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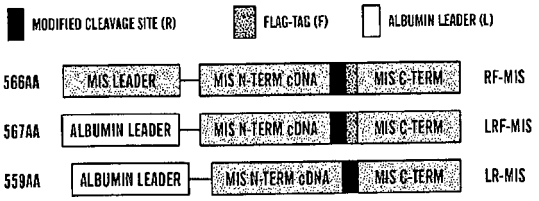

FIG. 1B

(57) Abstract: The present invention relates to modified recombinant human MIS protein which has improved cleavage and increased bioactivity and increased potency as compared to wild-type human MIS protein. Other aspects of the invention relate to methods to prevent and treat cancers, such as cancers that express the MIS receptor type II (MISRII) by administering to a subject a composition comprising a recombinant human MIS protein. Another aspect of the present invention relates to methods to lower plasma androgen levels in a subject, and/or for the treatment of a subject with a disease characterized by excess androgen. Another aspect provides pharmaceutical compositions and kits and methods for use comprising a recombinant human MIS protein. Another aspect of the present invention relates to methods to decrease the dose of a chemotherapeutic agent by administering the chemotherapeutic agent with the recombinant MIS protein that lowers the effective dose of the chemotherapeutic agent.

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MODIFIED MULLERIAN INHIBITING SUBSTANCE (MIS) PROTEINS AND USES THEREOF FOR THE TREATMENT OF DISEASES

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/777,135 filed March 12, 2013, the contents of which are incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 12, 2014, is named 030258-076964-PCT_SL.txt and is 28,114 bytes in size.

FIELD OF THE INVENTION

[0003] The present invention relates to modified recombinant human MIS protein which has improved cleavage and increased bioactivity and increased potency as compared to wild-type human MIS protein. In some aspects, the recombinant human MIS protein comprises at least one of the following: a modified Kex cleavage site for increased cleavage, a FLAG Tag, and a non-MIS leader sequence in place of the normal MIS leader sequence. Other aspects of the invention relate to methods, uses and kits comprising a recombinant human MIS protein for the treatment of cancers, such as those that expresses the MIS receptor type II (MISRII) or for the treatment of a disease characterized by excess androgen.

GOVERNMENT SUPPORT

[0004] This invention was made with government support under grant Number CA17393 awarded by the National Institutes of Health (NIH). The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0005] Mullerian Inhibiting Substance (MIS) also known as anti-Mullerian hormone (AMH), is a 140-kDa disulfide-linked homodimer glycoprotein member of the large transforming growth factor-β (TGFβ) multigene family of glycoproteins. The proteins in this gene family are all produced as dimeric precursors and undergo posttranslational processing for activation, requiring cleavage and dissociation to release bioactive C-terminal fragments. Similarly, the 140 kilodalton (kDa) disulfide-linked homodimer of MIS is proteolytically cleaved to generate its active C-terminal fragments.

[0006] The human MIS gene is located on chromosome 19, and its expression is sexually dimorphic. In males, MIS expression begins at 9 weeks gestation in the fetal testes and continues at high levels until puberty, when expression levels fall dramatically. In females, MIS is produced only

postnatally in granulosa cells from prepuberty through menopause at levels similar to adult males, after which expression ceases. In male fetuses MIS causes regression of the Mullerian ducts, the precursors to the Fallopian tubes, uterus, cervix, and upper third of the vagina.

[0007] MIS exerts its biologic effect after binding to a heterodimer of type I and type II single transmembrane spanning serine threonine kinase receptors, leading to cross phosphorylation of the GS box kinase domain of the type I receptor by the type II receptor. Subsequently, SMAD 1, 5 and 8 (but predominantly SMAD 8) are activated and, together with SMAD 4, regulate gene transcription. Only one MIS receptor type II (MISRII) gene has been identified in mice, rats, and rabbits, where in humans its gene localizes to chromosome 12. It is a 65-kDa protein which has been detected in embryonic and adult Mullerian structures, breast tissue, prostatic tissue, the gonads, motor neurons, and brain. In the fetus, mesoepithelial cells expressing MISRII in the coelomic epithelium covering the urogenital ridge migrate into and become part of the mesenchymal cells surrounding the Mullerian duct epithelium. Expression is also detected in the gonads, as wells as in the ovarian coelomic epithelium. Type I MIS receptors have been identified in mammals, with activin receptor-like kinase (ALK) 2 and 3 being the most likely candidates, depending upon animal species and the tissue examined.

[0008] In addition to its well established role in the regression of Mullerian ducts, MIS inhibits the proliferation of various human cancer cell lines *in vitro* and *in vivo.* The cell lines showing inhibition were derived from ovarian, cervical, endometrial, prostate and breast cancers. Toxicity has not been observed *in vivo* even when high concentrations of MIS are maintained systemically in rodents or in human patients with tumors secreting MIS for prolonged periods of time. These findings of relatively restricted receptor expression, anti-proliferative activity against cancer cells expressing the MIS RI and RII, and its apparent non-toxicity, taken together, make MIS an ideal reagent for use in combination with existing chemotherapeutic drugs for the treatment of ovarian cancer, which are known to become resistant to these conventional agents.

[0009] MIS acts through MIS Type II receptor cells to serve as a potent tumor suppressor of ovarian cancer initiation (Teixeira et al, unpublished). MIS can also target, as a receptor mediated event the stem/progenitor population of the ovarian cancer cell line (Meirelles et al, 2012; Wei et al, 2010). MIS can be used for the treatment of cancers, for example, expressing MISRII. MISRII is expressed in the majority of epithelial ovarian cancers (Masiakos et al. 1999; Bakkum-Gamez et al. 2008; Song et al. 2009).

[0010] MIS also inhibits growth of a variety of cancers *in vitro* and *in vivo,* without obvious toxicity after prolonged therapy *in vivo* (Pieretti-Vanmarcke et al. 2006b). Epithelial ovarian cancer recapitulates the original histology of the embryonic Mullerian ducts and its various subtypes (Scully 1977); for example, serous cystadenocarcinoma resembles embryonic Fallopian tube, endometrioid carcinoma, the endometrium, and mucinous carcinoma, the cervix. Also, MIS acts synergistically or additively with commonly used cancer drugs to control tumor growth (Pieretti-Vanmarcke et al. 2006a).

[0011] It has been previously reported that chemotherapeutic agents select for ovarian cancer stem cells, which are typically multi-drug resistant, and/or resistant to chemotherapeutics. In particular, there is a growing body of research reporting that ovarian cancers and cell lines are heterogeneous, with ovarian cancer stem cell populations that are resistant to chemotherapeutic drugs but remain responsive to MIS. MIS particularly targets ovarian cancer side population cells and a population of CD44+, CD24+, EpCam+ and E-Cadherin-negative cells with stem/progenitor characteristics that respond poorly to chemotherapeutic agents currently in clinical use for ovarian cancer (Wei et al, 2010). In particular, MIS has been shown to inhibit ovarian cancer cells both *in-vitro* and *in-vivo* and can specifically target and inhibit the growth of an ovarian cancer progenitor cell population enriched by the CD44+, CD24+, Ep-CAM+ and E-cadherin- cell surface markers. In order to accommodate clinical testing of MIS in ovarian cancer patients, the production of recombinant human MIS must be optimized to increase yield and purity.

[0012] However, the preparation resulting from purification of native or wild-type MIS is complex and the yield is low. Furthermore, the cleavage necessary to produce the active fragment of MIS is also inefficient. Human MIS protein is produced from a pre-proprotein, which comprises a leader sequence. The leader sequence (amino acids 1-25 of SEQ ID NO: 1) is cleaved off and the remaining preprotein (often called "holo-human MIS") must be post-translationally cleaved to result in a N-terminal and C-terminal domain. These covalently linked N-terminal and C-terminal domains form a monomer, and two identical monomers (comprising the N- and C-terminal domains) form together to generate a homodimer. Holo-human MIS is cleaved into its N- and C-terminal domains most likely by means of furin or a related prohormone convertase PC5, expressed in the gonads. Cleavage occurs primarily at a kex-like site characterized by R^{-4} XXR^{-1} with a serine in the +1 site, which makes the MIS cleavage site monobasic. The purified C-terminal domain is the biologically active moiety and cleavage is required for biological activity. A secondary cleavage site, whose significance is unknown, is observed less frequently at residues 229–230 (which corresponds to amino acid residues 254-255 of SEQ ID NO:1). Non-cleavable mutants of MIS are not biologically active and mutations in the human gene that truncate the carboxy-terminal domain lead to persistent Mullerian duct syndrome. The role of the amino-terminal domain *in vivo* may be to assist in protein folding and to facilitate delivery of the C-terminal peptide to its receptor. In one study (Cate, Pepinsky, et al.) addition of the N-terminal peptide was shown to enhance the biological activity of the C-terminal moiety in vitro, but the mechanism was unclear. The cleavage of recombinant MIS expressed by CHO cells is incomplete, thus cleavage with an exogenous serine protease such as plasmin is required to enhance bioactivity.

[0013] Accordingly, there is a need for a more efficient method to produce high concentrations of human MIS protein for use as a therapeutic biologic agent.

SUMMARY OF THE INVENTION

[0014] The present invention relates to modified recombinant human MIS protein which has improved cleavage and increased bioactivity and increased potency as compared to wild-type human

MIS protein, where the recombinant human MIS protein comprises a combination of the following: a modified Kex cleavage site for increased cleavage, and a non-MIS leader sequence in place of the normal MIS leader sequence, to improve the yield of bioactive protein with or without an, internal label, or Tag to facilitate its purification.

[0015] Accordingly, herein the inventors have engineered changes to the native human MIS amino acid sequence to do a combination of the following: (i) modify the primary cleavage site to increase cleavage and thus increase the potency and bioactivity of MIS, without insertion of a tag to facilitate its purification, and (ii) modify the endogenous leader sequence of MIS to increase yield of bioactive protein. Surprisingly, the addition of the leader sequence in combination with a modified primary cleavage site significantly increased both the yield of protein produced and the amount of cleavage from the primary cleavage site of the recombinant MIS protein. Furthermore, there is an unmet need to have a form of bioactive MIS that is labeled for use in receptor and other binding studies that will be very important both for the selection of patients for treatment and for addressing molecular mechanistic questions regarding the interaction of MIS in various receptor bearing tissues. In addition, the labeled ligand will be essential to determine if another receptor or other binding proteins exist in various tissues. Herein, the inventors demonstrate the production of an internally epitope tagged MIS that retains full bioactivity in the Mullerian duct regression assay. In one embodiment, the tag is a "FLAG" tag because of the availability of high quality reagents used for its detection and purification.

[0016] Herein, the inventors demonstrate that substitution of the MIS leader sequence to that of human serum albumin (HSA), combined with a modification of the primary endogenous cleavage site from RAQR/S (SEQ ID NO: 26) to RARR/S (SEQ ID NO: 27) results in greater expression, increased c-terminus cleavage and a reduction in unwanted cryptic internal cleavage when produced in CHO cells. Purified MIS containing these alterations retains its capacity to induce regression of the Mullerian duct in fetal rat embryonic urogenital ridge assays, and shows increased potency.

[0017] In another embodiment, the recombinant human MIS is engineered with a more efficient cleavage site at the carboxy-terminal end of the N-terminal domain, thereby eliminating the need for exogenous cleavage. This recombinant MIS protein can be used both as a therapeutic and as a probing molecule, without a tag for identification.

[0018] Importantly, the change in the endogenous leader sequence with another leader sequence, e.g., a human serum albumin (HSA) leader sequence increased production of the MIS protein. Surprisingly, the inventors demonstrate that the combination of the leader sequence and modified cleavage site increases cleavage from the primary cleavage site from 37% to over 80% which was unexpected, as an increase in protein yield is normally associated with decreased post-translational processing, including cleavage, because increased protein production typically saturates the available or endogeneous cleavage enzymes.

[0019] Accordingly, the present invention relates to a method of using a recombinant human MIS protein (e.g., the polypeptide and/or the nucleic acid encoding a recombinant human MIS protein)

or a functional fragment or derivative or variant thereof to treat cancer, for example, a cancer which expresses the MIS receptor II (MISRII).

[0020] Accordingly, one aspect of the present invention relates to a recombinant Mullerian Inhibiting Substance (MIS) protein comprising a combination of a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1, and a modification of at least one amino acid between residues 448-452 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification, wherein the recombinant MIS protein has increased cleavage and increased yield of production *in vitro* as compared to wild-type MIS protein corresponding to amino acid residues of SEQ ID NO: 1. In some embodiments, the recombinant MIS protein lacks a leader sequence. In these embodiments, the recombinant MIS protein can be produced from a pre-proprotein comprising a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1, wherein the leader sequence is cleaved off during production. In some embodiments, the recombinant MIS protein further comprises a Tag protein.

[0021] In some embodiments, a non-MIS leader sequence is an albumin leader sequence or a functional fragment thereof, for example, a human serum albumin (HSA) leader sequence or a fragment thereof. In some embodiments, the HSA leader sequence comprises the amino acid sequence of SEQ ID NO: 6 or a variant that is at least 80% homologous thereto, or a functional fragment, e.g., a fragment of the HSA sequence comprising at least 10 amino acids, or at least about 11, or at least 15 amino acids of SEQ ID NO: 6 or a variant that is at least 80% homologous thereto. In some embodiments, a fragment of the HSA leader sequence is selected from the group consisting of: MKWVTFISLLFLFSSAYS (SEQ ID NO: 13); MKWVTFISLLFLFSSAYSRGVFRR (SEQ ID NO: 6); MKWVSFISLLFLFSSAYS (SEQ ID NO:14).

[0022] In some embodiments, a non-MIS leader sequence is selected from a group consisting of: immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide (IgSP-tPA), murine immunoglobulin signal peptide (IgSP), a MPIF-1 signal sequence (MKVSVAALSCLMLVTALGSQA (SEQ ID NO: 15);a stanniocalcin signal sequence (MLQNSAVLLLLVISASA (SEQ ID NO:16); an invertase signal sequence (MLLQAFLFLLAGFAAKISA (SEQ ID NO:17); a yeast mating factor alpha signal sequence (K. lactis killer toxin leader sequence); a hybrid signal sequence (MKWVSFISLLFLFSSAYSRSLEKR (SEQ ID NO:18)); a HSA/MFα-1 hybrid signal sequence (MKWVSFISLLFLFSSAYSRSLDKR (SEQ ID NO:19)); a K. lactis killer/ MFα-1 fusion leader sequence (MNIFYIFLFLLSFVQGSLDKR (SEQ ID NO:20)); an immunoglobulin Ig signal sequence (MGWSCIILFLVATATGVHS (SEQ ID NO:21)); a Fibulin B precursor signal sequence (MERAAPSRRVPLPLLLLGGLALLAAGVDA (SEQ ID NO:22)); a clusterin precursor signal sequence (MMKTLLLFVGLLLTWESGQVLG (SEQ ID NO: 23)); and the insulin-like growth factor-binding protein 4 signal sequence (MLPLCLVAALLLAAGPGPSLG (SEQ ID NO:24)) or a functional fragment thereof.

[0023] In some embodiments, a modification of amino acid 450 of SEQ ID NO: 1 from Q to R increases the cleavage from the primary cleavage site in MIS as compared to the amount of cleavage in the absence of such a modification. In some embodiments, a recombinant MIS further comprises a modification of amino acid 452 of SEQ ID NO: 1 from S to R to increase cleavage as compared to in the absence of such a modification.

[0024] In some embodiments, the recombinant MIS protein disclosed herein comprises a tag which is a FLAG tag, for example, amino acid sequence DYKDDDDK (SEQ ID NO: 8), or a functional derivative or variant thereof. In some embodiments, a tag, e.g., FLAG tag is located after amino acid residue 452 of SEQ ID NO: 1 and before amino acid residue 453 of SEQ ID NO: 1. In some embodiments, the location of the tag, e.g., Flag Tag is between amino acid residue 452 and 453 of SEQ ID NO: 1. In some embodiments, the tag is located at the N-terminus of the C-terminal domain of MIS. In some embodiments, the tag is no longer than 50 amino acids, for example, no longer than about 50, or about 40, or about 30, or about 20, or about 10 amino acids in length or about 7 amino acids in length.

[0025] In some embodiments, a recombinant MIS protein described herein comprises the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3 or a functional fragment thereof, which can be encoded by nucleic acid sequences SEQ ID NO: 4 and 5 respectively.

[0026] Another aspect of the present invention relates to a pharmaceutical composition comprising a recombinant MIS protein as discussed herein and a pharmaceutically acceptable carrier.

[0027] Another aspect of the present invention relates to a polynucleotide encoding the recombinant MIS protein as discussed herein, e.g., where the polynucleotide corresponds to SEQ ID NO: 4 or SEQ ID NO: 5 or a nucleotide which has at least 95% sequence identity to the nucleic acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5 respectively. Another aspect of the technology described herein relates to a vector comprising the polynucleotide of SEQ ID NO: 4 or SEQ ID NO: 5 or a nucleotide which has at least 95% sequence identity to the nucleic acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5 respectively. In some embodiments, a vector is a viral vector or an expression vector, e.g., pcDNA 3.1, or alternative vectors for e.coli or bacteriophage. In some embodiments, a viral vector is selected from the group consisting of an adenoviral vector, a poxvirus vector and a lentiviral vector. In some embodiments, a viral vector is adeno-associated virus (AAV), for example, recombinant AAV serotype 9 (rAAV9).

[0028] In some embodiments, a vector comprises a nucleic acid sequence which encodes a recombinant MIS protein or fragment thereof which has at least 95% sequence identity to the nucleic acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5, and where the nucleic acid sequence is operatively linked to tissue- or cell-type specific promoter. In some embodiments, a host cell comprising such a vector is also encompassed in the present invention.

[0029] In some embodiments, the vector comprising the polynucleotides as discussed herein can express the recombinant MIS protein at a constant level over a desired period of time.

[0030] Another aspect of the present invention relates to a human MIS protein produced by post-translational processing of the recombinant human MIS protein as discussed herein.

[0031] Another aspect of the technology discussed herein relates to a pharmaceutical composition comprising the vector as discussed herein and a pharmaceutically acceptable carrier. Another aspect of the technology discussed herein relates a purified preparation, or substantially purified human MIS protein produced from the recombinant human MIS protein as discussed herein.

[0032] Another aspect of the technology as discussed herein relates to a method for treating a subject with cancer, comprising administering a composition comprising a recombinant MIS protein, wherein the recombinant MIS protein comprises a modification of at least one amino acid between residues 448-452 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification, with or without an internal tag protein. In some embodiments, the recombinant MIS protein has increased cleavage and increased yield of production *in vitro* as compared to wild-type MIS protein corresponding to amino acid residues of SEQ ID NO: 1.

[0033] In some embodiments, the recombinant human MIS protein (e.g., the polypeptide and/or the nucleic acid encoding a recombinant human MIS protein) as disclosed herein, or a functional fragment or derivative or variant thereof, can be used to treat a cancer. In some embodiments, the recombinant human MIS protein that can be used for cancer treatment comprises the amino acid residues 25-559 of SEQ ID NO: 2 or a functional fragment thereof. In some embodiments, the recombinant human MIS protein that can be used for cancer treatment comprises the amino acid residues 25-567 of SEQ ID NO: 3 or a functional fragment thereof. In some embodiments, the cancer is a MIS Responsive II (MISRII) cancer, or where the cancer expresses MISRII e.g., an ovarian cancer, or comprises an ovarian cancer cell, vulvar epidermal carcinoma cell, cervical carcinoma cell, endometrial edenocarinaoma cell and ovarian adenocarcinoma. In some embodiments, the cancer includes, but is not limited to any one of: breast cancer, lung cancer, head and neck cancer, bladder cancer, stomach cancer, cancer of the nervous system, bone cancer, bone marrow cancer, brain cancer, colon cancer, esophageal cancer, endometrial cancer, gastrointestinal cancer, gum cancer, kidney cancer, liver cancer, nasopharynx cancer, ovarian cancer, prostate cancer, pancreatic cancer, skin cancer, stomach cancer, testis cancer, tongue cancer, melanoma, ocular melanoma, or uterine cancer.

[0034] In some embodiments of the methods disclosed herein, the administration of the recombinant MIS protein is prior to, during, or after administration of an additional agent or cancer therapy.

[0035] In some embodiments, the expression of Mullerian Inhibiting Substance (MIS) receptor is measured in a biological sample obtained from the subject, e.g., a cancer or tumor tissue sample or a cancer cell or tumor cell, e.g., a biopsy tissue sample.

[0036] In some embodiments, the cancer is a chemotherapeutic-resistant or multi-drug resistant cancer, e.g., where the cancer is a paclitaxel, cisplatin, rapamycin, pyrazoloanthrone, or Doxorubicin-resistant cancer.

[0037] In some embodiments, a recombinant MIS can be administered by any route, e.g., via intravenous, intradermal, intramuscular, intraarterial, intralesional, percutaneous, or subcutaneous, or by aerosol administration. In some embodiments, administration is therapeutic or prophylactic administration. In all aspects as discussed herein, a subject is a mammal, e.g., a human.

[0038] In some embodiments, at least one additional agent is administered to the subject in combination with (e.g., before, during or after) administration of the recombinant human MIS, such as a therapeutic agent or chemotherapeutic agent, for example, a chemotherapeutic agent is selected from the group consisting of: paclitaxel, cisplatin, doxorubicin, rapamycin, pyrazoloanthrone, including but not limited to antra(1,9-cd)pyrazol-6(2H)-one (SP600125) or N1-methyl-1,9-pyrazoloanthrone (M-SP600125) or a functional derivative or functional analogue thereof. In some embodiments, a chemotherapeutic agent is a radiotherapeutic agent.

[0039] Another aspect of the technology as disclosed herein relates to a method of decreasing the dose of a chemotherapeutic agent for the treatment of cancer, the method comprising administering to the subject a therapeutically effective amount of a recombinant MIS protein, wherein the recombinant MIS protein comprises a modification of amino acid 450 of SEQ ID NO: 1 from Q to R, wherein the therapeutically effective dose of the chemotherapeutic agent in the presence of the recombinant MIS protein is lower as compared to the therapeutically effective dose of the chemotherapeutic agent alone. In some embodiments, the recombinant MIS protein optionally comprises a Tag protein.

[0040] Other aspects of the technology as disclosed herein relates to the use of a recombinant MIS protein for the manufacture of a medicament for treating cancer, wherein the recombinant MIS protein comprises a modification of amino acid 450 of SEQ ID NO: 1 from Q to R, and wherein the cancer expresses a Mullerian Inhibiting Substance (MIS) receptor.

[0041] Another aspect of the present invention relates to an article of manufacture comprising packaging material and a pharmaceutical composition comprising the recombinant MIS protein as discussed herein, wherein the packaging material comprises a label which indicates the pharmaceutical composition may be administered, for a sufficient term at an effective dose, for treating or reducing the risk of cancer which expresses a Mullerian Inhibiting Substance (MIS) receptor.

[0042] Other aspects of the technology as disclosed herein relates to a method of treating a subject affected with cancer, the method comprising assessing the expression and/or activity of Mullerian Inhibiting Substance Receptor II (MISRII) in a biological sample obtained from the subject, wherein a clinician reviews the results and if the results indicate the presence of expression and/or activity of MISRII, the clinician directs the subject to be treated with pharmaceutical composition comprising a recombinant MIS protein as disclosed herein.

[0043] Other aspects of the technology as disclosed herein relates to the use of a recombinant MIS protein to decrease the plasma serum levels of one or more androgens in a subject in need thereof, wherein the recombinant MIS protein comprises a modification of amino acid 450 of SEQ ID NO: 1

from Q to R and optionally a tag, e.g., a Flag tag. In some embodiments, the recombinant human MIS protein that can be used for decreasing androgen levels comprises the amino acid residues 25-559 of SEQ ID NO: 2 or a functional fragment thereof.

[0044] In some embodiments, one or more androgens is testosterone. In some embodiments, a subject in need thereof has benign prostatic hypertrophy, or prostate cancer or polycystic ovarian disease and/or precocious puberty. In alternative embodiments, a subject in need thereof has a disease or disorder selected from the group including, but not limited to; Benign Prostatic Hyperplasia (BPH), prostate carcinoma, testicular cancer, androgen dependent acne, male pattern baldness, precocious puberty, hyperandrogenism, hirsutism, virilization, Polycystic Ovary Syndrome (POCS), hyperandrogenism (HA) and insulin resistance (IR) and acanthosis nigricans (AN) (HIAR-AN) syndrome, ovarian hyperthecosis, follicular maturation arrest, atresia, anovulation, dysmenorrheal, dysfunctional uterine bleeding, infertility and androgen-producing tumors.

[0045] Other aspects of the technology as disclosed herein relates to a method to treat a disease or disorder characterized by androgenic dependency, comprising administering to a subject an effective amount of the pharmaceutical composition comprising a recombinant MIS protein as discussed herein, or a pharmaceutical composition comprising a preparation of MIS protein from the cleavage of the recombinant MIS protein as disclosed herein, wherein the pharmaceutical composition reduces the level of at least one androgen in the plasma serum of the subject and results in a decrease in at least one symptom of a disease or disorder characterized by androgenic dependency.

[0046] Other aspects of the technology as disclosed herein relates to a method to decrease the plasma level of one or more androgens in a subject, the method comprising administering an effective amount of a recombinant MIS protein, wherein the recombinant MIS protein comprises a modification of amino acid 450 of SEQ ID NO: 1 from Q to R, where the recombinant MIS optionally comprises a tag, and wherein the recombinant MIS protein decreases the plasma serum levels of one or more androgens in the subject.

[0047] In some embodiments, a subject has a disease or disorder characterized by androgenic dependency, for example, but not limited to a disease or disorder is selected from the group of; Benign Prostatic Hyperplasia (BPH), prostate carcinoma, testicular cancer, androgen dependent acne, male pattern baldness, precocious puberty, hyperandrogenism, hirsutism, virilization, Polycystic Ovary Syndrome (POCS), hyperandrogenism (HA) and insulin resistance (IR) and acanthosis nigricans (AN) (HIAR-AN) syndrome, ovarian hyperthecosis, follicular maturation arrest, atresia, anovulation, dysmenorrheal, dysfunctional uterine bleeding, infertility and androgen-producing tumors.

[0048] Other aspects of the technology as disclosed herein relates to a kit comprising a recombinant MIS protein as discussed herein, or preparation of a MIS protein produced by the post-translational processing of a recombinant MIS protein discussed herein, and a pharmaceutically acceptable carrier. In some embodiments, a kit can optionally comprise instructions of use of the

recombinant MIS protein for the treatment of cancer or treatment of an androgenic dependency disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

[0049] **Figs. 1A-1B** are schematic drawings showing the design of new recombinant MIS constructs with the albumin leader sequence. **Fig. 1A** shows the leader sequence of MIS (25 amino acids) and albumin (24 amino acids) have 20% identity and 5 conserved amino acids. **Fig. 1B** is a schematic drawing showing the design of the RF (modified cleavage site plus Flag tag), LRF(leader sequence plus modified cleavage site plus Flag tag), and LR (leader sequence plus modified cleavage site) constructs including the placement of the flag tag (F), the modified cleavage site (R), and the albumin leader (L).

[0050] **Fig. 2** shows MIS production and cleavage in CHOK1 clones stably transfected with recombinant human LR-MIS and LRF-MIS constructs. Western blot of 4% reduced SDS gels of media supernatant after 72h in culture using an anti-MIS goat polyclonal antibody targeting the c-terminus of MIS (1:200). Purified RF-MIS, CHO93 media and B9 media shown as positive controls.

[0051] **Figs. 3A-3B** show purified recombinant MIS analyzed by western blot of reduced SDS gels to estimate the amount of cleavage. Fig. 3A shows purified recombinant RF-MIS, LRF-MIS and WT-MIS is compared using an antibody against the N-terminus which can recognize holo MIS monomer, the cleaved N-terminus, and cryptic cleavage products containing part of the N-terminus. Fig. 3B shows detection of purified recombinant RF-MIS, LRF-MIS and WT-MIS using an antibody against the C-terminus which can recognize holo MIS monomer, the cleaved C-terminus, and cryptic cleavage products containing part of the C-terminus.

[0052] **Figs. 4A-4B** show the comparison of 5ug/ml (35uM) of WT, RF, and LRF recombinant MIS in a Mullerian duct regression bioassay. Recombinant human MIS produces was incubated for 72h with fetal rat uro-genital ridges. **Fig. 4A** shows representative sections from both the treated ridge and the untreated contralateral control ridge are compared for Mullerian duct regression. **Fig. 4B** is a histogram showing the frequency distribution of those scores in Fig. 4A. (LRF-MIS N=6, RF-MIS N=39). W, Wolffian duct; M, Müllerian duct.

[0053] **Figs. 5A-5B** show the amino acid of wild-type MIS protein (SEQ ID NO: 1) with the corresponding amino acid residues using conventional nomenclature of amino acid labeling (where the first numbered amino acid begins after the leader sequence). **Fig. 5A** shows the amino acid sequence of wild-type MIS protein of SEQ ID NO: 1, showing the leader sequence (in bold) and the primary and secondary cleavage sites highlighted. The corresponding amino acid numbering using conventional numbering is shown in brackets. **Fig. 5B** shows a Table indicating features on amino acid residues on SEQ ID NO: 1 which correspond with the amino acid residues using normal nomenclature of MIS (where the first numbered amino acid begins after the leader sequence). Fig. 5B discloses "RAQR/S" as SEQ ID NO: 26.

DETAILED DESCRIPTION OF THE INVENTION

[0054] The present invention relates to modified recombinant human MIS protein which has at least one of the following characteristics; improved cleavage, increased bioactivity, increased potency and can be produced at high yield as compared to the wild-type human MIS protein, where the recombinant human MIS protein comprises a combination of the following: a modified Kex cleavage site for increased cleavage and a non-MIS leader sequence in place of the normal MIS leader sequence, to improve the yield of bioactive protein. In some embodiments, this modified MIS is with or without an internal label, or Tag, to facilitate its purification.

[0055] Accordingly, herein the inventors have engineered changes to the native human sequence to increase endogenous cleavage and thus the potency of MIS. The inventors have also, optionally, inserted a tag to facilitate its purification.

[0056] The inventors have also additionally modified recombinant human MIS protein to comprise a non-MIS leader sequence instead of the 25 amino acid MIS leader sequence of amino acids 1-25 of SEQ ID NO:1. In some embodiments, the leader sequence comprises an albumin leader sequence, such as a human serum albumin sequence (HSA) or a functional fragment or variant thereof. In some embodiments, the leader sequence comprises 24 amino acids of SEQ ID NO: 6 or a functional fragment thereof, and replaces amino acid residues 1-25 of SEQ ID NO: 1. This addition, surprisingly, has further increased cleavage of the recombinant MIS protein. This combination has led to higher yield of a product that is more homogeneous, with increased potency due to increased cleavage. This combination of changes yields a recombinant human MIS variant that can meet a previously unmet need to have a form of bioactive MIS that is labeled for use in receptor and other binding studies that will be very important both for the selection of patients for treatment and for addressing molecular mechanistic questions regarding the interaction of MIS in various receptor bearing tissues. In addition, the labeled ligand will be essential to determine if another receptor or other binding proteins exist in various tissues. Herein, the inventors demonstrate the production of an internally epitope tagged MIS that retains full bioactivity in the Mullerian duct regression assay. In one embodiment, the tag is a "FLAG" tag because of the availability of high quality reagents used for its detection and purification.

[0057] As discussed herein, the present invention provides a method for treating a variety of conditions by administering an effective amount of a recombinant human MIS protein and functional fragments and derivatives thereof as disclosed herein to a subject in need thereof. Conditions that may be treated by the compounds of this invention, or a pharmaceutical composition containing the same, include any condition which is treated or reduces the symptoms by administration of human MIS or activation of MIS signaling or activation of MISRII, and thereby benefit from administration of a a recombinant human MIS protein and functional fragments and derivatives thereof. Representative conditions in this regard include, for example, but not limited to, cancers that express MIS receptors, for example cancer that express MISRII, for example, but not limited to ovarian, cervical and endometrial

cancer. Other conditions which can be treated with MIS or activation of MIS signalling reduces the symptoms are proliferative diseases such as cancer, or abnormally high androgen stages such as polycysic ovarian disease, precocious puberty, and other hyperandrogen disorders, such as testotoxicosis, or any androgen-dependent tumor such as prostate cancer.

Definitions:

[0058] For convenience, certain terms employed in the entire application (including the specification, examples, and appended claims) are collected here. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0059] The term "Mullerian Inhibiting Substance" and "MIS" are used interchangeably herein and is also known as anti-Müllerian hormone or AMH, refer to compounds and materials which are structurally similar to MIS. By "MIS" or "Mullerian Inhibiting Substance" is meant a polypeptide having an amino acid sequence at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% identical to amino acid residues 26-560 of SEQ ID NO: 1. The present invention is intended to include mutant forms of recombinant human MIS which have substantially the same, or greater biological activity as wild-type MIS. Examples of such mutant MIS molecules carrying a deletion, insertion, or alteration in the amino acid sequence of wild-type MIS (e.g., amino acid residues 26-560 of SEQ ID NO:1). Other forms of include substances are for example, salts, functional derivatives and aglycone forms of wild-type MIS and recombinant human MIS. Additionally, human recombinant MIS protein can be obtained using recombinant DNA technology, or from chemical synthesis of the MIS protein. For reference purposes only, the wild-type human MIS nucleic acid corresponds to RefSeq No: NM_000479, which are incorporated herein by reference.

[0060] The term "Mullerian Inhibiting Substance type II receptor" or "MISRII" are used interchangeably herein to refer to the type II receptor for MIS. The term MISRII is intended to encompass all MIS receptors substantially homologous to MISRII and functional derivatives of MISRII. MISRII is also known by the alias as AMHR2, and for reference purposes, the nucleic acid sequence of human MISRII corresponds to NM_020547 and GenBank No: AF172932 which are incorporated herein by reference

[0061] The term "wild type" refers to the naturally-occurring polynucleotide sequence encoding a protein, or a portion thereof, or protein sequence, or portion thereof, respectively, as it normally exists *in vivo*. Accordingly, as disclosed herein, the wild type amino acid sequence for the pre-proprotein of human MIS corresponds to SEQ ID NO: 1, where amino acid residues 1-25 correspond to the leader sequence. The proprotein of MIS comprises amino acid residues 26-560 of SEQ ID NO: 1 (e.g., lacking the 1-25 leader sequence), which is then post-translationally processed by cleavage as discussed herein to form a bioactive MIS homodimer.

[0062] The term "soluble MIS polypeptide" as used herein refers to a MIS polypeptide that does not comprise at least part of, or all of, the amino acids which allow it to functionally bind to the membrane.

[0063] By a "polynucleotide encoding MIS" is meant a polynucleotide encoding a polypeptide having at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity to any of the amino acid sequences corresponding to amino acid residues 26-560 of SEQ ID NO: 1.

[0064] The term "mutant" refers to any change in the genetic material of an organism, in particular a change (i.e., deletion, substitution, addition, or alteration) in a wild-type polynucleotide sequence or any change in a wild-type protein sequence. The term "variant" is used interchangeably with "mutant". Although it is often assumed that a change in the genetic material results in a change of the function of the protein, the terms "mutant" and "variant" refer to a change in the sequence of a wild-type protein regardless of whether that change alters the function of the protein (e.g., increases, decreases, imparts a new function), or whether that change has no effect on the function of the protein (e.g., the mutation or variation is silent). The term mutation is used interchangeably herein with polymorphism in this application.

[0065] The term "agent" or "compound" as used herein refers to a chemical entity or biological product, or combination of chemical entities or biological products, administered to a subject to treat or prevent or control a disease or condition. The chemical entity or biological product is preferably, but not necessarily a low molecular weight compound, but may also be a larger compound, or any organic or inorganic molecule, including modified and unmodified nucleic acids such as antisense nucleic acids, RNAi, such as siRNA or shRNA, peptides, peptidomimetics, receptors, ligands, and antibodies, aptamers, polypeptides, nucleic acid analogues or variants thereof. For example, an oligomer of nucleic acids, amino acids, or carbohydrates including without limitation proteins, oligonucleotides, ribozymes, DNAzymes, glycoproteins, siRNAs, lipoproteins, aptamers, and modifications and combinations thereof.

[0066] The term "nucleic acid" is well known in the art. A "nucleic acid" as used herein will generally refer to a molecule (i.e., strand) of DNA, RNA or a derivative or analog thereof, comprising a nucleobase. A nucleobase includes, for example, a naturally occurring purine or pyrimidine base found in DNA (e.g. an adenine "A," a guanine "G." a thymine "T" or a cytosine "C") or RNA (e.g. an A, a G. an uracil "U" or a C). The term "nucleic acid" encompasses the terms "oligonucleotide" and "polynucleotide," each as a subgenus of the term "nucleic acid." The term "oligonucleotide" refers to a molecule of between about 3 and about 100 nucleobases in length. The term "polynucleotide" refers to at least one molecule of greater than about 100 nucleobases in length. The term "nucleic acid" also refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or

DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides. The terms "polynucleotide sequence" and "nucleotide sequence" are also used interchangeably herein.

[0067] As used herein, the term "gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide, including both exon and (optionally) intron sequences. A "gene" refers to coding sequence of a gene product, as well as non-coding regions of the gene product, including 5'UTR and 3'UTR regions, introns and the promoter of the gene product. These definitions generally refer to a single-stranded molecule, but in specific embodiments will also encompass an additional strand that is partially, substantially or fully complementary to the single-stranded molecule. Thus, a nucleic acid may encompass a double-stranded molecule or a double-stranded molecule that comprises one or more complementary strand(s) or "complement(s)" of a particular sequence comprising a molecule. As used herein, a single stranded nucleic acid may be denoted by the prefix "ss", a double stranded nucleic acid by the prefix "ds", and a triple stranded nucleic acid by the prefix "is." The term "gene" refers to the segment of DNA involved in producing a polypeptide chain, it includes regions preceding and following the coding region as well as intervening sequences (introns) between individual coding segments (exons). A "promoter" is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain elements at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription of a nucleic acid sequence. The term "enhancer" refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence. An enhancer can function in either orientation and may be upstream or downstream of the promoter.

