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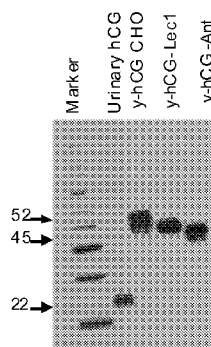


Figure 1

(57) Abstract: In one embodiment the present invention is directed to a recombinant human follicle stimulating hormone (FSH) antagonist having reduced N-linked glycosylation. In other embodiments, the present invention is directed to compositions comprising such FSH antagonists, such as combination compositions. In other embodiments the invention provides methods of treatment, including methods of treating ovarian cancer, and methods of increasing the efficacy of a chemotherapeutic agent, such as cisplatin.

RECOMBINANT HUMAN FOLLICLE STIMULATING HORMONE ANTAGONISTS AND METHODS OF USE THEREOF

[0001] This application claims the benefit of the filing date of U.S. Provisional Patent Application No. 61/152,474, filed February 13, 2009, the contents of which are hereby incorporated by reference.

[0002] The present invention was made with Government support under grant number awarded by National Institutes of Health with grant number NIDDK R01 DK063224. Therefore, the U.S. Government has certain rights in the invention.

[0003] This patent disclosure contains material which is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or the patent disclosure, as it appears in the U.S. Patent and Trademark Office patent file or records, but otherwise reserves any and all copyright rights.

[0004] Throughout this application, patent applications, published patent applications, issued and granted patents, texts, and literature references are cited. For the purposes of the United States and other jurisdictions that allow incorporation by reference, the disclosures of these publications are incorporated by reference into this application.

BACKGROUND OF THE INVENTION

[0005] Ovarian epithelial cancer (OEC) is the sixth most common cause of cancer and the fifth leading cause of cancer-related death among women in industrialized countries. The majority of women with ovarian cancer are diagnosed at a stage when the cancer has already metastasized due to an absence of specific symptoms and a lack of reliable screening mechanisms. Ovarian tumors exhibit resistance to conventional drugs such as cisplatin. Initial treatment for OEC is usually surgery followed by adjuvant platinum and taxane chemotherapy. Unfortunately, current therapeutic regimens are insufficient in treating OEC, and survival has improved only modestly over the past two decades. The development of novel therapies, both primary and adjuvant, are needed for women with OEC.

SUMMARY OF THE INVENTION

[0006] In one embodiment, the present invention provides recombinant human follicle stimulating hormone antagonists (hereinafter a "rhFSH-Ants") in which one or more of the N-linked glycosylation sites are disrupted. In one embodiment, the rhFSH-Ant is able to bind to the

follicle stimulating hormone (hereinafter “FSH”) receptor without activating the receptor, or with reduced ability to activate the receptor as compared to FSH, such that the rhFSH-Ant acts as a competitive antagonist of FSH.

[0007] In one embodiment, the invention provides a rhFSH-Ant comprising the amino acid sequence of SEQ ID NO:2. In one embodiment, the FSH antagonist comprises amino acid residues 19-252 of SEQ ID NO:2. SEQ ID NO:2 is the amino acid sequence of an exemplary rhFSH-Ant in which the asparagine (Asn) residues that correspond to amino acid positions 7 and 24 of the FSH beta subunit and the Asn residue that corresponds to amino acid position 52 of the FSH alpha subunit have been mutated to disrupt the N-linked glycosylation sites. The exemplary rhFSH-Ant of SEQ ID NO:2 is not mutated at the Asn residue that corresponds to amino Asn 78 of the FSH alpha subunit, such that the N-linked glycosylation site located at Asn 78 is intact. The exemplary FSH antagonist of SEQ ID NO:2, as illustrated in Figure 22, comprises an 18 amino acid leader peptide at amino acid residues 1 to 18, followed by a mutated FSH beta subunit at amino acid residues 19 to 129, an N4 peptide linker at amino acid residues 130 to 161, and a mutated FSH subunit from amino acid residues 162 onwards. In SEQ ID NO:2, because of the inclusion of the leader peptide and linker sequences, amino acid residue 25 corresponds to Asn 7 of the FSH beta subunit, amino acid residue 42 corresponds to Asn 72 of the FSH beta subunit, amino acid residue 213 corresponds to Asn 52 of the FSH alpha subunit, and amino acid residue 239 corresponds to Asn 78 of the FSH alpha subunit.

[0008] In one embodiment, the invention provides a rhFSH-Ant comprising a variant of the amino acid sequence of SEQ ID NO. 2 having at least about about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:2. In one embodiment, such variants do not comprise an Asn residue at amino acid positions 7 and 24 of the FSH beta subunit or at amino acid position 52 of the FSH alpha subunit, or at the positions that correspond thereto. In another embodiment, such variants retain an Asn residue at amino acid position 78 of the FSH alpha subunit, or at the position that corresponds thereto. In one embodiment, variants containing mutations of the amino acid sequence of SEQ ID NO. 2 retain the ability to bind to the FSH receptor but have no ability to activate the FSH receptor or reduced ability to activate the FSH receptor as compared to FSH.

[0009] In another embodiment, the invention provides a rhFSH-Ant encoded by the nucleic acid sequence of SEQ ID NO:1. SEQ ID NO:1 encodes the exemplary rhFSH-Ant of SEQ ID

NO:2, in which the asparagine (Asn) residues corresponding to amino acids 7 and 24 of the FSH beta subunit and the Asn residue corresponding to amino acid 52 of the FSH alpha subunit have been mutated to disrupt the N-linked glycosylation sites. The exemplary rhFSH-Ant encoded by SEQ ID NO:1 is not mutated at the amino acid residue that corresponds to Asn 78 of the alpha subunit, such that the N-linked glycosylation site at this Asn residue is intact.

[0010] In one embodiment, the invention provides a rhFSH-Ant encoded by a variant of the nucleic acid sequence of SEQ ID NO:1 having at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:1. In one embodiment, such variants do not encode an Asn residue at the amino acid residues corresponding to positions 7 and 24 of the FSH beta subunit or position 52 of the FSH alpha subunit. In another embodiment, such variants do encode an Asn residue at the residue that corresponds to amino acid position 78 of the FSH alpha subunit. In one embodiment, mutated variants of SEQ ID NO. 1 encode a rhFSH-Ant protein that retains the ability to bind to the FSH receptor but has no ability to activate the FSH receptor or has reduced ability to activate the FSH receptor as compared to FSH.

[0011] In another embodiment, the invention provides a rhFSH-Ant comprising an FSH beta subunit covalently linked to an FSH alpha subunit, wherein the beta subunit is mutated to disrupt the N-linked glycosylation sites at residues corresponding to Asn 7 and Asn 24, of the FSH beta subunit, and wherein the alpha subunit is mutated to disrupt the N-linked glycosylation site at the residue that corresponds to Asn 52 of the FSH alpha subunit. In one embodiment, the rhFSH is not mutated at the residue that corresponds to Asn 78 of the FSH alpha subunit, such that this N-linked glycosylation site is intact. In one embodiment, the C-terminus of the beta subunit is linked to the N-terminus of the alpha subunit by a linker. In another embodiment, the C-terminus of the alpha subunit is linked to the N-terminus of the beta subunit by a linker. In one embodiment the linker is a peptide linker. In one embodiment the linker comprises one or more glycosylation sites, such as N-linked glycosylation sites. In one embodiment the linker comprises 4 N-linked glycosylation sites. In one embodiment, the rhFSH-Ant comprises a leader peptide comprising a signal sequence that targets the polypeptide for excretion or secretion from the cell. In one embodiment, the rhFSH-Ant comprises half-life increasing moiety.

[0012] In one embodiment, the present invention provides a human chorionic gonadotropin (hereinafter "hCG") antagonist, such as a recombinant hCG antagonist (hereinafter a "rhHCG-Ant"). rhHCG-Ants that can be used in accordance with the methods of the present invention

include, but are not limited to, those described in U.S. Patent Publication No. 2008/0039372, the contents of which are incorporated by reference. In one embodiment, the rhCG-Ant is encoded by the nucleic acid of SEQ ID NO: 3. In one embodiment, the rhCG-Ant is encoded by a variant of SEQ ID NO: 3 having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO: 3.

[0013] In another embodiment, the invention provides a method for making a rhFSH-Ant comprising transforming a host cell with a vector comprising (i) the nucleic acid of SEQ ID NO:1, (ii) a nucleic acid having at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:1, (iii) a nucleic acid capable of expressing the amino acid sequence of SEQ ID NO:2, or (iv) a nucleic acid capable of expressing an amino acid sequence having at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:2. In another embodiment, the invention provides a method for making a rhCG-Ant comprising transforming a host cell with a vector comprising (i) the nucleic acid of SEQ ID NO:3, or (ii) a nucleic acid having at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:3. In one embodiment, the host cell can be a Chinese hamster ovary (CHO) cell. In another embodiment, the host cell is a bacterial cell, yeast cell, or insect cell. In one embodiment, the yeast cell is *picchia*. In another embodiment, the insect cell is susceptible to infection by a baculovirus.

[0014] In another embodiment, the invention provides a host cell expressing an amino acid sequence comprising the sequence of SEQ ID NO:2, or a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:2. In another embodiment, the invention provides a host cell expressing an amino acid sequence encoded by the sequence of SEQ ID NO:1, or a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:1. In another embodiment, the invention provides a host cell expressing an amino acid sequence encoded by the sequence of SEQ ID NO:3, or a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:3.

[0015] In another embodiment, the invention provides a method for inhibiting the activity of FSH comprising administering an effective amount of an FSH antagonist to a subject in need thereof. The FSH antagonist may be a small molecule drug or a protein or peptide-based FSH antagonist. In one embodiment, the FSH antagonist is a rhFSH-Ant as described above,. In one

embodiment, the FSH antagonist is administered orally, intravenously, intramuscularly, or subcutaneously.

[0016] In another embodiment, the present invention provides a pharmaceutical composition comprising a rhFSH-Ant. In another embodiment, the present invention provides a pharmaceutical composition comprising a rhFSH-Ant and an rhCG-Ant. In another embodiment, the present invention provides a pharmaceutical composition comprising a rhFSH-Ant and a chemotherapeutic agent. In another embodiment, the present invention provides a pharmaceutical composition comprising a rhCG-Ant and a chemotherapeutic agent. In another embodiment, the present invention provides a pharmaceutical composition comprising a rhFSH-Ant, a rhCG-Ant, and a chemotherapeutic agent. In some embodiments, the chemotherapeutic agent is cisplatin or a cisplatin analogue. In another embodiment, the present invention provides a pharmaceutical composition comprising a rhFSH-Ant and a contraceptive agent. In another embodiment, the present invention provides a pharmaceutical composition comprising a rhCG-Ant and a contraceptive agent. In another embodiment, the present invention provides a pharmaceutical composition comprising a rhFSH-Ant, a rhCG-Ant, and a contraceptive agent.

[0017] In another embodiment, the invention provides a method for inhibiting the growth of tumor cells and/or treating or slowing the progression of a cancer in a subject, the method comprising administering to a subject in need thereof an effective amount of an FSH antagonist or an hCG antagonist. The FSH or hCG antagonist may be a small molecule drug or a protein or peptide-based antagonist. In one embodiment, the antagonist is a rhFSH-Ant as described above. In one embodiment, the antagonist is a rhCG-Ant as described above. In one aspect, the tumor cell or cancer is an FSH-associated tumor cell or cancer, such as a tumor cell or cancer that secretes and/or is stimulated by FSH. In one embodiment, the tumor cells are ovarian tumor cells, such as ovarian epithelial tumor cells, and the cancer is an ovarian cancer, such as an epithelial ovarian cancer. In one embodiment, the FSH or hCG antagonist is administered after therapeutic surgical debulking of a tumor or tumor cells or of an organ or tissue comprising a tumor or tumor cells.

[0018] In another embodiment, the invention provides a method for inhibiting the growth of tumor cells and/or treating or slowing the progression of a cancer in a subject comprising administering to the subject an FSH or hCG antagonist and one or more hormones or chemotherapeutic agents. In one embodiment an FSH or hCG antagonist is administered to a

subject who has already been treated with, who is currently undergoing treatment with, or who will be treated with, one or more hormones or chemotherapeutic agents. In one embodiment the FSH or hCG antagonist and the one or more hormones or chemotherapeutic agents are administered together in a combination composition. In another embodiment the FSH or hCG antagonist and the one or more hormones or chemotherapeutic agents are administered using separate compositions. The FSH or hCG antagonist and the one or more hormones or chemotherapeutic agents can be administered concurrently or at different times. The FSH or hCG antagonist can be administered before or after administration of the one or more hormones or chemotherapeutic agents. Hormones that can be used in accordance with the present invention include, but are not limited to, gonadotropins (with or without a GnRH antagonist) and progesterone. In one embodiment the chemotherapeutic agent is selected from the group consisting of bleomycin, daunomycin, 5-FU, cytosine arabinoside, colchicine, cytochalasin B, daunorubicin, neocarzinostatin, suramin, doxorubicin, carboplatin, taxol, mitomycin C, vincristine, vinblastine, methotrexate, and cisplatin, and analogues, variants, or derivatives thereof. In a preferred embodiment, the chemotherapeutic agent is cisplatin or a cisplatin analogue. In one embodiment the cancer is an FSH-associated cancer, such as a cancer that secretes and/or is stimulated by FSH. In one embodiment, the cancer is ovarian cancer, such as an ovarian epithelial cancer. In one embodiment, the FSH or hCG antagonist and/or the one or more hormones or chemotherapeutic agents are administered after therapeutic surgical debulking of a tumor or tumor cells or of an organ or tissue comprising a tumor or tumor cells.

[0019] In another embodiment, the present invention provides a method for increasing the efficacy of a chemotherapeutic agent, the method comprising administering an FSH or hCG antagonist, such as a rhFSH-Ant or rhCG-Ant as described above, concurrently with administration of the chemotherapeutic agent (either by way of a combination composition or using separate compositions), before administration of the chemotherapeutic agent, and/or after administration of the chemotherapeutic agent. In one embodiment the chemotherapeutic agent is selected from the group consisting of bleomycin, daunomycin, 5-FU, cytosine arabinoside, colchicine, cytochalasin B, daunorubicin, neocarzinostatin, suramin, doxorubicin, carboplatin, taxol, mitomycin C, vincristine, vinblastine, methotrexate, and cisplatin, and analogues, variants or derivatives thereof. In a preferred embodiment, the chemotherapeutic agent is cisplatin or cisplatin analogue. In one embodiment the subject has cancer. In one embodiment the cancer is

an FSH-associated cancer, such as a cancer that secretes and/or is stimulated by FSH. In one embodiment, the cancer is ovarian cancer, such as an ovarian epithelial cancer. In one embodiment, the FSH or hCG antagonist and/or the chemotherapeutic agent are administered after therapeutic surgical debulking of a tumor or tumor cells or of an organ or tissue comprising a tumor or tumor cells.

[0020] In another aspect, the present invention provides a method for inhibiting the growth of tumor cells and/or treating or slowing the progression of a cancer in a subject comprising administering to the subject both an FSH antagonist and a human chorionic gonadotropin (hereinafter “hCG”) antagonist, such as a recombinant hCG antagonist (hereinafter a “rhHCG-Ant”). rhHCG-Ants that can be used in accordance with the methods of the present invention include, but are not limited to, those described in U.S. Patent Publication No. 2008/0039372, the contents of which are incorporated by reference. In one embodiment, the rhCG-Ant is encoded by the nucleic acid of SEQ ID NO: 3. In one embodiment, the rhCG-Ant is encoded by a variant of SEQ ID NO: 3 having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO: 3. In one embodiment an FSH antagonist is administered to a subject who has already been treated with, who is currently undergoing treatment with, or who will be treated with, an hCG antagonist. In one embodiment the FSH antagonist and the hCG antagonist are administered together in a combination composition. In other embodiment the FSH antagonist and the hCG antagonist are administered using separate compositions. The FSH antagonist and the hCG antagonist can be administered concurrently or at different times. The FSH antagonist can be administered before or after administration of the hCG antagonist. In one embodiment the cancer is an FSH-associated cancer, such as a cancer that secretes and/or is stimulated by FSH. In one embodiment, the cancer is ovarian cancer, such as an ovarian epithelial cancer. In one embodiment, the FSH antagonist and/or the hCG antagonist are administered after therapeutic surgical debulking of a tumor or tumor cells or of an organ or tissue comprising a tumor or tumor cells.

[0021] In another embodiment, the present invention provides a method for preventing pregnancy in a subject, the method comprising administering to the subject an effective amount of (i) an FSH antagonist, such as a rhFSH-Ant as described herein, (ii) an hCG antagonist, such as a rhCG-Ant as described herein, or (iii) an FSH antagonist and an hCG antagonist. In one embodiment, such methods comprise the administration of the FSH and/or hCG antagonist and

one or more other contraceptive agents. In one embodiment an FSH and/or hCG antagonist is administered to a subject who has already been treated with, who is currently undergoing treatment with, or who will be treated with, another contraceptive agent. In one embodiment the FSH and/or hCG antagonist and the other contraceptive agent administered together in a combination composition. In other embodiment the FSH and/or hCG antagonist and the other contraceptive agent are administered using separate compositions. The FSH and/or hCG antagonist and the other contraceptive agent can be administered concurrently or at different times. The FSH and/or hCG antagonist can be administered before or after administration of the other contraceptive agent. Other contraceptive agents that can be used in accordance with the methods of the invention include, but are not limited to progesterone and/or oestrogen based contraceptives that are currently used clinically.

[0022] In another embodiment, the present invention provides a method for treating ovarian hyperstimulation syndrome (OHSS) in a subject, the method comprising administering to the subject an effective amount of an FSH antagonist, such as a rhFSH-Ant as described herein. In one embodiment, both an FSH antagonist and an hCG antagonist are administered in such methods.

[0023] In those embodiments of the invention that involve methods of treatment, the FSH and/or hCG antagonists, such as the rhFSH-Ants and rhCG-Ants described herein, can be administered to a subject in an effective amount. In one embodiment, an effective amount is an amount ranging from about 3,000 IU to about 10,000 IU; or from about 2,500 IU to about 9,500 IU; or from about 3,500 IU to about 9,000 IU; or from about 4,000 IU to about 8,500 IU; or from about 4,500 IU to about 8,000 IU; or from about 5,000 IU to about 7,500 IU; or from about 5,500 IU to about 7,000 IU; or from about 6,000 IU to about 6,500 IU; or from about 2,000 IU to about 12,000 IU. In some embodiments, an effective amount of an FSH antagonist is an amount sufficient to inhibit FSH activity. In some embodiments, an effective amount of an hCG antagonist is an amount sufficient to inhibit hCG activity.

BRIEF DESCRIPTION OF THE FIGURES

[0024] **Figure 1:** Western blot (reducing conditions). The blot was probed with an anti-hCG α antibody. Lane 1 – molecular weight marker. Lane 2 - urinary hCG (the subunits are not yoked and thus only the α -subunit is visualized). Lanes 3-5 - yoked forms of hCG or hCG antagonist, as indicated.

[0025] **Figures 2A – 2B:** In vitro evaluation of hCG analogues using Chinese hamster ovary cells expressing the luteinizing hormone receptor (CHO-LHR cells). **2A.** Data from a receptor binding assay, which confirms that all of the analogues tested bind to the luteinizing hormone / chorionic gonadotropin receptor (LH/CGR) with equal affinity. **2B.** Data from a receptor activation assay. The data shows that rhCG treated with hydrogen fluoride to remove glycosylation (rhCG-HF) and rhCG-Ant were unable to activate the receptor while y-hCG expressed in glycosylation deficient mutant Lec-1 CHO cells (y-hCG -Lec1) activated the receptor, although at a significantly lower level as compared to rhCG.

[0026] **Figures 3A – 3B:** Blocking of activation of LHR by rhCG-Ant. Accumulation of cAMP in CHO-LHR cells. **3A.** Cells exposed to 10,000 mIU/mL rhCG. Cyclic adenosine monophosphate (cAMP) production is reduced as higher ratios of rhCG-Ant are added, with the maximum effect occurring at a ratio of 2X to 3X. **3B.** Cells exposed to increasing concentrations of rhCG only or a mixture of rhCG and 3X rhCG-Ant. Maximum cAMP response with rhCG-Ant is reduced to approximately 1/3 the response of rhCG alone.

[0027] **Figures 4A – 4C:** Both rhFSH (**4A**) and rhCG (**4B**) induce phosphorylation of Akt in CHO-K1 cells transfected with FSH or LH receptors respectively. **4C.** OVCAR-3 cells were evaluated for Akt phosphorylation by competitive Enzyme-Linked Immunoabsorbent Assay (CELISA). 5,000 cells were grown for 4 days in 0.5% serum overnight then for 1hr serum free before hCG was added to induce PI 3-kinase activity.

[0028] **Figure 5:** Western blot (reducing conditions) of FSHs with anti-hCG α . rFSH-N4 migrated at the highest molecular weight (MW), 65kD while rFSH-Ant migrated at a lower MW of around 40kD due to the removal of 3 carbohydrate sites.

[0029] **Figures 6A – 6B:** Binding and activation of FSH receptor using CHO-FSHR cells. **6A.** rhFSH-Ant and rhFSH bind the receptor with nearly equal affinity. **6B.** rhFSH-Ant has dramatically reduced ability to stimulate cAMP production.

[0030] **Figure 7:** *In Vitro* tubal formation assay. hCG stimulated the greatest response whereas y-hCG-lec1 with reduced carbohydrate was similar to the control treatment EGF and hCG-Ant had a statistically reduced response. * $p < 0.001$ compared to EGF, yhCG-lec1 and y-hCG-Ant-lec1 ** $p < 0.05$ compared to EGF, and y-hCG-lec1.

[0031] **Figures 8A – 8B:** Evaluation of ovarian cell lines: **8A.** Western blot of FSHR expression in ovarian cell lysates. CHO-FSHR, OVCAR-3, OVCAR-8 and SKOV3 cells showed

an FSHR band at 97kD. OVCAR-8 also had a band at 75kD. All cell lysates contained degraded FSHR at lower MWs. **8B.** Cell proliferation assay with OVCAR-3 confirmed exposure to FSH promotes cell proliferation.

[0032] Figure 9: Cytotoxicity assay with OVCAR 3 cells. Cells were incubated with increasing amounts of hCG or hCG-Ant for 72 hours. Toxicity was assessed with WST-1 reagent.

[0033] Figure 10: Cytotoxicity assay with OVCAR 3 cells in the presence of 2.5 μ M cisplatin and increasing amounts of hCG or hCG-Ant for 72hrs. Toxicity was assessed with WST-1 reagent.

[0034] Figure 11: rhCG-Ant blocks uhCG induced proliferation of JEG-3 cells. Cells were incubated for 4 days in complete media containing no hormone. 10IU/ml uhCG or 10IU/ml uhCG+rhCG-Ant. Proliferation was assessed with WST-1 reagent.

[0035] Figure 12: rhFSH-Ant enhances cisplatin induced apoptosis in OVCAR-3 cells. Cells were incubated for 24 hrs in 10 μ l cisplatin +/- 1IU/ml rhFSH or rhFSH-Ant. Caspase 3/7 activity was measured with the APO-ONE kit which utilizes a profluorescent substrate.

[0036] Figure 13: rhFSH-Ant enhances cisplatin induced apoptosis in OVCAR-3 cells. Cells were incubated for 24 hrs with 20 μ l cisplatin +/- 100mU/ml rhFSH or rhFSH-Ant. Caspase 3/7 activity was measured with the APO-ONE kit which utilizes a profluorescent substrate.

[0037] Figures 14A – 14B: Cytotoxicity assay. **14A.** HIO-80 cells and **14B.** Human embryonic kidney HEK-293 cells. Cells were incubated with 100mIU of rhFSH or rhFSH-Ant. Toxicity was assessed with WST-1 reagent.

[0038] Figure 15: OVCAR-3 tumor growth in BALB/c *nu/nu* mice.

[0039] Figure 16: Comparison of ovulated oocytes between treatment groups. hCG, as expected, increased the number of oocytes whereas rhCG-Ant dramatically reduced the number.

[0040] Figure 17: Corpus luteum formation was quantified by the presence of ovarian surface stigma. rhCG treatment produced significantly more stigma as compared to rhCG-Ant.

[0041] Figure 18: rhCG-Ant treated embryo morphology. E9.5 embryos are in different developmental stages.

[0042] Figure 19: Vascular permeability (VP) assay confirms treatment with hCG-Ant as monotherapy or following hCG treatment significantly reduced VP associated with high dose PMSG and hCG therapy. * $p < 0.001$ compared to hCG.

[0043] **Figure 20:** Diagram depicting a rhFSH-Ant provided by the invention. Site-directed mutagenesis was used to develop the rhFSH-antagonist with removal of three of the four N-linked glycosylation sites (Asn52, Asn7 and Asn24) to prevent FSH receptor activation. One site, Asn78, on the alpha subunit was left intact to enhance receptor binding. The effect is that the rhFSH-antagonist binds to, but does not activate, the receptor.

