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(54) Title: METHOD OF PRODUCTION OF GONADOTROPHIN

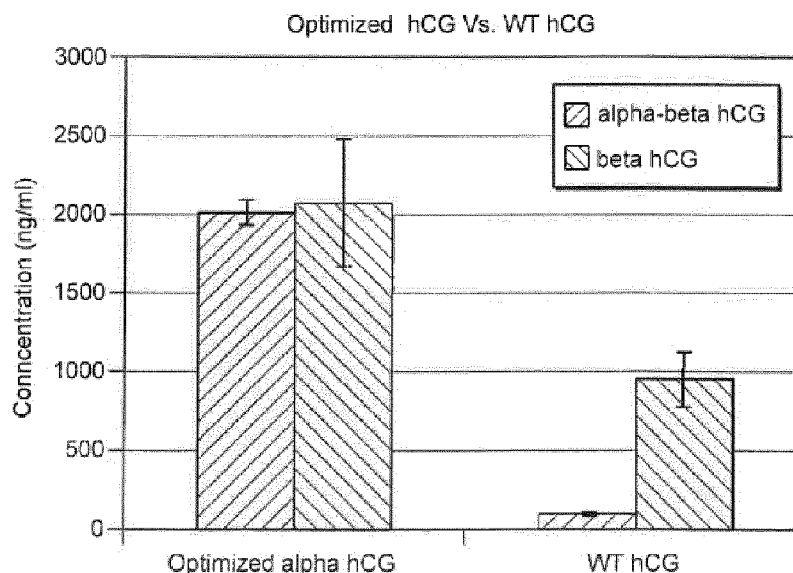


FIG. 7

(57) Abstract: A host cell characterized in that it comprises integrated into its genome a sequence coding for the a chain of hCG, and use of the host cell to produce recombinant hCG.

Method of production of gonadotrophin

The present invention relates to gonadotrophins for use in the treatment of infertility. In particular it relates to human chorionic gonadotrophin (hCG).

The gonadotrophins are a group of heterodimeric glycoprotein hormones which regulate gonadal function in the male and female. They include follicle stimulating hormone (FSH), luteinising hormone (LH) and chorionic gonadotrophin (CG).

Human chorionic gonadotrophin (hCG) is secreted mainly by human placenta during pregnancy and supports the maintenance of corpus luteum. hCG comprises a 92 amino acid alpha sub-unit (α chain), also common to the other glycoprotein hormones LH and FSH, and a 145 amino acid beta sub-unit (β chain) unique to hCG, which dictates the hormone specificity. Each sub-unit is post translationally modified by the addition of complex carbohydrate residues. The alpha sub-unit contains 2-N-linked glycosylation sites at amino acids 52 and 78 and the beta sub-unit contains 2-N-linked glycosylation sites at amino acids 13 and 30 and four O-linked glycosylation sites at amino acids 121, 127, 132 and 138.

hCG extracted from the urine of pregnant women [Choragon (Ferring)] has been used for many years in infertility treatment. The production of hCG extracted from urine involves the collection and processing of large amounts of urine. Ovitrelle (Serono), a recombinant version of hCG, is also available for use in infertility treatment. This recombinant product is expressed in Chinese hamster ovary (CHO) cells, and has a different pharmacokinetic profile to hCG produced from human urine.

There is considerable heterogeneity associated with hCG preparations which relates to differences in the amounts of various isoforms present. Individual hCG isoforms exhibit identical amino acid sequences but differ in the extent to which they are post-translationally modified; particular isoforms are characterised by heterogeneity of the carbohydrate branch structures and differing amounts of sialic acid (a terminal sugar) incorporation, both of which appear to influence the specific isoform bioactivity.

The glycosylation of recombinant hCG ("rhCG") products reflects the range of glycosyl-transferases present in the host cell line. The existing rhCG product, Ovitrelle, is derived from engineered Chinese hamster ovary cells (CHO cells). The range of glycan modifications in CHO derived rhCG are more limited than those found on the natural products, derived from urine. Examples of the

reduced glycan heterogeneity found in CHO derived rhCG include a lack of bisecting glucosamine and a reduced content of core fucosylation and acetyl lactosamine extensions. In addition, CHO cells are only able to add sialic acid using the $\alpha 2,3$ linkage (Kagawa *et al*, 1988, Takeuchi *et al*, 1988, Svensson *et al.*, 1990).

Human urinary hCG (that is, hCG extracted from the urine of pregnant women) which has been used to date in treatment of infertility is, in actual fact, placental hCG; hCG produced in the (human) placenta and then excreted in the urine. This hCG is extracted from urine for use as the pharmaceutical product. Bousfield *et al* (Rev Endocr Metab Disord (2011) 12:289-302) states that “while pituitary gonadotrophins possess both $\alpha 2,3$ - and $\alpha 2,6$ - linked sialic acid, placental hCG and recombinant gonadotrophins produced in Chinese hamster ovarian cells possess only $\alpha 2,3$ - linked sialic acid”. Thus, human urinary hCG (which is produced in the placenta) includes only $\alpha 2,3$ - sialylation (like the CHO cell line derived recombinant products). Thus, the known pharmaceutical compositions which include hCG include only $\alpha 2,3$ - linked sialic acid, but do not include $\alpha 2,6$ - linked sialic acid.

It has been demonstrated that a recombinant FSH preparation (Organon) differs in the amounts of FSH with an isoelectric point (pI) of below 4 (considered the acidic isoforms) when compared to pituitary, serum or post-menopausal urine FSH (Ulloa-Aguirre *et al.* 1995). The amount of acidic isoforms in the urinary preparations of FSH was much higher as compared to the recombinant products, Gonal-f (Serono) and Puregon (Organon) (Andersen *et al.* 2004). This must reflect a lower molar content of sialic acid in rFSH since the content of negatively-charged glycan modified with sulphate is low in FSH. The lower sialic acid content, compared to natural FSH, is a feature of both commercially available FSH products and therefore must reflect a limitation in the manufacturing process (Bassett and Driebergen, 2005). The circulatory life-time of FSH has been documented for materials from a variety of sources. Some of these materials have been fractionated on the basis of overall molecular charge, as characterised by their pI, in which more acid equates to a higher negative charge. The major contributor to overall molecular charge is the total sialic content of each FSH molecule. For instance, rFSH (Organon) has a sialic acid content of around 8 mol/mol, whereas urine-derived FSH has a higher sialic acid content (de Leeuw *et al.* 1996). The corresponding plasma clearance rates in the rat are 0.34 and 0.14 ml/min (Ulloa-Aguirre *et al.* 2003). In another example where a sample of recombinant FSH was split into high and low pI fractions, the *in vivo* potency of

the high pI (lower sialic acid content) fraction was decreased and it had a shorter plasma half-life (D'Antonio et al. 1999). The applicants have found that, similar to FSH, the known, CHO derived, recombinant hCG product (e.g. Ovitrelle) also has a lower amount of hCG with an isoelectric point (pI) of below 4 (considered the acidic isoforms) than urinary hCG, also reflecting a lower sialic acid content of the known rhCG product compared to urinary hCG.

The applicants have developed a human derived recombinant hCG (that is, a recombinant hCG which is produced or expressed in a human cell line, e.g. made by engineering a human cell line) which has a more acidic profile than the CHO cell line derived rhCG product, Ovitrelle, and which has a higher sialic acid content. This rhCG is the subject of International patent Application No. PCT/GB2010/001854, published as WO 2011/042688. Recombinant hCG with a mixture of both α 2,3 and α 2,6-linked sialic acid was made by engineering a human cell line to express both rhCG and α 2,3 sialyltransferase. The expressed product is highly acidic, having a sialic acid content [expressed in terms of a ratio of moles of sialic acid to moles of protein] of for example 19.1 mol/mol, and carries a mix of both α 2,3- and α 2,6-linked sialic acids; the latter provided by the endogenous sialyl transferase activity. The applicants' research indicates that the type of sialic acid linkage, α 2,3- or α 2,6-, can have a dramatic influence on biological clearance of hCG. Human cell lines, as opposed to CHO cell lines, can express recombinant hCG with sialic acids attached by both α 2,3 and α 2,6 linkages.

The cell line of WO 2011/042688 expresses human derived recombinant hCG well. However, the cell line also expresses a relatively high level of the free β chain (free beta sub unit), and purification (to remove this free β chain from product hCG) was required.

The applicants have now developed a new cell line which expresses both rhCG and α 2,3 sialyltransferase, and which produces rhCG with a reduced amount of the free β chain, thereby improving yield and reducing the level of product purification required (see Figure 7).

According to the present invention there is provided a (e.g. host) cell characterized in that it comprises integrated into its genome a (nucleic acid) sequence coding for the α chain of hCG selected from: a sequence according to SEQ ID NO: 1; a sequence which has at least 96.5 % homology with the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 96.5 % sequence identity with the sequence of SEQ ID NO:1); a sequence which has at least 97 % homology with (e.g. at least 98% or at least 99% homology with) the sequence of

SEQ ID NO:1 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ ID NO:1); a sequence according to SEQ ID NO. 4; a sequence which has at least 96.5 % homology with the sequence of SEQ ID NO: 4 (e.g. a sequence which has at least 96.5 % sequence identity with the sequence of SEQ ID NO:4); and a sequence which has at least 97 % homology with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:4 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ ID NO:4). Preferably the (nucleic acid) sequence coding for the α chain of hCG is not the sequence which is banked as AH007338 (that is, preferably, the (nucleic acid) sequence coding for the α chain of hCG is not that shown in SEQ ID NO:5). The cell may further comprise integrated into its genome a cDNA encoding an alpha-2,3-sialyltransferase, for example a sequence according to SEQ ID NO: 3. The cell may further comprise integrated into its genome a (nucleic acid) sequence coding for the β chain of hCG, for example the sequence according to SEQ ID NO:2.

