001054 and NM_001063), also called the C1 variant, or a derivative or variant, such as mutant transferrins (Mason et al., (1993) Biochemistry, 32, 5472; Mason et al., (1998), Biochem. J., 330, 35), truncated transferrins, transferrin lobes (Mason et al., (1996) Protein Expr. Purif., 8, 119; Mason et al., (1991) Protein Expr. Purif., 2, 214). Preferably the transferrin is a functional homologue of a serum transferrin. For example, the transferrin may have the sequence of SEQ ID No. 35 which is available from Novozymes under the trade mark Cell Prime (formerly available from Novozymes under the trade mark DeltaFerrin). Two or more of SEQ ID No. 33, SEQ ID No, 34 or SEQ ID No. 35 may be used in combination.

Sequences 33, 34 and 35 are provided below. The leader sequence of SEQ ID No. 34 is underlined (amino acids 1 to 19).

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20 VPDKTVRWCA VSEHEATKCQ SFRDHMKSVI PSDGPSVACV KKASYLDCIR AIAANEDAV
TLDAVLVYDA YLAPNNLKFV VAEFYGSKED PQTFYYAVAV VKKDGFQMN QLRGKKSCHT
GLGRSAGWNI PIGLLYCDLPM EPRKPLEKAQ ANFFSGSCAP CADGTDFQPL CQLPGCCGCS
TLNQYFGYSG AFKCLKDGAG DAVFVHKSTI FENLANKADR DQYELLCLDN TRKPVEYKRD
CHLAVQPSHT VVARSMSGKE DLIWELLNQA QEHFQGDKS QFQFLPSPHG KDLLFKDASAH
25 GFLKVPRFMD AKMYLGYEYV TAIRNLRGET CPEAPTDCEK PVKWCALSHH ERLKCEDWSV
NSVGKIECVS AETTEDCIAK IMNGEADAMS LGDGVFFYIIAG KCGLVFPVLA EYNKSNDCED
TPEAGYFAVA VVKKASDLT WDNLKGKKSC HTAVGRTAGW NIPMGLLYNK INHCRFDEFF
SEGCAPGSKK DSSLCLCMCG SSLNCLCEPNN KEGGYGXTGA FRCILVKE GDVAFVKHQTPVQ
NTGGKNPDAPK AKNLNEKDYE LLCLDGTRKP VEEYANCHLA RAPNHAWTR KDKEACVHKI
30 LRQQQHFLFGS NVTDGNSGFC LFRSETKDLL FRDHTVCLAK LHRRNTEKYE LGEEYVKAVG
NLRKCSTSSL LEACTFRRP [SEQ ID NO. 33]
MRLAVGALLV CAVLGLCLAV PDKTVRWCA VSEHEATKCQ SFRDHMKSVI PSDGPSVACV
KASYLDCIRA IAANEDAVAT LDALGLVDAY LAPNNLKPVV AEFYGSKEDP QTFYYAVAW
35 KKDSGFQMNQ LRGKKSCHTG LGRSAGWNNIP IGLLYCDLPE PRKPLEKAQ AIFFSGSCAPC
ADGDTDFQPLC QLCPGCGCST LNQYFGYSGA FKCLKDGAGD VAFKHLSTIF ENLANKADRD
QYELLCLDNTR RKPVDEYKDC HLAQVPSHTV VVARSMSGKE DLIWELLNQA QEHFQGDKSKE
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The transferrin may be a variant with reduced N- and O-linked glycosylation. Accordingly, in sequence NP_001054, N413 can be changed to any amino acid, preferably, Q, D, E or A; S415 can be changed to any amino acid except S or T, preferably, A; T613 can be changed to any amino acid except S or T, preferably, A; N611 can be changed to any amino acid; or combinations of the above. The co-ordinates given above relate to SEQ ID No. 33 (i.e. the sequence of NP_001054 without the 19 amino acid leader sequence). For clarity, the respective co-ordinates of SEQ ID No. 34 (i.e. the sequence of NP_001054 with the 19 amino acid leader sequence) are N432; S434; T632 and N630.

Concentrations of insulin-like factor and transferrin

The combination of the insulin-like factor and the Tf is synergistic. Thus, the insulin-like factor and the Tf may be used at concentrations where the yield of production of the desired protein is increased to a level greater than the combined increase in yield from the addition of the insulin-like factor or the Tf alone.
In particular, the cell culture medium may comprise the insulin-like factor at a concentration of 0.001-200 µg/ml (micro-g/ml), particularly 0.001-20 µg/ml (micro-g/ml), more particularly 0.01-0.2 µg/ml (micro-g/ml) for IGF-I, IGF-II and/or an IGF analogue, such as LONG® R3IGF-I, or 0.1 to 20 µg/ml (micro-g/ml) for insulin, and/or it may comprise the Tf at a concentration of 0.1-100 µg/ml (micro-g/ml), particularly 0.1-10 µg/ml (micro-g/ml), more particularly 0.5-5 µg/ml (micro-g/ml). An advantage of using a mixture of different insulin-like factors is that it may activate a number of different insulin receptors and therefore prove advantageous to cells undergoing culture.

Cell culture medium

The cell culture medium is generally serum-free and, in addition to IGF and Tf, generally comprises basal medium. Basal media may comprise, but are not limited to Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, alpha-modified Minimal Essential Medium (alpha.MEM), Glasgow's Minimal Essential Medium (G-MEM), and/or Iscove's Modified Dulbecco's Medium. The cell culture medium may also comprise one or more of the following components:

- Water.
- Osmolality regulators such as salts including NaCl, KCl, KNO₃. It is advantageous to have the osmolality range in the 150-450 milli-Osmols (mOsm) range, particular in the 200-400 mOsm range and more particular 290-350 mOsm.
- Buffers to maintain pH such as carbonates such as NaHCO₃; also chlorides, sulphates and phosphates such as CaCl₂x2H₂O, MgSO₄x7H₂O, NaHPO₄x2H₂O, or sodium pyruvate or HEPES, or MOPS. Buffers maintain the pH of the medium at pH 6-8, particularly pH 6.5-7.5 and most particularly 7.
- Energy sources including one or more of glucose, mannose, fructose, galactose and maltose. The energy source is present in an amount 250-20,000 mg/liter, particularly 500-15,000 mg/liter, and most particularly, 1000-10,000 mg/liter.
- Non-ferrous metal ions such as magnesium, copper and zinc, sodium, potassium and selenium. These are often added as salts such as chlorides and sulphates. Selenium particularly in the form of sodium selenite, Na₂SeO₃ and in a range between 0.004-0.08 mg/liter, particularly 0.009-0.02 mg/liter.
- Vitamins and enzyme co-factor vitamins (co-factors) include Vitamin B6 (pyridoxine), Vitamin B12 (cyanocobalamin), Vitamin K (biotin) present in an amount 0.005-0.75 mg/liter, particularly 0.01-0.5 mg/liter. Vitamin C (ascorbic acid) present in an amount
5-45 mg/liter, particularly 10-30 mg/liter. Vitamin B2 (riboflavin) present in amount 0.05-2 mg/liter, particularly 0.1-1 mg/liter. Vitamin B1 (thiamine), nicotinamide, Vitamin B5 (D calcium pantothenate, folic acid, i-insitol present in an amount 0.05-16 mg/liter, particularly 0.1-12 mg/liter, more particularly 0.2-8 mg/liter. Vitamin E present in an amount 0.1-20 µM (micromolar), particular 1-10 µM (micromolar), more particularly 2.5-7.5 µM (micromolar).

- Lipids and fatty acids, and lipid factors such as choline chloride, lipoic acid, oleic acid, myrisic acid, palmitic acid, stearic acid, Tween® 80, Pluronic® F-68, phosphatidylcholine or methyl lineoleate or arachadonic acid. The amount present in the range 0.00075-30 mg/liter, particularly 0.02-20 mg/liter, more particularly 0.05-10 mg/liter.

- Lipoproteins such as any lipophilic compound that can be, for example, carried in the plasma by apolipoproteins, including but not limited to cholesteryl esters, unesterified cholesterol, cholesterol, triglycerides, fatty acids and/or phospholipids. EX-CYTE®. The amount present in the range 0.0001-10% w/v, particularly 0.01-7.5% more particularly, 0.1-5%, even more particularly 1-3%.

- Compounds involved in lipid production for example alcoholumines such as ethanolamine. These may be present in an amount 1-30 µM (micromolar), particularly 3-20 µM (micromolar), more particularly 5-15 µM (micromolar). Alternatively, the ethanolamine may be present in an amount of 0.01-1000 mg/l.

- Amino acids, such as but not limited to glutamine. They may be present in an amount in the range of 0.01-1000mg/l or 1-300 mg/l, in particular 10-200 mg/l. L-glutamine is often present in higher concentrations in the range 250-750 mg/ml, particularly 400-600 mg/ml.

- It may be advantageous to include a pH indicator for example Phenol red sodium salt present in a range of 2.5-75 mg/l.

- Antibiotics such as polymyxin, neomycin, penicillin or streptomycin in an amount at a range from 2500-150,000 µl/l (micro-l/l).

- Growth factors, hormones including peptide hormones such as but not limited to insulin in an amount of 0.1-50 mg/l, in particular 1-25 mg/l, more in particularly 4-20 mg/l. Epidermal growth factor (EGF) in a range from 0.01-200 µg/l (micro-g/l), more particular 0.1-100 µg/liter. Platelet-derived growth factor (PDGF), thyroxine (T3), thrombin, interleukins such as IL2 and IL6, progesterone, testosterone, hydrocortisone. Any of the growth factors, for example EGF, IGF and analogues thereof, may be present at 0.001-200 µg/l (micro-g/l), for example at 0.001-20 µg/l (micro-g/l).
• Chelating agents, ion transporter and iron sources, radical scavengers such as EDTA, L-Ascorbic acid, α-Tocopherol (alpha-Tocopherol), present in a range of 0.001-1000 mg/l, in particular 0.1-30 mg/l, more particularly 0.25-15 mg/l, even more particularly 0.5-10 mg/l. Lactoferrin, chelated iron salts such as ferric citrate or ferric ammonium citrate.

• Protein or peptide digests, hydrolysates or extracts.
• Glycoproteins including fetuin.
• Putrescine, including as a salt such as HCl, which is known to play a role in maintaining the structure of the endoplasmic reticulum.

• Cell protectants such as polyethylene glycol, polyvinyl alcohol or pluronic polyols
• Hypoxanthine, thymidine, methotrexate for selection pressure regulation.
• Antioxidants

**Cell culture conditions**

The mammalian host cell is cultured under conditions permitting expression of the desired protein.

**Optional recovery and purification of desired protein**

After the fermentation process is ended, the polypeptide of interest may be recovered from the fermentation broth, using standard technology developed for the polypeptide of interest.

The relevant downstream processing technology to be applied depends on the nature of the polypeptide of interest.

A process for the recovery of a polypeptide of interest from a fermentation broth will typically (but is not limited to) involve some or all of the following steps:

1) pre-treatment of broth (e.g. flocculation)
2) removal of cells and other solid material from broth (primary separation)
3) capture of the polypeptide of interest
4) intermediate purification and final purification (final purification may also be referred to as ‘polishing’)
5) filtration
6) formulation and stabilization.
Apart from the unit operations listed above, a number of other recovery procedures and steps may be applied, e.g. pH-adjustments, variation in temperature, crystallization, treatment of the solution comprising the polypeptide of interest with active carbon, and use of various adsorbents.

**EXAMPLE 1:**

**Cell culture**

In the examples below, the cells used were derived by co-transfecting the CHO cell line DP-12 with a vector designed to co-express the variable light and heavy regions of the murine 6G4.2.5 monoclonal antibody (humanised) (ATCC CRL-12445). This cell line (herein referred to as CHO DP12 clone 1934) secretes recombinant IgG against human IL-8 under methotrexate (MTX) selection.