[0044] **Figure 21:** The nucleic acid sequence of a recombinant human FSH antagonist (SEQ ID NO:1).

[0045] **Figure 22:** The amino acid sequence of a recombinant human FSH antagonist (SEQ ID NO:2). The Asn residues at amino acid residues 25(7) and 42(24) of the FSH beta subunit and at amino acid residue 213 (52) of the beta subunit are mutated to Lys (K) (large bold type face). Asn 239 (78) of the FSH beta subunit (bold) is not mutated. The N4 linker is shown in italics. The 18 amino acid leader peptide sequence is underlined.

[0046] **Figure 23:** The nucleic acid sequence of an hCG-Ant (SEQ ID NO:3) – yoked hCG β N(13,30)K + α N(52)K. The mutated codons are underlined. (See also U.S. Patent Application Publication No. 2008/0039372, the contents of which are hereby incorporated by reference.)

DETAILED DESCRIPTION OF THE INVENTION

Definitions and Abbreviations

[0047] Numbers provided in parentheses herein are used to identify the corresponding numbered references provided in the Reference List that follows the Examples section of this application.

[0048] The abbreviation “FSH” is used herein to refer to follicle stimulating hormone.

[0049] The abbreviation “CG” is used herein to refer to chorionic gonadotropin.

[0050] The abbreviation “LH” is used herein to refer to luteinizing hormone.

[0051] The abbreviation “GnRH” is used herein to refer to gonadotropin releasing hormone.

[0052] The abbreviation “Ant” is used herein to refer to an antagonist. For example, FSHAnt or FSH-Ant refers to a follicle stimulating hormone antagonist.

[0053] The letter “h” is used herein to designate a human form of a protein or amino acid or nucleotide sequence. For example “hFSH” refers to human follicle stimulating hormone.

[0054] The lower case letter “r” is used herein to designate a recombinant form of a protein or amino acid or nucleotide sequence. For example rhFSH refers to recombinant human follicle stimulating hormone.

[0055] The upper case letter “R” is used herein to refer to a receptor. For example FSHR or FSH-R refers to a follicle stimulating hormone receptor.

[0056] The letter “y” is used herein to refer to a “yoked” protein or to nucleotide sequences that encode a “yoked” protein. A “yoked” protein is one in which alpha and beta subunit sequences are joined in a single peptide chain. For example, y-hCG refers to a yoked form of human chorionic gonadotropin in which the alpha and beta subunits of hCG are linked in a single peptide chain.

[0057] The nouns “tether,” “linker” and “yoke” are used interchangeably herein to refer to a structure used to join the alpha and beta subunits of a gonadotropin, such as hCG or FSH, such that they comprise a single chain. Typically the structure is a peptide chain.

[0058] The abbreviation “OHSS” is used herein to refer to ovarian hyperstimulation syndrome.

[0059] As used herein the term “about” is used herein to mean approximately, roughly, around, or in the region of. When the term “about” is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term “about” is used herein to modify a numerical value above and below the stated value by a variance of 20 percent up or down.

[0060] Other abbreviations and definitions are provided herein in the context in which they are used and/or are well known in the art.

Structure and glycosylation of gonadotropins

[0061] Glycoprotein hormone family members (TSH, LH, FSH and hCG) share a common α -subunit and differ in their hormone-specific β -subunit. Oligosaccharides on these glycoprotein hormones are important for many functions including proper folding, secretion, receptor binding and bioactivity (3). The most common carbohydrate chain added during post-translational processing is the N-linked carbohydrate. The recognition signal for N-linked glycosylation is the tri-peptide sequence Asn-X-Thr/Ser where X can be any amino acid residue except proline (4).

[0062] The common α -subunit contains two N-linked oligosaccharide chains at asparagine (Asn) residues 52 and 78. The β -subunit of hCG contains two N-linked oligosaccharide chains at Asn residues 13 and 30 as well as four O-linked sugars on the CTP. The O-linked oligosaccharides account for the long elimination half-life of hCG in humans (around 31 hours) compared to the other glycoprotein family members (around 11 hours) by reducing glomerular

filtration within the kidney (5). The β -subunit of FSH contains two N-linked oligosaccharide chains at Asn residues 7 and 24 (10, 15).

[0063] Chemical or genetic removal of the O-linked carbohydrates of hCG revealed that hCG was still able to bind to its receptor and stimulate biological activity, however, the half-life was significantly reduced (6, 7). In contrast, the N-linked oligosaccharides were found to be essential for hCG function. Loss of the β -chain N-linked carbohydrates at Asn 13 and 30 of hCG has been shown to reduce β -subunit secretion and dimerization (8) and diminish receptor activation. On the α -subunit, Asn 78 has been found to be involved in dimerization and to enhance receptor binding, whereas Asn 52 has been found to be essential for maintaining protein integrity within the cell and assisting in steroidogenesis and secretion (9). Importantly, Asn 52 of hCG has been found to be critical for normal receptor activation (10).

[0064] FSH mutant constructs lacking oligosaccharide attachments at any one of the Asn residues on the α - or β -subunit have similar affinities for the FSHR as wild-type constructs (16, 17). However, the N-linked oligosaccharides are important for FSHR activation. Specifically, carbohydrates at Asn 52, Asn 7 and Asn 24 are essential for signal transduction and steroidogenesis, while Asn 78 has only a minor role in FSHR activation. Previous studies have demonstrated that removing the oligosaccharide chains from both the α - and β -subunit produces the lowest bioactivity.

FSH Antagonists

[0065] In some embodiments, the present invention provides FSH antagonists, compositions comprising FSH antagonists, and methods of use of FSH antagonists. Any agent that has FSH antagonist activity can be used. For example, the FSH antagonist can be a small molecule FSH antagonist or a protein or peptide-based FSH antagonist. In preferred embodiments, the FSH antagonist is a modified form of FSH that binds to the FSH receptor but that either does not activate the FSH receptor or has reduced ability to activate the FSH receptor as compared to unmodified FSH. In preferred embodiments the modified forms of FSH have reduced N-linked glycosylation as compared to the naturally occurring form of FSH. N-linked sugars can be removed chemically, for example by chemical or enzymatic methods. Alternatively, N-linked glycosylation can be removed or reduced using recombinant DNA technology to disrupt N-linked glycosylation sites. Removing three of the four N-linked glycosylation sites of FSH (Asn residue 52 of the alpha subunit and at Asn residues 7 and 24 of the beta subunit) while leaving

Asn residue 78 of the alpha subunit intact results in a reduction in bioactivity without a reduction in receptor binding affinity (8, 15, 18), essentially converting FSH from an agonist to a potent antagonist (10, 15). Such mutant forms of FSH are suitable FSH antagonists for use in accordance with the present invention. In one embodiment, the present invention provides recombinant human FSH antagonists ("rhFSH-Ants") and methods of use of such antagonists.

[0066] In one embodiment, the invention provides a rhFSH-Ant comprising an FSH beta subunit and an FSH alpha subunit, wherein one or more of the N-linked glycosylation sites at Asn 7 and Asn 24 of the beta subunit and Asn 52 of the alpha subunit are disrupted to prevent or reduce N-linked glycosylation. In some embodiments one or more of these Asn residues is mutated to a lysine (Lys) residue. This mutation can be referred to as an Asn-to-Lys mutation or substitution. In one embodiment the rhFSH is not mutated at Asn 78 of the alpha subunit such that this N-linked glycosylation site is intact. In some embodiments, the FSH antagonists of the invention comprise an FSH beta subunit linked to an FSH alpha subunit. In one embodiment, the C-terminus of the beta subunit is linked to the N-terminus of the alpha subunit by a linker. In another embodiment, the C-terminus of the alpha subunit is linked to the N-terminus of the beta subunit by a linker. Such FSH antagonists wherein the alpha and beta subunits are linked are referred to herein as "yoked" antagonists. The linker can be any suitable structure. In some embodiments the linker is a peptide sequence. In some embodiments, a peptide linker can be used that itself comprises glycosylation sites, such as N-linked glycosylation sites. The inclusion of glycosylation sites within the linker can be useful to increase the protein's half-life (70, 78). . In one embodiment the linker comprise 4 N-linked glycosylation sites, referred to herein as an "N4 linker". In one embodiment, the rhFSH-Ant comprises a leader peptide comprising a signal sequence that targets the polypeptide for excretion or secretion from the cell. In one embodiment, the rhFSH-Ant comprises half-life increasing moiety. For example, in one embodiment, the CTP domain of hCG or sequences encoding N-linked carbohydrate sites, such as the N4 linker described herein, may be used to increase the half life of the FSH antagonists. For other exemplary half-life extending moieties that can be used in accordance with the present invention, see U.S. Patent Nos. 7,202,215 and 7,081,446, the contents of which are hereby incorporated by reference.

[0067] SEQ ID NO:2 (Figure 22) provides the amino acid sequence of an exemplary FSH antagonist having the beta subunit mutated to disrupt the N-linked glycosylation sites at

asparagine residues 7 and 24 of the FSH beta subunit (corresponding to asparagine residues 25 and 42 of SEQ ID NO:2) and the alpha subunit mutated to disrupt the N-linked glycosylation site at asparagine 52 of the FSH alpha subunit (corresponding to asparagine residue 213 of SEQ ID NO:2). The exemplary FSH antagonist of SEQ ID NO: 2 retains the N-linked glycosylation site at asparagine 78 of the alpha subunit (corresponding to asparagine residue 239 of SEQ ID NO:2) in non-mutated form. The exemplary FSH antagonist of SEQ ID NO:2 comprises a leader peptide (underlined in Fig 22) that begins with the methionine residue at position 1 and ends with cysteine residue at position 18. The leader peptide is a signal sequence that targets the polypeptide for excretion from the cell. The exemplary FSH antagonist of SEQ ID NO:2 also contains an N4 peptide linker - shown in italics in Figure 22- which begins with the glycine residue at position 130 and ends with serine residue at position 161 of SEQ ID NO:2. The mutated Asn residues in the alpha and beta subunits are depicted as a substituted Lys residue at position 25 of SEQ ID NO:2 (corresponds to Asn 7 in non-mutated beta subunit), a substituted Lys at position 42 of SEQ ID NO:2 (corresponds to Asn 24 in non-mutated beta subunit), and a substituted Lys at position 213 of SEQ ID NO:2 (corresponds to Asn 52 in non-mutated alpha subunit). Non-mutated Asn 78 on the alpha subunit corresponds to residue 239 of SEQ ID NO:2. The nucleotide sequence that encodes the exemplary FSH antagonist of SEQ ID NO. 2 is provided by SEQ ID NO:1, illustrated in Figure 21.

[0068] In the exemplary FSH antagonist of SEQ ID NO. 2, the α - and β -subunits are tethered using a peptide linker and 4 glycosylation sites (N4) are located within the tether (see Fig. 20) to increase the protein's half-life (70, 78).

[0069] In one embodiment, the present invention provides an FSH antagonist comprising the amino acid sequence of SEQ ID NO:2, or an amino acid sequence having at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:2. In preferred embodiments, the amino acids at positions corresponding to residues 25, 42 and 213 of SEQ ID NO:2 are not asparagine residues, and the amino acids at the position corresponding to residue 239 of SEQ ID NO:2 is an asparagine residue. In one embodiment, the asparagine residues in the N4 linker (residues 130-161 of SEQ ID NO:2) are not mutated. In one embodiment, FSH antagonists of the invention that are based on the sequence of SEQ ID NO: 2 can comprise various amino acid changes including, but not limited to, conservative amino acid changes. A conservative amino acid change involves, for example, substitution of an original amino acid

with an amino acid that is similar based on charge, polarity or structure. For example, conservative substitutions can be made between members within each of the following groups of amino acids: (1) amino acids with nonpolar, aliphatic R groups (glycine, alanine, valine, leucine, isoleucine and proline); (2) amino acids with aromatic R groups (phenylalanine, tyrosine and tryptophan); (3) amino acids with polar, uncharged R groups (serine, threonine, cysteine, methionine, asparagine and glutamine); (4) negatively charged R groups (aspartate and glutamate); and (5) amino acids with positively charged R groups (lysine, arginine and histidine).

[0070] The present invention also provides an FSH antagonist encoded by the nucleotide sequence of SEQ ID NO:1, or a nucleotide sequence having at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:1. Changes to the nucleotide sequence of SEQ ID NO. 1 may or may not result in changes in the amino acid sequence encoded. For example, because of the degeneracy of the genetic code, it is possible to make some mutations in the nucleotide sequence of SEQ ID NO. 1 that will not alter the amino acid encoded.

[0071] All of the methods described herein production of FSH and hCG antagonists can be used interchangeably. For example modifications such as linkers or half-life increasing moieties, and the various mutations and chemical or enzymatic modifications described for FSH antagonists can also be applied to hCG antagonists and vice versa.

hCG Antagonists

[0072] In some embodiments, the present invention provides hCG antagonists, compositions comprising hCG antagonists, and methods of use of hCG antagonists. hCG antagonists that can be used in accordance with the present invention are described in U.S. Patent Publication No. 2008/0039372, the contents of which are hereby incorporated by reference.

[0073] hCG is a placental hormone that maintains the steroid secretions of the corpus luteum during early pregnancy. It is a member of a family of glycoprotein hormones that are dimers formed from the noncovalent association of alpha subunits common to all members of the family and distinctive beta subunits that designate the target specificity of the different hormones. Both subunits are heavily glycosylated. The 92-residue alpha-chain (GenBank Accession No. 1HRPA) contains two sites of N-linked glycosylation, and the 145 residue beta-chain (GenBank Accession No. 1HRPB) has two N-linked glycosylation sites and four O-linked glycosylation sites on its unique carboxy-terminal extension (Pierce et al., Annu Rev Biochem 50, 465-495

(1981)). The O-linked glycosylation sites are not involved in the bioactivity of hCG. (Matzuk et al. "The biological role of the carboxyl-terminal extension of human chorionic gonadotropin beta-subunit." *Endocrinology*, 1990 January; 126(1):376-83.) Carbohydrate content in native hCG accounts for 30 to 35% of its 38,900 Da of molecular mass (Kessler et al., *J. Biol. Chem.* 254, 7901-7908 (1979)).

[0074] Any agent that has hCG antagonist activity can be used. For example, the hCG antagonist can be a small molecule hCG antagonist or a protein or peptide-based hCG antagonist. In preferred embodiments, the hCG antagonist is a modified form of hCG that binds to the hCG receptor but that either does not activate the hCG receptor or has reduced ability to activate the hCG receptor as compared to unmodified hCG. In preferred embodiments the modified forms of hCG have reduced N-linked glycosylation as compared to the naturally occurring form of hCG.

[0075] hCG which has a reduced number of carbohydrate residues relative to naturally occurring hCG binds strongly to its receptor, but adenylate cyclase activation and steroidogenesis are greatly impaired unless the alpha-chain is glycosylated at its first N-linked site (Matzuk et al., *J Biol Chem* 264, 2409-2414 (1989)). However, hCG deglycosylated by neuraminidase treatment retains significant biological activity (Moyle et al., *J Biol Chem* 250, 9163 (1975)), while hydrogen fluoride ("HF") treated hCG binds to the receptor but lacks biological efficacy (Chen et al., *J Biol Chem* 257, 1444 (1982)). It was found that HF-hCG could not activate cAMP or testosterone production in intact rat Leydig cells. Cyclic AMP activity assays are used to measure the biological activity of hCG.

[0076] In some embodiments, the present invention provides hCG antagonists that have a reduced number of carbohydrate residues relative to naturally occurring hCG. In one embodiment, the hCG antagonists comprise a reduced number of N-linked carbohydrates relative to naturally occurring hCG. In one embodiment, the hCG antagonists have their alpha and beta subunits linked together (fused, or yoked). In one embodiment, the hCG antagonists have three of the four N-linked carbohydrate sites disrupted by site directed mutagenesis. The N-linked carbohydrates that may be disrupted in the hCG antagonists of the present invention include sialic acid residues, N-acetylglucosamine residues, N-galactosamine residues, galactose residues, mannose residues, and fucose residues. The hCG antagonists of the present invention may have at least 50% to 90% less carbohydrates than naturally occurring hCG and has about 75% less carbohydrates than naturally occurring hCG. In particular, the hCG antagonists may have at least

55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97% or 99% less carbohydrates than naturally occurring hCG. This hCG antagonists can then be used as an effective hCG antagonist in vivo.

[0077] In one embodiment, hCG antagonists of the present invention may be produced in a host cell that is glycosylation deficient. "Glycosylation deficient" as used herein is defined as lacking the glycosylation machinery and/or ability to properly glycosylate hCG. For example, the glycosylation deficient host cell may lack a N-acetylglucosaminyl transferase enzyme. It will be appreciated by those of skill in the art that any suitable glycosylation deficient host cell may be used in the present invention. Non-limiting examples include Chinese hamster ovary ("CHO") cells, bacterial cells, yeast cells (e.g. *picchia* cells), and insect cells (e.g. insect cells that can be infected by a baculovirus). In one embodiment, the hCG antagonists of the present invention are produced by a Chinese hamster ovary (CHO) cell lacking the appropriate glycosylation machinery. For example, Lec 1 is a CHO cell line that is deficient in the enzyme N-acetylglucosaminyltransferase I (GlcNAc-TI) (Stanley et al., Cell 6, 121-128 (1975); Stanley, P., Glycobiology 2, 99-107 (1992)); therefore, it can be used to create deglycosylated hCG. Lec1 cells are unable to synthesize complex or hybrid-type N-linked glycans as they are unable to initiate the conversion of oligomannosyl to complex N-linked glycans. The lack of GlcNAc-TI in the Lec1 mutant is not lethal to CHO cells and enables researchers to use Lec1 cells to produce recombinant glycoproteins with truncated, simple oligomannosyl N-glycans (Stanley, P., Glycobiology 2, 99-107 (1992); Butters et al., Protein Sci. 8, 1696-1701 (1999)). Therefore, according to the present invention, the Lec1 cell line may be used to produce hCG antagonists.

[0078] In one embodiment hCG antagonists are made by removing N-linked sugars from hCG chemically or enzymatically, for example using hydrogen fluoride (HF) treatment, neuraminidase treatment, tunicamycin treatment, or treatment with endoglycosidases such as Endo H and Endo F. Suitable methods for treating hCG with HF are described in Chen et al., J Biol Chem 257, 1444 (1982), the contents of which are incorporated by reference. Suitable methods for treating hCG with neuraminidase are described in Moyle et al., J Biol Chem 250, 9163 (1975), the contents of which are incorporated by reference.

[0079] In one embodiment, hCG antagonists are made by disrupting N-linked glycosylation sites of hCG using recombinant DNA technology, such as site-directed mutagenesis. Removing three of the four N-linked glycosylation sites of hCG (Asn residue 52 of the alpha subunit and at

Asn residues 7 and 24 of the beta subunit) while leaving Asn residue 78 of the alpha subunit intact results in a reduction in bioactivity without a reduction in receptor binding affinity, essentially converting hCG from an agonist to a potent antagonist. Such mutant forms of hCG are suitable hCG antagonists for use in accordance with the present invention. In one embodiment, the present invention provides recombinant human hCG antagonists (“rhCG - Ants”) and methods of use of such antagonists.

[0080] In some embodiments, the present invention provides deglycosylated or partially deglycosylated recombinant hCG antagonists made using recombinant DNA technology techniques such as site directed mutagenesis. Such recombinant hCG antagonists, and methods of making them, are described in U.S. Patent Publication No. 2008/0039372, the contents of which are hereby incorporated by reference.

[0081] In some embodiments, the present invention provides recombinant hCG antagonists wherein the beta subunit is mutated to disrupt one or more of the N-linked glycosylation sites at asparagine (Asn) residue 13 of the hCG beta subunit, Asn residue 30 of the hCG beta subunit, and/or Asn 52 of the hCG alpha subunit, or at amino acid positions that correspond to one of these three N-linked glycosylation sites. In some embodiments all three of these N-linked glycosylation sites are mutated. In some embodiments, the the N-linked glycosylation site at Asn 78 of the alpha subunit, or the amino acid position that corresponds thereto, is not mutated.

[0082] In some embodiments one or more of the three Asn residues is mutated to a lysine (Lys) residue. Such a mutation is referred to herein as an Asn-to-Lys mutation or substitution.

[0083] In some embodiments, the hCG antagonists of the invention comprise an hCG beta subunit linked to a hCG alpha subunit. Such hCG antagonists wherein the alpha and beta subunits are linked are referred to herein as “yoked” antagonists. In one embodiment, the beta subunit is linked via its C-terminal end to the N-terminal end of the alpha subunit. In another embodiment, the alpha subunit is linked via its C-terminal end to the N-terminal end of the beta subunit. In some embodiments the alpha and beta subunits are linked directly with no intervening structure. In preferred embodiments, the alpha and beta subunits are joined via a intervening structure referred to as a linker.

[0084] The linker can be any suitable structure. In some embodiments the linker is a peptide sequence which forms a peptide linker. In some embodiments, a peptide linker can be used that

itself comprises glycosylation sites, such as N-linked glycosylation sites. The inclusion of glycosylation sites within the linker can be useful to increase the protein's half-life (70, 78).

[0085] The hCG antagonists of the invention can also include additional moieties capable of increasing a protein's half-life. For example, in one embodiment, the CTP domain of hCG or sequences encoding N-linked carbohydrate sites, such as the N4 linker described below, may be used to increase the half life of the hCG antagonists. For other exemplary half-life extending moieties that can be used in accordance with the present invention, see U.S. Patent Nos. 7,202,215 and 7,081,446, the contents of which are hereby incorporated by reference.

[0086] SEQ ID NO:3 (Figure 23) provides a nucleotide sequence that encodes an exemplary hCG antagonist having the beta subunit mutated to disrupt the N-linked glycosylation sites at asparagine residues 13 and 30 and the alpha subunit mutated to disrupt the N-linked glycosylation site at asparagine 52. The exemplary hCG antagonist encoded by SEQ ID NO: 3 retains the N-linked glycosylation site at asparagine 78 in non-mutated form. The exemplary hCG antagonist encoded by SEQ ID NO:3 also contains linker sequence joining the alpha and beta subunits within a single peptide chain.

[0087] In one embodiment, the present invention provides a hCG antagonist encoded by the nucleotide sequence of SEQ ID NO:3, or a nucleotide sequence having at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:3. Changes to the nucleotide sequence of SEQ ID NO. 3 may or may not result in changes in the amino acid sequence encoded. For example, because of the degeneracy of the genetic code, it is possible to make some mutations in the nucleotide sequence of SEQ ID NO. 3 that will not alter the amino acid encoded.

[0088] The exemplary hCG antagonist encoded by SEQ ID NO:3 has the following characteristics: 1) the alpha and beta subunits are tethered together, 2) Asn 78 is not mutated (this residue can be important for dimer formation and receptor binding) and 3) Asn 52, 13 and 30 are mutated to prevent receptor (LHR) activation. Data presented in the Examples section of this application demonstrates that the recombinant hCG antagonist encoded by SEQ ID NO:3 is able to bind to, but not activate, the LHR and that the antagonist competitively blocks rhCG activity in an *in vitro* system - confirming that recombinant hCG antagonist is capable of antagonizing native hormone.

[0089] All of the methods described herein for production of FSH and hCG antagonists can be used interchangeably. For example modifications such as linkers or half-life increasing moieties, and the various mutations and chemical or enzymatic modifications described for hCG antagonists can also be applied to FSH antagonists and vice versa.

Methods of Producing FSH and hCG antagonists

[0090] The FSH and hCG antagonists described herein can be made using standard molecular biology techniques for the production of recombinant proteins. For example, in one embodiment an FSH or hCG antagonist of the invention can be made by transforming a host cell with a vector comprising a nucleic acid encoding FSH or hCG antagonist. In one embodiment the FSH and/or hCG antagonists of the invention are present in an expression vector. The term "expression vector" refers to a plasmid, virus or other vehicle known in the art that can be manipulated by insertion or incorporation of a nucleic acid encoding a FSH or hCG antagonist of the invention. Expression vectors can comprise a selectable marker to ascertain successful incorporation of the intended nucleic acid. Suitable selectable markers include, but are not limited to, green fluorescent protein (GFP), antibiotic resistance genes, such as for ampicillin, tetracycline, neomycin, zeocin, and/or hygromycin resistance genes, and recessive markers such as thymidine kinase (TK), dihydrofolate reductase (DHFR), adenine phosphoribosyl transferase (APRT) and/or hypoxanthine phosphoribosyl transferase genes. Nucleic acid sequences encoding the FSH and/or hCG antagonists of the invention can be operatively linked to expression control sequences including, but not limited to, promoters, enhancers, transcription terminators, a start codon (i.e., ATG), splicing signals, a stop codon, and leader sequences. Nucleic acid sequences encoding the FSH and/or hCG antagonists of the invention can also be operatively linked to other sequences, including, but not limited to, sequences that encode markers or tags, such as those that can be used to facilitate detection and/or purification of the FSH and/or hCG antagonists. Examples of such markers or tags include, but are not limited to fluorescent tags (such as GFP), and myc, T7, GST, HA (hemagglutinin), V5, His, and FLAG tags. In preferred embodiments, the FSH and hCG antagonists of the invention are isolated/purified prior to use. For example, the FSH and hCG antagonists of the invention may be purified from the supernatant of cells that express FSH and hCG antagonists from an expression vector. Standard methods for purifying proteins may be used, including, but not limited to affinity chromatography methods.

