HU000030652T2

(19) **HU** (11) Lajstromszám: **E 030 652** (13) **T2**

MAGYARORSZÁG
Szellemi Tulajdon Nemzeti Hivatala

EURÓPAI SZABADALOM
SZÖVEGÉNEK FORDÍTÁSA

(21) Magyar ügyszám: **E 14 178729**
(22) A bejelentés napja: **2009. 04. 16.**

(51) Int. Cl.: ***C07K 14/59*** *(2006.01)*
A61K 38/00 *(2006.01)*
A61K 38/24 *(2006.01)*

(96) Az európai bejelentés bejelentési száma:
EP 20090178729
(97) Az európai bejelentés közzétételi adatai:
EP 2808340 A1 **2014. 12. 03.**
(97) Az európai szabadalom megadásának meghirdetési adatai:
EP 2808340 B1 **2016. 08. 17.**

(30) Elsőbbségi adatok:

45424 P	**2008. 04. 16.**	**US**
08251528	**2008. 04. 25.**	**EP**

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(54) **Rekombináns, alfa-2,3- és alfa-2,5-szializációt tartalmazó FSH**

Az európai szabadalom ellen, megadásának az Európai Szabadalmi Közlönyben való meghirdetésétől számított kilenc hónapon belül, felszólalást lehet benyújtani az Európai Szabadalmi Hivatalnál. (Európai Szabadalmi Egyezmény 99. cikk(1))

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 2 808 340 B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
17.08.2016 Bulletin 2016/33

(21) Application number: **14178729.1**

(22) Date of filing: **16.04.2009**

(51) Int Cl.:
C07K 14/59 $^{(2006.01)}$ ***A61K 38/24*** $^{(2006.01)}$
A61K 38/00 $^{(2006.01)}$

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(54) **RECOMBINANT FSH INCLUDING ALPHA 2,3- AND ALPHA 2,6-SIALYLATION**
RECOMBINANTES FSH MIT ALPHA 2,3- AND ALPHA 2,6-SIALYLIERUNG
FSH RECOMBINANTE COMPRENANT LA SIALYLATION ALPHA 2,3 ET ALPHA 2,6

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(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK TR

(30) Priority: **16.04.2008 US 45424 P**
25.04.2008 EP 08251528

(43) Date of publication of application:
03.12.2014 Bulletin 2014/49

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
13193214.7 / 2 722 339
09733497.3 / 2 268 666

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- **FLACK M R ET AL: "Increased biological activity due to basic isoforms in recombinant human follicle-stimulating hormone produced in a human cell line.", THE JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM SEP 1994, vol. 79, no. 3, September 1994 (1994-09), pages 756-760, XP002494715, ISSN: 0021-972X**

Remarks:
The file contains technical information submitted after the application was filed and not included in this specification

EP 2 808 340 B1

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Printed by Jouve, 75001 PARIS (FR)

Description

[0001] The present invention relates to gonadotrophins for use in the treatment of infertility. In particular it relates to follicle stimulating hormone (FSH).

[0002] 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).

[0003] FSH is naturally secreted by the anterior pituitary gland and functions to support follicular development and ovulation. FSH comprises a 92 amino acid alpha sub-unit, also common to the other glycoprotein hormones LH and CG, and a 111 amino acid beta sub-unit unique to FSH that confers the biological specificity of the hormone (Pierce and Parsons, 1981). Each sub-unit is post translationally modified by the addition of complex carbohydrate residues. Both subunits carry 2 sites for N-linked glycan attachment, the alpha sub-unit at amino acids 52 and 78 and the beta sub-unit at amino acid residues 7 and 24 (Rathnam and Saxena, 1975, Saxena and Rathnam, 1976). FSH is thus glycosylated to about 30% by mass (Dias and Van Roey. 2001. Fox *et al.* 2001).

[0004] FSH purified from post-menopausal human urine has been used for many years in infertility treatment; both to promote ovulation in natural reproduction and to provide oocytes for assisted reproduction technologies. Two recombinant versions of FSH, Gonal-F (Serono) and Puregon (Organon) became available in the mid-1990's. These are both expressed in Chinese hamster ovary (CHO) cells (Howles, 1996).

[0005] There is considerable heterogeneity associated with FSH preparations which relates to differences in the amounts of various isoforms present. Individual FSH 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.

[0006] Glycosylation of natural FSH is highly complex. The glycans in naturally derived pituitary FSH can contain a wide range of structures that can include combinations of bi-, tri- and tetra-antennary glycans (Pierce and Parsons, 1981. Ryan *et al.,* 1987. Baenziger and Green, 1988). The glycans can carry further modifications: core fucosylation, bisecting glucosamine, chains extended with acetyl lactosamine, partial or complete sialylation, sialylation with α2,3 and α2,6 linkages, and sulphated galactosamine substituted for galactose (Dalpathado *et al.,* 2006). Furthermore, there are differences between the distributions of glycan structures at the individual glycosylation sites. A comparable level of glycan complexity has been found in FSH derived from the serum of individuals and from the urine of post-menopausal women (Wide *et* al., 2007).

[0007] The glycosylation of recombinant FSH products reflects the range of glycosyl-transferases present in the host cell line. Existing rFSH products are derived from engineered Chinese hamster ovary cells (CHO cells). The range of glycan modifications in CHO derived rFSH are more limited than those found on the natural products, derived either from pituitary extracts or urine. Examples of the reduced glycan heterogeneity found in CHO derived rFSH include a lack of bisecting glucosamine and a reduced content of core fucosylation and acetyl lactosamine extensions (Hard *et al.,* 1990). In addition, CHO cells are only able to add sialic acid using the α2,3 linkage (Kagawa *et al,* 1988, Takeuchi *et al,* 1988, Svensson *et al.,* 1990). This is different from naturally produced FSH which contains glycans with a mixture of α2,3 and α2,6-linked sialic acid.

[0008] 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 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 the 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).

[0009] There is a large body of scientific work which analyses and tries to explain the variations in FSH glycosylation between individuals and changes over the course of an ovulation cycle. One of the major discussions relates to the observation that FSH concentration and sialic acid content both decrease during the pre-ovulatory phase of the cycle. The decreased sialic acid content results in a more basic FSH which is both cleared more rapidly and, in vitro at least, is more potent at the target receptor (Zambrano *et al.* 1996). The question as to the biological relevance of these changes and how they may be involved in selecting the dominant follicle remains unresolved (reviewed by Ulloa-Aguirre, 2003).

[0010] 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 pl, in which more acid equates to a higher negative charge. As previously stated 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). It has also been reported that the more basic FSH circulating during the later stages of the ovulation cycle is due to the down-regulation of α2,3 sialyltransferase in the anterior pituitary which is caused by increasing levels of estradiol (Damian-Matsumara *et al.* 1999. Ulloa-Aguirre *et al.* 2001). Results for the α2,6 sialyltransferase have not been reported.

[0011] The total sialic acid content of FSH and rFSH is not directly comparable since sialic acids are commonly linked in two ways. Pituitary/ serum/ urinary FSH contain both α2,3 and α2,6-linked sialic acid, with a predominance of the former. However, CHO cell derived recombinants only contain α2,3 (Kagawa *et al,* 1988, Takeuchi *et al,* 1988, Svensson *et al.,* 1990). This is another difference between natural and current recombinant products in addition to the lower overall sialic acid content of the latter.

[0012] CHO cells are commonly used for the production of pharmaceutical human recombinant proteins. Structural analysis has identified that sialic acid is exclusively attached by a α2,3-linkage. (Kagawa *et al,* 1988, Takeuchi *et al,* 1988, Svensson *et al.,* 1990). Many human glycoproteins contain a mixture of both α2,3- and α2,6-linkages. Therefore recombinant proteins expressed using the CHO system will differ from their natural counterparts in their type of terminal sialic acid linkages. This is an important consideration in the production of biologicals for pharmaceutical use since the carbohydrate moieties may contribute to the pharmacological attributes of the molecule.

[0013] It is desirable to have a rFSH product that more closely replicates or mimics the physiochemical and pharmacokinetic profile of the product produced from human urine. It is desirable to have a rFSH product that has improved pharmacokinetic property or properties compared to the known recombinant product.

[0014] According to the present invention there is provided recombinant FSH ("rFSH" or "recFSH") including α2, 3 sialylation and α2, 6 sialylation wherein 80% or more of the total sialylation is α2,3-sialylation and wherein from 5% to 20% of the the total sialylation is α2,6-sialylation. The rFSH (or rFSH preparation) of the invention may have 5% or less of the total sialylation being α2,8-sialylation, for example 0.1-4% of the total sialylation may be α2,8- sialylation.

[0015] The applicants have found that the type of sialic acid linkage, α2,3- or α2,6-, can have a dramatic influence on biological clearance of FSH. Human cell lines, as opposed to CHO cell lines, can express recombinant FSH with sialic acids attached by both α2,3 and α2,6 linkages. In Example 4 a recombinant FSH cell line was made which expressed FSH containing glycans with low levels of both α2,3- and α2,6-linked sialic acid (Figure 6). This basic material, with limited sialic acid content (Figure 4) was cleared very quickly from the circulation in rat as would be predicted (Figure 7). The cell line was then subjected to a second engineering step with the addition of the gene encoding for the α2,6-sialyltransferase (Example 5). The resulting rFSH was highly sialylated showing sialic acid content and pI distribution comparable with urinary FSH (Figure 5). However, the material was cleared very rapidly from circulation of rats at a rate comparable to the original material which had low sialic acid content (Figure 8). This was an unexpected observation since it is known that a proportion of sialic acid on natural and biologically active FSH is α2,6-linked. The clearance of the α2,6-sialylated rFSH was found to be mediated by the asialoglycoprotein (ASGP) receptor found in the liver (Example 9). This was demonstrated by transient blockade of the ASGP receptors using an excess of another substrate for the receptor. With the receptor blocked by asialofetuin, the expected clearance for the highly-sialylated material was restored (Figure 9). This was maintained for several hours until the blockade was overcome and the α2,6 linked highly sialylated rFSH resumed its rapid clearance.

[0016] Recombinant FSH with a mixture of both α2,3 and α2,6-linked sialic acid was made by engineering a human cell line to express both rFSH and α2,3 sialyltransferase (Example 4 and 5). The expressed product is highly acidic and carries a mix of both α2,3- and α2,6-linked sialic acids; the latter provided by the endogenous sialyl transferase activity (Figure 6). This has two advantages over rFSH expressed in conventional CHO cells: first the material is more highly sialylated due to the combined activities of the two sialyltransferases; and secondly the material more closely resembles the natural FSH. This is likely to be more biologically appropriate compared to CHO cell derived recombinant products that have produce only α2,3 linked sialic acid (Kagawa *et al,* 1988, Takeuchi *et al,* 1988, Svensson *et al.,* 1990) and have decreased sialic acid content (Ulloa-Aguirre *et al.* 1995., Andersen *et al.* 2004).

[0017] The applicants have surprisingly found that rFSH of the invention may more closely replicate or mimic the physiochemical and pharmacokinetic profile of the natural human urinary product than other recombinant products. In other words, rFSH of the invention may be closer to the "natural" FSH. This may have significant advantages regarding dosing etc. Further, a more "natural" or more "human" product may be more desirable to the patient, who may desire therapy, although in a sense artificial, to be as "natural" as possible. There may be other advantages (e.g. pharmacokinetic advantages) in a recombinant product having carbohydrate (e.g. glycan) structure which is closer to natural (e.g. human urinary) FSH than other recombinant products.

[0018] The invention is thus a recombinant version of FSH which carries a mix of α2,3 and α2,6 sialic acid and therefore more closely resembles natural FSH. It is expected that the use of this compound for controlled ovarian stimulation, in IVF techniques, and ovulation induction will result in a more natural stimulation of the ovary compared to existing recombinant products.