[0068] As used herein, the term "gene product(s)" is used to refer to include RNA transcribed from a gene (e.g., mRNA), or a polypeptide encoded by a gene or translated from RNA.

[0069] The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Peptides, oligopeptides, dimers, multimers, and the like, are also composed of linearly arranged amino acids linked by peptide bonds, and whether produced biologically, recombinantly, or synthetically and whether composed of naturally occurring or non-naturally occurring amino acids, are included within this definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include co-translational (e.g., leader sequence cleavage of amino acids 1-25 of SEQ ID NO:1) and post-translational modifications of the polypeptide, such as, for example, disulfide-bond formation, glycosylation, acetylation, phosphorylation, proteolytic cleavage (e.g., cleavage by furins or metalloproteases and prohormone convertases (PCs)), and the like. Furthermore, for purposes of the present invention, a "polypeptide" encompasses a protein that includes modifications, such as deletions, additions, and substitutions (generally conservative in nature as would be known to a person in the art), to the native sequence, as long as the protein maintains the desired activity. These modifications can be deliberate, as through site-directed mutagenesis, or can be accidental, such as through mutations of

hosts that produce the proteins, or errors due to PCR amplification or other recombinant DNA methods. Polypeptides or proteins are composed of linearly arranged amino acids linked by peptide bonds, but in contrast to peptides, has a well-defined conformation. Proteins, as opposed to peptides, generally consist of chains of 50 or more amino acids. For the purposes of the present invention, the term "peptide" as used herein typically refers to a sequence of amino acids of made up of a single chain of D- or L- amino acids or a mixture of D- and L-amino acids joined by peptide bonds. Generally, peptides contain at least two amino acid residues and are less than about 50 amino acids in length.

[0070] The incorporation of non-natural amino acids, including synthetic non-native amino acids, substituted amino acids, or one or more D-amino acids into the peptides (or other components of the composition, with exception for protease recognition sequences) is desirable in certain situations. D-amino acid-containing peptides exhibit increased stability *in vitro* or *in vivo* compared to L-amino acid-containing forms. Thus, the construction of peptides incorporating D-amino acids can be particularly useful when greater in vivo or intracellular stability is desired or required. More specifically, D-peptides are resistant to endogenous peptidases and proteases, thereby providing better oral trans-epithelial and transdermal delivery of linked drugs and conjugates, improved bioavailability of membrane-permanent complexes (see below for further discussion), and prolonged intravascular and interstitial lifetimes when such properties are desirable. The use of D-isomer peptides can also enhance transdermal and oral trans-epithelial delivery of linked drugs and other cargo molecules. Additionally, D-peptides cannot be processed efficiently for major histocompatibility complex class II-restricted presentation to T helper cells, and are therefore less likely to induce humoral immune responses in the whole organism. Peptide conjugates can therefore be constructed using, for example, D-isomer forms of cell penetrating peptide sequences, L-isomer forms of cleavage sites, and D-isomer forms of therapeutic peptides. In some embodiments, a recombinant human MIS protein is comprised of D- or L-amino acid residues, as use of naturally occurring L-amino acid residues has the advantage that any break-down products should be relatively non-toxic to the cell or organism.

[0071] In yet a further embodiment, a recombinant human MIS protein or fragments or derivatives thereof can be a retro-inverso peptides. A "retro-inverso peptide" refers to a peptide with a reversal of the direction of the peptide bond on at least one position, i.e., a reversal of the amino- and carboxy-termini with respect to the side chain of the amino acid. Thus, a retro-inverso analogue has reversed termini and reversed direction of peptide bonds while approximately maintaining the topology of the side chains as in the native peptide sequence. The retro-inverso peptide can contain L-amino acids or D-amino acids, or a mixture of L-amino acids and D-amino acids, up to all of the amino acids being the D-isomer. Partial retro-inverso peptide analogues are polypeptides in which only part of the sequence is reversed and replaced with enantiomeric amino acid residues. Since the retro-inverted portion of such an analogue has reversed amino and carboxyl termini, the amino acid residues flanking the retro-inverted portion are replaced by side-chain-analogous α-substituted geminal-diaminomethanes and malonates, respectively. Retro-inverso forms of cell penetrating peptides have been found to work

as efficiently in translocating across a membrane as the natural forms. Synthesis of retro-inverso peptide analogues are described in Bonelli, F. et al., Int J Pept Protein Res. 24(6):553-6 (1984); Verdini, A and Viscomi, G. C., J. Chem. Soc. Perkin Trans. 1:697-701 (1985); and U.S. Patent No. 6,261,569, which are incorporated herein in their entirety by reference. Processes for the solid-phase synthesis of partial retro-inverso peptide analogues have been described (EP 97994-B) which is also incorporated herein in its entirety by reference.

[0072] The term "fragment" of a peptide, polypeptide or molecule as used herein refers to any contiguous polypeptide subset of the molecule. The term "protein fragment" as used herein includes both synthetic and naturally-occurring amino acid sequences derivable from the naturally occurring amino acid sequence of MIS (SEQ ID NO:1). The protein is said to be "derivable from the naturally-occurring amino acid sequence of a recombinant human MIS protein" if it can be obtained by fragmenting the recombinant human MIS protein, or if it can be synthesized based upon a knowledge of the sequence of the naturally occurring amino acid sequence or of the genetic material (DNA or RNA) which encodes this sequence. Accordingly, a "fragment" of a molecule, is meant to refer to any polypeptide subset of the molecule. In some embodiments, a functional fragment of recombinant human MIS comprises at least the C-terminal domain and at least the N-terminal domain. . In some embodiments, a functional fragment comprises a portion of the C-terminal and/or a portion (e.g., fragment) of the N-terminal domain of the recombinant human MIS protien. Fragments of a recombinant human MIS protein which have the activity at least or greater than the wildtype MIS protein of SEQ ID NO: 1 as disclosed herein and which are soluble are also encompassed for use in the present invention.

[0073] Fragments of a recombinant human MIS protein, for example functional fragments of SEQ ID NO: 2 or 3 useful in the methods as disclosed herein have at least 30% the activity as that of a polypeptide of SEQ ID NO: 2 or 3 *in vivo*, e.g., to cause Mullerian duct regression in an Mullerian duct regression bioassay as disclosed herein in the Examples. Stated another way, a functional fragment of a recombinant human MIS protein is a fragment of any of SEQ ID NO: 2 or 3 which, alone or as a fusion protein can result in at least 30% of the same activity as compared to SEQ ID NO: 2 or 3 to bind and activate MISRII, or cause Mullerian duct regression in a Mullerian duct regression bioassay as disclosed herein (see Fig. 4). Fragments as used herein can be soluble (i.e. not membrane bound). A "fragment" can be at least about 6, at least about 9, at least about 15, at least about 20, at least about 30, least about 40, at least about 50, at least about 100, at least about 250, at least about 300 nucleic or amino acids, and all integers in between. Exemplary fragments include C-terminal truncations, N-terminal truncations, or truncations of both C- and N-terminals (e.g., deletions of, for example, at least 1, at least 2, at least 3, at least 4, at least 5, at least 8, at least 10, at least 15, at least 20, at least 25, at least 40, at least 50, at least 75, at least 100 or more amino acids deleted from the N-termini, the C-termini, or both). One of ordinary skill in the art can create such fragments by simple deletion analysis. Such a fragment of SEQ ID NO:2 or 3 can be, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids or

more than 10 amino acids, such as 15, 30, 50, 100 or more than 100 amino acids deleted from the N-terminal and/or C-terminal of SEQ ID NO: 2 or 3, respectively. Persons of ordinary skill in the art can easily identify the minimal peptide fragment of SEQ ID NO: 2 or 3 useful in the methods and compositions as disclosed herein, or fusion proteins as disclosed herein, by sequentially deleting N- and/or C-terminal amino acids from SEQ ID NO: 2 or 3, or sequentially deleting N-and C-terminal amino acids from recombinant human MIS protein and assessing the function of the resulting peptide fragment, alone or when it is cleaved. One can create functional fragments with multiple smaller fragments. These can be attached by bridging peptide linkers. One can readily select linkers to maintain wild type conformation. One of ordinary skill in the art can easily assess the function of recombinant human MIS protein as disclosed herein to activate MISRII or in the Mullerian duct regression bioassay, as disclosed herein as compared to a recombinant human MIS protein corresponding to SEQ ID NO: 2 or 3. Using such an *in vivo* assay, if the fragment of the recombinant human MIS protein has at least 30% of the biological activity of the recombinant human MIS protein corresponding to SEQ ID NO:2 or 3 as disclosed herein, then the fragment is considered a valid recombinant human MIS protein-fragment and can used in the compositions and methods as disclosed herein. In some embodiments, a fragment of SEQ ID NO: 2 or 3 can be less than 200, or less than 150 or less than 100, or less than 50, or less than 20 amino acids of SEQ ID NO: 2 or 3. In some embodiments, a fragment of SEQ ID NO: 2 or 3 is less than 100 peptides in length. However, as stated above, the fragment must be at least 6 amino acids, at least about 9, at least about 15, at least about 20, at least about 30, at least about 40, at least about 50, at least about 100, at least about 250, at least about 500 nucleic acids or amino acids, or any integers in between.

[0074] The term "derivative" as used herein refers to peptides which have been chemically modified, for example but not limited to by techniques such as ubiquitination, labeling, pegylation (derivatization with polyethylene glycol) or addition of other molecules. A molecule also a "derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties can improve the molecule's solubility, absorption, biological half life, etc. The moieties can alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., MackPubl., Easton, PA (1990).

[0075] The term "functional" when used in conjunction with "derivative" or "variant" or "fragment" refers to a polypeptide which possess a biological activity (either functional or structural) that is substantially similar to a biological activity of the polypeptide which it is a functional derivative, variant or functional fragment thereof. The term functional derivative is intended to include the fragments, analogues or chemical derivatives of a molecule. By "substantially similar" in this context is meant that the biological activity, e.g., activation of MISRII is at 25% or at least 35%, or at least 50% as active as a reference polypeptide, e.g., a corresponding wild-type MIS polypeptide or recombinant human MIS protein , and preferably at least 60% as active, 70% as active, 80% as active, 90% as active,

95% as active, 100% as active or even higher (i.e., the variant or derivative has greater activity than the wild-type), e.g., 110% as active, 120% as active, or more. Stated another way, a "substantially similar" functional fragment of a recombinant human MIS protein in this context is meant that at least 25%, at least 35%, at least 50% of the relevant or desired biological activity of a corresponding recombinant human MIS protein is retained. In the instance of a functional fragment or peptide of a recombinant human MIS protein as disclosed herein (e.g., SEQ ID NO: 2 or 3), a functional fragment of SEQ ID NO: 2 or 3 would be a protein or peptide comprising a portion of SEQ ID NO: 2 or 3 which retained an activity to activate MISRII, or in the Mullerian duct regression bioassay, as disclosed herein in the Examples; preferably the fragment of SEQ ID NO: 2 or 3 that retains at least 25%, at least 35%, at least 50% at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 100% or even higher (i.e., the variant or derivative has greater activity than the wild-type MIS of SEQ ID NO: 1 or of a recombinant human MIS protein of SEQ ID NO 2 or 3), e.g., at least 110%, at least 120%, or more activity compared to the full length SEQ ID NO: 2 or 3 to activate MISRII or cause Mullerian duct regression in the Mullerian duct regression bioassay as disclosed herein. As another example, in the instance of a fragment of MIS (e.g., amino acids 26-560 of SEQ ID NO: 1) would be a protein or peptide comprising a portion of amino acids 26-560 of SEQ ID NO: 1 which retained an activity for Mullerian duct regression, preferably the fragment of amino acids 26-560 of SEQ ID NO: 1 retains at least 25%, at least 35%, at least 50% at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 100% or even higher (i.e., the variant or derivative has greater activity than the wild-type), e.g., at least 110%, at least 120%, or more activity compared to the full length amino acids 26-560 of SEQ ID NO: 1 to cause Mullerian duct regression in an mullerian duct regression bioassay as disclosed herein in the Examples. As an alternative example, a fragment of a HSA leader sequence of SEQ ID NO: 6 would be a protein or peptide comprising a portion of SEQ ID NO: 6 which retained at least 25%, at least 35%, at least 50% at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 100% or even higher (i.e., the variant or derivative has greater activity than the wild-type HSA sequence), e.g., at least 110%, at least 120%, or more activity compared to the full length HSA sequence of SEQ ID NO: 6, as determined by an assay, for example as disclosed in U.S. Patent 5,759,802 which is incorporated herein in its entirety by reference.

[0076] The term "functional derivative" and "mimetic" or "biologically active variant" or "biologically active fragment" are used interchangeably, and refers to a compound which possess a biological activity (either functional or structural) that is substantially similar to a biological activity of the entity or molecule its is a functional derivative of (e.g., the recombinant human MIS protein). The term functional derivative is intended to include the fragments, variants, analogues or chemical derivatives of a molecule.

[0077] The term "functional derivatives" is intended to include the "fragments," "variants," "analogs," or "chemical derivatives" of a molecule. A molecule is said to be "substantially similar" to another molecule if both molecules have substantially similar structures or if both molecules possess a

similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the structure of one of the molecules not found in the other, or if the sequence of amino acid residues is not identical. An "analog" of a recombinant human MIS protein is meant to refer to a molecule substantially similar in function to either the entire molecule or to a fragment thereof. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties can improve the molecule's solubility, absorption, biological half life, etc. The moieties can alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., MackPubl., Easton, PA(1990).

[0078] A "variant" of a recombinant human MIS protein is meant to refer to a molecule substantially similar in structure and function to either the entire molecule, or to a fragment thereof. Accordingly, the term "variant" as used herein refers to a peptide or nucleic acid that differs from the naturally occurring polypeptide or nucleic acid by one or more amino acid or nucleic acid deletions, additions, substitutions or side-chain modifications, yet retains one or more specific functions or biological activities of the naturally occurring molecule. Amino acid substitutions include alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may be classified as "conservative", in which case an amino acid residue contained in a polypeptide is replaced with another naturally occurring amino acid of similar character either in relation to polarity, side chain functionality or size. Substitutions encompassed by the present invention may also be "non conservative", in which an amino acid residue which is present in a peptide is substituted with an amino acid having different properties, such as naturally-occurring amino acid from a different group (e.g., substituting a charged or hydrophobic amino; acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-conventional amino acid. In some embodiments amino acid substitutions are conservative. Also encompassed within the term variant when used with reference to a polynucleotide or polypeptide, refers to a polynucleotide or polypeptide that can vary in primary, secondary, or tertiary structure, as compared to a reference polynucleotide or polypeptide, respectively (e.g., as compared to a wild- type polynucleotide or polypeptide). A "variant" of a recombinant human MIS protein is meant to refer to a molecule substantially similar in structure and function, i.e. where the function is the ability to activate MISRII.

[0079] For example, a variant of a recombinant human MIS protein can contain a mutation or modification that differs from a reference amino acid in SEQ ID NO: 2 or 3. In some embodiments, a variant of SEQ ID NO: 2 or 3 is a fragment of SEQ ID NO: 2 or 3 as disclosed herein. In some embodiments, a variant can be a different isoform of SEQ ID NO: 2 or 3 or can comprise different isomer amino acids. Variants can be naturally-occurring, synthetic, recombinant, or chemically modified polynucleotides or polypeptides isolated or generated using methods well known in the art. Variants can include conservative or non-conservative amino acid changes, as described below.

Polynucleotide changes can result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence. Variants can also include insertions, deletions or substitutions of amino acids, including insertions and substitutions of amino acids and other molecules) that do not normally occur in the peptide sequence that is the basis of the variant, for example but not limited to insertion of ornithine which do not normally occur in human proteins.

[0080] The term "conservative substitution," when describing a polypeptide, refers to a change in the amino acid composition of the polypeptide that does not substantially alter the polypeptide's activity. For example, a conservative substitution refers to substituting an amino acid residue for a different amino acid residue that has similar chemical properties. Conservative amino acid substitutions include replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine. "Conservative amino acid substitutions" result from replacing one amino acid with another having similar structural and/or chemical properties, such as the replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine. Thus, a "conservative substitution" of a particular amino acid sequence refers to substitution of those amino acids that are not critical for polypeptide activity or substitution of amino acids with other amino acids having similar properties (e.g., acidic, basic, positively or negatively charged, polar or non-polar, etc.) such that the substitution of even critical amino acids does not reduce the activity of the peptide, (i.e. the ability of the peptide to reduce T-reg cells and/or decrease inflammatory cytokines as disclosed herein). Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, the following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). (See also Creighton, Proteins, W. H. Freeman and Company (1984).) In some embodiments, individual substitutions, deletions or additions that alter, add or delete a single amino acid or a small percentage of amino acids can also be considered "conservative substitutions" is the change does not reduce the activity of the MIS protein (i.e. the ability of a recombinant human MIS protein or variant to cause Mullerian duct regression *in vivo,* which can be determined using the Mullerian Duct regression bioassay as disclosed herein). Insertions or deletions are typically in the range of about 1 to 5 amino acids. The choice of conservative amino acids may be selected based on the location of the amino acid to be substituted in the peptide, for example if the amino acid is on the exterior of the peptide and expose to solvents, or on the interior and not exposed to solvents.

[0081] In alternative embodiments, one can select the amino acid which will substitute an existing amino acid based on the location of the existing amino acid, i.e. its exposure to solvents (i.e. if the amino acid is exposed to solvents or is present on the outer surface of the peptide or polypeptide as compared to internally localized amino acids not exposed to solvents). Selection of such conservative amino acid substitutions are well known in the art, for example as disclosed in Dordo et al, J. Mol Biol,

1999, 217, 721-739 and Taylor et al, J. Theor. Biol. 119(1986);205-218 and S. French and B. Robson, J. Mol. Evol. 19(1983)171. Accordingly, one can select conservative amino acid substitutions suitable for amino acids on the exterior of a protein or peptide (i.e. amino acids exposed to a solvent), for example, but not limited to, the following substitutions can be used: substitution of Y with F, T with S or K, P with A, E with D or Q, N with D or G, R with K, G with N or A, T with S or K, D with N or E, I with L or V, F with Y, S with T or A, R with K, G with N or A, K with R, A with S, K or P.

[0082] In alternative embodiments, one can also select conservative amino acid substitutions encompassed suitable for amino acids on the interior of a protein or peptide, for example one can use suitable conservative substitutions for amino acids on the interior of a protein or peptide (i.e. the amino acids are not exposed to a solvent), for example but not limited to, one can use the following conservative substitutions: where Y is substituted with F, T with A or S, I with L or V, W with Y, M with L, N with D, G with A, T with A or S, D with N, I with L or V, F with Y or L, S with A or T and A with S, G, T or V. In some embodiments, non-conservative amino acid substitutions are also encompassed within the term of variants. A variant of a recombinant human MIS protein, for example a variant of SEQ ID NO: 2 or 3 is meant to refer to any molecule substantially similar in structure and function to either the entire molecule of SEQ ID NO:2 or 3, or to a fragment thereof.

[0083] The terms "homology", "identity" and "similarity" refer to the degree of sequence similarity between two peptides or between two optimally aligned nucleic acid molecules. Homology and identity can each be determined by comparing a position in each sequence which can be aligned for purposes of comparison. For example, it is based upon using a standard homology software in the default position, such as BLAST, version 2.2.14. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by similar amino acid residues (e.g., similar in steric and/or electronic nature such as, for example conservative amino acid substitutions), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of similar or identical amino acids at positions shared by the compared sequences, respectfully. A sequence which is "unrelated" or "non-homologous" shares less than 40% identity, though preferably less than 25% identity with the sequences as disclosed herein.

[0084] As used herein, the term "sequence identity" means that two polynucleotide or amino acid sequences are identical (i.e., on a nucleotide-by-nucleotide or residue-by-residue basis) over the comparison window. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T. C, G. U. or I) or residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the comparison window (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

[0085] The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide or amino acid sequence, wherein the polynucleotide or amino acid comprises a sequence that has at least 85% sequence identity, preferably at least 90% to 95% sequence identity, more usually at least 99% sequence identity as compared to a reference sequence over a comparison window of at least 18 nucleotide (6 amino acid) positions, frequently over a window of at least 24-48 nucleotide (8-16 amino acid) positions, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the sequence which can include deletions or additions which total 20 percent or less of the reference sequence over the comparison window. The reference sequence can be a subset of a larger sequence. The term "similarity", when used to describe a polypeptide, is determined by comparing the amino acid sequence and the conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

[0086] As used herein, the terms "homologous" or "homologues" are used interchangeably, and when used to describe a polynucleotide or polypeptide, indicates that two polynucleotides or polypeptides, or designated sequences thereof, when optimally aligned and compared, for example using BLAST, version 2.2.14 with default parameters for an alignment (see herein) are identical, with appropriate nucleotide insertions or deletions or amino-acid insertions or deletions, in at least 70% of the nucleotides, usually from about 75% to 99%, and more preferably at least about 98 to 99% of the nucleotides. The term "homolog" or "homologous" as used herein also refers to homology with respect to structure and/or function. With respect to sequence homology, sequences are homologs if they are at least 50%, at least 60 at least 70%, at least 80%, at least 90%, at least 95% identical, at least 97% identical, or at least 99% identical. Determination of homologs of the genes or peptides of the present invention can be easily ascertained by the skilled artisan.

[0087] The term "substantially homologous" refers to sequences that are at least 90%, at least 95% identical, at least 96%, identical at least 97% identical, at least 98% identical or at least 99% identical. Homologous sequences can be the same functional gene in different species. Determination of homologs of the genes or peptides of the present invention can be easily ascertained by the skilled artisan.

[0088] A molecule is said to be "substantially similar" to another molecule if both molecules have substantially similar structures or if both molecules possess a similar biological activity, for example if both molecules are able to activate MISRII. Thus, provided that two molecules possess a similar activity, (i.e. a variant of a recombinant human MIS protein which can activate MISRII similar to that of the MIS protein which corresponds to SEQ ID NO: 1, or recombinant human MIS protein which corresponds to SEQ ID NO: 2 or 3) are considered variants and are encompassed for use as disclosed herein, even if the structure of one of the molecules not found in the other, or if the sequence of amino acid residues is not identical. Thus, provided that two molecules possess a similar biological activity, they are considered variants as that term is used herein even if the structure of one of the molecules not found in the other, or if the sequence of amino acid residues is not identical. In particular,

the term "substantially similar", when used to define a recombinant human MIS protein comprising a functional variant of recombinant human MIS protein as compared to the recombinant human MIS protein encoded by SEQ ID NO:2 or 3, means that a particular subject sequence, for example, a recombinant human MIS protein variant or derivative sequence, varies from the sequence of the natural (or wild-type) MIS of SEQ ID NO: 1 or recombinant human MIS protein (i.e. encoded by SEQ ID NO: 2 or 3), by one or more substitutions, deletions, or additions, although the net effect of which is to retain at least some of the biological activity found in the recombinant human MIS protein as disclosed herein. As such, nucleic acid and amino acid sequences having lesser degrees of similarity but comparable biological activity to recombinant human MIS protein are considered to be equivalents. In determining polynucleotide sequences, all subject polynucleotide sequences capable of encoding substantially similar amino acid sequences are considered to be substantially similar to a reference polynucleotide sequence, regardless of differences in codon sequence. A nucleotide sequence is "substantially similar" to a specific nucleic acid sequence of SEQ ID NO:4 or 5 as disclosed herein if: (a) the nucleotide sequence is hybridizes to the coding regions of the natural MIS nucleic acid, or (b) the nucleotide sequence is capable of hybridization to nucleotide sequence of a recombinant human MIS protein encoded by SEQ ID NO: 4 or 5 under moderately stringent conditions and has biological activity similar to the recombinant human MIS protein; or (c) the nucleotide sequences which are degenerative as a result of the genetic code to the nucleotide sequences defined in (a) or (b). Substantially similar proteins will typically be greater than about 80% similar to the corresponding sequence of the native protein.

[0089] The term "substantial similarity" in the context of polypeptide sequences, indicates that the polypeptide comprises a sequence with at least 60% sequence identity to a reference sequence, or 70%, or 80%, or 85% sequence identity to the reference sequence, or most preferably 90% identity over a comparison window of about 10-20 amino acid residues. In the context of amino acid sequences, "substantial similarity" further includes conservative substitutions of amino acids. Thus, a polypeptide is substantially similar to a second polypeptide, for example, where the two peptides differ by one or more conservative substitutions.

[0090] In one embodiment, the term "human homolog" to a gene transcript refers to a DNA sequence that has at least about 55% homology to the full length nucleotide sequence of the sequence of a recombinant human MIS protein gene as encoded by the genome of humans or an animal, for example mouse or transgenic animal. In one embodiment, the term "human homolog" to a protein identified as associated with a recombinant human MIS protein refers to an amino acid sequence that has 40% homology to the full length amino acid sequence of the protein identified as associated with a recombinant human MIS protein as encoded by the genome of the transgenic animal of the present invention, more preferably at least about 50%, still more preferably, at least about 60% homology, still more preferably, at least about 70% homology, even more preferably, at least about 75% homology, yet more preferably, at least about 80% homology, even more preferably at least about 85% homology, still

more preferably, at least about 90% homology, and more preferably, at least about 95% homology. As discussed above, the homology is at least about 50% to 100% and all intervals in between (i.e., 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, etc.). Determination of the human homologs of the genes of the present invention may be easily ascertained by the skilled artisan.

[0091] The term "conservative substitution," when describing a polypeptide, refers to a change in the amino acid composition of the polypeptide that does not substantially alter the polypeptide's activity. Thus, a "conservative substitution" of a particular amino acid sequence refers to substitution of those amino acids that are not critical for polypeptide activity or substitution of amino acids with other amino acids having similar properties (e.g., acidic, basic, positively or negatively charged, polar or non-polar, etc.) such that the substitution of even critical amino acids does not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, the following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). (See also Creighton, Proteins, W. H. Freeman and Company (1984).) In addition, individual substitutions, deletions or additions that alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservative substitutions."

[0092] As used herein, the term "nonconservative" refers to substituting an amino acid residue for a different amino acid residue that has different chemical properties. The nonconservative substitutions include, but are not limited to aspartic acid (D) being replaced with glycine (G); asparagine (N) being replaced with lysine (K); or alanine (A) being replaced with arginine (R).

[0093] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[0094] Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith and Waterman (Adv. Appl. Math. 2:482 (1981), which is incorporated by reference herein), by the homology alignment algorithm of Needleman and Wunsch (J. Mol. Biol. 48:443-53 (1970), which is incorporated by reference herein), by the search for similarity method of Pearson and Lipman (Proc. Natl. Acad. Sci. USA 85:2444-48 (1988), which is incorporated by reference herein), by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection. (See generally Ausubel et al. (eds.), Current Protocols in Molecular Biology, 4th ed., John Wiley and Sons, New York (1999)).

[0095] One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show the percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle (J. Mol. Evol. 25:351-60 (1987), which is incorporated by reference herein). The method used is similar to the method described by Higgins and Sharp (Comput. Appl. Biosci. 5:151-53 (1989), which is incorporated by reference herein). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

[0096] Another example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described by Altschul et al. (J. Mol. Biol. 215:403-410 (1990), which is incorporated by reference herein). (See also Zhang et al., Nucleic Acid Res. 26:3986-90 (1998); Altschul et al., Nucleic Acid Res. 25:3389-402 (1997), which are incorporated by reference herein). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information internet web site. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al. (1990), supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-9 (1992), which is incorporated by reference herein) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0097] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul, Proc. Natl. Acad. Sci. USA 90:5873-77 (1993), which is incorporated by reference herein). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more typically less than about 0.01, and most typically less than about 0.001.

[0098] The term "insertions" or "deletions" are typically in the range of about 1 to 5 amino acids. The variation allowed can be experimentally determined by producing the peptide synthetically while systematically making insertions, deletions, or substitutions of nucleotides in the sequence using recombinant DNA techniques.

[0099] The term "substitution" when referring to a peptide, refers to a change in an amino acid for a different entity, for example another amino acid or amino-acid moiety. Substitutions can be conservative or non-conservative substitutions.

[00100] An "analog" of a molecule such as a recombinant human MIS protein, for example SEQ ID NO: 2 or 3 refers to a molecule similar in function to either the entire molecule or to a fragment thereof. The term "analog" is also intended to include allelic, species and induced variants. Analogs typically differ from naturally occurring peptides at one or a few positions, often by virtue of conservative substitutions. Analogs typically exhibit at least 80 or 90% sequence identity with natural peptides. Some analogs also include unnatural amino acids or modifications of N or C terminal amino acids. Examples of unnatural amino acids are, for example but not limited to; acedisubstituted amino acids, N-alkyl amino acids, lactic acid, 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ-N-methylarginine. Fragments and analogs can be screened for prophylactic or therapeutic efficacy in transgenic animal models as described below.

[00101] By "covalently bonded" is meant joined either directly or indirectly (e.g., through a linker) by a covalent chemical bond.

[00102] The term "fusion protein" as used herein refers to a recombinant protein of two or more proteins. Fusion proteins can be produced, for example, by a nucleic acid sequence encoding one protein is joined to the nucleic acid encoding another protein such that they constitute a single open-reading frame that can be translated in the cells into a single polypeptide harboring all the intended proteins. The order of arrangement of the proteins can vary. As a non-limiting example, the nucleic acid sequence encoding the recombinant human MIS-fusion protein is derived from the nucleotide sequence of encoding a recombinant human MIS protein or a functional derivative fragment or variant thereof, fused in frame to an end, either the 5' or the 3' end, of a gene encoding a first fusion partner, such as a IgG1 Fc fragment. In this manner, on expression of the gene, the recombinant human MIS

protein or functional derivative fragment or variant thereof is functionally expressed and fused to the N-terminal or C-terminal end of the IgG1 Fc. In certain embodiments, modification of the polypeptide probe is such that the functionality of the recombinant human MIS protein or a functional derivative fragment or variant thereof remains substantially unaffected in terms of its biological activity by fusion to the first fusion partner, such as IgG1 Fc.

[00103] By "specifically binds" or "specific binding" is meant a compound or antibody that recognizes and binds a desired polypeptide but that does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which naturally includes a polypeptide of the invention.

[00104] By "substantially pure" or is meant a nucleic acid, polypeptide, or other molecule that has been separated from the components that naturally accompany it. Typically, a polypeptide is substantially pure when it is at least about 60%, or at least about 70%, at least about 80%, at least about 90%, at least about 95%, or even at least about 99%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. For example, a substantially pure polypeptide may be obtained by extraction from a natural source, by expression of a recombinant nucleic acid in a cell that does not normally express that protein, or by chemical synthesis.

[00105] By "enhanced proteolytic stability" is meant a reduction of in the rate or extent of proteolysis of a peptide sequence by at least about 2%, at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% as compared to a control sequence under the same conditions (e.g., *in vivo* or in an *in vitro* system such as in a cell or cell lysate). A peptide with enhanced proteolytic stability may contain any modification, for example, insertions, deletions, or point mutations which reduce or eliminate a site subject to proteolytic cleavage at a particular site. Sites of proteolytic cleavage may be identified based on known target sequences or using computer software (e.g., software described by Gasteiger et al., *Protein Identification and Analysis Tools on the ExPASy Server*. In John M. Walker, ed. *The Proteomics Protocols Handbook*, Humana Press (2005)). Alternatively, proteolytic sites can be determined experimentally, for example, by Western blot for the protein following expression or incubation in a cellular system or cellular lysate, followed by sequencing of the identified fragments to determine cleavage sites.

[00106] The term "recombinant" as used herein to describe a nucleic acid molecule, means a polynucleotide of genomic, cDNA, viral, semisynthetic, and/or synthetic origin, which, by virtue of its origin or manipulation, is not associated with all or a portion of the polynucleotide with which it is associated in nature. The term recombinant as used with respect to a protein or polypeptide, means a polypeptide produced by expression of a recombinant polynucleotide. The term recombinant as used with respect to a host cell means a host cell into which a recombinant polynucleotide has been introduced. Recombinant is also used herein to refer to, with reference to material (e.g., a cell, a nucleic

acid, a protein, or a vector) that the material has been modified by the introduction of a heterologous material (e.g., a cell, a nucleic acid, a protein, or a vector).

[00107] The terms "subject" and "individual" are used interchangeably herein, and refer to an animal, for example a human, to whom treatment, including prophylactic treatment, with the pharmaceutical composition according to the present invention, is provided. The term "subject" as used herein refers to human and non-human animals. The term "non-human animals" and "non-human mammals" are used interchangeably herein includes all vertebrates, e.g., mammals, such as non-human primates, (particularly higher primates), sheep, dog, rodent (e.g. mouse or rat), guinea pig, goat, pig, cat, rabbits, cows, and non-mammals such as chickens, amphibians, reptiles etc. In one embodiment, the subject is human. In another embodiment, the subject is an experimental animal or animal substitute as a disease model. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered. Examples of subjects include humans, dogs, cats, cows, goats, and mice. The term subject is further intended to include transgenic species. The term subject also encompasses a mammal, for example, a human, to whom treatment, such as therapeutic treatment and/or prophylactic treatment with a composition comprising a recombinant human MIS protein as disclosed herein is provided.

[00108] The term "tissue" is intended to include intact cells, blood, blood preparations such as plasma and serum, bones, joints, muscles, smooth muscles, and organs.

[00109] The term "disease" or "disorder" is used interchangeably herein, refers to any alternation in state of the body or of some of the organs, interrupting or disturbing the performance of the functions and/or causing symptoms such as discomfort, dysfunction, distress, or even death to the person afflicted or those in contact with a person. A disease or disorder can also related to a distemper, ailing, ailment, amlady, disorder, sickness, illness, complaint, inderdisposion, affection.

[00110] The term "malignancy" and "cancer" are used interchangeably herein, refers to diseases that are characterized by uncontrolled, abnormal growth of cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. The term is also intended to include any disease of an organ or tissue in mammals characterized by poorly controlled or uncontrolled multiplication of normal or abnormal cells in that tissue and its effect on the body as a whole. Cancer diseases within the scope of the definition comprise benign neoplasms, dysplasias, hyperplasias as well as neoplasms showing metastatic growth or any other transformations like e.g. leukoplakias which often precede a breakout of cancer.

[00111] As used herein, the term "tumor" refers to a mass of transformed cells that are characterized, at least in part, by containing angiogenic vasculature. The transformed cells are characterized by neoplastic uncontrolled cell multiplication which is rapid and continues even after the stimuli that initiated the new growth has ceased. The term "tumor" is used broadly to include the tumor parenchymal cells as well as the supporting stroma, including the angiogenic blood vessels that infiltrate the tumor parenchymal cell mass. Although a tumor generally is a malignant tumor, i.e., a

cancer having the ability to metastasize (i.e. a metastatic tumor), a tumor also can be nonmalignant (i.e. non-metastatic tumor). Tumors are hallmarks of cancer, a neoplastic disease the natural course of which is fatal. Cancer cells exhibit the properties of invasion and metastasis and are highly anaplastic.

[00112] As used herein, the term "metastases" or "metastatic tumor" refers to a secondary tumor that grows separately elsewhere in the body from the primary tumor and has arisen from detached, transported cells, wherein the primary tumor is a solid tumor. The primary tumor, as used herein, refers to a tumor that originated in the location or organ in which it is present and did not metastasize to that location from another location. As used herein, a "malignant tumor" is one having the properties of invasion and metastasis and showing a high degree of anaplasia. Anaplasia is the reversion of cells to an immature or a less differentiated form, and it occurs in most malignant tumors.

[00113] The term "therapy resistant cancer" as used herein refers to a cancer present in a subject which is resistant to, or refractory to at least two different anti-cancer agents such as chemotherapy agents, which means, typically a subject has been treated with at least two different anti-cancer agents that did not provide effective treatment as that term is defined herein.

[00114] The term 'sensitize' or 'sensitizes' used interchangeably herein, refers to making the cell sensitive, or susceptible to other secondary agents, for example other pro-drugs or other environmental effects such as radiation etc.

[00115] As used herein, the terms "treat" or "treatment" or "treating" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow the development of the disease, such as slow down the development of a tumor, the spread of cancer, or reducing at least one effect or symptom of a condition, disease or disorder associated with inappropriate proliferation or a cell mass, for example cancer. Treatment is generally "effective" if one or more symptoms or clinical markers are reduced as that term is defined herein. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a measurable lessening of one or more symptoms or measurable markers of a disease or disorder (e.g., cancer) and/or a cessation of at least slowing of progress or worsening of symptoms that would be expected in absence of treatment. Measurable lessening includes any statistically significant decline in a measurable marker or symptom. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already diagnosed with cancer, as well as those likely to develop secondary tumors due to metastasis. In some embodiments, treatment can be prophylactic treatment.

[00116] The term "effective amount" as used herein refers to the amount of a recombinant human MIS protein as disclosed herein, to alleviate at least one or more symptom of the disease or

disorder, and relates to a sufficient amount of pharmacological composition to provide the desired effect. The phrase "therapeutically effective amount" as used herein, e.g., a pharmaceutical composition comprising at least one recombinant human MIS protein as disclosed herein means a sufficient amount of the composition to treat a disorder, at a reasonable benefit/risk ratio applicable to any medical treatment. The term "therapeutically effective amount" therefore refers to an amount of the composition as disclosed herein that is sufficient to effect a therapeutically or prophylacticly significant reduction in a symptom or clinical marker associated with a cancer or a cancer-mediated condition.