Chemotherapeutic Agents

[0091] In some embodiments, the present invention provides compositions comprising an FSH antagonist and a chemotherapeutic agent. In other embodiments, the present invention provides methods of treatment that comprise administration of an FSH antagonist and a chemotherapeutic agent. In one embodiment, the chemotherapeutic agent is selected from the group consisting of bleomycin, daunomycin, 5-FU, cytosine arabinoside, colchicine, cytochalasin B, daunorubicin, neocarcinostatin, suramin, doxorubicin, carboplatin, taxol, mitomycin C, vincristine, vinblastine, methotrexate, and cisplatin, and analogues, variants, or derivatives thereof.

[0092] In one embodiment, the chemotherapeutic agent is an alkylating agent. In one embodiment, the chemotherapeutic agent is a platinum-based chemotherapeutic agent. In one embodiment, the chemotherapeutic agent is cisplatin or a cisplatin analogue.

[0093] Cisplatin, which is also referred to as cis-PtCl₂(NH₃)₂, cisplatinum, cis-diamminedichloroplatinum(II) (CDDP), Platinol®, and Platinol®-AQ, is a platinum-based alkylating agent used to treat various types of cancers, including sarcomas, some carcinomas (e.g. small cell lung cancer, and ovarian cancer), lymphomas, and germ cell tumors. Cisplatin can cause crosslinking of DNA which ultimately triggers apoptosis.

[0094] Cisplatin and cisplatin analogues include, but are not limited to, carboplatin, ormaplatin, tetraplatin, oxaliplatin, DWA2114R, enloplatin, lobaplatin, CI-973 [NK-121], 254-S, JM-216, and liposome-entrapped cis-bis-neodecanoato-trans-R,R-1,2-diaminocyclohexane platinum (II) [LNDDP]. For further cisplatin analogues that can be used in accordance with the methods of the invention see, for example, Weiss et al. "New cisplatin analogues in development. A review" *Drugs*. 1993, 46(3): 360-77, and Murray et al. "Interaction of 11 cisplatin analogues with DNA: characteristic pattern of damage with monofunctional analogues." *Biochimica et Biophysica Acta/Gene Structure and Expression*, 1997, 1354(3): 261-271, the contents of which are hereby incorporated by reference. Other cisplatin analogues are known to those of skill in the art and encompassed by the present invention.

Compositions

[0095] In some embodiments, the present invention provides pharmaceutical compositions comprising an FSH and/or an hCG antagonist of the invention. The pharmaceutical composition can be, for example, an aqueous solution, a non-aqueous solution, a suspension or an emulsion. The pharmaceutical composition can comprise, in addition to the FSH and/or an hCG antagonist,

one or more other pharmaceutically acceptable components including, but not limited to, solvents (such as aqueous or non-aqueous solvents), diluents, carriers, vehicles, excipients, surfactants, adjuvants, preservatives, stabilizers, wetting agents, emulsifying agents, antibacterial agents, antifungal agents, sugars, salts, agents that promote sustained release of the active compounds, agents that facilitate or limit absorption, and the like. One of skill in the art can readily select suitable agents for inclusion in the pharmaceutical compositions of the invention, for example by consulting "Remington's Pharmaceutical Sciences", Gennaro, A.R., 18th Edition, Mack Publishing Co., Easton, PA, the contents of which are hereby incorporated by reference.

[0096] In some embodiments, the present invention provides combination compositions comprising an FSH and/or an hCG antagonist of the invention and one or more additional active agent. In some embodiments, the combination compositions of the present invention comprise an FSH antagonist and an hCG antagonist. In another embodiment, the combination compositions of the present invention comprise an FSH and/or an hCG antagonist and a chemotherapeutic agent. In another embodiment, the combination compositions of the present invention comprise an FSH antagonist, an hCG antagonist, and a chemotherapeutic agent. FSH antagonists, hCG antagonists, and chemotherapeutic agents that can be used in accordance with the present invention are described above. In other embodiments, the combination compositions comprise an FSH and/or an hCG antagonist, and a contraceptive agent, such as an oestrogen- or progesterone-based contraceptive agent.

[0097] In some embodiments, the compositions of the present invention contain an effective amount of the active agent(s). An effective amount of an FSH antagonist can be an amount that is sufficient to bind to the FSH receptor and to reduce FSH activity by competitive inhibition, i.e. an FSH-antagonizing amount. One of skill in the art can readily determine such amounts, for example by using the methods described herein, such as in the Examples, to measure receptor binding and/or to measure the amount of FSH receptor activation (for example as indicated by cAMP levels, PI3-kinase activity, or Akt kinase activity). An effective amount can be an amount that is sufficient to reduce FSH activity completely, or an amount that is sufficient to reduce FSH activity by about 98% or more, or by about 95% or more, or by about 90% or more, or by about 80% or more, or by about 70% or more, or by about 60% or more, or by about 50% or more, or by about 40% or more, or by or by about 30% or more, or by about 20% or more. In one embodiment, an effective amount of an FSH antagonists is an amount ranging from about 3,000

IU to about 10,000 IU; or from about 2,500 IU to about 9,500 IU; or from about 3,500 IU to about 9,000 IU; or from about 4,000 IU to about 8,500 IU; or from about 4,500 IU to about 8,000 IU; or from about 5,000 IU to about 7,500 IU; or from about 5,500 IU to about 7,000 IU; or from about 6,000 IU to about 6,500 IU; or from about 2,000 IU to about 12,000 IU. The Examples section of this application provides cell lines and assays that can be used to determine an effective amount of an FSH antagonist of the invention. An effective amount of an FSH antagonist can also be determined by performing experiments in an animal, such as in a mouse or in a primate and by then scaling the dose to provide an amount that would be effective in a human, according to normal pharmaceutical practice. The effective amount for any individual subject may also be adjusted based on factors such as, for example, the subject's weight, age, health, and other factors.

[0098] An effective amount of an hCG antagonist can be an amount that is sufficient to bind to the hCG receptor and to reduce hCG activity by competitive inhibition. One of skill in the art can readily determine such amounts, for example by using the methods described herein, such as in the Examples, to measure receptor binding and/or to measure the amount of hCG receptor activation (for example as indicated by cAMP levels, PI3-kinase activity, or Akt kinase activity). An effective amount can be an amount that is sufficient to reduce hCG activity completely, or an amount that is sufficient to reduce hCG activity by about 98% or more, or by about 95% or more, or by about 90% or more, or by about 80% or more, or by about 70% or more, or by about 60% or more, or by about 50% or more, or by about 40% or more, or by or by about 30% or more, or by about 20% or more. In one embodiment, an effective amount of an hCG antagonist is an amount ranging from about 3,000 IU to about 10,000 IU; or from about 2,500 IU to about 9,500 IU; or from about 3,500 IU to about 9,000 IU; or from about 4,000 IU to about 8,500 IU; or from about 4,500 IU to about 8,000 IU; or from about 5,000 IU to about 7,500 IU; or from about 5,500 IU to about 7,000 IU; or from about 6,000 IU to about 6,500 IU; or from about 2,000 IU to about 12,000 IU. The Examples section of this application provides cell lines and assays that can be used to determine an effective amount of an hCG antagonist of the invention. An effective amount of an hCG antagonist can also be determined by performing experiments in an animal, such as in a mouse or in a primate and by then scaling the dose to provide an amount that would be effective in a human, according to normal pharmaceutical practice. The effective amount for

any individual subject may also be adjusted based on factors such as, for example, the subject's weight, age, health, and other factors.

[0099] In embodiments directed to combination compositions, an effective amount of the active agent may be lower than the amount that would be effective in a non-combination composition. For example, the inventors have discovered that FSH-antagonists and chemotherapeutic agents, such as cisplatin, can have a synergistic effect and/or that an FSH antagonist can increase the efficacy of a chemotherapeutic agent, such as cisplatin. In such situations, a lower amount of the chemotherapeutic agent and/or the FSH-antagonist can be used in a combination composition than might be needed in a non-combination composition. Similarly, FSH-antagonists and hCG-antagonists can have a synergistic effect. In such situations, a lower amount of the FSH antagonist and/or the hCG-antagonist can be used in a combination composition than might be in a non-combination. One of skill in the art can readily determine the amount of each active agent to use, for example by using the methods described herein, such as in the Examples, to measure receptor binding and/or to measure the amount of hCG and/or FSH receptor activation (for example as indicated by cAMP levels, PI3-kinase activity, or Akt kinase activity) following administration of a combination composition, and/or by by using the methods described herein, such as in the Examples, to measure the efficacy of a chemotherapeutic agent, for example by measuring tumor cell apoptosis, tumor cell viability, tumor cell proliferation, tumor growth, tumor size, angiogenesis, tubal formation, and the like. The Examples section of this application provides cell lines and assays that can be used to determine an effective amount of active agents in a combination composition of the invention.

Methods of Treatment

[00100] In some embodiments, the present invention provides methods for inhibiting FSH activity. In other embodiments, the present invention provides methods for inhibiting tumor cell growth, and/or methods of treating cancer, including, but not limited to methods for treating ovarian epithelial cancer and/or other FSH-associated tumors. In other embodiments, the present invention provides methods for enhancing the efficacy of chemotherapeutic agents, including cisplatin and cisplatin analogues. In other embodiments, the present invention provides methods for treating ovarian hyperstimulation syndrome. In other embodiments, the present invention provides contraceptive methods. All of the above are referred to as methods of treatment herein.

[00101] The methods of treatment of the invention involve administration of an FSH antagonist and/or an hCG antagonist, either alone or in combination with one or more additional agents such as hormones and/or chemotherapeutic agents, as described herein. Also contemplated by the present invention are methods that comprise administration or delivery of a nucleic acid that encodes an FSH antagonist (and/or an hCG antagonist), either alone or in combination with one or more additional agents such as hormones and/or chemotherapeutic agents, as described herein. The compositions of the invention may be administered by any suitable route, including by systemic or local administration. Contemplated delivery routes include, but are not limited to, intravenous delivery, intra-arterial delivery, intranasal delivery, subcutaneous delivery, transdermal delivery, oral delivery, and any other suitable delivery route known in the art. In preferred embodiments, the compositions of the invention may be administered locally to the desired site of action, such as to the ovaries or to an ovarian tumor. The compositions of the invention can be administered locally by local injection, or by implantation of a preparation (such as a slow or sustained release hydrogel, or a suppository, or a cream) or a device (such as a drug-releasing pump or a drug-coated device) that comprises a composition of the invention, at the desired site. In such local delivery embodiments, any method known in the art suitable for local delivery of a therapeutic agent to the ovaries or to an ovarian tumor may be used. In some embodiments, preparations and devices comprising the compositions of the invention of the invention may be implanted locally at the desired site of action by a doctor. One of skill in the art can readily select a suitable means of administration of the compositions of the invention without undue experimentation.

[00102] In one embodiment, the subjects to be treated using the compositions and methods of the invention can be suffering from, or at risk of developing, a condition associated with excessive FSH or hCG activity, or a condition that is triggered by or associated with FSH or FSH or hCG activity. Examples of such conditions include, but are not limited to, FSH-associated tumors, such as cancer of the ovary, and in particular ovarian epithelial cancer. Other such conditions include OHSS. In other embodiments, the subjects to be treated using the compositions and methods of the invention can be subjects in need of a contraceptive agent.

[00103] Unless stated otherwise, the terms “treat” “treated” or “treatments” include curative methods, methods aimed at reducing the severity and/or duration of symptoms, methods of preventing symptoms, methods of reducing the recurrence of symptoms, and in the case of

contraceptives, methods of inhibiting or reducing the incidence of pregnancy, or methods of inhibiting ovarian follicle development, or inhibiting ovulation, or inhibiting male spermatogenesis.

[00104] The subjects to be treated in accordance with the present invention may be any human or other mammalian species. In preferred embodiments, the subjects are humans. In certain embodiments, the subjects are female humans that are afflicted with, have previously been afflicted with, or are at risk of developing, an FSH or hCG related condition, such as ovarian cancer or OHSS, or that are in need of a contraceptive agent.

SEQUENCE LISTING

[00105] The instant application contains a Sequence Listing which has been submitted electronically, and which is hereby incorporated by reference in its entirety .

[00106] The following Examples further illustrate certain embodiments of the present invention. These Examples are set forth to aid in the understanding of the invention, and should not be construed to limit in any way the scope of the invention as defined herein.

EXAMPLES

[00107] Numbers provided in parentheses herein are used to identify the corresponding numbered publications found in the Reference List that follows these Examples.

EXAMPLE 1: PRODUCTION AND TESTING OF RECOMBINANT HCG ANTAGONISTS

[00108] A yoked form of hCG (y-hCG) comprising the alpha and beta subunits joined in a single peptide chain was produced. The coding sequence of y-hCG was contained within the baculovirus expression vector pVL1393. The insert encodes the leader peptide and mature β -chain sequence of human chorionic gonadotropin followed by the coding region of the mature α -chain. The two coding regions are linked together, in frame, by the hexanucleotide, GAATTC. The y-hCG insert was excised from pVL1393 and sub-cloned into the mammalian expression vector, QCXIN (BD Biosciences Clontech, Palo Alto, CA). The expression vector was transfected into CHO cells using standard techniques. For protein production, transfected cells were grown in suspension cultures as previously described (70). Y-hCG analogues were purified by adsorption and elution from an hCG monoclonal antibody (B107) (71) column that was

prepared by coupling 5 mg purified B107 to 1ml CNBr-Sepharose-4B (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions (71). After applying the cell supernatant, the column was washed in 50 bed volumes of PBS followed by 2 bed volumes of distilled water. The y-hCG analogues were eluted in 3–4 bed volumes of 1 M acetic acid and immediately dried *en vacuo* in a Speed-Vac (Savant Instruments, Holbrook, NY). This analogue was important for demonstrating that the reduced biological effects associated with the rhCG-Ant were not attributable to merely linking the α - and β -chains of hCG, but rather were a result of the reduction of the carbohydrate content of the protein.

[00109] An original experimental design was to produce an hCG antagonist by transfecting the y-hCG construct into CHO-lec1 cells (Pamela Stanley, Albert Einstein University), which are CHO cells lacking *N*-acetylglucosaminyltransferase I (GlcNAc-TI) activity and thus do not synthesize complex or hybrid N-glycans. Western blot analysis confirmed the y-hCG produced from CHO-lec1 cells did contain less carbohydrate than y-hCG produced in CHO-K1 cells (Fig 1). Binding studies established that y-hCG-lec1 did bind rhLH/CGRs (Fig 2A). However, y-hCG-lec1 had significant residual hCG activity as determined by a cAMP assay using CHO cells transfected with rhLH/CGRs (CHO-LHR) (Fig 2B). In contrast, hydrogen fluoride (HF) treated hCG (chemical deglycosylation) was unable to stimulate activity. Thus, simply utilizing CHO-lec-1 cells to produce a potential rhCG-Ant was not used in the experiments described herein.

[00110] Site-directed mutagenesis was used to selectively disrupt the signal sequence for N-linked glycosylation at sites Asn 13 and 30 on the β -subunit and Asn 52 on the α -subunit, carbohydrates essential for receptor activation (rhCG-Ant). This construct retained the glycosylation site Asn 78, which is important for receptor binding (18). Acquisition of specific mutations was monitored at each step by dideoxy DNA sequencing. The α - and β -subunit were physically linked (yoked) as a single contiguous protein to assist in purification. The resultant sequence, r-hCG β -N13K, N30K + α -N52K was subcloned into the expression vector pQCXIN (Clontech Laboratories, Inc., Mountain View, CA). Clonal cell lines that expressed the construct were prepared by introducing the plasmid into the Chinese hamster ovary cell line, CHO-K1 by transfection using FuGENE 6 transfection reagent (Roche Diagnostics, Inc., Indianapolis, IN). Clonal cell lines containing the plasmid were obtained by limiting dilution in the presence of the selection agent G-418. Western blot analyses confirmed the reduced carbohydrate content of rhCG-Ant as compared to y-hCG-lec-1 (Fig 1).

In Vitro Binding and Receptor Activation Assays for rhCG-Ant

[00111] Using CHO cells expressing the LH/CG receptor (CHO-LHR) receptor binding and activation studies were performed (72). Both rhCG and rhCG-Ant bind with equal affinity to the hLH/hCGR (Fig. 2A). *In vitro* bioactivity studies with CHO-LHR cells reveal that while hCG is able to activate the LH/CGR as indicated by an increase in intracellular cAMP levels, rhCG-Ant was unable stimulate cAMP levels above baseline (Fig. 2B). These data indicate that rhCG-Ant is able to bind but not activate the LHR.

In Vitro Antagonist Assays for rhCG-Ant

[00112] *In vitro* antagonist assays for rhCG-Ant were performed. Using CHO-LHR cells, activation studies were performed in which rhCG and rhCG-Ant were added to the cell mixture. Ratios of rhCG-Ant were evaluated to determine what was required to limit activation by a high dose of rhCG. A dose of 10,000mIU/mL of rhCG was added in conjunction with rhCG-Ant (mIU/mL) at 1X (10,000), 1.5X (15,000), 2X (20,000) and 3X (30,000). Fig 3A demonstrates that even when rhCG-Ant was administered at an equal dose to rhCG (10,000mIU/mL) there was a decrease in cAMP induction. The cAMP production decreased as rhCG-Ant concentration increased. Based upon these data the cAMP receptor activation study was repeated with a range of rhCG (3; 10; 30; 100; 300; 1000; 3,000; 10,000; 30,000; 100,000; 300,000) and 3X more rhCG-Ant (range 9-900,000mIU/mL). As seen in Fig 3B, rhCG-Ant significantly inhibited rhCG at 30mIU/mL and continued to inhibit to the highest dose, 300,000mIU/mL. Based upon these studies, rhCG-Ant is able to antagonize cAMP production associated with LH/CGR activation. In a non-cancer study, the hCG-Ant has been used in rats as a potential new treatment for Ovarian Hyperstimulation Syndrome (OHSS) to antagonize the deleterious angiogenic effects of the hCG given to trigger final oocyte maturation (73).

[00113] As described above, when CHO-LHR cells are exposed to hCG in the presence of rhCG-Ant, there is a significant reduction in hCG-stimulated cAMP production. While a ratio of 1:1 rhCG and rhCG-Ant was effective in reducing activity, a ratio of 1:3 rhCG to rhCG-Ant appeared to promote the largest reduction in cAMP production. These experiments can be repeated to obtain further support for these results. The efficacy of hCG-Ant can also be evaluated at different hCG to rhCG-Ant ratios. For example, the following hCG to rhCG-Ant ratios can be performed: 2:1, 3:1 and 4:1 ratios to determine the minimum amount of rhCG-Ant required to produce a reduction in cAMP production. Furthermore, experiments can be designed

to determine whether larger amounts of rhCG-Ant (1:4, 1:5 or 1:10) can completely abrogate cAMP production.

[00114] Common pharmacological gonadotropin preparations such as urinary hCG, urinary menotropins and PMSG (pregnant mare serum gonadotropin) are also able to activate the CHO-LHR cells due to the presence of LH or hCG in the preparations. Thus, the invention provides methods to determine if rhCG-Ant is capable of limiting or inhibiting the LH activity of these commonly used gonadotropin therapies.

[00115] CHO-LHR cells can be cultured and treated with urinary hCG, menotropin or PMSG at a high dose of (1,000 mIU/mL) using methods of the invention. Concurrently the following therapies can also be examined: 1,000IU/mL gonadotropin analogue plus the rhCG-Ant at 1,000 mIU/mL(1X), 2,000 mIU/mL(2X), and 3,000 mIU/mL(3X). If required, additional ratios of gonadotropin to rhCG-Ant can be evaluated. Once the optimal ratio for reducing cAMP production is identified for each analogue, the different gonadotropin therapies can be studied at a broad range of concentrations, from 1 to 300,000mIU/mL with concomitant ratio of rhCG-Ant to determine the capacity of rhCG-Ant to limit gonadotropin activity across multiple concentrations.

In Vitro Evaluation of rhCG-Ant Activity on the PI3-Kinase Pathway

[00116] In vitro assays were performed to evaluate the effect of hCG-Ant on the PI3-kinase pathway. The LH/CGR can activate two divergent intracellular secondary messenger pathways, cAMP and PI 3-kinase (74). The secondary messenger for the PI 3-kinase pathway is phosphorylation of Akt. CHO-LHR cells were cultured and treated with gonadotropin analogues at a concentration of 1,000mIU/mL. Twenty minutes later cells were harvested and the Akt kinase assay kit was employed for evaluating the PI 3-kinase pathway. Experiments were carried out to assess whether FSH and LH can activate the PI3 kinase pathway in CHO-FSHR and CHO-LHR, respectively, by evaluating phosphorylation of the downstream messenger Akt. Both hCG and FSH induced PI 3-kinase activity, for their respective receptors (Fig 4A and B). Next, OVCAR-3 cells were evaluated, 5,000 cells/well were plated in a 96 well plate and incubated overnight in complete medium (Ham's F12, 10% FCS, 400ug/ml G418 and antibiotics). Cells were then serum-starved by washing and incubating for 4 hours in serum free medium. Medium was replaced with serum-free medium +/- 100mIU/ml rhCG and incubated for varying timepoints (Fig 4C). Cells were immediately fixed by replacing the medium with 4%

formaldehyde in TBS and incubating for 20 min at room temperature. Amounts of total Akt and phosphorylated Akt (pAkt) were estimated in each well using a cell-based ELISA (CELISA) according to the manufacturer's instructions (Millipore, Billerica, MA). The highest ratio of Akt was observed at the 10 minute incubation timepoint (Fig 4C). Experiments can be performed to optimize dose and incubation times before the antagonists are evaluated. In addition, experiments can be carried out to evaluate whether known PI 3-kinase and Akt kinase inhibitors suppress activation to ensure that rhCG-Ant and rhFSH-Ant suppress the intracellular pathways to the same degree as small molecule inhibitors.

[00117] CHO-LHR cells can be cultured and treated with gonadotropin analogues at a concentration of 1,000mIU/mL. 30 minutes later cells can be harvested and the Akt kinase assay kit can be employed for evaluating the PI 3-kinase pathway. rhCG-Ant is predicted to completely inhibit the PI 3-kinase activity. These experiments can be performed with PI 3-kinase and Akt kinase inhibitors as controls to ensure that rhCG-Ant suppresses the intracellular pathways to the same degree as small molecule inhibitors.

EXAMPLE 2: PRODUCTION AND TESTING OF RECOMBINANT HUMAN FSH ANTAGONISTS

rhFSH-Ant

[00118] An antagonist expression vector for hFSH was prepared by substituting lysine (Lys or K) codons for the Asn codons for 3 of the 4 native N-linked glycosylation sites in human rhFSH-N4. The rhFSH-N4 construct contains a N4 sequence linking the α - and β -subunits of FSH together. Embedded within the linker sequence is the code for 4 additional N-linked carbohydrates (N4) essential for increasing the half-life of the protein (70). Point mutations were introduced sequentially by site-directed mutagenesis (75) at Asn 7 and Asn 24 in the β -chain sequence and Asn 52 in the α -chain in rhFSH-N4 to inhibit N-linked glycosylation at these sites. Acquisition of specific mutations was monitored at each step by dideoxy DNA sequencing. The resultant sequence, rhFSH-N4 β -N7, 24K + α -N52K (SEQ ID NO:2) was subcloned into the expression vector pQCXIN (Clontech Laboratories, Inc., Mountain View, CA). Clonal cell lines that express the construct were prepared by introducing the plasmid into the CHO-K1 (76) by transfection using FuGENE 6 (Roche Diagnostics, Inc., Indianapolis, IN). Clonal cell lines containing the plasmid were obtained by limiting dilution in the presence of the selection agent

G-418. Ectopic gene expression was detected in supernatants by Western blotting using an α -chain hCG antibody (77).

[00119] CHO cells transfected with the rhFSH-N4-Ant (herein referred to as rhFSH-Ant) were grown in spinners (78, 79). Cell supernatant was collected, concentrated using a SpeedVac and evaluated by Western blot analysis (Fig 5). The decrease in molecular weight of rhFSH-Ant compared to rhFSH-N4 reflects a reduction in the carbohydrate content of the protein.