[0019] According to the present invention there is provided recombinant FSH ("rFSH" or "recFSH") including α2,3-

and a2,6-sialylation wherein 80% or more of the total sialylation is α2,3-sialylation and wherein from 5% to 20% of the the total sialylation is α2,6-sialylation. The rFSH or rFSH preparation may optionally further include α2, 8 sialylation.

[0020] In embodiments of the invention, the rFSH may be present as a single isoform or as a mixture of isoforms.

[0021] The rFSH according to the invention may have a sialic acid content [expressed in terms of a ratio of moles of sialic acid to moles of protein] of 6 mol/mol or greater.

[0022] The rFSH according to the invention has 80% or more of the total sialylation being α2,3-sialylation. For example, 90% or more of the total sialylation may be α2,3-sialylation. The rFSH of the invention has 5 to 20% of the total sialylation being α2,6-sialylation. The rFSH of the invention may have 5% or less of the total sialylation being α2,8-sialylation. For example 2.5% or less of the total sialylation may be α2,8- sialylation. The rFSH (or rFSH preparation) may include α2,8-sialylation in an amount which is from 0.1 to 4% of the total sialylation, for example from 0.5 to 3% of the total sialylation, for example from 0.5 to 2.5% of the total sialylation. By sialylation it is meant the amount of sialic residues present on the FSH carbohydrate structures. α2,3-sialylation means sialylation at the 2,3 position (as is well known in the art) and α2,6 sialylation at the 2,6 position (also well known in the art). Thus "% of the total sialylation may be α 2,3 sialylation" refers to the % of the total number of sialic acid residues present in the FSH which are sialylated in the 2,3 position. The term "% of the total sialylation being α2,6-sialylation" refers to the % of the total number of sialic acid residues present in the FSH which are sialylated in the 2,6 position.

[0023] Recombinant FSH expressed in Chinese hamster ovary (CHO) cells includes exclusively α 2, 3 sialylation (Kagawa *et al,* 1988, Takeuchi *et al.* 1988, Svensson *et al.* 1990).

[0024] The rFSH of the invention may be produced or expressed in a human cell line. This may simplify (and render more efficient) the production method because manipulation and control of e.g. 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 rFSH produced than in production of known rFSH products; more acidic rFSH is produced and separation/removal of basic FSH is less problematic. The rFSH may be produced or expressed in a Per.C6 cell line, a Per.C6 derived cell line or a modified Per.C6 cell line. The cell line may be modified using α2,3-sialyltransferase. The cell line may be modified using α2,6-sialyltransferase. Alternatively or additionally, the rFSH may include α2,6-linked sialic acids (α2,6 sialylation) provided by endogenous sialyl transferase activity [of the cell line].

[0025] The rFSH may be produced using α2,3- and/or α2,6-sialyltransferase. The rFSH may be produced using α2,3-sialyltransferase. The rFSH may include α2,6-linked sialic acids (α2,6 sialylation) provided by endogenous sialyl transferase activity.

[0026] According to the present invention in a further aspect there is provided a method of production of rFSH and/or an rFSH preparation as described herein (according to aspects of the invention) comprising the step of producing or expressing the rFSH in a human cell line, for example a Per.C6 cell line, a Per.C6 derived cell line or a modified Per.C6 cell line, for example a cell line which has been modified using α2,3-sialyltransferase.

[0027] The rFSH 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 rFSH of the invention may have glycans with sialylation presence on mono-antennary and/or di-antennary and/or tri-antennary and/or tetra-antennary structures.

[0028] According to the present invention in a further aspect there is provided a pharmaceutical composition comprising rFSH including α2,3- and a2,6-sialylation wherein 80% or more of the total sialylation is α2,3-sialylation and wherein from 5% to 20% of the the total sialylation is a2,6-sialylation (e.g. as set out above). The pharmaceutical composition may further comprise hCG and/or LH.

[0029] hCG can be obtained by any means known in the art. hCG as used herein includes human-derived and recombinant hCG. Human-derived hCG can be purified from any appropriate source (e.g. urine, and placenta) by any method known in the art. Methods of expressing and purifying recombinant hCG are well known in the art.

[0030] 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. Methods of expressing and purifying recombinant LH are known in the art.

[0031] The pharmaceutical composition may be for 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 used, for example, in medical indications where known FSH preparations are used. The pharmaceutical compositions of the present invention can be formulated into well-known compositions for any route of drug administration, e.g. oral, rectal, parenteral, transdermal (e.g. patch technology), intravenous, intramuscular, subcutaneous, intrasusternal, 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 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.

[0032] Examples of suitable aqueous and non-aqueous pharmaceutical carriers, diluents, solvents or vehicles include water, ethanol, 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.

[0033] The compositions of the present invention also can contain additives such as but not limited to preservatives, wetting agents, emulsifying agents, and dispersing agents. Antibacterial and antifungal agents can be included to prevent growth of microbes and includes, for example, 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.

[0034] In some cases, to effect prolonged action it is desirable to slow the absorption of FSH (and other active ingredients, if present) from subcutaneous or intramuscular injection. This can be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of FSH 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 FSH combination form is accomplished by dissolving or suspending the FSH combination in an oil vehicle.

[0035] Injectable depot forms can be made by forming microencapsule matrices of the FSH (and other agents, if present) in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of FSH to polymer and the nature of the particular polymer employed, the rate of FSH release can be controlled. Examples of other biodegradable polymers include polyvinylpyrrolidone, poly(orthoesters), poly(anhydrides) etc. Depot injectable formulations are also prepared by entrapping the FSH in liposomes or microemulsions which are compatible with body tissues.

[0036] 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 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.

[0037] Injectable formulations can be supplied as a product having pharmaceutical compositions containing FSH (optionally with hCG, LH etc.) If there is more than one active ingredient (i.e. FSH and e.g. hCG 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 containing either FSH, hCG, or a combination of both FSH and hCG, the syringes packaged in a blister package or other means to maintain sterility. A product can optionally contain instructions for using the FSH and hCG formulations.

[0038] The pH and exact concentration of the various components of the pharmaceutical composition are adjusted in accordance with routine practice in this field. See GOODMAN and GILMAN's THE PHARMACOLOGICAL BASIS FOR THERAPEUTICES, 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 especially preferred embodiment, the parenteral compositions are supplied in dosage unit form for ease of administration and uniformity of dosage.

Detailed description of the invention

[0039] 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 a plasmid map of the pFSHalpha/beta expression vector;
Figure 2 shows the α2,3-sialyltransferase (ST3GAL4) expression vector;
Figure 3 shows the α2,6-sialyltransferase (ST6GAL1) expression vector;
Figure 4 shows Isoelectric focussing of recombinant FSH produced by Per.C6 cells stably expressing FSH;
Figure 5 shows example of clones analysed by isoelectric focussing of recombinant FSH produced by Per.C6 cells stably expressing FSH after engineering with α2,3- or α2,6-sialyltransferase;
Figure 6 shows analysis of the Sialic acid linkages of Per.C6 FSH;
Figure 7 shows metabolic clearance rates (MCRs) of Per.C6 FSH samples;
Figure 8 shows MCRs of α2,6-sialytransferase engineered Per.C6 FSH samples;
Figure 9 shows MCRs of α2,6-sialytransferase engineered Per.C6 FSH samples;
Figure 10 shows MCRs of α2,3-sialytransferase engineered Per.C6 FSH samples;
Figure 11 shows ovarian weight augmentation by Per.C6 rFSH clones of parental Per.C6 rFSH, according to the method of Steelman and Pohley (1953);
Figure 12 shows ovarian weight augmentation by Per.C6 rFSH clones of engineered (α2,6-sialyltransferase) Per.C6 rFSH; and
Figure 13 shows ovarian weight augmentation by Per.C6 rFSH clones of engineered (α2,3-sialyltransferase) Per.C6 rFSH.

Sequence Selection

Human FSH

[0040] The coding region of the gene for the FSH alpha polypeptide was used to according to Fiddes and Goodman. (1981). The sequence is banked as AH007338 and at the time of construction there were no other variants of this protein sequence. The sequence is referred herein as SEQ ID 1.

[0041] The coding region of the gene for FSH beta polypeptide was used according to Keene *et al* (1989). The sequence is banked as MM_000510 and at the time of construction there were no other variants of this protein sequence. The sequence is referred herein as SEQ ID 2

Sialyltransferase

[0042] α2,3-Sialyltransferase - The coding region of the gene for beta-galactoside alpha-2,3-sialyltransferase 4 (α2,3-sialyltransferase, ST3GAL4) was used according to Kitagawa and Paulson (1994). The sequence is banked as L23767 and referred herein as SEQ ID 3.

[0043] α2,6-Sialyltransferase - The coding region of the gene for beta-galactosamide alpha-2,6-sialyltransferase 1 (α2,6-sialyltransferase, ST6GAL1) was used according to Grundmann *et al.* (1990). The sequence is banked as NM_003032 and referred herein as SEQ ID 4.

EXAMPLES

Example 1 Construction of the FSH expression vector

[0044] The coding sequence of FSH alpha polypeptide (AH007338, SEQ ID 1) and FSH beta polypeptide (NM_003032, SEQ ID 2) were amplified by PCR using the primer combinations FSHa-fw and FSHa-rev and FSHb-fw and FSHb-rec respectively.

FSHa-fw 5'-CCAGGATCCGCCACCATGGATTACTACAGAAAAATATGC-3'
FSHa-rev 5'-GGATGGCTAGCTTAAGATTTGTGATAATAAC-3'
FSHb-fw 5'-CCAGGCGCGCCACCATGAAGACACTCCAGTTTTTC-3'
FSHb-rev 5'-CCGGGTTAACTTATTATTCTTTCATTTCACCAAAGG-3'

[0045] The resulting amplified FSH beta DNA was digested with the restriction enzymes *Asc*I and *Hpa*I and inserted into the *Asc*I and *Hpa*I sites on the CMV driven mammalian expression vector carrying a neomycin selection marker. Similarly the FSH alpha DNA was digested with *Bam*HI and *Nhe*I and inserted into the sites BamHI and *Nhe*I on the expression vector already containing the FSH beta polypeptide DNA.

[0046] The vector DNA was used to transform the DH5α strain of *E.coli.* Sixty colonies were picked for amplification and fifty seven contained the vector containing both FSH alpha and beta. Twenty of these were selected for sequencing and all contained the correct sequences according to SEQ ID 1 and SEQ ID 2. Plasmid pFSH A+B#17 was selected for transfection (Figure 1).

Example 2 Construction of the ST3 expression vector

[0047] The coding sequence of beta-galactoside alpha-2,3-sialyltransferase 4 (ST3, L23767, SEQ ID 3) was amplified by PCR using the primer combination 2,3STfw and 2,3STrev.

2,3STfw 5'-CCAGGATCCGCCACCATGTGTCCTGCAGGCTGGAAGC-3'
2,3STrev 5'-TTTTTTTCTTAAGTCAGAAGGACGTGAGGTTCTTG-3'

[0048] The resulting amplified ST3 DNA was digested with the restriction enzymes *Bam*HI and *Afl*II and inserted into the *Bam*HI and *Afl*II sites on the CMV driven mammalian expression vector carrying a hygromycin resistance marker. The vector was amplified as previously described and sequenced. Clone pST3#1 (Figure 2) contained the correct sequence according SEQ ID 3 and was selected for transfection.

Example 3 Construction of the ST6 expression vector

[0049] The coding sequence of beta-galactosamide alpha-2,6-sialyltransferase 1 (ST6, NM_003032, SEQ ID 4) was amplified by PCR using the primer combination 2,6STfw and 2,6STrev.