[00117] A therapeutically or prophylatically significant reduction in a symptom is, e.g. at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 125%, at least about 150% or more in a measured parameter as compared to a control or non-treated subject. Measured or measurable parameters include clinically detectable markers of disease, for example, elevated or depressed levels of a biological marker, as well as parameters related to a clinically accepted scale of symptoms or markers for a disease or disorder. It will be understood, however, that the total daily usage of the compositions and formulations as disclosed herein will be decided by the attending physician within the scope of sound medical judgment. The exact amount required will vary depending on factors such as the type of disease being treated.

[00118] With reference to the treatment of a subject with a cancer with a pharmaceutical composition comprising at least one recombinant human MIS protein as disclosed herein, the term "therapeutically effective amount" refers to the amount that is safe and sufficient to prevent or delay the development and further growth of a tumor or the spread of metastases in cancer patients. The amount can thus cure or cause the cancer to go into remission, slow the course of cancer progression, slow or inhibit tumor growth, slow or inhibit tumor metastasis, slow or inhibit the establishment of secondary tumors at metastatic sites, or inhibit the formation of new tumor metastases. The effective amount for the treatment of cancer depends on the tumor to be treated, the severity of the tumor, the drug resistance level of the tumor, the species being treated, the age and general condition of the subject, the mode of administration and so forth. Thus, it is not possible to specify the exact "effective amount". However, for any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using only routine experimentation. The efficacy of treatment can be judged by an ordinarily skilled practitioner, for example, efficacy can be assessed in animal models of cancer and tumor, for example treatment of a rodent with a cancer, and any treatment or administration of the compositions or formulations that leads to a decrease of at least one symptom of the cancer, for example a reduction in the size of the tumor or a slowing or cessation of the rate of growth of the tumor indicates effective treatment. In embodiments where the compositions are used for the treatment of cancer, the efficacy of the composition can be judged using an experimental animal model of cancer, e.g., wild-type mice or rats, or preferably, transplantation of tumor cells. When using an experimental animal model, efficacy of treatment is evidenced when a reduction in a symptom of the cancer, for example a reduction in the

size of the tumor or a slowing or cessation of the rate of growth of the tumor occurs earlier in treated, versus untreated animals. By "earlier" is meant that a decrease, for example in the size of the tumor occurs at least 5% earlier, but preferably more, e.g., one day earlier, two days earlier, 3 days earlier, or more.

[00119] As used herein, the term "treating" when used in reference to a cancer treatment is used to refer to the reduction of a symptom and/or a biochemical marker of cancer, for example a significant reduction in at least one biochemical marker of cancer would be considered an effective treatment. Examples of such biochemical markers of cancer include CD44, telomerase, TGF-α, TGF-β, erbB-2, erbB-3, MUC1, MUC2, CK20, PSA, CA125 and FOBT. A reduction in the rate of proliferation of the cancer cells by at least about 10% would also be considered effective treatment by the methods as disclosed herein. As alternative examples, a reduction in a symptom of cancer, for example, a slowing of the rate of growth of the cancer by at least about 10% or a cessation of the increase in tumor size, or a reduction in the size of a tumor by at least about 10% or a reduction in the tumor spread (i.e. tumor metastasis) by at least about 10% would also be considered as affective treatments by the methods as disclosed herein. In some embodiments, it is preferred, but not required that the therapeutic agent actually kill the tumor.

[00120] The term "prophylactically effective amount" refers to an amount of a recombinant human MIS protein or functional fragment or variant thereof which is effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, e.g., to prevent the onset of cancer in a subject who is at risk of developing cancer. Typically, since a prophylactic dose of a recombinant human MIS protein or functional fragment or variant thereof is administered to a subject prior to, or at an earlier stage of a cancer, or to a subject who has a genetic predisposition to get cancer, for example, but by no way a limitation, to a subject that has a mutation in a gene which increases the likelihood of the subject getting ovarian cancer. In some embodiments, a prophylactically effective amount is less than the therapeutically effective amount. A prophylatically effective amount of a recombinant human MIS protein or functional fragment or variant thereof is also one in which any toxic or detrimental effects of the compound are outweighed by the beneficial effects.

[00121] As used herein, the terms "prevent," "preventing" and "prevention" refer to the avoidance or delay in manifestation of one or more symptoms or measurable markers of a disease or disorder, e.g., of an autoimmune disease. A delay in the manifestation of a symptom or marker is a delay relative to the time at which such symptom or marker manifests in a control or untreated subject with a similar likelihood or susceptibility of developing the disease or disorder. The terms "prevent," "preventing" and "prevention" include not only the avoidance or prevention of a symptom or marker of the disease, but also a reduced severity or degree of any one of the symptoms or markers of the disease, relative to those symptoms or markers in a control or non-treated individual with a similar likelihood or susceptibility of developing the disease or disorder, or relative to symptoms or markers likely to arise based on historical or statistical measures of populations affected by the disease or disorder. By

"reduced severity" is meant at least a 10% reduction in the severity or degree of a symptom or measurable disease marker, relative to a control or reference, e.g., at least 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99% or even 100% (i.e., no symptoms or measurable markers).

[00122] As used herein, the terms "administering," and "introducing" are used interchangeably herein and refer to the placement of the agents of metabolic regulators of the present invention into a subject by a method or route which results in at least partial localization of a recombinant human MIS protein at a desired site. The compounds of the present invention can be administered by any appropriate route which results in an effective treatment in the subject. In some embodiments, for the treatment of a cancer, the recombinant human MIS protein can be placed directly at, or near the site of the tumor or alternatively administered systemically.

[00123] A "composition" or "pharmaceutical composition" are used interchangeably herein refers to a composition that usually contains an excipient, such as a pharmaceutically acceptable carrier that is conventional in the art and that is suitable for administration to cells. The cells may be part of a subject, for example for therapeutic, diagnostic, or prophylactic purposes. The cells may also be cultured, for example cells as part of an assay for screening potential pharmaceutical compositions, and the cells may be part of a transgenic animal for research purposes. The composition can also be a cell culture, in which a polypeptide or polynucleotide encoding a metabolic regulator of the present invention is present in the cells and/or in the culture medium. In addition, compositions for topical (e.g., oral mucosa, respiratory mucosa) and/or oral administration can form solutions, suspensions, tablets, pills, capsules, sustained-release formulations, oral rinses, or powders, as known in the art and described herein. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, University of the Sciences in Philadelphia (2005) Remington: *The Science and Practice of Pharmacy with Facts and Comparisons,* 21st Ed.

[00124] The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and intrasternal injection and infusion. The phrases "systemic administration," "administered systemically", "peripheral administration" and "administered peripherally" as used herein mean the administration of a recombinant human MIS protein such that it enters the animal's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

[00125] The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00126] The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in maintaining the activity of or carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body. In addition to being "pharmaceutically acceptable" as that term is defined herein, each carrier must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation. The pharmaceutical formulation contains a compound of the invention in combination with one or more pharmaceutically acceptable ingredients. The carrier can be in the form of a solid, semi-solid or liquid diluent, cream or a capsule. These pharmaceutical preparations are a further object of the invention. Usually the amount of active compounds is between 0.1-95% by weight of the preparation, preferably between 0.2-20% by weight in preparations for parenteral use and preferably between 1 and 50% by weight in preparations for oral administration. For the clinical use of the methods of the present invention, targeted delivery composition of the invention is formulated into pharmaceutical compositions or pharmaceutical formulations for parenteral administration, e.g., intravenous; mucosal, e.g., intranasal; enteral, e.g., oral; topical, e.g., transdermal; ocular, e.g., via corneal scarification or other mode of administration. The pharmaceutical composition contains a compound of the invention in combination with one or more pharmaceutically acceptable ingredients. The carrier can be in the form of a solid, semi-solid or liquid diluent, cream or a capsule.

[00127] The term "oncogene" as used herein refers to a nucleic acid sequence encoding, or polypeptide, of a mutated and/or overexpressed version of a normal gene that in a dominant fashion can release the cell from normal restraints on growth and thus alone or in concert with other changes, contribute to a cells tumorigenicity. Examples of oncogenes include; gp40 (v-fms); p21 (ras); p55 (v-myc); p65 (gag-jun); pp60 (v-src);, v-abl; v-erb; v-erba; v-fos etc. A proto-oncogene refers to the normal expression of a nucleic acid expressing the normal, cellular equivalent of an oncogene, typically these genes are usually a gene involved in the signaling or regulation of cell growth.

[00128] The term "regeneration" means regrowth of a cell population, organ or tissue, and in some embodiments after disease or trauma.

[00129] The term "vectors" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked; a plasmid is a species of the genus encompassed by "vector". The term "vector" typically refers to a nucleic acid sequence containing an origin of replication and other entities necessary for replication and/or maintenance in a host cell. Vectors capable of directing the expression of genes and/or nucleic acid sequence to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome, and typically comprise entities for stable or transient expression or the encoded DNA. Other expression vectors can be used in the methods as disclosed herein for example, but are not limited to, plasmids, episomes, bacterial artificial chromosomes, yeast artificial

chromosomes, bacteriophages or viral vectors, and such vectors can integrate into the host's genome or replicate autonomously in the particular cell. A vector can be a DNA or RNA vector. Other forms of expression vectors known by those skilled in the art which serve the equivalent functions can also be used, for example self replicating extrachromosomal vectors or vectors which integrates into a host genome. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". Expression vectors can result in stable or transient expression of the DNA. An exemplary expression vector for use in the present invention is pcDNA3.1.

[00130] The term "viral vectors" refers to the use as viruses, or virus-associated vectors as carriers of the nucleic acid construct into the cell. Constructs may be integrated and packaged into non-replicating, defective viral genomes like Adenovirus, Adeno-associated virus (AAV), or Herpes simplex virus (HSV) or others, including reteroviral and lentiviral vectors, for infection or transduction into cells. The vector may or may not be incorporated into the cells genome. The constructs may include viral sequences for transfection, if desired. Alternatively, the construct may be incorporated into vectors capable of episomal replication, e.g EPV and EBV vectors.

[00131] As used herein, a "promoter" or "promoter region" or "promoter element" used interchangeably herein, refers to a segment of a nucleic acid sequence, typically but not limited to DNA or RNA or analogues thereof, that controls the transcription of the nucleic acid sequence to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences which modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be *cis*-acting or may be responsive to *trans*-acting factors. Promoters, depending upon the nature of the regulation may be constitutive or regulated.

[00132] The term "regulatory sequences" is used interchangeably with "regulatory elements" herein refers element to a segment of nucleic acid, typically but not limited to DNA or RNA or analogues thereof, that modulates the transcription of the nucleic acid sequence to which it is operatively linked, and thus act as transcriptional modulators. Regulatory sequences modulate the expression of gene and/or nucleic acid sequence to which they are operatively linked. Regulatory sequence often comprise "regulatory elements" which are nucleic acid sequences that are transcription binding domains and are recognized by the nucleic acid-binding domains of transcriptional proteins and/or transcription factors, repressors or enhancers etc. Typical regulatory sequences include, but are not limited to, transcriptional promoters, inducible promoters and transcriptional elements, an optional operate sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences to control the termination of transcription and/or translation. Regulatory sequences can be a single regulatory sequence or multiple regulatory sequences, or modified regulatory sequences or

fragments thereof. Modified regulatory sequences are regulatory sequences where the nucleic acid sequence has been changed or modified by some means, for example, but not limited to, mutation, methylation etc.

[00133] The term "operatively linked" as used herein refers to the functional relationship of the nucleic acid sequences with regulatory sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of nucleic acid sequences, typically DNA, to a regulatory sequence or promoter region refers to the physical and functional relationship between the DNA and the regulatory sequence or promoter such that the transcription of such DNA is initiated from the regulatory sequence or promoter, by an RNA polymerase that specifically recognizes, binds and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it may be necessary to modify the regulatory sequence for the expression of the nucleic acid or DNA in the cell type for which it is expressed. The desirability of, or need of, such modification may be empirically determined. Enhancers need not be located in close proximity to the coding sequences whose transcription they enhance. Furthermore, a gene transcribed from a promoter regulated in trans by a factor transcribed by a second promoter may be said to be operatively linked to the second promoter. In such a case, transcription of the first gene is said to be operatively linked to the first promoter and is also said to be operatively linked to the second promoter.

[00134] As used herein, the term "biological sample" also refers to a cell or population of cells or a quantity of tissue or fluid from a subject. Most often, the sample has been removed from a subject, but the term "biological sample" can also refer to cells or tissue analyzed *in vivo,* i.e. without removal from the subject. Often, a "biological sample" will contain cells from a subject, but the term can also refer to non-cellular biological material, such as non-cellular fractions of blood, saliva, or urine, that can be used to measure protein phosphorylation levels. In some embodiments, a "biological sample" or "tissue sample" refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, blood, plasma, serum, tumor biopsy, urine, stool, sputum, spinal fluid, pleural fluid, nipple aspirates, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, cells (including but not limited to blood cells), tumors, organs, and also samples of *in vitro* cell culture constituent. In some embodiments, a biological sample is from a resection, bronchoscopic biopsy, or core needle biopsy of a primary, secondary or metastatic tumor, or a cellblock from pleural fluid. In addition, fine needle aspirate biological samples are also useful. In some embodiments, a biological sample is primary ascite cells. Samples can be fresh, frozen, fixed or optionally paraffin-embedded, frozen or subjected to other tissue preservation methods, including for example methods to preserve the phosphorylation status of polypeptides in the biological sample. A biological sample can also mean a sample of biological tissue or fluid that comprises protein or cells. Such samples include, but are not limited to, tissue isolated from subjects or animals. Biological samples may also include sections of tissues such as biopsy and autopsy samples, frozen sections taken for histological purposes, blood, plasma, serum, sputum, stool, tears, mucus, hair, and skin. Biological

samples also include explants and primary and/or transformed cell cultures derived from patient tissues. A biological sample may be provided by removing a sample of cells from subject, but can also be accomplished by using previously isolated cells (e.g., isolated by another person, at another time, and/or for another purpose), or by performing the methods of the invention *in vivo*. Archival tissues, such as those having treatment or outcome history may also be used. Biological samples include, but are not limited to, tissue biopsies, scrapes (e.g. buccal scrapes), whole blood, plasma, serum, urine, saliva, cell culture, or cerebrospinal fluid. Biological samples also include tissue biopsies, cell culture. The biological sample can be obtained by removing a sample of cells from a subject, but can also be accomplished by using previously isolated cells (e.g. isolated by another person), or by performing the methods of the invention *in vivo*. Such samples include, but are not limited to, whole blood, cultured cells, primary cell preparations, sputum, amniotic fluid, tissue or fine needle biopsy samples, peritoneal fluid, and pleural fluid, among others. In some embodiments a biological sample is taken from a human patient, and in alternative embodiments the biological sample is taken from any mammal, such as rodents, animal models of diseases, commercial animals, companion animals, dogs, cats, sheep, cattle, and pigs, etc. The biological sample can be pretreated as necessary for storage or preservation, by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used. The biological sample can in certain circumstances be stored for use prior to use in the assay as disclosed herein. Such storage can be at +4C or frozen, for example at -20C or -80C, provided suitable cryopreservation agents are used to maintain cell viability once the cells are thawed.

[00135] The term "reduced" or "reduce" or "decrease" or "lower" as used herein generally means a decrease by a statistically significant amount relative to a reference. However, for avoidance of doubt, "reduced" means statistically significant decrease of at least 10% as compared to a reference level, for example a decrease by at least 20%, at least 30%, at least 40%, at least t 50%, or least 60%, or least 70%, or least 80%, at least 90% or more, up to and including a 100% decrease (i.e. absent level as compared to a reference sample), or any decrease between 10-100% as compared to a reference level, as that term is defined herein. The term "decrease" or "inhibition" used in the context of the level of expression or activity of a gene refers to a reduction in protein or nucleic acid level or activity in a cell, a cell extract, or a cell supernatant. For example, such a decrease may be due to reduced RNA stability, transcription, or translation, increased protein degradation, or RNA interference. Preferably, this decrease is at least about 5%, at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 80%, or even at least about 90% of the level of expression or activity under control conditions.

[00136] The term "low" as used herein generally means lower by a statically significant amount; for the avoidance of doubt, "low" means a statistically significant value at least 10% lower than a reference level, for example a value at least 20% lower than a reference level, at least 30% lower

than a reference level, at least 40% lower than a reference level, at least 50% lower than a reference level, at least 60% lower than a reference level, at least 70% lower than a reference level, at least 80% lower than a reference level, at least 90% lower than a reference level, up to and including 100% lower than a reference level (i.e. absent level as compared to a reference sample).

[00137] The terms "increased" or "increase" as used herein generally mean an increase by a statically significant amount; for the avoidance of doubt, "increased" means a statistically significant increase of at least 10% as compared to a reference level, including an increase of at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100% or more, including, for example at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 10-fold increase or greater as compared to a reference level, as that term is defined herein. The term an "increase" as used in the context of the expression or activity of a gene or protein is meant a positive change in protein or nucleic acid level or activity in a cell, a cell extract, or a cell supernatant. For example, such a increase may be due to increased RNA stability, transcription, or translation, or decreased protein degradation. Preferably, this increase is at least 5%, at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 80%, at least about 100%, at least about 200%, or even about 500% or more over the level of expression or activity under control conditions.

[00138] The term "high" as used herein generally means a higher by a statically significant amount relative to a reference; for the avoidance of doubt, "high" means a statistically significant value at least 10% higher than a reference level, for example at least 20% higher, at least 30% higher, at least 40% higher, at least 50% higher, at least 60% higher, at least 70% higher, at least 80% higher, at least 90% higher, at least 100% higher, at least 2-fold higher, at least 3-fold higher, at least 4-fold higher, at least 5-fold higher, at least 10-fold higher or more, as compared to a reference level.

[00139] The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

[00140] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean ±1%. The present invention is further explained in detail by the following examples, but the scope of the invention should not be limited thereto.

[00141] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims. Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

Mullerian Inhibiting Substance (MIS)

[00142] Without wishing to be bound by theory, the Mullerian Inhibiting Substance (MIS) is a member of the TGFβ multigene family of glycoproteins. The proteins in this gene family are all produced as dimeric precursors and undergo posttranslational processing for activation, requiring cleavage and dissociation to release bioactive C-terminal fragments. MIS is a 140-kDa dimer which consists of identical 70 kDa disulfide-linked monomers, each composed of a 57kDa N-terminal domain and a 12.5 kDa carboxyl-terminal (C-terminal). Thus, MIS comprises 2 identical monomers (and thus is termed a "homodimer"), each monomer comprising two domains, the N-terminal and C-terminal domain, which are held in non-covalent association. The purified C-terminal domain is the biologically active moiety and cleavage is required for activity. The N-terminal domain may assist with protein folding *in vivo* and facilitate delivery of the C-terminal peptide to its receptor, e.g., MISRI and MISRII. A non-cleavable mutant of MIS is biologically inactive.

[00143] The carboxy-terminal active domain shares amino acid homology with other TGFb family members, such as TGF-B 1, 2, and 3, inhibin, activin, and bone morphogenetic proteins, as well as a member of Growth and Differentiation Factors (GDFs). The structure of the MIS carboxy-terminal domain is supported by seven cysteines involved both in intra- and intermolecular disulfides bridges that lead to its structural stability, as revealed by homology to the three dimensional structure of TGFb using molecular modeling (Lorenzo, Donahoe, et al., unpublished data).

[00144] Like other TGFb family members, MIS can be cleaved by plasmin which generates its amino- and carboxy-terminal domains. This proteolytic process is required for its physiological activity and occurs at a site in a position similar to the dibasic cleavage site found in the sequence of TGFb. The resultant products are tightly associated in a non-covalent complex that dissociates at low pH; therefore, technically complex and time-demanding protocols with plasmin treatment and molecular size exclusion chromatography are required to enhance or complete the separation of the carboxy terminus from the amino terminus.

[00145] MIS contains two major cleavage sites that are sensitive to plasmin; the primary monobasic site which is located at amino acid position 426-427 of human wild-type MIS (corresponding to amino acid 451-452 of SEQ ID NO:1 herein). Cleavage at this site, which releases the active carboxy-terminal domain of MIS, resembles a consensus furin cleavage site. A secondary cleavage site (referred to as "R/S"), identified by amino-terminal sequencing of MIS fragments is located at residues 229-230 in the amino-terminal domain of wild-type MIS (corresponding to amino acids 254-255 of SEQ ID NO: 1). This site contains an R/S, but otherwise does not follow the consensus Arg–X–(Arg/Lys)–Arg for furin cleavage. Separation of purified carboxy-terminal from amino-terminal MIS after digestion with exogenous plasmin previously used molecular size-exclusion chromatography under acidic conditions. This technique requires extreme care to control MIS digestion, since long incubations of MIS in plasmin produced the carboxy- terminal MIS domain plus other fragments of 22 and 34 kDa, due to cleavage both at the primary and secondary sites, are extremely

difficult to separate from one another by size exclusion. Since all fragments generated after plasmin digestion are glycosylated, except the carboxy-terminal domain, wheat-germ lectin affinity can be used as an alternative to size chromatography separation to purify the carboxy- terminal domain of MIS. After plasmin cleavage, the resulting fragments can be loaded onto a wheat germ lectin column at pH 3.5 in order to dissociate the amino- and carboxy-terminal domains, as disclosed in Lorenzo et al., J. Chromatography, (2001), 776; 89-98, which is incorporated herein its entirety by reference.

[00146] Accordingly, to overcome several issues with respect to avoiding the production of fragments of MIS, e.g., both the carboxy- terminal MIS domain plus a 22 and 34 kDa fragments due to cleavage both at the primary and secondary sites, the inventors have modified the primary cleavage site at amino acid position 426–427 of human wild-type MIS (corresponding to amino acid 451-452 of SEQ ID NO:1 herein). To aid the purification of the C-terminal domain without the need for complicated methods using wheat-germ lectin affinity or size chromatography columns, the most flexible domain of the C-terminus , the inventors have included a tag at the N-terminus of the C-terminal domain.

[00147] Furthermore, the wild-type MIS protein is produced as a prohormone comprising a N-terminal leader sequence, which corresponds to amino acid residues 1-25 of SEQ ID NO: 1. Processing of the mature hormone MIS protein can involve the proteolytic cleavage and removal of the leader sequence (e.g., amino acids 1-25 of SEQ ID NO: 1), the cleavage of the MIS protein at the primary site to generate the N-terminal and C-terminal domains, and the formation of these domains into a monomer, which is disulfide linked by inter- and intrachain disulfide bonds to an identical monomer to form the bioactive homodimer MIS protein.

Leader Sequences

[00148] Without wishing to be bound by theory, leader sequences improve expression and/or secretion of a polypeptide of interest in a host cell, and are useful for the recombinant production of proteins. Generally, as an efficient method for secreting a desired protein by a genetic engineering procedure, a method is known wherein a fused protein comprising the desired protein (e.g., MIS) and a prepropeptide (signal peptide + propeptide) is expressed in a host cell and then intracellularly cleaved (processed) by enzymes of the host, and then, extracellularly secreted. According to this process, however, the fused protein must be cleaved twice by enzymes of the host to be a mature protein, resulting in lower yield of the mature protein and contamination of the mature protein with residual fused protein.

[00149] Accordingly, secreted proteins are expressed initially inside the cell in a precursor form containing a leader sequence ensuring entry into the secretory pathway. Such leader sequences, also referred to as signal peptides, direct the expressed product across the membrane of the endoplasmic reticulum (ER). Signal peptides are generally cleaved off by signal peptidases during translocation to the ER. Once entered in the secretory pathway, the protein is transported to the Golgi apparatus. From the Golgi the protein can follow different routes that lead to compartments such as the cell vacuole or

the cell membrane, or it can be routed out of the cell to be secreted to the external medium (Pfeffer and Rothman (1987) Ann. Rev. Biochem. 56:829-852).

[00150] For Industrial production of a secreted protein, the protein to be produced needs to be secreted efficiently from the host cell or the host organism. The signal peptide may be, e.g., the native signal peptide of the protein to be produced, a heterologous signal peptide, or a hybrid of native and heterologous signal peptide. However, several problems are encountered with the use of currently known signal peptides. One problem often encountered when producing a human protein from a non-human host cell or organism is that the native signal peptide does not ensure efficient translocation and/or cleavage of the signal peptide. This leads to low rates of protein secretion and/or to secretion of mature proteins that display N-terminal extensions due to an incorrect cleavage of the signal peptide. Thus the choice of the signal peptide is of great importance for industrial production of a protein.

[00151] In addition of leader sequences directing the secretion of the protein, a precursor form can comprise supplemental leader sequences that are cleaved during maturation. These supplemental leader peptides, named propeptides, usually follow the signal peptide. Virtually all peptide hormones, numerous bioactive protein (for example, growth factors, receptors and cell-adhesion molecules, and including MIS), and many bacterial toxins and viral envelope glycoproteins comprise a propeptide that is post-translationally excised to generate the mature and biologically active protein (Seidah and Chretien (1999) Brain Res. 848:45-62).

[00152] Peptides are further cleaved by enzymes named proprotein convertases. Mammalian proprotein convertases include, e.g., the subtilisin convertases PCSK1, PCSK2 and furin. Furin is ubiquitously expressed and located in the trans-Golgi network. Furin proteolytically activates large numbers of proproteins substrates in secretory pathway compartments. (Thomas (2002) Nat Rev Mol Cell Biol. 3:753-766). More specifically, furin localizes to the Trans Golgi Network, a late Golgi structure that is responsible for sorting secretory pathway proteins to their final destinations, including the cell surface, endosomes, lysosomes and secretory granules. The site that furin cleaves has been extensively studied. The cleavage site is positioned after the carboxyl-terminal arginine of the consensus sequence R-X-L/R-R, wherein X may represent any amino acid (Nakayama (1997) Biochem. J 327:625-635). The cleavage efficiency is increased when X is a lysine, a valine, an isoleucine or an alanine (Watanabe et al (1992) J Biol. Chem. 267:8270-8274).

[00153] In some embodiments, the recombinant human MIS protein comprises a modified leader sequence in place of the wild-type leader sequence of the MIS protein of SEQ ID NO:1. In some embodiments, the native leader sequence of amino acid residues 1-25 of SEQ ID NO: 1 is replaced with a non-MIS leader sequence, for example, but not limited to an albumin leader sequence, or functional fragment thereof. In some embodiments, the non-MIS leader sequence is a human serum albumin sequence (HSA), for example, a leader sequence corresponding to SEQ ID NO:6, which is encoded by nucleic acids corresponding to SEQ ID NO: 7.

[00154] In some embodiments, a HSA sequence is a functional fragment of SEQ ID NO: 6, for example, or at least 23, or at least 22, or at least 21, or at least 20, or at least 19, or at least 18, or at least 17, or at least 16, or at least 15, or at least 14, or at least 13, or at least 12, or at least 11, or at least 10, or less than 10 consecutive or non-consecutive amino acids of SEQ ID NO:6. Modified versions of HSA leader sequence are also encompassed for use in the present invention and are disclosed in US Patent 5,759,802 which is incorporated herein in its entirety by reference. In some embodiments, a functional fragment of HSA leader sequence is MKWVTFISLLFLFSSAYS (SEQ ID NO: 13) or variations therefor, which are disclosed in EP patent EP2277889 which is incorporated herein in its entirety. Variants of the pre-pro region of the HSA signal sequence (e.g., MKWVTFISLLFLFSSAYSRGVFRR, SEQ ID NO: 6) include fragments, such as the pre region of the HSA signal sequence (e.g., MKWVTFISLLFLFSSAYS, SEQ ID NO:13) or variants thereof, such as, for example, MKWVSFISLLFLFSSAYS, (SEQ ID NO:14)

[00155] In some embodiments, the leader sequence is a leader sequence is at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% identical to amino acid residues of SEQ ID NO: 6.

[00156] The HSA leader sequence as used herein resulted in an expected increased yield (both higher concentration and higher production) of the recombinant human MIS protein (see Fig. 2 and 3). However, the presence of the HSA leader sequence also resulted in a surprising and unexpected increase in cleavage from the primary cleavage site (corresponding to cleavage at 451/452 of SEQ ID NO: 1 (or 426/427 of conventional amino acid nomenclature of wild-type human MIS protein) (see Fig. 2 and 3). This increased yield and increased cleavage was surprising because with an increased yield (and therefore more protein produced by the cell), one would expect a decreased cleavage as the activity of the available cleavage enzymes becomes saturated and overextended. However, this was not the case - in fact the exact opposite occurred where with increased protein production there was increased cleavage from the primary cleavage site.

[00157] Other leader sequences are encompassed for use in a recombinant human MIS protein as disclosed herein, e.g., to replace amino acids 1-25 of SEQ ID NO: 1. Such leader sequences are well known in the art, and include the leader sequences comprising an immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide (IgSP-tPA), as disclosed in US 2007/0141666, which is incorporated herein in its entirety by reference. Numerous other signal peptides are used for production of secreted proteins. One of them is a murine immunoglobulin signal peptide (IgSP, EMBL Accession No. M13331). IgSP was first identified in 1983 by Loh et al. (Cell. 33:85-93). IgSP is known to give a good expression in mammalian cells. For example. EP patent No. 0382762 discloses a method of producing horseradish peroxidase by constructing a fusion polypeptide between IgSP and horseradish peroxidase.

[00158] Other leader sequences include, for example, but not limited to, the MPIF-1 signal sequence (e.g., amino acids 1-21 of GenBank Accession number AAB51134) MKVSVAALSCLMLVTALGSQA (SEQ ID NO: 15); the stanniocalcin signal sequence (MLQNSAVLLLLVISASA, SEQ ID NO:16); the invertase signal sequence (e.g., MLLQAFLFLLAGFAAKISA, SEQ ID NO:17); the yeast mating factor alpha signal sequence (e.g., K. lactis killer toxin leader sequence); a hybrid signal sequence (e.g., MKWVSFISLLFLFSSAYSRSLEKR, SEQ ID NO:18); an HSA/MFα-1 hybrid signal sequence (also known as HSA/kex2) (e.g., MKWVSFISLLFLFSSAYSRSLDKR, SEQ ID NO:19); a K. lactis killer/ MFα-1 fusion leader sequence (e.g., MNIFYIFLFLLSFVQGSLDKR, SEQ ID NO:20); the Immunoglobulin Ig signal sequence (e.g., MGWSCIILFLVATATGVHS, SEQ ID NO:21); the Fibulin B precursor signal sequence (e.g., MERAAPSRRVPLPLLLLGGLALLAAGVDA, SEQ ID NO:22); the clusterin precursor signal sequence (e.g., MMKTLLLFVGLLLTWESGQVLG, SEQ ID NO: 23); and the insulin-like growth factor-binding protein 4 signal sequence (e.g., MLPLCLVAALLLAAGPGPSLG, SEQ ID NO:24).

[00159] Where it is desirable to produce recombinant MIS in a bacterial system, leader sequences can include bacterial leader sequences as disclosed in US Application 2011/0020868. A number of other secretion signals have been described for use in expressing recombinant polypeptides or proteins. See, for example, U.S. Pat. No. 5,914,254; U.S. Pat. No. 4,963,495; European Patent No. 0 177 343; U.S. Pat. No. 5,082,783; PCT Publication No. WO 89/10971; U.S. Pat. No. 6,156,552; U.S. Pat. Nos. 6,495,357; 6,509,181; 6,524,827; 6,528,298; 6,558,939; 6,608,018; 6,617,143; U.S. Pat. Nos. 5,595,898; 5,698,435; and 6,204,023; U.S. Pat. No. 6,258,560; PCT Publication Nos. WO 01/21662, WO 02/068660 and U.S. Application Publication 2003/0044906; U.S. Pat. No. 5,641,671; and European Patent No. EP 0 121 352, which are incorporated herein in their entirety by reference.

Modified cleavage sites

[00160] As discussed herein, the preparation of a MIS protein for preclinical use is complex and inefficient. Human MIS protein is produced from a pre-proprotein, which comprises a leader sequence. The leader sequence (amino acids 1-25 of SEQ ID NO: 1) is cleaved off and the remaining protein (often called "holo-human MIS"), and corresponding to amino acid residues 26-560 of SEQ ID NO:1, must be additionally post-translationally cleaved to result in an N-terminal and an C-terminal domain. These N-terminal and C-terminal domains form a monomer, and two identical monomers (comprising the N- and C-terminal domains) form together to generate a homodimer. Holo-human MIS is cleaved into its N- and C-terminal domains most likely by means of furin or a related prohormone convertase PC5, expressed in the gonads. Cleavage occurs primarily at a kex-like site characterized by R^{-4} XXR^{-1} with a serine in the +1 site, which makes the MIS cleavage site monobasic, but more furin/hex consensus. The purified C-terminal domain is the biologically active moiety and cleavage is required for biological activity. A secondary cleavage site, whose significance is unknown, is observed less

frequently at residues 229–230 (which correspond to amino acids 254-255 of SEQ ID NO: 1). Non-cleavable mutants of MIS are not biologically active and mutations in the human gene that truncate the carboxy-terminal domain lead to persistent Mullerian duct syndrome. The cleavage of recombinantly expressed MIS protein by CHO cells is incomplete and inefficient, thus cleavage with an exogenous serine protease such as plasmin is required to enhance bioactivity.

[00161] Herein, the inventors have modified the kex-like site characterized by R^{-4} XXR^{-1} with an R in the -2 site, which makes the monobasic MIS cleavage site more like a consensus Kex/Furin recognition site. In particular, in one embodiment, the recombinant human MIS is produced from a proprotein where the amino acid residue at position 450 of SEQ ID NO: 1 has been changed from a Q (glutamine or Gln) to a R (arginine, or Arg). This mutation can be referred to as Q450R of SEQ ID NO:1. This corresponds to a change in amino acid residue 425 (Q425R) of MIS which is numbered with conventional protein numbering, where the first numbered amino acid begins *after* the leader sequence.

[00162] This change in amino acid sequence of Q450R of SEQ ID NO:1 allows for production of a highly purified cleaved preparation of human MIS protein which has full bioactivity.

[00163] In alternative embodiments, the primary cleavage site in the MIS protein, e.g., the monobasic site which is located at amino acid position 426–427 of human wild-type MIS (corresponding to amino acid 451-452 of SEQ ID NO:1 herein) can be modified to an amino acid recognition site which is recognized by a different cleavage enzyme. For example, the primary cleavage site in the MIS protein, e.g., the monobasic site which is located at amino acid position 426–427 can be modified to an amino acid sequence which is recognized by a protease or peptidase, such as pro-hormone convertases (PC's), or other cleaving agents expressed by a cell and found in surrounding tissue, or produced by a microbe capable of establishing an infection in a mammal. Enzyme-cleavable peptides can, but are not required to, contain one or more amino acids in addition to the amino acid recognition sequence; additional amino acids can be added to the amino terminal, carboxy terminal, or both the amino and carboxy terminal ends of the recognition sequence. Means of adding amino acids to an amino acid sequence, e.g., in an automated peptide synthesizer, as well as means of detecting cleavage of a peptide, e.g., by chromatographic analysis for the amino acid products of such cleavage, are well known to ordinarily skilled artisans given the teachings of this invention.

[00164] Prohormone protein convertases constitute a family of serine proteases structurally related to bacterial subtilisins and to yeast kexin. Several eukaryotic members of this family are currently known. Pro-hormone Convertases (PC's) cleave precursor polypeptides at specific basic residues, most often after selected paired basic residues, to generate bioactive peptide and proteins. Many members of the insulin family of proteins (e.g. Insulin, Igf-1) are substrates for PC's.

Tags to enhance purification

[00165] In some embodiments, a recombinant MIS protein comprises at least one internal label or "tag". In some embodiments the tag can be, for example, a c-myc, poly histidine, or FLAG tag. In some embodiments, the tag is a FLAG tag, for example, a FLAG tag of SEQ ID NO:8. A FLAG tag can be encoded by the nucleic acid of SEQ ID NO 9.

[00166] In some embodiments, the tag on the recombinant human MIS protein is internal at the carboxy terminus immediately downstream from the cleavage site. As it is the most flexible part of the C-terminus and not involved in binding to receptor and rendering specificity, as are the "fingertips" of the C-terminus (Papakostas et al, 2010, Lorenzo et al, 2002). In some embodiments, the labeling at this site is most likely to preserve biologic activity. In some embodiments, a tag, e.g., a FLAG tag is located after the primary cleavage site, e.g., after amino acid 450 of SEQ ID NO: 1 (corresponding to amino acid residue 425 of conventional protein nomenclature). In some embodiments, a tag is located between amino acid residues 452 and 453 of SEQ ID NO: 1 (which corresponds with amino acid residues 427 and 428 under normal amino acid nomenclature of MIS protein).

[00167] In alternative embodiments, the tag or label is located at any position between sequence 450 and 560 of SEQ ID NO: 1. In some embodiments, the tag is inserted 2 amino acid residues after the modified amino acid at position 450 of SEQ ID NO: 1. However, a position of the tag at the N-terminus of the C-terminal domain of MIS is preferred, as it location at the C-terminus of the C-terminal domain renders the C-terminal domain totally inactive, significantly reducing the bioactivity of the MIS protein.

[00168] In some embodiments, a recombinant MIS protein comprises more than one tag, e.g., for example, at least 2 or at least 3, or at least 4 or more than 4 tags. In some embodiments, the tags are sequential (e.g., one after another) and in some embodiments, they are dispersed (e.g., intermittent) in the recombinant human MIS protein. Preferably, the tags do not interfere or substantially affect the bioactivity of the recombinant MIS protein function at binding and activating MISRII. In some embodiments, where the recombinant MIS protein comprises more than one tag, the tags are the same tag. In alternative embodiments, where the recombinant MIS protein comprises more than one tag, the tags are different tags, for example, a recombinant MIS protein can comprise a FLAG tag and a histidine tag. The small size of the Flag tag allows it to be contained in the flexible, non binding N-terminal domain of the C-terminus. Accordingly, in some embodiments, any tag known to a person of ordinary skill in the art can be used in place of the Flag Tag, for example a tag of between about 5-10 amino acids, or between about 10-15 amino acids, or a tag between about 15-20 amino acids, or a tag between 20-30 amino acids, or a tag between about 30-50 amino acids. In some embodiments, a tag greater than 50 amino acids in length is not recommended, as the tag may sterically hinder the flexible N-terminus of the C-terminal domain, and thus inhibit the bioactivity of the recombinant MIS protein.