[00120] In the above protocol, CHO cells stably transfected with the rhFSH-Ant expression vector are grown in culture spinners as previously described (78). Expression levels mandate that cell supernatant must be concentrated 20X on an Amicon concentrator fitted with a 10K cutoff membrane. Affinity purification of rhFSH-Ant from concentrated supernatant was accomplished using an immunoaffinity column (polyclonal anti-RCM α -antibody (77)). After applying the cell supernatant, the column is washed with 50 bed volumes of PBS followed by 2 bed volumes of distilled water. RhFSH-Ant was eluted with 3 or 4 bed volumes of 1 mol/l acetic acid and immediately dried on a SpeedVac concentrator (Savant Instruments, Holbrook, NY, USA). FSH protein concentration were measured using Immulite (Diagnostic Product Corp., Los Angeles, CA) and confirmed with FSH RIA as previously described (70). Protein purity can be confirmed by silver stain. Purified aliquots of the current rhFSH-Ant analogue can be sent for amino acid sequencing and carbohydrate content by MALDI analyses (70, 79).

In vitro binding and receptor activation assays for rhFSH-Ant

[00121] Using CHO-FSHR cells receptor binding and activation studies were performed as previously described (79). As seen in Fig 6A, rhFSH and rhFSH-Ant bind the FSHR with equal affinity. As illustrated in Fig 6B, treatment with rhFSH increased cAMP production while rhFSH-Ant had significantly reduced cAMP production. This demonstrates that rhFSH-Ant binds FSHR with no activation at lower doses and significantly reduced activation at higher doses. Purification of the rhFSH-Ant can remove contaminants within the cell supernatant that may be promoting cAMP production. The rhFSH-Ant of SEQ ID NO:2 may have some residual activity and further modification of the protein construct can be performed.

[00122] RhFSH-Ant can be added to CHO-FSHR cells at increasing protein concentrations and the competitive receptor binding assay can be performed as previously described (79). *In Vitro* binding analyses of rhFSH-Ant has confirmed the rhFSH-Ant provided by the invention has a similar receptor-binding pattern as rhFSH.

[00123] To evaluate rhFSH-Ant receptor activation, purified protein can be incubated with CHO-FSHR cells for 30 minutes, cells can be harvested and the level of cAMP produced can be measured by standard RIA (70). Previous experience with the cAMP assay demonstrates a sigmoidal increase in cAMP production with increasing concentrations of FSH. Preliminary data shows that rhFSH-Ant induces lower levels of cAMP. Experiments can be carried out to identify an analogue that does not activate FSHR and does not increase cAMP production even at high concentrations.

In vitro evaluation of rhFSH-Ant antagonism of FSHR activation

[00124] As described for rhCG-Ant in Example 1, rhFSH (1,000mIU/ml) can be administered to CHO-FSHR cells in combination with rhFSH-Ant at a range of concentrations: 1,000mIU/ml (1X), 2,000mIU/ml (2X), 3,000mIU/ml (3X) and 4,000 mIU/ml (4X) to confirm rhFSH-Ant is able to reduce rhFSH activity and potentially identify the optimal ratio of rhFSH-Ant required to maximally inhibit rhFSH activity. Once an optimal ratio has been identified, the experiment can be repeated with a range of rhFSH concentrations: 1 to 300,000 mIU/ml. These studies can be extended to examine the efficacy of rhFSH-Ant to inhibit the following gonadotropin therapies: uhFSH, urinary menotropins and PMSG in CHO-FSHR cells, to confirm rhFSH-Ant is capable of limiting cAMP production promoted by these other FSH species.

In vitro evaluation of rhFSH-Ant activity on PI 3kinase pathway

[00125] To confirm that the PI 3-kinase pathway is activated by rhFSH (87), CHO-FSHR cells can be cultured, gonadotropin hormones can be added to the media for 30 minutes, cells can be harvested and activation of a downstream mediator, Akt, can be measured using an Akt kinase assay kit (Cell Signaling Technology, Danvers, MA). If rhFSH increases Akt kinase activity, one can examine whether all gonadotropin analogues activate this pathway in CHO-FSHR cells and determine if rhFSH-Ant can reduce activation of the PI 3-kinase pathway *in vitro* as monotherapy antagonist to recombinant and urinary gonadotropin preparations.

Half-life evaluation of rhCG-Ant and rhFSH-Ant

[00126] For each analogue, six adult Sprague-Dawley (SD) rats can be dosed with 40IU of antagonist dissolved in sterile 0.9% saline, administered as an IV bolus through the tail vein. The rats can be anesthetized with inhaled isoflurane for blood collection. Serial blood samples can be collected at 0, 1, 30 min, 1, 2, 4, 6, 12, 24, and 48 h. After dosing and serial blood sampling, two blinded observers can be present to record any visible bleeding or behavioral changes

suggesting acute toxicity. The plasma can be collected and stored at -80°C and analyzed by Immulite (Siemens Diagnostics, Deerfield, IL).

[00127] Pharmacokinetic analysis can be performed using data from individual rats for which the mean and standard error of the mean (SEM) can be calculated for each group. The elimination rate constant (KE) can be estimated by linear regression of the blood or plasma concentrations in the log-linear terminal phase. In order to estimate the blood or plasma concentrations (C_0) immediately after treatment dosing, a two-compartmental model can be fitted to the plasma concentration *versus* time data using PK Solutions Version 5.1 (Summit Research Services, Montrose, CO). The estimated C_0 can then be used with the actual measured plasma concentrations to determine the area under the plasma concentration time curve (AUC). The $\text{AUC}_{0-\infty}$ can be calculated using the combined log-linear trapezoidal rule for data from time of dosing to the last measured concentration, plus the quotient of the last measured concentration divided by KE. Non-compartmental pharmacokinetic methods can be used to calculate clearance (CL by dividing dose by $\text{AUC}_{0-\infty}$) and volume of distribution (Vd_β by dividing CL by).

EXAMPLE 3: TUMOR EFFECTS OF HCG AND FSH ANTAGONISTS

[00128] Gonadotropins are emerging as prominent players in cancer biology. Many ovarian cancers (OC) express FSH or LH/CG or both (1). Choriocarcinoma, a uterine tumor in women, and often present in mixed testicular cancer tumors secretes hCG. Moreover, many non-reproductive tumors secrete hCG and express gonadotropin receptors. The role of gonadotropins in promoting tumor growth is not yet clearly defined, but there is evidence to suggest the secreted hormones promote tumor growth, protect tumors from normal apoptotic degradation, and advance tumor invasion (2).

[00129] Currently, the therapies used in the treatment of ovarian epithelial cancer (OEC) are highly toxic and demonstrate only limited efficacy for remission, thus any potential new therapies for controlling this lethal disease need to be aggressively explored. Although, choriocarcinoma is a lethal tumor that does respond to chemotherapy, most survivors are young with long post-treatment life expectancies and the long-term consequences of toxic chemotherapy is an unknown and a source of significant anxiety. The antagonists provided by the invention may be used as adjuvant therapies that can reduce the amount of chemotherapy required to control the disease can minimize their potential long-term negative effects enabling patients a better quality of life.

[00130] OEC is the sixth most common cancer and the fifth leading cause of cancer-related death among women in industrialized countries. The majority of women with ovarian cancer (60–65%) are diagnosed at a stage when the cancer has already metastasized due to an absence of specific symptoms and a lack of reliable screening modalities. Most patients present at stages III and IV classification (International Federation of Gynecology and Obstetrics (FIGO)), which is associated with 5-year survival rates of only 28% and 16%, respectively (19). Initial treatment for OEC is usually surgical cytoreduction followed by adjuvant platinum and taxane chemotherapy. Unfortunately, these regimens are insufficient in treating OEC as survival has improved only modestly over the past two decades. It is clear that the development of novel therapies, both primary and adjuvant, are needed for women with OEC (20).

[00131] A growing body of evidence indicates that reproductive hormones influence the incidence and aggressiveness of OEC (21). The “gonadotropin hypothesis” asserts that the excessive levels of gonadotropins associated with ovulatory surges during the reproductive years and the loss of gonadal negative feedback during the menopause plays an important role in the development and/or progression of OEC (22–27). The hypothesis is supported by the observations that ovarian tumors occur in transgenic or knockout animal models that exhibit high levels of circulating FSH and LH, analogous to the postmenopausal state in women (28–31).

[00132] The gonadotropin hypothesis of OEC is further supported by several epidemiological studies. First, a close temporal association exists between the increased incidence of OEC and the rise in circulating gonadotropins (32). Gonadotropin levels are particularly high 2–3 years after menopause, when concentrations of FSH and LH reach a peak of 10–20 times (50–100 mIU/ml) and 3–4 times (20–50 mIU/ml) the values recorded during the proliferative phase of the menstrual cycle, respectively, after which there is a gradual but slight decline in both gonadotropins (32). In turn, the risk of developing OEC increases during early menarche when ovulation occurs more frequently and late menopause when patients have been exposed to elevated gonadotropins for a prolonged period of time (33, 34). The fact that a history of pregnancy and oral contraceptive use decreases the risk of OEC favors the gonadotropin hypothesis because both factors are associated with lower levels of gonadotropins and inhibition of ovulation (35–37). Moreover, although limited, evidence now suggests that treatment of infertile women with ovulation induction agents elevates their overall exposure to gonadotropins increasing their risk of OEC (38–43).

[00133] At the molecular level, because FSHR expression increases from presumed precursor lesions (ovarian epithelial inclusions) to benign ovarian epithelial tumors to borderline tumors, it appears likely that FSH may play a pivotal role in early tumor transformation (44). Furthermore, overexpression of the FSHR in non-tumorigenic SV40 Tag immortalized OSE cells not only accelerated the proliferation rate of these cells, but also activated the ERK1/2 MAPK pathway leading to an increased expression of several proto-oncogenes including epidermal growth factor receptor (EGFR), c-myc, and HER-2/neu (45). As OECs de-differentiate, the levels of FSHR and LHR expression declined, a common phenomenon in tumorigenesis. However, 94% of borderline tumors and 60% of epithelial carcinomas still expressed high levels of FSHR advocating for FSH activity as a potential target of intervention in OEC therapy (46).

[00134] A primary reason the overall 5-year survival rate for advanced OEC patients is relatively low (20–30%) is due to tumor resistance to conventional cytotoxic drugs such as cisplatin (47, 48). For OVCAR-3 cells (ovarian epithelial adenoma) treatment with cisplatin leads to cell cycle arrest and increased apoptosis. Pre-treatment of OVCAR-3 cells with FSH was found to significantly reduce the effectiveness of cisplatin and was associated with a concomitant reduction in tumor cell apoptosis (49, 50). Thus, the persistent presence of FSH in the OEC environment is potentially limiting the effectiveness of standard OEC chemotherapy. The data presented herein shows that cisplatin accelerated apoptosis of OVCAR-3 cells. Co-administration of rhFSH with cisplatin inhibited apoptosis, thereby improving the survival of the cancer cells. However, when rhFSH-Ant was added to cisplatin, apoptosis increased above the level observed with cisplatin monotherapy. Further experiments can be conducted to determine if rhFSH-Ant can block the survival-promoting effect of rhFSH in the cancer cells. An inhibitor of FSH signal transduction can be a valuable preventive and/or adjunctive treatment in OEC.

[00135] An rhFSH-Ant can be used in OEC patients who had recently completed their surgical debulking. This timing seems most appropriate, in that FSH appears to reduce chemosensitivity (50, 51). The nature and timing of this therapy is especially relevant given that bilateral oophorectomies which accompany the debulking process increase pituitary FSH release due to the removal of any remaining ovarian induced negative feedback from estradiol and inhibin, potentially worsening the prognosis. After conventional chemotherapy, an rhFSH-Ant can be used as a long-term adjunctive agent, analogous to tamoxifen for breast carcinoma, for the suppression of residual OEC. The possibility also exists that an rhFSH-Ant can have a broader

prophylactic application in high risk patients, such as those with BRCA mutations (51) or even the nulliparous (52). The invention provides gonadotropin antagonists that can be used as an adjuvant therapy in cancer patients that have undergone surgical debulking (for example, tumor removal or ovary removal) in order to prevent or delay the recurrence of the tumor.

[00136] Less common gonadotropin-sensitive tumors are those classified as gestational trophoblastic neoplasia (GTN) (53). These tumors secrete hCG, however they secrete multiple forms of hCG including hyperglycosylated, nicked, free β and β -core. These tumors also express the LH/CGR (54). In general these tumors are treatable with tamoxifen chemotherapy, which is relatively safe and preserves future fertility. There is a small group of patients with persistent neoplasia that is more challenging to treat. Initial studies in mice have demonstrated that administration of hCG antibodies can be used for tumor location (55) and limiting tumor growth and metastases (56). Thus, rhCG-Ant may be a potential adjuvant therapy for GTN patients with recurrent or resistant tumors. One of the more challenging tasks for physicians treating these patients is detecting metastases. GTN can become an aggressive tumor with very early distant metastases. For metastases that are not chemosensitive, surgical extraction can be beneficial; however, localization of the metastases is challenging. Because rhCG-Ant binds but does not activate the LH/CGR, it could be labeled with a radioactive marker and transformed into an excellent method for visualizing metastases *via* PET scanning for surgical resection or could even treat such tumors potentially *via* concentrated radioablation as is routinely done with thyroid disease. The invention provides methods for using the gonadotropin antagonists in methods to visualize or target cells expressing gonadotropin receptors. In one embodiment, the antagonist can be labeled with a detectable label to facilitate visualization. In another embodiment, the antagonist can be used to deliver a therapeutic molecule or drug to cells.

[00137] Choriocarcinoma represents the most aggressive variant of testicular non-seminomatous germ cell (GC) tumors in adult males (57). These tumors are highly vascular and exhibit rapid growth with early hematological spread. Thus, it is not unusual for patients to present with metastases at the time of diagnosis. Although common sites for metastases include lung, liver and brain, there are case reports of metastases to other sites such as stomach (58), heart (59), bone (60), uvea (61) and skin (62). More than 90% of non-seminoma germ cell tumors (NSGCT) contain a mixture of various histological types including choriocarcinoma, embryonal carcinoma, teratomas, and yolk sac tumors (63). Syncytiotrophoblast cells within

germ cell tumors can produce hCG and elevated hCG can be detected within both seminoma (30%) and non-seminoma germ cell tumors (50%). However, very high serum concentrations of hCG (over 1,000 IU/L)(64) occur almost exclusively in NSGCT and when found in conjunction with rapid metastases this represents either a pure choriocarcinoma or a mixed testicular germ cell tumor with a choriocarcinoma component (65, 66).

[00138] Surgical and chemotherapy advances in the treatment of disseminated germ cell tumors has dramatically improved patient care with 80% of patients attaining a disease-free status and approximately 70% of patients becoming long-term survivors (67). However, this signals that 20-30% of patients are not responding to current therapies. Certainly one area of concern is choriocarcinoma tumors which can metastasize so rapidly that by the time the cancer is diagnosed the tumor has spread extensively and patients are not strong enough and/or do not have adequate time to respond to current treatments. Therefore, developing a new therapy that can rapidly inhibit the growth of choriocarcinoma tumors would potentially extend the patient's life sufficiently, allowing time to respond to traditional therapies. Following treatment of the initial cancer, there is continued concern for tumor recurrence; therefore, therapy that would reduce the risk of relapse is another important goal in the treatment of patients with choriocarcinoma testicular cancer. Thus, the compositions and methods provided by the invention can be use in targeting tumor hCG production as a mechanism for limiting the growth of choriocarcinoma and preventing tumor recurrence. In patients with high levels of serum hCG, the rate of serum hCG decline is used to monitor the patient's response to chemotherapy (68). Persistent hCG elevation after chemotherapy indicates lingering disease even with no radiological evidence of residual tumor (63). Furthermore, following successful treatment, monitoring hCG levels can help to detect tumor recurrence often before there is any clinical or radiological evidence of relapse (69). rhCG-Ant can also be employed as an adjuvant therapy to allow administration of chemotherapy at lower, less toxic doses. Chemotherapy toxicity is a serious concern for testicular cancer patients even if they are diagnosed early because these patients tend to be young and live long lives. Thus, the long-term consequences of chemotherapy are a significant concern for these patients.

[00139] Numerous cancers secrete gonadotropins or express gonadotropin receptors or both, including, ovarian epithelial cancer (OEC) and gestational trophoblastic tumors, specifically choriocarcinoma. The compositions provided by the invention can be used to confirm that

gonadotropin binding to gonadotropin receptors induces the growth and metastases of the immortalized ovarian cell lines. There are a number of gonadotropin-sensitive ovarian cancer cell lines available for evaluation as well as immortalized ovarian surface epithelial (OSE) lines that can be used in the the methods of the invention to address the importance of gonadotropins to tumor physiology by comparing control groups to experimental groups which are supplemented with rhFSH and hCG with or without their respective antagonists. Tumors of ovarian cancer patients can be screened for the expression of FSHR and LHR to characterize the potential gonadotropin sensitivity of both early and advanced stage tumors of various histological subtypes. The role of hCG can be studied as well as the effect of rhCG-Ant on gestational trophoblast tumors, specifically choriocarcinoma.

In vitro tubal formation assay

[00140] Because an adequate blood supply is necessary for tumor growth and hCG has been reported to enhance blood vessel invasion and proliferation (80) the angiogenic potential of hCG and rhCG-Ant was investigated. The tubal formation assay measures the effect of different growth factors on promoting blood vessel formation within an *in vitro* system (81). hCG was predicted to stimulate tubal formation while the rhCG-Ant would inhibit. A 400 μ l aliquot of collagen gel was added to each well of a 24-well plate and allowed to gel for at least 1 hour at 37°C. After gelation, human umbilical vascular endothelial cells (HUVEC) (82) were plated on the gel ($1-1.2 \times 10^5$ cells/well) in human endothelial SFM Basal growth medium (Invitrogen, Carlsbad, CA) containing 20ng/ml of EGF (Invitrogen, Carlsbad, CA) and incubated at 37°C for 3 hrs. HUVEC were subsequently covered with 400 μ l of collagen gel and further incubated for 3 hrs at 37°C. The gel was then covered with SFM supplemented with 20ng/ml EGF and either 1) hCG, 2) y-hCG in lec1 cells or 3) y-hCG-Ant in lec1 cells. All hCG analogues were added at a concentration of 15 IU/ml. Cells were allowed to grow for 5 days and then evaluated for tubal formation using a phase-contrast microscope (Fig 7). Tubal length was quantified using ImagePro Plus version 4.01 software (Media Cybernetics, Silver Spring, MD).

[00141] The greatest tubal formation was seen with hCG treatment ($831 \mu\text{m}^2 \pm 54$) while the control treatment, EGF, stimulated at a significantly lower level (475 ± 35), as expected. The y-hCG in lec1 cells (see Example 1) did not stimulate tubal formation above the level of EGF confirming that loss of full-length N-linked carbohydrates significantly decreased hCG bioactivity, although y-hCG-lec1 did have moderate activity in the receptor activation assay.

The y-hCG-Ant produced in lec1 cells failed to promote vessel development and inhibited the minimal vessel formation promoted by the presence of EGF, demonstrating a potent antagonism for tumor blood vessel development and tumor formation. These studies can be repeated with rhCG-Ant produced from CHO K-1 cells (see Example 5).

Ovarian cell lines expression of FSH receptors

[00142] Many ovarian cancer cell lines are known to express either FSH, LH or both FSH and LH receptors (1). Examples of cell lines reported to contain FSHR include: HI0-80 and the cancer line SKOV3 (83). Examples of OEC cell lines containing both FSH and LH receptors include: OVCAR-3, OVCA 420, OVCA 429, OVCA 432, OVCA 433, BG1 and CaOV3. Examples of non-reproductive cell lines reported to express LHR include the melanoma lines, MRI-H255 and MRI-H187 (83).

[00143] To confirm ovarian cell lines expression of FSHRs, 4 different lines were cultured: CHO-FSHR, OVCAR-3, OVCAR8 and SKOV3. When cultures reached 80% confluence, cells were harvested, protein extracted and gel electrophoresed. Following transfer, the membrane was probed with FSHR 106.105 monoclonal antibody (Dr. James Dias, Wadsworth Institute, Albany NY). As seen in Fig 8A, all 4 cell lines express the FSHR; the 97kD band represents the mature form, previously reported to have an apparent molecular weight of 87kD (84). Others have confirmed the presence of FSHR in OVCAR-3 and to a lesser degree in SKOV3 (49). These cell lines can be used for the OEC studies testing rhFSH-Ant described in these Examples.

FSH promotes cell proliferation in OVCAR-3 cell line

[00144] To confirm that OVCAR-3 cells were responsive to FSH, the WST-1 cell proliferation assay was performed. Briefly, OVCAR-3 cells were plated at 5×10^3 cells/well in a 96-well plate and exposed to either media or media containing 1000mIU/mL of recombinant- or urinary-hFSH for 4 days. As demonstrated in Fig 8B the OVCAR-3 cells increased proliferation in response to rhFSH and a greater increase with uhFSH. This observation confirms that OVCAR-3 is highly responsive to FSH with an increase in the proliferation of the tumor cells (Fig 8B)

rhCG-Ant toxicity on OVCAR-3 cells

[00145] OVCAR-3 cells were grown to confluency in a 96 well plate in complete medium (Medium 199/MCDB 105, 4% FCS, 10ug/ml insulin and antibiotics). Medium was replaced with 75ul complete medium, 1 or 10IU/ml rhCG or 1 or 10IU/ml rhCG-Ant in the presence (2.5μM) or absence of cisplatin and the plate was incubated at 37°C, 5% CO₂ for 72hr. 10ul of

WST-1 reagent (Roche, Indianapolis, IN) was added and the plate was incubated at 37°C for 2-4 hr. Metabolically active cells converted this substrate to a colored compound and absorbance was measured at 450nm. While rhCG or hCG-Ant treatment had no measurable difference in toxicity (Fig 9), when 2.5µM cisplatin was added in the presence of 10IU of hCG-Ant a slight increase in toxicity was observed (Fig 10). The presence of hormone appears to reduce the efficacy of the cisplatin, as has been reported for other hormones (50).

rhCG-Ant blocks proliferation of JEG-3 choriocarcinoma cells

[00146] A 96 well plate was seeded with 1500 JEG-3 cells/well and allowed to attach for 3 hrs in complete medium (MEM with Earle's salts, non-essential amino acids and pyruvate with the addition of 10% FCS and antibiotics). Medium was removed and replaced with 100ul test solutions in complete medium. Plain medium, 10 IU/ml uhCG and 10 IU/ml uhCG + 100 IU/ml rhCG-Ant were tested. The plate was grown for 96 hr and solutions were changed daily. On the final day, each well received 10ul of WST-1 reagent (Roche, Indianapolis, IN), the plate was incubated for 3-4 hours at 37°C and absorbance was measured at 450nm. While uhCG significantly stimulated proliferation, rhCG-Ant inhibited the proliferation associated with uhCG (Fig 11).

Use of rhCG-Ant to alter expression of tumor invasion genes during *in vitro* tumor proliferation

[00147] The choriocarcinoma cell line, JEG-3, was grown in culture for 3 days in the presence of either hCG or rhCG-Ant. To confirm that treatment with rhCG-Ant could inhibit tumor progression and metastases, JEG-3 cells were harvested and evaluated by RT-PCR for expression of matrix metalloproteinases (MMP)-1 and MMP-7, proteins that facilitate membrane invasion. Exposure to rhCG-Ant reduced expression of MMP-1 (51.95 ± 15.51 vs. 85.99 ± 10.56 ; 40%) and MMP-7 (66.27 ± 13.01 vs. 116.50 ± 16.48 ; 43%) compared to treatment with hCG respectively ($p < 0.05$). Treatment with rhCG-Ant vs. hCG increased the expression of metastin, a repressor of metastases (5.44 ± 2.29 vs. 4.63 ± 1.32 ; +17%). This data shows that hCG antagonism is capable of altering gene expression in a choriocarcinoma cell line resulting in the downregulation of genes associated with tumor invasion. RhCG-Ant may play a role as an adjuvant therapy in hCG sensitive tumors. From these initial studies it is predicted that rhFSH-Ant may similarly inhibit the progression of ovarian cancer

rhFSH-Ant enhanced apoptosis in OVCAR-3 cells

[00148] A black 96 well plate was seeded with 2000 cells/well and allowed to grow for 72 hr in complete medium (RPMI 1640, 20% FCS, 10ug/ml insulin and antibiotics). Medium was removed and replaced with 50ul of test solutions in complete medium. Test solutions were 1) medium only; 2) medium + 10 μ M cisplatin (Sigma); 3) 1 IU/ml rhFSH + cisplatin and 4) 1 IU/ml rhFSH-Ant + cisplatin. The plate was incubated for 24 hr and then apoptosis was assessed by measuring relative caspase 3/7 activity, cysteine aspartic acid specific proteases which are active in apoptosis. Caspase activity was measured with the Apo-ONE kit according to the manufacturer's instructions (Promega, Madison, WI). Briefly, the kit reagent lyses the cells and a profluorescent substrate is converted to a fluorescent molecule by caspase 3/7 and emitted fluorescence at 528 nm is measured using an excitation wavelength of 485nm. Cisplatin therapy alone enhanced apoptosis. In the presence of rhFSH, cisplatin had a minimal effect on apoptosis confirming similar findings by others (50). However, co-administration of cisplatin and rhFSH-Ant increased apoptosis rates to levels higher than observed with cisplatin alone at two different concentrations (Figs 12 (10 μ M) and 13 (20 μ M)). Thus, rhFSH-Ant appears to enhance the potency of this chemotherapeutic agent.

rhFSH-Ant impairs viability of HIO-80 cells

[00149] HIO-80 cells were grown to confluency in a 96 well plate in complete medium (Medium 199/MCDB 105, 4% FCS, 10ug/ml insulin and antibiotics). Medium was replaced with 75ul complete medium and 100mIU/ml rhFSH or 100mIU/ml rhFSH-Ant and the plate was incubated at 37°C, 5% CO₂ for 72hr. 10ul of WST-1 reagent (Roche, Indianapolis, IN) was added and the plate was incubated at 37°C for 2-4 hr. Metabolically active cells converted this substrate to a colored compound and absorbance was measured at 450nm. While rhFSH was comparable to medium alone and had no measurable affect on the HIO-80 cells, rhFSH-Ant killed 60% of the cells (Fig 14A).