2,6STfw 5'-CCAGGATCCGCCACCATGATTCACACCAACCTGAAG-3'
2,6STrev 5'-TTTTTTTCTTAAGTTAGCAGTGAATGGTCCGG-3'

[0050] The resulting amplified ST6 DNA was digested with the restriction enzymes *Bam*HI and *Afl*II and inserted into the *Bam*HI and *Afl*II sites on the CMV driven mammalian expression vector carrying a hygromycin resistance marker. The vector was amplified as previously described and sequenced. Clone pST6#11 (Figure 3) contained the correct sequence according SEQ ID 4 and was selected for transfection.

Example 4 Stable expression of pFSH A+B in PER.C6 cells. Transfection isolation and screening of clones.

[0051] Per.C6 clones producing FSH were generated by expressing both polypeptide chains of FSH from a single plasmid (see Example 1).

[0052] To obtain stable clones a liposome based transfection agent with the pFSH A+B construct. Stable clones were selected in VPRO supplemented with 10% FCS and containing G418. Three weeks after transfection G418 resistant clones grew out. A total of 250 clones were selected for isolation. The isolated clones were cultured in selection medium until 70-80% confluent. Supernatants were assayed for FSH protein content using an FSH selective ELISA and pharmacological activity at the FSH receptor in cloned cell line, using a cAMP accumulation assay. Clones (98) expressing functional protein were progressed for culture expansion to 24 well, 6 well and T80 flasks.

[0053] Studies to determine productivity and quality of the material from seven clones were initiated in T80 flasks to generate sufficient material. Cells were cultured in supplemented media as previously described for 7 days and the supernatant harvested. Productivity was determined using the FSH selective ELISA. The isoelectric profile of the material was determined (Example 6). Representative samples are shown in Figure 4. The information from the IEF was used to select clones for metabolic clearance rate analysis (Example 9). Clones (005, 104, 179, 223, 144) with sufficient productivity and quality were selected for sialyltransferase engineering.

Example 5 Level of sialylation is increased in cells that over express α2,3- or α2,6-sialyltransferase. Stable expression of pST3 or pST6 in FSH expressing PER.C6 cells; Transfection isolation and screening of clones.

[0054] Per.C6 clones producing highly sialylated FSH were generated by expressing α2,3 sialyltransferase or α2,6 sialyltransferase from separate plasmids (see Examples 2 and 3) in Per.C6 cells already expressing both polypeptide chains of FSH (see Example 4). Four clones produced from PER.C6® cells as set out in Example 4 were selected for their characteristics including productivity, good growth profile, production of functional protein, and produced FSH which included some sialylation.

[0055] Stable clones were generated as previously described in Example 4. A total of 202 clones from the α2,3-sialyltransferase program and 210 clones from the α2,6-sialyltransferase program were isolated, expanded and assayed. The final clone number for the α2,3- study was 12 and 30 for the α2,6- study.

[0056] The α2,3-sialyltransferase clones were adapted to serum free media and suspension conditions.

[0057] As before clones were assayed using a FSH selective ELISA, functional response in an FSH receptor cell line, IEF (Example 6), metabolic clearance rate (Example 9) and Steelman Pohley analysis (Example 10). Results were compared to a commercially available recombinant FSH (Gonal-f, Serono) and the parental FSH Per.C6 cell lines. Representative samples are shown in Figure 5. Some clones did not demonstrate an increase in sialylation but it can be seen that FSH produced by most of the clones has significantly improved sialylation (i.e. on average more FSH isoforms with high numbers of sialic acids) compared to FSH expressed without α2,3- or α2,6- sialyltransferase.

[0058] In conclusion expression of FSH together with sialyltransferase in Per.C6 cells results in increased levels of sialylated FSH compared to cells expressing FSH only.

Example 6 Analysis of the pi of Per.C6 produced FSH isoforms by isoelectric focussing.

[0059] 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 the molecules migrate.

[0060] 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 FSH isoforms subtly alters the pI point for each isoform, which can be exploited using this technique to visualise the Per.C6 FSH isoforms from each clone.

[0061] The isoelectric points of the Per.C6 produced FSH isoforms in cell culture supernatants were analyzed using isoelectric focussing. Cell culture media from Per.C6 FSH clones was produced as described in Example 4 and 5.

[0062] Per.C6 FSH samples were separated on Novex® IEF Gels containing 5% polyacrylamide under native conditions on a pH 3.0 -7.0 gradient in an ampholyte solution pH 3.0 - 7.0.

[0063] Proteins were transferred onto supported nitrocellulose and visualised using a primary anti-FSHα monoclonal antibody, secondary anti-mouse IgG alkaline phosphatase conjugated antibody and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) reagent to visualise the bands.

[0064] As indicated in Figures 4 and 5, the bands represent isoforms of FSH containing different numbers of sialic acid molecules.

[0065] Using this method clones producing FSH isoforms with a higher number of sialic acid molecules were identified. Engineering with α2,3- or α2,6- sialyltransferase resulted in clones with more sialic acid and a lower pI.

Example 7 Analysis of the Sialic acid linkages of Per.C6 FSH

[0066] Glycoconjugates were analyzed using a lectin based glycan differentiation method. With this method glycoproteins and glycoconjuagtes bound to nitrocellulose can be characterized. Lectins selectively recognize a particular moiety, for example α2,3 linked sialic acid. The lectins applied are conjugated with the steroid hapten digoxigenin which enables immunological detection of the bound lectins.

[0067] Purified Per.C6 FSH from a parental clone (no additional sialyltransferase), a α2,3-sialyltransferase engineered clone and a α2,6-sialyltransferase engineered clone were separated using standard SDS-PAGE techniques. A commercially available recombinant FSH (Gonal-f, Serono) was used as a standard.

[0068] Sialic acid was analyzed using the DIG Glycan Differentiation Kit (Cat. No. 11 210 238 001, Roche) according to the manufacturers instructions. Positive reactions with Sambucus nigra agglutinin (SNA) indicated terminally linked (2-6) sialic acid. Positive reactions with Maackia amurensis agglutinin II (MAA): indicated terminally linked (α2-3) sialic acid

[0069] In summary the parental clone 005 contained low levels of both α2,3- and α2,6- sialic acid. The clones engineered with α2,3-sialyltransferase contained high levels of α2,3- sialic acid linkages and low levels of α2,6- sialic acid linkages. Clones engineered with α2,6-sialyltransferase contained high levels of α2,6- sialic acid linkages and low levels of α2,3-sialic acid linkages. The standard control Gonal-f only contains α2,3- sialic acid linkages. This is consistent with what is known about recombinant proteins produced in Chinese Hamster ovary (CHO) cells (Kagawa *et al,* 1988, Takeuchi *et al,* 1988, Svensson *et al.,* 1990).

[0070] In conclusion, engineering of Per.C6 FSH cells with α2,3- or α2,6-sialyltransferase successfully increased the number of sialic acid molecules conjugated to the FSH in the sample.

Example 8a Quantification of total Sialic acid

[0071] 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.

[0072] The total sialic acid on purified rFSH (Example 11) was measured using an enzymatic sialic acid quantification kit according to the manufacturers protocol (Sigma, Sialic-Q). In short N-acetylneuraminic acid aldolase catalyses sialic acid to N-acetylmannoasine and pyruvic acid. The pyruvic acid can be reduced to lactic acid by β-NADH and lactic dehydrogenase. B-NADH oxidation can be accurately measured spectrophotometrically.

[0073] Protein concentration was measured in microtiter plates using a commercial bicinchoninic acid (BCA) assay kit (Sigma, B 9643) based on the Lowry method (Lowry *et al,* 1951).

[0074] The total sialic acid content of Per.C6 FSH was measured and found to be greater than 6 mol/mol.

Example 8b Quantification of relative amounts of α2,3, α2,6 and α2,8 sialic acid

[0075] The relative percentage amounts of α2,3, α2,6 and α2,8 sialic acid on purified rFSH (Example 11) were measured using known techniques.

[0076] Each sample of rFSH was immobilized (gel block), washed, reduced, alkylated and digested with PNGase F overnight. The N-glycans were then extracted and processed. N-glycans for NP-HPLC and WAX-HPLC analysis were labelled with the fluorophore 2AB as detailed in Royle et al. The N-glycans were run on normal phase (NP) HPLC on a TSK amide column (as detailed in Royle et al) with retention times expressed in glucose units (GU).

[0077] Samples of the extracted, pooled, glycans (extracted as above) were digested with different sialidases to determine the linkages. NAN 1 (recombinant sialidase) releases α2,3 linked non-reducing terminal sialic acids (NeuNAc and NeuNGc), ABS *(Arthrobacter ureafaciens sialidase*) releases α2,3, α2,6 and α2,8 linked non-reducing terminal sialic acids (NeuNAc and NeuNGc). Samples were analysed by NP-HPLC, to allow comparison of the undigested sample with that digested with NAN1 and that digested with ABS. Comparison of the three NP-HPLC traces (undigested, NAN1 digested, ABS digested) shows that digestion with ABS and NAN1 give different results. This indicates that the samples have sialic acids with α2,3, α2,6 and α2,8 linkages. The relative percentages were calculated from structures present in the undigested glycan pools and were found to be in the ranges 65% - 85% (e.g. 77.75%) for α2,3 sialylation; 15 to 35% (e.g. 21.46%) for α2,6 sialylation; and 0.1 to 3% for α2,8 sialylation.

Example 8c Quantification of relative amounts mono, di, tri and tetra antennary sialylated structures

[0078] The relative percentage amounts of mono, di, tri and tetra sialylated structures on glycans extracted from purified rFSH (Example 11) were measured using known techniques.

[0079] Each sample of rFSH was immobilized (gel block), washed, reduced, alkylated and digested with PNGase F overnight. The N-glycans were then extracted and processed. N-glycans for NP-HPLC and WAX-HPLC analysis were labelled with the fluorophore 2AB as detailed in Royle et al.

[0080] Weak anion exchange (WAX) HPLC to separate the N-glycans by charge (Example 8b) was carried out as set out in Royle et al, with a Fetuin N-glycan standard as reference. Glycans were eluted according to the number of sialic acids they contained. All samples included mono (1 S), di(2S), tri(3S) and tetra(4S) sialylated structures. The relative amounts of sialylated structures were found to be in the following ratios (1 S:2S:4S:4S): 9-15%: 27-30%: 30-36%: 25-29 % (for example 10.24:28.65:35.49:25.62).

Example 9 Determination of the metabolic clearance rates of rFSH

[0081] To determine the metabolic clearance rate (MCR) of Per.C6 FSH samples conscious female rats (3 animals per clone) were injected into the tail vein at time zero with a bolus of rFSH (1 - 10 μg/rat, based on ELISA quantification of samples, DRG EIA 1288). Blood samples (400 μl) were taken from the tip of the tail at 1, 2, 4, 8, 12, 24 and 32 hours after test sample injection. Serum was collected by centrifugation and assayed for FSH content by ELISA (DRG EIA 1288).

[0082] The asialoglycoprotein receptor (ASGP-R) recognizes desialyated (galactose-terminated) glycoproteins such as asialofetuin (ASF). (Pricer and Ashwell, 1971. Van Lenten and Ashwell, 1972). The ASGP receptor and the bound desialyated glycoprotein are internalized into the cell where the receptor is recycled and the ligand is degraded (Regoeczi *et al,* 1978, Steer and Ashwell, 1980).

[0083] To investigate if Per.C6 FSH material was cleared via this mechanism, the ASGP-R was saturated with asialofetuin. The metabolic clearance rate of parental, α2,6 or α2,3-sialyltransferase engineered material was determined as described with co administration of a minimum 7500-fold molar excess of asialofetuin to saturate the ASGP-R for 1-2 h.