[00169] In some embodiments, a tag-labeled, e.g., FLAG tagged recombinant human MIS protein, such as the LRF recombinant human MIS protein as disclosed herein (see Fig. 1) can be eluted

by a single step to produce highly purified efficiently cleaved preparation with full bioactivity. When scaled-up, this purification of recombinant human MIS protein will be suitable for clinical applications; furthermore it will be useful for various binding assays in both clinical and experimental settings. Internal labeling of MIS during translation has proved to be more effective than labeling after purification of the protein as iodination or biotinylation greatly reduced MIS bioactivity. Surprisingly, the inventors have discovered that the LRF recombinant human MIS protein construct is more bioactive than the wild-type MIS. Inserting the FLAG tag sequence has several other distinct advantages. First, its unique amino acid domain is not present in any other gene (except for mouse brain phosphatase), thus making the anti-FLAG antibody very specific. Second, the elution of the protein with the 3x FLAG peptide is specific for the FLAG MIS and not other proteins that bind non-specifically to the agarose beads.

[00170] Surprisingly, a FLAG-tagged, cleavage optimized recombinant human MIS (e.g., a RF recombinant human MIS or RARR/S (SEQ ID NO: 27) FLAG MIS) was bioactive whereas a FLAG-tagged, non-cleavage optimized recombinant human MIS (e.g., RAQR/R (SEQ ID NO: 28) FLAG MIS) was not when compared to native human MIS or to the previously prepared untagged RAQR/R (SEQ ID NO: 28) MIS. As it is likely that the presence of the acidic FLAG tag so close to the cleavage site may impair the degree of cleavage, thus causing loss of activity. Thus, the inventors did not anticipate enhanced cleavage with the addition of the Flag tag. Moreover, the holo RAQR/R FLAG MIS ("RAQR/R" disclosed as SEQ ID NO: 28) preparation in CHO (or HEK) cells is not bioactive, as no endogenous processing occurs with the RAQR/R (SEQ ID NO: 28) cleavage site in contrast to what was reported by Kurian (Cancer Res., 1995. 1;343-349) when the construct lacked the FLAG tag. On the other hand, the retention of the serine at position 428 and the conversion of the monobasic site to dibasic (corresponding to Q>R at amino acid position 425 using conventional protein nomenclature), or Q>R at position 450 of SEQ ID NO: 1) makes the endogenous cleavage more efficient and very specific. Furthermore, a tag such as a FLAG MIS is a powerful tool for binding studies, and can be used to immunoprecipitate the endogenous MISRII without crosslinking. Accordingly, in some embodiments, a labeled recombinant human MIS protein, e.g., a MIS with an internal FLAG is useful in an efficient method for producing a highly pure and biologically active internally labeled form of MIS, which can be used for scale-up for preclinical and clinical use, for the study of MIS binding proteins and for tracking in pharmacokinetic studies.

Variants of a human recombinant MIS protein.

[00171] In some embodiments, a recombinant human MIS protein as disclosed can have a modification in the core MIS protein sequence, e.g., amino acids residues 26-560 of SEQ ID NO: 1 (including a modification of amino acid residue 450 from Q to R of SEQ ID NO: 1) and/or the insertion of a tag at the beginning of the C-terminal domain). Such variants are considered to be homologous to wild-type MIS protein.

[00172] As used herein, the term "polypeptide" refers to a polymer of amino acids and its equivalent and does not refer to a specific length of the product; thus, peptides, oligopeptides and proteins are included within the definition of a polypeptide. A derivative is a polypeptide having conservative amino acid substitutions, as compared with another sequence. Derivatives further include other modifications of proteins, including, for example, modifications such as glycosylations, acetylations, phosphorylations, and the like.

[00173] In some embodiments, a recombinant human MIS protein is at least 75%, at least 80%, at least 85%, at least 90% or at least 95% similar to the homologous recombinant human MIS protein. As used herein, "similarity" or "percent similarity" in the context of two or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or conservative substitutions thereof, that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms, or by visual inspection. By way of example, a first amino acid sequence can be considered similar to a second amino acid sequence when the first amino acid sequence is at least 50%, 60%, 70%, 75%, 80%, 90%, or even 95% identical, or conservatively substituted, to the second amino acid sequence when compared to an equal number of amino acids as the number contained in the first sequence, or when compared to an alignment of polypeptides that has been aligned by a computer similarity program known in the art, as discussed below.

[00174] Homologues and functional derivatives and functional fragments of MIS of SEQ ID NO: 1 are also encompassed for use in the present invention, and can also be identified, for example, by expression of MIS from an expression library. (See, e.g., Sambrook et al. (2001). Molecular cloning: a laboratory manual, 3rd ed. (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press); Ausubel et al., supra.) A mutated endogenous gene sequence can be referred to as a heterologous transgene; for example, a transgene encoding a mutation in MIS which is not known in naturally-occurring genomes is a heterologous transgene with respect to murine and non-murine, e.g., human species. A MIS protein, such as, for example, those disclosed in U.S. Patent Publication Nos. 5,427,780, 5,359,033 and 5,661,126 (the disclosures of which are incorporated by reference herein).

[00175] The variation in primary structure of core human MIS protein sequence (e.g., amino acids residues 26-560 of SEQ ID NO: 1 (including a modification of amino acid residue 450 from Q to R of SEQ ID NO: 1) and/or the insertion of a tag at the beginning of the N-terminal domain of the C-terminal domain), or functional fragment, or a homologue are encompassed for use in the present invention, for instance, may include deletions, additions and substitutions. The substitutions may be conservative or non-conservative. The differences between a recombinant human MIS protein and a variant generally conserve desired properties, mitigate or eliminate undesired properties and add desired or new properties. For example, variants of a recombinant human MIS protein can have superior activity as compared to wild-type MIS protein.

[00176] It will be appreciated by those of skill that the core human MIS protein sequence (e.g., amino acids residues 26-560 of SEQ ID NO: 1) of a recombinant human MIS protein as disclosed herein can be readily manipulated to alter the amino acid sequence of a protein. A gene encoding the MIS protein or a functional fragment, homologue or variant thereof, can be manipulated by a variety of well known techniques for *in vitro* mutagenesis, among others, to produce variants of the naturally occurring human protein or fragment thereof, herein referred to as variants or muteins, may be used in accordance with the invention.

Other modifications to a recombinant human MIS protein

[00177] The recombinant human MIS protein useful in the present invention can also be modified at their amino termini, for example, so as to increase their hydrophilicity. Increased hydrophobicity enhances exposure of the peptides on the surfaces of lipid-based carriers into which the parent peptide-lipid conjugates have been incorporated. Polar groups suitable for attachment to peptides so as to increase their hydrophilicity are well known, and include, for example and without limitation: acetyl ("Ac"), 3- cyclohexylalanyl ("Cha"), acetyl-serine ("Ac Ser"), acetyl-seryl-serine ("Ac-Ser-Ser-"), succinyl ("Suc"), succinyl-serine ("Suc-Ser"), succinyl-seryl-serine ("Suc-Ser-Ser"), methoxy succinyl ("MeO-Suc"), methoxy succinyl-serine ("MeO-Suc-Ser"), methoxy succinyl-seryl-serine ("MeO-Suc-Ser-Ser") and seryl-serine ("Ser-Ser-") groups, polyethylene glycol ("PEG"), polyacrylamide, polyacrylomorpholine, polyvinylpyrrolidine, a polyhydroxyl group and carboxy sugars, e.g., lactobionic, N-acetyl neuraminic and sialic acids, groups. The carboxy groups of these sugars would be linked to the N-terminus of the peptide via an amide linkage. Presently, the preferred N- terminal modification is a methoxy-succinyl modification.

[00178] In some embodiments, a recombinant human MIS protein can be fused to one or more fusion partners. In certain embodiments, one of the fusion partners is the Fc protein (e.g., mouse Fc or human Fc). The fusion protein may further include a second fusion partner such as a purification or detection tag, for example, proteins that may be detected directly or indirectly such as green fluorescent protein, hemagglutinin, or alkaline phosphatase), DNA binding domains (for example, GAL4 or LexA), gene activation domains (for example, GAL4 or VP16), purification tags, or secretion signal peptides (e.g., preprotyrypsin signal sequence).

[00179] In one embodiment, a recombinant human MIS protein fusion protein useful in the methods and compositions as disclosed herein can comprise a human Fc protein or a functional fragment thereof. Accordingly, in one embodiment, a recombinant human MIS protein fusion protein useful in the methods and compositions as disclosed herein can comprises a human Fc molecule as the first fusion partner, where the Fc fragment can be SEQ ID NO: 10 or functional variants or functional derivatives thereof, where SEQ ID NO: 10 is as follows:

LELVPRGSGDPIEGRGGGGGDPKSCDKPHTCPLCPAPELLGGPSVFLFPPKPKDTLMISRTP
EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL

NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKATPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN
HYTQKSLSLSPGK

[00180] Variations and modifications to a recombinant human MIS protein and vectors can be used to increase or decrease recombinant human MIS protein expression, and to provide means for targeting. For example, a recombinant human MIS protein can be linked with a molecular targeting molecule for targeting cancer cells or ovarian cells, to make the recombinant human MIS protein specific for cancers or tissue specific to the ovary, respectively.

[00181] In one embodiment, a recombinant human MIS protein is fused to a second fusion partner, such as a carrier molecule to enhance its bioavailability. Such carriers are known in the art and include poly (alkyl) glycol such as poly ethylene glycol (PEG). Fusion to serum albumin can also increase the serum half-life of therapeutic polypeptides.

[00182] In some embodiments, a recombinant human MIS protein can also be fused to a second fusion partner, for example, to a polypeptide that targets the product to a desired location, or, for example, a tag that facilitates its purification, if so desired. In some embodiments, tags and fusion partners can be designed to be cleavable, if so desired. Another modification specifically contemplated is attachment, e.g., covalent attachment, to a polymer. In one aspect, polymers such as polyethylene glycol (PEG) or methoxypolyethylene glycol (mPEG) can increase the *in vivo* half-life of proteins to which they are conjugated. Methods of PEGylation of polypeptide agents are well known to those skilled in the art, as are considerations of, for example, how large a PEG polymer to use.

[00183] In some embodiments, a recombinant human MIS protein or functional fragment thereof is modified to achieve adequate circulating half-lives, which impact dosing, drug administration and efficacy. Many approaches have been undertaken with the aim to increase the half-life of biotherapeutics. Small proteins below 60 kD are cleared rapidly by the kidney and therefore do not reach their target. This means that high doses are needed to reach efficacy. The modifications to a recombinant human MIS protein and fragments encompassed in the methods of the present invention to increase the half-life of proteins in circulation include: PEGylation; conjugation or genetic fusion with proteins, e.g., transferrin (WO06096515A2), albumin, growth hormone (US2003104578AA); conjugation with cellulose (Levy and Shoseyov, 2002); conjugation or fusion with Fc fragments; glycosylation and mutagenesis approaches (Carter, 2006), which are incorporated herein by reference.

[00184] In the case of PEGylation, polyethylene glycol (PEG) is conjugated to a recombinant human MIS protein or fragment, which can be for example a plasma protein, antibody or antibody fragment. The first studies regarding the effect of PEGylation of antibodies were performed in the 1980s. The conjugation can be done either enzymatically or chemically and is well established in the art (Chapman, 2002; Veronese and Pasut, 2005). With PEGylation the total size can be increased, which reduces the chance of renal filtration. PEGylation further protects from proteolytic degradation and slows the clearance from the blood. Further, it has been reported that PEGylation can reduce

immunogenicity and increase solubility. The improved pharmacokinetics by the addition of PEG is due to several different mechanisms: increase in size of the molecule, protection from proteolysis, reduced antigenicity, and the masking of specific sequences from cellular receptors. In the case of antibody fragments (Fab), a 20-fold increase in plasma half-life has been achieved by PEGylation (Chapman, 2002).

[00185] To date there are several approved PEGylated drugs, e.g., PEG–interferon alpha2b (PEG-INTRON) marketed in 2000 and alpha2a (Pegasys) marketed in 2002. A PEGylated antibody fragment against TNF alpha, called Cimzia or Certolizumab Pegol, was filed for FDA approval for the treatment of Crohn's disease in 2007 and has been approved on April 22, 2008. A limitation of PEGylation is the difficulty in synthesizing long monodisperse species, especially when PEG chains over 1000 kD are needed. For many applications, polydisperse PEG with a chain length over 10000 kD is used, resulting in a population of conjugates having different length PEG chains, which need extensive analytics to ensure equivalent batches between productions. The different length of the PEG chains may result in different biological activities and therefore different pharmacokinetics. Another limitation of PEGylation is a decrease in affinity or activity as it has been observed with alpha-interferon Pegasys, which has only 7% of the antiviral activity of the native protein, but has improved pharmacokinetics due to the enhanced plasma half-life.

[00186] In some embodiments, a recombinant human MIS protein or fragment thereof is conjugated with a long lived protein, e.g. albumin, which is 67 kD and has plasma half-life of 19 days in human (Dennis et al., 2002). Albumin is the most abundant protein in plasma and is involved in plasma pH regulation, but also serves as a carrier of substances in plasma. In the case of CD4, increased plasma half-life has been achieved after fusing it to human serum albumin (Yeh et al., 1992). Other examples for fusion proteins are insulin, human growth hormone, transferrin and cytokines (Ali et al., 1999; Duttaroy et al., 2005; Melder et al., 2005; Osborn et al., 2002a; Osborn et al., 2002b; Sung et al., 2003) and see (US2003104578A1, WO06096515A2, and WO07047504A2, herein incorporated in entirety by reference).

[00187] The effect of glycosylation on plasma half-life and protein activity has also been extensively studied. In the case of tissue plasminogen activator (tPA) the addition of new glycosylation sites decreased the plasma clearance, and improved the potency (Keyt et al., 1994). Glycoengineering has been successfully applied for a number of recombinant proteins and immunoglobulins (Elliott et al., 2003; Raju and Scallon, 2007; Sinclair and Elliott, 2005; Umana et al., 1999). Further, glycosylation influences the stability of immunoglobulins (Mimura et al., 2000; Raju and Scallon, 2006).

[00188] In some embodiments, a recombinant human MIS protein or fragments thereof can be fused to the Fc fragment of an IgG (Ashkenazi and Chamow, 1997). The Fc fusion approach has been utilized, for example in the Trap Technology developed by Regeneron (e.g. IL1 trap and VEGF trap). The use of albumin to extend the half-life of peptides has been described in US2004001827A1. Positive effects of albumin have also been reported for Fab fragments and scFv-HSA fusion protein (Smith et

al., 2001). It has been demonstrated that the prolonged serum half-life of albumin is due to a recycling process mediated by the FcRn (Anderson et al., 2006; Chaudhury et al., 2003; Smith et al., 2001).

[00189] In some embodiments, a recombinant human MIS protein is conjugated to a biotinylated Fc protein, as disclosed in US application 2010/0209424, which is incorporated herein in its entirety by reference.

[00190] As used herein, the term "conjugate" or "conjugation" refers to the attachment of two or more entities to form one entity. For example, the methods of the present invention provide conjugation of a recombinant human MIS protein (i.e. SEQ ID NO: 2 or 3 or fragments or derivatives or variants thereof) joined with another entity, for example a moiety such as a first fusion partner that makes the recombinant human MIS protein stable, such as Ig carrier particle, for example IgG1 Fc. The attachment can be by means of linkers, chemical modification, peptide linkers, chemical linkers, covalent or non-covalent bonds, or protein fusion or by any means known to one skilled in the art. The joining can be permanent or reversible. In some embodiments, several linkers can be included in order to take advantage of desired properties of each linker and each protein in the conjugate. Flexible linkers and linkers that increase the solubility of the conjugates are contemplated for use alone or with other linkers as disclosed herein. Peptide linkers can be linked by expressing DNA encoding the linker to one or more proteins in the conjugate. Linkers can be acid cleavable, photocleavable and heat sensitive linkers. Methods for conjugation are well known by persons skilled in the art and are encompassed for use in the present invention.

[00191] According to the present invention, a recombinant human MIS protein (i.e. SEQ ID NO: 2 or 3 or fragments, derivatives or variants thereof), can be linked to the first fusion partner via any suitable means, as known in the art, see for example U.S. Patent Nos. 4,625,014, 5,057,301 and 5, 514,363, which are incorporated herein in their entirety by reference. For example, a recombinant human MIS proteincan be covalently conjugated to the IgG1 Fc, either directly or through one or more linkers. In one embodiment, a recombinant human MIS protein as disclosed herein is conjugated directly to the first fusion partner (e.g. Fc), and in an alternative embodiment, a recombinant human MIS protein as disclosed herein can be conjugated to a first fusion partner (such as IgG1 Fc) via a linker, e.g. a transport enhancing linker.

[00192] A large variety of methods for conjugation of a recombinant human MIS protein as disclosed herein with a first fusion partner (e.g. Fc) are known in the art. Such methods are e.g. described by Hermanson (1996, Bioconjugate Techniques, Academic Press), in U.S. 6,180,084 and U.S. 6,264,914 which are incorporated herein in their entirety by reference and include e.g. methods used to link haptens to carriers proteins as routinely used in applied immunology (see Harlow and Lane, 1988, "Antibodies: A laboratory manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). It is recognized that, in some cases, a recombinant human MIS protein can lose efficacy or functionality upon conjugation depending, e.g., on the conjugation procedure or the chemical group utilized therein. However, given the large variety of methods for conjugation the skilled person is able to find a

conjugation method that does not or least affects the efficacy or functionality of the entities, such as a recombinant human MIS protein to be conjugated.

[00193] Suitable methods for conjugation of a recombinant human MIS protein as disclosed herein with a first fusion partner (e.g. Fc) include e.g. carbodimide conjugation (Bauminger and Wilchek, 1980, Meth. Enzymol. 70: 151-159). Alternatively, a moiety can be coupled to a targeting agent as described by Nagy et al., Proc. Natl. Acad. Sci. USA 93:7269-7273 (1996), and Nagy et al., Proc. Natl. Acad. Sci. USA 95:1794-1799 (1998), each of which are incorporated herein by reference. Another method for conjugating one can use is, for example sodium periodate oxidation followed by reductive alkylation of appropriate reactants and glutaraldehyde crosslinking.

[00194] One can use a variety of different linkers to conjugate a recombinant human MIS protein as disclosed herein with a first fusion partner (e.g. Fc), for example but not limited to aminocaproic horse radish peroxidase (HRP) or a heterobiofunctional cross-linker, e.g. carbonyl reactive and sulfhydryl- reactive cross-linker. Heterobiofunctional cross linking reagents usually contain two reactive groups that can be coupled to two different function targets on proteins and other macromolecules in a two or three-step process, which can limit the degree of polymerization often associated with using homobiofunctional cross-linkers. Such multi-step protocols can offer a great control of conjugate size and the molar ratio of components.

[00195] The term "linker" refers to any means to join two or more entities, for example a recombinant human MIS protein as disclosed herein with a first fusion partner (e.g. Fc). A linker can be a covalent linker or a non-covalent linker. Examples of covalent linkers include covalent bonds or a linker moiety covalently attached to one or more of the proteins to be linked. The linker can also be a non-covalent bond, e.g. an organometallic bond through a metal center such as platinum atom. For covalent linkages, various functionalities can be used, such as amide groups, including carbonic acid derivatives, ethers, esters, including organic and inorganic esters, amino, urethane, urea and the like. To provide for linking, the effector molecule and/or the probe can be modified by oxidation, hydroxylation, substitution, reduction etc. to provide a site for coupling. It will be appreciated that modification which do not significantly decrease the function of a recombinant human MIS protein as disclosed herein or the first fusion partner (e.g. Fc) are preferred.

[00196] *Targeting*. In some embodiments, a recombinant human MIS protein, or functional fragment, or a homologue for use in the methods and compositions as disclosed herein can be targeted to a cancer or ovarian cells via a targeting ligand. A targeting ligand is a molecule, e.g., small molecule, protein or fragment thereof that specifically binds with high affinity to a target, e.g., a cell-surface marker on a pre-selected cell, such as a surface protein such as a receptor that is present to a greater degree on the pre-selected cell target than on any other body tissue. Accordingly, in some embodiments, a recombinant human MIS protein for use in the compositions and methods as disclosed herein can be fused to a Fc and/or optionally also to a targeting molecule. In some embodiments, a nucleic acid encoding a targeting ligand can be fused to a nucleotide encoding a recombinant human MIS protein or

fragment or homologue or variant thereof. Another example of a targeting ligand is a group of cadherin domains from a human cadherin. A targeting ligand component attached to a recombinant human MIS protein can include a naturally occurring or recombinant or engineered ligand, or a fragment thereof, capable of binding the pre- selected target cell.

[00197] Further examples of targeting ligands also include, but are not limited to, antibodies and portions thereof that specifically bind a pre-selected cell surface protein with high affinity. By "high affinity" is meant an equilibrium dissociation constant of at least molar, as determined by assay methods known in the art, for example, BiaCore analysis. In one embodiment, the targeting ligand may also comprise one or more immunoglobulin binding domains isolated from antibodies generated against a selected tissue-specific surface protein or target tissue- specific receptor. The term "immunoglobulin or antibody" as used herein refers to a mammalian, including human, polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen, which, in the case of the present invention, is a tissue-specific surface protein, a target tissue-specific receptor, or portion thereof. If the intended targeting fusion polypeptide will be used as a mammalian therapeutic, immunoglobulin binding regions should be derived from the corresponding mammalian immunoglobulins. If the targeting fusion polypeptide is intended for non-therapeutic use, such as for diagnostics and ELISAs, the immunoglobulin binding regions may be derived from either human or non-human mammals, such as mice. The human immunoglobulin genes or gene fragments include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant regions, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, lgG, 1gM, IgA, lgD, and IgE, respectively. Within each lgG class, there are different isotypes (e.g. lgG1, lgG2, etc.). Typically, the antigen- binding region of an antibody will be the most critical in determining specificity and affinity of binding.

[00198] An exemplary immunoglobulin (antibody) structural unit of human lgG, comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one light chain (about 25 kD) and one heavy chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100-110 or more amino acids primarily responsible for antigen recognition. The terms "variable light chain" (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively. Antibodies exist as intact immunoglobulins, or as a number of well- characterized fragments produced by digestion with various peptidases. For example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'2, a dimer of Fab which itself is a light chain joined to VH-CH by a disulfide bond. The F(ab)'2 may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'2 dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region. While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by using recombinant DNA methodology.

Thus, the terms immunoglobulin or antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized de novo using recombinant DNA methodologies (e.g., single chain Fv)(scFv)) or those identified using phase display libraries (see, for example, McCafferty et al. (1990) Nature 348:552-554). In addition, the fusion polypeptides of the invention include the variable regions of the heavy (VH) or the light (VL) chains of immunoglobulins, as well as tissue-specific surface protein and target receptor-binding portions thereof. Methods for producing such variable regions are described in Reiter, et al. (1999) J. Mol. Biol. 290:685-698.

[00199] Methods for preparing antibodies are known to the art. See, for example, Kohler & Milstein (1975) Nature 256:495-497; Harlow & Lane (1988) Antibodies: a Laboratory Manual, Cold Spring Harbor Lab., Cold Spring Harbor, NY). The genes encoding the heavy and light chains of an antibody of interest can be cloned from a cell, e.g., the genes encoding a monoclonal antibody can be cloned from a hybridoma and used to produce a recombinant monoclonal antibody. Gene libraries encoding heavy and light chains of monoclonal antibodies can also be made from hybridoma or plasma cells. Random combinations of the heavy and light chain gene products generate a large pool of antibodies with different antigenic specificity. Techniques for the production of single chain antibodies or recombinant antibodies (US Patent No. 4,946778; US Patent No. 4,816,567) can be adapted to produce antibodies used in the fusion polypeptides and methods of the instant invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express human or humanized antibodies. Alternatively phage display technology can be used to identify antibodies, antibody fragments, such as variable domains, and heteromeric Fab fragments that specifically bind to selected antigens.

[00200] Screening and selection of preferred immunoglobulins (e.g., antibodies) can be conducted by a variety of methods known to the art: Initial screening for the presence of monoclonal antibodies specific to a tissue- specific or target receptor may be conducted through the use of ELISA-based methods or phage display, for example. A secondary screen is preferably conducted to identify and select a desired monoclonal antibody for use in construction of the tissue-specific fusion polypeptides of the invention. Secondary screening may be conducted with any suitable method known to the art. One method, termed "Biosensor Modification- Assisted Profiling" ("BiaMAP") (US patent publication 2004/101920), allows rapid identification of hybridoma clones producing monoclonal antibodies with desired characteristics. More specifically, monoclonal antibodies are sorted into distinct epitope-related groups based on evaluation of antibody: antigen interactions.

Production of Recombinant human MIS proteins

[00201] Recombinant human MIS proteins as disclosed herein, and functional fragments and derivatives thereof can be obtained by any suitable method. For example, polypeptides can be produced using conventional recombinant nucleic acid technology such as DNA or RNA, preferably DNA. Guidance and information concerning methods and materials for production of polypeptides using

recombinant DNA technology can be found in numerous treatises and reference manuals. See, e.g., Sambrook et al, 1989, Molecular Cloning - A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press; Ausubel et al. (eds.), 1994, Current Protocols in Molecular Biology, John Wiley & Sons, Inc.; Innis et al. (eds.), 1990 PCR Protocols, Academic Press.

[00202] Alternatively, recombinant human MIS proteins or functional fragments thereof can be obtained directly by chemical synthesis, e.g., using a commercial peptide synthesizer according to vendor's instructions. Methods and materials for chemical synthesis of polypeptides are well known in the art. See, e.g., Merrifield, 1963, "Solid Phase Synthesis," J. Am. Chem. Soc. 83:2149 -2154.

[00203] In some embodiments, a recombinant human MIS protein, or functional fragment or derivative or variant thereof can be expressed in the cell following introduction of a DNA encoding the protein, e.g., a nucleic acid encoding recombinant human MIS proteins or homologues or functional derivatives thereof, e.g., in a conventional expression vector as disclosed herein or by a catheter or by cells transformed with the nucleic acid *ex vivo* and transplanted into the subject.

Assays to determine the activity of the recombinant human MIS protein

[00204] In one embodiment, an Organ Culture Assay System can be used to assay the bioactivity of a human recombinant MIS protein as disclosed herein. The assay system used was described by Donahoe et al, J. Surg. Res., 23, 141-148, 1977 which is the Mullerian regression organ culture assay. The urogenital ridge was dissected from the 14-day female rat embryo and transferred to an organ culture dish (Falcon, 3010). Specimens were placed on stainless-steel grids coated with a thin layer of 2% agar and incubated for 72 hr at 37.degree. C. in 5% CO.sub.2 and 95% air over 2 ml of culture medium [CMRL 1066 containing 10% fetal calf serum, 1% penicillin (10,000 units/ml)] or a 1:1 mixture of culture medium and the supernatant or gradient fraction to be tested. The incubated tissue was then coated with a mixture of 2% agar and albumin at 44.degree. C., fixed in buffered formaldehyde, dehydrated in ethanol, cleaned in xylene, and embedded in paraffin. Eight-micrometer serial sections were stained with hematoxylin and eosin for viewing by light microscopy. Sections from the cephalic end of the Mullerian duct were assigned a coded number and graded for regression (Donahoe et al, Biol. Reprod., 15, 329-334, 1976) on a scale of 0 to V. Five slides with six to eight sections per slide were read for each assay. A grade of activity was listed as the nearest whole number to the mean. A test group for the fractionation procedures represents at least 10 assays. If the mean fell midway between two numbers, then both numbers were listed. Grade 0 refers to no regression. The Mullerian duct, which is lined with columnar epithelial cells whose nuclei have a basilar orientation, has a widely patent lumen. Grade I is minimal regression. The duct is slightly smaller, and either the surrounding mesenchyme is condensed around the duct as seen in plastic sections or there is a clear area around the duct as seen in paraffin sections. Grade II refers to mild regression. The duct is smaller, and the mesenchymal condensation or the clear area around the duct is more pronounced. The nucleii of the shorter epithelial cells loose their basilar orientation. Grade III is moderate regression. The duct is very

small and disorganized. The tip of the urogenital ridge develops poorly distal to the Wolffian duct. Grade IV is severe regression. The duct is replaced by a whorl of cells. Grade V refers to complete regression. No remnant of the duct can be detected. Positive tissue controls, using fetal testis, and negative tissue controls, where the Mullerian ducts were incubated alone or with muscle were included in each experiment. Mullerian ducts exposed to extracts from nontesticular tissue, to inactive testicular fractions, or to saline served as biochemical controls. Aliquots of all fractions were dialyzed against distilled water and freeze-dried, and protein content was measured.

Delivery of recombinant human MIS protein

[00205] Methods known in the art for the therapeutic delivery of a recombinant human MIS protein and/or nucleic acids encoding the same can be used for treating a disease or disorder, such as cancer in a subject, e.g., cellular transfection, gene therapy, direct administration with a delivery vehicle or pharmaceutically acceptable carrier, indirect delivery by providing recombinant cells comprising a nucleic acid encoding a targeting fusion polypeptide of the invention.

[00206] In some embodiments, the recombinant human MIS protein is cleaved *in vitro* to form a bioactive halo-dimer of MIS, comprising two identical monomers, each consisting of the N-terminal domain and the C-terminal domain, and then administered to a subject.

[00207] Various delivery systems are known and can be used to administer a recombinant human MIS protein (before or after it has been cleaved into its bioactive form) to a subject, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction can be enteral or parenteral and include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, pulmonary, intranasal, intraocular, epidural, and oral routes. A recombinant human MIS protein can be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions comprising a recombinant human MIS protein, before or after cleavage into its bioactive form, into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

Methods to treat proliferative diseases and cancer

[00208] One aspect of the present invention provides methods for treating cancers, e.g., cancers which express MISRII in a subject. Accordingly, one aspect of the present invention relates generally

to a method of treating a proliferative disease or disorder in a subject, where the proliferative disease or disorder is associated with cells that express a MIS receptor, for example cells expressing MISRII. In some embodiments, the proliferative disease or disorder is cancer, where the cancer or cancer cells express at least one MIS receptor, for example cancer or cancer cells expressing MISRII. The method of the present invention comprises the administration of an effective amount of a recombinant human MIS protein as disclosed herein or a functional fragment or derivative thereof to a subject in with a proliferative disorder, where the cells associated with the proliferative disorder express at least one MIS receptors, for example the cells express MISRII. For example, an effective amount of a recombinant human MIS protein as disclosed herein or a functional fragment is administered to a subject with a cancer expressing at least one MIS receptors, for example expressing MISRII. Thus, by using the methods of the present invention, one can intervene in the proliferative disease, for example cancer, ameliorate the symptoms, and in some cases cure the disease. In some embodiments, the recombinant human MIS protein that can be used for treating proliferative diseases and cancer comprises the amino acid sequence residues 25-559 of SEQ ID NO: 2 or a functional fragment thereof.

[00209] Examples of such diseases where proliferation of cells expressing at least one MIS receptors, for example expressing MISRII is the cause of disease are cancers, for example cervical cancer and ovarian cancer. In some embodiments, the cancer expressing at least one MIS receptor, for example MISRII is a cancer cell. In some embodiments, such a cancer cell expressing at least one MIS receptors, for example expressing MISRII is, for example but not limited to, an ovarian cancer cell, vulvar epidermal carcinoma cell, cervical carcinoma cell, endometrial edenocarinaoma cell, ovarian adenocarcinoma.

[00210] In alternative embodiments, the cancer expressing at least one MIS receptor, for example cancers expressing MISRII are for example but not limited to; breast cancer, lung cancer, head and neck cancer, bladder cancer, stomach cancer, cancer of the nervous system, bone cancer, bone marrow cancer, brain cancer, colon cancer, esophageal cancer, endometrial cancer, gastrointestinal cancer, genital-urinary cancer, stomach cancer, lymphomas, melanoma, glioma, bladder cancer, pancreatic cancer, gum cancer, kidney cancer, retinal cancer, liver cancer, nasopharynx cancer, ovarian cancer, oral cancers, bladder cancer, hematological neoplasms, follicular lymphoma, cervical cancer, multiple myeloma, osteosarcomas, thyroid cancer, prostate cancer, colon cancer, prostate cancer, skin cancer, stomach cancer, testis cancer, tongue cancer, or uterine cancer.

[00211] In alternative embodiments, the present invention relates to the use of a recombinant human MIS protein as disclosed herein or a functional fragment or derivative or variant thereof for the treatment of any disorder where administration of the MIS protein or a nucleic acid encoding MIS protein or activation MISRII is whole or part of the therapeutic regime.

[00212] In some embodiments, the cancer is a MIS-responsive cancer, for example but not limited to ovarian cancer and cervical cancer. In some embodiments, the cancer expresses MISRII, for example but not limited to ovarian cancer and cervical cancer. In some embodiments, the disorder is a

disorder associated with excess androgen states, for example as disclosed in US Patent No. 6,673,352, which is incorporated in its entirety herein by reference. In some embodiments, the methods of the present invention are used in the treatment of prostatic cancer, polycysic ovarian disease, benign prostatic hypertrophy and precocious puberty.

[00213] In some embodiments, the cancer is a chemotherapeutic-resistant or multi-drug resistant cancer, e.g., where the cancer is a paclitaxel, cisplatin, rapamycin, pyrazoloanthrone, or Doxorubicin-resistant cancer.

[00214] In a related embodiment, a tissue to be treated is a tumor tissue expressing at least one MIS receptor, for example expressing MISRII of a subject, for example the tumor tissue is, but not limited to a solid tumor, a metastases, a skin cancer, a breast cancer, an ovarian cancer, an cervical cancer, a hemangioma or angiofibroma and the like cancer. Typical solid tumor tissues treatable by the pharmaceutical composition of the invention, includes for example, but not limited to tumors of the lung, pancreas, breast, colon, laryngeal, ovarian, and the like tissues. In some embodiment, the solid tumor tissue treatable by the present methods include thyroid, and the cancer type is medullary thyroid cancer.

[00215] In a related embodiment, the invention contemplates the practice of the method of administering a composition comprising a recombinant human MIS protein as disclosed herein or a functional fragment in conjunction with other therapies such as conventional chemotherapy directed against solid tumors and for control of establishment of metastases. For example, a chemotherapeutic agent used in chemotherapy include, but is not limited to, paclitaxel, cisplatin, doxorubicin, rapamycin, pyrazoloanthrone, including but not limited to antra(1,9-cd)pyrazol-6(2H)-one (SP600125) or N1-methyl-1,9-pyrazoloanthrone (M-SP600125) or a functional derivative or functional analogue thereof. In some embodiments, a chemotherapeutic agent is a radiotherapeutic agent. The administration of the compounds described herein is typically conducted prior to and/or at the same time and/or after chemotherapy, although it is also encompassed within the present invention to inhibit cell proliferation after a regimen of chemotherapy at times where the tumor tissue will be responding to the toxic assault by inducing angiogenesis to recover by the provision of a blood supply and nutrients to the tumor tissue. In addition, the pharmaceutical compositions of the invention for the treatment of proliferative disorders, for example cancer, can be administrated prophylatically and/or before the development of a tumor, if the subject has been identified as to have a risk of developing cancer, for example to subjects that are positive for biomarkers of cancer cells or tumors. Insofar as the present methods apply to inhibition of cell proliferation, the methods can also apply to inhibition of tumor tissue growth, to inhibition of tumor metastases formation, and to regression of established tumors.

[00216] In some embodiments, the expression of Mullerian Inhibiting Substance (MIS) receptor is measured in a biological sample obtained from the subject, e.g., a cancer or tumor tissue sample or a cancer cell or tumor cell, e.g., a biopsy tissue sample.

[00217] The presence of MISRII in the cells in fluids such as blood may be indicative of the presence of cancer. The presence of MISRII in fluids or sites not near a tumor may be indicative of metastasis. In some such embodiments, the compounds of the present invention are administered to the subject, and in some embodiments the compounds of the present invention are administered to the subject in a pharmaceutical composition comprising one or more additional therapies.

[00218] The inventive methods disclosed herein provide for the parenteral and oral administration of a recombinant human MIS protein as disclosed herein or a functional fragment or derivative thereof, in combination with other pharmaceutical compositions to subjects in need of such treatment. Parenteral administration includes, but is not limited to, intravenous (IV), intramuscular (IM), subcutaneous (SC), intraperitoneal (IP), intranasal, and inhalant routes. In the method of the present invention, a recombinant human MIS protein as disclosed herein or a functional fragment or analogs thereof are preferably administered orally. IV, IM, SC, and IP administration may be by bolus or infusion, and may also be by slow release implantable device, including, but not limited to pumps, slow release formulations, and mechanical devices. The formulation, route and method of administration, and dosage will depend on the disorder to be treated and the medical history of the subject. In general, a dose that is administered by subcutaneous injection will be greater than the therapeutically-equivalent dose given intravenously or intramuscularly. Preferably, the dose of compounds of the present invention will be administered at doses from about 0.1mg to about 250 mg. In some embodiments, the dose of compounds of the present invention will be from about 1mg to about 60mg.

[00219] The methods of the present invention for treating cancer expressing at least one MIS receptor, for example expressing MISRII, are useful for treatment of proliferation-related diseases or cancer, which is associated with cells expressing at least one MIS receptor, for example MISRII, comprising contacting a tissue in which proliferation is occurring, or is at risk for occurring, with a composition comprising a therapeutically effective amount of a recombinant human MIS protein as disclosed herein or a functional fragment or functional derivatives thereof.