The frozen cryovials of CHO DP12 clone 1934 cells were thawed in a 37°C water bath. The thawed cells were transferred into a T75 tissue culture flask containing 10-20ml warmed sterile culture medium (DMEM/F12 containing 10% FBS (fetal bovine serum), 4.5g/l glucose, 2mg/l recombinant human insulin, 20OnM MTX and 2mM GlutaMax™). The flask was incubated at 37°C/5% CO₂ overnight. The culture medium was changed the following day, whilst also checking flasks for cell growth and absence of contamination.

Cells were used for an assay when they were between 70 and 90% confluent (i.e. covering 70-90% of the base of the flask). When the cells were 70-90% confluent, the growth medium was aspirated and the cells washed with 10ml of warmed Dulbecco’s phosphate buffered saline (D-PBS). After aspiration of the D-PBS, 2ml of warmed trypsin solution was added and the flask incubated at 37°C for 2 minutes. The flask was then gently tapped to detach the monolayer of cells and 8ml of warm culture media was added to resuspend the cells. The cells were washed in serum-free and growth factor-free medium (SFM; DMEM/F12, 4.5g/l glucose, 20OnM MTX and 2mM GlutaMax™) and the number of viable cells determined by the trypan blue exclusion method. The cells were then prepared by adding the required volume of cells to an appropriate volume of fresh SFM and mixed by pipetting gently up and down.
Determination of growth and productivity of adherent CHO cells in SFM with the addition of recombinant supplements.

Assay set-up

1000 cells (in 180 µl (micro-l) SFM) were added to each well of a 96-well culture plate using a multi-channel pipette, excluding the perimeter of the plate. The perimeter wells of each plate were filled with medium only to minimise evaporation of the test wells during the assay incubation period. The plates were incubated at room temperature in the biological safety cabinet for 1 hour to allow cells to evenly attach. All controls and supplements were tested at least in triplicate. GlutaMax™ (and/or other additives) was added to the SFM on the day of use.

A negative control (SFM) and positive control (SFM + 0.1% or 1% FBS) were included in each assay. Controls were prepared fresh on the day of use in 15 or 50ml centrifuge tubes. Supplements were prepared as 10 or 20x concentrates to allow for dilution when they were added to the medium in the 96-well plate. All supplements were prepared in sterile siliconised glass vials. Supplements and controls were at room temperature before addition to the wells. Moving from left to right across the plate and from low to high sample concentration, controls and supplements (20 µl (micro-l)) were added to the appropriate wells in triplicate. The plates were incubated at 37°C/5% CO₂ for 6 or 10 days.

Recombinant supplements examined were LONG® R3IGF-I (range 10-400ng/ml), human transferrin (DeltaFerrin™) (range of 0.625-20 µg/ml (micro-g/l)) or combinations of the two at optimal concentrations (as determined from earlier experiments). DeltaFerrin has the sequence ….. [SEQ ID No. 35]
Harvesting

After the appropriate number of days the plates were centrifuged at 179 RCF (in g forces) for 10 minutes at room temperature. 100µl of conditioned medium was aseptically transferred using a multi-channel pipette to a separate 96-well plate for analysis of the IgG concentration. The remaining medium was removed by gently inverting the plate, then gently blotted dry over a pad of absorbent paper, to avoid dislodging the cells. To examine cell growth, the cell numbers in each well were estimated indirectly by either: 1) measuring the metabolic activity of the cells in each well or, 2) measuring the total cellular DNA, as described below.

Metabolic activity of viable cells measured using reduction of a tetrazolium compound, MTS.

10µl (micro-l) of fresh SFM (37°C) and 20µl (micro-l) of the MTS dye (CellTiter96 Aqueous One Solution Cell Proliferation Assay, Promega) were added to each well under test including the three blank wells using a multi-channel pipette. Plates were incubated at 37°C/5% CO₂ whilst shaking for 2hr± (plus or minus) 15min. The optical density was read on a microplate reader at room temperature at 490nm (background readings at 650nm were subtracted).

Total DNA using CyQuant NF dye

100µl of CyQuant dye (CyQuantNF Cell Proliferation Assay Kit, Invitrogen) was added to each well under test including three blank wells using a multi-channel pipette. The plates were incubated at 37°C/5% CO₂ whilst shaking for 1hr ± (plus or minus) 15min. The fluorescent intensity of each well was then read on a microplate reader at room temperature with excitation at 485nm and emission detection at 530nm.

Quantification of IgG secretion into conditioned media.

The productivity of the cells was determined by measuring the concentration of the IgG secreted into the harvested medium as described above. (Bethyl Laboratories, Montgomery, USA). This ELISA was performed with reagents supplied in the kit and following the manufacturer's instructions and is described briefly below.
Reagents and Disposables from Bethyl used in the ELISA Human IgG ELISA Quantitation kit:

- **Coating Antibody**: Goat anti-Human IgG-affinity purified; Concentration: 1 mg/ml; Catalogue No: A80-104A; Working Dilution: 1/100
- **Calibrator**: Human Reference Serum; Catalogue No: RS10-110; Concentration: 4.0 mg/ml IgG; Working range: 500 - 7.8 ng/ml
- **HRP Detection Antibody**: Goat anti-Human IgG-HRP conjugate; Concentration: 1 mg/ml; Catalogue No: A80-104P; Working Dilution: 1:200,000 - 1:400,000

ELISA starter accessory package:

- **Microtiter Wells**: Nunc MaxiSorp C bottom well Modules & Frames, #445101
- **Coating Buffer**: Carbonate-bicarbonate buffer capsules, Sigma Chemical #C3041
- **Wash Solution**: 50 mM Tris buffered saline, pH 8.0, 0.05% Tween® 20; Sigma Chemical #T9039
- **Postcoat Solution**: 50 mM Tris buffered saline, pH 8.0, 1% BSA; Sigma Chemical #T6789
- **Sample/Conjugate Diluent**: Use Postcoat Solution plus 10% Tween® 20; Sigma Chemical #P7949
- **Enzyme Substrate**: 1 X TMB Peroxidase Substrate 60mL, KPL catalogue 37-00-78; 1 X Peroxidase Substrate Solution B 60mL, KPL 37-00-79; 10% Tween 20 Bethyl catalogue E108