[0084] The material produced by the parental Per.C6 FSH clones contained some longer MCR material but a high percentage was cleared quickly (Figure 7). The lead clone 005 which contained the most sialylated material was engineered using α2,6- or α2,3-sialyltransferase (Example 5). Although the clones engineered with α2,6-sialyltransferase demonstrated increased sialylation (Figure 5) there was no improvement in the MCR (Figure 7). Blockade of the ASGR restored the MCR of the α2,6 material to that of the standard demonstrating that even with increased α2,6 linkages the material is cleared quickly (Figure 8). Engineering with α2,3-sialyltransferase resulted in clones with comparable MCR to the standard (Figure 9) and varying sialic content was consistent with what is known for the isoforms of FSH (Figure 10).

Example 10 Steelman-Pohley *in vivo* assay

[0085] To demonstrate increasing sialic acid content on FSH results in an increased biological effect, the increase in ovarian weights in rats by highly sialylated FSH such as produced in Example 5 was examined.

[0086] The increase in ovarian weights due to the Per.C6 rFSH clones were analysed according to the method of Steelman and Pohley (1953). Per.C6 rFSH from filtered cell media samples was quantified by ELISA (DRG,EIA-1288). The samples (Per.C6 rFSH) and standards (Gonal-f rFSH) were tested at five different doses (3 animals/dose). Gonal-f was dosed at 50, 100, 200, 400, and 800 ng/rat, The sample doses were calculated using their AUC values relative to Gonal-f, typically 0.05 - 10 μg/rat.

[0087] In conclusion, the undersialylated material produced by the parental Per.C6 FSH clones (Figure 11) was not as potent in the ovarian weight augmentation assay as the commercially available rFSH. Sialyltransferase engineering to add additional α2,6- linkages increased the sialic acid content but did not improve potency in the *in vivo* assay (Figure 12). However, additional α2,3- linkages significantly improved potency (Figure 13) and the two recombinant FSH preparations (Per.C6 and CHO-derived) display very similar profiles in this assay.

Example 11 Production and purification overview

[0088] A procedure was developed to produce FSH in PER.C6 cells that were cultured in suspension in serum free medium. The procedure is described below and was applied to several FSH-producing PER.C6 cell lines.

[0089] FSH from the parental clone 005, α2,3- clone 007 and α2,6 clone 059 was prepared using a using a modification of the method described by Lowry *et al.* (1976).

[0090] For the production of PER.C6-FSH, the cell lines were adapted to a serum- free medium, i.e., Excell 525 (JRH Biosciences). The cells were first cultured to form a 70%-90% confluent monolayer in a T80 culture flask. On passage the cells were re-suspended in the serum free medium, Excell 525 + 4 mM L-Glutamine, to a cell density of 0.3×10^6 cells/ml. A 25 ml cell suspension was put in a 250 ml shaker flask and shaken at 100 rpm at 37°C at 5% CO_2. After reaching a cell density of > 1×10^6 cells/ml, the cells were sub-cultured to a cell density of 0.2 or 0.3×10^6 cells/ml and further cultured in shaker flasks at 37°C, 5% CO_2 and 100 rpm.

[0091] For the production of FSH, the cells were transferred to a serum- free production medium, i.e., VPRO (JRH Biosciences), which supports the growth of PER.C6 cells to very high cell densities (usually > 10^7 cells/ml in a batch culture). The cells were first cultured to > 1×10^6 cells/ml in Excell 525, then spun down for 5 min at 1000 rpm and subsequently suspended in VPRO medium + 6 mM L-glutamine to a density of 1×10^6 cells/ml. The cells were then cultured in a shaker flask for 7-10 days at 37°C, 5% CO2 and 100 rpm. During this period, the cells grew to a density of > 10^7 cells/ml. The culture medium was harvested after the cell viability started to decline. The cells were spun down for 5 min at 1000 rpm and the supernatant was used for the quantification and purification of FSH. The concentration of FSH was determined using ELISA (DRG EIA 1288).

[0092] Thereafter, purification of FSH was carried out using a modification of the method described by Lowry *et al.* (1976). This was achieved by chromatography on DEAE cellulose, gel filtration on Sephadex G100, adsorption chromatography on hydroxyapatite, and preparative polyacrylamide electrophoresis.

[0093] During all chromatographic procedures, the presence of immunoreactive FSH was confirmed by RIA (DRG EIA 1288) and IEF (Example 6).

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[0094]

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SEQ ID 1
Follicle stimulating hormone alpha polypeptide
Accession number AH007338
Nucleotide sequence of FSH alpha

1 ATGGATTACT ACAGAAAATA TGCAGCTATC TTTCTGGTCA CATTGTCGGT GTTTCTGCAT
61 GTTCTCCATT CCGCTCCTGA TGTGCAGGAT TGCCCAGAAT GCACGCTACA GGAAAACCCA
121 TTCTTCTCCC AGCCGGGTGC CCCAATACTT CAGTGCATGG GCTGCTGCTT CTCTAGAGCA
181 TATCCCACTC CACTAAGGTC CAAGAAGACG ATGTTGGTCC AAAAGAACGT CACCTCAGAG
241 TCCACTTGCT GTGTAGCTAA ATCATATAAC AGGGTCACAG TAATGGGGGG TTTCAAAGTG
301 GAGAACCACA CGGCGTGCCA CTGCAGTACT TGTTATTATC ACAAATCTTA A

Protein sequence of FSH alpha

1 MKTLQFFFLF CCWKAICCNS CELTNITIAI EKEECRFCIS INTTWCAGYC YTRDLVYKDP
61 ARPKIQKTCT FKELVYETVR VPGCAHHADS LYTYPVATQC HCGKCDSDST DCTVRGLGPS
121 YCSFGEMKE

SEQ ID 2
Follicle stimulating hormone beta polypeptide
Accession number NM_000510
Nucleotide sequence of FSH beta

1 ATGAAGACAC TCCAGTTTTT CTTCCTTTTC TGTTGCTGGA AAGCAATCTG CTGCAATAGC
61 TGTGAGCTGA CCAACATCAC CATTGCAATA GAGAAAGAAG AATGTCGTTT CTGCATAAGC

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241 GTGCCCGGCT GTGCTCACCA TGCAGATTCC TTGTATACAT ACCCAGTGGC CACCCAGTGT

301 CACTGTGGCA AGTGTGACAG CGACAGCACT GATTGTACTG TGCGAGGCCT GGGGCCCAGC

361 TACTGCTCCT TTGGTGAAAT GAAAGAATAA

Protein sequence of FSH beta

1 MKTLQFFFLF CCWKAICCNS CELTNITIAI EKEECRFCIS INTTWCAGYC YTRDLVYKDP

61 ARPKIQKTCT FKELVYETVR VPGCAHHADS LYTYPVATQC HCGKCDSDST DCTVRGLGPS

121 YCSFGEMKE

SEQ ID 3
Beta-galactoside alpha-2,3-sialyltransferase 4
Accession Number L23767
Nucleotide sequence of ST3GAL4

1 ATGTGTCCTG CAGGCTGGAA GCTCCTGGCC ATGTTGGCTC TGGTCCTGGT CGTCATGGTG

61 TGGTATTCCA TCTCCCGGGA AGACAGGTAC ATCGAGCTTT TTTATTTTCC CATCCCAGAG

121 AAGAAGGAGC CGTGCCTCCA GGGTGAGGCA GAGAGCAAGG CCTCTAAGCT CTTTGGCAAC

181 TACTCCCGGG ATCAGCCCAT CTTCCTGCGG CTTGAGGATT ATTTCTGGGT CAAGACGCCA

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301 GCCATCACCA GCTCCTCCAT CCCCAAGAAC ATCCAGAGCC TCAGGTGCCG CCGCTGTGTG

361 GTCGTGGGGA ACGGGCACCG GCTGCGGAAC AGCTCACTGG GAGATGCCAT CAACAAGTAC

421 GATGTGGTCA TCAGATTGAA CAATGCCCCA GTGGCTGGCT ATGAGGGTGA CGTGGGCTCC

481 AAGACCACCA TGCGTCTCTT CTACCCTGAA TCTGCCCACT TCGACCCCAA AGTAGAAAAC

541 AACCCAGACA 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

781 GGCCTGTTGG CCATCACGCT GGCCCTCCAC CTCTGTGACT TGGTGCACAT TGCCGGCTTT

841 GGCTACCCAG ACGCCTACAA CAAGAAGCAG ACCATTCACT ACTATGAGCA GATCACGCTC

901 AAGTCCATGG CGGGGTCAGG CCATAATGTC TCCCAAGAGG CCCTGGCCAT TAAGCGGATG

961 CTGGAGATGG GAGCTATCAA GAACCTCACG TCCTTCTGA

Protein Sequence of ST3GAL4

1 MCPAGWKLLA MLALVLVVMV WYSISREDRY IELFYFPIPE KKEPCLQGEA ESKASKLFGN

61 YSRDQPIFLR LEDYFWVKTP SAYELPYGTK GSEDLLLRVL AITSSSIPKN IQSLRCRRCV

121 VVGNGHRLRN SSLGDAINKY DVVIRLNNAP VAGYEGDVGS KTTMRLFYPE SAHFDPKVEN

181 NPDTLLVLVA FKAMDFHWIE TILSDKKRVR KGFWKQPPLI WDVNPKQIRI LNPFFMEIAA

241 DKLLSLPMQQ PRKIKQKPTT GLLAITLALH LCDLVHIAGF GYPDAYNKKQ TIHYYEQITL

301 KSMAGSGHNV SQEALAIKRM LEMGAIKNLT SF

SEQ ID 4

Beta-galactosamide alpha-2,6-sialyltransferase 1

Accession number NM_003032
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1 ATGATTCACA CCAACCTGAA GAAAAAGTTC AGCTGCTGCG TCCTGGTCTT TCTTCTGTTT
61 GCAGTCATCT GTGTGTGGAA GGAAAAGAAG AAAGGGAGTT ACTATGATTC CTTTAAATTG
121 CAAACCAAGG AATTCCAGGT GTTAAAGAGT CTGGGGAAAT TGGCCATGGG GTCTGATTCC
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241 CTCAGAGGCC TAGCCAAGGC CAAACCAGAG GCCTCCTTCC AGGTGTGGAA CAAGGACAGC
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361 AAGTACAAAG TGTCCTACAA GGGGCCAGGA CCAGGCATCA AGTTCAGTGC AGAGGCCCTG
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1201 TTCCGGACCA TTCACTGCTA A

0p-
Protein Sequence of ST6GAL1

1 MIHTNLKKKF SCCVLVFLLF AVICVWKEKK KGSYYDSFKL QTKEFQVLKS LGKLAMGSDS
61 QSVSSSSTQD PHRGRQTLGS LRGLAKAKPE ASFQVWNKDS SSKNLIPRLQ KIWKNYLSMN
121 KYKVSYKGPG PGIKFSAEAL RCHLRDHVNV SMVEVTDFPF NTSEWEGYLP KESIRTKAGP
181 WGRCAVVSSA GSLKSSQLGR EIDDHDAVLR FNGAPTANFQ QDVGTKTTIR LMNSQLVTTE
241 KRFLKDSLYN EGILIVWDPS VYHSDIPKWY QNPDYNFFNN YKTYRKLHPN QPFYILKPQM
301 PWELWDILQE ISPEEIQPNP PSSGMLGIII MMTLCDQVDI YEFLPSKRKT DVCYYYQKFF
361 DSACTMGAYH PLLYEKNLVK HLNQGTDEDI YLLGKATLPG FRTIHC

[0095] There have been disclosed hereinbefore the recombinant FSHs, recombinant FSH preparations, pharmaceutical compositions, uses and methods defined by the following numbered paragraphs:

1. Recombinant FSH (rFSH) including α2,3- and α2,6-sialylation.

2. Recombinant FSH according to paragraph 1 having a sialic acid content [expressed in terms of a ratio of moles of sialic acid to moles of protein] of 6 mol/mol or greater.