Evaluation of rhFSH-Ant toxicity on different cell lines

[00150] All cells were grown to confluency in a 96 well plate in complete medium (Medium 199/MCDB 105, 4% FCS, 10ug/ml insulin and antibiotics). Medium was replaced with 75ul complete medium, 20 or 100mIU/ml rhFSH or 20 or 100mIU/ml rhFSH-Ant and each plate CHO-K1, HEK-293, JEG-3, HIO-80 and OVCAR-3 was incubated at 37°C, 5% CO₂ for 72hr. 10ul of WST-1 reagent (Roche, Indianapolis, IN) was added and the plate was incubated at 37°C for 2-4 hr. Metabolically active cells converted this substrate to a colored compound and

absorbance was measured at 450 nm. While FSH-Ant did significantly kill ovarian cells neither rhFSH or FSH-Ant treatment had a statistically significant effect on the metabolism of the non-ovarian cells HEK-293 (Fig 14B). Therefore FSH-Ant treatment appears to be safe to non-ovarian cells at these concentrations.

In vitro evaluation of rhFSH-Ant and rhCG-Ant on ovarian cancer cell lines – cell growth, inhibition and cytotoxicity assays

[00151] Two ovarian cancer cell lines, OVCAR-3 and OVCAR-8, have been identified that express FSHR as well as LHR and 2 CHO cell lines transfected with hFSHR or hLHR have been produced as non-cancerous controls. An immortalized ovarian epithelial cell line, HIO-80, provides another control cell line. OVCAR-3 is derived from the ascites of a patient resistant to adriamycin, melphalan and cisplatin and expresses both estrogen and FSH receptors, consistent with the tumor being sensitive to hormonal changes (90). OVCAR-8 was derived from ascites cells from a cisplatin-resistant patient. IGROV-1 cells, which are a paclitaxel resistant human ovarian cancer cell line, can also be tested. Each cell line can be propagated in the recommended media.

[00152] To evaluate the effect of different therapies on cell proliferation and apoptosis, cells can be harvested at 80% confluence and plated in 96-well plates at 1×10^4 cells per well in 200 μ l of media per well (91). At 24 hours after plating, each of the cell lines can be treated with either rhFSH, rhFSH-Ant, hCG, rhCG-Ant, paclitaxel, cisplatin, doxorubicin, AzadC or rapamycin (Sigma-Aldrich, St. Louis, MO) at concentrations ranging from 10^{-9} μ g/mL to 1 μ g/mL (92). After a 24h incubation, the Cell Titer Blue cell viability assay (Promega, Madison, WI) can be used to quantify viable cell number by recording fluorescence ($560_{\text{Ex}}/590_{\text{Em}}$) induced by the conversion of a resorufin based reagent to resazurin in a fluorometer. The same cells can then be assessed for caspase 3/7 activity, a marker of apoptosis, by assessing the fluorescence ($485_{\text{Ex}}/527_{\text{Em}}$) one hour after the addition of Apo-One Caspase reagent (Promega, Madison, WI). The IC₅₀s for rhFSH, rhFSH-Ant, hCG, rhCG-Ant and the cytotoxic agents can be established for all cell lines. The above experiment can then be repeated with combination therapies of gonadotropin antagonists and each chemotherapeutic agent, and then the IC₅₀ dose of the therapeutic reagent alone can be compared to the IC₅₀ of gonadotropin antagonists plus chemotherapy to determine if the antagonists are capable of enhancing the cytotoxic effects of these chemotherapeutic agents.

In vitro evaluation of rhCG-Ant on gestational trophoblast cell lines – cell growth inhibition and cytotoxicity assays

[00153] We have identified an hCG producing and hCG sensitive cell line, JEG-3. The effect of hCG on cell proliferation and apoptosis can be evaluated and compared to the effect of treatment with rhCG-Ant. The JEG-3 cells are known to secrete hCG into the culture media so combination therapies with hCG and rhCG-Ant to confirm antagonist activity may not be required since the cells provide an internal control. However, since the secreted hCG is hyperglycosylated (64), combination therapy with other hCG preparations may be tested.

In vitro cell migration assay

[00154] OVCAR-3 and JEG-3 cell lines can be cultured under normal conditions. Migration and invasion assays can be performed as previously described (93). Briefly, 5×10^4 cells can be placed in the upper compartment of a Boyden chamber and allowed to equilibrate for 12 hours. Concentrations of hCG or hCG-Ant for JEG-3 cells or rhFSH and rhFSH-Ant for OVCAR-3 cells can be added to the lower chamber at concentrations ranging from 10-100,000mIU/mL. Chambers can be incubated for 24 hours at 37°C. Non-migrating cells on the upper chamber are removed, the filters are then removed, H&E stained and the numbers of migrating cells on the lower portion of the filter are quantified.

Pattern of FSHR and LHR expression in epithelial ovarian tumors

[00155] The expression of FSHR and LHR in ovarian cancer can be determined as described herein. Standard IHC staining can be performed to detect FSHR and LHR in tissue microarrays. Staining of > 10% of epithelial cells can be considered positive. All samples should be blinded and independently evaluated by two investigators. A third investigator can score discordant cases. This analysis will allow determination of the differential expression of FSHR and LHR in ovarian cancer. Further identification of specific sub-types and tumor stages associated with increased FSHR expression will allow better focused research efforts on tumors most likely to respond to rhFSH-Ant therapy.

Statistical Analysis

[00156] For comparison of two sets having parametric characteristics, univariate two-tailed t-tests can be used. rhFSH-Ant, drug and combinations can be compared with vehicle as a control: each drug alone can then be compared with treatment and in combination. In addition, two-way factorial ANOVA can be used to test for an interaction between rhFSH-Ant and drug being

tested. If an interaction is found and the IC₅₀ is greater than for either treatment alone, the interaction can be classified as synergistic. If an interaction is found and the inhibition is less than for either treatment alone, then the interaction can be classified as antagonistic or competitive. If no interaction is found ($p > 0.05$), but the effect is greater than either treatment alone, then the effect of the combined treatments can be considered additive.

Studies to determine if either rhFSH-Ant or rhCG-Ant can act alone or synergistically to alter cancer growth and metastases in female nude mice

[00157] Xenograph mouse model: OVCAR-3 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a water-saturated atmosphere with 5% CO/95% air. OVCAR-3 cells (5×10^6) were injected ip in 500 μ L of RPMI-1640 medium in the back region of the nude 8-week-old female BALB/c *nu/nu* mice (85). All mice are housed separately and examined twice weekly for tumor development. Abdominal girth was measured every third day to monitor tumor growth and ascites formation. For the studies described in this Example, treatment should not be initiated until tumors reach 2 mm in their longest diameter (Fig 15).

[00158] *In vivo* evaluation of rhFSH-Ant as a novel therapy for OEC: *In vivo* studies on the effect of FSH inhibition can be performed using the OVCAR-3 cell line. OVCAR-3 can be grown to 80% confluence, detached and resuspended to a concentration of 5×10^6 cells/mL. Four week old, athymic, female nude mice (Taconic, Germantown, NY) maintained in a barrier facility can be injected ip with 5×10^6 OVCAR-3 cells. Every day animals can be checked for survival. Every third day the abdominal girth of the experimental mice can be assessed to monitor tumor growth (using a caliper) and ascites formation. Tumor volume can be calculated using the equation $V \text{ (mm}^3\text{)} = A \times B^2/2$, where A is the largest diameter and B is the smallest diameter. Mice can be sacrificed on day 28 to remove the tumor for measuring its weight.

[00159] Using methods of the invention, two sets of *in vivo* experiments can be performed, the first to determine the effect of rhFSH-Ant on limiting tumor growth and promoting regression of established tumors, the second to examine rhFSH-Ant's ability to inhibit initial tumor infiltration and development. To assess the efficacy of rhFSH-Ant on established tumors, animals can receive injections of rhFSH-Ant or control vehicle beginning 14 days after ip tumor injection. To ensure that the physiological effects on tumor are not due to an indirect effect of rhFSH-Ant to block estradiol or progesterone production but rather due to a direct effect of blocking the FSHR,

two additional groups can also be included: treatment with tamoxifen, an anti-estrogen therapy and treatment with RU486, an anti-progesterone therapy. After 28 days of treatment the animals can be sacrificed. Blood can be collected for measurement of serum FSH, estradiol, and progesterone levels. Tumors can be extirpated and weighed and the volume of ascites recorded. A portion of the intra-abdominal tumors can be preserved in paraformaldehyde and a portion of tumor can be snap frozen for RNA analysis. The liver, lung and brains of the animals can be examined for evidence of parenchymal metastases. Tumors can be stained with H&E for histological evaluation. IHC can be performed to assess for cell proliferation with PCNA, apoptosis with TUNEL, blood vessel density with CD31, and VEGF expression. Using RT-PCR the tumors can be probed for expression of FSHR, LH/CGR, VEGF, Metastin, a metastasis suppression gene, and MMP proteins, promoters of basement membrane degradation and thereby tumor infiltration. Treatment with rhFSH-Ant is expected to reduce the amount of tumor extirpated by the greatest amount. While the tamoxifen and RU486 animals may present with smaller tumors than the rhFSH treated animals their tumors are anticipated to still be larger than the rhFSH-Ant group. This molecular analysis can help determine if the reduced tumor growth is secondary to poor vascular development, limited proliferation, increased apoptosis or reduced tissue invasion capacity.

[00160] If decreased vascularization plays a pivotal role in the efficacy of rhFSH-Ant anti-tumor activity, the experiment described above can be repeated and vascular architecture can be examined with FITC-labeled lycopersicon lectin. Mice can be anesthetized and injected with intravenous FITC-labeled lycopersicon lectin (Jackson Immunology Research, West Grove, PA). After 10 minutes. mice are perfused with 4% paraformaldehyde in PBS. Tumors can then be harvested, fixed and immersed in 30% sucrose. Vessel visualization occurs through uptake of fluorescent lycopersicon lectin.

[00161] The data can show that OVCAR-3 cells can be inhibited with a combination of rhCG-Ant and rhFSH-Ant. The above experiment can be repeated with combination therapy using the optimal dose identified by the IC₅₀ studies. Serum can be examined for hormone levels as above.

[00162] Experiments can be designed to assess the ability of rhFSH-Ant to inhibit tumor implantation and development. As before, 4 week old, athymic female nude mice can be injected ip with 5×10^6 OVCAR-3 cells. Daily rhFSH-Ant, tamoxifen or RU486 treatment or

vehicle can begin at the time of OVCAR-3 injection. In this experiment one can look at the interval before the development of a clinically significant tumor. The animals can be assigned randomly for sacrifice on Day 7, 14, or 28. Growth curves can then be constructed for controls and rhFSH-Ant treated animals. An analysis of histology, proliferation, apoptosis and angiogenesis can be performed as described above. Serum can be examined for hormone levels as above.

Effect of an rhFSH-Ant in combination with chemotherapeutic and hormonal agents for OEC:

[00163] *In vivo* studies can be performed using the OVCAR-3 cell line injected into nude mice. Animals can receive injections of rhFSH-Ant in combination with chemotherapeutic/hormonal agent or control vehicle beginning 7 days after ip tumor injection. Dosing of rhFSH-Ant and chemotherapeutic agents can be based on the results of the above *in vitro* IC50 dosing experiments. After 21 days of treatment, on day 28, animals can be sacrificed. Tumors can be extirpated and weighed and the volume of ascites recorded. Proliferation, apoptosis and angiogenesis can be measured as above.

[00164] Survival Studies: As described above nude mice can be inoculated with OVCAR-3 tumor cells. Based on the above *in vivo* experiments an optimal dose of saline, rhFSH-Ant or rhFSH-Ant plus chemotherapeutic agents can be administered into animals daily, beginning on Day 7. The animals can be evaluated daily for survival and the tumor can be measured every 3 days. Animals can not be sacrificed at 21 days as the previous experiments, but instead followed for the length of survival. If animals are observed to be sick or in pain they can be sacrificed to avoid suffering. Kaplan-Meiers curve can be calculated for each group of animals. Animals receiving rhFSH-Ant or rhFSH-Ant plus chemotherapy are predicted to have a longer survival rate than saline treated mice.

[00165] Evaluation of rhCG-Ant capacity to alter choriocarcinoma tumor growth and metastases *in vivo*: As described above, studies to evaluate tumor growth, tumor regression, metastases and survival can be performed in nude mice injected with JEG-3 tumor cells. Again, tamoxifen and RU486 can be used to ensure the alterations in tumor growth are due to a direct effect of rhCG-Ant and not a secondary effect through reduction of steroid hormone production. These tumors are quite aggressive and can develop as rapidly as 2 weeks; thus, the timing for the experiments may require alteration. Patients with low risk gestational trophoblastic tumors are commonly treated with single-agent methotrexate chemotherapy. Methotrexate alone can be

studied and compared in combination with rhCG-Ant to determine if hCG-Ant can improve tumor sensitivity to chemotherapy.

EXAMPLE 4: REPRODUCTIVE EFFECTS OF HCG AND FSH ANTAGONISTS

[00166] The discovery and isolation of the gonadotropin hormones (follicle stimulating hormone (FSH), leutinizing hormone (LH) and human chorionic gonadotropin (hCG)) was fundamental to understanding the control and physiological events of reproduction. FSH was identified for its ability to promote the growth of ovarian follicles, while LH was key for leutiniziation of granulosa cells to support corpus luteum formation (Greenwald GS 1966 Ovarian follicular development and pituitary FSH and LH content in the pregnant rat. *Endocrinology* 79:572-578). The discovery of hCG aided in deciphering why the corpus luteum (CL) persisted during pregnancy but regressed when pregnancy was not achieved (Strott CA, Yoshimi T, Ross GT, Lipsett MB 1969 Ovarian physiology: relationship between plasma LH and steroidogenesis by the follicle and corpus luteum; effect of HCG. *J Clin Endocrinol Metab* 29:1157-1167).

[00167] Molecular and genetic analyses suggest that the activities of FSH and LH are far more complex and interconnected than originally described. For example, according to an early two-cell model of ovarian steroidogenesis, LH receptors (LHR) were located on theca cells and LH secretion initiated steroidogenesis, FSH receptors (FSHR) were located on granulosa cells to promote conversion of androgens to estrogens which was associated with granulosa cell proliferation and antral follicle growth (Liu YX, Hsueh AJ 1986 Synergism between granulosa and theca-interstitial cells in estrogen biosynthesis by gonadotropin-treated rat ovaries: studies on the two-cell, two-gonadotropin hypothesis using steroid antisera. *Biol Reprod* 35:27-36). LHRs are also present on granulosa cells and their expression is induced by activation of FSHR (Ascoli M, Fanelli F, Segaloff DL 2002 The lutropin/choriogonadotropin receptor, a 2002 perspective. *Endocr Rev* 23:141-174). FSH and LH work together to regulate steroid hormone production. In rodents, administration of rhFSH enhanced large follicle development but not concomitant theca development, whereas administration of LH/hCG is associated with thick theca but smaller follicles (Trousdale RK, Pollak SV, Klein J, Lobel L, Funahashi Y, Feirt N, Lustbader JW 2007 Single-chain bifunctional vascular endothelial growth factor (VEGF)-follicle-stimulating hormone (FSH)-C-terminal peptide (CTP) is superior to the combination therapy of recombinant VEGF plus FSH-CTP in stimulating angiogenesis during ovarian

folliculogenesis. *Endocrinology* 148:1296-1305). Optimal estradiol production requires the presence of both FSH and LH in rodents as well as humans.

[00168] Both FSH and LH have been developed into pharmaceutical therapies to enhance reproduction for infertile men and women. Today, administration of exogenous gonadotropins is an essential element in assisted reproduction therapy (ART) for both genders as well as in the agricultural community. However, further research on gonadotropins has also uncovered clinical pathologies associated with endogenous over-expression of the hormones or exogenous administration of high doses of gonadotropin therapies. For example, with polycystic ovarian (PCO) disease there is a disruption of the normal ratio of FSH and LH secreted by the pituitary leading to a higher ratio of LH to FSH. PCO is also defined by a disruption of normal menstrual cycling, with elevated estradiol (E2) multiple cysts in the ovary and infertility (Franks S 1989 Polycystic ovary syndrome: a changing perspective. *Clin Endocrinol (Oxf)* 31:87-120).

[00169] High-dose gonadotropin therapy during ART increases the risk for Ovarian Hyperstimulation Syndrome (OHSS), a rare but serious complication for female infertility patients. Hundreds of clinical protocols to prevent or limit the risk of OHSS have been tested with moderate success. Most protocols include monitoring E2 levels and canceling further FSH administration once a threshold has been exceeded or withholding hCG and “coasting”, or bypassing exogenous hCG by administering GnRH agonist to prompt a smaller, endogenous LH surge (Franks S 1989 Polycystic ovary syndrome: a changing perspective. *Clin Endocrinol (Oxf)* 31:87-120; Whelan JG, 3rd, Vlahos NF 2000 The ovarian hyperstimulation syndrome. *Fertil Steril* 73:883-896). Unfortunately, once hCG has been administered to optimize retrieving large follicles, its effects cannot be reversed should OHSS develop. The current treatment for OHSS is “supportive care”. Thus, developing therapies that can block hCG activity and limit OHSS progression would greatly benefit the reproductive community.

[00170] In the farm community a common and inexpensive gonadotropin therapy employed to enhance fertility is pregnant mare serum gonadotropin (PMSG). Although this gonadotropin is a powerful stimulator of follicle development, inappropriate persistence of gonadotropin activity following ovulation can produce problems such as early corpus luteum (CL) regression, rapid embryo transport through the fallopian tubes, lower implantation rates and overall reduction in fertility. Researchers have attempted, with some success, to reduce unwanted PMSG activity by altering the dosing schedule and administration of PMSG or gonadotropin-specific antibodies

(Pintado B, A G-A, B PL 1998 Superovulatory response of Murciana goats to treatments based on PMSG/Anti-PMSG or combined FSH/PMSG administration. *Theriogenology* 50:357-36; Gonzalez A, Wang H, Carruthers TD, Murphy BD, Mapletoft RJ 1994 Superovulation in the cow with pregnant mare serum gonadotrophin: effects of dose and antipregnant mare serum gonadotrophin serum. *Can Vet J* 35:158-162). These treatments can present concerns for future fertility of the animals. As a result, many ranchers have abandoned PMSG to use pituitary FSH. Unfortunately the pituitary preparation is derived from cows or pigs and there is a significant concern for injecting brain products for fear of spreading prion disease. Recombinant therapies are safer but more costly and for large animals the cost may be prohibitive. Development of a gonadotropin antagonist that can reduce the unwanted activity of PMSG may be a more cost-effective treatment than rhFSH therapy for extended periods.

[00171] The invention provides potent recombinant gonadotropin antagonists and methods to explore their potential for antagonizing endogenous and exogenous gonadotropin activity, the invention will expand the understanding of the complex roles of FSH and LH in reproduction.

[00172] FSH β and FSHR knockout mice demonstrated that FSH is essential for progression from the large pre-antral follicle to the large antral follicle (Matzuk MM 2000 Revelations of ovarian follicle biology from gene knockout mice. *Mol Cell Endocrinol* 163:61-66 ; Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P 1998 Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Natl Acad Sci U S A* 95:13612-13617; Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM 2000 The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology* 141:1795-1803). In the absence of FSH activity, no antral follicles were present. Administration of exogenous gonadotropins stimulated a range of large antral follicles consistent with the ovaries capacity to respond to FSH. However, the adult ovaries were extremely small suggesting that FSH is important earlier in development, thus this model is not definitive for examining the role of FSH in the normal adult. Hypophysectomized adult animals also present problems with understanding the role of FSH and LH in an intact system because of the affect on the thyroid and adrenal axes. Alternatively, rodents with an intact pituitary-gonadotropin axis allow for normal ovarian development and the antagonists can be applied as

physiologic probes to specifically block FSH and/or LH activity at different stages of adult reproduction.

[00173] The LH β and LHR knockout mice had a more limited disruption of follicle development compared to the FSH β deficiency mice (Zhang FP, Poutanen M, Wilbertz J, Huhtaniemi I 2001 Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Mol Endocrinol* 15:172-183; Ma X, Dong Y, Matzuk MM, Kumar TR 2004 Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. *Proc Natl Acad Sci U S A* 101:17294-17299; Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV 2001 Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. *Mol Endocrinol* 15:184-200). Lack of LH gene expression was associated with a loss of final follicle maturation, an increase in the antrum and rapid increase in follicle growth. Lack of endogenous LH surge also limited, but did not fully abrogate ovulation, which was deemed the primary reason for female infertility in these mice. In humans, LH and hCG bind to and activate a common receptor, the LHR. LH is not requisite for normal follicle development, but the LH surge is important for promoting final follicle maturation and ovulation.

[00174] Recently, the GnRH receptor was localized to the rodent ovary and within the ovary a longer transcript of LH was secreted compared to the pituitary LH hormone. These findings indicate that the ovary contains an internal gonadotropin axis for fine-tuning of paracrine gonadotropin activity (Schirman-Hildesheim TD, Gershon E, Litichever N, Galiani D, Ben-Aroya N, Dekel N, Koch Y 2008 Local production of the gonadotropic hormones in the rat ovary. *Mol Cell Endocrinol* 282:32-38). Studying the effect of rFSH-Ant and rhCG-Ant on rodent folliculogenesis, ovulation and early pregnancy offers a unique opportunity to study the roles of FSH and LH on ovarian function in intact adult animals. Administration of gonadotropin antagonists is predicted to reduce large follicle production, limit antrum formation and prevent ovulation which will confirm the results elucidated with genetically modified mice. However, these studies can also be used to determine if novel alterations of reproduction are elucidated, particularly with regards to expression of other reproductive hormones including estradiol, progesterone, inhibins and activins.

[00175] The compositions and methods provided by the invention can be used to evaluate the effect of exogenous high-dose gonadotropin therapy on reproduction. This will be a useful model

for evaluating changes in the reproductive system associated with ART treatments. OHSS is a serious side-effect of infertility therapies. As presented in Example 29 (also see Fig. 19), rhCG-Ant is shown to be effective at decreasing the vascular permeability (VP) associated with high dose gonadotropin use, using the rat OHSS model.

[00176] In rodents, administration of recombinant FSH as well as PMSG is associated with a significant alteration in fertility. In these studies, rhFSH altered the maternal hormone concentration, changed the uterine environment and delayed fetal development (Kelley RL, Kind KL, Lane M, Robker RL, Thompson JG, Edwards LJ 2006 Recombinant human follicle-stimulating hormone alters maternal ovarian hormone concentrations and the uterus and perturbs fetal development in mice. *Am J Physiol Endocrinol Metab* 291:E761-770). A reduction in implantation as well as fetal development has also been reported (Ertzeid G, Storeng R 2001 The impact of ovarian stimulation on implantation and fetal development in mice. *Hum Reprod* 16:221-225). These studies, however, did not clearly elucidate if the effect of high dose gonadotropins was directly related to gonadotropins acting on the embryo or if it was secondary to adverse effects on CL function or the uterine environment. Studies to evaluate in vitro maturation of the embryos exposed to different levels of gonadotropins hormones will be helpful in addressing the question of direct effect. To assess uterine environment one can conduct a series of embryo transfer experiments to allow embryos collected from hyperstimulated rodents to mature in the uterus of normally cycling adult female surrogate mothers (Kelley RL, Kind KL, Lane M, Robker RL, Thompson JG, Edwards LJ 2006 Recombinant human follicle-stimulating hormone alters maternal ovarian hormone concentrations and the uterus and perturbs fetal development in mice. *Am J Physiol Endocrinol Metab* 291:E761-770; Ertzeid G, Storeng R 2001 The impact of ovarian stimulation on implantation and fetal development in mice. *Hum Reprod* 16:221-225; Van der Auwera I, D'Hooghe T 2001 Superovulation of female mice delays embryonic and fetal development. *Hum Reprod* 16:1237-1243; Long CR, Lamberson WR, Bates RO 1991 Genetic correlations among reproductive traits and uterine dimensions in mice. *J Anim Sci* 69:99-103; Edwards LJ, Kind KL, Armstrong DT, Thompson JG 2005 Effects of recombinant human follicle-stimulating hormone on embryo development in mice. *Am J Physiol Endocrinol Metab* 288:E845-851).