The coating antibody (diluted in Coating buffer) was added to the appropriate number of microtitre wells (Nunc MaxiSorp) and the plates incubated in a sealed humidified container at 4°C overnight or for 60 minutes at room temperature. After incubation the plate was washed 3 times with Wash Solution. After the addition of Blocking Solution (Postcoat) the plate was incubated at room temperature for 30 minutes on a platform shaker. The Blocking (Postcoat) Solution was removed and the plate washed 3 times with Wash Solution. Calibrators and samples were diluted in the appropriate volume of Sample Diluent as provided in the kit. The dilution factor was based on the expected concentration of the IgG, so as to fall within the concentration range of the standards. Quality Control (QC) samples were prepared and diluted in the same manner as the test samples. 100µl (micro-l) of standard or sample was transferred to the assigned wells and the plate incubated for 60 minutes on a platform shaker at room temperature.

After incubation, each well was washed 5 times with Wash Solution. 100µl (micro-l) of HRP
Conjugate (diluted 1:200,000) was added to each well and incubated for 60 minutes on a platform shaker at room temperature. The HRP (horse radish peroxidase) Conjugate was removed and each well washed 5 times with Wash Solution. 100µl (micro-l) of TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate solution was added to each well and incubated for 10 minutes. The reaction was stopped by adding 100µl (micro-l) of 2M H2SO4 to each well. The optical density was recorded using a microplate reader at wavelengths 450nm for TMB and 650nm for subtraction of background. The concentration of IgG was calculated from the standard curve followed by multiplying the result by the applicable dilution factor.

Measurable levels of LONG®R3IGF-I in different culture media

The aim of this experiment was to determine if the apparent synergistic effect of LONG®R3IGF-I and DeltaFerrin™ was due to sustained levels of LONG®R3IGF-I during the culture period. We had previously demonstrated that bovine serum albumin (BSA) at 0.01% (w/v) was able to protect LONG®R3IGF-I from non-specific adsorption to culture vessel or plate surfaces. Thus, allowing for greater availability of LONG®R3IGF-I to bind to cell-surface receptors. We investigated if DeltaFerrin™ was having a similar effect. LONG®R3IGF-I concentrations in the media were determined using a commercially available ELISA (LONG®R3IGF-I ELISA, IDS).

LONG®R3IGF-I samples at 10 and 50ng/ml in the presence or absence of 0.01% BSA or 5µg/ml DeltaFerrin™ were prepared in culture medium (DMEM/F12, 4.5g glucose, 2mM GlutaMax™). These samples were then added to 96-well tissue culture plates and incubated at 37°C/5% CO2 in the presence of CHO K1 cells (500 cells/well) in a total volume of 200µl. Final concentrations examined were 10 and 50ng/ml LONG®R3IGF-I ± (plus or minus) 0.01% (w/v) BSA, 5µg/ml DeltaFerrin™ with either 10 or 50ng/ml LONG®R3IGF-I ± (plus or minus) 0.01% (w/v) BSA.

Following the 3 day incubation, 130µl (micro-l) of each sample was transferred to LoBind 1.5ml tubes (Eppendorf, minimal non-specific adsorption to these tubes was previously demonstrated). LONG®R3IGF-I concentrations were evaluated using the LONG®R3IGF-I ELISA following the manufacturer’s instructions with the exception of the preparation of the calibrator for the standard curves. Calibrator was diluted in medium ± (plus or minus) BSA ± (plus or minus) DeltaFerrin™ as appropriate and not Calibrator Diluent as specified in the kit.
Statistical Analysis of Data

The IgG expression levels were transformed by taking their logs, then analysed by ANOVA and regression to determine a linear model based on LONG®R3IGF-I, DeltaFerrin™, and an interaction term between the two factors. A significance level of p<0.05 was used for the analysis.

Synergistic action of combination of transferrin and IGF-I in increasing recombinant expression of monoclonal antibody by CHO cells

CHO DP12 clone 1934 cells expressing a monoclonal antibody (IgG) against hIL-8 were grown in 96-well plates in SFM supplemented with IGF-I analogue (LONG®R3IGF-I; L®R3, LR3) at 25 or 100ng/ml and/or transferrin (DeltaFerrin™; DF, rTF) at 2.5 or 5µg/ml (micro-g/ml). After 10 days of incubation at 37°C/5%CO2 the conditioned medium was removed and the IgG concentration was measured by a specific ELISA.

Table 1: Productivity of CHO DP12 cells grown with differing concentrations of LONG®R3IGF-I and transferrin.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Productivity Average</th>
<th>Stdev*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFM</td>
<td>186</td>
<td>87</td>
</tr>
<tr>
<td>25ng/ml LR3</td>
<td>298</td>
<td>52.4</td>
</tr>
<tr>
<td>100 ng/ml LR3</td>
<td>467</td>
<td>85.6</td>
</tr>
<tr>
<td>2.5 µg/ml (micro-g/ml) rTF</td>
<td>1038</td>
<td>115.4</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF</td>
<td>1025</td>
<td>83.6</td>
</tr>
<tr>
<td>2.5µg/ml (micro-g/ml) rTF + 25ng/ml LR3</td>
<td>3427</td>
<td>369.8</td>
</tr>
<tr>
<td>2.5µg/ml (micro-g/ml) rTF + 100ng/ml LR3</td>
<td>4778</td>
<td>671</td>
</tr>
<tr>
<td>5 µg/ml rTF (micro-g/ml) + 25ng/ml LR3</td>
<td>3671</td>
<td>576.3</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF + 100 ng/ml LR3</td>
<td>4998</td>
<td>593.5</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 1) show that LONG®R3IGF-I significantly (p<0.05) increased productivity over that of SFM by 1.6-fold and 2.5-fold at 25 and 100ng/ml, respectively. DeltaFerrin™, at a concentration of 2.5µg/ml (micro-g/ml), significantly (p<0.05) increased the production of IgG by 5.5-fold. However, there was no further increase at the higher concentration of 5µg/ml (micro-g/ml). The combination of LONG®R3IGF-I and DeltaFerrin™ resulted in up to a 26-fold
increase in productivity, which represents a synergistic effect (p<0.05) over the addition of their effects (2.5 fold +5.5 fold = 8 fold) when used separately.