The host cell may be, for example, a PER.C6® cell, a HT1080 cell, a GT-5s cell etc.. Preferably the cell is a PER.C6® cell such as a PER.C6® cell deposited under ECACC no. 96022940.

According to the present invention there is provided a PER.C6® cell such as a PER.C6® cell deposited under ECACC no. 96022940, characterized in that it further comprises integrated into its genome a (nucleic acid) sequence coding for the α chain of hCG. The (nucleic acid) sequence coding for the α chain of hCG may be selected from: a sequence according to SEQ ID NO: 1; a sequence which has at least 90 % homology with the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 90 % sequence identity with the sequence of SEQ ID NO:1); a sequence which has at least 97 % homology with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ ID NO:1); a sequence according to SEQ ID NO. 4; a sequence which has at least 90 % homology with the sequence of SEQ ID NO: 4 (e.g. a sequence which has at least 90 % sequence identity with the sequence of SEQ ID NO:4); and a sequence which has at least 97 % homology with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:4 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ

ID NO:4). Preferably the (nucleic acid) sequence coding for the α chain of hCG is not the sequence which is banked as AH007338. The cell may further comprise integrated into its genome a cDNA encoding an alpha-2,3-sialyltransferase, for example a sequence according to SEQ ID NO: 3. The cell
5 may further comprise integrated into its genome a (nucleic acid) sequence coding for the β chain of hCG, for example the sequence according to SEQ ID NO:2.

It will be appreciated that the term "a sequence coding for the α chain of hCG" or "a (nucleic acid) sequence coding for the α chain of hCG" used herein also refers to a sequence coding for the α chain of FSH (or a sequence coding for
10 the α chain of LH) because the α chain of hCG is the same as that for FSH (and LH).

The term "nucleic acid sequence" herein relates to any nucleic acid molecule that codes for polypeptides such as peptides, proteins etc. These nucleic acid molecules may be made of DNA, RNA or analogues thereof.
15 However, nucleic acid molecules being made of DNA are preferred.

The person skilled in the art is clearly aware that modification of a starting nucleotide sequence describes the process of optimization with respect to codon usage. The changes introduced can be easily identified by comparing the modified sequence and the starting sequence. Moreover, both sequences (that
20 is, the starting sequence and the optimised sequence) will code for the same amino acid sequence.

The amino acid sequence of the α -chain of human hCG is disclosed in WO2011/042688 (referred to as SEQ ID1 in that document), and is that described in Fiddes and Goodman 1979. It is banked under AH007388, and is
25 shown as SEQ ID NO. 5 below. The amino acid sequence of the β -chain of human hCG is depicted in SEQ ID No.2, and is banked as NP_000728. These amino acid sequences correspond to the wild-type amino acid sequences of the α - and the β -chain of human hCG. The terms "wild-type nucleic acid sequence" or "starting nucleic acid sequence" for the purposes of the present invention
30 relate to a nucleic acid sequence which is intended to be used for (over)expression in a host cell and which has not been adapted to the codon usage in the host cell, but is the actual wild-type nucleic acid sequence coding for the protein. The term "optimized nucleic acid sequence" for the purposes of the present invention relates to a sequence that has been modified for expression in
35 a host cell by adapting the sequence of the nonmodified/starting nucleic acid sequence. An optimized nucleic acid sequence codes for a protein having the same amino acid sequence as the protein encoded by the non-modified

sequence. The applicants have developed a optimised sequences which code for the α -chain of hCG (see e.g. SEQ ID No. 1 and 4, Figure 1).

According to the present invention in a further aspect there is provided a polynucleotide sequence (e.g. a polynucleotide sequence coding for the α chain of hCG) comprising a sequence selected from a nucleic acid sequence according to SEQ ID NO: 1; a nucleic acid sequence according to SEQ ID NO: 4; a nucleic acid sequence with at least 97% homology with (e.g. at least 98% or at least 99% homology with) the nucleic acid of SEQ ID NO: 1 (e.g. a nucleic acid sequence with at least 97% sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the nucleic acid of SEQ ID NO: 1); and a nucleic acid sequence with at least 97% homology with (e.g. at least 98% or at least 99% homology with) the nucleic acid of SEQ ID NO: 4 (e.g. a nucleic acid sequence with at least 97% sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the nucleic acid of SEQ ID NO: 4).

According to the present invention in a further aspect there is provided a method for producing recombinant hCG in a cell, comprising culturing the cell in a suitable medium and harvesting the recombinant protein (hCG) from said cell and/or said medium (e.g. harvesting the recombinant hCG from the cell culture supernatant), wherein the cell (host cell) comprises integrated into its genome a (nucleic acid) sequence coding for the α chain of hCG selected from: a sequence according to SEQ ID NO: 1; a sequence which has at least 90 % homology with the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 90 % sequence identity with the sequence of SEQ ID NO:1); a sequence which has at least 97 % homology with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ ID NO:1); a sequence according to SEQ ID NO. 4; a sequence which has at least 90 % homology with the sequence of SEQ ID NO: 4 (e.g. a sequence which has at least 90 % sequence identity with the sequence of SEQ ID NO:4); and a sequence which has at least 97 % homology with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:4 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ ID NO:4).

Preferably the (nucleic acid) sequence coding for the α chain of hCG is not the sequence which is banked as AH007338. The cell may further comprise integrated into its genome a cDNA encoding an alpha-2,3-sialyltransferase, for example a sequence according to SEQ ID NO: 3. The cell may further comprise

integrated into its genome a (nucleic acid) sequence coding for the β chain of hCG, for example the sequence according to SEQ ID NO:2. The method may include a further step or steps of purifying the recombinant hCG obtained from said cell and/or said medium (e.g. purifying the recombinant hCG from the cell culture supernatant).

The (host) cell may be, for example, a PER.C6® cell, a HT1080 cell, a GT-5s cell etc.. Preferably the cell is a PER.C6® cell such as a PER.C6® cell deposited under ECACC no. 96022940.

According to the present invention in a further aspect there is provided a method for producing recombinant hCG in a cell, comprising culturing the cell in a suitable medium and harvesting the recombinant protein (hCG) from said cell and/or said medium (e.g. harvesting the recombinant hCG from the cell culture supernatant), wherein the cell is a PER.C6® cell (such as a PER.C6® cell deposited under ECACC no. 96022940) comprising integrated into its genome a (nucleic acid) sequence coding for the α chain of hCG. The (nucleic acid) sequence coding for the α chain of hCG may be selected from: a sequence according to SEQ ID NO: 1; a sequence which has at least 90 % homology with the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 90 % sequence identity with the sequence of SEQ ID NO:1); a sequence which has at least 97 % homology with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ ID NO:1); a sequence according to SEQ ID NO. 4; a sequence which has at least 90 % homology with the sequence of SEQ ID NO: 4 (e.g. a sequence which has at least 90 % sequence identity with the sequence of SEQ ID NO:4); and a sequence which has at least 97 % homology with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:4 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ ID NO:4). Preferably the (nucleic acid) sequence coding for the α chain of hCG is not the sequence which is banked as AH007338. The cell may further comprise integrated into its genome a cDNA encoding an alpha-2,3-sialyltransferase, for example a sequence according to SEQ ID NO: 3. The cell may further comprise integrated into its genome a (nucleic acid) sequence coding for the β chain of hCG, for example the sequence according to SEQ ID NO:2. The method may include a further step or steps of purifying the recombinant hCG obtained from said cell and/or said medium (e.g. purifying the recombinant hCG from the cell culture

supernatant).

The term "host cell" for the purposes of the present invention refers to any cell that is commonly used for expression, i.e. transcription and translation of nucleic acid sequences for the production of e.g. polypeptides. In particular, the
5 term "host cell" or "organism" relates to prokaryotes, lower eukaryotes, plants, insect cells or mammalian cell culture systems. Preferably, the host cell is a mammalian cell, more preferably the host cell is a human cell, even more preferably a PERC6® cell.

The term "recombinant nucleic acid molecule" within the meaning of the
10 present invention is intended to comprise all kinds of nucleic acid molecules which are capable of being introduced into a host cell and effecting the expression of a nucleic acid sequence which is contained within the recombinant nucleic acid molecule. The term includes, for example, plasmid vectors and viral vectors (e.g. adenoviral, lentiviral and retroviral vectors), as are well known in the
15 art.

According to the present invention in a further aspect there is provided a recombinant nucleic acid molecule comprising a (first) nucleic acid sequence coding for the α chain of hCG, which (first) nucleic acid sequence is selected from a sequence according to SEQ ID NO: 1; a sequence which has at least 96.5 %
20 homology with the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 96.5 % sequence identity with the sequence of SEQ ID NO:1); a sequence which has at least 97 % homology with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence
25 identity with) the sequence of SEQ ID NO:1); a sequence according to SEQ ID NO. 4; a sequence which has at least 96.5 % homology with the sequence of SEQ ID NO: 4 (e.g. a sequence which has at least 96.5 % sequence identity with the sequence of SEQ ID NO:14; and a sequence which has at least 97 %
30 homology with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:4 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ ID NO:4). Preferably, the recombinant nucleic acid molecule of this aspect of the present invention comprises both an optimised nucleic acid sequence coding for the α -chain of human hCG and a nucleic acid sequence coding for the β -chain of
35 hCG. Preferably, the (first) nucleic acid sequence is under the control of a promoter which is active in a host cell. Preferably the recombinant nucleic acid molecule further comprises a second nucleic acid sequence coding for the β

chain of hCG. The second nucleic acid sequence coding for the β chain of hCG may be the sequence according to SEQ ID No. 2.