[00177] Administration of antagonists to counter-act the effect of high dose gonadotropin therapies will be essential for understanding the adverse effects on the ovary, CL formation and

uterine environment associated with exogenous gonadotropin use. Studies aimed at evaluating the effect of PMSG will be useful for the farm community which relies on the use of PMSG for ovarian hyperstimulation.

[00178] The invention also provides compositions and methods to examine the potential for rhFSH-Ant or rhCG-Ant or the combination to be a potent method for female contraception. While the studies can be designed towards understanding the early reproductive process, the ultimate goal of reproduction is the live birth of healthy progeny. If rhFSH-Ant and rhCG-Ant are to be developed as therapies for assisting reproduction, effect of these analogues on actual pregnancy rates can be determined. The FSH-Ant and hCG-Ant could be used soon after ovulation to limit continued excessive gonadotropin activity. This short-term use could improve the pregnancy outcome in the same cycle. Thus, studies can be designed to examine the effect of single-dose antagonist administration on pregnancy rates.

[00179] If FSH-Ant and hCG-Ant are able to block antral follicle production, ovulation and early embryo development they potentially could be potent contraceptive agents which will require their examination at a higher dose for a more prolonged period of time. Approximately 6 million women in the U.S. become pregnant every year, but only half are intended. There is an overwhelming need for safe and effective birth control, preferably with tolerable side effects and a simple administration protocol, factors that would greatly increase compliance. Steroid based combination contraceptive pills are the most common form of reversible birth control in the U.S. They are safe and effective, but require daily dosing and often precipitate unpleasant side effects. Moreover, the estrogen component is linked to a small but significant increased risk of thromboembolic phenomena, hypertension or diminished glucose tolerance.

Medroxyprogesterone acetate, a progesterone-only hormone contraceptive therapy is popular because it is administered every 3 months but has even more persistent side effects including irregular bleeding and significant weight gain. Not surprisingly, avoiding or minimizing steroid hormone exposure has been a trend in the evolution of contraception development for the last fifty years.

[00180] Proof of principle has already been established that an FSH antagonist can successfully function as a female contraceptive agent. When a synthetic molecule capable of blocking FSHR activity through a non-competitive pathway was administered to randomly cycling female rats, normal ovulation was inhibited (Arey BJ, Deecher DC, Shen ES, Stevis PE, Meade EH, Jr.,

Wrobel J, Frail DE, Lopez FJ 2002 Identification and characterization of a selective, nonpeptide follicle-stimulating hormone receptor antagonist. *Endocrinology* 143:3822-3829; Arey BJ, Stevis PE, Deecher DC, Shen ES, Frail DE, Negro-Vilar A, Lopez FJ 1997 Induction of promiscuous G protein coupling of the follicle-stimulating hormone (FSH) receptor: a novel mechanism for transducing pleiotropic actions of FSH isoforms. *Mol Endocrinol* 11:517-526). However, the disadvantage of using the synthetic molecule for long-term use in the clinical setting includes, low potency for this molecule and its potentially harmful composition, a derivative of the toxic chemical naphthalene. Therefore, a safe, potent and long-acting FSH antagonist would be an important breakthrough in women's reproductive health. In one embodiment of the invention, an rhFSH-Ant analogue could be administered as a depot-injection like medroxyprogesterone acetate to antagonize FSH activity, inhibit follicular recruitment and ultimately prevent ovulation without the side effects associated with steroid-based therapies.

[00181] The presence and localization of LH/CGRs in human endometrium was first reported in the early 1990's (Bernardini L, Moretti-Rojas I, Brush M, Rojas FJ, Balmaceda JP 1995 Status of hCG/LH receptor and G proteins in human endometrium during artificial cycles of hormone replacement therapy. *J Soc Gynecol Investig* 2:630-635; Bhattacharya S, Banerjee J, Sen S, Manna PR 1993 Human chorionic gonadotropin binding sites in the human endometrium. *Acta Endocrinol (Copenh)* 129:15-19). Subsequent research confirmed the profile pattern of LH/CGR expression by endometrial epithelium correlates with the theoretical timing of the implantation window. Whereas an embryo could potentially be implanted in any human tissue, the endometrium is the only tissue in which implantation can only occur during a finite period. HCG is the signal by which the embryo announces its presence to the maternal organism even before it implants. Current studies suggest that hCG can positively enhance implantation through specific interactions with the LH/CGR at the materno-fetal interface including a coordinated series of cellular events leading to apposition, adhesion and finally invasion of the blastocyst into the uterine wall (Perrier d'Hauterive S, Berndt S, Tsampalas M, Charlet-Renard C, Dubois M, Bourgain C, Hazout A, Foidart JM, Geenen V 2007 Dialogue between blastocyst hCG and endometrial LH/hCG receptor: which role in implantation? *Gynecol Obstet Invest* 64:156-160).

[00182] If the antagonists do not accumulate in the ovary at a high enough concentration to fully inhibit large follicle development, the invention provides that the dosing of the rhFSH-Ant

can be adjusted to identify the optimal dose required to competitively inhibit endogenous FSH. Similar studies can be performed for rhCG-Ant.

[00183] Since hCG has a critical role in implantation, tolerance of the embryo, decidual differentiation and remodeling, as well as in placentation, any therapy that would potentially block LH/CGRs in the endometrium at the time of implantation may lead to a reduction in embryo implantation, placentation or development. Thus, the invention provides methods to determine the effect of the rhCG-Ant on the uterine environment, specifically during the implantation window. The expression profile of LHR distribution can be examined in rodent endometrium and the effect of rhCG-Ant therapy on embryo implantation and fetal development can be determined.

[00184] The compositions and methods of the present invention can be used to elucidate the role of specific gonadotropins during folliculogenesis into pregnancy using two pathways, for example. First, the effect of endogenous gonadotropin activity can be evaluated by using gonadotropin antagonists to block hormone activity at different stages of the reproductive cycle and evaluate the downstream effects on fertility. Activity can be blocked during the follicular phase to evaluate the effect on large follicle growth and ovulation; antagonists can be administered in the peri-ovulatory period to inhibit normal ovulation; and finally antagonists can be administered during the early pregnancy stage to examine the effects on embryo development.

[00185] The use of exogenous hormones to promote fertility is steadily increasing. Thus, the invention provides a gonadotropin antagonist that can be used to limit the activity of exogenous gonadotropins to potentially reduce unwanted side effects from these powerful therapies. For a subset of women, the use of exogenous gonadotropins leads to unwanted and potentially dangerous side effects such as Ovarian Hyperstimulation Syndrome (OHSS). Therefore, one can evaluate the ability of rhFSHAnt and rhCG-Ant to limit the ovarian stimulation effects of exogenous hormone therapies and the potentially harmful uterine side effects stimulated by high dose hormone therapies. Gonadotropin antagonists may provide a unique therapy for female contraception. In contrast to the studies evaluating the effect of antagonist therapy on the reproductive cycle, for the contraception studies, antagonists can be administered for longer durations and at higher doses. One can study the effects of high dose antagonist therapy on implantation, fetal development and pregnancy rates. The invention provides methods to evaluate the efficacy of gonadotropin antagonists compared to other currently utilized hormone

contraception methods. Such methods can be used in proof of principle studies to study the therapeutic potential of the gonadotropin antagonists provided by the invention

Experiments

[00186] Studies were designed to determine the effect of endogenous FSH and LH on folliculogenesis, ovulation, CL function and uterine receptivity during early pregnancy by evaluating the effect of rhFSH-Ant and rhCG-Ant administration in rodents.

Effect of rhCG-Ant on ovarian folliculogenesis

[00187] Day 21 female SD rats were randomized into 3 groups (n=6 per group): 1) Saline; 2) hCG (20IU) or 3) rhCG-Ant (20IU). Rats were injected ip at 0 and 24 hours. At 48 hours, animals were anesthetized, blood collected by cardiac puncture, ovaries extirpated and weighed. One ovary was preserved in 4% paraformaldehyde and then embedded in paraffin, serial sectioned, H&E stained and large antral follicles were quantified. The contralateral ovary was fixed in OCT, frozen, sectioned and used for evaluation of apoptosis. While contraceptive studies have not yet been conducted in female rodents, promising results from a study in male mice offers proof of principle.

Effect of rhCG-Ant on male mouse spermatogenesis

[00188] Adult male C57Bl/6J males age 10 weeks were randomized into 3 groups (n=3 per group): 1) saline; 2) hCG (10IU); and 3) hCG-Ant (10IU). Males were injected daily for a total of 10 days and then sacrificed on Day 11. Testes were removed, weighed, embedded in paraffin and sectioned. At least 3 slides approximately 10 sections apart were H & E stained. There was no significant difference in testicular weight, but there was a trend for increased weight in the hCG treated males. There was no significant difference in testosterone levels between groups, but hCG animals had higher testosterone and interestingly, rhCG-Ant testosterone levels were below saline. Histological evaluations of testicular tubules were staged based on a system previously described (Russell LD, Ren HP, Sinha Hikim I, Schulze W, Sinha Hikim AP 1990 A comparative study in twelve mammalian species of volume densities, volumes, and numerical densities of selected testis components, emphasizing those related to the Sertoli cell. Am J Anat 188:21-3). Fifty tubules per testes were evaluated. Saline treated males, as anticipated, exhibited normal sperm development. The hCG treated animals produced all stages of sperm development; however, the number of spermatocytes per cell was noticeably increased as compared to saline. In contrast, the rhCG-Ant treated males exhibited a blockage of sperm development at step 12

with a noticeable absence of steps 13-16 spermatids. These experiments confirm that the rhCG-Ant is biologically active and disrupting the normal physiology of sperm development in adult male mice.

rhFSH-Ant and rhCG-Ant inhibition of large antral follicle development

[00189] 6-week-old SD female rats can be used. Vaginal smears can be performed to confirm animals are cycling regularly. At the beginning of estrus phase animals can be randomized to one of the follow treatment groups: 1) saline or 2) rhFSHAnt at 20IU. Therapies can be administered at 0 and 24 hours. The animals can be sacrificed at 48 hours, blood collected and ovaries extirpated. One ovary can then be serial sectioned, H & E stained and the number of large antral follicles quantified. Size of the large antral follicles can also be measured using ImagePro Analysis System.

[00190] To determine if the follicles present are healthy or undergoing atresia, the TUNEL assay (Negoescu A, Guillermet C, Lorimier P, Brambilla E, Labat-Moleur F 1998 Importance of DNA fragmentation in apoptosis with regard to TUNEL specificity. Biomed Pharmacother 52:252-258; Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, Brambilla C, Brambilla E 1996 In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem 44:959-968) can be performed on the contralateral ovary to identify apoptotic cells. Follicles with greater than 10 apoptotic granulosa cells can be categorized as atretic follicles. Serum E2 levels can be quantified by Immulite, a proven reliable method for measuring rodent E2 levels (Rodriguez HA, Kass L, Varayoud J, Ramos JG, Ortega HH, Durando M, Munoz-De-Toro M, Luque EH 2003 Collagen remodelling in the guinea-pig uterine cervix at term is associated with a decrease in progesterone receptor expression. Mol Hum Reprod 9:807-813). Serum thyroid hormone levels can also be measured to evaluate the effect of antagonist on thyroid function. RhFSH-Ant is predicted to limit large follicle development, increase follicle atresia and inhibit E2 and progesterone secretion. Serum levels of inhibin and activin can also be measured by ELISA.

[00191] The study can be repeated with the following treatment groups to evaluate the role of LH on large follicle development: 1) Saline and 2) rhCG-Ant (20IU). Injections can be given ip at 0 and 24 hours. Animals can be sacrificed at 48 hours. Previous research confirms that in rodents LH activity is not essential for antral follicle production but is important for E2 production, the final stage of antrum production and pre-ovulatory, rapid growth of large

follicles. Thus, antral follicles are expected to be predominately at the lower end of the antral follicle size spectrum in the rhCG-Ant treated animals. Blocking LH activity is anticipated to inhibit E2 production.

[00192] Ovulation inhibition, in vitro embryo development, corpus luteum formation and endometrial evaluation following rhFSH-Ant and hCG-Ant treatment: Four-week-old C57Bl/6J female mice can be evaluated for regularly cycling by vaginal smears. At the initiation of the proestrus stage, mice can be randomized to receive either saline or FSH-Ant (5IU) and then randomized again to receive either hCG or hCG-Ant at 46 hours creating 4 treatment groups: 1) saline followed by hCG; 2) saline followed by rhCG-Ant; 3) rhFSH-Ant followed by hCG; or 4) rhFSH-Ant followed by rhCG-Ant. Females can be mated with males of proven fertility overnight and the following morning assessed for the presence of vaginal coital plugs, sacrificed and their ovaries and fallopian tubes extirpated and placed into culture media. The ovaries and uterus can be weighed. The ovaries can be examined for the presence of stigma (marker of ovulation and CL formation) and then both ovary and uterus can be preserved in 4% paraformaldehyde for histological evaluation. Ovaries can be examined for the presence of CL formation by IHC with PECAM to confirm normal vascular development and TUNEL stain to evaluate for early CL regression. IHC can also be performed on the uterus to characterize FSHR and LHR in the endometrium and alterations of receptor localization following treatment with antagonists.

[00193] The fallopian tubes can be flushed and ovulated eggs collected and counted. The eggs can then be cultured for 96 hours in appropriate media to determine how many eggs progress to the blastocyst stage indicative of fertilization and normal early embryo development. Saline followed by hCG therapy results in the release of approximately 5 eggs per mouse while rhFSH followed by hCG stimulates the release of approximately 10 eggs per mouse (Trousdale RK, Yu B, Pollak SV, Husami N, Vidali A, Lustbader JW 2008 Efficacy of native and hyperglycosylated follicle-stimulating hormone analogs for promoting fertility in female mice. Fertil Steril Feb 2. [Epub ahead of print]). The saline plus rhCG-Ant mice are predicted to release zero eggs. Based on the prediction that rhFSH-Ant can limit large follicle development; zero eggs are expected to be released following rhFSH-Ant therapy whether it is followed by hCG or rhCG-Ant. If any eggs are released following either rhFSH-Ant or rhCG-Ant or both, they are not expected to progress to blastocysts.

Ability of the rhFSH-Ant and rhCG-Ant to limit the effect of exogenously administered gonadotropins on folliculogenesis, ovulation, CL function and uterine receptivity during early pregnancy in rodents

[00194] Ovarian folliculogenesis studies: Day 21 female SD rats were randomized into 2 groups (n=5 per group): 1) rhFSH+hCG or 2) rhFSH+hCG+rhCG-Ant. rhFSH and hCG were injected ip at a dose of 20IU at 0 and 24 hours; rhCG-Ant was injected at 2X dose (40IU) at 0 and 24 hours. At 48 hours, animals were anesthetized, blood collected by cardiac puncture, ovaries extirpated and weighed. One ovary was preserved in 4% paraformaldehyde and then embedded in paraffin, serial sectioned, H&E stained and then large antral follicles were quantified. The contralateral ovary was fixed in OCT, frozen, sectioned and used for evaluation of apoptosis. HCG is essential for late antral follicle development. As expected, administration of rhCG-Ant did not completely inhibit the development of large antral follicles, but did reduce the number of follicles advancing to the large follicle stage and increased the rate of follicle atresia consistent with antagonistic activity for rhCG-Ant.

[00195] Ovulation and Corpus Luteum Development: 21d old female Wistar rats (n=9) were treated with 10 IU of PMSG. 48 hrs after injection, rats were randomized to receive one of the following treatments: 1) 0.1 mL of saline (control), 2) hCG (10IU), or 3) rhCG-Ant (10IU). 16 hrs after treatment animals were sacrificed, blood collected and the ovaries and fallopian tubes were extirpated.

[00196] To evaluate the effect of hCG on ovulation, fallopian tubes were flushed to remove the ovulated eggs and then placed in hyaluronidase to remove cumulus cells and the number of eggs per ovary was quantified (Fig. 16). Because PMSG has both FSH and LH activity in rodents, even saline-treated animals have some baseline ovulation. Treatment with hCG significantly increased the number of ovulated oocytes whereas treated with rhCG-Ant reduced the number of oocytes to below the level present with saline. This suggests that rhCG-Ant has significantly reduced hCG activity and it may inhibit LH activity associated with PMSG therapy.

[00197] CL formation was evaluated by quantification of ovarian surface stigma (see Fig. 17). The mean stigma per ovary: Saline, 7; hCG, 11.5 and rhCG-Ant, 3. Hormone analysis confirmed that the hCG treated rodents had elevated progesterone levels >7, consistent with ovulation whereas the rhCG-Ant treated rodents had progesterone levels less than 7 consistent with limited or anovulatory levels. Data indicates the stigma reduction is due to blocking ovulation; however

further studies can be conducted to determine if reduced stigma is due to a block in ovulation, poor CL formation, rapid regression of CL or a combination of events.

[00198] In vitro blastocyst formation: 4-week-old female C57Bl/6J mice were treated ip with 5IU of PMSG. 46hrs later mice were divided into 3 treatment groups: 1) hCG (5IU) 2) rhCG-Ant (5IU) or 3) rhCG (5IU followed by rhCG-Ant (5IU) 4 hrs later. Females were mated with males of proven fertility overnight. The following morning, females were sacrificed, eggs retrieved and cultured for 72 hours as previously described (Trousdale RK, Yu B, Pollak SV, Husami N, Vidali A, Lustbader JW 2008 Efficacy of native and hyperglycosylated follicle-stimulating hormone analogs for promoting fertility in female mice. Fertil Steril Feb 2. [Epub ahead of print]). At 72 hours eggs were quantified and embryo stage was determined. With hCG treatment a mean of 11.8 eggs per mouse progressed to at least the 4 cell stage as compared to 1.08 eggs per mouse with rhCG-Ant therapy. Whereas treatment with hCG followed by rhCG-Ant did not reduce the mean number of eggs per mouse progressing to at least the 4 cell stage, 9.9, which was statistically equivalent to treatment with hCG alone.

[00199] Evaluation of rhCG-Ant on early embryo development in vivo: 4-week-old female CD1 mice were treated with 5IU of PMSG. 46hrs later mice were divided into 3 treatment groups: 1) hCG (5IU); 2) hCGAnt LD (5IU); or 3) hCG-Ant HD (60IU). Females were allowed to mate with male CD1 mice of proven fertility overnight and checked for plugs the next morning. At embryo day (ED) 9.5, females were sacrificed, uteri were extirpated and implantation sites were quantified and embryos were collected. In the hCG group the mean number of implantation sites per mouse was 22, for the LD rhCG-Ant group the mean was 15, while in the HD rhCG-Ant group the mean implantation sites was only 3. More importantly, the embryos in the hCG treatment group were all at a similar stage of development. In contrast, the embryos in the rhCG-Ant group were at a wide range of developmental stages and many of the implantation sites demonstrated evidence of embryo resorption (Fig. 18). Based on these limited studies, rhCG-Ant may have an adverse effect on embryo implantation with disruption in normal embryo development.

[00200] Alterations of vascular permeability associated with high dose exogenous gonadotropin treatment: In humans, one risk for exogenous gonadotropin treatment is the development of OHSS. A major contributor to the symptoms of OHSS is increased vascular permeability (VP). The rodent OHSS model employs prolonged, high dose exposure to gonadotropins leading to an

increase in VP. The purpose of this study was to determine if treatment with rhCG-Ant could reduce the VP associated with the high dose gonadotropin treatment.

[00201] Briefly, 21D Wistar rats were hyperstimulated with PMSG for 4 consecutive days (10 IU). On Day 5, rats were treated with either 1) hCG (30 IU), 2) rhCG-Ant (30 IU) or 3) hCG then rhCG-Ant (30 IU) on day 6. On day 7, VP studies were conducted. Animals were anesthetized by isoflurane inhalation. A fixed volume (0.2 mL) of 5 mM Evans Blue (EB) dye diluted in distilled water was injected via the femoral vein. Twenty minutes after dye injection, the peritoneal cavity was filled with 5 mL of 0.9% saline (21C, pH 6) and massaged for 20 seconds. Subsequently, the fluid was extracted gently from the abdominal cavity to prevent tissue or vessel damage. After centrifugation at 900 x g for 12 minutes, EB concentration was measured at 600 nm by spectrophotometer. The level of extravasated dye present in the recovered fluid was expressed as μg per 100 g body weight (Gomez R, Gonzalez-Izquierdo M, Zimmermann RC, Novella-Maestre E, Alonso-Muriel I, Sanchez-Criado J, Remohi J, Simon C, Pellicer A 2006 Low-dose dopamine agonist administration blocks vascular endothelial growth factor (VEGF)-mediated vascular hyperpermeability without altering VEGF receptor 2-dependent luteal angiogenesis in a rat ovarian hyperstimulation model. *Endocrinology* 147:5400-5411; Gomez R, Simon C, Remohi J, Pellicer A 2003 Administration of moderate and high doses of gonadotropins to female rats increases ovarian vascular endothelial growth factor (VEGF) and VEGF receptor-2 expression that is associated to vascular hyperpermeability. *Biol Reprod* 68:2164-2171). As seen in Fig. 19 hCG significantly increased VP compared to saline treated animals. Treatments with rhCG-Ant or hCG followed by rhCG-Ant were both able to significantly reduce the level of VP. RT-PCR also confirmed that VEGF expression, a mediator for VP, was significantly decreased with administration of rhCG-Ant.

[00202] rhFSH-Ant inhibition of large antral follicles: FSH and hyperglycosylated FSH are potent promoters of large ovarian follicle development. When adult female mice or rats are injected with FSH at any point in their reproductive cycle, the exogenous FSH enhances follicle development with a significant increase in large antral follicle production 46-48 hours later. To evaluate the effect of rhFSH-Ant on follicle development day 21 SD female rats (n=7 per group) can be injected at 0 and 24hrs with either 1) rhFSH at 20IU or 2) rhFSH (20IU)+rhFSH-Ant at 40IU (2X rhFSH therapy). The animals can be sacrificed at 48 hours, blood collected and ovaries extirpated. One ovary can then be serial sectioned, H & E stained and the number of large antral

follicles can be quantified. Size of the large antral follicles can be measured using ImagePro Analysis System. To determine if the follicles present are healthy or undergoing atresia, the TUNEL assay (Negoescu A, Guillermet C, Lorimier P, Brambilla E, Labat-Moleur F 1998 Importance of DNA fragmentation in apoptosis with regard to TUNEL specificity. Biomed Pharmacother 52:252-258; Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, Brambilla C, Brambilla E 1996 In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem 44:959-968) can be performed on contralateral ovary to identify apoptotic cells. Serum E2 levels can be quantified by Immulite (Rodriguez HA, Kass L, Varayoud J, Ramos JG, Ortega HH, Durando M, Munoz-De-Toro M, Luque EH 2003 Collagen remodelling in the guinea-pig uterine cervix at term is associated with a decrease in progesterone receptor expression. Mol Hum Reprod 9:807-813). rhFSH-Ant, even if LH is present, is predicted to inhibit the number of ovarian follicles advancing to the antral follicle phase, inhibit serum E2 levels and substantially increase the number of large follicles entering atresia.

[00203] rhCG-Ant inhibition of exogenously administered hCG for ovulation and CL formation in ovarian stimulation protocols: Day 21 C57Bl/6J mice can be treated with rhFSH 5IU ip at 0 and 24 hrs. At 46hrs mice can receive either 1) hCG (5IU) or 2) hCG (5IU)+hCG-Ant (10IU) (2X rhCG). Females can be mated with males of proven fertility overnight. The following morning females can be checked for plugs as confirmation of copulation. Females can be sacrificed; blood collected and ovaries and uterus can be extirpated. Ovarian stigma can be quantified. Ovarian and uterine histology can be characterized as described in the Examples. Serum progesterone can be measured to confirm elevation consistent with ovulation. Fallopian tubes can be flushed to quantify number of eggs ovulated. Eggs can then be cultured for 96 hours to determine the number progressing to the blastocyst stage. The uterus can be sectioned for histopathological evaluation. C/EBP beta has been reported as a possible downstream regulator of hCG and can be assayed for by IHC (Sterneck E, Tessarollo L, Johnson PF 1997 An essential role for C/EBPbeta in female reproduction. Genes Dev 11:2153-2162; Wang W, Bergh A, Damber JE 2007 Increased expression of CCAAT/enhancer-binding protein beta in proliferative inflammatory atrophy of the prostate: relation with the expression of COX-2, the androgen receptor, and presence of focal chronic inflammation. Prostate 67:1238-1246) as well as COX-2 (Motola S, Popliker M, Tsafiriri A 2008 Response of follicle cells to ovulatory stimuli within the

follicle and in primary culture. Mol Cell Endocrinol 282:26-31). Because PMSG is a more potent gonadotropin than rhFSH in mice; the study can be repeated with mice receiving PMSG (5IU) at time 0 followed 46 hours later with either 1) hCG (5IU) or 2) hCG (5IU)+hCG-Ant (10IU).