**EXAMPLE 2:**

**Cell culture**

In the example below, the cells used were derived by co-transfecting the CHO cell line DXB1 1 with a vector designed to co-express the variable light and heavy regions of the murine 23F2G monoclonal antibody (humanised) (ATCC CRL-1 1397™). This cell line (herein referred to as B13-24) secretes recombinant IgG against beta chain (CD 18) of human leukocytes integrins.

The frozen cryovials of CHO B13-24 cells were thawed in a 37°C water bath. The thawed cells were transferred into a T75 tissue culture flask containing 10-20ml warmed sterile culture medium (DMEM/F12 containing 10% FBS and 2mM GlutaMax™). The flask was incubated at 37°C/5% CO₂ overnight. The culture medium was changed the following day, whilst also checking flasks for cell growth and absence of contamination.

Table 2: Relative growth of CHO B13-24 cells with different concentrations of LONG®R³IGF-I and transferrin.

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Relative growth (Average)</th>
<th>Stdev*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFM</td>
<td>0.060</td>
<td>0.011</td>
</tr>
<tr>
<td>SFM + 100 ng/ml LR3</td>
<td>0.465</td>
<td>0.060</td>
</tr>
<tr>
<td>SFM + 5 µg/ml (micro-g/ml) rTF</td>
<td>0.113</td>
<td>0.013</td>
</tr>
<tr>
<td>SFM + 5 µg/ml (micro-g/ml) rTF + 100 ng/ml LR3</td>
<td>1.329</td>
<td>0.023</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 2) show that LONG®R³IGF-I (LR3) at a concentration 100 ng/ml significantly (p<0.05) increased cell growth over that of SFM by 7.8-fold. DeltaFerrin™ (rTF), at a concentration of 5µg/ml (micro-g/ml), significantly (p<0.05) increased the growth by 1.9-fold. The combination of LONG®R³IGF-I and DeltaFerrin™ resulted in a 22-fold increase in cell growth, which represents a synergistic effect (p<0.05) over the addition of their effects (7.8 fold + 1.9 fold = 9.7 fold) when used separately.
The productivity assessed in parallel with the cell growth demonstrated the similar synergism when LONG®R³IGF-I and DeltaFerrin™ were used in combination.

Table 3: Productivity of CHO B13-24 cells grown in different concentrations of LONG®R³IGF-I and transferrin (IgG (ng/ml))

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Productivity (Average)</th>
<th>Stdev*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFM</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>SFM + 100 ng/ml LR3</td>
<td>32.5</td>
<td>4.1</td>
</tr>
<tr>
<td>SFM + 5 µg/ml (micro-g/ml) rTF</td>
<td>21.5</td>
<td>3.2</td>
</tr>
<tr>
<td>SFM + 5 µg/ml (micro-g/ml) rTF + 100 ng/ml LR3</td>
<td>157.8</td>
<td>6.2</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 3) show that LONG®R³IGF-I (LR3) at a concentration 100 ng/ml significantly (p<0.05) increased productivity over that of SFM by 3.2-fold. DeltaFerrin™ (rTF), at a concentration of 5µg/ml (micro-g/ml), significantly (p<0.05) increased the productivity by 2.1-fold. The combination of LONG®R³IGF-I and DeltaFerrin™ resulted in a 15.8-fold increase in productivity, which represents a synergistic effect (p<0.05) over the addition of their effects (3.2 fold +2.2 fold = 5.4 fold) when used separately.

**EXAMPLE 3:**

**Cell Culture**

Hybridoma cells were removed from frozen stock and grown in DMEM/F12 (SAFC, Catalogue 51445C) in an incubator at 37°C, 5% CO₂. The growth medium was supplemented with Glutamax™ (2mM) and 10% FBS. Cells were adapted to serum free medium by transferring them to DMEM/F12 containing Glutamax™, ITS and Probumin™. The presence of Probumin™ and ITS allowed cells to survive in the absence of FBS until required for assay.

Cells were washed twice in DMEM/F12 containing Glutamax™. Using the Trypan Blue (SIGMA T8154) exclusion method and a haemocytometer, cells were counted and plated into Corning 96 well plates at 1500 cells/well. Plates were incubated at 37°C / 5% CO₂ for approximately one hour while samples were prepared. A negative control (SFM) and positive
control (SFM + 10% FBS) were included in each assay.

Recombinant supplements examined were LONG®R³IGF-I at concentrations 25 or 100ng/ml, human transferrin (DeltaFerrin™) at concentrations 1 or 5 µg/ml (micro-g/ml) or combinations of the two. Plates were incubated at 37°C, 5% CO₂ for 6-10 days. Cell growth was assessed using either the CyQuant™ NFor MTS method (refer to Example 1).