The second nucleic acid sequence may be under the control of a separate promoter which is active in a host cell. The first nucleic acid sequence and/or
5 the second nucleic acid sequence may be under the control of a viral promoter (e.g. a CMV promoter).

According to the present invention in a further aspect there is provided a host cell comprising or including a recombinant nucleic acid molecule as set out above (e.g. a host cell comprising a recombinant nucleic acid molecule
10 comprising a (first) nucleic acid sequence coding for the α chain of hCG, which (first) nucleic acid sequence is selected from a sequence according to SEQ ID NO: 1; a sequence which has at least 96.5 % homology with the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 96.5 % sequence identity with the sequence of SEQ ID NO:1); a sequence which has at least 97 % homology
15 with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:1 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ ID NO:1); a sequence according to SEQ ID NO. 4; a sequence which has at least 96.5 % homology with the sequence of SEQ ID NO: 4 (e.g. a sequence which has at
20 least 96.5 % sequence identity with the sequence of SEQ ID NO:4); and a sequence which has at least 97 % homology with (e.g. at least 98% or at least 99% homology with) the sequence of SEQ ID NO:4 (e.g. a sequence which has at least 97 % sequence identity with (e.g. at least 98% or at least 99% sequence identity with) the sequence of SEQ ID NO:4).

25 The applicants have found that the cells (host cells), cell lines incorporating these host cells, recombinant nucleic acid molecules, polynucleotides and methods of the invention may produce recombinant hCG in high yield and purity (e.g. with little free beta chain).

According to the present invention in a further aspect there is provided a
30 recombinant hCG ("rhCG" or "rechCG") which includes α 2,3- and α 2,6-sialylation, wherein the recombinant hCG is produced in a cell as described and claimed herein. The recombinant hCG may have a sialic acid content [expressed in terms of a ratio of moles of sialic acid to moles of protein] of from 12 mol/mol to 20 mol/mol, for example from 12 mol/mol to 15.5 mol/mol, for example 12
35 mol/mol to 14.9 mol/mol. The rhCG (or rhCG preparation) may have a sialic acid content of 12.5 mol/mol to 14.5 mol/mol, for example a sialic acid content of 12.8 mol/mol to 13.2 mol/mol. The rhCG (or rhCG preparation) according to the

invention may have a sialic acid content of 13 mol/mol.

In examples of the invention, the rhCG may be present as a single isoform or as a mixture of isoforms.

The recombinant hCG (or rhCG preparation) produced by the methods
5 and cells of the invention includes α 2,3- and α 2,6-sialylation. By sialylation it is meant the amount of sialic residues present on the hCG carbohydrate structures. α 2,3-sialylation means sialylation at the 2,3 position (as is well known in the art); and α 2,6 sialylation means sialylation at the 2,6 position (also well known in the art). Thus, herein, the wording “% of the total sialylation may be α 2,3 sialylation”
10 refers to the % of the total number of sialic acid residues present in the hCG which are sialylated in the 2,3 position. The term “% of the total sialylation being α 2,6-sialylation” refers to the % of the total number of sialic acid residues present in the hCG which are sialylated in the 2,6 position. The term “% of the total sialylation being α 2,8-sialylation” refers to the % of the total number of sialic acid
15 residues present in the hCG which are sialylated in the 2,8 position.

The rhCG (or rhCG preparation) produced by the methods and cells according to the invention may have 1% to 99% of the total sialylation being α 2,3-sialylation. The rhCG (or rhCG preparation) produced by the methods and cells according to the invention may have 1% to 90% of the total sialylation being α 2,3-
20 sialylation. The rhCG (or rhCG preparation) according to the invention may have 10% or more of the total sialylation being α 2,3-sialylation, for example, 10% to 90% of the total sialylation may be α 2,3-sialylation. For example, 20, 30, 40, 45, 50, 55, 60, 70, 80 or 90% or more of the total sialylation may be α 2,3-sialylation. 45% to 80% of the total sialylation may be α 2,3-sialylation, for example 50% to
25 70% of the total sialylation may be α 2,3-sialylation, for example 55 to 65% of the total sialylation may be α 2,3-sialylation. The rhCG (or rhCG preparation) may include α 2,3-sialylation in an amount which is from 65 to 95% of the total sialylation, for example from 70 to 90% of the total sialylation, for example from 85 to 90% of the total sialylation.

30 The rhCG (or rhCG preparation) produced by the methods and cells of the invention may have 1 to 99% of the total sialylation being α 2,6-sialylation. The rhCG (or rhCG preparation) of the invention may have 5 to 50% of the total sialylation being α 2,6-sialylation. For example 5 to 45%, for example 6 to 40%, for example 7 to 30%, for example 8 to 20% of the total sialylation may be α 2,6-
35 sialylation. The rhCG (or rhCG preparation) may include α 2,6-sialylation in an amount which is from 20-75% of the total sialylation, for example, 30-60% of the total sialylation, for example 35-45% of the total sialylation. The rhCG (or rhCG

preparation) may include α 2,6-sialylation in an amount which is from 5 to 35% of the total sialylation, for example from 10 to 20% of the total sialylation. For example 11-55% of the total sialylation may be α 2,6- sialylation.

5 The rhCG or rhCG preparation produced by the methods and cells may optionally further include α 2,8 sialylation. The rhCG (or rhCG preparation) of the invention may have 5% or less of the total sialylation being α 2,8-sialylation, for example 0 to 4%, e.g. 0.1-4% of the total sialylation may be α 2,8- sialylation. The rhCG (or rhCG preparation) of the invention may have no α 2,8-sialylation.

10 The rhCG (or rhCG preparation) produced by the methods and cells according to the invention may have a sialic acid content (amount of sialylation per hCG molecule) of (based on the mass of protein, rather than the mass of protein plus carbohydrate) of 6% or greater (e.g. between 6% and 15%, e.g. between 7% and 13%, e.g. between 8% and 12%, e.g. between 11% and 15%, e.g. between 12% and 14%) by mass.

15 The rhCG (or rhCG preparation) may be produced or expressed in a human cell line, for example a PER.C6® cell line, a HT1080 cell line etc.. The rhCG (or rhCG preparation) may be produced or expressed in a human derived cell line or a modified human cell line, for example a PER.C6® derived cell line or modified PER.C6® cell line, a modified HT1080 cell line or HT1080 derived cell
20 line etc.. In a preferred example, the rhCG is produced or expressed in a PER.C6® cell line, a PER.C6® derived cell line or a modified PER.C6® cell line. rhCG which is produced or expressed in a human cell line (e.g. a PER.C6® cell line, a HT1080 cell line, a GT-5s cell line) will include some α 2,6-linked sialic acids (α 2,6 sialylation) provided by endogenous sialyl transferase activity [of the
25 cell line] and will include some α 2,3-linked sialic acids (α 2,3 sialylation) provided by endogenous sialyl transferase activity [of the cell line]. The cell line may be modified using α 2,3-sialyltransferase. Alternatively or additionally, the cell line may be modified using α 2,6-sialyltransferase. This may simplify (and render more efficient) the production method because manipulation and control of e.g.
30 the cell growth medium to retain sialylation may be less critical than with known processes. The method may also be more efficient because there is little basic rhCG produced compared to production of known rhCG products; more acidic rhCG is produced and separation/removal of basic hCG is less problematic.

35 The rhCG may be produced using an α 2,3- sialyltransferase, for example produced using a human cell line modified using an α 2,3-sialyltransferase. The rhCG may include α 2,6-linked sialic acids (α 2,6 sialylation) provided by endogenous sialyl transferase activity. The rhCG may be produced using an

α 2,3- and/or an α 2,6-sialyltransferase, for example produced using a human cell line modified using an α 2,3-sialyltransferase and/or an α 2,6-sialyltransferase.

According to the present invention in a further aspect there is provided a recombinant hCG or a recombinant hCG preparation as described herein which
5 is produced or expressed by the methods described herein.

The rhCG structure contains glycan moieties. Branching can occur with the result that the glycan may have 1, 2, 3, 4 or more terminal sugar residues or “antennae”, as is well known in the art. The rhCG according to aspects of the invention of the invention may have glycans with mono-antennary and/or di-
10 antennary and/or tri-antennary and/or tetra-antennary glycan structures. The rhCG may include mono-antennary and/or bi-antennary and/or tri-antennary and/or tetra-antennary glycan structures, for example with relative amounts as follows: 0.1 to 3% mono-antennary; 65% to 85% bi-antennary; 15 to 25% tri-antennary and 0.5 to 1.5% tetra-antennary (e.g. as shown by WAX analysis of
15 charged glycans).

According to the present invention in a further aspect there is provided a pharmaceutical composition comprising a recombinant hCG (rhCG) having α 2,3-sialylation and α 2,6-sialylation, wherein the recombinant hCG is produced by the methods and/or cells disclosed herein. The rhCG may have a sialic acid content
20 [expressed in terms of a ratio of moles of sialic acid to moles of protein] of from 12 mol/mol to 15.5 mol/mol, for example 12 mol/mol to 14.9 mol/mol (e.g. as set out above). The rhCG may have a sialic acid content of 12.5 mol/mol to 14.5 mol/mol, for example a sialic acid content of 12.8 mol/mol to 13.2 mol/mol. Preferably the rhCG according to the invention has a sialic acid content of 13
25 mol/mol.