[00220] In some embodiments, the subject treated by the methods of the present invention in its many embodiments is a human subject, although it is to be understood that the principles of the invention indicate that the invention is effective with respect to all mammals. In this context, a mammal is understood to include any mammalian species in which treatment of diseases associated with cancer or a proliferative-related disorder is desirable, particularly agricultural and domestic mammalian species, as well as transgenic animals.

Uses

[00221] In another embodiment, the present invention provides a method for treating a variety of conditions by administering an effective amount of a recombinant human MIS protein or functional derivatives thereof of the invention to a subject in need thereof. Conditions that may be treated by the

compounds of this invention, or a pharmaceutical composition containing the same, include any condition which is treated or reduces the symptoms by administration of MIS or activation of MIS signaling or activation of MISRII, and thereby benefit from administration of a recombinant human MIS protein or functional derivatives thereof. Representative conditions in this regard include, for example, but not limited to, cancers that express MIS receptors, for example cancer that express MISRII, for example, but not limited to ovarian, cervical and endometrial cancer. Other conditions which can be treated with MIS or activation of MIS signalling reduces the symptoms are, for example, rheumatoid arthritis, proliferative diseases such as cancer, treatment of prostatic cancer, polycysic ovarian disease, benign prostatic hypertrophy and precocious puberty and other hyperandrogen disorders such as testotoxicosis.

[00222] Accordingly, the present invention relates to the use of a recombinant human MIS protein or functional derivatives thereof for the treatment of any disorder where administration of the MIS protein or a nucleic acid encoding MIS protein or a functional derivative of MIS or activation MISRII is whole, or part, of the therapeutic regime. In some embodiments, the recombinant human MIS protein that can be used comprises the amino acid residues 25-559 of SEQ ID NO: 2 or a functional fragment thereof.

[00223] In some embodiments, the methods of the present invention are directed to use of a recombinant human MIS protein or functional derivatives thereof with other therapeutic agents, for example chemotherapy agents, wherein the chemotherapy agents, for example paclitaxel or MIS can be used at a lower dose that results in decreased side effects.

Uses of a recombinant human MIS protein or functional derivatives or analogues thereof for the treatment of excess androgen states

[00224] In another embodiment, a recombinant human MIS protein or functional derivatives or analogues thereof, can be used for the treatment of a disorder associated with excess androgen production in a subject. The inventors have previously demonstrated that the administration of MIS protein and/or MIS nucleic acid decreases levels of androgen in a subject, and decreases serum levels of androgen in a subject, as disclosed in U.S. Patent 6,673,352 and U.S. Patent Application 10/683,346, which are incorporated herein in their entirety by reference. Transgenic mice that overexpress MIS have also shown to have decreased serum testosterone concentrations, and administration of MIS results in decreased serum testosterone levels (Sriraman et al., J Androl. 2001, 22(5):750-8 and Trbovich et al., PNAS, 2001 Mar 13;98(6):3393-7). MIS has also been demonstrated to suppress both androgen-stimulated growth and androgen-independent survival of cells, and MIS regulates prostate growth by suppressing testicular testosterone synthesis also direct regulates androgen-induced gene expression and growth in the prostate at the cellular level (Trann et al, Mol Endocrinol. 2006, 20(10):2382-91).

[00225] Androgen stimulates or controls the development and maintenance of masculine characteristics in vertebrates by binding to androgen receptors. Androgens are also known as

androgenic hormones or testoids, and are also the precursor of all estrogens, the female sex hormones. The primary and most well-known androgen is testosterone.

[00226] Without wishing to be bound by theory, excessive androgen production by the adrenal glands and/or the ovary, results in androgen excess and can result from increased local tissue sensitivity to circulating androgens. Androgen excess affects different tissues and organ systems, causing clinical conditions ranging from acne to hirsutism to frank virilization.

[00227] Hyperandrogenism, which refers to the excess production and secretion of androgens and precursors, is a common and sometimes serious endrocrinopathy for women of reproductive age. The excess androgens and precursors originate from the adrenal glands and ovaries in various proportions and manifest in varying effects depending on the amount of excess androgen. Clinical manifestations range from hirsutism (excessive hair growth of male pattern, sometimes accompanied by acne) to virilization (clitorimegaly, temporal balding, deepening of voice, or enhanced musculature).

[00228] Hyperandrogenism occurs as part of a wide spectrum of disease manifestations, including polycystic ovary syndrome (PCOS) which is a variable combination of hirsutism, infertility, obesity, insulin resistance and polycystic ovaries, the HAIR-AN syndrome (hyperandrogenism, insulin resistance and acanthosis nigricans), ovarian hyperthecosis (HAIR-AN with hyperplasia of luteinized theca cells in ovarian stroma), and other manifestations of high intraovarian androgen concentrations (e. g., follicular maturation arrest, atresia, anovulation, dysmenorrhea, dysfunctional uterine bleeding, infertility), androgenproducing tumors (virilizing ovarian or adrenal tumors).

[00229] Hirsutism is excessive recognizable hair growth characterized by an increase in the number and length of terminal hairs in androgen-sensitive areas. Racial, familial, genetic, and ethnic differences all affect the occurrence of hirsutism. Hirsutism is difficult to quantitate. The entire body needs to be inspected and the findings must be documented carefully. Particular attention should be directed to the chin, lip, sideburns, breasts, and sternum, the midline between the umbilicus and the pubis and the thigh.

[00230] Ferriman and Gallwey published a rating scale for grading hirsutism and is commonly known by persons of ordinary skill in the art. This scale allows the physician to measure a response to therapy objectively. This system is the most widely used and evaluates body areas for absent-to-severe hirsutism with scores of 0-4, respectively. Scores of 8 and higher are consistent with a diagnosis of hirsutism. This scale does not measure the thickness of the hair, which is another way of objectively assessing excess hair. Scoring systems are a useful aid in quantifying hirsutism and in evaluating treatment response. Even with scores greater than 8, the patient provides the definition. From a clinical standpoint, the patient can determine if he or she notices a difference. Photographs are helpful for documentation and for following the progress of therapy.

[00231] Virilization is relatively uncommon; it occurs with extreme hyperandrogenism. Virilization is characterized by temporal balding, breast atrophy, androgenic muscle development, clitoral hypertrophy, amenorrhea, deepening of the voice, and extreme hirsutism.

[00232] Current medical therapies for women are directed against the adrenals, the ovaries or the androgen receptor. Glucocorticoid therapy is directed against the adrenal glands but is limited, in some cases, by unwanted suppression of cortisol synthesis. GnRH therapy is directed against the ovaries, but is expensive, and its long-term effects are unknown. Further, therapy using oral contraceptives may be unsuitable because most contain progestins with androgenic activity.

[00233] Because the abnormal production of androgens is implicated in the pathways of many diseases and/or disorders for which there are no acceptable treatments, a need exists to find small molecules to inhibit the production of gonadotropins and/or androgens in mammals for their treatment and/or prophylaxis.

[00234] Accordingly, in one embodiment, a recombinant human MIS protein or functional derivatives or analogues thereof, can be used for the treatment of a disorder associated with excess androgen production in a subject. In some embodiments, the recombinant human MIS protein that can be used comprises the amino acid residues 25-559 of SEQ ID NO: 2 or a functional fragment thereof.

[00235] The term "androgen" is used herein to mean steroids that encourage the development of male sex characteristics and include the steroid derivatives of androstane including, testosterone, androstenedione, and analogs.

[00236] As used herein, a disease state or disorder characterized by "androgenic dependency" is a disease state which is exacerbated by, or caused by, insufficient, excessive, inappropriate or unregulated androgen production. Examples of such diseases in men include, but are not limited to, BPH, metastatic prostatic carcinoma, testicular cancer, androgen dependent acne, male pattern baldness and precocious puberty in boys. Examples of such diseases in women include, but are not limited to, hyperandrogenism, hirsutism, virilization, POCS, HAIR-AN syndrome, ovarian hyperthecosis, follicular maturation arrest, atresia, anovulation, dysmenorrhea, dysfunctional uterine bleeding, infertility, androgen-producing tumors.

[00237] As used herein, "androgen inhibiting" refers to an effective amount of an the pyrazoloanthrone or functional derivatives or analogues thereof as defined herein, such as SP600125, which will cause a decrease in the in vivo levels of the androgen to normal or sub-normal levels, when administered to a subject for the prophylaxis or treatment of a disease state which is exacerbated by, or caused by, excessive or unregulated androgen production.

[00238] In some embodiments, a recombinant human MIS protein or functional derivatives or analogues thereof as disclosed herein, can be used to treat prostate cancer. The impact of androgens on prostate carcinoma is known, as is the treatment of prostate cancer by androgen deprivation, including androgen blockade and inhibition of androgen synthesis (Huggins et al.,Archs. Surg., Vol. 43, pp. 209-223 (1941)). J.Steroid Biochem. Molec. Biol., Vol. 37, pp.349-362 (1990)). In addition, steroid hormones are widely used as contraceptives. Anti-spermatogenic agents are male contraceptives that inhibit spermatogenesis, the process leading to mature spermatazoa. Drugs that interfere in this process include androgens and anti-androgens. Since the anti-androgenic effects of a recombinant human MIS

protein or functional derivatives or analogues thereof as disclosed herein are reversible, the recombinant human MIS protein can also be used as a male contraceptive agent. Korolkovas, A., Essentials Of Medicinal Chemistry, Second Edition, pp.1032 (1988).

[00239] In some embodiments, other agents can be used in combination with the pharmaceutical compositions comprising a recombinant human MIS protein or functional derivatives or analogues thereof as disclosed herein for the treatment of excess androgen in a subject. In some embodiments, the agents function to lower the serum-free androgen levels and blocking the peripheral androgen action. Examples of such agents include, but are not limited to, suppression of ovarian androgens by administration of estrogens and/or progestins (i.e., contraceptive pill) or GnRH agonist and add-back estrogen therapy; suppression of adrenal androgens by administration of glucocorticoids (such as dexamethasone, prednisolone), antiandrogens (such as spironolactone, flutamide, cyproterone acetate), 5α-reductase inhibitor (such as finasteride), bromocriptine, and insulin-sensitizing drugs (such as metformin, thiazolidinediones).

[00240] Subjects amenable to treatment with a recombinant human MIS protein or functional derivatives or analogues thereof by the methods as disclosed herein are subjects that have been identified with a disease or disorder associated with excess androgen levels, such as, for example disorders such as, but not limited to BPH, prostate carcinoma, benign prostic hypertrophy, testicular cancer, androgen dependent acne, male pattern baldness, precocious puberty, hyperandrogenism, hirsutism, virilization, POCS, HIAR-AN syndrome, ovarian hyperthecosis, follicular maturation arrest, atresia, anovulation, dysmenorrheal, dysfunctional uterine bleeding, infertility and androgen-producing tumors.

[00241] In some embodiments, subjects amenable to treatment with a recombinant human MIS protein orfunctional derivatives or analogues thereof by the methods as disclosed herein are subjects with congenical adrenal hyperplasma (CAH), which can be commonly identified by one of ordinary skill in the art. CAH is most typically an autosomal recessive disorder where the enzyme 21-hydrolase is missing or functionally deficent. Alternatively subjects with CAH can have a loss and/or reduction in the function of 11α-hydroxylase enzyme and/or a 3α-hydroxy-steroid dehydrogenase enzyme. When these enzymes are missing or functioning at low levels, the body cannot make adequate amounts of the adrenal steroid hormones cortisol and aldosterone. High levels of ACTH that stimulate adrenal hyperplasia and hypersecretion of androgen precursors for cortisol and aldosterone synthesis ensue. CAH can appear in utero or develop postnatally. Pseudohermaphroditism may be present at birth.

[00242] The 21-hydroxylase deficiency is the most common autosomal-recessive disorder (more common than cystic fibrosis) and manifests itself with elevated levels of 17-hydroxyprogesterone. The 11a-hydroxylase deficiency is characterized by elevated levels of 11-deoxy-cortisol (compound S) and results in elevated levels of deoxycorticosterone (DOC), a mineralocorticoid. Hypertension and hypokalemia can be a prominent feature of 11a-hydroxylase deficiency. Another form of CAH, 3a-hydroxy-steroid dehydrogenase deficiency, results in elevated levels of pregnenolone,

17-hydroxy-pregnenolone, and DHEA. This condition is lethal if not detected because no corticosteroids are synthesized.

[00243] A partial defect in the above enzymes that manifests after puberty results in elevated levels of adrenal steroids via the same mechanism. The elevations are not as marked as they are with the congenital condition and this condition is referred to as nonclassical (maturity-onset or late-onset) CAH. Accordingly, in some embodiments, subjects amenable to treatment with a recombinant human MIS protein or functional derivatives or analogues thereof by the methods as disclosed herein are subjects with nonclassical (maturity-onset or late onset) CAH.

[00244] In some embodiments, subjects amenable to treatment with a recombinant human MIS protein or functional derivatives or analogues thereof by the methods as disclosed herein are female subjects with testosterone levels about or exceeding 2.0 ng/mL (200 ng/dL, 8.92 nmol/L) or at least about 2.5 times the upper limit of the reference range. In some embodiments, such subjects have Sertoli-Leydig cell tumors, hilus cell tumors, and lipoid cell (adrenal rest) tumors are the most common. Sertoli-Leydig cell tumors reach palpable size at the time of clinical diagnosis, whereas hilar cell and lipoid cell tumors are difficult to detect by any means because of their small size.

[00245] In some embodiments, subjects amenable to treatment with a recombinant human MIS protein or functional derivatives or analogues thereof by the methods as disclosed herein are subjects with tumors of the adrenal glands (adenomas, carcinomas), which secrete elevated levels of androgens. In such embodiments, such subjects amenable to treatment by the methods as disclosed herein can be identified by having a DHEAS level of about or exceeding 7 μg/mL (18 μmol/L).

[00246] Other subjects that are amenable to the methods of treatment of excess androgen states as disclosed herein include, for example, classical and nonclassical (late-onset) CAH, cushing syndrome, where subjects with Cushing syndrome secrete elevated androgens, Hyperandrogenic, insulin resistance, and acanthosis nigricans (HAIR-AN) syndrome. In some embodiments, other subjects amenable to the methods of treatment of excess androgen states as disclosed herein include, for example, subjects with mild androgenic disorders, such as, but not limited to, Ovulatory PCOS (Ovulatory hyperandrogenic subjects with polycystic ovary at ultrasonography), Idiopathic hyperandrogenism (an Ovulatory hyperandrogenic subject but with normal ovaries at ultrasonography); Idiopathic hirsutism (subjects with an androgenic phenotype with normal androgens).

[00247] Reference testosterone levels and DHEAS levels are commonly known by persons of ordinary skill in the art, and are disclosed in Guay et al, International Journal of Impotence Research (2004) 16, 112–120, which is incorporated herein in its entirety by reference. Briefly, normal androgen levels in women between the ages of 20 and 49 years range between; DHEAS; about 195.6-140.4 ug/dl; serum testosterone about 51.5-33.7 ng/dl and free testosterone 1.51-1.03pg/ml. Accordingly, subjects amenable to the treatment of the pyrazoloanthrone or functional derivatives or analogues thereof by the methods as disclosed herein have at least about a 20%, or at least about a 30% or at least about a 40% or at least about a 50%, or at least about a 60% or at least about a 70%, or at least about a 80%, or at least

about a 90%, or at least about a 100% or greater increase in DHEAS or serum testosterone, or free testosterone levels as compared to the highest range value of the normal value for DHEAS (195.6μg/dl), serum testosterone (51.5ng/dl), free testostereone (1.51 pg/ml). In some embodiments, subjects amenable to the treatment of the pyrazoloanthrone or functional derivatives or analogues thereof by the methods as disclosed herein have at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold, or at least about a 10-fold or greater increase in DHEAS or serum testosterone, or free testosterone levels as compared to the highest range value of the normal value for DHEAS (195.6μg/dl), serum testosterone (51.5ng/dl), free testostereone (1.51 pg/ml).

[00248] DHEAS can be measured by one of ordinary skill in the art using a kit from by Diagnostic Products Corporation of Los Angeles, California, USA. Cross-reactivity has previously been determined as being 100% for DHEAS and 0.121% with androstenedione, 15% with 9-hydroxyandrostenedione, 0.046% with estrone 3 sulfate, 0.55% with androsterone sulfate, 0.5% with DHEA and negligible for all other steroids tested. Free Testosterone can be measured by one of ordinary skill in the art using was measured using the Coat a Count Kits of Diagnostic Products Corporation, Los Angeles, California, USA. Cross-reactivity has previously been determined to be 0.41% for dihydrotestosterone, 0.01% for androstenedione, 0.10% for methyl testosterone ando0.01% for all other steroids tested. Total serum testosterone levels can be measured by one of ordinary skill in the art using with the Immunochem serum testosterone kit of ICN Biomedicals Inc., Diagnostic Division of Costa Mesa, California, USA.

[00249] The assays to determine serum pregnenolone and 17-hydroxypregnenolone can be performed by one of ordinary skill in the art from the kit from Quest Laboratory in Tarzana, California, USA. Free Androgen Index (FAI) can be calculated using the following formula: (Total testosterone ng/dl x 0.0347)/(SHBG nmol/l) x100 = FAI.

Administration of Pharmaceutical compositions

[00250] A recombinant human MIS protein or derivative or functional fragment thereof can be administered by any route known in the art or described herein, for example, oral, parenteral (e.g., intravenously or intramuscularly), intraperitoneal, rectal, cutaneous, nasal, vaginal, inhalant, skin (patch), or ocular. The recombinant human MIS protein or derivative or functional fragment protein may be administered in any dose or dosing regimen.

[00251] With respect to the therapeutic methods of the invention, it is not intended that the administration of a recombinant human MIS protein or polynucleotide encoding such a recombinant human MIS protein or functional fragment thereof be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intraperitoneal, intravesicular, intraarticular, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to treat an autoimmune disease or immune-related disorder as disclosed herein. An effective amount, e.g., a therapeutically effective dose

of a recombinant human MIS protein may be administered to the patient in a single dose or in multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one hour, three hours, six hours, eight hours, one day, two days, one week, two weeks, or one month. For example, a composition comprising a recombinant human MIS protein agent can be administered for, e.g., 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, or more weeks. It is to be understood that, for any particular subject, specific dosage regimes should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions. For example, the dosage of the therapeutic can be increased if the lower dose does not provide sufficient therapeutic activity.

[00252] While the attending physician ultimately will decide the appropriate amount and dosage regimen, an effective amounts of a recombinant human MIS protein or derivative or functional fragment thereof can provided at a dose of 0.0001, 0.01, 0.01 0.1, 1, 5, 10, 25, 50, 100, 500, or 1,000 mg/kg. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test bioassays or systems. In some embodiments, doses of a recombinant human MIS protein are about 1pg/kg to 10mg/kg (body weight of patient) although lower and higher doses can also be administered.

[00253] In some embodiments, reference ranges for doses of recombinant human MIS are estimated from reference groups in the United States, and are disclosed in Antimullerian Hormone (AMH), Serum from Mayo Medical Laboratories. Retrieved April 2012. In some embodiments, female subjects can be administered the following doses of recombinant human MIS: females younger than 24 months: Less than 5 ng/mL; females 24 months to 12 years: Less than 10 ng/mL; females 13–45 years: 1 to 10 ng/mL; females older than 45 years: Less than 1 ng/mL. In some embodiments, male subjects can be administered the following doses of recombinant human MIS; males younger than 24 months: 15 to 500 ng/m; males between 24 months to 12 years: 7 to 240 ng/mL; males older than 12 years: 0.7 to 20 ng/mL. It is noted that MIS measurements may be less accurate if the person being measured is vitamin D deficient.

[00254] Additionally, as additivity, synergy, or competition has been demonstrated with MIS and rapamycin, AzadC, doxorubicin, cisplatin, and paclitaxel, recombinant human MIS as disclosed herein can be administered in combination with selective targeted therapies, for example to achieve greater activity against ovarian cancer than the use of recombinant human MIS or the chemotherapeutic agent used alone.

[00255] Dosages for a particular patient or subject can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol). A physician may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. The dose administered to a patient is sufficient to effect a beneficial therapeutic response in the patient over time, or, e.g., to reduce symptoms, or other appropriate activity, depending on the application. The dose is determined by the

efficacy of the particular formulation, and the activity, stability or serum half-life of a recombinant human MIS protein or functional derivatives or functional fragments thereof as disclosed herein, and the condition of the patient, the autoimmune disease to be treated, as well as the body weight or surface area of the patient to be treated. The size of the dose is also determined by the existence, nature, and extent of any adverse side- effects that accompany the administration of a particular vector, formulation, or the like in a particular subject. Therapeutic compositions comprising a recombinant human MIS protein or functional derivatives or functional fragments thereof are optionally tested in one or more appropriate *in vitro* and/or *in vivo* animal models of disease, such a an Mullerian duct regression bioassay as disclosed herein in the Examples, and known to persons of ordinary skill in the art, to confirm efficacy, tissue metabolism, and to estimate dosages, according to methods well known in the art. In particular, dosages can be initially determined by activity, stability or other suitable measures of treatment vs. non-treatment (e.g., comparison of treated vs. untreated cells or animal models), in a relevant assay. Formulations are administered at a rate determined by the LD50 of the relevant formulation, and/or observation of any side-effects of a recombinant human MIS protein or functional derivatives or functional fragments thereof at various concentrations, e.g., as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

[00256] In determining the effective amount of a recombinant human MIS protein or functional derivatives or functional fragments thereof to be administered in the treatment or prophylaxis of a disease, the physician evaluates circulating plasma levels, formulation toxicities, and progression of the disease. The selected dosage level will also depend upon a variety of factors including the activity of the particular compound of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

[00257] In some embodiments, a recombinant human MIS protein as disclosed herein can be administered at a dose in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners.

[00258] Dosage regimens of a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can be adjusted to provide the optimum desired response (e.g. a therapeutic or prophylactic response). For example, a single bolus can be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage.

[00259] Furthermore, actual dosage levels of a recombinant human MIS protein in a pharmaceutical composition can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular subject, composition, and mode of administration, without being toxic to the subject. A pharmaceutical composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can be a "therapeutically effective amount" and/or a "prophylactically effective amount". In general, a suitable daily dose of a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein will be that amount of the a recombinant human MIS protein which is the lowest dose effective to produce a therapeutic effect, such as a reduction of a symptom of a proliferative disorder or cancer as disclosed herein. Such an effective dose will generally depend upon the factors described above.

[00260] If desired, the effective daily dose of a composition comprising a recombinant human MIS protein or functional fragment or variant thereof can be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

[00261] The dosage level administered to a subject can be constant over a desired period of time, for example, at least 1 week, at least 2 weeks, at least 3 weeks, at least 1 month, at least 2 months, at least 3 months, at least 6 months, at least 1 year, or at least 5 years. Alternatively, the dosage level administered to a subject can vary depending on the progression of the condition being treated.

[00262] It is to be noted that dosage values may vary with the type and severity of the cancer to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

[00263] The efficacy and toxicity of the compound can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose is effective in 50% of the population) and LD50 (the dose is lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. An appropriate experimental model which can be used includes determining a the dose can be use of the mullerian duct regression bioassay as disclosed herein in the examples, or a in vivo cancer model which is commonly known by ordinary skill in the art. *In vivo* cancer models are discussed in Frese et al.,"*Maximizing mouse cancer models*" Nat Rev Cancer. 2007 Sep;7(9):645-58 and Santos et al., *Genetically modified mouse models in cancer studies.* Clin Transl Oncol. 2008 Dec;10(12):794-803, and "Cancer stem cells in mouse models of cancer", 6th Annual MDI Stem Cell Symposium, MDI Biological Lab, Salisbury Cove, ME, August 10-11, 2007" which are incorporated herein in their entirety by reference.

[00264] For example, a therapeutically effective amount can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in other subjects. Generally, the therapeutically effective amount is dependent of the desired therapeutic effect. For example, the therapeutically effective amount of a recombinant human MIS protein can be assessed in a mouse model of cancer, or using the Mullerian Duct Regression bioassay as disclosed herein in the Examples and Fig. 4.

[00265] A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. It is also noted that humans are treated generally longer than the mice or other experimental animals exemplified herein, which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses may be single doses or multiple doses over a period of several days, but single doses are preferred.

[00266] In some embodiments, a recombinant human MIS protein (e.g., proteins or nucleic acids encoding a recombinant human MIS protein or fragments thereof) can be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

[00267] After formulation with an appropriate pharmaceutically acceptable carrier in a desired dosage, a pharmaceutical composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can be administered to a subject. A pharmaceutical a composition comprising a recombinant human MIS protein or functional fragment or variant thereof can be administered to a subject using any suitable means. In general, suitable means of administration include, but are not limited to, topical, oral, parenteral (e.g., intravenous, subcutaneous or intramuscular), rectal, intracisternal, intravaginal, intraperitoneal, ocular, or nasal routes.

[00268] In a specific embodiment, it may be desirable to administer the pharmaceutical composition comprising a recombinant human MIS protein locally to the area in need of treatment; this may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, e.g., by injection, by means of a catheter, or by means of an implant, the implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, fibers, or commercial skin substitutes. In some embodiments, a recombinant human MIS protein as disclosed herein can be applied to the muscle using topical creams, patches, intramuscular injections and the like.

[00269] In some embodiments, a recombinant human MIS protein can be administered to a subject orally (e.g., in capsules, suspensions or tablets) or by parenteral administration. Conventional methods for oral administration include administering a recombinant human MIS protein in any one of the following; tablets, suspensions, solutions, emulsions, capsules, powders, syrups and the like are usable. Known techniques that deliver a recombinant human MIS protein orally or intravenously and retain the biological activity are preferred. Parenteral administration can include, for example, intramuscular, intravenous, intraarticular, intraarterial, intrathecal, subcutaneous, or intraperitoneal administration. A recombinant human MIS protein can also be administered orally, transdermally, topically, by inhalation (e.g., intrabronchial, intranasal, oral inhalation or intranasal drops) or rectally. Administration can be local or systemic as indicated. Agents, e.g., nucleic acid agents which encode a recombinant human MIS protein or functional fragment thereof can also be delivered using a vector, e.g., a viral vector by methods which are well known to those skilled in the art.

[00270] When administering a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein parenterally, it will generally be formulated in a unit dosage injectable form (e.g., solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

[00271] The term "Dosage unit" form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding a recombinant human MIS protein an active agent for the treatment of sensitivity in individuals.

[00272] The pharmaceutically acceptable compositions comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can be suspended in aqueous vehicles and introduced through conventional hypodermic needles or using infusion pumps.

Pharmaceutical Compositions

[00273] In some embodiments, a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can be formulated in any suitable means, e.g., as a sterile injectable solution, e.g., which can be prepared by incorporating the recombinant human MIS protein in the required amount of the appropriate solvent with various of the other ingredients, as desired.

[00274] A pharmacological formulation of a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicles, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of delivery systems useful in the present invention include those presented in U.S. Pat. Nos: 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447, 224; 4,439,196 and 4,475,196. Other such implants, delivery systems, and modules are well known to those skilled in the art.

[00275] Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Non-aqueous vehicles such a cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, may also be used as solvent systems for compound compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, e.g., parabens, chlorobutanol, phenol and sorbic acid. In many cases, it will be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the compounds.

[00276] In another embodiment, a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can comprise lipid-based formulations. Any of the known lipid-based drug delivery systems can be used in the practice of the invention. For instance, multivesicular liposomes, multilamellar liposomes and unilamellar liposomes can all be used so long as a sustained release rate of the encapsulated active compound can be established. Methods of making controlled release multivesicular liposome drug delivery systems are described in PCT Application Publication Nos: WO 9703652, WO 9513796, and WO 9423697, the contents of which are incorporated herein by reference.

[00277] The composition of the synthetic membrane vesicle is usually a combination of phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. Examples of lipids useful in synthetic membrane vesicle production include phosphatidylglycerols, phosphatidylcholines, phosphatidylserines, phosphatidylethanolamines, sphingolipids, cerebrosides, and gangliosides, with preferable embodiments including egg phosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidyleholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, and dioleoylphosphatidylglycerol.

[00278] In preparing lipid-based vesicles containing a recombinant human MIS protein or functional fragment or variant thereof, such variables as the efficiency of active compound encapsulation, labiality of the active compound, homogeneity and size of the resulting population of vesicles, active compound-to-lipid ratio, permeability, instability of the preparation, and pharmaceutical acceptability of the formulation should be considered.

[00279] In another embodiment, a recombinant human MIS protein can be delivered in a vesicle, in particular a liposome (see Langer (1990) Science 249:1527-1533). In yet another embodiment, a recombinant human MIS protein can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer (1990) supra). In another embodiment, polymeric materials can be used (see Howard et al. (1989) J. Neurosurg. 71:105). In another embodiment where the active agent of the invention is a nucleic acid encoding a recombinant human MIS protein, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by use of a retroviral vector (see, for example, U.S. Pat. No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

[00280] Prior to introduction, a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can be sterilized, by any of the numerous available techniques of the art, such as with gamma radiation or electron beam sterilization.

[00281] In another embodiment of the invention, a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein, can be administered and/or formulated in conjunction (e.g., in combination) with any other therapeutic agent. For purpose of administration, a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein is preferably formulated as a pharmaceutical composition. Pharmaceutical compositions of the present invention comprise a compound of this invention and a pharmaceutically acceptable carrier, wherein the compound is present in the composition in an amount which is effective to treat the condition of interest. Appropriate concentrations and dosages can be readily determined by one skilled in the art.

[00282] Pharmaceutically acceptable carriers are familiar to those skilled in the art. For compositions formulated as liquid solutions, acceptable carriers include saline and sterile water, and may optionally include antioxidants, buffers, bacteriostats and other common additives. The compositions can also be formulated as pills, capsules, granules, or tablets which contain, in addition to a compound of this invention, diluents, dispersing and surface active agents, binders, and lubricants. One skilled in this art may further formulate the compounds of this invention in an appropriate manner,

and in accordance with accepted practices, such as those disclosed in Remington's Pharmaceutical Sciences, Gennaro, Ed., Mack Publishing Co., Easton, Pa. 1990.

[00283] The compositions of the present invention can be in any form. These forms include, but are not limited to, solutions, suspensions, dispersions, ointments (including oral ointments), creams, pastes, gels, powders (including tooth powders), toothpastes, lozenges, salve, chewing gum, mouth sprays, pastilles, sachets, mouthwashes, aerosols, tablets, capsules, transdermal patches, that comprise one or more resolvins and/or protectins or their analogues of the invention.

[00284] Formulations of a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can be prepared by a number or means known to persons skilled in the art. In some embodiments the formulations can be prepared for administration as an aerosol formulation, e.g., by combining (i) a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein in an amount sufficient to provide a plurality of therapeutically effective doses; (ii) the water addition in an amount effective to stabilize each of the formulations; (iii) the propellant in an amount sufficient to propel a plurality of doses from an aerosol canister; and (iv) any further optional components e.g. ethanol as a cosolvent; and dispersing the components. The components can be dispersed using a conventional mixer or homogenizer, by shaking, or by ultrasonic energy. Bulk formulation can be transferred to smaller individual aerosol vials by using valve to valve transfer methods, pressure filling or by using conventional cold-fill methods. It is not required that a stabilizer used in a suspension aerosol formulation be soluble in the propellant. Those that are not sufficiently soluble can be coated onto the drug particles in an appropriate amount and the coated particles can then be incorporated in a formulation as described above.

[00285] In certain embodiments, a composition comprising a recombinant human MIS protein as disclosed herein can be administered to a subject as a pharmaceutical composition with a pharmaceutically acceptable carrier. In certain embodiments, these pharmaceutical compositions optionally further comprise one or more additional therapeutic agents. In certain embodiments, the additional therapeutic agent or agents are autoimmune disease or drugs, such as immune suppressants and the like. In some embodiments, an additional therapeutic agent is a cortiosteriod. In some embodiments, an additional therapeutic agent is selected from the group consisting of Prednisone, methylprednisolone, Kenalog, Medrol Oral, Medrol (Pak) Oral, Depo-Medrol Inj, prednisolone Oral, Solu-Medrol Inj, hydrocortisone Oral, Cortef Oral, Solu-Medrol IV, cortisone Oral, Celestone Soluspan Inj, Orapred ODT Oral, Orapred Oral, Prelone Oral, methylprednisolone acetate Inj , Prednisone Intensol Oral, betamethasone acet & sod phos Inj, Veripred, Celestone Oral, methylprednisolone sodium succ IV, methylprednisolone sodium succ Inj, Millipred Oral, Solu-Medrol (PF) Inj, Solu-Cortef Inj, Aristospan Intra-Articular Inj, hydrocortisone sod succinate Inj, prednisolone sodium phosphate Oral, methylprednisolone sod suc(PF) IV, Solu-Medrol (PF) IV, triamcinolone hexacetonide Inj, A-Hydrocort Inj, A-Methapred Inj, Millipred DP Oral, Flo-Pred Oral, Aristospan Intralesional Inj, betamethasone Oral, methylprednisolone sod suc(PF) Inj, hydrocortisone sod succ (PF) Inj, Solu-Cortef

(PF) Inj, prednisolone acetate Oral, dexamethasone in 0.9 % NaCl IV, Rayos, levothyroxine. Of course, such therapeutic agents are which are known to those of ordinary skill in the art can readily be substituted as this list should not be considered exhaustive or limiting.

[00286] Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions. Examples of pharmaceutically acceptable antioxidants include: water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfate, sodium sulfite and the like; oil- soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

[00287] Formulations of the present invention include those suitable for intravenous, oral, nasal, topical, transdermal, buccal, sublingual, rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

[00288] Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil- in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an active ingredient. A compound of the present invention may also be administered as a bolus, electuary or paste.

[00289] In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; humectants, such as glycerol; disintegrating agents, such as agar- agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; solution retarding agents, such as paraffin; absorption accelerators, such as quaternary ammonium compounds; wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; absorbents, such as kaolin and bentonite clay; lubricants, such a talc, calcium stearate, magnesium stearate, solid

polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

[00290] A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

[00291] The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

[00292] Liquid dosage forms for oral administration of the compounds of the invention include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs.

[00293] In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

[00294] Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters,

microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

[00295] In some instances, a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can be in a formulation suitable for rectal or vaginal administration, for example as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore release the active compound. Suitable carriers and formulations for such administration are known in the art.

[00296] Dosage forms for the topical or transdermal administration of a recombinant human MIS protein of this invention, e.g., for muscular administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. A recombinant human MIS protein or functional fragment or variant thereof as disclosed herein may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

[00297] The ointments, pastes, creams and gels may contain, in addition to an active compound of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof. Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

[00298] Transdermal patches have the added advantage of providing controlled delivery of a recombinant human MIS protein of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the compound in the proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the active compound in a polymer matrix or gel.

[00299] Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more compounds of the invention in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

[00300] Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such

as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

[00301] These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

[00302] In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

[00303] Injectable depot forms are made by forming microencapsulated matrices of the subject compounds in biodegradable polymers such as polylactide- polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

[00304] In certain embodiments, a recombinant human MIS protein or functional fragment or variant thereof can be isolated and/or purified or substantially purified by one or more purification methods described herein or known by those skilled in the art. Generally, the purities are at least 90%, in particular 95% and often greater than 99%. In certain embodiments, the naturally occurring compound is excluded from the general description of the broader genus.

[00305] In some embodiments, the composition comprises at least one a recombinant human MIS protein in combination with a pharmaceutically acceptable carrier. Some examples of materials which can serve as pharmaceutically acceptable carriers include, without limitation: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum

hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations.

[00306] In certain embodiments, a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically acceptable salts with pharmaceutically acceptable bases. The term "pharmaceutically acceptable salts, esters, amides, and prodrugs" as used herein refers to those carboxylate salts, amino acid addition salts, esters, amides, and prodrugs of the compounds of the present invention which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of patients without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio, and effective for their intended use of the compounds of the invention. The term "salts" refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention.

[00307] These salts can be prepared in situ during the final isolation and purification of the compounds or by separately reacting the purified compound in its free base form with a suitable organic or inorganic acid and isolating the salt thus formed. These may include cations based on the alkali and alkaline earth metals, such as sodium, lithium, potassium, calcium, magnesium and the like, as well as non-toxic ammonium, quaternary ammonium, and amine cations including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. (See, for example, Berge S. M., et al., "Pharmaceutical Salts," J. Pharm. Sci., 1977;66:1-19 which is incorporated herein by reference).

[00308] The term "pharmaceutically acceptable esters" refers to the relatively non-toxic, esterified products of the compounds of the present invention. These esters can be prepared in situ during the final isolation and purification of the compounds, or by separately reacting the purified compound in its free acid form or hydroxyl with a suitable esterifying agent. Carboxylic acids can be converted into esters via treatment with an alcohol in the presence of a catalyst. The term is further intended to include lower hydrocarbon groups capable of being solvated under physiological conditions, e.g., alkyl esters, methyl, ethyl and propyl esters.

[00309] As used herein, "pharmaceutically acceptable salts or prodrugs" are salts or prodrugs that are, within the scope of sound medical judgment, suitable for use in contact with the tissues of patients without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio, and effective for their intended use. These compounds include the zwitterionic forms, where possible, of r compounds of the invention.

[00310] The term "salts" refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention. These salts can be prepared in situ during the final isolation and purification of the compounds or by separately reacting the purified compound in its free base form with a suitable organic or inorganic acid and isolating the salt thus formed. These may include cations based on the alkali and alkaline earth metals, such as sodium, lithium, potassium,

calcium, magnesium and the like, as well as non-toxic ammonium, quaternary ammonium, and amine cations including, but not limited to ammonium, tetramethylanunonium, tetraethyl ammonium, methyl amine, dimethyl amine, trimethylamine, triethylamine, ethylamine, and the like (see, e.g., Berge S. M., et al. (1977) J. Pharm. Sci. 66, 1, which is incorporated herein by reference).