The productivity of the cells was determined by measuring the concentration of IgG secreted into the harvested medium using protein A affinity chromatography. Details of the method are presented below:

Column: Vydac® Venture® A, 50 x 4.6 mm
Column Temperature: 23°C
Mobile Phase A: 20mM phosphate, 150mM sodium chloride, pH 7.4 (Binding Buffer, pH 7.4)
Mobile Phase B: 0.1 M sodium citrate, pH 3.2 (Elution Buffer, pH 3.2)
Fluorescence Detector: Excitation at 275 nm; Emission at 303 nm
Gradient: 100% A first 5 minutes; 0-100% B in 7 minutes.
Total analysis time = 20 minutes
Flow gradient: 0.3 - 1.0 ml/min first 5 minutes; 0.5 - 1.0 ml/min in 7 minutes

Synergistic action of a combination of transferrin (rTF) and IGF-I analogue (LR3) in increasing cell growth and recombinant expression of monoclonal antibody by SP2/0 cells

SP2/0 clone cells expressing a monoclonal antibody (IgG) against TGF 3 were grown in 96-well plates in SFM supplemented with LONG®R³IGF-I at 25 or 100ng/ml and/or recombinant transferrin at 1 or 5µg/ml (micro-g/ml). After 6-10 days of incubation at 37°C/5%CO₂ the cell growth was assessed (CyQuant™ NF Cell Proliferation Assay), conditioned medium was removed and the IgG concentration was measured by affinity HPLC. Selected results are presented in Table 4 below.
Table 4: Relative growth and productivity of SP2/0 cells grown with LONG®R3IGF-I and transferrin.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Relative Growth</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Stdev*</td>
</tr>
<tr>
<td>SFM</td>
<td>2166</td>
<td>119</td>
</tr>
<tr>
<td>100 ng/ml LR3</td>
<td>2273</td>
<td>217</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF</td>
<td>6434</td>
<td>1548</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF + 100 ng/ml LR3</td>
<td>12075</td>
<td>3047</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 4) show that LONG®R3IGF-I at 100ng/ml (similarly to 25ng/ml, not presented) did not cause any significant effect on growth or productivity of SP2/0 cells compared to SFM. Recombinant transferrin, at a concentration of 5.0µg/ml (micro-g/ml), significantly (p<0.05) increased the cell growth and production of IgG by 3.0-fold and 5.6-fold, respectively. The combination of LONG®R3IGF-I and recombinant transferrin resulted in an increase in cell growth and productivity by 5.6-fold and 14.0-fold, respectively, which represents a synergistic effect (p<0.05) over the addition of their effects. The combination of LONG®R3IGF-I at 25 ng/ml and recombinant transferrin at 1.0 µg/ml (micro-g/ml) demonstrated a synergistic effect (p<0.05) as well (results are not presented).

**EXAMPLE 4:**

Synergistic action of a combination of transferrin (rTF) and IGF-I or insulin in increasing cell growth and recombinant expression of monoclonal antibody by DP12 cells

CHO DP12 clone 1934 cells expressing a monoclonal antibody (IgG) against hlL-8 were grown in 96-well plates in SFM supplemented with IGF-I at 25 or 100ng/ml or insulin at 0.1, 1 or 10 µg/ml (micro-g/ml) and/or recombinant transferrin at 1 or 5µg/ml (micro-g/ml). After 6-10 days of incubation at 37°C/5%CO2 the cell growth was assessed (MTS dye) and conditioned medium was removed and the IgG concentration was measured by a specific ELISA. Selected results are presented in Tables 5, 6 and 7 (below).
Table 5: Relative growth and productivity of CHO DP 12 cells grown with IGF-I and transferrin.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Relative Growth</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Stdev*</td>
</tr>
<tr>
<td>SFM</td>
<td>0.227</td>
<td>0.014</td>
</tr>
<tr>
<td>100 ng/ml IGF-I</td>
<td>0.422</td>
<td>0.038</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF</td>
<td>0.324</td>
<td>0.023</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF + 100 ng/ml IGF-I</td>
<td>1.255</td>
<td>0.026</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 5) show that IGF-I significantly (p<0.05) increased growth and productivity over those of SFM by 1.9-fold and 2.1-fold, respectively. Recombinant transferrin, at a concentration of 5µg/ml (micro-g/ml), significantly (p<0.05) increased growth and production of IgG by 1.4 and 2.3-fold, respectively. The combination of IGF-I and recombinant transferrin resulted in a 5.5-fold increase in cell growth, which represents a synergistic effect (p<0.05) over the addition of their effects (1.9 fold + 1.4 fold = 3.3 fold) when used separately. The combination of IGF-I and recombinant transferrin resulted in a 9.9-fold increase in productivity, which represents a synergistic effect (p<0.05) over the addition of their effects (2.1 fold + 2.3 fold = 4.4 fold) when used separately. The combination of IGF-I at 25 ng/ml and recombinant transferrin at 1.0 µg/ml (micro-g/ml) demonstrated a synergistic effect (p<0.05) too (results are not presented).

Table 6: Relative growth and productivity of CHO DP 12 cells grown with insulin and transferrin.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Relative Growth</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Stdev*</td>
</tr>
<tr>
<td>SFM</td>
<td>0.334</td>
<td>0.013</td>
</tr>
<tr>
<td>100 ng/ml Insulin</td>
<td>0.683</td>
<td>0.039</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF</td>
<td>0.402</td>
<td>0.029</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF + 100 ng/ml Insulin</td>
<td>1.238</td>
<td>0.044</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 6) show that insulin significantly (p<0.05) increased growth and productivity over those of SFM by 2.0-fold and 2.3-fold, respectively. Recombinant transferrin, at a concentration of 5µg/ml (micro-g/ml), significantly (p<0.05) increased growth and the production of IgG by 1.2 and 2.1-fold, respectively. The combination of insulin and
recombinant transferrin resulted in a 3.7-fold increase in cell growth, which represents a synergistic effect (p<0.05) over the addition of their effects (2.0 fold + 1.2 fold = 3.2 fold) when used separately. The combination of insulin and recombinant transferrin resulted in a 10.2-fold increase in productivity, which represents a synergistic effect (p<0.05) over the addition of their effects (2.3 fold + 2.1 fold = 4.4 fold) when used separately.

The combination of insulin at 1 or 10 µg/ml (micro-g/ml) and recombinant transferrin at 1.0 µg/ml (micro-g/ml) or 5 µg/ml (micro-g/ml) demonstrated a synergistic effect (p<0.05) on cell productivity. Selected results are presented in table below.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFM</td>
<td>1780</td>
</tr>
<tr>
<td>10 µg/ml (micro-g/ml) Insulin</td>
<td>3675</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF</td>
<td>3673</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF + 10 µg/ml (micro-g/ml) Insulin</td>
<td>13445</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

Table 7: Productivity of CHO DP 12 cells grown with insulin and transferrin.