3. Recombinant FSH according to paragraph 1 or paragraph 2 having a sialic acid content of between 6 mol/mol and 15 mol/mol.

4. Recombinant FSH according to any preceding paragraph wherein 10% or more of the total sialylation is α2,3-sialylation.

5. Recombinant FSH according to any preceding paragraph which includes α2,3-sialylation in an amount which is from 65 to 85% of the total sialylation.

6. Recombinant FSH according to any preceding paragraph which includes α2,3-sialylation in an amount which is from 70 to 80% of the total sialylation.

7. Recombinant FSH according to any preceding paragraph wherein 50% or less of the total sialylation is α2,6-sialylation.

8. Recombinant FSH according to any preceding paragraph which includes a2,6-sialylation in an amount which is from 15 to 35% of the total sialylation.

9. Recombinant FSH according to any preceding paragraph which includes a2,6-sialylation in an amount which is from 20 to 30% of the total sialylation,

10. Recombinant FSH according to any preceding paragraph which further includes α2,8-sialylation.

11. Recombinant FSH according to any preceding paragraph wherein the sialic acid content is 6% or greater by mass.

12. Recombinant FSH according to any preceding paragraph produced or expressed in a human cell line.

13. Recombinant FSH according to any preceding paragraph produced or expressed in a Per.C6 cell line, a Per.C6 derived cell line or a modified Per.C6 cell line.

14. Recombinant FSH according to paragraph 12 or 13 wherein the cell line has been modified using α2,3-sialyltransferase.

15. Recombinant FSH according to any of paragraphs 12 to 14 which includes α2,6-linked sialic acids (α2,6 sialylation) provided by endogenous sialyl transferase activity.

16. Recombinant FSH expressed in a human cell line.

17. Recombinant FSH according to paragraph 16 wherein 10% or more of the total sialylation is α2,3-sialylation.

18. Recombinant FSH according to paragraph 16 or 17 which includes α2,3-sialylation in an amount which is from 65 to 85% of the total sialylation.

19. Recombinant FSH according to any of paragraphs 16 to 18 wherein 50% or less of the total sialylation is α2,6-sialylation.

20. Recombinant FSH according to any of paragraphs 16 to 19 which includes α2,6-sialylation in an amount which is from 15 to 35% of the total sialylation.

21. Recombinant FSH according to any of paragraphs 16 to 20 which includes a sialic acid content [expressed in terms of a ratio of moles of sialic acid to moles of protein] of 6 mol/mol or greater.

22. Recombinant FSH according to any of paragraph 16 or 21 including α2,3-sialylation and α2,6-sialylation.

23. A recombinant FSH preparation including α2,3- and α2,6-sialylation.

24. A preparation according to paragraph 23 which is a pharmaceutical preparation.

25. A preparation according to paragraph 23 or 24 having a sialic acid content [expressed in terms of a ratio of moles of sialic acid to moles of protein] of 6 mol/mol or greater.

26. A preparation according to any of paragraphs 23 to 25 wherein 10% or more of the total sialylation is α2,3-sialylation.

27. A preparation according to any of paragraphs 23 to 26 which includes α2,3-sialylation in an amount which is from 65 to 85% of the total sialylation.

28. A preparation according to any of paragraphs 23 to 27 wherein 50% or less of the total sialylation is α2,6-sialylation.

29. A preparation according to any of paragraphs 23 to 28 which includes a2,6-sialylation in an amount which is from 15 to 35% of the total sialylation.

30. A preparation according to any of paragraphs 23 to 29 produced or expressed in a human cell line.

31. A pharmaceutical composition comprising rFSH including α2,3-sialylation and α2,6-sialylation.

32. A pharmaceutical composition according to paragraph 31 wherein 10% or more of the total sialylation is α2,3-sialylation.

33. A pharmaceutical composition according to paragraphs 31 or 32 which includes α2,3-sialylation in an amount which is from 65 to 85% of the total sialylation.

34. A pharmaceutical composition according to any of paragraphs 31 to 33 wherein 50% or less of the total sialylation is α2,6-sialylation.

35. A pharmaceutical composition according to any of paragraphs 31 to 33 which includes a2,6-sialylation in an amount which is from 15 to 35% of the total sialylation.

36. A pharmaceutical composition comprising rFSH according to any of paragraphs 1 to 22 and/or a preparation according to any of paragraphs 23 to 30.

37. A pharmaceutical composition according to any of paragraphs 31 to 36 further comprising hCG and/or LH.

38. A pharmaceuticalcomposition according to any of paragraphs 31 to 37 for use in the treatment of infertility.

39. A method of treatment of infertility comprising a step of administering to a subject a composition comprising rFSH according to any of paragraphs 1 to 22 and/or a preparation according to any of paragraphs 23 to 30 and/or a pharmaceutical composition according to any of paragraphs 31 to 37.

40. Use of rFSH according to any of paragraphs 1 to 22 and/or an rFSH preparation according to any of paragraphs 23 to 30 in the manufacture of a medicament for the treatment of infertility.

41. A method of production of rFSH according to any of paragraphs 1 to 22 and/or an rFSH preparation according to any of paragraphs 23 to 30 comprising the step of producing or expressing the rFSH in a human cell line.

SEQUENCE LISTING

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<150> US 61/045424

<151> 2008-04-16

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tactcccggg atcagcccat cttcctgcgg cttgaggatt atttctgggt caagacgcca 240

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Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val Arg
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Glu Glu Cys Arg Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly
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Tyr Cys Tyr Thr Arg Asp Leu Val Tyr Lys Asp Pro Ala Arg Pro Lys
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Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val Arg
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Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val
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Ser Leu Arg Cys Arg Arg Cys Val Val Val Gly Asn Gly His Arg Leu
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Lys Thr Thr Met Arg Leu Phe Tyr Pro Glu Ser Ala His Phe Asp Pro
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Lys Val Glu Asn Asn Pro Asp Thr Leu Leu Val Leu Val Ala Phe Lys
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Arg Asp His Val Asn Val Ser Met Val Glu Val Thr Asp Phe Pro Phe
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Asn Thr Ser Glu Trp Glu Gly Tyr Leu Pro Lys Glu Ser Ile Arg Thr
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Lys Arg Phe Leu Lys Asp Ser Leu Tyr Asn Glu Gly Ile Leu Ile Val
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Trp Asp Pro Ser Val Tyr His Ser Asp Ile Pro Lys Trp Tyr Gln Asn
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Pro Asp Tyr Asn Phe Phe Asn Asn Tyr Lys Thr Tyr Arg Lys Leu His
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Claims

1. Recombinant FSH (rFSH) including α2,3- and a2,6-sialylation wherein 80% or more of the total sialylation is α2,3-sialylation and wherein from 5% to 20% of the the total sialylation is α2,6-sialylation.

2. Recombinant FSH according to claim 1 expressed in a human cell line.

3. A pharmaceutical composition comprising rFSH according to claim 1 or claim 2.

4. A pharmaceutical composition according to claim 3 further comprising hCG and/or LH.

5. A pharmaceutical composition according to claim 3 or 4 for use in the treatment of infertility.

6. A method of production of rFSH according to any of claims 1 or 2 comprising the step of producing or expressing the rFSH in a human cell line.

7. A method according to claim 6 wherein the cell line has been modified using α2,3-sialyltransferase and/or α2,6-sialyltransferase.

Patentansprüche

1. Rekombinantes FSH (rFSH) mit α2,3- und α2,6-Sialylierung, wobei 80 % oder mehr der Gesamtsialylierung α2,3-Sialylierung sind und wobei 5 bis 20 % der Gesamtsialylierung α2,6-Sialylierung sind.

2. Rekombinantes FSH nach Anspruch 1, exprimiert in einer humanen Zelllinie.

3. Pharmazeutische Zusammensetzung, die rFSH nach Anspruch 1 oder Anspruch 2 umfasst.

4. Pharmazeutische Zusammensetzung nach Anspruch 3, die ferner hCG und/ oder LH umfasst.

5. Pharmazeutische Zusammensetzung nach Anspruch 3 oder 4 zur Verwendung bei der Behandlung von Unfruchtbarkeit.

6. Verfahren zum Produzieren von rFSH nach Anspruch 1 oder 2, das den Schritt des Erzeugens oder Exprimierens des rFSH in einer humanen Zelllinie beinhaltet.

7. Verfahren nach Anspruch 6, wobei die Zelllinie mit α2,3- und/oder α2,6-Sialylierung modifiziert wurde.

Revendications

1. FSH recombinante (rFSH) avec sialylation sur α2,3 et α2,6, dans laquelle 80% ou plus de la sialylation totale est une sialylation sur α2,3 et de 5 à 20% de la sialylation totale est une sialylation sur α2,6.

2. FSH recombinante selon la revendication 1, exprimée dans une lignée de cellules humaines.

3. Composition pharmaceutique comprenant une rFSH selon la revendication 1 ou la revendication 2.

4. Composition pharmaceutique selon la revendication 3, comprenant en outre de la hCG et/ou de la LH.

5. Composition pharmaceutique selon la revendication 3 ou 4, destinée à être utilisée dans le traitement de l’infertilité.

6. Procédé de production de rFSH selon l’une quelconque des revendications 1 ou 2 comprenant l’étape de production ou d’expression de la rFSH dans une lignée de cellules humaines.

7. Procédé selon la revendication 6 dans lequel la lignée de cellules a été modifiée par α2,3-sialyltransférase et/ou α2,6-sialytransférase.

Figs 1, 2 and 3: Plasmid maps of the pFSHalpha/beta ,pST3 and pST6 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, FSH A = Follicle stimulating hormone alpha polypeptide, FSH B = Follicle stimulating hormone beta polypeptide, ST3GAL4 = α2,3-sialyltransferase, ST6GAL1 = α2,6-sialyltransferase, ColEl = ColEl origin of replication, Amp = ampicillin resistance marker.