[00311] The term "prodrug" refers to compounds or agents that are rapidly transformed *in vivo* to yield the active recombinant human MIS protein, e.g., a biologically active or functional active MIS protein or nucleic acid (e.g., mRNA, DNA, MOD-RNA) which encodes a functionally active MIS protein. In some embodiments, a recombinant human MIS protein prodrug can be activated by hydrolysis in blood, e.g., via cleavage of a leader sequence, and or cleavage at the primary cleavage site to result in the N-terminal and C-terminal domains for production of a bioactive MIS protein, similar to how insulin is activated from its proprotein into an active insulin protein. A thorough discussion is provided in T. Higachi and V. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in Bioreversible Carriers in: Drug Design, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987, both of which are hereby incorporated by reference. As used herein, a prodrug is a compound that, upon *in vivo* administration, is metabolized or otherwise converted to the biologically, pharmaceutically or therapeutically active form of the compound. The prodrug may be designed to alter the metabolic stability or the transport characteristics of a recombinant human MIS protein, to mask side effects or toxicity, or to alter other characteristics or properties of the recombinant human MIS protein.

[00312] By virtue of knowledge of pharmacodynamic processes and drug metabolism or post-translational protein processing of MIS *in vivo,* once a pharmaceutically active compound is identified, those of skill in the pharmaceutical art generally can design a recombinant human MIS protein prodrug which can be activated *in vivo* to increase levels of a bioactive MIS protein in the subject (see, e.g., Nogrady (1985) Medicinal Chemistry A Biochemical Approach, Oxford University Press, N.Y., pages 388-392). Conventional procedures for the selection and preparation of suitable prodrugs are described, for example, in "Design of Prodrugs," ed. H. Bundgaard, Elsevier, 1985. Suitable examples of prodrugs include methyl, ethyl and glycerol esters of the corresponding acid.

[00313] As discussed herein, in some embodiments a composition comprising a recombinant human MIS protein or functional fragment or variant thereof as disclosed herein can be conjugated or covalently attached to a targeting agent to increase their tissue specificity and targeting to a cell, for example a muscle cells. Targeting agents can include, for example without limitation, antibodies, cytokines and receptor ligands, as discussed in the section entitled "targeting." In some embodiments, the targeting agent is overexpressed on the cells to be targeted, for example the muscle cells as compared to non-muscle cells.

[00314] Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of

the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of ordinary skill in the art.

Gene therapy

[00315] In some embodiments, a nucleic acid encoding a recombinant human MIS protein or functional fragment thereof as disclosed herein, can be suitably administered as a vector, e.g., a viral vector.

[00316] In some embodiments, a nucleic acid encoding a recombinant human MIS protein can be effectively used in treatment by gene therapy. See, generally, for example, U.S. Pat. No. 5,399,346, which is incorporated herein by reference. The general principle is to introduce the polynucleotide into a target cell in a patient, and where it is transcribed into the protein.

[00317] Entry into the cell can be facilitated by suitable techniques known in the art such as providing the polynucleotide in the form of a suitable vector, or encapsulation of the polynucleotide in a liposome.

[00318] A desired mode of gene therapy is to provide the polynucleotide in such a way that it will replicate inside the cell, enhancing and prolonging the desired effect. Thus, the polynucleotide is operably linked to a suitable promoter, such as the natural promoter of the corresponding gene, a heterologous promoter that is intrinsically active in liver, neuronal, bone, muscle, skin, joint, or cartilage cells, or a heterologous promoter that can be induced by a suitable agent.

[00319] Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can be used to produce recombinant constructs for the expression of a recombinant human MIS protein or a functional derivative or functional variant or functional fragment thereof as disclosed herein. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. These vectors can be viral vectors such as adenovirus, adeno-associated virus, pox virus such as an orthopox (vaccinia and attenuated vaccinia), avipox, lentivirus, murine moloney leukemia virus, etc.

[00320] Alternatively, in some embodiments, a plasmid expression vector can be used. Plasmid expression vectors include, but are not limited to, pcDNA3.1, pET vectors (Novagen ®), pGEX vectors (GE Life Sciences), and pMAL vectors (New England labs. Inc.) for protein expression in E. coli host cell such as BL21, BL21(DE3) and AD494(DE3)pLysS, Rosetta (DE3), and Origami(DE3) ((Novagen ®); the strong CMV promoter-based pcDNA3.1 (Invitrogen ™ Inc.) and pCIneo vectors (Promega) for expression in mammalian cell lines such as CHO, COS, HEK-293, Jurkat, and MCF-7; replication incompetent adenoviral vector vectors pAdeno X, pAd5F35, pLP-Adeno-X-CMV (Clontech ®), pAd/CMV/V5-DEST, pAd-DEST vector (Invitrogen ™ Inc.) for adenovirus-mediated gene transfer and expression in mammalian cells; pLNCX2, pLXSN, and pLAPSN retrovirus vectors for use with the Retro-X ™ system from Clontech for retroviral-mediated gene transfer and expression in mammalian

cells; pLenti4/V5-DEST™, pLenti6/V5-DEST™, and pLenti6.2/V5-GW/lacZ (INVITROGEN™ Inc.) for lentivirus-mediated gene transfer and expression in mammalian cells; adenovirus-associated virus expression vectors such as pAAV-MCS and pAAV-IRES-hrGFP for adeno-associated virus-mediated gene transfer and expression in mammalian cells; BACpak6 baculovirus (Clontech ®) and pFastBac™ HT (Invitrogen ™ Inc.) for the expression in Spodopera frugiperda 9 (Sf9) and Sf11 insect cell lines; pMT/BiP/V5-His (Invitrogen ™ Inc.) for the expression in Drosophila Schneider S2 cells; Pichia expression vectors pPICZα , pPICZ, pFLDα and pFLD (Invitrogen ™ Inc.) for expression in Pichia pastoris and vectors pMETα and pMET for expression in P. methanolica; pYES2/GS and pYD1 (Invitrogen ™ Inc.) vectors for expression in yeast Saccharomyces cerevisiae. Recent advances in the large scale expression heterologous proteins in Chlamydomonas reinhardtii are described by Griesbeck C. et. al. 2006 Mol. Biotechnol. 34:213-33 and Fuhrmann M. 2004, Methods Mol Med. 94:191-5. Foreign heterologous coding sequences are inserted into the genome of the nucleus, chloroplast and mitochodria by homologous recombination. The chloroplast expression vector p64 carrying the most versatile chloroplast selectable marker aminoglycoside adenyl transferase (aadA), which confer resistance to spectinomycin or streptomycin, can be used to express foreign protein in the chloroplast. Biolistic gene gun method is used to introduce the vector in the algae. Upon its entry into chloroplasts, the foreign DNA is released from the gene gun particles and integrates into the chloroplast genome through homologous recombination.

[00321] Viral vector systems which can be utilized in the present invention include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors; (c) adeno- associated virus vectors; (d) herpes simplex virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; (i) pox virus vectors such as an orthopox, e.g., vaccinia virus vectors or avipox, e.g. canary pox or fowl pox; and (j) a helper-dependent or gutless adenovirus. In a preferred embodiment, the vector is an adenovirus. Replication-defective viruses can also be advantageous.

[00322] The vector may or may not be incorporated into the cells genome. The constructs may include viral sequences for transfection, if desired. Alternatively, the construct may be incorporated into vectors capable of episomal replication, e.g., EPV and EBV vectors.

[00323] Constructs for the expression of a nucleic acid encoding a recombinant human MIS protein as disclosed herein., e.g., DNA, MOD-RNA or RNAa, can generally be operatively linked to regulatory elements, e.g., promoters, enhancers, etc., to ensure the expression of the construct in target cells. Other specifics for vectors and constructs are described in further detail below.

[00324] Typical regulatory sequences include, but are not limited to, transcriptional promoters, inducible promoters and transcriptional elements, an optional operate sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences to control the termination of transcription and/or translation. Included in the term "regulatory elements" are nucleic acid sequences such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operatively linked. In some examples, transcription of a

recombinant gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of a protein. In some instances the promoter sequence is recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required for initiating transcription of a specific gene.

[00325] Regulatory sequences can be a single regulatory sequence or multiple regulatory sequences, or modified regulatory sequences or fragments thereof. Modified regulatory sequences are regulatory sequences where the nucleic acid sequence has been changed or modified by some means, for example, but not limited to, mutation, methylation etc. Regulatory sequences useful in the methods as disclosed herein are promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type- specific, tissue-specific or inducible by external signals or agents (e.g. enhancers or repressors); such elements may be located in the 5' or 3' regions of the native gene, or within an intron.

[00326] As used herein, the term "tissue-specific promoter" means a nucleic acid sequence that serves as a promoter, i.e., regulates expression of a selected nucleic acid sequence operably linked to the promoter, and which selectively affects expression of the selected nucleic acid sequence in specific cells of a tissue, such as cells of ovarian origin.

[00327] The term "constitutively active promoter" refers to a promoter of a gene which is expressed at all times within a given cell. Exemplary promoters for use in mammalian cells include cytomegalovirus (CMV), and for use in prokaryotic cells include the bacteriophage T7 and T3 promoters, and the like. The term "inducible promoter" refers to a promoter of a gene which can be expressed in response to a given signal, for example addition or reduction of an agent. Non-limiting examples of an inducible promoter are "tet-on" and "tet-off" promoters, or promoters that are regulated in a specific tissue type.

[00328] In a specific embodiment, viral vectors that contain nucleic acid sequences e.g., DNA, MOD-RNA or RNAa encoding a recombinant human MIS protein or functional fragment thereof as disclosed herein can be used. For example, a retroviral vector can be used (see Miller et al., Meth. Enzymol. 217:581-599 (1993)). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding a recombinant human MIS protein are cloned into one or more vectors, which facilitate delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., Biotherapy 6:291-302 (1994), which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., J. Clin. Invest. 93:644-651 (1994); Kiem et al., Blood 83:1467-1473 (1994); Salmons and Gunzberg, Human

Gene Therapy 4:129-141 (1993); and Grossman and Wilson, Curr. Opin. in Genetics and Devel. 3:110-114 (1993).

[00329] The production of a recombinant retroviral vector carrying a gene of interest is typically achieved in two stages. First, sequence encoding a recombinant human MIS protein or a functional derivative or functional variant or functional fragment thereof, alone or fused to -Fc can be inserted into a retroviral vector which contains the sequences necessary for the efficient expression of the metabolic regulators (including promoter and/or enhancer elements which can be provided by the viral long terminal repeats (LTRs) or by an internal promoter/enhancer and relevant splicing signals), sequences required for the efficient packaging of the viral RNA into infectious virions (e.g., a packaging signal (Psi), a tRNA primer binding site (-PBS), a 3' regulatory sequence required for reverse transcription (+PBS)), and a viral LTRs). The LTRs contain sequences required for the association of viral genomic RNA, reverse transcriptase and integrase functions, and sequences involved in directing the expression of the genomic RNA to be packaged in viral particles.

[00330] Following the construction of the recombinant retroviral vector, the vector DNA is introduced into a packaging cell line. Packaging cell lines provide viral proteins required in trans for the packaging of viral genomic RNA into viral particles having the desired host range (e.g., the viral-encoded core (gag), polymerase (pol) and envelope (env) proteins). The host range is controlled, in part, by the type of envelope gene product expressed on the surface of the viral particle. Packaging cell lines can express ecotrophic, amphotropic or xenotropic envelope gene products. Alternatively, the packaging cell line can lack sequences encoding a viral envelope (env) protein. In this case, the packaging cell line can package the viral genome into particles which lack a membrane-associated protein (e.g., an env protein). To produce viral particles containing a membrane-associated protein which permits entry of the virus into a cell, the packaging cell line containing the retroviral sequences can be transfected with sequences encoding a membrane-associated protein (e.g., the G protein of vesicular stomatitis virus (VSV)). The transfected packaging cell can then produce viral particles which contain the membrane-associated protein expressed by the transfected packaging cell line; these viral particles which contain viral genomic RNA derived from one virus encapsidated by the envelope proteins of another virus are said to be pseudotyped virus particles.

[00331] Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, Current Opinion in Genetics and Development 3:499-503 (1993) present a review of adenovirus-based gene therapy. Bout et al., Human Gene Therapy 5:3-10 (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Another preferred viral vector is a pox virus such as a vaccinia virus, for example an attenuated vaccinia such as Modified Virus Ankara (MVA) or NYVAC,

an avipox such as fowl pox or canary pox. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., Science 252:431-434 (1991); Rosenfeld et al., Cell 68:143-155 (1992); Mastrangeli et al., J. Clin. Invest. 91:225-234 (1993); PCT Publication WO94/12649; and Wang, et al., Gene Therapy 2:775-783 (1995). In another embodiment, lentiviral vectors are used, such as the HIV based vectors described in U.S. Patent Nos. 6,143,520; 5,665,557; and 5,981,276, which are herein incorporated by reference. In some embodiments, a viral vector such as an Adeno-associated virus (AAV) vector is used. Exemplary AAV vectors are disclosed in Walsh et al., Proc. Soc. Exp. Biol. Med. 204:289-300 (1993); U.S. Pat. No. 5,436,146 which is incorporated herein by reference; Gao et al., Gene Therapy 2005, 5, 285-297; Vandenberghe et al., Gene Therapy 2009, 16, 311-319; Gao et al., PNAS 2002, 99, 11854-11859; Gao et al., PNAS 2003, 100, 6081-6086; Gao et al., J. of Virology 2004, 78, 6381-6388; Molecular Cloning: A Laboratory Manual (4^{th} edition) ed. by M. Green and J. Sambrook. In some embodiments, the AAV vector is an AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.10, AAV2.5. It should be noted that the selection of a particular type of AAV vectors can depend on the target tissue.

[00332] In some embodiments, when a recombinant human MIS protein encoded by a viral vector is expressed endogenously in a subject, the expression level of the recombinant human MIS protein disclosed herein can be constant over a desired period of time, for example, at least 1 week, at least 2 weeks, at least 3 weeks, at least 1 month, at least 2 months, at least 3 months, at least 6 months, at least 1 year, or at least 5 years. In some embodiments, the expression of the recombination human MIS protein disclosed herein can be sustained at or above a therapeutically effective dosage level over a desired period of time.

[00333] Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

[00334] U.S. Pat. No. 5,676,954 (which is herein incorporated by reference) reports on the injection of genetic material, complexed with cationic liposome carriers, into mice. U.S. Pat. Nos. 4,897,355, 4,946,787, 5,049,386, 5,459,127, 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication NO: WO 94/9469 (which are herein incorporated by reference) provide cationic lipids for use in transfecting DNA into cells and mammals. U.S. Pat. Nos. 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication NO: WO 94/9469 (which are herein incorporated by reference) provide methods for delivering DNA-cationic lipid complexes to mammals. Such cationic lipid complexes or nanoparticles can also be used to deliver protein.

[00335] A gene or nucleic acid sequence can be introduced into a target cell by any suitable method. For example, a recombinant human MIS protein construct can be introduced into a cell by transfection (e.g., calcium phosphate or DEAE-dextran mediated transfection), lipofection,

electroporation, microinjection (e.g., by direct injection of naked DNA), biolistics, infection with a viral vector containing a muscle related transgene, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, nuclear transfer, and the like. A nucleic acid encoding a recombinant human MIS protein can be introduced into cells by electroporation (see, e.g., Wong and Neumann, Biochem. Biophys. Res. Commun. 107:584-87 (1982)) and biolistics (e.g., a gene gun; Johnston and Tang, Methods Cell Biol. 43 Pt A:353-65 (1994); Fynan et al., Proc. Natl. Acad. Sci. USA 90:11478-82 (1993)).

[00336] In certain embodiments, a gene or nucleic acid sequence encoding a recombinant human MIS protein can be introduced into target cells by transfection or lipofection. Suitable agents for transfection or lipofection include, for example, calcium phosphate, DEAE dextran, lipofectin, lipfectamine, DIMRIE C, Superfect, and Effectin (Qiagen), unifectin, maxifectin, DOTMA, DOGS (Transfectam; dioctadecylamidoglycylspermine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOTAP (1,2-dioleoyl-3-trimethylammonium propane), DDAB (dimethyl dioctadecylammonium bromide), DHDEAB (N,N-di-n-hexadecyl-N,N-dihydroxyethyl ammonium bromide), HDEAB (N-n-hexadecyl-N,N-dihydroxyethylammonium bromide), polybrene, poly(ethylenimine) (PEI), and the like. (See, e.g., Banerjee et al., Med. Chem. 42:4292-99 (1999); Godbey et al., Gene Ther. 6:1380-88 (1999); Kichler et al., Gene Ther. 5:855-60 (1998); Birchaa et al., J. Pharm. 183:195-207 (1999)).

[00337] Methods known in the art for the therapeutic delivery of agents such as proteins and/or nucleic acids can be used for the delivery of a polypeptide or nucleic acid encoding a recombinant human MIS protein to a subject, e.g., cellular transfection, gene therapy, direct administration with a delivery vehicle or pharmaceutically acceptable carrier, indirect delivery by providing recombinant cells comprising a nucleic acid encoding a targeting fusion polypeptide of the invention.

[00338] Various delivery systems are known and can be used to directly administer therapeutic polypeptides such as a recombinant human MIS protein and/or a nucleic acid encoding a recombinant human MIS protein as disclosed herein, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, and receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432). Methods of introduction can be enteral or parenteral and include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, pulmonary, intranasal, intraocular, epidural, and oral routes. The agents may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local.

[00339] In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, e.g., by injection, by means of a catheter, or by means of an implant, the implant being of a porous, non-porous,

or gelatinous material, including membranes, such as sialastic membranes, fibers, or commercial skin substitutes.

[00340] In another embodiment, the active agent can be delivered in a vesicle, in particular a liposome (see Langer (1990) Science 249:1527-1533). In yet another embodiment, the active agent can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer (1990) supra). In another embodiment, polymeric materials can be used (see Howard et al. (1989) J. Neurosurg. 71:105).

[00341] Thus, a wide variety of gene transfer/gene therapy vectors and constructs are known in the art. These vectors are readily adapted for use in the methods of the present invention. By the appropriate manipulation using recombinant DNA/molecular biology techniques to insert an operatively linked recombinant human MIS protein encoding nucleic acid segment into the selected expression/delivery vector, many equivalent vectors for the practice of the methods described herein can be generated.

[00342] It will be appreciated by those of skill that cloned genes readily can be manipulated to alter the amino acid sequence of a protein. The cloned gene for recombinant human MIS protein can be manipulated by a variety of well-known techniques for in vitro mutagenesis, among others, to produce variants of the naturally occurring human protein, herein referred to as muteins or variants or mutants of a recombinant human MIS protein, which may be used in accordance with the methods and compositions described herein.

[00343] The variation in primary structure of muteins of a recombinant human MIS protein useful in the invention, for instance, may include deletions, additions and substitutions. The substitutions may be conservative or non-conservative. The differences between the natural protein and the mutein generally conserve desired properties, mitigate or eliminate undesired properties and add desired or new properties.

[00344] Remington's Pharmaceutical sciences Ed. Germany, Merk Publishing, Easton, PA, l 995 (the contents of which are hereby incorporated by reference), discloses various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Some examples of materials which can serve as pharmaceutically acceptable carriers include, but are not limited to, sugars such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; malt; gelatin; talc; excipients such as cocoa butter and: suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols; such a propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide;; water; isotonic saline; Ringer's solution, ethyl alcohol, and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium sulfate, as well as coloring agents, releasing agents, coating

agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator.

Kits

[00345] The invention also provides kits or pharmaceutical packages that comprise a recombinant human MIS protein or functional variant or functional fragment or fusion protein thereof for use in the prevention and/or treatment of a proliferative disease or disorder, e.g., cancer or disease of excess androgen as disclosed herein. The kit can comprise a recombinant human MIS protein composition in the form of, for example, tablets, capsules, or lyophilized powders, and can optionally include instructions for using a recombinant human MIS protein for the treatment of cancer or disease associated with androgen dependency. A composition comprising a recombinant human MIS protein or functional variant or functional fragment or fusion protein thereof can be provided in the kits or packages in a bottle or another appropriate form (e.g., a blister pack). Optionally, the kits or pharmaceutical packages can also include other pharmaceutically active agents (see, e.g., the agents listed above, such as other agents used for treatment of autoimmune diseases and disorders), and/or materials used in administration of the drug(s), such as diluents, needles, syringes, applicators, and the like.

[00346] Various embodiments of the disclosure could also include permutations of the various elements recited in the claims as if each dependent claim was a multiple dependent claim incorporating the limitations of each of the preceding dependent claims as well as the independent claims. Such permutations are expressly within the scope of this disclosure.

[00347] While the invention has been particularly shown and described with reference to a number of embodiments, it would be understood by those skilled in the art that changes in the form and details may be made to the various embodiments disclosed herein without departing from the spirit and scope of the invention and that the various embodiments disclosed herein are not intended to act as limitations on the scope of the claims. All references cited herein are incorporated in their entirety by reference.

[00348] Each of the applications and patents cited in this text, as well as each document or reference cited in each of the applications and patents (including during the prosecution of each issued patent; "application cited documents"), and each of the PCT and foreign applications or patents corresponding to and/or claiming priority from any of these applications and patents, and each of the documents cited or referenced in each of the application cited documents, are hereby expressly incorporated herein by reference and may be employed in the practice of the invention. More generally, documents or references are cited in this text, either in a Reference List before the claims, or in the text itself; and, each of these documents or references ("herein cited references"), as well as each document or reference cited in each of the herein cited references (including any manufacturer's specifications, instructions, etc.), is hereby expressly incorporated herein by reference.

[00349] Some embodiments of the invention are listed in the following paragraphs:

1. A recombinant Mullerian Inhibiting Substance (MIS) protein comprising a modification of at least one amino acid between residues 448-452 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification.
2. The recombinant MIS protein of paragraph 1, further comprising a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1, andwherein the recombinant MIS protein has increased cleavage and increased yield of production *in vitro* as compared to wild-type MIS protein corresponding to amino acid residues of SEQ ID NO: 1.
3. The recombinant MIS protein of paragraph 1 or 2, wherein the recombinant MIS protein further comprises a Tag protein.
4. The recombinant MIS protein of paragraph 2, wherein the non-MIS leader sequence is an albumin leader sequence or a functional fragment thereof.
5. The recombinant MIS protein of paragraph 4, wherein the albumin leader sequence is a human serum albumin (HSA) leader sequence or a fragment thereof.
6. The recombinant MIS protein of paragraph 5, wherein the HSA leader sequence comprises the amino acid sequence of SEQ ID NO: 6 or a variant that is at least 80% homologous thereto.
7. The recombinant MIS protein of paragraph 5, wherein a fragment of the HSA leader sequence comprises at least 10 amino acids of SEQ ID NO: 6 or a variant that is at least 80% homologous thereto.
8. The recombinant MIS protein of paragraph 5, wherein the HSA leader sequence comprises at least 15 amino acids of SEQ ID NO: 6, or a variant that is at least 80% homologous thereto.
9. The recombinant MIS protein of paragraph 5, wherein the HSA leader sequence comprises at least 11 amino acids of SEQ ID NO: 6, or a variant that is at least 80% homologous thereto.
10. The recombinant MIS protein of paragraph 5, wherein a fragment of the HSA leader sequence is selected from the group consisting of: MKWVTFISLLFLFSSAYS (SEQ ID NO: 13); MKWVTFISLLFLFSSAYSRGVFRR (SEQ ID NO: 6); MKWVSFISLLFLFSSAYS (SEQ ID NO:14).
11. The recombinant MIS protein of paragraph 2, wherein the non-MIS leader sequence is selected from a group consisting of: immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide (IgSP-tPA), murine immunoglobulin signal peptide (IgSP), a MPIF-1 signal sequence (MKVSVAALSCLMLVTALGSQA (SEQ ID NO: 15);a stanniocalcin signal sequence (MLQNSAVLLLLVISASA (SEQ ID NO:16)); an invertase signal sequence (MLLQAFLFLLAGFAAKISA (SEQ ID NO:17)); a yeast mating factor alpha signal sequence (K. lactis killer toxin leader sequence); a hybrid signal sequence (MKWVSFISLLFLFSSAYSRSLEKR, (SEQ ID NO:18)); a HSA/MFα-1 hybrid signal sequence (MKWVSFISLLFLFSSAYSRSLDKR (SEQ ID NO:19)); a K. lactis killer/ MFα-1

fusion leader sequence (MNIFYIFLFLLSFVQGSLDKR (SEQ ID NO:20)); an immunoglobulin Ig signal sequence (MGWSCIILFLVATATGVHS (SEQ ID NO:21)); a Fibulin B precursor signal sequence (MERAAPSRRVPLPLLLLGGLALLAAGVDA (SEQ ID NO:22)); a clusterin precursor signal sequence (MMKTLLLFVGLLLTWESGQVLG (SEQ ID NO: 23)); and the insulin-like growth factor-binding protein 4 signal sequence (MLPLCLVAALLLAAGPGPSLG (SEQ ID NO:24)) or a functional fragment thereof.

12. The recombinant MIS protein of paragraph 1, comprising a modification of amino acid 450 of SEQ ID NO: 1 from Q to R to increase cleavage as compared to in the absence of such a modification.
13. The recombinant MIS protein of paragraph 1, further comprising a modification of amino acid 452 of SEQ ID NO: 1 from S to R to increase cleavage as compared to in the absence of such a modification.
14. The recombinant MIS protein of paragraph 3, wherein the tag is a FLAG tag.
15. The recombinant MIS protein of paragraph 14, wherein the FLAG tag comprises amino acid sequence DYKDDDDK (SEQ ID NO: 8), or a functional derivative or variant thereof.
16. The recombinant MIS protein of paragraph 14, wherein the FLAG tag is located after amino acid residue 452 of SEQ ID NO: 1 and before amino acid residue 453 of SEQ ID NO: 1.
17. The recombinant MIS protein of paragraph 14, wherein the FLAG tag is located between amino acid residue 452 and 453 of SEQ ID NO: 1.
18. The recombinant MIS protein of paragraph 1, which comprises the amino acid sequence of SEQ ID NO: 2 or a functional fragment thereof.
19. The recombinant MIS protein of paragraph 1, which comprises the amino acid sequence of SEQ ID NO: 3 or a functional fragment thereof.
20. The recombinant MIS protein of paragraph 18, which is encoded by nucleic acid sequence of SEQ ID NO: 4.
21. The recombinant MIS protein of paragraph 19, which is encoded by nucleic acid sequence of SEQ ID NO: 5.
22. A pharmaceutical composition comprising the recombinant MIS protein of any of paragraphs 1 to 21 and a pharmaceutically acceptable carrier.
23. A polynucleotide encoding the recombinant MIS protein of any of paragraphs 1 to 21.
24. The polynucleotide of paragraph 23, wherein the nucleotide corresponds to SEQ ID NO: 4 or a nucleotide which has at least 95% sequence identity to the nucleic acid sequence of SEQ ID NO: 4.
25. The polynucleotide of paragraph 23, wherein the nucleotide corresponds to SEQ ID NO: 5 or a nucleotide which has at least 95% sequence identity to the nucleic acid sequence of SEQ ID NO: 5.
26. A vector comprising the polynucleotide of paragraphs 23-25.

27. The vector of paragraph 26, wherein the vector is a viral vector or an expression vector.
28. The vector of paragraph 27, wherein the expression vector is pcDNA 3.1 or cDNA or genome vector for bacteria (e.g., e coli) or bacteriophage.
29. The vector of paragraph 27, wherein the viral vector is selected from the group consisting of an adenoviral vector, a poxvirus vector and a lentiviral vector.
30. The vector of paragraph 27, wherein the viral vector is an adeno-associated vector (AAV).
31. The vector of paragraph 30, wherein the AAV is AAV9.
32. The vector of any of paragraphs 26 to 31, wherein the nucleic acid sequence encodes a recombinant MIS protein or fragment thereof which has at least 95% sequence identity to the nucleic acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5, wherein the nucleic acid sequence is operatively linked to tissue- or cell-type specific promoter.
33. A human MIS protein produced by post-translational processing of the recombinant human MIS protein of paragraph 1.
34. A host cell comprising the vector of any of the paragraphs 26 to 32.
35. A pharmaceutical composition comprising the vector of any of the paragraphs 26 to 32 and a pharmaceutically acceptable carrier.
36. A purified preparation of human MIS protein produced from the recombinant human MIS protein of any of paragraphs 1 to 21.
37. A method for treating a subject with cancer, comprising administering a composition comprising a recombinant MIS protein, wherein the recombinant MIS protein comprises a modification of at least one amino acid between residues 448-452 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification.
38. The method of paragraph 37, wherein the recombinant MIS protein has increased cleavage and increased yield of production *in vitro* as compared to wild-type MIS protein corresponding to amino acid residues of SEQ ID NO: 1.
39. The method of paragraph 37, wherein the recombinant MIS protein is produced from a pre-proprotein comprising a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1.
40. The method of paragraph 37, wherein the recombinant MIS protein further comprises a Tag protein.
41. The method of paragraph 39, wherein the non-MIS leader sequence is an albumin leader sequence or a functional fragment thereof.
42. The method of paragraph 41, wherein the albumin leader sequence is a human serum albumin (HSA) leader sequence or a fragment thereof.
43. The method of paragraph 37, wherein the recombinant MIS protein comprises a modification of amino acid 450 of SEQ ID NO: 1 from Q to R to increase cleavage as compared to in the absence of such a modification.

44. The method of paragraph 40, wherein the tag is a FLAG tag comprising amino acid sequence of SEQ ID NO: 8 or a functional fragment thereof.
45. The method of paragraph 37, wherein the recombinant MIS protein comprises the amino acid residues 25-559 of SEQ ID NO: 2 or a functional fragment thereof.
46. The method of paragraph 37, wherein the recombinant MIS protein comprises the amino acid residues 25-567 of SEQ ID NO: 3 or a functional fragment thereof.
47. The method of paragraph 37, wherein the cancer is a MIS Responsive II cancer.
48. The method of paragraph 37, wherein the cancer is ovarian cancer.
49. The method of paragraph 37, wherein the cancer is a chemotherapeutic-resistant or multi-drug resistant cancer.
50. The method of paragraph 37, wherein the administration of the recombinant MIS protein is prior to, during, or after administration of an additional agent or cancer therapy.
51. The method of paragraph 37, wherein the cancer expresses Mullerian Inhibiting Substance Receptor II (MISRII).
52. The method of paragraph 51, wherein the expression of Mullerian Inhibiting Substance (MIS) receptor is measured in a biological sample obtained from the subject.
53. The method of paragraph 52, wherein the biological sample is a cancer or tumor tissue sample or a cancer cell or tumor cell.
54. The method of paragraph 52, wherein the biological sample is a biopsy tissue sample.
55. The method of paragraph 37 , wherein the cancer is an ovarian cancer cell, vulvar epidermal carcinoma cell, cervical carcinoma cell, endometrial edenocarinaoma cell and ovarian adenocarcinoma cell.
56. The method of paragraph 37, wherein the cancer is selected from the group consisting of: breast cancer, lung cancer, head and neck cancer, bladder cancer, stomach cancer, cancer of the nervous system, bone cancer, bone marrow cancer, brain cancer, colon cancer, esophageal cancer, endometrial cancer, gastrointestinal cancer, gum cancer, kidney cancer, liver cancer, nasopharynx cancer, ovarian cancer, prostate cancer, skin cancer, stomach cancer, testis cancer, tongue cancer, melanoma, ocular melanoma, or uterine cancer.
57. The method of paragraph 49, wherein the multi-drug resistant cancer is a paclitaxel- or Doxorubicin-resistant cancer.
58. The method of paragraph 37, wherein administering is intravenous, intradermal, intramuscular, intraarterial, intralesional, percutaneous, or subcutaneous, or by aerosol.
59. The method of paragraph 37, wherein administering is prophylactic administration.
60. The method of paragraph 37, wherein administering is therapeutic administration.
61. The method of paragraph 37, wherein the subject is a mammal.
62. The method of paragraph 61, wherein the mammal is a human.

63. The method of paragraph 37, wherein at least one additional agent is administered to the subject in combination with (e.g., before, during or after) administration of the recombinant human MIS.
64. The method of paragraph 63, wherein the additional agent is a therapeutic agent or chemotherapeutic agent.
65. The method of paragraph 64, wherein the chemotherapeutic agent is selected from the group consisting of: paclitaxel, cisplatin, doxorubicin, rapamycin, pyrazoloanthrone.
66. The method of paragraph 64, wherein the chemotherapeutic agent is a radiotherapeutic agent.
67. The method of paragraph 64, wherein the chemotherapeutic agent is a pyrazoloanthrone.
68. The method of paragraph 67, wherein the pyrazoloanthrone is antra(1,9-cd)pyrazol-6(2H)-one (SP600125) or a functional derivative or functional analogue thereof.
69. A method of decreasing the dose of a chemotherapeutic agent for the treatment of cancer, the method comprising administering to the subject a therapeutically effective amount of a recombinant MIS protein, wherein the recombinant MIS protein comprises a modification of amino acid 450 of SEQ ID NO: 1 from Q to R, and wherein the therapeutically effective dose of the chemotherapeutic agent in the presence of the recombinant MIS protein is lower as compared to the therapeutically effective dose of the chemotherapeutic agent alone.
70. The method of paragraph 69, wherein the recombinant MIS protein further comprises a Tag protein.
71. Use of recombinant MIS protein for the manufacture of a medicament for treating cancer, wherein the recombinant MIS protein comprises a modification of amino acid 450 of SEQ ID NO: 1 from Q to R, and wherein the cancer expresses a Mullerian Inhibiting Substance (MIS) receptor.
72. The use of paragraph 71, wherein the recombinant MIS protein further comprises a Tag protein.
73. The use of paragraph 71, wherein the Mullerian Inhibiting Substance (MIS) receptor is MIS type II receptor or a homologue or functional fragment thereof.
74. An article of manufacture comprising packaging material and a pharmaceutical composition comprising the recombinant MIS protein of any of paragraphs 1 to 21, wherein the packaging material comprises a label which indicates the pharmaceutical composition may be administered, for a sufficient term at an effective dose, for treating or reducing the risk of cancer which expresses a Mullerian Inhibiting Substance (MIS) receptor.
75. A method of treating a subject affected with cancer, the method comprising assessing the expression and/or activity of Mullerian Inhibiting Substance Receptor II (MISRII) in a biological sample obtained from the subject, wherein a clinician reviews the results and if the results indicate the presence of expression and/or activity of MISRII, the clinician directs the subject to be treated with pharmaceutical composition of paragraph 22 or 35.
76. The method of paragraph 75, wherein the biological sample is a tissue sample.

77. The method of paragraph 76, wherein the tissue sample is a cancer or tumor tissue sample or a cancer cell or tumor cell.
78. The method of paragraph 76, wherein the biological sample is a biopsy tissue sample.
79. The method of paragraph 75, wherein the cancer is an ovarian cancer cell, vulvar epidermal carcinoma cell, cervical carcinoma cell, endometrial edenocarinaoma cell and ovarian adenocarcinoma cell.
80. The method of paragraph 75, wherein the cancer is breast cancer, lung cancer, head and neck cancer, bladder cancer, stomach cancer, cancer of the nervous system, bone cancer, bone marrow cancer, brain cancer, colon cancer, esophageal cancer, endometrial cancer, gastrointestinal cancer, gum cancer, kidney cancer, liver cancer, nasopharynx cancer, ovarian cancer, prostate cancer, skin cancer, stomach cancer, testis cancer, tongue cancer, melanoma, ocular melanoma or uterine cancer.
81. Use of a recombinant MIS protein to decrease the plasma serum levels of one or more androgens in a subject in need thereof, wherein the recombinant MIS protein comprises a modification of amino acid 450 of SEQ ID NO: 1 from Q to R.
82. The use of paragraph 81, wherein the recombinant MIS protein further comprises a Tag protein.
83. The use of paragraph 81, wherein one or more androgens is testosterone.
84. The use of paragraph 81, wherein the subject in need thereof has benign prostatic hypertrophy.
85. The use of paragraph 81, wherein the subject in need thereof has prostate cancer.
86. The use of paragraph 81, wherein the subject in need thereof has polycystic ovarian disease and/or precocious puberty.
87. The use of paragraph 81, wherein the subject in need thereof has a disease or disorder selected from the group consisting of: Benign Prostatic Hyperplasia (BPH), prostate carcinoma, testicular cancer, androgen dependent acne, male pattern baldness, precocious puberty, hyperandrogenism, hirsutism, virilization, Polycystic Ovary Syndrome (POCS), hyperandrogenism (HA) and insulin resistance (IR) and acanthosis nigricans (AN) (HIAR-AN) syndrome, ovarian hyperthecosis, follicular maturation arrest, atresia, anovulation, dysmenorrheal, dysfunctional uterine bleeding, infertility and androgen-producing tumors.
88. A method to treat a disease or disorder characterized by androgenic dependency, comprising administering to a subject an effective amount of the pharmaceutical composition of paragraph 22 or 35, wherein the pharmaceutical composition reduces the level of at least one androgen in the plasma serum of the subject and results in a decrease in at least one symptom of a disease or disorder characterized by androgenic dependency.
89. A method to decrease the plasma level of one or more androgens in a subject, the method comprising administering an effective amount of a recombinant MIS protein, wherein the recombinant MIS protein comprises a modification of amino acid 450 of SEQ ID NO: 1 from Q

to R, and wherein the recombinant MIS protein decreases the plasma serum levels of one or more androgens in the subject.