Insulin at concentration 10 µg/ml (micro-g/ml) (Table 7) significantly (p<0.05) increased IgG production over that of SFM by 2.1-fold. Recombinant transferrin, at a concentration of 5µg/ml (micro-g/ml), significantly (p<0.05) increased the production of IgG by 2.1-fold. The combination of insulin and recombinant transferrin resulted in a 7.6-fold increase in productivity, which represents a synergistic effect (p<0.05) over the addition of their effects (2.1 fold + 2.1 fold = 4.2 fold) when used separately.

EXAMPLE 5:

**Synergistic action of a combination of recombinant transferrin (rTF) and IGF-I or Insulin in increasing cell growth and recombinant expression of monoclonal antibody by SP2/0 cells**

SP2/0 clone cells expressing a monoclonal antibody (IgG) against TGF 3 were grown in 96-well plates in SFM supplemented with IGF-I at 25 or 100ng/ml or insulin at 0.1, 1 or 10 µg/ml (micro-g/ml) and/or recombinant transferrin at 1 or 5µg/ml (micro-g/ml). After 6-10 days of incubation at 37°C/5%CO2 the cell growth was assessed (MTS), conditioned medium was
removed and the IgG concentration was measured by an affinity HPLC. Selected results are presented in Tables 8 and 9 below.

Table 8: Relative growth and productivity of SP2/0 cells grown with IGF-I and transferrin.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Relative Growth</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFM</td>
<td>0.099</td>
<td>1055</td>
</tr>
<tr>
<td>100 ng/ml IGF-I</td>
<td>0.088</td>
<td>0</td>
</tr>
<tr>
<td>5 µg/ml rTF (micro-g/ml)</td>
<td>0.375</td>
<td>8108</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF + 100 ng/ml IGF-I</td>
<td>0.592</td>
<td>11654</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 8) show that IGF-I at 100ng/ml (similarly to 25ng/ml, not presented) did not cause any significant effect on growth or productivity of SP2/0 cells compared to SFM.

Recombinant transferrin, at a concentration of 5.0µg/ml (micro-g/ml), significantly (p<0.05) increased the cell growth and production of IgG by 3.8-fold and 7.7-fold, respectively. The combination of IGF-I and recombinant transferrin resulted in an increase in cell growth and productivity by 6.0-fold and 11.0-fold, respectively, which represents a synergistic effect (p<0.05) over the addition of their effects. The combination of IGF-I at 25 ng/ml and recombinant transferrin at 1.0 µg/ml (micro-g/ml) also demonstrated a synergistic effect (p<0.05) (results are not presented).

Table 9: Relative growth and productivity of SP2/0 cells grown with insulin and transferrin.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Relative Growth</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFM</td>
<td>0.115</td>
<td>475</td>
</tr>
<tr>
<td>10 µg/ml (micro-g/ml) Insulin</td>
<td>0.110</td>
<td>418</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF</td>
<td>0.377</td>
<td>7248</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF + 10 µg/ml (micro-g/ml) Insulin</td>
<td>0.491</td>
<td>13148</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 9) show that insulin at 10 µg/ml (micro-g/ml) (similarly to 0.1 and 1 µg/ml (micro-g/ml), not presented) did not cause any significant effect on growth or productivity of SP2/0 cells compared to SFM. Recombinant transferrin, at a concentration of 5.0µg/ml
(micro-g/ml), significantly (p<0.05) increased the cell growth and production of IgG by 3.3-fold and 15.3-fold, respectively. The combination of insulin and recombinant transferrin resulted in an increase in cell growth and productivity by 4.3-fold and 27.7-fold, respectively, which represents a synergistic effect (p<0.05) over the addition of their effects. The combination of insulin at 0.1 and 1 µg/ml (micro-g/ml) and recombinant transferrin at 5.0 µg/ml (micro-g/ml) also demonstrated a synergistic effect (p<0.05) (results are not presented).

EXAMPLE 6:

Synergistic action of a combination of recombinant transferrin (rTF) and IGF-I or its analogue or insulin in increasing cell growth and recombinant expression of monoclonal antibody by SP2/0 cells - confirmation in mini-bioreactor model

Prior to the assay, cells were grown as for Example 3. For the experiment cells were washed once in DMEM/F12 containing 2mM GlutaMAX™ and counted using the Trypan Blue exclusion method. A total of 10^5 cells were seeded into each experimental CultiFlask 50ml disposable bioreactor (Sartorius, cat. no. 167050) and topped up to 10ml with medium containing GlutaMAX™ (SFM) supplemented with 100ng/ml LONG®R3IGF-I (LR3), 100ng/ml IGF-I, 100 ng/ml insulin or rTF alone or insulin-like factors in combination with recombinant transferrin (rTF).

After 5-6 days of incubation at 37°C/5%CC>2 the cell growth (number of viable cells per ml) was assessed using the ViaCount assay on the Guava EasyCyte™ PCA-96 (The cell suspension samples were diluted to the optimal analysis concentration range using PBS. Guava ViaCount Flex Reagent was added and the samples were left to incubate for 15 minutes at room temperature, protected from light). The productivity (IgG concentration, ng/ml) was measured by an affinity HPLC. Selected results are presented in Tables 10 to 12, below.
Table 10: Growth and productivity of SP2/0 cells grown with LONG\textsuperscript{R}IGF-I and transferrin in mini-bioreactor model.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Growth</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Stdev*</td>
</tr>
<tr>
<td>SFM</td>
<td>92646</td>
<td>6355</td>
</tr>
<tr>
<td>100 ng/ml LR3</td>
<td>1919</td>
<td>19</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF</td>
<td>607558</td>
<td>17060</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF + 100 ng/ml LR3</td>
<td>944589</td>
<td>69293</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 10) show that LONG\textsuperscript{R}IGF-I at 100ng/ml did not cause any positive effect on growth or productivity of SP2/0 cells compared to SFM.