The pharmaceutical composition may further comprise FSH and/or LH.

FSH can be obtained by any means known in the art. FSH as used herein includes human-derived and recombinant FSH. Human-derived FSH can be purified from any appropriate source (e.g. urine) by any method known in the art.
30 The FSH may be recombinant FSH – for example expressed in a human cell line. Methods of expressing and purifying recombinant FSH are well known in the art.

LH can be obtained by any means known in the art. LH, as used herein, includes human-derived and recombinant LH. Human-derived LH can be purified from any appropriate source (e.g. urine) by any method known in the art.
35 Methods of expressing and purifying recombinant LH are known in the art.

The pharmaceutical composition may be for, or for use in, the treatment of infertility, e.g. for use in e.g. assisted reproductive technologies (ART), ovulation

induction or intrauterine insemination (IUI). The pharmaceutical composition may be for, or for use in, triggering final maturation / ovulation and luteinization, the stimulation of follicular development in women with severe LH and FSH deficiency, and/or in luteal support (rhCG/rLH). The pharmaceutical composition
5 may be for, or for use in, inducing monofollicular development in anovulatory WHO Type II women (e.g. rhCG with rLH), for enhancing multifollicular response with LH priming in patients undergoing COH (e.g. rhCG with rLH), and for supplementation to COH to improve implantation/pregnancy rates (e.g. rhCG with rLH), and/or increasing pregnancy rates in assisted reproductive technologies.
10 The pharmaceutical composition may be for, or for use in, the prevention of miscarriages, and/or prematurity prevention. The pharmaceutical composition may be for, or for use in, the treatment of endometriosis.

The pharmaceutical composition may be used, for example, in medical indications where known hCG preparations are used. The present invention also
15 provides the use of rhCG and/or an rhCG preparation described herein (according to aspects of the invention) for, or in the manufacture of a medicament for, the treatment of infertility.

The pharmaceutical compositions of the present invention may be formulated into well-known compositions for any route of drug administration, e.g.
20 oral, rectal, parenteral, transdermal (e.g. patch technology), intravenous, intramuscular, subcutaneous, intracisternal, intravaginal, intraperitoneal, local (powders, ointments or drops) or as a buccal or nasal spray. A typical composition comprises a pharmaceutically acceptable carrier, such as aqueous solution, non toxic excipients, including salts and preservatives, buffers and the
25 like, as described in Remington's Pharmaceutical Sciences fifteenth edition (Matt Publishing Company, 1975), at pages 1405 to 1412 and 1461 – 87, and the national formulary XIV fourteenth edition (American Pharmaceutical Association, 1975), among others. Examples of suitable aqueous and non-aqueous pharmaceutical carriers, diluents, solvents or vehicles include water, ethanol,
30 polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (such as olive oil), and injectible organic esters such as ethyl oleate. The compositions of the present invention also can contain additives such as but not limited to preservatives, wetting agents, emulsifying agents, and dispersing agents.
35 Antibacterial and antifungal agents can be included to prevent growth of microbes and includes, for example, m-cresol, benzyl alcohol, paraben, chlorobutanol, phenol, sorbic acid, and the like. Furthermore, it may be desirable to include

isotonic agents such as sugars, sodium chloride, and the like.

In some cases, to effect prolonged action it is desirable to slow the absorption of hCG (and other active ingredients, if present) from subcutaneous or intramuscular injection. This can be accomplished by the use of a liquid
5 suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of hCG then depends upon its rate of dissolution which, in turn, can depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered hCG combination form is accomplished by dissolving or suspending the hCG combination in an oil vehicle. Injectable
10 depot forms can be made by forming microencapsule matrices of the hCG (and other agents, if present) in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of hCG to polymer and the nature of the particular polymer employed, the rate of hCG release can be controlled. Examples of other biodegradable polymers include polyvinylpyrrolidone,
15 poly(orthoesters), poly(anhydrides) etc. Depot injectable formulations are also prepared by entrapping the hCG in liposomes or microemulsions which are compatible with body tissues. Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved
20 or dispersed in sterile water or other sterile injectable medium just prior to use. Injectable formulations can be supplied in any suitable container, e.g. vial, pre-filled syringe, injection cartridges, and the like.

Formulations (e.g. injectable formulation can be supplied as a product having pharmaceutical compositions containing hCG (optionally with FSH, LH
25 etc.) If there is more than one active ingredient (i.e. hCG and e.g. FSH or LH) these may be suitable for administration separately or together. If administered separately, administration can be sequential. The product can be supplied in any appropriate package. For example, a product can contain a number of pre-filled syringes or vials containing either hCG, FSH, or a combination of both FSH and
30 hCG. The syringes or vials may be packaged in a blister package or other means to maintain sterility. A product can optionally contain instructions for using the hCG and FSH formulations.

The pH and exact concentration of the various components of the pharmaceutical composition are adjusted in accordance with routine practice in
35 this field. See GOODMAN and GILMAN's THE PHARMACOLOGICAL BASIS FOR THERAPEUTICS, 7th ed. In a preferred embodiment, the compositions of the invention are supplied as compositions for parenteral administration. General

methods for the preparation of the parenteral formulations are known in the art and are described in REMINGTON; THE SCIENCE AND PRACTICE OF PHARMACY, supra, at pages 780-820. The parenteral compositions can be supplied in liquid formulation or as a solid which will be mixed with a sterile injectable medium just prior to administration. In an embodiment, the parenteral compositions are supplied in dosage unit form for ease of administration and uniformity of dosage.

Detailed description of the invention

The present invention will now be described in more detail with reference to the following Examples and to the attached drawings in which:

- Figure 1 shows the nucleic acid and derived amino acid sequences of the optimised hCG alpha sequence;
- Figure 2 shows the nucleic acid and derived amino acid sequences of hCG beta sequence;
- Figure 3 shows the nucleic acid and derived amino acid sequences of ST3GAL4
- Figure 4 shows a plasmid map of the pHCGalpha/beta expression vector;
- Figure 5 shows the α 2,3-sialyltransferase (ST3GAL4) expression vector;
- Figure 6 shows the detection of rhCG Isoforms by IEF stained with Coomassie Blue in compositions according to the invention (Lanes 4, 5, 6 and 7, 15 μ g per lane); the CHO derived composition of the prior art, Ovitrelle (Lane 2, 15 μ g); and a human urinary product obtained from pregnant women Novarel (Lane 3, 15 μ g); and Figure 7 compares the concentration of hCG ("alpha-beta hCG") and free beta chain ("beta hCG") produced using the cells of the invention including the "Optimised" nucleic sequence of the alpha sub unit, compared with concentration of hCG ("alpha-beta hCG") and free beta chain ("beta hCG") produced in comparative example cells including the WT (wild-type) alpha sub unit;

Examples 1 to 7

Overview of cell line development process

A single plasmid carrying hCG alpha and beta chains, each driven from a separate CMV promoter, was transfected into PER.C6 cells by electroporation.

This plasmid carried a neomycin resistance gene cassette (Fig 4) allowing for selection of transfectants using G418 (Geneticin). Pools of transfectants were expanded and then subjected to limiting dilution cloning in 96 well plates under

G418 selection. Clonal lines were expanded and high expressing colonies were identified based on hCG titre in the cell culture supernatant.

Five lead clones were selected, based on high productivity, and each was then transfected with a second plasmid expressing the α 2,3-sialyltransferase gene ST3GAL4 along with the Hygromycin selection maker (Fig 5). Each of the five
5 transfection pools was expanded and cell culture supernatants were assessed for hCG concentration and *in vivo* pharmacokinetics (PK).

Promising pools were then subjected to another round of limited dilution cloning. The resulting clones were expanded and cell culture supernatants were
10 assessed for hCG concentration and exposed galactose on the hCG protein (by lectin ELISA). Those with a low level of exposed galactose were subjected to further assays including *in vivo* PK. Clones that produced hCG with optimum PK profiles, had low exposed galactose and expressed the protein at high levels were assessed and selected for productivity and growth characteristics.

15 **Example 1: Sequence selection and plasmid vectors**

The coding region of the gene for the hCG alpha polypeptide is shown in Fig 1. The coding region of the gene for the hCG alpha polypeptide sequence is referred to herein SEQ ID NO: 1.

The coding region of the gene for hCG beta polypeptide was used
20 according to Fiddes and Goodman (1980). The sequence is banked as NP_000728 and is consistent with the protein sequences of CGbeta3, CGbeta5 and CGbeta7. The sequence is referred to herein as SEQ ID NO: 2

The coding region of the gene for beta-galactoside alpha-2,3-sialyltransferase 4 (α 2,3-sialyltransferase, ST3GAL4) was used according to
25 Kitagawa and Paulson (1994). The sequence is banked as L23767 and referred to herein as SEQ ID NO: 3.

Two plasmids were used: the first, pHCG, co-expresses hCG alpha and beta chains; and the second, expresses the sialyltransferase gene ST3GAL4.

30 **Example 2a: Construction of the hCG expression vector**

pHCG is a synthetic alpha chain of hCG, optimised for codon usage in mammalian cells including the native hCG alpha signal peptide. It may be engineered by methods well known in the art.