90. The method of paragraph 89, wherein the recombinant MIS protein further comprises a Tag protein.
91. The method of paragraph 89, wherein the subject has a disease or disorder characterized by androgenic dependency.
92. The method of any of paragraphs 89 to 91 , wherein the disease or disorder is selected from the group consisting of: Benign Prostatic Hyperplasia (BPH), prostate carcinoma, testicular cancer, androgen dependent acne, male pattern baldness, precocious puberty, hyperandrogenism, hirsutism, virilization, Polycystic Ovary Syndrome (POCS), hyperandrogenism (HA) and insulin resistance (IR) and acanthosis nigricans (AN) (HIAR-AN) syndrome, ovarian hyperthecosis, follicular maturation arrest, atresia, anovulation, dysmenorrheal, dysfunctional uterine bleeding, infertility and androgen-producing tumors.
93. A kit comprising a recombinant MIS protein of any of paragraphs 1-21 and a pharmaceutically acceptable carrier.
94. The kit of paragraph 93, optionally further comprising instructions of use of the recombinant MIS protein for the treatment of cancer or treatment of an androgenic dependency disorder.

[00350] The invention can be understood more fully by reference to the following detailed description and illustrative examples, that are intended to exemplify non-limiting embodiments of the invention.

EXAMPLES

[00351] The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

[00352] The description of the present invention has been presented for purposes of illustration and description, but is not intended to be exhaustive or limiting of the invention to the form disclosed. The scope of the present invention is limited only by the scope of the following claims. Many modifications and variations will be apparent to those of ordinary skill in the art. The embodiment described and shown in the Figs. was chosen and described in order to best explain the principles of the invention, the practical application, and to enable others of ordinary skill in the art to understand the invention for various embodiments with various modifications as are suited to the particular use contemplated.

[00353] ***Materials and methods***

[00354] **Constructs and plasmid cloning.**

[00355] *WT-MIS: pBG311 vector with genomic sequence of MIS.* The vector was constructed as previously described (Cate et al, 1986). Briefly, the genomic sequence of human MIS was sub-cloned

into a pBG311 expression vector from chMIS33 which was isolated from a human cosmid library using a bovine cDNA probe (Cate et al. 1986).

[00356] *RF-MIS: pcDNA 3.1 and pAAV-IRES-NEO vectors containing MIS cDNA with native MIS leader sequence, modified cleavage site, and flag tag.* The coding sequence of MIS, present in a pcDNA3.1 vector containing a FLAG-labeled full-length human MIS cDNA sequence previously described (Papakostas et al, 2010) was subcloned into a pAAV-IRES-Neo expression vector at an ECORV site. This coding sequence contains a FLAG-epitope inserted after a modified cleavage site at position 428 (RARR/S) (SEQ ID NO: 27) (Papakostas et al, 2010).

[00357] *LR-MIS: pcDNA 3.1 vector containing MIS cDNA with human serum albumin leader sequence and modified cleavage site.* The pcDNA3.1 vector containing a full-length human MIS cDNA sequence containing a modified cleavage site, as previously described (Papakostas et al, 2010) was used to incorporate the albumin leader sequence. The albumin leader was cloned in the place of the MIS leader using a forward primer containing an EcoRV site:

*CGAGATAC***ATGAAGTGGGTGAGCTTCATCAGCCTGCTGTTCCTGTTCAGCAGCGCTTA CTCCCGCGGTGTGTTCCGGCGCA**AGAGCAGAGGAGCCAGCTGTG (SEQ ID NO: 11) (with the nucleic acid encoding the leader sequence highlighted in **bold**) and a backward primer at position 451-432 of MIS GCTCCTGGAACCTCAGCGAG (SEQ ID NO: 12).

[00358] *LRF-MIS: pcDNA 3.1 vector containing MIS cDNA with human serum albumin leader sequence, modified cleavage site and Flag tag.* The pcDNA3.1 vector containing a full-length human MIS cDNA sequence containing a modified cleavage site and a flag tag, as previously described (Papakostas et al, 2010) was used to incorporate the albumin leader sequence as described above.

[00359] **Transfections and cloning:**

[00360] *Wild-type MIS* (WT-MIS). The WT-MIS construct (pBG311) along with pSV2DHFR was previously transfected in DHFR- CHO cells and the B9 clone was selected as the highest expresser as previously described (Cate et al, 1986).

[00361] *RARR/S-Flag MIS (RF-MIS) ("RARR/S" disclosed as SEQ ID NO: 27):* The RF-MIS construct (in pAAV-IRES-NEO) was transfected in CHO-S cells using Fugene 6 (Roche) according to the manufacturer's protocol and the CHO93 stably expressing clone was selected under geneticin selection (550ug/ml) as the highest expresser determined by western blot.

[00362] *LR-MIS.* The LR-MIS construct (in pcDNA3.1) was transfected in CHO-K1 cells using lipofectamine 2000 (invitrogen), according to the manufacturer's protocol. Clones were selected in 800ug/ml of geneticin, and the highest expressers as determined by western blot (LR8, 11 and 22) were chosen for further study.

[00363] *LRF-MIS.* The LRF-MIS construct (in pcDNA3.1) was transfected in CHO-K1 cells using lipofectamine 2000 (invitrogen), according to the manufacturer's protocol. Clones were selected in 800ug/ml of geneticin (G418), and the highest expressers as determined by western blot (LRF8, 18 and 22) were chosen for further study.

[00364] **Media and culture conditions:**

[00365] *B9 clone.* B9 is grown in roller bottles (1700cm^2) with 250ml of alpha MEM-supplemented with 5% female fetal calf serum (FFCS) (Biologos), 0.24μM methotrexate, 2nM glutamine, 100U/ml penicillin and 100ug/ml streptomycin (Invitrogen) maintained confluent for several months in 5% CO2, at 37C while media is collected every 3-4 days. Media is screened by western and MIS ELISA to monitor and measure production.

[00366] *CHO93 clone.* CHO93 is grown in roller bottles (1700cm^2) with 250ml of DMEM:F12 supplemented with 10% FFCS , 550ug/ml of geneticin, 2nM glutamine, 100U/ml penicillin and 100ug/ml streptomycin (Invitrogen) maintained confluent for several months in 5% CO2, at 37C while media is collected every 3-4 days. Media is screened by western and MIS ELISA to monitor and measure production.

[00367] *LR8, 11, 22 and LRF8, 18, 22 clones.* Both LR and LRF clones are grown in roller bottles (1700cm^2) with 250ml of DMEM supplemented with 10% FFCS , 800ug/ml of geneticin, 2nM glutamine, 100U/ml penicillin and 100ug/ml streptomycin (Invitrogen) maintained confluent for several months in 5% CO2, at 37C while media is collected every 7 days. Media is screened by western and MIS ELISA to monitor and measure production.

[00368] **Purification of MIS.**

[00369] *Purification using immunoaffitnity anti-Flag beads.* RF-MIS and LRF-MIS, which contain a flag tag, are isolated from serum-containing media collected from roller bottles of stably expressing clones of CHO (CHO93, LRF8, LRF18, LRF22) as described above. Collected media is spun down to discard dead cells and the supernatant is collected into 500ml containers and stored in -20C until purification. For purification, media is thawed at 4C overnight and then incubated with anti-FLAG agarose beads (SIGMA, 500μl packed beads/500ml media), mixing with rotation overnight at 4C. Subsequently, the beads are precipitated at 13000 rpm, for 10 seconds and washed extensively (7X) with cold1X Tris Buffered Saline (TBS) (SIGMA). All reagents are kept on ice. RF-MIS and LRF-MIS is eluted with 50μg of 3X FLAG peptide (SIGMA)/500μl beads in 1X TBS at 25C for 45 minutes with rotation. The beads are spun down at 13000 rpm, for 10 seconds at room temperature and the supernatant containing the FLAG MIS is collected, aliquoted, and stored in low protein binding Eppendorf tubes (VWR) at -80C for subsequent use.

[00370] *Purification using anti-MIS 6E11 immunoaffinity column.*

[00371] The 6E11 MIS monoclonal antibody column was produced as previously described (Ragin et al, 1992). Briefly, a 5ml immunoaffinity column was constructed using approximately 50mg of protein A -sepharose (Sigma Chemical Co., St Louis, MO)- purified mouse monoclonal anti-human rhMIS antibody, as previously described. [Ragin 1992, Hudson 1990], covalently attached to 5ml packed Affigel-10 agarose resin (Biorad Laboratories, Richmont, CA) per manufacturer's instructions (approximately 80% coupling efficiency). The column was blocked with ethonalamine and equilibrated with 50ml of 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4 and 200 ml

concentrated (10X, serum free) conditioned media loaded at 1 column vol/h at 4oC. After loading, the column was washed with 10 column volumes of 20mM Hepes, pH 7.4. A pre-elution step employed 1 column volume containing 0.5M NaCl in 20mM Hepes, pH 7.4. Elution of bound rhMIS was achieved using 1M Acetic Acid in 20mM Hepes, pH 3.0. The majority of the rhMIS eluted in a 2-5ml fraction, post 2ml void volume fraction. Eluted rhMIS was immediately neutralized with NaOH to a pH between 7.0 and 7.4. The acid eluted fractions were dialyzed overnight versus 0.02M Hepes, pH7.4. The resulting rhMIS was analyzed for total protein by Bradford method (Bradford, 1976) and for rhMIS concentrations by an enzyme-linked immunoassay (Hudson 1990) and examined by polyacrylamide gel electrophoresis (Weber 1969), Western blot analysis (Towbin 1979), in vitro Mullerian duct regression bioassays and tumor antiproliferative assays (Chin 1991).

[00372] Electrophoresis and Western Blotting

[00373] Samples were reduced with 100mM Dithiothreitol in 1x Laemmli buffer (0.0625mM Tris pH 6.8, 2% (w/v) SDS stock, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue) and heat denatured on a thermoblock at 70 °C for 10 min. Samples were run on a 4-12% Tris-Bis NuPage Novex "mini" gel (Invitrogen) at 130V with 1X MES running buffer (Invitrogen). Gels were stained with coomassie stain (0.3% Brilliant Blue R-250, 45% Methanol, 10% Acetic Acid in H2O) for 15 min at room temperature with agitation. Subsequently, they were agitated overnight at room temperature in de-staining solution (20% methanol, 10% acetic acid in H2O) with agitation.

[00374] For Western blot analysis, gels were transferred onto PVDF (Millipore) membranes, previously equilibrated in 1x NuPage transfer buffer (Invitrogen) containing 12% (v/v) methanol, at 25V for 45 min and at 35V for another 45 min. Membranes were blocked with 1x PBS, 0.1% Tween-20 containing 5% nonfat dry milk for 30 min at room temperature and probed with horseradish peroxidase conjugated mouse monoclonal anti-FLAG M2 antibody (SIGMA) (1:1000), goat C20 anti-MIS c-terminus antibody (Santa Cruz) (1:200) or rabbit MGH4 anti-MIS n-terminus MIS antibody (custom) (1:1000). Blots were washed two times 5 min each at room temperature with 1x PBS, Tween-20 0.1%, and incubated with appropriate secondary antibody if necessary, and washed three times (5 mins). Proteins bands were visualized with the ECL kit detection system (Perkin-Elmer) onto Kodak Biomax MR film.

[00375] Animals and Organ Cultures:

[00376] The standard organ culture bioassay for Mullerian Inhibiting Substance (MIS) was performed as described previously (Donahoe, 1977). Briefly, female urogenital ridges from timed pregnant rats at E14.5 (Harlan) were dissected and cultured on agar coated stainless steel grids mounted above fortified Cambridge Medical Research Laboratories (CMRL) 1066 media (Life Technologies) supplemented with 10% FFCS (to avoid an effect of bovine MIS in male serum), 1% penicillin/streptomycin, 1% L-Glutamine, 1% Fungizone (Invitrogen), and 1 nM testosterone (Sigma). After incubation for 72 hours in humidified 5% CO_2 at 37°C, the specimens were fixed in Zamboni buffer (15% formaldehyde solution, and 5% picric acid), and embedded in paraffin, and 8-um sections

of the cephalic end were stained with hematoxylin and eosin. The sections were then scored from 0 (no regression) to 5 (complete regression), by two experienced observers. Cultures were carried out with purified RF-MIS, LRF-MIS or WT-MIS at a final concentration of 5μg/ml, and at lower doses of 3, 2, and 1μg/ml.

EXAMPLE 1

[00377] Purification of Mullerian Inhibiting Substance (MIS) protein for preclinical efficacy (Pieretti-Vanmarcke et al. 2006), has predominantly been done from conditioned media from CHO cells transfected with a genomic clone (Cate et al. 1986). The media was then immunoaffinity purified (Ragin et al. 1992) using a mouse monoclonal antibody (Hudson et al. 1990) or purified by serial chromatography (Lorenzo et al. 2002). Biologic activity was detected in an embryonic organ culture Mullerian duct regression assay (Donahoe et al. 1977) and immunoactivity detected by an ELISA (Hudson et al. 1990) using monoclonal and polyclonal antibodies raised to human MIS. The transfected CHO cells were subsequently adapted to serum free conditions and suspension culture (MacLaughlin/Stafford/Dean, Donahoe unpublished), clonally selected, scaled, and purified as above. Western analysis confirmed 25-30% cleavage to yield the homodimerized C-terminus bioactive moeity which was held in noncovalent association with the homodimerized N terminus, with cleavage at the Kex-like, primary cleavage site at amino acid residues 426-427, and secondary cleavage at amino acid positions 229-230. Bands on reduced electrophoretic gels at 70, 55, 34, 24, and 12.5 kDa were all MIS fragments, as determined by amino acid sequencing (Ragin et al. 1992; Lorenzo et al. 2002), and representative of predicted Kex and dibasic cleavage products.

[00378] To optimize cleavage and the primary cleavage site at amino acid position 427, the recognition sequence was mutagenized to create a dibasic cleavage site; the RAQR/R (SEQ ID NO: 28) variant was bioactive (Kurian et al, 1994). Position 425 (corresponding to amino acid residue 450 of SEQ ID NO: 1) was then mutagenized to create a more consensus Kex cleavage site (Nachtigal & Ingraham 1996) (Hosaka et al. 1991), RARR/S (SEQ ID NO: 27), and an 8 amino acid Flag (DYKDDDDK) (SEQ ID NO:8) tag was added just downstream of the first serine in the C-terminus to aid in detection and purification. Expression of this variant resulted in improved cleavage and increased bioactivity. By comparison, when the C-terminal arginine (Kurian et al 1994) was followed by Flag, the protein produced by this construct was bioinactive (Papakostas et al 2010); thus, the serine appeared to be important for preservation of bioactivity. The RARR/S (SEQ ID NO: 27) Flag construct (Papakostas et al 2010) was transfected into CHO cells and improved cleavage and preservation of bioactivity confirmed (Papakostas et al, 2010). The modification of the cleavage site increased the cleavage to over 50-60% (Papakostas et al, 2010).

[00379] To scale expression, the MIS RARR/S (SEQ ID NO: 27) Flag construct was further modified to substitute the endogenous MIS leader sequence with that of human serum albumin (HSA). HSA is the most abundant protein in plasma and is produced at a very high rate by the liver to achieve a

blood concentration ranging from 3.4 to 5.4g/dL (Farrugia 2010). The production and processing of HSA is finely tuned to allow efficient maturation and secretion of the protein. HSA, like MIS is synthesized as a prepro-protein, which contains a leader sequence that is subsequently cleaved during maturation. This HSA leader sequence consists of only 24 AA, is not immunogenic in humans, and is removed during protein processing. Here the inventors demonstrate that substitution of the MIS leader sequence with that of HSA increases production, and unexpectedly, cleavage, which correlates with increased potency of the recombinant human MIS product.

EXAMPLE 2

[00380] Previous efforts to scale up production of human recombinant MIS led us to develop a new construct featuring the cDNA of hMIS with a modified cleavage site at position 427/428 inserted into pcDNA3.1 (Papakostas et al, 2010). By substituting the modified RARR/S (SEQ ID NO: 27) for the endogenous RAQR/S (SEQ ID NO: 26) (noted as R in constructs), and inserting a Flag tag immediately downstream of the cleavage site (noted as F in construct) (**Table 1**) (**Fig. 1**), the inventors demonstrated increased cleavage of the tagged C-terminus (Papakostas et al, 2010). Furthermore, the recombinant RARR/S-Flag MIS ("RARR/S" disclosed as SEQ ID NO: 27) (referred to herein as "RF-MIS") protein retained bioactivity in the fetal rat urogenital ridge assay (Papakostas et al, 2010). To overcome low expression yields, the backbone vector of RF-MIS was switched to pAAV-IRES-Neo, and cloned into CHO-S cells, and screened under high Geneticin concentration. The resulting expression vector is polycistronic and includes an internal ribosomal entry site (IRES) driving expression of the neomycin resistance cassette downstream of MIS, allowing for better selection of high expressers. The highest expressing clone, CHO93, was subsequently scaled up for production using roller bottles and recombinant RF-MIS was purified using anti-flag M2 immunoaffinity beads (**Table 2**). However, while RF-MIS has increased cleavage of the active C-terminus, and importantly, less internal cryptic cleavage (**Fig. 2**)(**Fig. 3**), the yield and production of the cDNA clone CHO93 (0.16pg/cell/day) remains much lower than that of the genomic clone B9 (10.59pg/cell/day)(**Table 3**), although it is unclear whether this is due to the expression vector, the CHO cells, the nature of the drug selection, or the type of message produced (cDNA versus genomic MIS).

[00381] To improve production, the original R-MIS and RF-MIS construct in pcDNA3.1 vectors were modified by substituting the 24 AA of the HSA leader sequence (pre-pro peptide) (herein noted as L in constructs) to the 25AA MIS leader to create the "LR" and "LRF" constructs (**Table 1**) (**Fig. 1**).

Table 1: List of modifications to the MIS wild-type sequence and corresponding nomenclature.

Notation	Native	Modification (shown in BOLD)	Position (AA) (normal protein	Position on SEQ ID NO: 1	Purpose

			nomenclature)		
R	RAQR/S (SEQ ID NO: 26)	RARR/S (SEQ ID NO: 27)	423-427	448-452	Furin/Kex2 census site for improved cleavage
F	n/a	FLAG Tag (DYKDDDDK) (SEQ ID NO: 8)	Located between 427-428	Located between 452-453 of SEQ ID NO: 1	C-terminus FLAG tag for easier purification and tracking.
L	MIS Leader Sequence	Albumin Leader Sequence	1-25	1-25	Increased production, secretion and cleavage.

[00382] HSA leader sequence fusion has been shown to increase production of recombinant interleukins (Carter et al, 2010) and TNF-alpha (Maeda Y et al 1997), and has been suggested as a way to produce proteins otherwise difficult to express and to scale. Furthermore, HSA is known to also enhance secretion of fused proteins such as human lysozyme in yeast expression system with Pichia pastoris (Xiong et al, 2008). The three highest stably expressing clones in CHOK1 were selected for further analysis: LR8/11/18 and LRF8/18/22 (**Fig. 2**). Both cloning efficiency and expression levels were greater for the LR clones than the LRF clones, suggesting the Flag tag may make expression less efficient. Similarly to CHO93, all LR and LRF clones have reduced peptide fragments resulting from internal cryptic cleavage at position 229, when compared to the wild type (WT-MIS) protein produced by B9. Unexpectedly, they also appear to have greater proportion of cleaved C-terminus (**Fig. 2 and 3**). This increased cleavage could be explained by the strong evolutionary pressures on the albumin leader for efficient processing in the trans-golgi network and transport to secretory vesicles, since albumin is endogenously secreted at much higher rate than MIS (Rothschild et al. 1988). LRF18 was chosen for characterization since it is the highest expressing LRF clone, and can be purified and tracked using the Flag-tag (**Table 2**).

Table 2: List of constructs and cell line clones producing MIS and corresponding purification methods.

Construct	**Clones**	**Vector**	**Cell Line**	**Purification**
MIS	B9	MIS WT genomic sequence in pBG311 plasmid.	CHO cells lacking the DHFR gene.	Immunoaffinity using 6E11 monoclonal antibody against MIS or serial chromatography.
RF-MIS	CHO93	MIS cDNA sequence inserted into pAAV-	CHO-S	Immunoaffinity using M2 monoclonal antibody

		IRES-Neo plasmid.		against FLAG tag.
LR-MIS	LR8 LR11 LR18	MIS cDNA sequence inserted into pcDNA3.1plasmid.	CHO-K1	Immunoaffinity using 6E11 monoclonal antibody against MIS or serial chromatography.
LRF-MIS	LRF8 LRF18 LRF22	MIS cDNA sequence inserted into pcDNA3.1plasmid.	CHO-K1	Immunoaffinity using M2 monoclonal antibody against FLAG tag.

[00383] When cultured for 24 hours in flasks, the concentration of MIS, as detected by ELISA, is greater in the media of B9 (WT-MIS) (15μg/ml) than in the media of clones (LR8: 3.04μg/ml); LR11: 11.66μg/ml; LR22: 6.28μg/ml) (Table 3). The highest producing clone of LR, LR11 secretes 3.24pg/cell/day of MIS while the WT clone B9 produces 10.58pg/cell/day, however, LR11 cells grow much more compact fashion, conversely, the highest expressing clone of LRF, LRF18 has both higher concentration (1.1μg/ml) and higher production (0.26pg/cell/day) than RF-MIS (CHO93) with (0.67μg/ml) and (0.15pg/cell/day) (**Table3**). Thus, the addition of the HSA leader increases the production of the flag-tagged MIS product but not the untagged product. However, as the flag-tagged constructs clearly do not produce as much as the untagged ones, the flag tag may be interfering with protein stability or expression. Coomassie stains and western blot show that the product purified from LRF18 by anti-flag immunoaffinity purification has fewer bands representative of internal cleavage (Ragin 1992) than the MIS purified from WT-MIS (B9) using anti-MIS affinity purification (**Fig. 3**).

Table 3: Purification yield from MIS from various constructs.

	WT-MIS	**RF-MIS**	**LRF-MIS**	**LR-MIS**
MIS concentration (μg/ml) at 24 hours	15	0.67	1.10	11.67
Production (pg/cell/day)	10.59	0.15	0.26	3.24
Purification yield (% w/w)	15%	20%	20%	15%
Percent cleavage	20%	50%	90%	90%

[00384] Since the C-terminus of MIS has previously been shown to be the active moiety (Pepinski 1988, Maclaughlin et al 1992), increased cleavage should correlate with greater bioactivity in the rat UGR assay. Here, the inventors demonstrate that that LRF-MIS is able to fully regress the Mullerian duct at 5μg/ml (35μM) and show greater activity than RF-MIS and WT-MIS at these concentrations, which only display partial regression (**Fig. 4**). Furthermore LRF-MIS continues to

display full regression even at lower doses, down to 2μg/ml, a dose at which WT-MIS no longer shows any activity (data not shown). Accordingly, the presence of the leader sequence (L) in the LRF-MIS recombinant human MIS protein results in a dose-dependently decrease the regression of the Mullerian ducts, as compared to the RF-MIS construct, indicating that this construct has a higher potency and is more active than the RF-MIS construct.

[00385] Taken together, the inventors demonstrate herein that the LR product results in a greater yield of production with increased cleavage and higher bioactivity or potency.

[00386] Accordingly, the inventors demonstrate that the HSA leader sequence surprisingly resulted in an increased yield (both higher concentration and higher production) of the recombinant human MIS protein (see Fig. 2 and 3). Furthermore, the presence of the HSA leader sequence also resulted in an unexpectedly increase in cleavage from the primary cleavage site (corresponding to cleavage at 451/452 of SEQ ID NO: 1 (or 426/427 of conventional amino acid nomenclature of wild-type human MIS protein) (see Fig. 2 and 3). This increased yield and increased cleavage was surprising because with an increased yield (and therefore more protein produced by the cell), one would expect a decreased cleavage as the activity of the available cleavage enzymes becomes saturated and overextended. However, this was not the case - in fact the exact opposite occurred where with increased protein production there was increased cleavage from the primary cleavage site.

[00387] This is particularly unexpected as the effect of the leader sequence, which is not located anywhere near the cleavage site of the primary cleavage site of MIS, was not expected to have an effect on increased cleavage as the leader sequence is typically cleaved first before the post-translation cleavage of the proprotein MIS.

[00388] Furthermore, the leader sequence also resulted in less cleavage from the secondary cleavage site (located between amino acid residues 229/230 of normal wild-type MIS numbering or corresponding to residues 254/255 of SEQ ID NO: 1). This is also surprising, considering that there was no modification to the secondary cleavage site.

[00389] Additionally, the presence of the leader sequence also increased the production and yield even when a FLAG tag is present in the recombinant human MIS protein. (The FLAG tag significantly decreases the yield as shown in Table 3). This again was a surprising discovery, as the leader sequence is not located anywhere near the FLAG tag and it would not be expected that such a modification to the leader sequence would increase the production yield of a protein comprising a FLAG tag.

Example 3

[00390] *LR11* is grown in 5 layer flask with 250ml of DMEM or in 10 layer flasks (1700cm^2) with 500ml media supplemented with 10% FFCS, 800ug/ml of geneticin, 2nM glutamine, 100U/ml penicillin and 100ug/ml streptomycin (Invitrogen) maintained confluent for several months in 5% CO2, at 37C. Once a week, the media is replaced with a serum-free media which omits FFCS and replaces it

with non-essential amino acids (NEAA) and ITS (insulin, transferring, selenium) supplements for 72h. The media is then concentrated 10X using tangential flow osmosis membranes. Using these methods media of 4-5ug/ml is concentrated to 25-50ug/ml, and effective purification yield of LR-MIS rises to approximately 30%.

Table 4: Purification yield from MIS from various constructs using a new serum-free media purification protocol.

	WT-MIS	RF-MIS	LRF-MIS	LR-MIS
MIS concentration (μg/ml) at 24 hours	16.821	1.236	2.149	4.866
Production (pg/cell/day)	7.597	0.254	0.430	1.142
Concentration in serum-free media at 24h	1.528	0.223	0.457	1.411
Purification yield (% w/w)	15%	20%	20%	30%
Percent cleavage in serum-free media	25%	50%	37%	79%

REFERENCES

[00391] Each of the applications and patents cited in this text, as well as each document or reference cited in each of the applications and patents (including during the prosecution of each issued patent; "application cited documents"), and each of the PCT and foreign applications or patents corresponding to and/or claiming priority from any of these applications and patents, and each of the documents cited or referenced in each of the application cited documents, are hereby expressly incorporated herein by reference and may be employed in the practice of the invention. More generally, documents or references are cited in this text, either in a Reference List before the claims, or in the text itself; and, each of these documents or references ("herein cited references"), as well as each document or reference cited in each of the herein cited references (including any manufacturer's specifications, instructions, etc.), is hereby expressly incorporated herein by reference. Accordingly, the references are each incorporated herein in their entirety by reference.

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SEQUENCE LISTING:

SEQ ID NO: 1 MIS (560AA) - amino acid sequence (underlined identifies native MIS leader sequence)
mrdlpltsla lvlsalgall gtealraeep avgtsglifr edldwppgsp qeplclvalg
gdsngssspl rvvgalsaye qaflgavqra rwgprdlatf gvcntgdrqa alpslrrlga
wlrdpggqrl vvlhleevtw eptpslrfqe pppggagppe lallvlypgp gpevtvtrag
lpgaqslcps rdtrylvlav drpagawrgs glaltlqprg edsrlstarl qallfgddhr
cftrmtpall llprsepapl pahgqldtvp fppprpsael eesppsadpf letltrlvra
lrvpparasa prlaldpdal agfpqglvnl sdpaalerll dgeeplllll rptaattgdp
aplhdptsap watalarrva aelqaaaael rslpglppat apllarllal cpggpgglgd
plralllka lqglrvewrg rdprgpgra**q** rsagataadg pcalrelsvd lraersvlip
etyqanncqg vcgwpqsdrn prygnhvvll lkmqvrgaal arppccvpta yagkllisls
eerisahhvp nmvatecgcr

SEQ ID NO: 2 LR (559AA) BOLD indicates-albumin leader sequence; Underlined identifies the Modified cleavage site
mkwvtfisll flfssaysrg vfrr raeep avgtsglifr edldwppgsp qeplclvalg
gdsngssspl rvvgalsaye qaflgavqra rwgprdlatf gvcntgdrqa alpslrrlga
wlrdpggqrl vvlhleevtw eptpslrfqe pppggagppe lallvlypgp gpevtvtrag
lpgaqslcps rdtrylvlav drpagawrgs glaltlqprg edsrlstarl qallfgddhr
cftrmtpall llprsepapl pahgqldtvp fppprpsael eesppsadpf letltrlvra
lrvpparasa prlaldpdal agfpqglvnl sdpaalerll dgeeplllll rptaattgdp
aplhdptsap watalarrva aelqaaaael rslpglppat apllarllal cpggpgglgd
plralllka lqglrvewrg rdprgpgra**R** rsagataadg pcalrelsvd lraersvlip
etyqanncqg vcgwpqsdrn prygnhvvll lkmqvrgaal arppccvpta yagkllisls
eerisahhvp nmvatecgcr

SEQ ID NO: 3 LRF (567AA) Italicized indicates Flag tag (**DYKDDDDK (SEQ ID NO: 8)**)
mkwvtfisll flfssaysrg vfrr raeep avgtsglifr edldwppgsp qeplclvalg
gdsngssspl rvvgalsaye qaflgavqra rwgprdlatf gvcntgdrqa alpslrrlga
wlrdpggqrl vvlhleevtw eptpslrfqe pppggagppe lallvlypgp gpevtvtrag
lpgaqslcps rdtrylvlav drpagawrgs glaltlqprg edsrlstarl qallfgddhr
cftrmtpall llprsepapl pahgqldtvp fppprpsael eesppsadpf letltrlvra
lrvpparasa prlaldpdal agfpqglvnl sdpaalerll dgeeplllll rptaattgdp
aplhdptsap watalarrva aelqaaaael rslpglppat apllarllal cpggpgglgd
plralllka lqglrvewrg rdprgpgra**R** rs***DYKDDDDK*** agataadg pcalrelsvd
lraersvlip etyqanncqg vcgwpqsdrn prygnhvvll lkmqvrgaal arppccvpta
yagkllisls eerisahhvp nmvatecgcr

SEQ ID NO: 4 LR - nucleic acid sequence
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CGGGTGGTGGGGGCTCTAAGCGCCTATGAGCAGGCCTTCCTGGGGGCCGTGCAGAGGGCCCGCTGGGGC
CCCCGAGACCTGGCCACCTTCGGGGTCTGCAACACCGGTGACAGGCAGGCTGCCTTGCCCTCTCTACGG
CGGCTGGGGGCCTGGCTGCGGGACCCTGGGGGGCAGCGCCTGGTGGTCCTACACCTGGAGGAAGTGACC
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GCGGAACTCGAGGAGTCGCCACCCAGCGCAGACCCCTTCCTGGAGACGCTCACGCGCCTGGTGCGGGCG
CTGCGGGTCCCCCCGGCCCGGGCCTCCGCGCCGCGCCTGGCCCTGGATCCGGACGCGCTGGCCGGCTTC
CCGCAGGGCCTAGTCAACCTGTCGGACCCCGCGGCGCTGGAGCGCCTACTCGACGGCGAGGAGCCGCTG
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GTGCTGCTGCTGAAGATGCAGGCCCGTGGGGCCGCCCTGGCGCGCCCACCCTGCTGCGTGCCCACCGCC
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GCCACCGAGTGTGGCTGCCGGTGA

SEQ ID NO: 5 LRF - nucleic acid sequence
ATGAAGTGGGTGAGCTTCATCAGCCTGCTGTTCCTGTTCAGCAGCGCTTACTCCCGCGGTGTGTTCCGC
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GCACTGCTGTTCGGCGACGACCACCGCTGCTTCACACGGATGACCCCGGCCCTGCTCCTGCTGCCGCGG
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CGCGGGCGGGATCCGCGCGGGCCGGGTCGGGCAC**G**GCGCAGC***gactacaaggatgacgacgacaag***GCG
GGGGCCACCGCCGCCGACGGGCCGTGCGCGCTGCGAGCTCAGCGTAGACCTCCGCGCCGAGCGCTCC
GTACTCATCCCCGAGACCTACCAGGCCAACAATTGCCAGGGCGTGTGCGGCTGGCCTCAGTCCGACCGC
AACCCGCGCTACGGCAACCACGTGGTGCTGCTGCTGAAGATGCAGGCCCGTGGGGCCGCCCTGGCGCGC
CCACCCTGCTGCGTGCCCACCGCCTACGCGGGCAAGCTGCTCATCAGCCTGTCGGAGGAGCGCATCAGC
GCGCACCACGTGCCCAACATGGTGGCCACCGAGTGTGGCTGCCGGTGA

SEQ ID NO: 6 HSA Leader Sequence (amino acid sequence):
mkwvtfisll flfssaysrg vfrr

SEQ ID NO: 7 - HSA Leader Sequence (nucleic acid sequence):
ATGAAGTGGGTGAGCTTCATCAGCCTGCTGTTCCTGTTCAGCAGCGCTTACTCCCGCGGTGTGTTCCGC
CGCAGAGCA

SEQ ID NO: 8 - FLAG tag (amino acid sequence):
DYKDDDDK

SEQ ID NO: 9 - FLAG tag (nucleic acid sequence):
gactacaaggatgacgacgacaag

CLAIMS

1. A recombinant Mullerian Inhibiting Substance (MIS) protein comprising a combination of a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1, and a modification of at least one amino acid between residues 447-451 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification, wherein the recombinant MIS protein has increased cleavage and increased yield of production *in vitro* as compared to wild-type MIS protein corresponding to amino acid residues of SEQ ID NO: 1.
2. The recombinant MIS protein of claim 1, wherein the recombinant MIS protein further comprises a Tag protein.
3. The recombinant MIS protein of claim 1, wherein the recombinant MIS protein comprises at least a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1 and a modification of at least one amino acid between residues 447-451 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification.
4. The recombinant MIS protein of claim 1, wherein the non-MIS leader sequence is an albumin leader sequence or a functional fragment thereof.
5. The recombinant MIS protein of claim 4, wherein the albumin leader sequence is a human serum albumin (HSA) leader sequence or a fragment thereof.
6. The recombinant MIS protein of claim 5, wherein the HSA leader sequence comprises the amino acid sequence of SEQ ID NO: 6 or a variant that is at least 80% homologous thereto.
7. The recombinant MIS protein of claim 5, wherein a fragment of the HSA leader sequence comprises at least 10 amino acids of SEQ ID NO: 6 or a variant that is at least 80% homologous thereto.
8. The recombinant MIS protein of claim 5, wherein the HSA leader sequence comprises at least 15 amino acids of SEQ ID NO: 6, or a variant that is at least 80% homologous thereto.
9. The recombinant MIS protein of claim 5, wherein the HSA leader sequence comprises at least 11 amino acids of SEQ ID NO: 6, or a variant that is at least 80% homologous thereto.
10. The recombinant MIS protein of claim 5, wherein a fragment of the HSA leader sequence is selected from the group consisting of: MKWVTFISLLFLFSSAYS (SEQ ID NO: 13); MKWVTFISLLFLFSSAYSRGVFRR (SEQ ID NO: 6); MKWVSFISLLFLFSSAYS (SEQ ID NO:14).
11. The recombinant MIS protein of claim 1, wherein the non-MIS leader sequence is selected from a group consisting of: immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide (IgSP-tPA), murine immunoglobulin signal peptide (IgSP), a MPIF-1 signal sequence (MKVSVAALSCLMLVTALGSQA (SEQ ID NO: 15);a stanniocalcin signal

sequence (MLQNSAVLLLLVISASA (SEQ ID NO:16)); an invertase signal sequence (MLLQAFLFLLAGFAAKISA (SEQ ID NO:17)); a yeast mating factor alpha signal sequence (K. lactis killer toxin leader sequence); a hybrid signal sequence (MKWVSFISLLFLFSSAYSRSLEKR, (SEQ ID NO:18)); a HSA/MFα-1 hybrid signal sequence (MKWVSFISLLFLFSSAYSRSLDKR (SEQ ID NO:19)); a K. lactis killer/ MFα-1 fusion leader sequence (MNIFYIFLFLLSFVQGSLDKR (SEQ ID NO:20)); an immunoglobulin Ig signal sequence (MGWSCIILFLVATATGVHS (SEQ ID NO:21)); a Fibulin B precursor signal sequence (MERAAPSRRVPLPLLLLGGLALLAAGVDA (SEQ ID NO:22)); a clusterin precursor signal sequence (MMKTLLLFVGLLLTWESGQVLG (SEQ ID NO: 23)); and the insulin-like growth factor-binding protein 4 signal sequence (MLPLCLVAALLLAAGPGPSLG (SEQ ID NO:24)) or a functional fragment thereof.

12. The recombinant MIS protein of claim 1, comprising a modification of amino acid 449 of SEQ ID NO: 1 from Q to R to increase cleavage as compared to in the absence of such a modification.
13. The recombinant MIS protein of claim 1, further comprising a modification of amino acid 451 of SEQ ID NO: 1 from S to R to increase cleavage as compared to in the absence of such a modification.
14. The recombinant MIS protein of claim 1, wherein the tag is a FLAG tag.
15. The recombinant MIS protein of claim 14, wherein the FLAG tag comprises amino acid sequence DYKDDDDK (SEQ ID NO: 8), or a functional derivative or variant thereof.
16. The recombinant MIS protein of claim 14, wherein the FLAG tag is located after amino acid residue 451 of SEQ ID NO: 1 and before amino acid residue 452 of SEQ ID NO: 1.
17. The recombinant MIS protein of claim 14, wherein the FLAG tag is located between amino acid residue 451 and 452 of SEQ ID NO: 1.
18. The recombinant MIS protein of claim 1, which comprises the amino acid sequence of SEQ ID NO: 2 or a functional fragment thereof.
19. The recombinant MIS protein of claim 1, which comprises the amino acid sequence of SEQ ID NO: 3 or a functional fragment thereof.
20. The recombinant MIS protein of claim 18, which is encoded by nucleic acid sequence of SEQ ID NO: 4.
21. The recombinant MIS protein of claim 19, which is encoded by nucleic acid sequence of SEQ ID NO: 5.
22. A pharmaceutical composition comprising the recombinant MIS protein of any of claims 1 to 21 and a pharmaceutically acceptable carrier.
23. A polynucleotide encoding the recombinant MIS protein of any of claims 1 to 21.