Figure 1. FSH expression vector

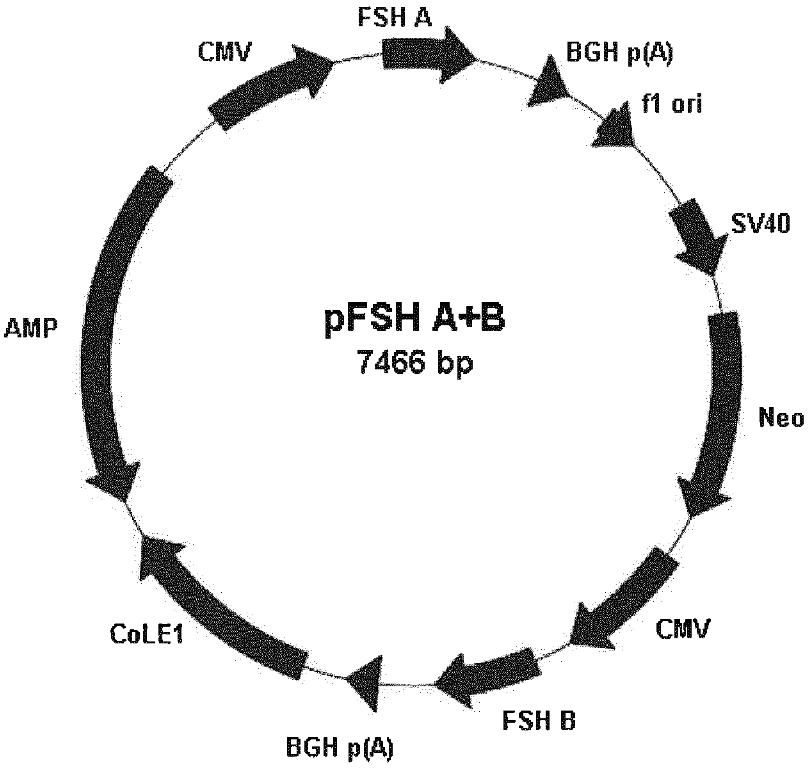


Figure 2. α2,3-sialyltransferase (ST3GAL4) expression vector

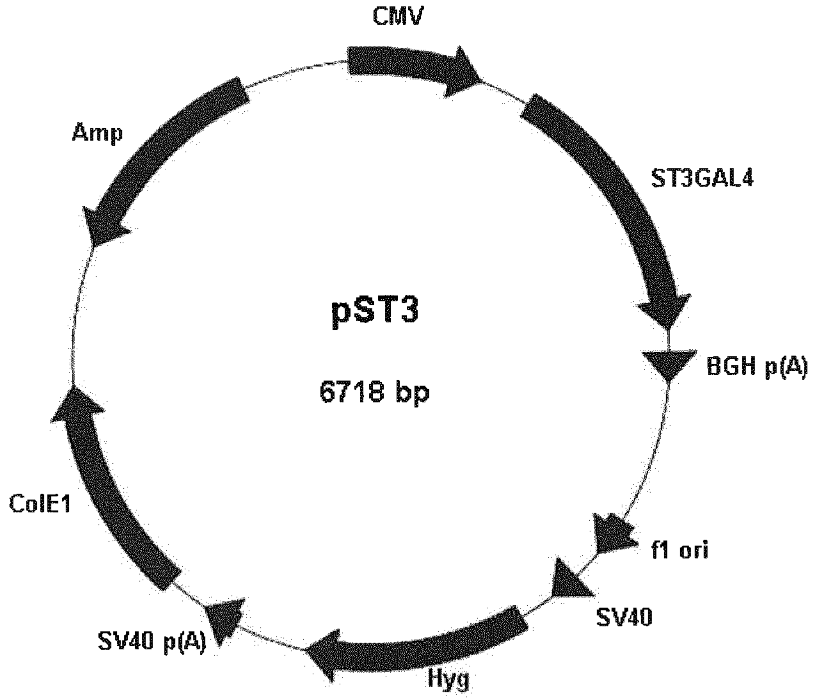


Figure 3. α2,6-sialyltransferase (ST6GAL1) expression vector

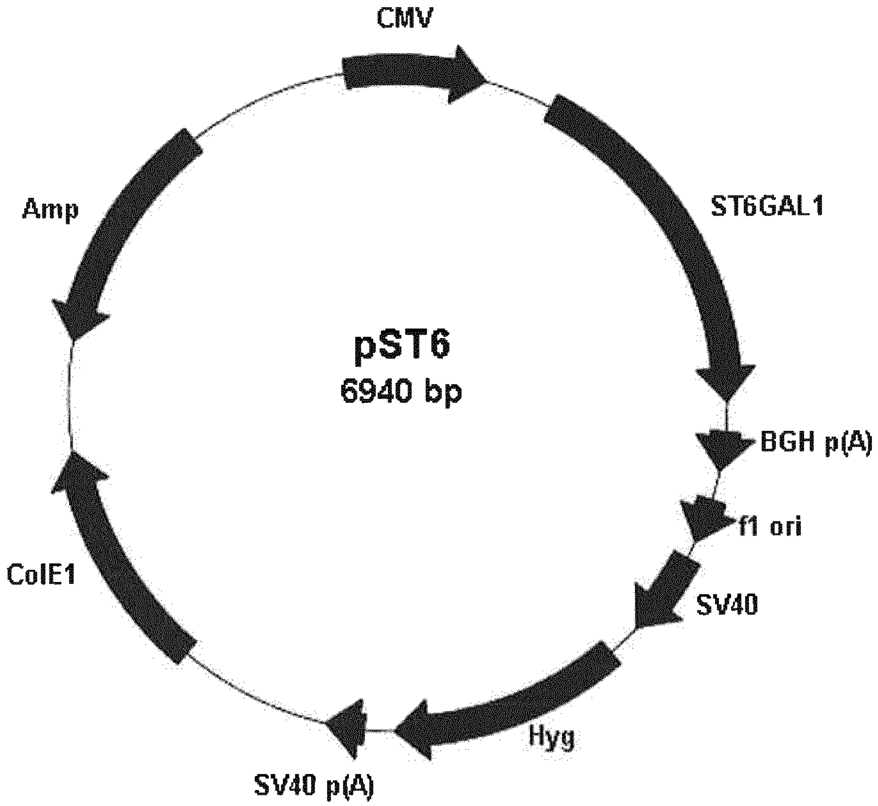


Figure 4 Isoelectric focussing of recombinant FSH produced by Per.C6 cells stably expressing FSH. Cell culture supernatants separated under native conditions on a pH 3.0 -7.0 gradient. Clone 005 is representative of the five clones taken forward for sialytransferase engineering. Clones containing less acidic isoforms were discarded.

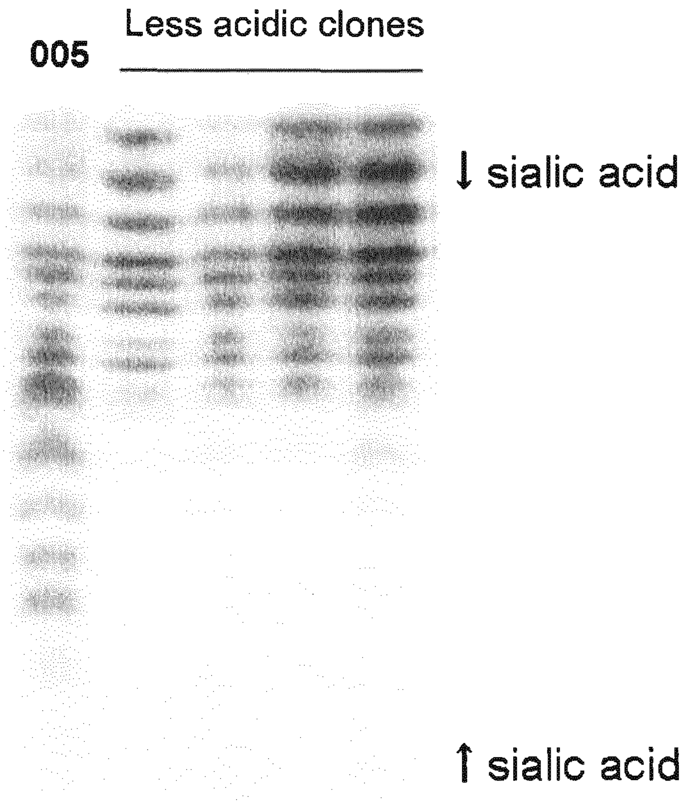


Figure 5 Example of clones analysed by isoelectric focussing of recombinant FSH produced by Per.C6 cells stably expressing FSH after engineering with α2,3- or α2,6-sialyltransferase. Cell culture supernatants separated under native conditions on a pH 3.0 -7.0 gradient. Clone 005 is the parental Per.C6 FSH cell line. Clones displaying basic or mixed profiles were discontinued (*). The remaining clones demonstrate successful engineering with a sialyltransferase to increase the number of sialic acid molecules on FSH.

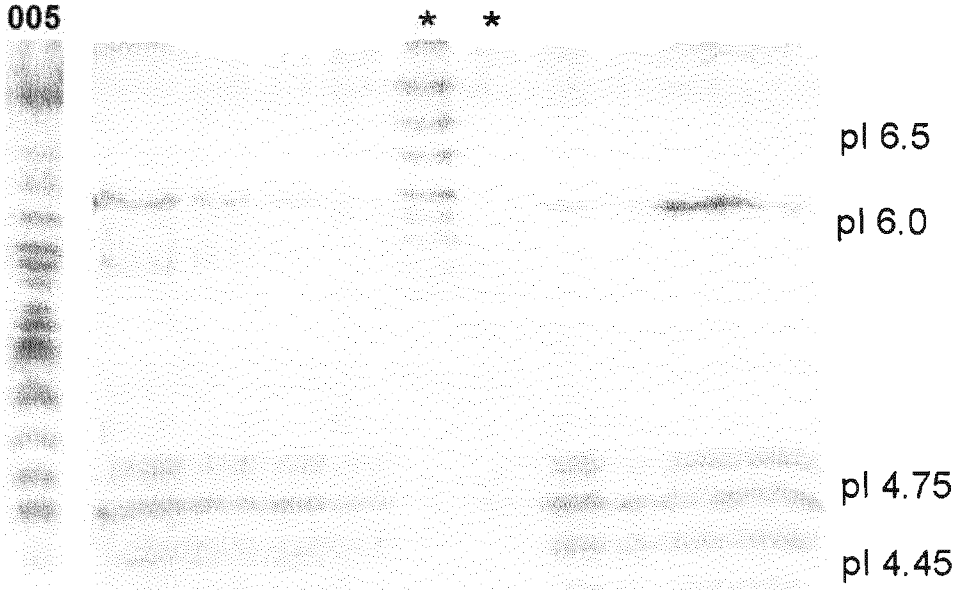
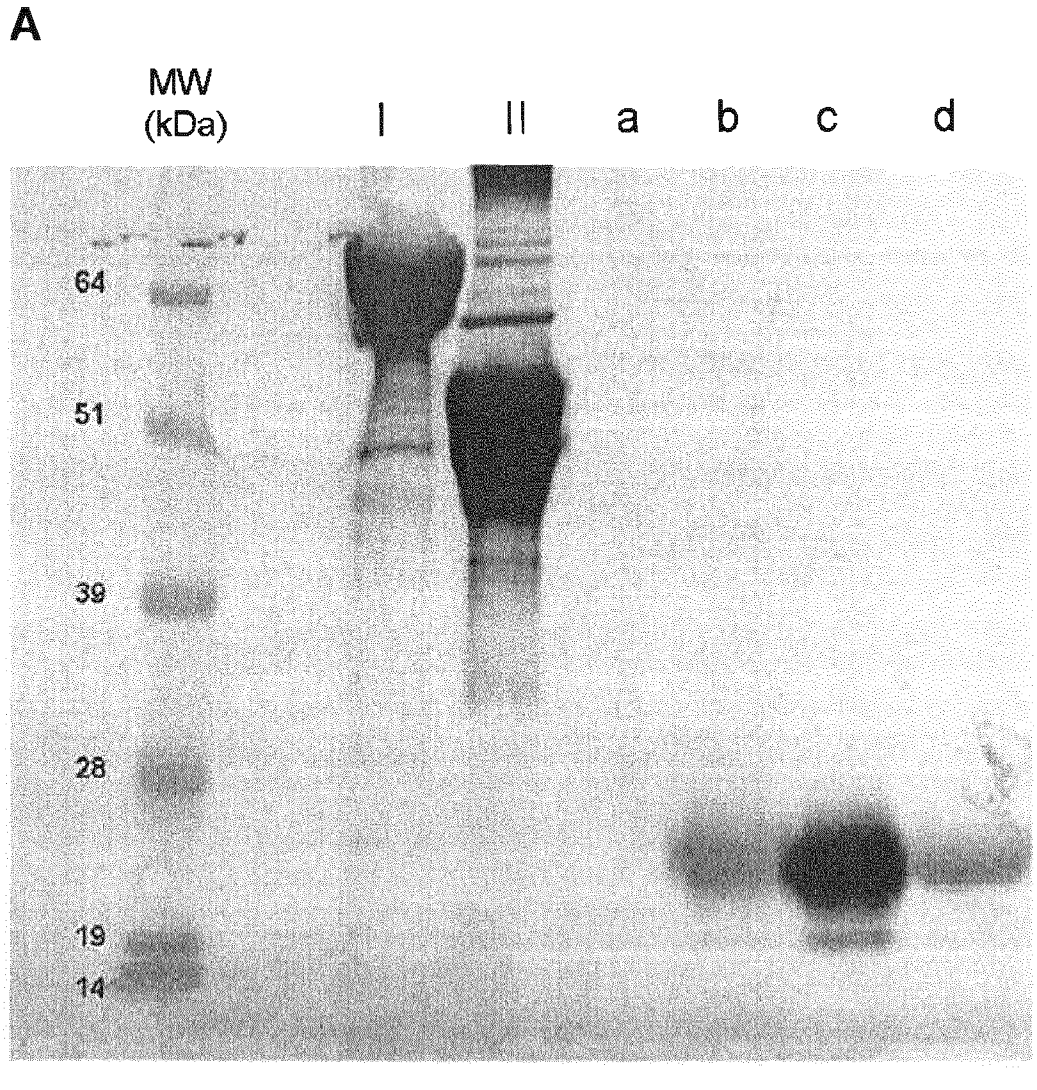