The coding sequence of hCG alpha polypeptide (SEQ ID NO: 1) and hCG
35 beta polypeptide (NP_000728, SEQ ID NO: 2) were amplified by PCR using methods well known in the art (see, for example, International Patent Application

published as WO2011/042688). The phCG alpha DNA was digested with *Bam*HI and *Nhe*I and inserted into the sites *Bam*HI and *Nhe*I on a CMV driven mammalian expression vector (Crucell vector pcDNA3002Neo). This placed the gene in the correct orientation for expression driven by a CMVie promoter with a downstream BGH polyA signal. The native hCG beta gene including native signal peptide was digested with the restriction enzymes *As*cl and *H*paI and inserted into the *As*cl and *H*paI sites so that expression would be driven by a second CMVie promoter with an additional downstream BGH poly A signal. The vector backbone also included a neomycin resistance maker as well as elements required for selection and replication in prokaryotic cells. The vector was amplified and sequenced using general methods are known in the art. Sequences of the optimised hCG alpha and native (wild-type) beta chains are given in Figures 1 and 2, respectively.

All colonies selected for sequencing contained the correct sequences according to SEQ ID NO: 1 and SEQ ID NO: 2. Plasmid phCG A+B was selected for transfection (Figure 4).

Example 2b: Construction of the ST3 expression vector

The ST3GAL4 gene is expressed in pST3. The coding sequence of beta-galactoside alpha-2,3-sialyltransferase 4 (ST3, L23767, SEQ ID NO: 3, Fig 3) was amplified by PCR using the primer combination 2,3STfw and 2,3STrev.

2,3STfw 5'-CCAGGATCCGCCACCATGTGTCCTGCAGGCTGGAAGC-3'

2,3STrev 5'-TTTTTTTCTTAAGTCAGAAGGACGTGAGGTTCTTG-3'

The resulting amplified ST3 DNA was digested with the restriction enzymes *Bam*HI and *A*flII and inserted into the *Bam*HI and *A*flII sites on the CMV driven mammalian expression vector carrying a hygromycin resistance marker (vector pcDNA3002Neo) so that it is located downstream of CMVie promoter and upstream of BGH polyA sequence. The vector backbone also included a hygromycin resistance maker as well as elements required for selection and replication in prokaryotic cells. The vector was amplified as previously described and sequenced. Clone pST3#1 (Figure 5) contained the correct sequence according to SEQ ID NO: 3 and was selected for transfection.

Example 3 - Plasmid Transfection

The plasmid phCG (Fig 4), which has a single *P*vul site located in the prokaryotic beta-lactamase gene, was linearised with *P*vul (New England Biolabs

Cat. No. R0150). Linearized plasmid DNA was transfected into PER.C6® cells as follows.

Cell cultures were maintained in complete PERMAB medium (CD4PERMAB (Hyclone Cat. No. SH30871.01) supplemented with L-glutamine to
5 3mM final concentration (Invitrogen Cat. No. 25030-123) and Pluronic F68 at 1.0g/L final concentration (Invitrogen Cat. No. 24040-032) in 250ml Erlenmeyer flasks. Cells were maintained in a shaking incubator (Kuhner Climo-shaker ISF1-X) set at 100rpm, 5% CO₂ and 37°C, for at least 14 days prior to transfection. 48 hours prior to transfection, cells were transferred into fresh medium at a density
10 of 0.5x10⁶ cells/ml.

On the day of transfection, cells were counted in a Beckman Coulter ViCell XR to determine cell density and to ensure viability was >90%. Cells were harvested by centrifugation and resuspended in fresh PERMAB medium before being mixed with linearized phCG DNA. The cell/DNA mix was electro-shocked in
15 the chamber of an electroporator set at 250V for 5msec, before being quickly transferred to 10ml of pre-warmed PERMAB medium. This process was repeated a total of 6 times and all 6 transfections were pooled into a single T-175cm² tissue culture flask. The flask was placed in a static incubator set at 37°C, 5% CO₂. After 48 hours, cells were resuspended in the appropriate volume of selective PERMAB
20 (complete PERMAB medium + G418 (125µg/ml)) to give a viable cell density of 0.5x10⁶/ml.

The pool culture was passaged twice weekly, maintaining cells at a density of 0.3x10⁶ cell/ml in selective medium until cell viability had increased to >50%. At this point, cells were transferred to shaking cultures in 250ml Erlenmeyer flasks.
25 After several weeks in shaking culture, pool supernatant was sampled and assayed for hCG concentration. Once it was established that the pool was positive for hCG expression, cells were prepared for limited dilution cloning.

A cell suspension at 0.3 viable cells/ml was prepared in PERMAB medium supplemented with G-418 at 125µg/ml. The cell suspension was dispensed into
30 96 well flat bottomed tissue culture plates at 200µl/well and incubated in a humidified atmosphere at 37°C, 5% CO₂ (Binder CB150). Plates were scanned regularly using the Genetix Clone Select Imager to track the growth of cells in each well.

After two weeks, 535 wells were identified that contained actively growing
35 colonies of cells. Supernatants from these wells were sampled and assayed for hCG using a commercial kit (DRG diagnostics HCG ELISA Cat. No. EIA1469). Based on the results from these assays, a total of 162 of the colonies were

transferred into 24 well plates containing 0.5ml/well selective PERMAB medium. When cells in the wells were near to confluency, supernatant from each of the 162 wells was sampled and assayed for hCG levels. Based on these results, 91 of the best expressing cell lines were selected for expansion into T-25 flasks. These cell
5 lines were again grown to near confluence, at which point supernatants were sampled and assayed for hCG as above. Based on these results, the 58 best expressing cell lines were expanded into T-75 flasks. Specific production rate (SPR) analysis was performed on each of these 58 cell lines by methods known in the art and specific productivity was expressed in pg/cell/day.

10 Thus, in this Example a plasmid incorporating hCG alpha and beta chains was transfected into PER.C6® cells and transfectants were selected using medium containing G418. Growing colonies of cells were screened for hCG concentration in the supernatant and those expressing the highest level were expanded further. After subsequent rounds of expansion and screening, 20 clones that expressed
15 hCG were selected for growth and productivity studies. Based on the results of these studies, 5 clones were selected for transfection with a second plasmid incorporating the ST3GAL4 gene.

Example 4 - Transfection with the ST3GAL4 plasmid pST3

20 Stable clones were generated as previously described in Example 3.

The five best clones produced by the method as described in Example 3 were selected for transfection with the ST3GAL4 plasmid pST3 (see Example 2, Figure 3 and 5). Transfection was performed by electroporation using the same method described in Example 3, except that transformants were selected in
25 complete PERMAB medium supplemented with Hygromycin at 0.5µg/ml instead of G418. Using this method, five pools were obtained that expressed hCG.

Samples of supernatants from the transfection pools were assayed in a rat pharmacokinetic model and, based on this data along with data from the RCA-lectin binding assay (measuring exposed galactose on the hCG chains), a single
30 pool was selected for further limited dilution cloning, by methods known in the art.

This dilution cloning yielded >600 clones each of which was also screened for hCG expression and the degree of exposed galactose as measured by RCA-lectin binding. Those clones with the lowest levels of exposed galactose were expanded further and samples were subjected to *in vivo* PK and corroborating
35 lectin binding data. Five clones derived from the single pool were further assessed for growth and productivity characteristics.

Each of these clones was expanded and a seed stock cell bank was made following standard cryopreservation procedures. Stocks from the seed stock cell bank were thawed and found to be viable.

5 **Example 5 - Analysis by isoelectric focussing.**

Electrophoresis is defined as the transport of charged molecules through a solvent by an electrical field. The mobility of a biological molecule through an electric field will depend on the field strength, net charge on the molecule, size and shape of the molecule, ionic strength and properties of the medium through which
10 the molecules migrate.

Isoelectric focusing (IEF) is an electrophoretic technique for the separation of proteins based on their pI. The pI is the pH at which a protein has no net charge and will not migrate in an electric field. The sialic acid content of the hCG isoforms subtly alters the pI point for each isoform, which can be exploited using this
15 technique to visualise the Per.C6 hCG isoforms from each clone.

The isoelectric points of the Per.C6 produced hCG isoforms were analyzed using isoelectric focussing. Per.C6 hCG was produced as described in Example 6.

Per.C6 hCG samples were separated on Novex® IEF Gels containing 5%
20 polyacrylamide under native conditions on a pH 3.0 -7.0 gradient in an ampholyte solution pH 3.0 – 7.0. Proteins were visualised using Coomassie Blue staining, using methods well known in the art.

Figure 6 shows the detection of rhCG Isoforms by IEF stained with Coomassie Blue in compositions produced according to the invention produced
25 from the cloned cell lines made by the method set out in Examples 6 (Lanes 4, 5, 6 and 7, 15µg per lane); the CHO derived composition of the prior art, Ovitrelle (Lane 2, 15µg); and a human urinary product obtained from pregnant women Novarel (Lane 3, 15µg). The bands represent isoforms of hCG containing different numbers of sialic acid molecules. Figure 6 indicates that human cell line derived
30 recombinant hCG engineered with $\alpha 2,3$ - sialyltransferase (compositions according to the invention) is more acidic than Ovitrelle and urinary hCG from pregnant women.

Figure 7 compares the concentration of hCG (“alpha-beta hCG”) and free beta chain (“beta hCG”) produced using the cells of the invention (that is, using cell
35 lines made by the method set out in Examples 1 to 4) which include the “Optimised” alpha sub unit, compared with concentration of hCG (“alpha-beta hCG”) and free beta chain (“beta hCG”) produced in comparative example cells including the WT

(wild-type) alpha sub unit. Figure 7 shows that the cells of the invention express high amounts of hCG and low excess of the free beta subunit. In other words, the yield and purity of recombinant hCG produced by the cells and methods of the invention is markedly improved.