[00204] Effect of hCG-Ant on in vivo early embryo development following exogenous gonadotropin therapy: As described in Preliminary Data female C57Bl/6J 4 weeks old can be treated with PMSG (5IU), 46 hours later they can be randomized into one of the following groups: 1) hCG (5IU); 2) rhCG-Ant (5IU); 3) hCG (5IU) followed 4 hours later by rhCG-Ant (10IU). Females can be mated with males of proven fertility overnight. The following morning males and females can be separated. On Day ED 9.5, females can be sacrificed; the uterus can be examined for number of implantation sites and early embryo development. Since rhCG-Ant can reduce the number of ovulated eggs, rhCG-Ant animals are anticipated to have fewer implantation sites. Abnormal fetal development is also anticipated. For the hCG followed by rhCG-Ant animals, ovulation is predicted to occur and therefore more implantation sites are expected. Experiments can be designed to evaluate whether rhCG-Ant therapy after hCG can produce similar fetal abnormalities as noted with rhCG-Ant monotherapy or if enough hCG activity can be preserved to support the CL function and allow for normal embryo development.

Evaluation of rhFSH-Ant and rhCG-Ant on pregnancy and a potential role in female contraception.

[00205] Disruption of normal estrus cycle: To determine if administration of rhFSH-Ant or rhCG-Ant alters the natural ovulatory cycle, C57Bl/6J female mice (n=10 per group) age 4 weeks can be treated ip with either: 1) saline; 2) rhFSH-Ant (5IU) or rhCG-Ant (5IU) every 48 hrs for 16 days (approximately 4 estrus cycles). The animals can also undergo daily vaginal smear analyses to determine the length of their reproductive cycle in response to each of the therapies. Mice treated with gonadotropin antagonists are predicted to have significant cycle asynchrony and possible complete cessation of cycling.

[00206] In vivo evaluation of rhFSH-Ant and rhCG-Ant on pregnancy rates following ovarian stimulation protocols: Three studies can be preformed. First C57Bl/6J females (n=20 per group) can be treated with either 1) rhFSH (5IU) or 2) rhFSH-Ant (5IU), 46 hours later mice can be injected with hCG to promote ovulation, mated with males of proven fertility overnight. The following morning males and females can be separated, the females can be observed for 21 days for evidence of pregnancy, delivery of pups, weight of pups and size of litters. In the second

study, C57Bl/6J females can be treated with 5IU of PMSG, 46 hours later the mice can be treated with either: 1) hCG (5IU); 2) rhCG-Ant (5IU) or 3) rhCG (5IU) then 4 hours later rhCG-Ant (10IU). Females can be mated with C57Bl/6J males of proven fertility overnight then observed for 21 days for evidence of pregnancy. For the third study, females can be treated with rhFSH-Ant (5IU), 46 hours later they can be treated with 1) rhCG-Ant or 2) hCG then rhCGAnt 4 hours later, mated overnight and then followed for 21 days for evidence of pregnancy. The groups that receive rhFSH-Ant are anticipated to have lower pregnancy rates compared to rhFSH animals, and the rhCG-Ant group is anticipated to exhibit significantly lower pregnancy rates compared to hCG treated animals. That the combined antagonist treated animals are predicted to have a zero pregnancy rate.

[00207] Embryo transfer experiments: C57Bl/6J donor female mice can undergo ovarian stimulation with PMSG (5IU ip) then treated 46 hours later with either 1) hCG (5IU); 2) rhCG-Ant (5IU) or 3) hCG (5IU) followed 4 hours later by rhCG-Ant (10IU). The females can then be mated with C57Bl/6J males of known fertility. Surrogate mothers in estrus can be mated with vasectomized males to induce pseudopregnancy. On day 4, hCG or rhCG-Ant blastocysts can be transferred into the uteri of wild-type pseudopregnant recipients (Paria BC, Huet-Hudson YM, Dey SK 1993 Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. Proc Natl Acad Sci U S A 90:10159-10162). The surrogate mothers can be followed for evidence of pregnancy including delivery and litter size. Methods can be used to determine if rhCG-Ant has any adverse effect on blastocyst implantation and fetal development. If rhCG-Ant therapy results in a reduction of delivery rate, the study can be repeated and surrogate mothers sacrificed on Day 1, 2, 3, 5, 7, and 14 to evaluate at which time point rhCG-Ant therapy is adversely affecting pregnancy.

[00208] In vivo evaluation of rhFSH-Ant and rhCG-Ant as contraceptive therapy in female mice: To determine if prolonged, high dose exposure to gonadotropin antagonists can act as a long-term contraceptive in females, 90 female C57Bl6J mice 6 weeks of age can be randomized to receive 5IU ip of either 1) saline; 2) rhFSH-Ant or 3) rhCG-Ant for a total of 16 days (approximately 4 estrus cycles). 2 days after initiating hormone therapy the females can be housed with C57Bl/6J males of previously proven fertility. The females can remain with the males for a total of 7 days and then separated and continue to receive hormone treatment for another 2 days. The females can then be observed for evidence of pregnancy including increased

weight gain and delivery of pups. About 85% of control C57Bl/6J females become pregnant after 7 days of co-habitation with males. The pregnancy rate for the antagonist treated females is expected to be zero percent.

[00209] Following demonstration that rhFSH-Ant and rhCG-Ant reduces pregnancy rates, the study can be repeated, but animals can be sacrificed at the following time points following separation from males: 24 hours, 48 hours, 72 hours, day 7, day 14, day 21, and females can undergo an autopsy to determine if the reduction in pregnancy is due to reduction of embryos produced (flushing of fallopian tubes and/or uterine horns), interruption of implantation (Song H, Han K, Lim H 2007 Progesterone supplementation extends uterine receptivity for blastocyst implantation in mice. *Reproduction* 133:487-493) or early fetal demise followed by a formal necropsy with a veterinary pathologist. If warranted, embryo transfer experiments about can be performed.

[00210] Comparison of gonadotropin antagonists to estradiol/progesterone as a contraceptive in mice: To compare the efficacy and safety of rhFSH-Ant and rhCG-Ant compared to currently available contraceptive methods, 60 C57Bl/6J animals can be treated with SC injections of either 1) rhFSH-Ant; 2) rhCG-Ant; 3) combined estradiol/progesterone therapy; or 4) depo-provera daily for 16 days. One week after initiating hormone therapy, females can be housed with males of proven fertility for 7 days as above and followed for evidence of pregnancy. The dose and timing of rhFSH-Ant therapy may be altered for optimal contraceptive capacity based on the results above for half-life and ovulatory disruption studies.

Statistical Analysis:

[00211] For ovarian weights, follicle counts, serum hormone levels, egg retrieval, blastocysts development and pregnancy outcomes, two-way ANOVA analysis for comparison of multiple groups can be performed. For non-parametric data Kruskal-Wallis test can be calculated. All calculations can be performed with Prism (GraphPad Software Inc, San Diego, CA), where P values <0.05 can be considered statistically significant.

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CLAIMS**What is claimed is:**

1. A method for treating an ovarian tumor, the method comprising administering to a subject in need thereof an effective amount of an FSH antagonist and a chemotherapeutic agent, wherein the FSH antagonist comprises:

a) SEQ ID NO:2., or

(b) a variant of SEQ ID NO:2 that binds to the FSH receptor and in which the Asn residues at amino acid positions 25, 42, and 213 have been mutated to prevent N-linked glycosylation,

and wherein the chemotherapeutic agent is cisplatin or a cisplatin analogue.

2. The method of claim 1, wherein the administering occurs after surgical debulking of ovarian tumor cells.

3. The method of claim 1, wherein the cisplatin analogue is selected from the group consisting of: carboplatin, ormaplatin, tetraplatin, oxaliplatin, DWA2114R, enloplatin, lobaplatin, CI-973 [NK-121], 254-S, JM-216, and liposome-entrapped cis-bis-neodecanoato-trans-R,R-1,2-diaminocyclohexane platinum (II) [LNDDP].

4. The method of claim 1, wherein the FSH antagonist is administered concurrently with the chemotherapeutic agent.

5. The method of claim 1, wherein the FSH antagonist is administered before the chemotherapeutic agent.

6. The method of claim 1, wherein the FSH antagonist is administered after the chemotherapeutic agent.

7. The method of claim 1, further comprising administration of an hCG antagonist.

8. The method of claim 7, wherein the hCG antagonist is encoded by SEQ ID NO:3 or a variant of SEQ ID NO:3 that encodes an hCG antagonist that binds to the hCG receptor and in which the Asn residues at amino acid positions 13 and 30 of the beta subunit and position 52 of the alpha subunit have been mutated to prevent N-linked glycosylation.

9. A method for treating an ovarian tumor, the method comprising administering to a subject in need thereof an effective amount of an hCG antagonist and a chemotherapeutic agent, wherein the hCG antagonist is encoded by:

a) SEQ ID NO:3., or

(b) a variant of SEQ ID NO:3 that encodes a protein that binds to the hCG receptor and in which the Asn residues corresponding to amino acids 13 and 30 of the hCG beta subunit, and amino acid 52 of the hCG alpha subunit have been mutated to prevent N-linked glycosylation,

and wherein the chemotherapeutic agent is cisplatin or a cisplatin analogue.

10. The method of claim 9, wherein the administering occurs after surgical debulking of ovarian tumor cells.

11. The method of claim 9, wherein the cisplatin analogue is selected from the group consisting of: carboplatin, ormaplatin, tetraplatin, oxaliplatin, DWA2114R, enloplatin, lobaplatin, CI-973 [NK-121], 254-S, JM-216, and liposome-entrapped cis-bis-neodecanoato-trans-R,R-1,2-diaminocyclohexane platinum (II) [LNDDP].

12. The method of claim 9, wherein the hCG antagonist is administered concurrently with the chemotherapeutic agent.

13. The method of claim 9, wherein the hCG antagonist is administered before with the chemotherapeutic agent.

14. The method of claim 9, wherein the hCG antagonist is administered after the chemotherapeutic agent.
15. The method of claim 9, further comprising administration of an FSH antagonist.
16. The method of claim 15, wherein the FSH antagonist comprises SEQ ID NO:2, or a variant of SEQ ID NO:2 that encodes an FSH antagonist that binds to the FSH receptor and in which Asn residues 25, 42, and 213 have been mutated to prevent N-linked glycosylation.
17. A method for preventing pregnancy in a subject, the method comprising administering to the subject an effective amount of an FSH antagonist, wherein the FSH antagonist comprises:
 - a) SEQ ID NO:2., or
 - (b) a variant of SEQ ID NO:2 that binds to the FSH receptor and in which the Asn residues at amino acid positions 25, 42, and 213 have been mutated to prevent N-linked glycosylation.
18. The method of claim 17, further comprising administering to the subject an effective amount of an hCG antagonist, wherein the hCG antagonist is encoded by:
 - a) SEQ ID NO:3., or
 - (b) a variant of SEQ ID NO:3 that encodes a protein that binds to the hCG receptor and in which the Asn residues corresponding to amino acids 13 and 30 of the hCG beta subunit, and amino acid 52 of the hCG alpha subunit have been disrupted to prevent N-linked glycosylation.
19. A composition comprising an FSH antagonist, wherein the FSH antagonist comprises:
 - a) SEQ ID NO:2., or
 - (b) a variant of SEQ ID NO:2 that binds to the FSH receptor and in which the Asn residues at amino acid positions 25, 42, and 213 have been mutated to prevent N-linked glycosylation.

20. An composition comprising an FSH antagonist and a chemotherapeutic agent, wherein the FSH antagonist comprises:
- a) SEQ ID NO:2., or
 - (b) a variant of SEQ ID NO:2 that binds to the FSH receptor and in which the Asn residues at amino acid positions 25, 42, and 213 have been mutated to prevent N-linked glycosylation,
- and wherein the chemotherapeutic agent is cisplatin or a cisplatin analogue.

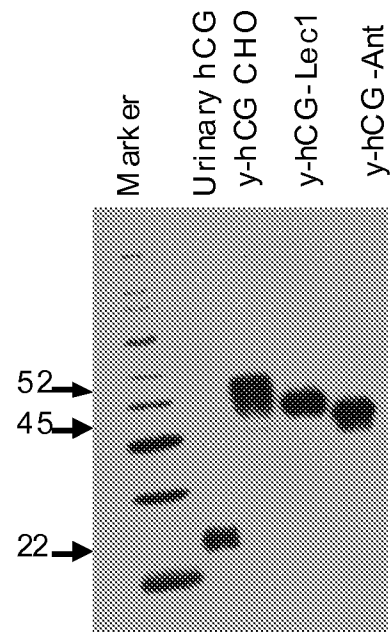
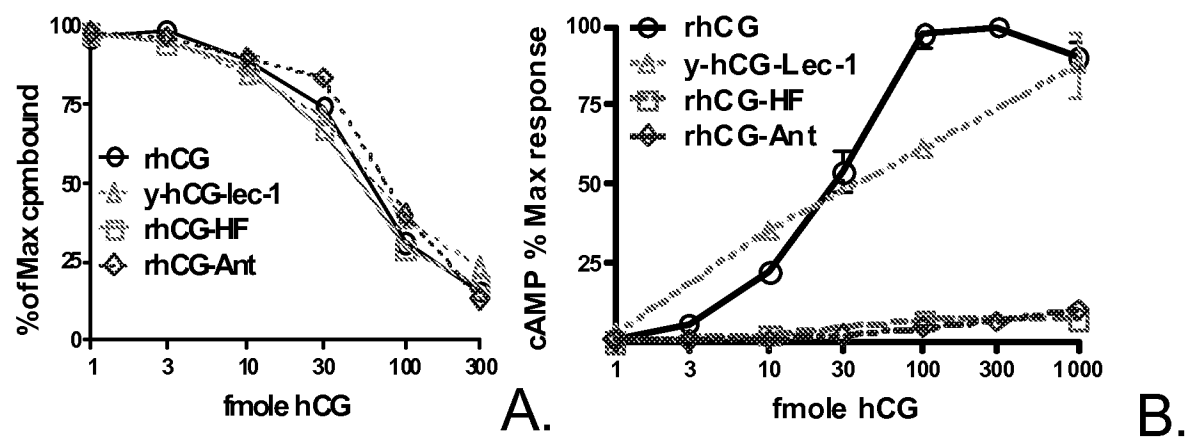
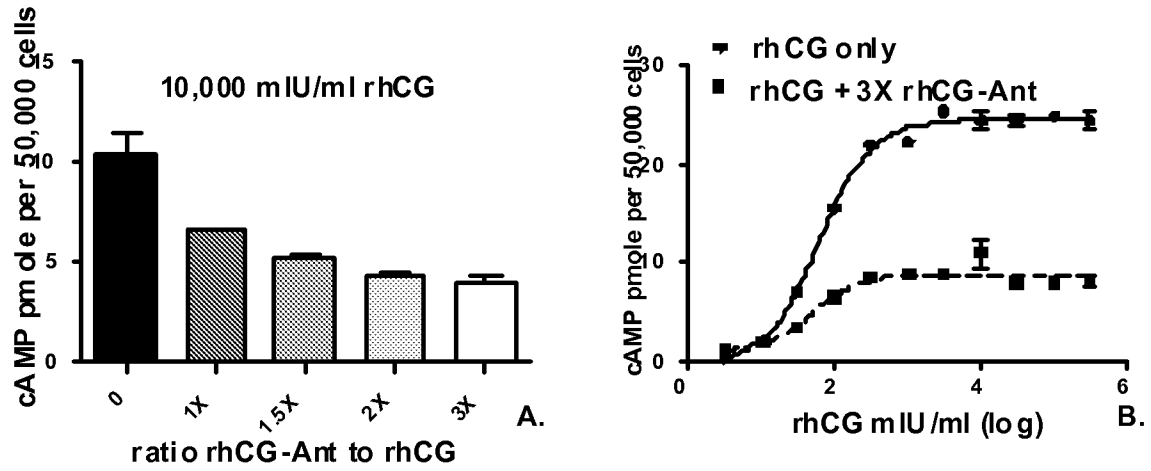


Figure 1

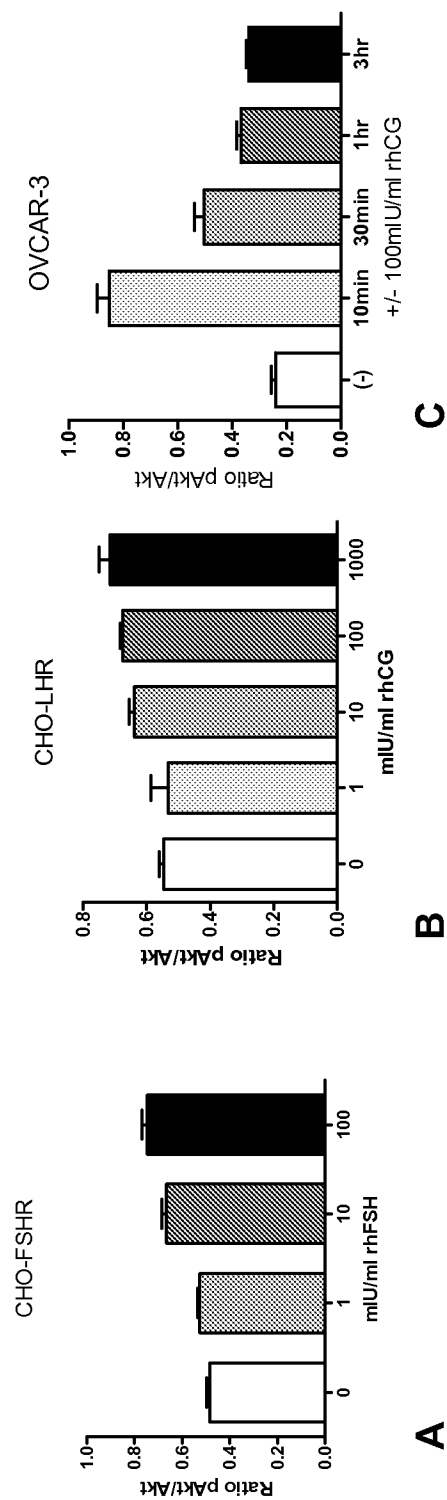


Figures 2A – 2B

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Figures 3A – 3B



Figures 4A – 4C

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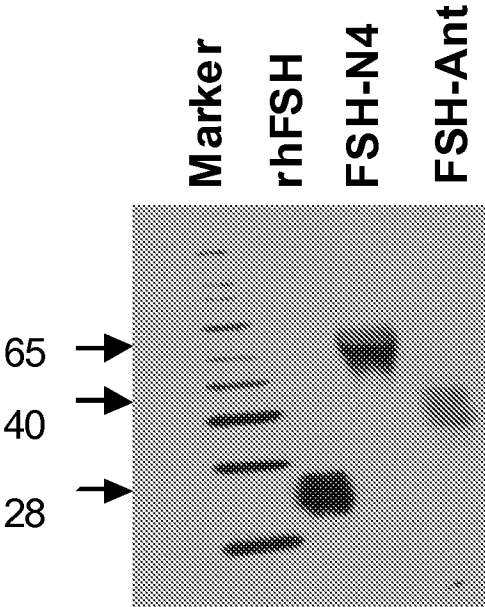
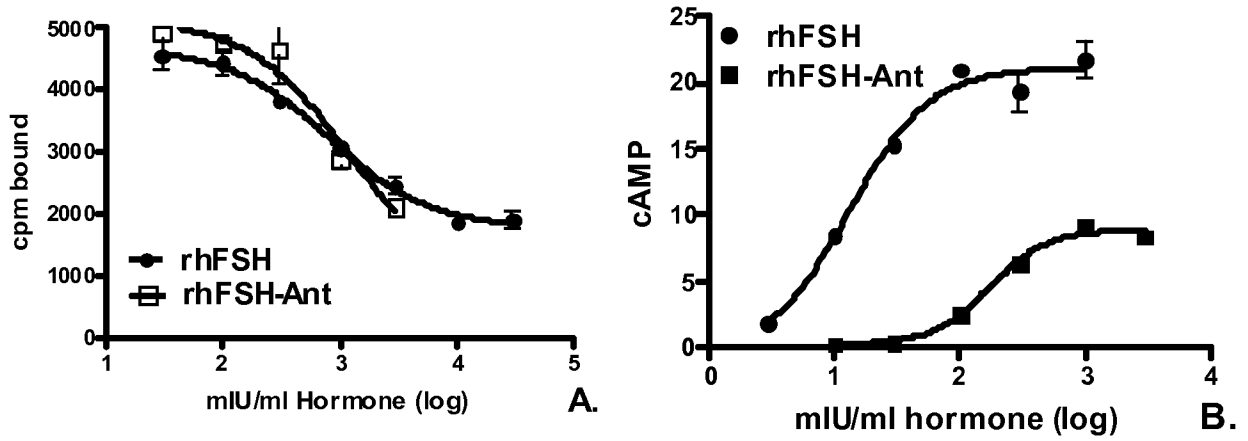
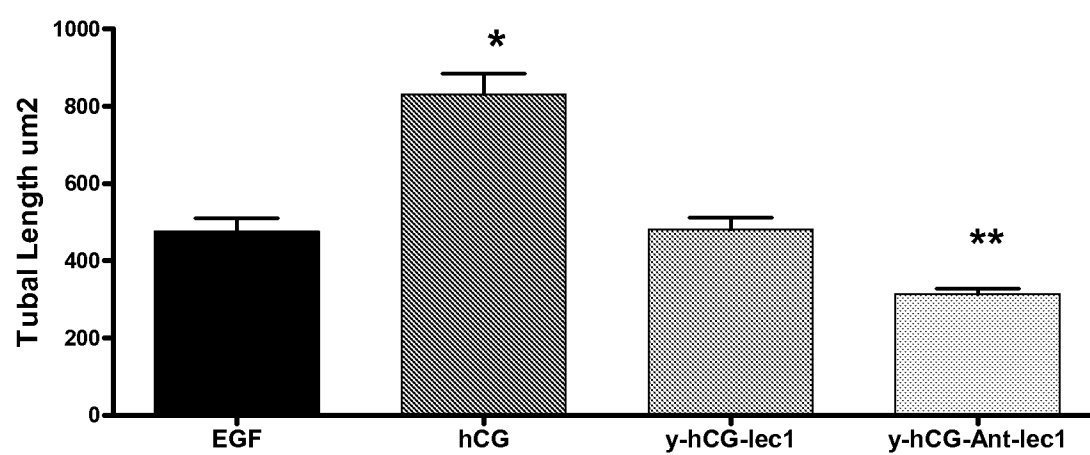


Figure 5

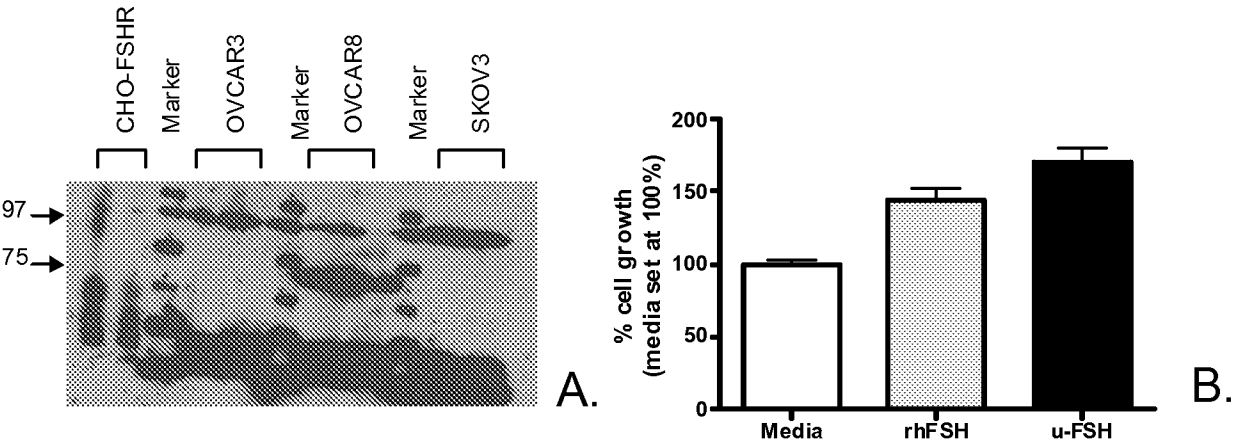
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**Figures 6A – 6B**