Figure 6 Analysis of the Sialic acid linkages of Per.C6 FSH. Purified Per.C6 FSH was separated by SDS PAGE on duplicate gels, transferred to nitrocellulose and visualised using the DIG Glycan Differentiation Kit (Cat. No. 11 210 238 001, Roche) according to the manufacturers instructions. Positive reactions with Sambucus nigra agglutinin (SNA) indicated terminally linked (2–6) sialic acid (**A**). Positive reactions with Maackia amurensis agglutinin (MAA): indicated terminally linked (2–3) sialic acid (**B**). Lane I manufacturers control containing α2,6 linkages only. Lane II manufacturers control containing α2,6 and α2,3 linkages. Sample a, commercial CHO derived recombinant FSH (Gonal-f, Serono). Sample b, parental Per.C6 rFSH, no sialyl-transferase engineering. Sample c, Per.C6 rFSH with α2,6-sialyltransferase engineering. Sample d, Per.C6 rFSH with α2,3-sialyltransferase engineering



B

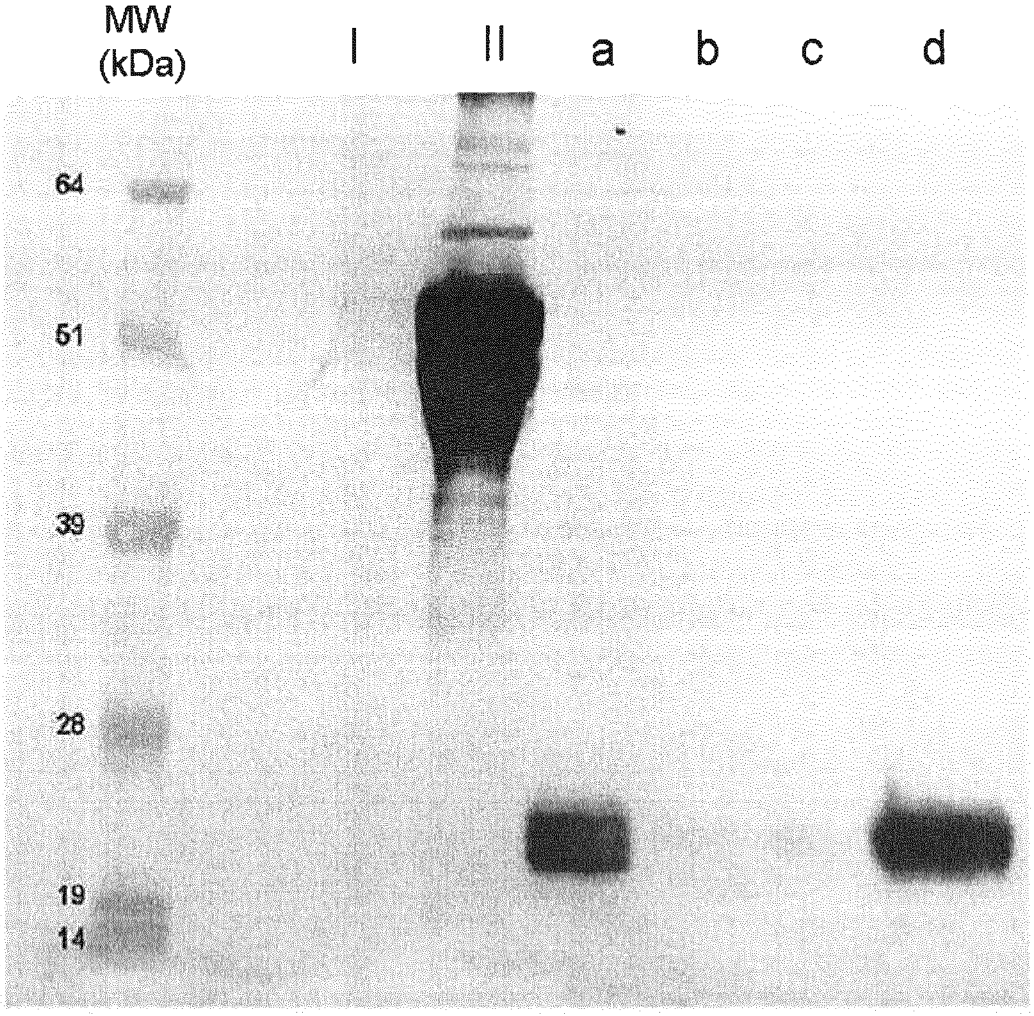


Figure 7 Metabolic clearance rates of Per.C6 FSH samples. Female rats (3 animals per clone) were injected into the tail vein at time zero with a bolus of rFSH (1 - 10 µg/rat). Blood samples collected over time were assayed for FSH content by ELISA.

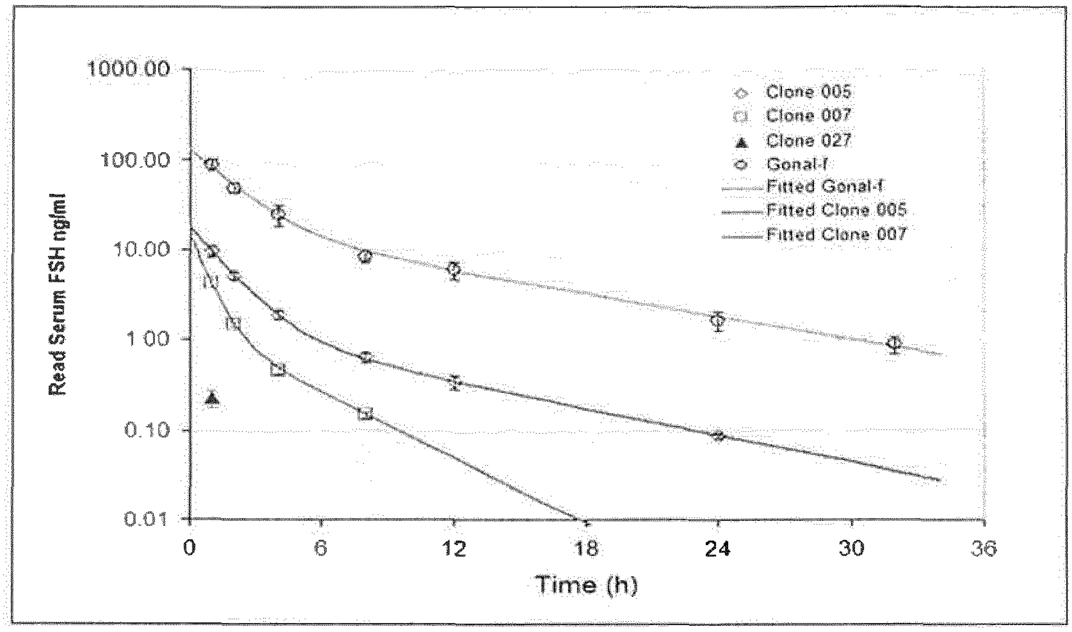


Figure 8 Metabolic clearance rates of α2,6-sialyltransferase engineered Per.C6 FSH samples. Female rats (3 animals per clone) were injected into the tail vein at time zero with a bolus of rFSH (1 - 10 µg/rat). Blood samples collected over time were assayed for FSH content by ELISA.

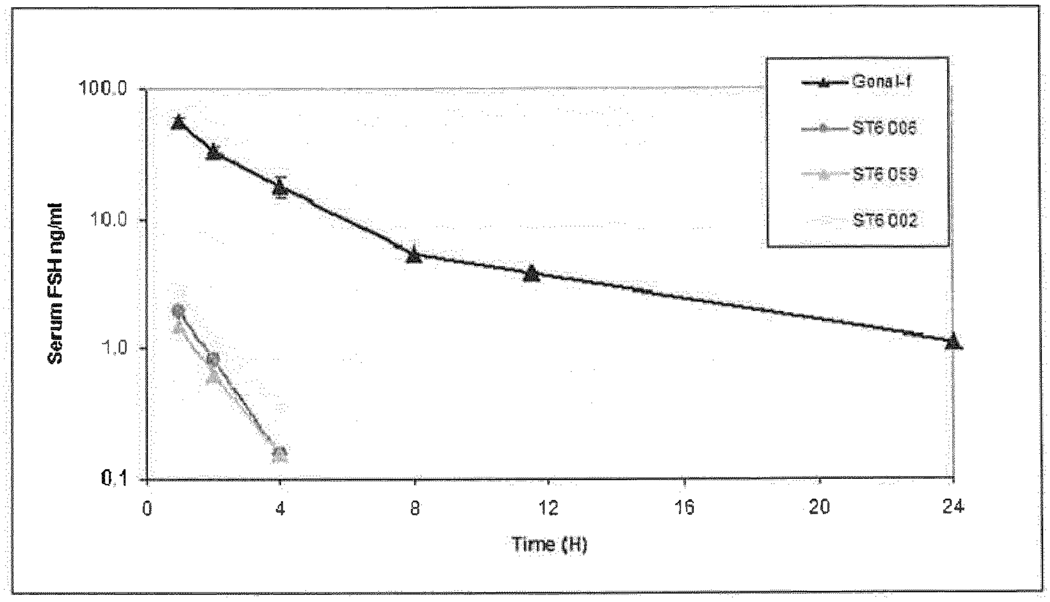


Figure 9 Metabolic clearance rates of α2,6-sialyltransferase engineered Per.C6 FSH samples with co administration of a 7500-fold molar excess of asialofetuin to saturate the ASGP-R for 1-2 h.