5 Table A indicates the percentage of free β -hCG Subunits in semi-purified samples of various batches of hCG produced using the cells of the invention (that is, using cell lines made by the method set out in Examples 1 to 4) which include the "Optimised" alpha sub unit ("Opt. alpha hCG"), compared with the percentage of free β -hCG Subunits in semi-purified samples of hCG produced in comparative
10 example cells including the WT (wild-type) alpha sub unit ("WT alpha hCG"), as determined by Hydrophobic Phenyl-5PW HPLC chromatography.

Table A shows that the cells of the invention express high amounts of hCG and a rather lower excess of the free beta subunit (5.7 – 10.6%) compared to cells including the WT alpha sub unit (64 – 66%). In other words, the yield and purity of
15 recombinant hCG produced by the cells and methods of the invention is markedly improved.

Table A

Clone	% Free β -hCG (PHE – 5PW)
WT alpha hCG (1G2)	64.1
WT alpha hCG (1G2)	66.0
Opt. alpha hCG (13.8)	5.7
Opt. alpha hCG (29)	7.5
Opt. alpha hCG (29)	6.6
Opt. alpha hCG (29)	10.6

Example 6 - Production and purification overview

20 A procedure was developed to produce recombinant hCG in PER.C6 cells that were cultured in suspension in serum free medium. The procedure is described below and was applied to several hCG-producing PER.C6 cell lines.

Recombinant hCG from an α 2,3-sialyltransferase transfected clone was prepared using the methods of Examples 1 to 4 described above.

25 The cells were grown in shaker flasks in 6GRO medium (SAFC) until a cell density of 1×10^6 to 3×10^6 cells/ml was achieved. The cells were transferred to a

5L glass stirred tank bioreactor with a density of about 1×10^6 cells/ml. The bioreactor worked in perfusion mode using a Proper1 media (Lonza).

Thereafter, purification of the product rhCG was carried out using various ultrafiltration steps, anion and cation exchange capture chromatography, hydrophobic chromatography and pseudo-affinity chromatography, by methods well known in the art.

During all chromatographic procedures, the presence of immunoreactive recombinant hCG was confirmed by ELISA (DRG EIA 1469) and IEF (Example 5).

10 **Example 7 – Sialic Acid Content**

Sialic acid is a protein-bound carbohydrate considered to be a mono-saccharide and occurs in combination with other mono-saccharides like galactose, mannose, glucosamine, galactosamine and fucose. The total sialic acid on purified rhCG according to the invention was measured using a method based on the method of Stanton *et. al.* (J. Biochem. Biophys. Methods. 30 (1995), 37 – 48).

The total sialic acid content of samples of Per.C6 recombinant hCG modified with $\alpha 2,3$ - sialyltransferase (produced by the methods of Example 4 and 6) were measured and the results are in Table 1 below [expressed in terms of a ratio of moles of sialic acid to moles of protein].

Example 8 – hCG Bioassay according to USP

A hCG Bioassay was carried out, in order to determine the hCG specific activity, for each of the samples of Table 1 below. The activity was measured according to USP (USP Monographs: Chorionic Gonadotropin, USPC Official 8/1/09-11/30/09), using Ovitrelle as a standard. Ovitrelle has a biological activity of 26,000 IU/mg (Curr Med Res Opin. 2005 Dec; 21(12): 1969 – 76). The acceptance limit was $>21,000$ IU hCG/mg. The biological activity for samples of human cell line derived hCG recombinant hCGs engineered with $\alpha 2,3$ - sialyltransferase are shown in Table 1.

Table 1

Sample	1	2	3	4	5	6
Sialic acid (SA) content ($\mu\text{mol SA} / \mu\text{mol hCG}$)	13	13	13	13	13	15
Potency IU/mg	25363	25009	24904	24623	25645	26623

% Potency (Ovitrelle 26000 IU/mg)	97.5	96.2	95.8	94.7	98.6	102.4
Sample	7	8	9	10	11	
Sialic acid (SA) content ($\mu\text{mol SA} / \mu\text{mol hCG}$)	14	14	13	13	13	
Potency IU/mg	27227	26142	26539	26181	24230	
% Potency (Ovitrelle 26000 IU/mg)	104.7	100.5	102	101	93.2	

As seen above, the potency is similar to, and may be greater than, Ovitrelle (see e.g Sample 7).

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- 25

Optimised hCG alpha

5 Nucleotide sequence of optimised hCG alpha (**SEQ ID NO: 1**)

1 ATGGACTACT ACCGGAAGTA CGCCGCCATC TTCCTGGTGA CCCTGAGCGT
GTTCTGTCAC

61 GTGCTGCACA GCGCCCCTGA CGTGCAGGAC TGCCCCGAGT GCACCCTGCA

10 GGAAAACCCC

121 TTCTTCAGCC AGCCTGGCGC CCCTATCCTG CAGTGCATGG GCTGCTGCTT
CAGCAGAGCC

181 TACCCACCCC CCCTGCGGAG CAAGAAAACC ATGCTGGTGC AGAAAAACGT
GACCAGCGAG

15 241 AGCACCTGCT GCGTGCCCAA GAGCTACAAC CGGGTGACCG TGATGGGCGG
CTTCAAGGTG

301 GAGAACCACA CCGCCTGCCA CTGCAGCACC TGCTACTACC ACAAGTCCT

Protein sequence of hCG optimised alpha

20 1 MDYYRKYAAI FLVTLVFLH VLHSAPDVQD CPECTLQENP FFSQPGAPIL
QCMGCCFSRA

61 YPTPLRSKKT MLVQKNVTSE STCCVAKSYN RVTVMGGFKV ENHTACHCST CYYHKS

25

Human Chorionic Gonadotrophin beta polypeptide

Accession number NP_000728

30 Nucleotide sequence of hCG beta (**SEQ ID NO: 2**)Nucleotide sequence

1 ATGGAGATGT TCCAGGGGCT GCTGCTGTTG CTGCTGCTGA GCATGGGCGG

35 GACATGGGCA

61 TCCAAGGAGC CGCTTCGGCC ACGGTGCCGC CCCATCAATG CCACCCTGGC
TGTGGAGAAG

121 GAGGGCTGCC CCGTGTGCAT CACCGTCAAC ACCACCATCT GTGCCGGCTA
CTGCCCCACC

40 181 ATGACCCGCG TGCTGCAGGG GGTCTGCCG GCCCTGCCTC AGGTGGTGTG
CAACTACCGC

241 GATGTGCGCT TCGAGTCCAT CCGGCTCCCT GGCTGCCCCG GCGGCGTGAA
CCCCGTGGTC

301 TCCTACGCCG TGGCTCTCAG CTGTCAATGT GCACTCTGCC GCCGCAGCAC

45 CACTGACTGC

361 GGGGGTCCCA AGGACCACCC CTTGACCTGT GATGACCCCC GCTTCCAGGA
 CTCCTCTTCC
 421 TCAAAGGCCC CTCCCCCAG CCTTCCAAGT CCATCCCGAC TCCCGGGGCC
 CTCGGACACC
 5 481 CCGATCCTCC CACAATAA

Protein sequence of hCG beta

1 MEMFQGLLLL LLLSMGGTWA SKEPLRPRCR PINATLAVEK EGCPVCITVN
 10 TTICAGYCPT
 61 MTRVLQGVLP ALPQVVCNYR DVRFESIRLP GCPRGVNPVV SYAVALSCQC
 ALCRRSTTDC
 121 GGPKDHPLTC DDPRFQDSSS SKAPPPSLPS PSRLPGPSDT PILPQ

15

Beta-galactoside alpha-2,3-sialyltransferase 4

Accession Number L23767

20 Nucleotide sequence of ST3GAL4 (SEQ ID NO: 3)

1 ATGTGTCCTG CAGGCTGGAA GTCCTGGCC ATGTTGGCTC TGGTCCTGGT
 CGTCATGGTG
 61 TGGTATTCCA TCTCCCGGGA AGACAGGTAC ATCGAGCTTT TTTATTTTCC
 25 CATCCCAGAG
 121 AAGAAGGAGC CGTGCCTCCA GGGTGAGGCA GAGAGCAAGG CCTCTAAGCT
 CTTTGGCAAC
 181 TACTCCCGGG ATCAGCCCAT CTCCTGCGG CTTGAGGATT ATTTCTGGGT
 CAAGACGCCA
 30 241 TCTGCTTACG AGCTGCCCTA TGGGACCAAG GGGAGTGAGG ATCTGCTCCT
 CCGGGTGCTA
 301 GCCATCACCA GTCCTCCAT CCCCAGAAGC ATCCAGAGCC TCAGGTGCCG
 CCGCTGTGTG
 361 GTCGTGGGGA ACGGGCACCG GCTGCGGAAC AGCTCACTGG GAGATGCCAT
 35 CAACAAGTAC
 421 GATGTGGTCA TCAGATTGAA CAATGCCCCA GTGGCTGGCT ATGAGGGTGA
 CGTGGGCTCC
 481 AAGACCACCA TGCCTCTCTT CTACCCTGAA TCTGCCCCACT TCGACCCCAA
 AGTAGAAAAC
 40 541 AAGCCAGACA CACTCCTCGT CCTGGTAGCT TTCAAGGCAA TGGACTTCCA
 CTGGATTGAG
 601 ACCATCCTGA GTGATAAGAA GCGGGTGCGA AAGGGTTTCT GGAAACAGCC
 TCCCCTCATC