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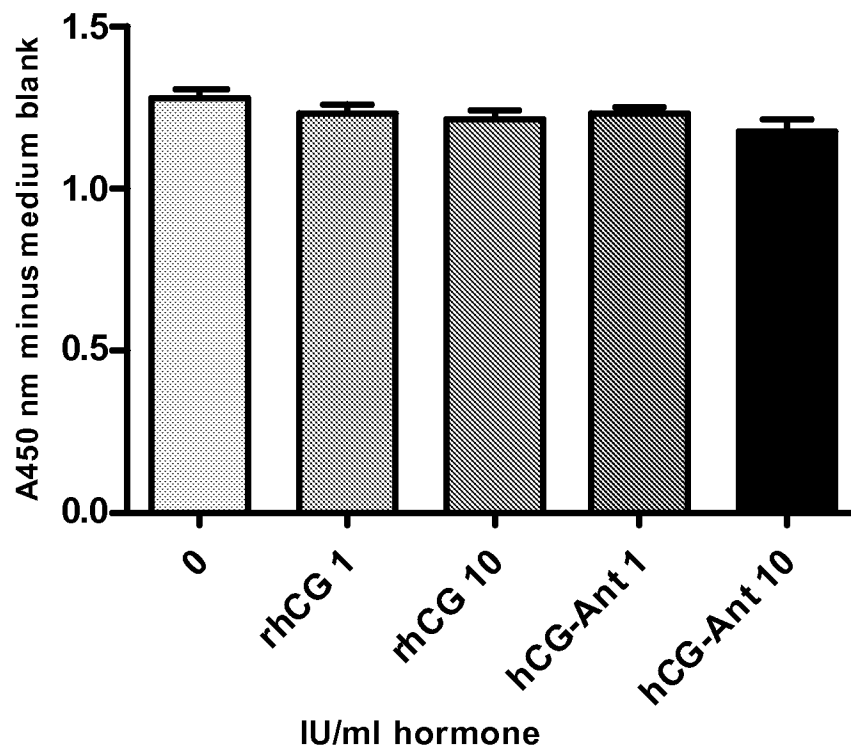
**Figure 7**

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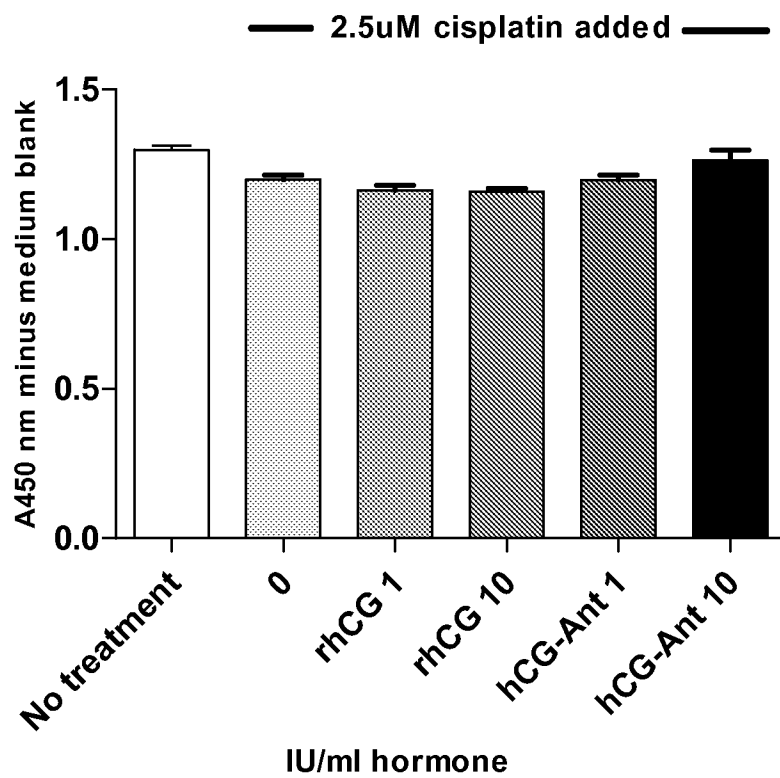


Figures 8A – 8B

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**Figure 9**

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**Figure 10**

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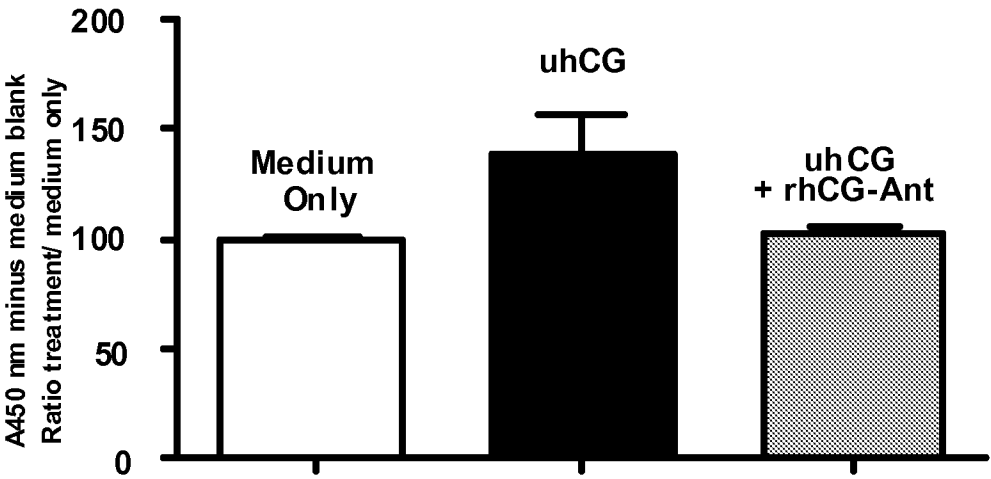
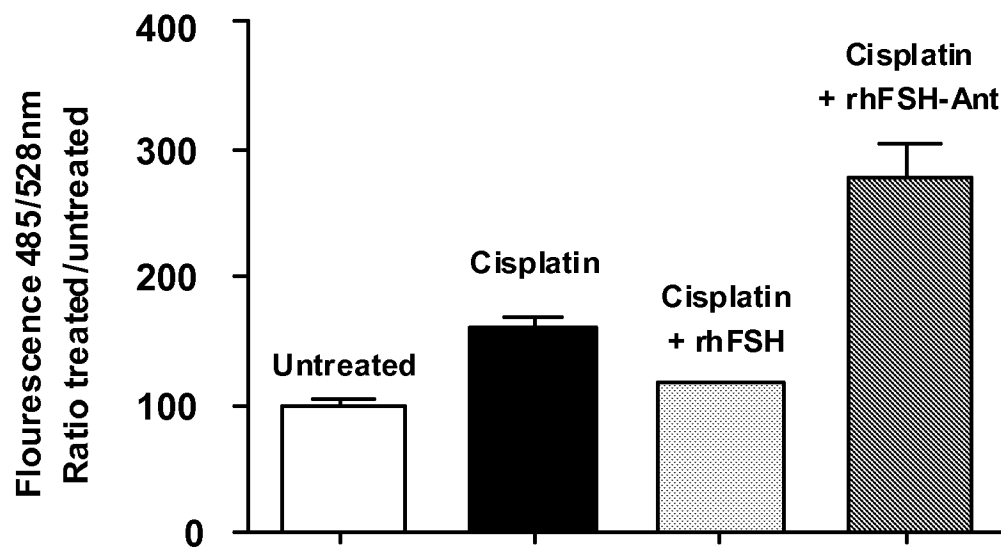
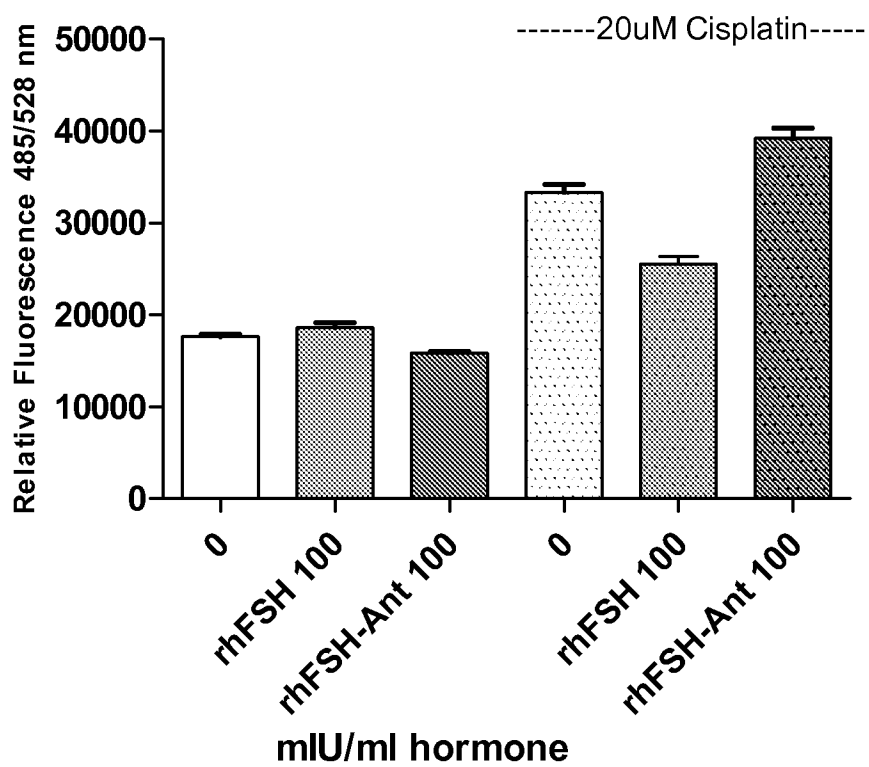


Figure 11

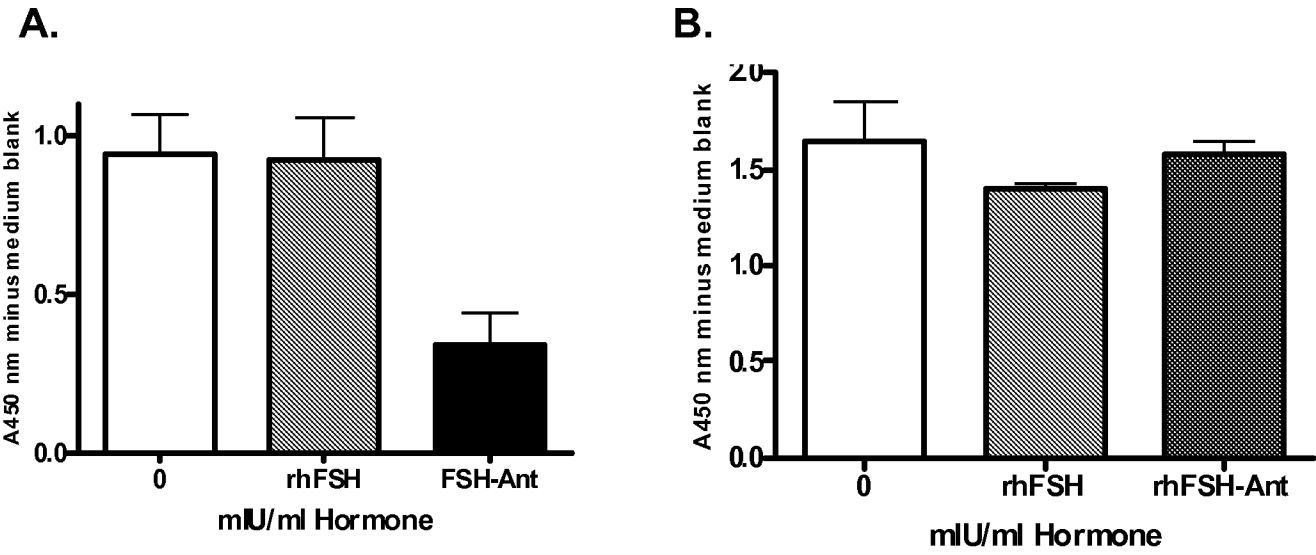
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**Figure 12**

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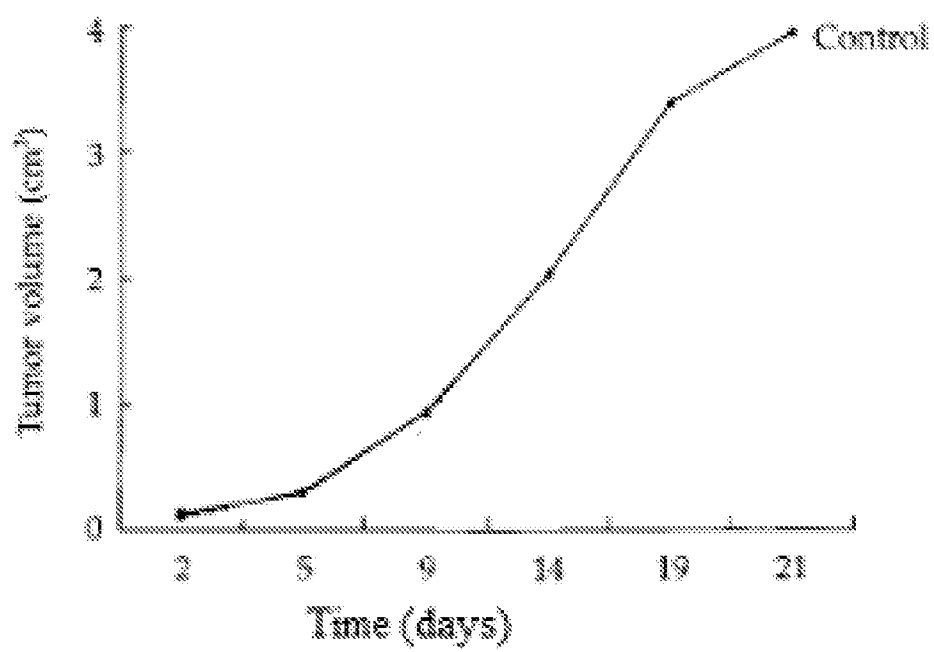
**Figure 13**

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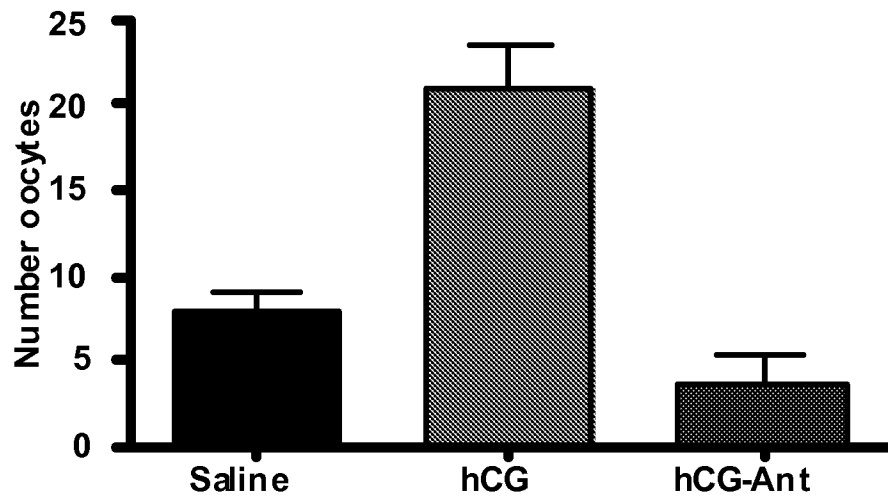


Figures 14A – 14B

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**Figure 15**

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**Figure 16**

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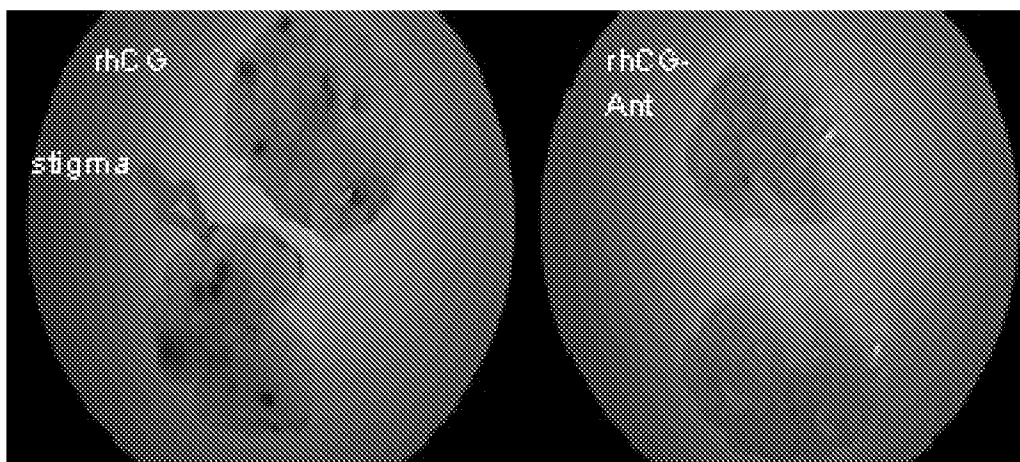


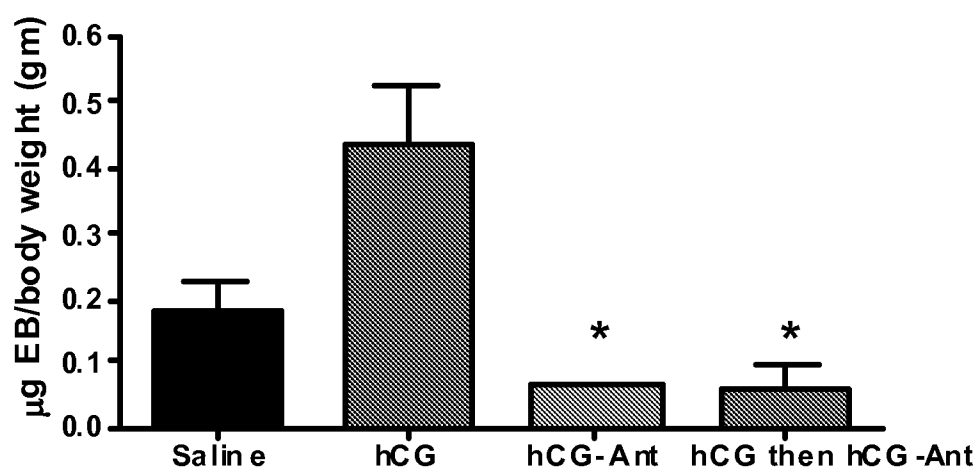
Figure 17

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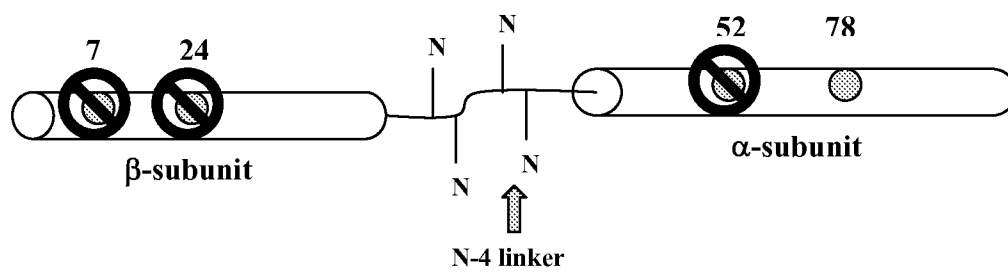


Figure 18

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**Figure 19**

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**Figure 20**

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ATGAAGACACTCCAGTTTTTCTTCCTTTTCTGTTGCTGGAAAGCAATCTGCTGCAATA
GCTGTGAGCTGACCAAAATCACCATTGCAATAGAGAAAGAAGAATGTCGTTTCTGC
ATAAGCATCAAAACCACTTGGTGTGCTGGCTACTGCTACACCAGGGATCTGGTGTAT
AAGGACCCAGCCAGGCCCAAAATCCAGAAAACATGTACCTTCAAGGAACTGGTATA
TGAAACAGTGAGAGTGCCCGGCTGTGCTCACCATGCAGATTCCTTGTATACATACCC
AGTGGCCACCCAGTGTCCTGTGGCAAGTGTGACAGCGACAGCACTGATTGTACTGT
GCGAGGCCTGGGGCCCAGCTACTGCTCCTTTGGTGAAATGAAAGAAGGATCCGGAT
CGAACGCGACGGGGTCAGGTTCTAATGCAACTTCAAGATCTGGATCGAACGCGACG
GGGTCAGGTTCTAATGCAACTTCAACTAGTGCTCCTGATGTGCAGGATTGCCCAGAA
TGCACGCTACAGGAAAACCCATTCTTCTCCCAGCCGGGTGCCCCAATACTTCAGTGC
ATGGGCTGCTGCTTCTCTAGAGCATATCCCCTCCACTAAGGTCCAAGAAGACGATG
TTGGTCCAAAAGAAAGTCACCTCAGAGTCCACTTGCTGTGTAGCTAAATCATATAAC
AGGGTCACAGTAATGGGGGGTTTCAAAGTGGAGAACCACACGGCGTGCCACTGCAG
TACTTGTTATTATCACAAATCTTAA (SEQ ID NO:1)

Figure 21

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$\beta 7$ $\beta 24$
MKTLQFFFLFCCWKAICCNSCELT $\beta 7$ KITIAIEKEECRFCISI $\beta 24$ KTTWCAGYCYTRDLVYKDP
ARPKIQKTCTFKELVYETVRVPGCAHHADSLYTYPVATQCHCGKCDSSTDCTVRGLG
PSYCSFGEMKEGSGSNATGSGSNATSRSGSNATGSGSNATSTSAPDVQDCPECTLQENPFFS
QPGAPILQCMGCCFSRAYPTPLRSKKTMLVQK $\alpha 52$ VTSESTCCVAKSYNRVTVMGGFKVE
NHTACHCSTCYHHK (SEQ ID NO:2) $\alpha 78$

Figure 22

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ATGGGAAGCTTCGTGGATCCAGACAAGG⁻CAGGGGACGCACCAAGGATGGAGATGTT
CCAGGGGGCTGCTGCTGTTGCTGCTGCTGAGCATGGGCGGGACATGGGCATCCAAGG
AGCCGCTTCGGCCACGGTGCCGCCCCATCAAAAGCCACCCTGGCTGTGGAGAAGGAG
GGCTGCCCCGTGTGCATCACCGTCAAAGACCACCATCTGTGCCGGCTACTGCCCCACC
ATGACCCGCGTGCTGCAGGGGGTCTTGCCGGCCCTGCCTCAGGTGGTGTGCAACTAC
CGCGATGTGCGCTTCGAGTCCATCCGGCTCCCTGGCTGCCCCGCGCGGGCGTGAACCCC
GTGGTCTCCTACGCCGTGGCTCTCAGCTGTCAATGTGCACTCTGCCGCCGCAGCACC
ACTGACTGCGGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCCGCTTCCAG
GACTCCTCTTCCTCAAAGGCCCCCTCCCCCAGCCTTCCAAGCCCATCCCGACTCCCG
GGGCCCTCGGACACCCCGATCCTCCCACAAGAATTCGCTCCTGATGTGCAGGATTGC
CCAGAATGCACGCTACAGGAAAACCCATTCTTCTCCCAGCCGGGTGCCCCAATACTT
CAGTGCATGGGCTGCTGCTTCTCTAGAGCATATCCCACTCCACTAAGGTCCAAGAAG
ACGATGTTGGTCCAAAAGAAAGTCACCTCAGAGTCCACTTGCTGTGTAGCTAAATCA
TATAACAGGGTCACAGTAATGGGGGGTTTCAAAGTGGAGAACCACACGGGCGTGCCA
CTGCAGTACTTGTTATTATCACAAATCTTAA (SEQ ID NO:3).

Figure 23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/24304

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/16, C07K 14/59 (2010.01)

USPC - 514/8, 530/397, 530/350

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)

USPC: 514/8, 530/397, 530/350

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

USPC: 514/2, 530/399

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

Electronic data bases searched: PubWEST (EPAB, JPAB, PGPB, USPT); Google Scholar, GenCore Sequence Search (AA)

Search terms: FSH antagonist, hCG antagonist, ovarian cancer, cisplatin, cisplatin analog, contraceptive\$, SEQ ID NOs: 2, 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 6,238,890 B1 (BOIME et al.) 29 May 2001 (29.05.2001). Especially SEQ ID NO: 12 and col 31 ln2 8-29.	19 ----- 1-8, 16-18, 20
Y	ABDENNEBI et al., Generating FSH antagonists and agonists through immunization against FSH receptor N-terminal decapeptides. J Mol Endocrinol, April 1999, Vol 22, No 2, Pages 151-159. Especially pg 157 right col para 2	17,18
Y	PARROTT et al., Expression and actions of both the follicle stimulating hormone receptor and the luteinizing hormone receptor in normal ovarian surface epithelium and ovarian cancer. Mol Cell Endocrinol, 14 February 200,1 Vol 172, No 1-2, Pages 213-22. Especially abstract, pg 218 fig 2A.	1-16, 20
Y	ERICKSON et al., Cyclophosphamide, cisplatin, and leuprolide acetate in patients with debulked stage III or IV ovarian carcinoma. Gynecol Oncol, August 1994, Vol 54, No2, Pages 196-200. Abstract only.	1-16, 20
Y	US 2008/0039372 A1 (LUSTBADER) 14 February 2008 (14.02.2008). Especially para [0015]; SEQ ID NO: 1	8-16,18

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 April 2010 (16.04.2010)

Date of mailing of the international search report

27 APR 2010

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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