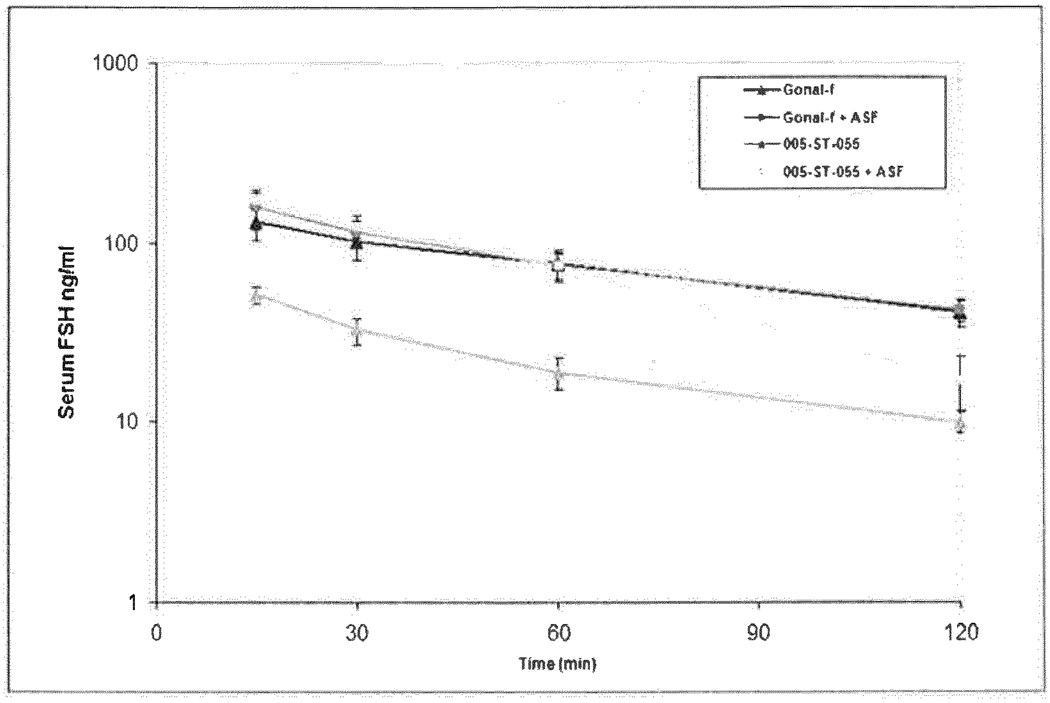


Figure 10 Metabolic clearance rates of α2,3-sialyltransferase engineered Per.C6 FSH samples. Samples were chosen for their sialic acid content based on their IEF profile.

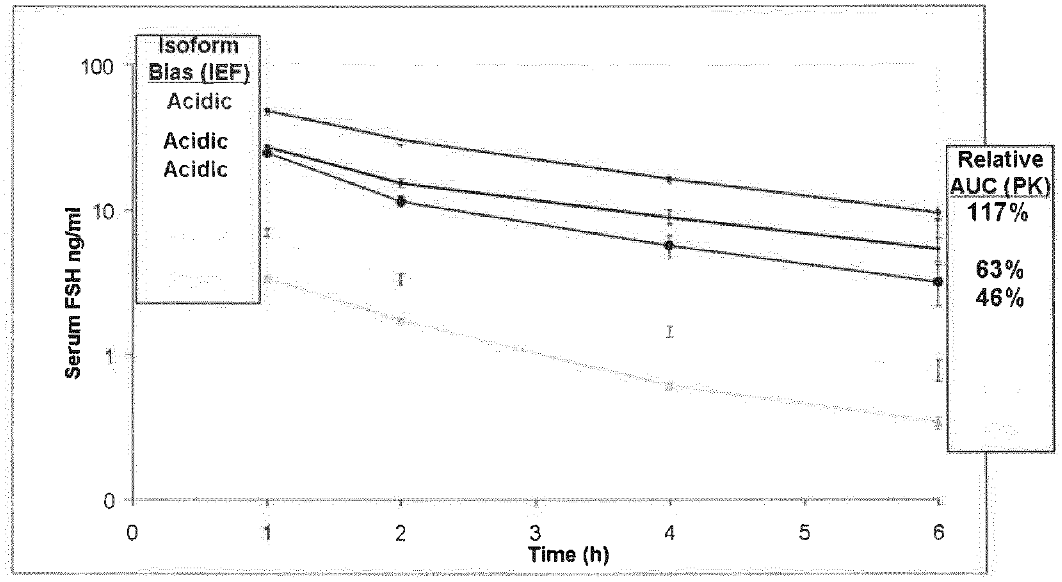


Figure 11 Ovarian weight augmentation according to the method of Steelman and Pohley (1953). Per.C6 rFSH and standards (Gonal-f rFSH) were tested at different doses (3 rats/dose).

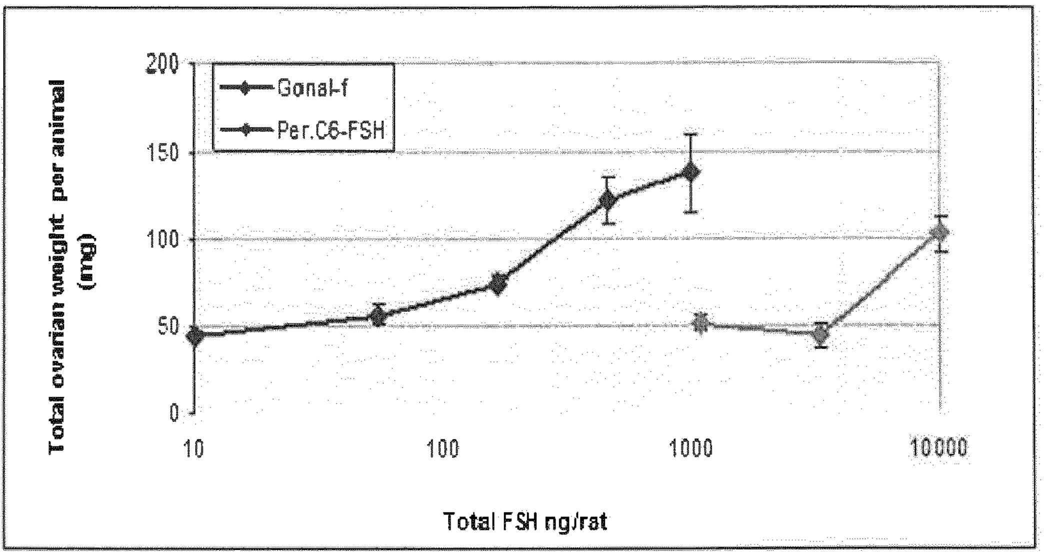


Figure 12 Ovarian weight augmentation according to the method of Steelman and Pohley (1953). α2,6-sialyltransferase engineered Per.C6 rFSH and standards (Gonal-f rFSH) were tested at different doses (3 rats/dose).

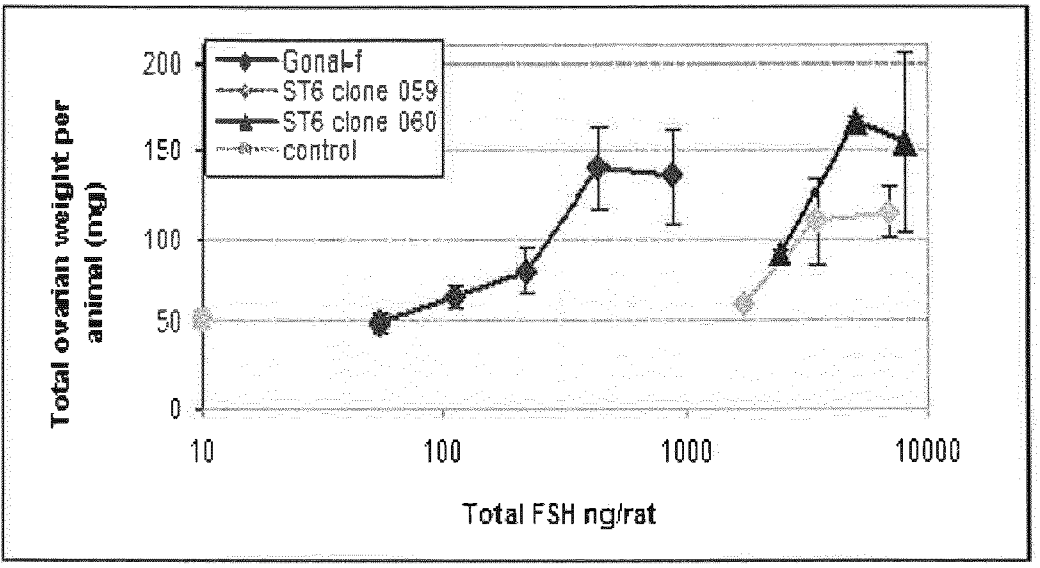
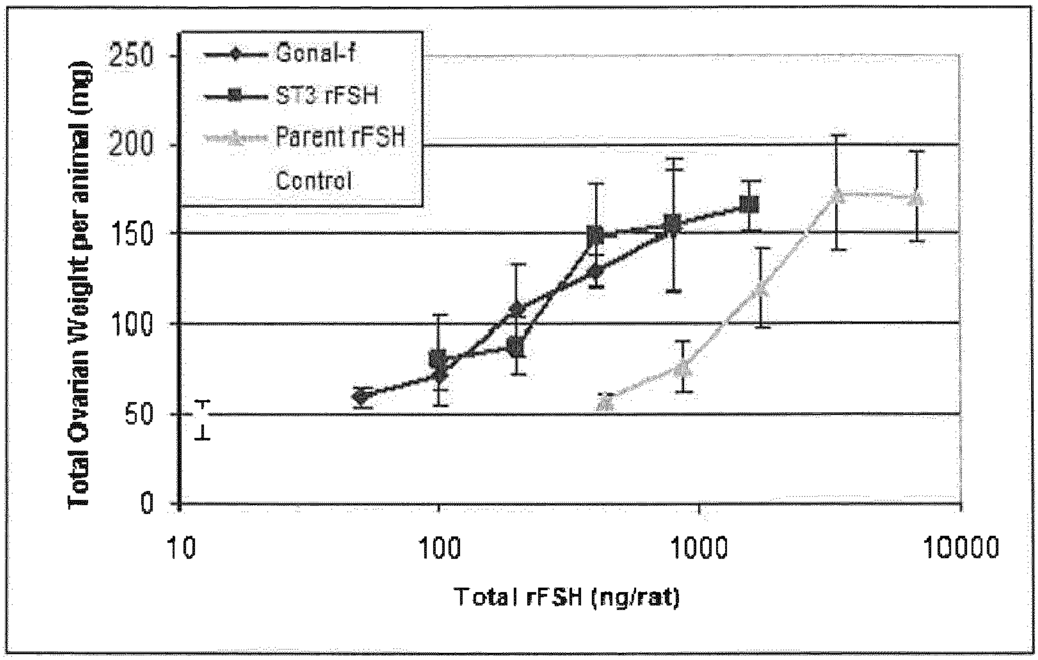


Figure 13 Ovarian weight augmentation according to the method of Steelman and Pohley (1953). Parental Per.C6 rFSH, α2,3-sialyltransferase engineered Per.C6 rFSH and standards (Gonal-f rFSH) were tested at five different doses (3 rats/dose).



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REKOMBINÁNS, ALFA-2,3- ÉS ALFA-2,5-SZIALIZÁCIÓT TARTALMAZÓ FSH

Szabadalmi igénypontok

1. Rekombináns FSH (rFSH, follikulas stimuláló hormon), amely α2,3- és α2,6-szializációt tartalmaz, ahol az összes szializáció 80%-a vagy nagyobb aránya α2,3-szializáció és ahol az összes szializáció 5-20%-a α2,6-szializáció.

2. Az 1. igénypont szerinti rekombináns FSH, amely humán sejtvonalban expresszált.

3. Gyógyászati készítmény, amely 1. vagy 2. igénypont szerinti rFSH-t tartalmaz.

4. A 3. igénypont szerinti gyógyászati készítmény, amely hCG-t és/vagy LH-t is tartalmaz.

5. A 3. vagy 4. igénypont szerinti gyógyászati készítmény meddőség kezelésében történő alkalmazásra.

6. Eljárás 1. vagy 2. igénypont szerinti rFSH előállítására, amely tartalmazza az rFSH termelését vagy expresszálását humán sejtvonalban.

7. A 6. igénypont szerinti eljárás, ahol a sejtvonal α2,3-szialiltranszferáz és/vagy α2,6- szialiltranszferáz alkalmazásával módosított.