	661	TGGGATGTCA	ATCCTAAACA	GATTCGGATT	CTCAACCCCT	TCTTCATGGA
		GATTGCAGCT				
	721	GACAAACTGC	TGAGCCTGCC	AATGCAACAG	CCACGGAAGA	TTAAGCAGAA
		GCCCACCACG				
5	781	GGCCTGTTGG	CCATCACGCT	GGCCCTCCAC	CTCTGTGACT	TGGTGCACAT
		TGCCGGCTTT				
	841	GGCTACCCAG	ACGCCTACAA	CAAGAAGCAG	ACCATTCACT	ACTATGAGCA
		GATCACGCTC				
	901	AAGTCCATGG	CGGGGTCAGG	CCATAATGTC	TCCCAAGAGG	CCCTGGCCAT
10		TAAGCGGATG				
	961	CTGGAGATGG	GAGCTATCAA	GAACCTCACG	TCCTTCTGA	

Protein Sequence of ST3GAL4

15	1	MCPAGWKLLA	MLALVLVVMV	WYSISREDRY	IELFYFPIPE	KKEPCLQGEA
		ESKASKLFGN				
	61	YSRDQPIFLR	LEDYFWVKTP	SAYELPYGTK	GSEDLLLRVL	AITSSSIPKN
		IQSLRCRRCV				
	121	VVGNGHRLRN	SSLGDAINKY	DVVIRLNNAP	VAGYEGDVGS	KTTMRLFYPE
20		SAHFDPKVEN				
	181	NPDTLVLVA	FKAMDFHWIE	TILSDKKRV	KGFWKQPPLI	WDVNPQIRI
		LNPFFMEIAA				
	241	DKLLSLPMQQ	PRKIKQKPTT	GLLAITLALH	LCDLVHIAGF	GYPDAYNKKQ
		TIHYEQITL				
25	301	KSMAGSGHNV	SQEALAIKRM	LEMGAIKNLT	SF	

Optimised hCG alpha chain

Nucleotide sequence of optimised hCG alpha chain (SEQ ID NO: 4)

30	1	CCCCCTGACG	TGCAGGACTG	CCCCGAGTGC	ACCCTGCAGG	AAAACCCCTT
		CTTCAGCCAG				
	61	CCTGGCGCCC	CTATCCTGCA	GTGCATGGGC	TGCTGCTTCA	GCAGAGCCTA
		CCCCACCCCC				
35	121	CTGCGGAGCA	AGAAAACCAT	GCTGGTGCAG	AAAAACGTGA	CCAGCGAGAG
		CACCTGCTGC				
	181	GTGGCCAAGA	GCTACAACCG	GGTGACCGTG	ATGGGCGGCT	TCAAGGTGGA
		GAACCACACC				
40	241	GCCTGCCACT	GCAGCACCTG	CTACTACCAC	AAGTCCT	

Protein sequence of hCG optimised alpha chain

1 APDVQDCPEC TLQENPFFSQ PGAPILQCMG CCFSRAYPTP LRSKKTMLVQ
KNVTSESTCC

5 61 VAKSYNRVTV MGGFKVENHT AHCSTCYH KS

hCG alpha polypeptide

Accession number AH007338

10

Nucleotide sequence of hCG alpha (**SEQ ID NO: 5**)

1 ATGGATTACT ACAGAAAATA TGCAGCTATC TTTCTGGTCA CATTGTCGGT
GTTTCTGCAT

15 61 GTTCTCCATT CCGCTCCTGA TGTGCAGGAT TGCCCAGAAT GCACGCTACA
GGAAAACCCA

121 TTCTTCTCCC AGCCGGGTGC CCCAATACTT CAGTGCATGG GCTGCTGCTT
CTCTAGAGCA

181 TATCCCACTC CACTAAGGTC CAAGAAGACG ATGTTGGTCC AAAAGAACGT
20 CACCTCAGAG

241 TCCACTTGCT GTGTAGCTAA ATCATATAAC AGGGTCACAG TAATGGGGGG
TTTCAAAGTG

301 GAGAACCACA CGGCGTGCCA CTGCAGTACT TGTTATTATC ACAAATCTTA A

25 Protein sequence of hCG alpha

1 MDYYRKYAAI FLVTLVFLH VLHSAPDVQD CPECTLQENP FFSQPGAPIL
QCMGCCFSRA

61 YPTPLRSKKT MLVQKNVTSE STCCVAKSYN RVTVMGGFKV ENHTACHCST CYYHKS

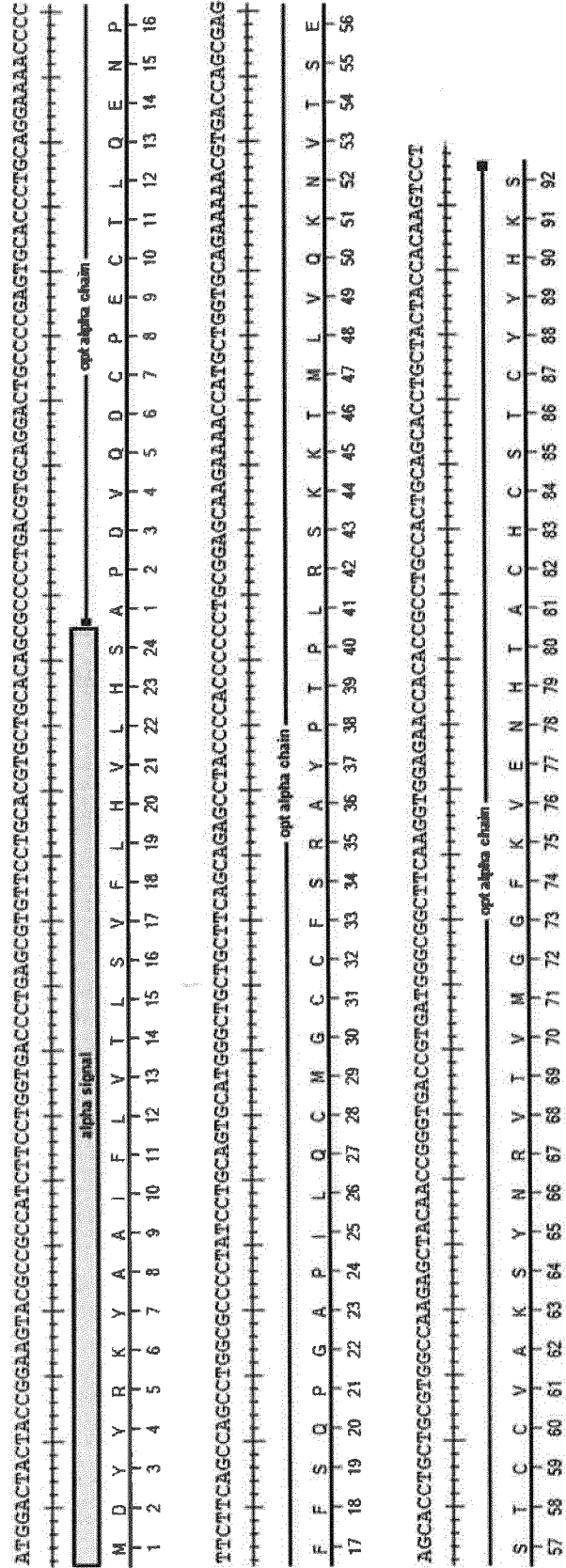
30

Claims

1. A polynucleotide sequence comprising a nucleic acid sequence according to SEQ ID NO: 1.
- 5 2. A polynucleotide sequence comprising a nucleic acid sequence according to SEQ ID NO: 4.
3. A polynucleotide sequence comprising a nucleic acid sequence with at least 97% sequence identity with the nucleic acid of SEQ ID NO: 1.
- 10 4. A polynucleotide sequence comprising a nucleic acid sequence with at least 97% sequence identity with the nucleic acid of SEQ ID NO: 4.
5. A cell characterized in that it comprises integrated into its genome a sequence coding for the α chain of hCG selected from: a sequence according to SEQ ID NO: 1; a sequence which has at least 96.5 % homology with the sequence of SEQ ID NO:1; a sequence which has at least 97 % homology with the sequence of SEQ ID NO:1; a sequence according to SEQ ID NO. 4; a sequence which has at least 96.5 % homology with the sequence of SEQ ID NO: 4; and a sequence which has at least 97 % homology with the sequence of SEQ ID NO:4.
- 15 6. A cell according to claim 5 which is a PER.C6® cell such as a PER.C6® cell deposited under ECACC no. 96022940.
7. A PER.C6® cell such as a PER.C6® cell deposited under ECACC no. 96022940, characterized in that it further comprises integrated into its genome a sequence coding for the α chain of hCG.
- 25 8. A cell according to any of claims 5 to 7 characterized in that it further comprises integrated into its genome a cDNA encoding alpha-2,3-sialyltransferase .
9. A cell according to any of claims 5 to 8 characterized in that it further comprises integrated into its genome a nucleic acid sequence coding for the β chain of hCG.
- 30

10. A method for producing recombinant protein in a cell, comprising culturing a cell according to any of claims 5 to 9 in a suitable medium and harvesting the recombinant protein from said cell and/or said medium.
- 5 11. A method for producing recombinant hCG in a cell, comprising culturing the cell in a suitable medium and harvesting the recombinant protein (hCG) from said cell and/or said medium, wherein the cell is a PER.C6® cell comprising integrated into its genome a sequence coding for the α chain of hCG.
- 10 12. A recombinant hCG ("rhCG" or "rechCG") which includes α 2,3- and α 2,6-sialylation, wherein the recombinant hCG is produced in a cell according to any of claims 5 to 9.