Recombinant transferrin, at a concentration of 5.0µg/ml (micro-g/ml), significantly (p<0.05) increased the cell growth and production of IgG by 6.6-fold and 2.0-fold, respectively, compared to SFM. The combination of LONG\textsuperscript{R}IGF-I and recombinant transferrin resulted in an increase in cell growth and productivity by 10.2-fold and 2.5-fold, respectively, compared to SFM. This represents a positive synergistic effect (p<0.05) over effect of rTF.

Table 11: Growth and productivity of SP2/0 cells grown with IGF-I and transferrin in mini-bioreactor model.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Growth</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Stdev*</td>
</tr>
<tr>
<td>SFM</td>
<td>92646</td>
<td>6355</td>
</tr>
<tr>
<td>100 ng/ml IGF-I</td>
<td>35</td>
<td>50</td>
</tr>
<tr>
<td>5 µg/ml rTF (micro-g/ml)</td>
<td>607558</td>
<td>17060</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF + 100 ng/ml IGF-I</td>
<td>877375</td>
<td>117532</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 11) show that IGF-I at 100ng/ml did not cause any positive effect on growth or productivity of SP2/0 cells compared to SFM.

Recombinant transferrin, at a concentration of 5.0µg/ml (micro-g/ml), significantly (p<0.05) increased the cell growth and production of IgG by 6.6-fold and 2.0-fold, respectively, compared to SFM. The combination of IGF-I and recombinant transferrin resulted in an increase in cell growth and productivity by 9.5-fold and 2.2-fold, respectively, compared to
SFM. This represents a positive synergistic effect (p<0.05) over effect of rTF.

Table 12: Relative growth and productivity of SP2/0 cells grown with insulin and transferrin in mini-bioreactor model.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Growth</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Stdev*</td>
</tr>
<tr>
<td>SFM</td>
<td>72223</td>
<td>2492.6</td>
</tr>
<tr>
<td>100 ng/ml Insulin</td>
<td>49709</td>
<td>243.2</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF</td>
<td>366441</td>
<td>10244.6</td>
</tr>
<tr>
<td>5 µg/ml (micro-g/ml) rTF + 100 ng/ml Insulin</td>
<td>583425</td>
<td>24721.2</td>
</tr>
</tbody>
</table>

*Stdev: Standard Deviation

The results (Table 12) show that insulin at 100ng/ml did not cause any positive effect on growth or productivity of SP2/0 cells compared to SFM.

Recombinant transferrin, at a concentration of 5.0µg/ml (micro-g/ml), significantly (p<0.05) increased the cell growth and production of IgG by 5.1-fold and 1.4-fold, respectively, compared to SFM. The combination of insulin and recombinant transferrin resulted in an increase in cell growth and productivity by 8.1-fold and 1.6-fold, respectively, compared to SFM. This represents a positive synergistic effect (p<0.05) over the effect of rTF.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure, including definitions will be controlling.
CLAIMS

1. A process for the production of a desired protein, comprising:
   a) transfecting a mammalian host cell with a DNA sequence encoding the desired protein, and
   b) culturing the transfected host cell in a cell culture medium comprising an insulin-like factor and a transferrin (Tf) under conditions permitting expression of the desired protein.

2. The process of claim 1 wherein the combination of the insulin-like factor and the Tf increases the yield of production of the desired protein to a level greater than the sum of the increase in yield from the addition of the insulin-like factor or the Tf separately.

3. The process of any preceding claim wherein the combination of the insulin-like factor and the Tf increases the growth of the mammalian host cell to a level greater than the sum of the increase in yield from the addition of the insulin-like factor or the Tf separately.

4. The process of any preceding claim wherein the cell culture medium comprises the insulin-like factor at a concentration of 0.001-200 µg/ml (micro-g/ml).

5. The process of any preceding claim wherein the cell culture medium comprises the Tf at a concentration of 0.1-100 µg/ml (micro-g/ml).

6. A cell culture medium comprising:
   a) basal medium
   b) an insulin-like factor at a concentration of 0.001-200 µg/ml (micro-g/ml); and
   c) a transferrin at a concentration of 0.1-100 µg/ml (micro-g/ml).

7. A process or cell culture medium according to any preceding claim wherein the insulin-like factor is at a concentration of 0.001-20 µg/ml (micro-g/ml).

8. A process of cell culture medium according to any preceding claim wherein the transferrin is at a concentration of 0.1-10 µg/ml (micro-g/ml).

9. The process or method of any preceding claim wherein the insulin-like factor is one or more of insulin and IGF.
10. The process or method of claim 9 wherein the insulin-like factor has a sequence according to one or more of SEQ ID No 1, SEQ ID No 2, SEQ ID No 3, SEQ ID No 4, SEQ ID No 5, SEQ ID No 6, SEQ ID No 7, SEQ ID No 8, SEQ ID No 9, SEQ ID No 10, SEQ ID No 11, SEQ ID No 12, SEQ ID No 13, SEQ ID No 14, SEQ ID No 15, SEQ ID No 16, SEQ ID No 17, SEQ ID No 18, SEQ ID No 19, SEQ ID No 20, SEQ ID No 21, SEQ ID No 22, SEQ ID No 23, SEQ ID No 24, SEQ ID No 25, SEQ ID No 26, SEQ ID No 27, SEQ ID No 28, SEQ ID No 29, SEQ ID No 30, SEQ ID No 31 and SEQ ID No 32.

11. The process or method of claim 10 wherein the insulin-like factor has a sequence according to SEQ ID No 6.

12. The process or method of claim 10 wherein the insulin-like factor has a sequence according to SEQ ID No 3 and/or SEQ ID No 4.

13. The process or method of claim 10 wherein the insulin-like factor has a sequence according to SEQ ID No 1.

14. The process or method of claim 10 wherein the insulin-like factor has a sequence according to SEQ ID No 28.

15. The process of method of any preceding claim wherein the transferrin has a sequence according to one or more of SEQ ID No. 35, SEQ ID No. 34 and SEQ ID No. 33.