Fig 1: Optimised hCG alpha:



Figs 4 and 5: Plasmid maps of the phCGalpha/beta and pST3 expression vectors. CMV = Cytomegalovirus promoter, BGHp(A) = Bovine Growth Hormone poly-adenylation sequence, fl ori = fl origin of replication, SV40 = Simian Virus 40 promoter, Neo = Neomycin resistance marker, Hyg = Hygromycin resistance marker, SV40 p(A) = Simian Virus 40 poly-adenylation sequence, hCG α = human chorionic gonadotropin alpha polypeptide, hCG β = human chorionic gonadotropin beta polypeptide, ST3GAL4 = α 2,3-sialyltransferase, ST6GAL1 = α 2,6-sialyltransferase, ColEI = ColEI origin of replication, Amp = ampicillin resistance marker.

Figure 4. phCG expression vector

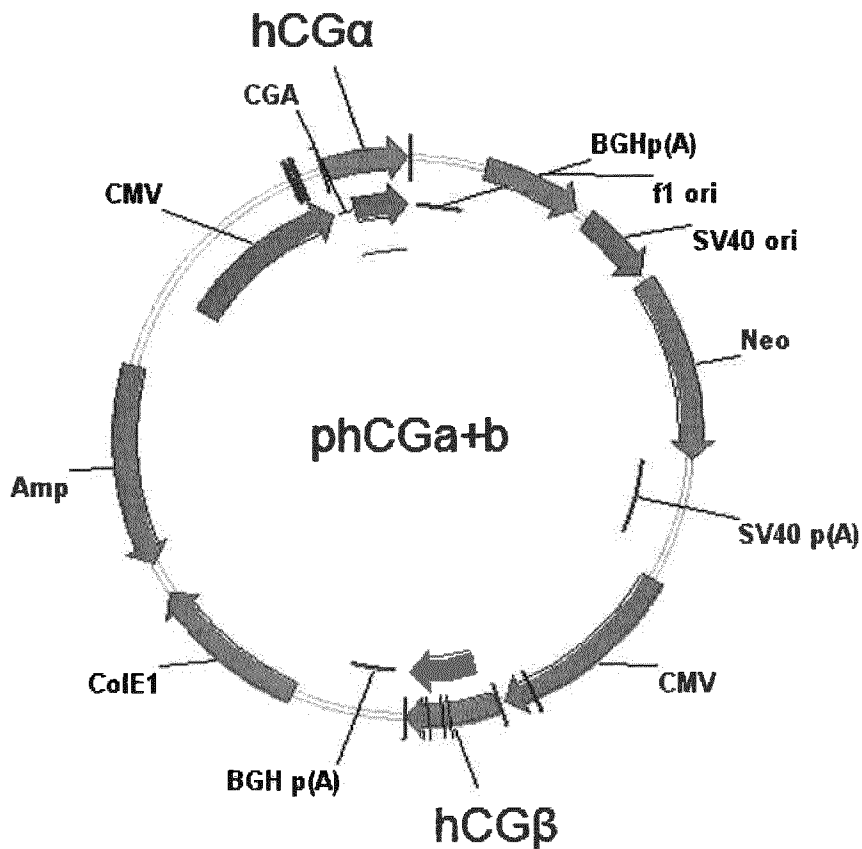
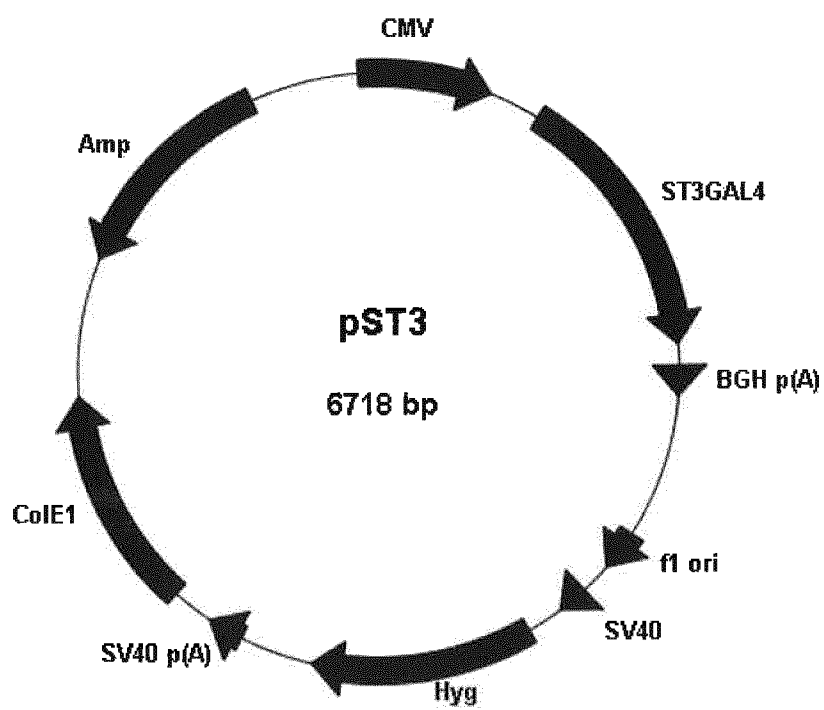


Figure 5. α 2,3-sialyltransferase (ST3GAL4) expression vector

6/6

Figure 6: shows the detection of rhCG Isoforms by IEF stained with Coomassie Blue in compositions according to the invention (Lanes 4, 5, 6 and 7, 15 μ g per lane); the CHO derived composition of the prior art, Ovitrelle (Lane 2, 15 μ g); and a human urinary product obtained from pregnant women Novarel (Lane 3, 15 μ g); IEF marker (Lane 1 (pI's from top: 7.0, 6.8, 6.5, 6.0, 5.1 and 4.75 respectively))

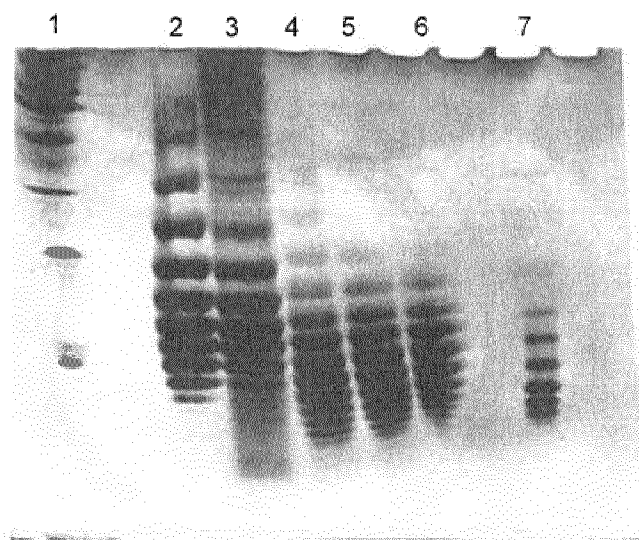


FIG. 6

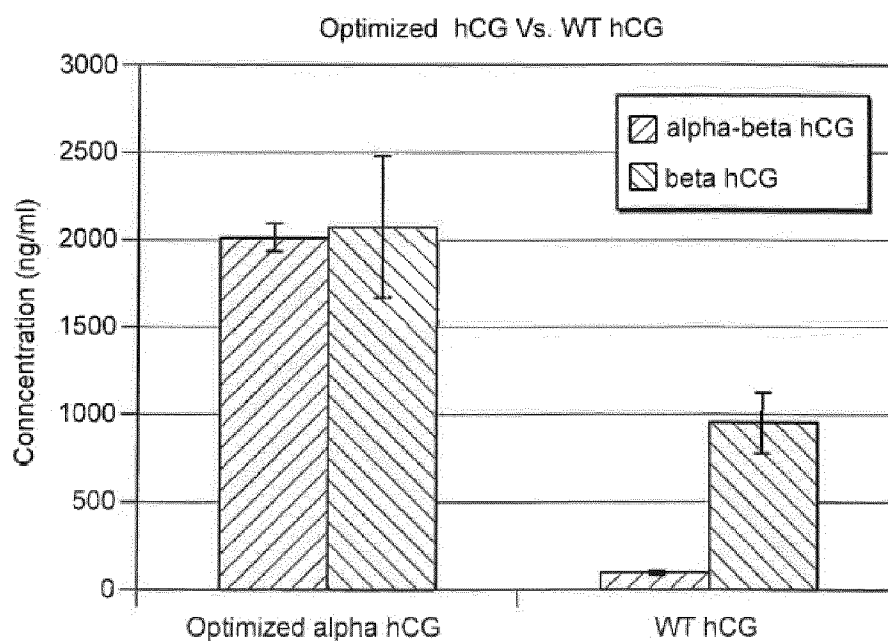


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/059006

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/59
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/042688 A1 (FERRING INT CT SA [CH]; COTTINGHAM IAN [CH]; PLAKSIN DANIEL [CH]; WHIT) 14 April 2011 (2011-04-14)	12
Y	page 22, paragraph 1; figures 2, 4; example 8	1,2,5-11
X	----- WO 2013/151666 A2 (MODERNA THERAPEUTICS [US]) 10 October 2013 (2013-10-10) sequence 1926 & DATABASE EMBL [Online] 28 January 2014 (2014-01-28), "Sequence 1926 from Patent W02013151666.", retrieved from EBI accession no. EM PAT:JC113281 Database accession no. JC113281 sequence ----- -/--	3,4



Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

6 June 2016

Date of mailing of the international search report

20/06/2016

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Lanzrein, Markus

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/059006

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/059006

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