24. The polynucleotide of claim 23, wherein the nucleotide corresponds to SEQ ID NO: 4 or a nucleotide which has at least 95% sequence identity to the nucleic acid sequence of SEQ ID NO: 4.
25. The polynucleotide of claim 23, wherein the nucleotide corresponds to SEQ ID NO: 5 or a nucleotide which has at least 95% sequence identity to the nucleic acid sequence of SEQ ID NO: 5.
26. A vector comprising the polynucleotide of claims 23-25.
27. The vector of claim 26, wherein the vector is a viral vector or an expression vector.
28. The vector of claim 27, wherein the expression vector is pcDNA 3.1 or cDNA or genome vector for bacteria (e.g., e coli) or bacteriophage.
29. The vector of claim 27, wherein the viral vector is selected from the group consisting of an adenoviral vector, a poxvirus vector and a lentiviral vector.
30. The vector of any of claims 26 to 29, wherein the nucleic acid sequence encodes a recombinant MIS protein or fragment thereof which has at least 95% sequence identity to the nucleic acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5, wherein the nucleic acid sequence is operatively linked to tissue- or cell-type specific promoter.
31. A human MIS protein produced by post-translational processing of the recombinant human MIS protein of claim 1.
32. A host cell comprising the vector of any of the claims 26 to 30.
33. A pharmaceutical composition comprising the vector of any of the claims 26 to 30 and a pharmaceutically acceptable carrier.
34. A purified preparation of human MIS protein produced from the recombinant human MIS protein of any of claims 1 to 21.
35. A method for treating a subject with cancer, comprising administering a composition comprising a recombinant MIS protein, wherein the recombinant MIS protein comprises a combination of a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1 and a modification of at least one amino acid between residues 447-451 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification, wherein the recombinant MIS protein has increased cleavage and increased yield of production *in vitro* as compared to wild-type MIS protein corresponding to amino acid residues of SEQ ID NO: 1.
36. The method of claim 35, wherein the recombinant MIS protein further comprises a Tag protein.
37. The method of claim 35, wherein the recombinant MIS protein comprises at least a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1 and a modification of at least one amino acid between residues 447-451 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification.

38. The method of claim 35, wherein the non-MIS leader sequence is an albumin leader sequence or a functional fragment thereof.
39. The method of claim 38, wherein the albumin leader sequence is a human serum albumin (HSA) leader sequence or a fragment thereof.
40. The method of claim 35, wherein the recombinant MIS protein comprises a modification of amino acid 449 of SEQ ID NO: 1 from Q to R to increase cleavage as compared to in the absence of such a modification.
41. The method of claim 35, wherein the tag is a FLAG tag comprising amino acid sequence of SEQ ID NO: 8 or a functional fragment thereof.
42. The method of claim 35, wherein the cancer is a MIS Responsive II cancer.
43. The method of claim 35, wherein the cancer is ovarian cancer.
44. The method of claim 35, wherein the cancer is a chemotherapeutic-resistant or multi-drug resistant cancer.
45. The method of claim 35, wherein the administration of the recombinant MIS protein is prior to, during, or after administration of an additional agent or cancer therapy.
46. The method of claim 35, wherein the cancer expresses Mullerian Inhibiting Substance Receptor II (MISRII).
47. The method of claim 46, wherein the expression of Mullerian Inhibiting Substance (MIS) receptor is measured in a biological sample obtained from the subject.
48. The method of claim 47, wherein the biological sample is a cancer or tumor tissue sample or a cancer cell or tumor cell.
49. The method of claim 47, wherein the biological sample is a biopsy tissue sample.
50. The method of claim 35 , wherein the cancer is an ovarian cancer cell, vulvar epidermal carcinoma cell, cervical carcinoma cell, endometrial edenocarinaoma cell and ovarian adenocarcinoma cell.
51. The method of claim 35, wherein the cancer is selected from the group consisting of: breast cancer, lung cancer, head and neck cancer, bladder cancer, stomach cancer, cancer of the nervous system, bone cancer, bone marrow cancer, brain cancer, colon cancer, esophageal cancer, endometrial cancer, gastrointestinal cancer, gum cancer, kidney cancer, liver cancer, nasopharynx cancer, ovarian cancer, prostate cancer, skin cancer, stomach cancer, testis cancer, tongue cancer, melanoma, ocular melanoma, or uterine cancer.
52. The method of claim 44, wherein the multi-drug resistant cancer is a paclitaxel- or Doxorubicin-resistant cancer.
53. The method of claim 35, wherein administering is intravenous, intradermal, intramuscular, intraarterial, intralesional, percutaneous, or subcutaneous, or by aerosol.
54. The method of claim 35, wherein administering is prophylactic administration.
55. The method of claim 35, wherein administering is therapeutic administration.

56. The method of claim 35, wherein the subject is a mammal.
57. The method of claim 56, wherein the mammal is a human.
58. The method of claim 35, wherein at least one additional agent is administered to the subject in combination with (e.g., before, during or after) administration of the recombinant human MIS.
59. The method of claim 58, wherein the additional agent is a therapeutic agent or chemotherapeutic agent.
60. The method of claim 59, wherein the chemotherapeutic agent is selected from the group consisting of: paclitaxel, cisplatin, doxorubicin, rapamycin, pyrazoloanthrone.
61. The method of claim 59, wherein the chemotherapeutic agent is a radiotherapeutic agent.
62. The method of claim 59, wherein the chemotherapeutic agent is a pyrazoloanthrone.
63. The method of claim 62, wherein the pyrazoloanthrone is antra(1,9-cd)pyrazol-6(2H)-one (SP600125) or a functional derivative or functional analogue thereof.
64. A method of decreasing the dose of a chemotherapeutic agent for the treatment of cancer, the method comprising administering to the subject a therapeutically effective amount of a recombinant MIS protein, wherein the recombinant MIS protein comprises a combination of a modification of amino acid 449 of SEQ ID NO: 1 from Q to R, and a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1, wherein the therapeutically effective dose of the chemotherapeutic agent in the presence of the recombinant MIS protein is lower as compared to the therapeutically effective dose of the chemotherapeutic agent alone.
65. The method of claim 64, wherein the recombinant MIS protein further comprises a Tag protein.
66. Use of recombinant MIS protein for the manufacture of a medicament for treating cancer, wherein the recombinant MIS protein comprises a combination of a modification of amino acid 449 of SEQ ID NO: 1 from Q to R, and a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1, and wherein the cancer expresses a Mullerian Inhibiting Substance (MIS) receptor.
67. The use of claim 66, wherein the recombinant MIS protein further comprises a Tag protein.
68. The use of claim 66, wherein the Mullerian Inhibiting Substance (MIS) receptor is MIS type II receptor or a homologue or functional fragment thereof.
69. An article of manufacture comprising packaging material and a pharmaceutical composition comprising the recombinant MIS protein of any of claims 1 to 21, wherein the packaging material comprises a label which indicates the pharmaceutical composition may be administered, for a sufficient term at an effective dose, for treating or reducing the risk of cancer which expresses a Mullerian Inhibiting Substance (MIS) receptor.
70. A method of treating a subject affected with cancer, the method comprising assessing the expression and/or activity of Mullerian Inhibiting Substance Receptor II (MISRII) in a biological sample obtained from the subject, wherein a clinician reviews the results and if the

results indicate the presence of expression and/or activity of MISRII, the clinician directs the subject to be treated with pharmaceutical composition of claim 22 or 33.

71. The method of claim 70, wherein the biological sample is a tissue sample.
72. The method of claim 71, wherein the tissue sample is a cancer or tumor tissue sample or a cancer cell or tumor cell.
73. The method of claim 71, wherein the biological sample is a biopsy tissue sample.
74. The method of claim 70, wherein the cancer is an ovarian cancer cell, vulvar epidermal carcinoma cell, cervical carcinoma cell, endometrial edenocarinaoma cell and ovarian adenocarcinoma cell.
75. The method of claim 70, wherein the cancer is breast cancer, lung cancer, head and neck cancer, bladder cancer, stomach cancer, cancer of the nervous system, bone cancer, bone marrow cancer, brain cancer, colon cancer, esophageal cancer, endometrial cancer, gastrointestinal cancer, gum cancer, kidney cancer, liver cancer, nasopharynx cancer, ovarian cancer, prostate cancer, skin cancer, stomach cancer, testis cancer, tongue cancer, melanoma, ocular melanoma or uterine cancer.
76. Use of a recombinant MIS protein to decrease the plasma serum levels of one or more androgens in a subject in need thereof, wherein the recombinant MIS protein comprises a combination of a modification of amino acid 449 of SEQ ID NO: 1 from Q to R, and a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1.
77. The use of claim 76, wherein the recombinant MIS protein further comprises a Tag protein.
78. The use of claim 76, wherein one or more androgens is testosterone.
79. The use of claim 76, wherein the subject in need thereof has benign prostatic hypertrophy.
80. The use of claim 76, wherein the subject in need thereof has prostate cancer.
81. The use of claim 76, wherein the subject in need thereof has polycystic ovarian disease and/or precocious puberty.
82. The use of claim 76, wherein the subject in need thereof has a disease or disorder selected from the group consisting of: Benign Prostatic Hyperplasia (BPH), prostate carcinoma, testicular cancer, androgen dependent acne, male pattern baldness, precocious puberty, hyperandrogenism, hirsutism, virilization, Polycystic Ovary Syndrome (POCS), hyperandrogenism (HA) and insulin resistance (IR) and acanthosis nigricans (AN) (HIAR-AN) syndrome, ovarian hyperthecosis, follicular maturation arrest, atresia, anovulation, dysmenorrheal, dysfunctional uterine bleeding, infertility and androgen-producing tumors.
83. A method to treat a disease or disorder characterized by androgenic dependency, comprising administering to a subject an effective amount of the pharmaceutical composition of claim 22 or 33, wherein the pharmaceutical composition reduces the level of at least one androgen in the

plasma serum of the subject and results in a decrease in at least one symptom of a disease or disorder characterized by androgenic dependency.

84. A method to decrease the plasma level of one or more androgens in a subject, the method comprising administering an effective amount of a recombinant MIS protein, wherein the recombinant MIS protein comprises a combination of a modification of amino acid 449 of SEQ ID NO: 1 from Q to R, and a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1, and wherein the recombinant MIS protein decreases the plasma serum levels of one or more androgens in the subject.
85. The method of claim 84, wherein the recombinant MIS protein further comprises a Tag protein.
86. The method of claim 84, wherein the subject has a disease or disorder characterized by androgenic dependency.
87. The method of any of claims 84 to 86 , wherein the disease or disorder is selected from the group consisting of: Benign Prostatic Hyperplasia (BPH), prostate carcinoma, testicular cancer, androgen dependent acne, male pattern baldness, precocious puberty, hyperandrogenism, hirsutism, virilization, Polycystic Ovary Syndrome (POCS), hyperandrogenism (HA) and insulin resistance (IR) and acanthosis nigricans (AN) (HIAR-AN) syndrome, ovarian hyperthecosis, follicular maturation arrest, atresia, anovulation, dysmenorrheal, dysfunctional uterine bleeding, infertility and androgen-producing tumors.
88. A kit comprising a recombinant MIS protein of any of claims 1-21 and a pharmaceutically acceptable carrier.
89. The kit of claim 88, optionally further comprising instructions of use of the recombinant MIS protein for the treatment of cancer or treatment of an androgenic dependency disorder.

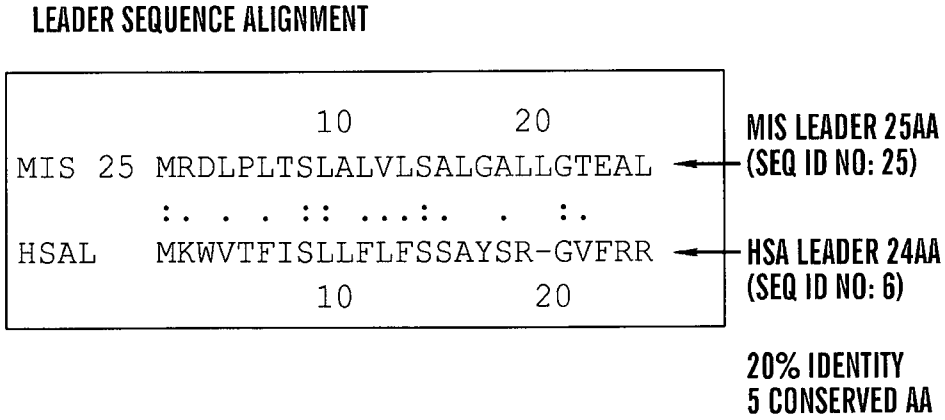
LEADER SEQUENCE ALIGNMENT
10 20
MIS 25 MRDLPLTSLALVLSALGALLGTEAL
:. . . :: ...:. . :.
HSAL MKWVTFISLLFLFSSAYSR-GVFRR
10 20
MIS LEADER 25AA
(SEQ ID NO: 25)
HSA LEADER 24AA
(SEQ ID NO: 6)
20% IDENTITY
5 CONSERVED AA

FIG. 1A

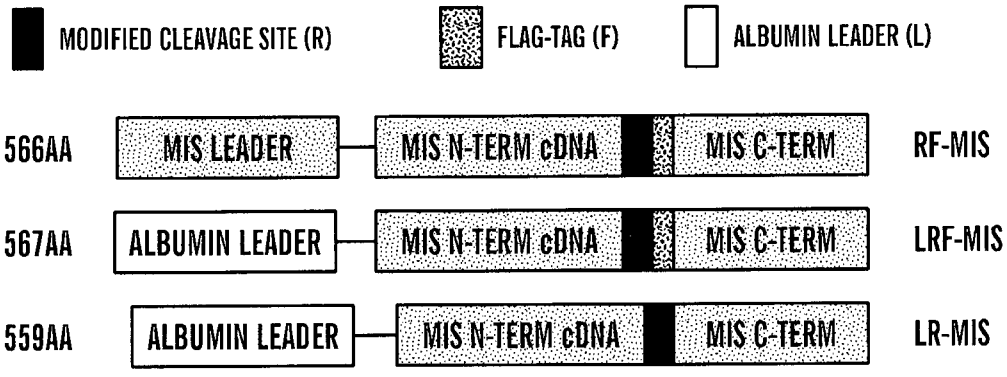
MODIFIED CLEAVAGE SITE (R)
FLAG-TAG (F)
ALBUMIN LEADER (L)
566AA
MIS LEADER
MIS N-TERM cDNA
MIS C-TERM
RF-MIS
567AA
ALBUMIN LEADER
MIS N-TERM cDNA
MIS C-TERM
LRF-MIS
559AA
ALBUMIN LEADER
MIS N-TERM cDNA
MIS C-TERM
LR-MIS

FIG. 1B

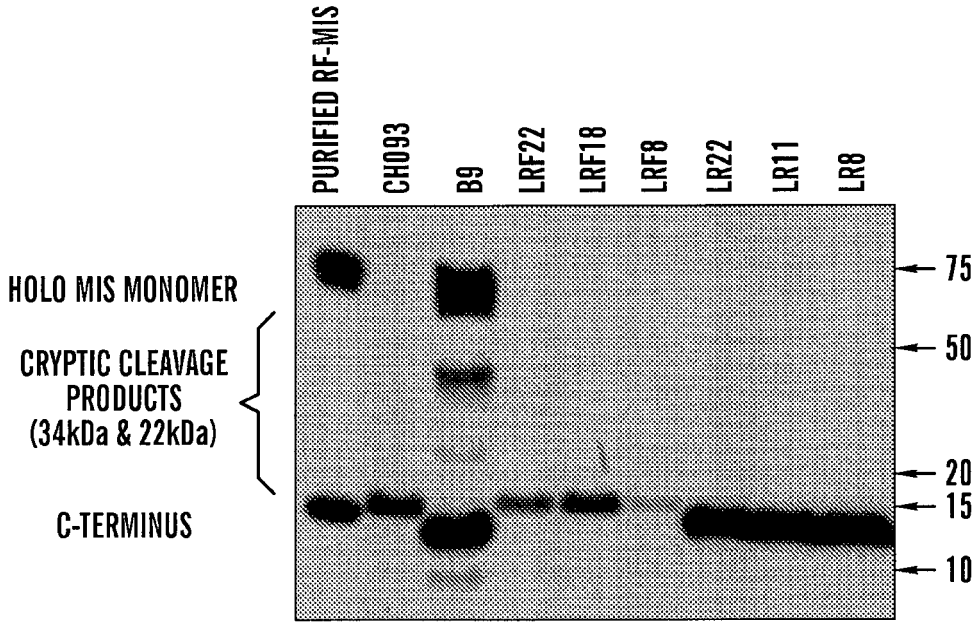
PURIFIED RF-MIS
CHO93
B9
LRF22
LRF18
LRF8
LR22
LR11
LR8
HOLO MIS MONOMER
CRYPTIC CLEAVAGE PRODUCTS (34kDa & 22kDa)
C-TERMINUS
75
50
20
15
10

FIG. 2

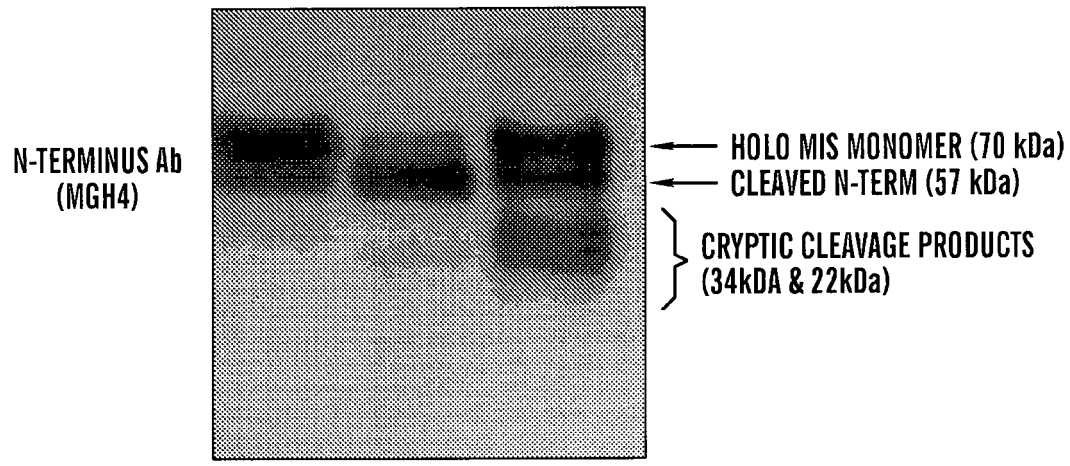
N-TERMINUS Ab
(MGH4)
HOLO MIS MONOMER (70 kDa)
CLEAVED N-TERM (57 kDa)
CRYPTIC CLEAVAGE PRODUCTS
(34kDA & 22kDa)

FIG. 3A

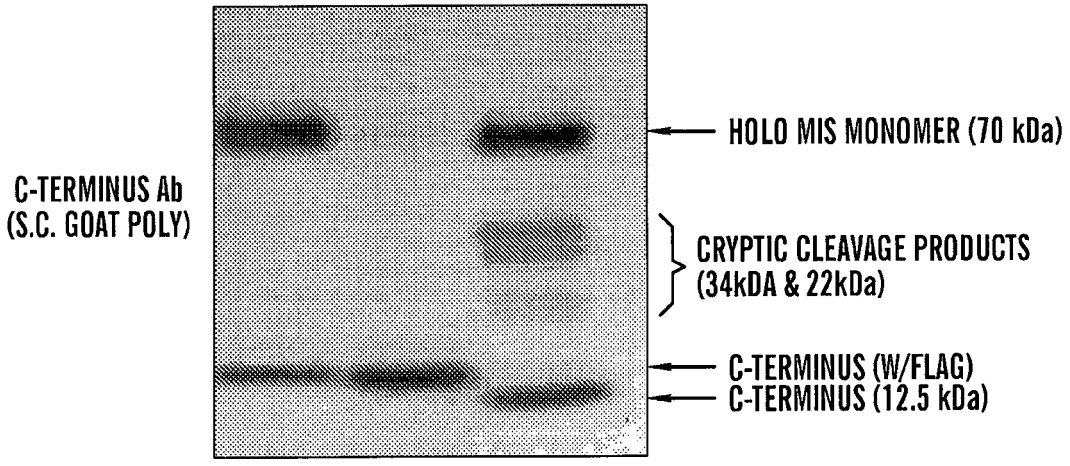
C-TERMINUS Ab
(S.C. GOAT POLY)
HOLO MIS MONOMER (70 kDa)
CRYPTIC CLEAVAGE PRODUCTS
(34kDA & 22kDa)
C-TERMINUS (W/FLAG)
C-TERMINUS (12.5 kDa)

FIG. 3B

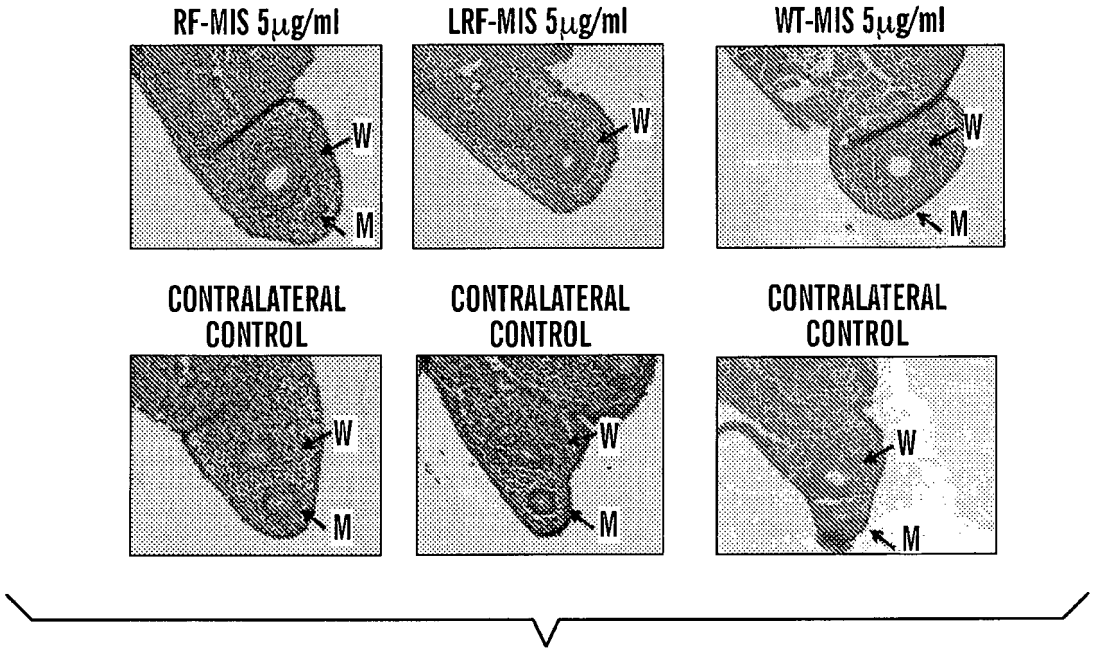
RF-MIS 5μg/ml
LRF-MIS 5μg/ml
WT-MIS 5μg/ml
W
M
CONTRALATERAL CONTROL
CONTRALATERAL CONTROL
CONTRALATERAL CONTROL

FIG. 4A

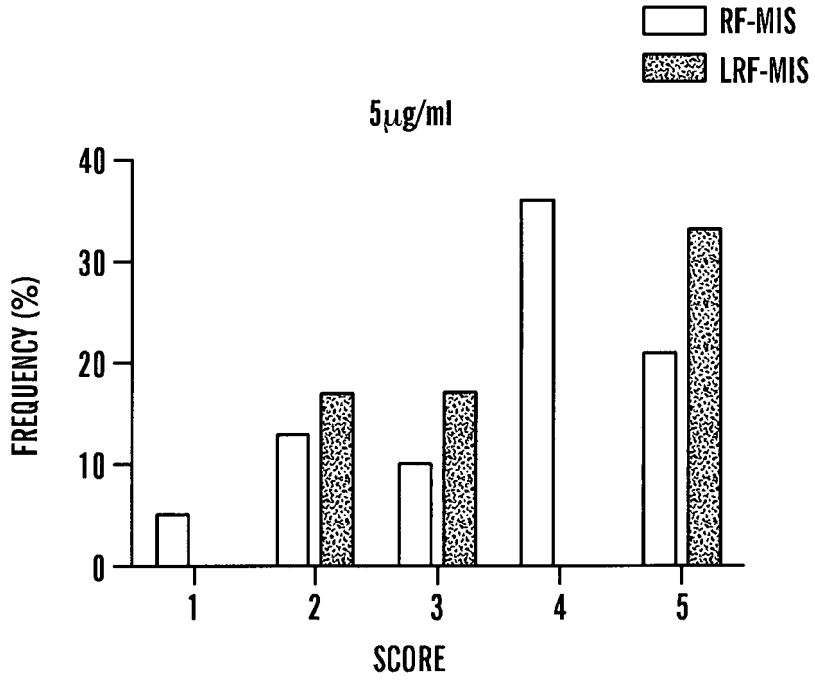
RF-MIS
LRF-MIS
5μg/ml
FREQUENCY (%)
40
30
20
10
0
1
2
3
4
5
SCORE

FIG. 4B

(Conventional Numbering)

mrdlpltsla lvlsalgall gtealraeep avgtsglifr edldwppgsp qeplclvalg 60 (35)
LEADER SEQUENCE (1-25 of SEQ ID NO:1)

gdsngssspl rvvgalsaye qaflgavqra rwgprdlatf gvcntgdrqa alpslrrlga 120 (95)

wlrdpggqrl vvlhleevtw eptpslrfqe pppggagppe lallvlypgp gpevtvtrag 180 (155)

lpgaqslcps rdtrylvlav drpagawrgs glaltlqprg edsrlstarl qallfgddhr 240 (215)

cftrmtpall llprsepapl pahgqldtvp fppprpsael eesppsadpf letltrlvra 300 (275)
Secondary cleavage site (254/255 of SEQ ID NO: 1)

lrvpparasa prlaldpdal agfpqglvnl sdpaalerll dgeeplllll rptaattgdp 360 (335)

aplhdptsap watalarrva aelqaaaael rslpglppat apllarllal cpggpgglgd 420 (395)

plrallllka lqglrvewrg rdprgpg**raq rs**agataadg pcalrelsvd lraersvlip 480 (455)
Primary cleavage recognition sequence (448-452 of SEQ ID NO: 1)

etyqanncqg vcgwpqsdrn prygnhvvll lkmqvrgaal arppccvpta yagkllisls 540 (515)

eerisahhvp nmvatecgcr 560 (535) (SEQ ID NO:1)

FIG. 5A

	LOCATION ON SEQ ID NO: 1	LOCATION ON NORMAL NOMENCLATURE OF MIS (FIRST AMINO ACID IS AFTER THE LEADER SEQUENCE)
LEADER SEQUENCE:	1-25	AMINO ACID RESIDUES -24-0
PRIMARY CLEAVAGE RECOGNITION SEQUENCE	448-452 (RAQR/S)	423-427
PRIMARY CLEAVAGE SITE	BETWEEN 451 AND 452 (451/452)	BETWEEN 426 AND 427 (426/427)
CHANGE OF PRIMARY CLEAVAGE RECOGNITION SEQUENCE	CHANGE OF AMINO ACID 450 FROM a Q TO an R (Q450R)	CHANGE OF AMINO ACID 425 FROM a Q TO an R (Q425R)
SECONDARY CLEAVAGE RECOGNITION SITE	BETWEEN 254 AND 255 (254/255)	BETWEEN 229 AND 230 (229/230)

FIG. 5B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/024010

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/22 (2014.01)
USPC - 424/198.1
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)
IPC(8) - A61K 38/22, 48/00; A61P 5/28, 15/00, 08 (2014.01)
USPC - 424/198.1; 514/9.7, 9.8, 10.2, 21.2; 530/324, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 38/22, 48/00 (2014.06)

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2001/019387 A1 (DONAHOE et al.) 22 March 2001 (22.03.2001) entire document	1-5, 12, 14-17, 22-23, 31, 34-69, 76-82, 84-89
Y	PAPAKOSTAS et al. "Development of an efficiently cleaved, bioactive, highly pure FLAG-tagged recombinant human Mullerian Inhibiting Substance," Protein Expr Purif. 13 September 2009 (13.09.2009), Vol. 70, No. 1, Pgs. 32-38. entire document	1-5, 12, 14-17, 22-23, 31, 34-69, 76-82, 84-89
Y	PIERETTI-VANMARCKE et al. "Mullerian Inhibiting Substance enhances subclinical doses of chemotherapeutic agents to inhibit human and mouse ovarian cancer," Proc Natl Acad Sci, 14 November 2006 (14.11.2006), Vol.103, No. 46, Pgs. 17426-17431. entire document	64-65
Y	ZOU et al. "Overexpression of human transforming growth factor-beta1 using a recombinant CHO cell expression system," Protein Expr Purif. 01 October 2004 (01.10.2004), Vol. 37, No. 2, Pgs. 265-272. entire document	4-5
Y	US 2010/0233689 A1 (TEIXEIRA et al) 16 September 2010 (16.09.2010) entire document	61-62
A	WO 2009/012357 A2 (DONAHOE et al) 22 January 2009 (22.01.2009) entire document	3

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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	Date of mailing of the international search report
05 August 2014	29 AUG 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/024010

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 26-30, 32, 33, 70-75, 83
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Extra Sheets

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5, 12, 14-17, 19, 22-23, 31, 34-69, 76-82, and 84-89, limited to SEQ ID NO:3.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/024010

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs:1 and 3 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/024010

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-25, 31, 34-69, 76-82, and 84-89 are drawn to a recombinant Mullerian Inhibiting Substance (MIS) protein, and a method for treating a subject with cancer, a method of decreasing the dose of a chemotherapeutic agent for the treatment of cancer, and a method for decreasing the plasma serum levels of one or more androgens in a subject in need thereof, said methods comprising the same.

The first invention of Group I+ is restricted to a recombinant Mullerian Inhibiting Substance (MIS) protein, and a method for treating a subject with cancer, a method of decreasing the dose of a chemotherapeutic agent for the treatment of cancer, and a method for decreasing the plasma serum levels of one or more androgens in a subject in need thereof, said methods comprising the same, wherein the recombinant MIS protein is selected to be SEQ ID NO:3, wherein SEQ ID NO:3 comprises non-MIS human serum albumin (HSA) leader sequence of SEQ ID NO:6 in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1, modification of amino acid 450 of SEQ ID NO: 1 from Q to R (amino acid residue 449 of SEQ ID NO:1 is not Q), and further comprises Tag protein of SEQ ID NO:8, wherein the recombinant MIS protein of SEQ ID NO:3 has increased cleavage and increased yield of production in vitro as compared to wild-type MIS protein corresponding to amino acid residues of SEQ ID NO: 1. It is believed that claims 1-5, 10, 12, 14-17, 19, 21-25, 31, 34-69, 76-82, and 84-89 read on this first named invention and thus these claims will be searched without fee to the extent that they read on SEQ ID NO:3.

Applicant is invited to elect additional recombinant Mullerian Inhibiting Substance (MIS) proteins with specified SEQ ID NO to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be a recombinant Mullerian Inhibiting Substance (MIS) protein, and a method for treating a subject with cancer, a method of decreasing the dose of a chemotherapeutic agent for the treatment of cancer, and a method for decreasing the plasma serum levels of one or more androgens in a subject in need thereof, said methods comprising the same, wherein the recombinant MIS protein is selected to be SEQ ID NO:2. Additional recombinant Mullerian Inhibiting Substance (MIS) proteins will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element, requiring the selection of alternatives for the recombinant Mullerian Inhibiting Substance (MIS) protein "comprising a combination of a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1, and a modification of at least one amino acid between residues 44 7-451 of SEQ ID NO: 1" and "the HSA leader sequence comprises at least 11 amino acids of SEQ ID NO: 6, or a variant that is at least 80% homologous thereto" and "wherein the non-MIS leader sequence is selected from a group consisting of: immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide (IgSP-tPA), murine immunoglobulin signal peptide (IgSP), a MPIF -1 signal sequence (MKVSV AALSCLML VT ALGSQA (SEQ ID NO: 15); a stanniocalcin signal 14857650.6 -106- Attorney Docket No. 030258-076964-PCT sequence (MLQNSAVLLLLVISASA (SEQ ID NO:16)); an invertase signal sequence (MLLQAFLFLLAGF AAKISA (SEQ ID NO: 17)); a yeast mating factor alpha signal sequence (K. lactis killer toxin leader sequence); a hybrid signal sequence (MKWVSFISLLFLFSSAYSRSLEKR, (SEQ ID NO:18)); a HSA/MFa-1 hybtid signal sequence (MKWVSFISLLFLFSSA YSRSLDKR (SEQ ID NO: 19)); a K. lactis killer/ MFa-1 fusion leader sequence (MNIFYIFLFLLSFVQGSLDKR (SEQ ID NO:20)); an innnunoglobulin Ig signal sequence (MGWSCIILFLVATATGVHS (SEQ ID NO:21)); a Fibulin B precursor signal sequence (MERAAPSRRVPLPLLLLGGLALLAAGVDA (SEQ ID NO:22)); a clusterin precursor signal sequence (MMKTLLLFVGLLLTWESGQVLG (SEQ ID NO: 23)); and the insulin-like growth factor-binding protein 4 signal sequence (MLPLCLVAALLLAAGPGPSLG (SEQ ID NO:24)) or a functional fragment thereof".

The Groups I+ share the technical features of a recombinant Mullerian Inhibiting Substance (MIS) protein, and methods comprising the same, said MIS protein comprising a combination of a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1, and a modification of at least one amino acid between residues 447-451 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification, wherein the recombinant MIS protein has increased cleavage and increased yield of production in vitro as compared to wild-type MIS protein corresponding to amino acid residues of SEQ ID NO: 1, wherein the modification of at least one amino acid between residues 447-451 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification is modification of amino acid 450 of SEQ ID NO: 1 from Q to R; a method for treating a subject with cancer, comprising administering a composition comprising the recombinant MIS protein; a method of decreasing the dose of a chemotherapeutic agent for the treatment of cancer, the method comprising administering to the subject a therapeutically effective amount of the recombinant MIS protein, wherein the therapeutically effective dose of the chemotherapeutic agent in the presence of the recombinant MIS protein is lower as compared to the therapeutically effective dose of the chemotherapeutic agent alone; use of recombinant MIS protein for the manufacture of a medicament for treating cancer, and wherein the cancer expresses a Mullerian Inhibiting Substance (MIS) receptor; a use of a recombinant MIS protein to decrease the plasma serum levels of one or more androgens in a subject in need thereof; a method to decrease the plasma level of one or more androgens in a subject, the method comprising administering an effective amount of the recombinant MIS protein, and wherein the recombinant MIS protein decreases the plasma serum levels of one or more androgens in the subject. However, these shared technical features do not represent a contribution over the prior art.

Specifically, WO 2001/19387 A1 to Donahoe et al. discloses a recombinant Mullerian Inhibiting Substance (MIS) protein (Mullerian Inhibiting Substance (MIS) can be obtained from a mammalian source or through the use of recombinant DNA technology, Pg. 7, Lns. 23-24), and methods comprising the same, said MIS protein comprising a combination of a non-MIS leader sequence or a functional fragment thereof in place of the MIS leader sequence of amino acids 1-25 of SEQ ID NO: 1 (the polynucleotide encoding MIS contains a secretory signal sequence that facilitates secretion of the protein. Typically, the signal sequence is positioned in the coding region of the polynucleotide to be expressed towards or at the 5' end of the coding region. The signal sequence may be homologous or heterologous to the polynucleotide of interest, Pg. 27, Lns. 11-15; the MIS DNA sequences of this invention may be fused in the same reading frame in an expression vector of this invention to at least a portion of a DNA sequence coding for at least one eukaryotic or prokaryotic signal sequence, Pg. 9, Lns. 28-30 and Pg. 10, Ln. 1), and a modification of at least one amino acid to increase cleavage as compared to in the absence of a modification, wherein the recombinant MIS protein has increased cleavage and increased yield of production in vitro as compared to wild-type MIS protein corresponding to amino acid residues of SEQ ID NO: 1 (the methods of the present invention can be practiced using mutant forms of the C-terminal fragment of MIS which have substantially the same biological activity as the C-terminal fragment of MIS. Examples of such mutant forms would be C-terminal fragment of MIS molecules carrying a deletion, insertion, or alteration of amino acid sequence, Pg. 7, Lns. 14-18; improve possible cleavage at amino acid residue 443 to release an active C-terminal fragment, Pg. 10, Lns. 16-17; replacement of one or a combination of codons leading to amino acid replacement ...may alter its properties in a useful way, e.g., increase the stability, increase the solubility or increase the therapeutic activity, Pg. 10, Lns. 24-26); a method for treating a subject with cancer (methods of treating prostate cancer, Pg. 1, Lns. 17-18), comprising administering a composition comprising the recombinant MIS protein (the method comprising administering an effective amount of MIS to a patient, Pg. 2, Lns. 16-18); use of recombinant MIS protein for the manufacture of a medicament for treating cancer, and wherein the cancer expresses a Mullerian Inhibiting Substance (MIS) receptor (methods of treating prostate cancer, Pg. 1, Lns. 17-18; method of treating a condition or disease characterized by an excess of one or more androgens, the method comprising administering an effective amount of MIS to a patient, Pg. 2, Lns. 16-18); a use of a recombinant MIS protein to decrease the plasma serum levels of one or more androgens in a subject in need thereof (a method of decreasing the plasma level of one or more androgens, Pg. 2, Lns. 23-24); a method to decrease the plasma level of one or more androgens in a subject, the method comprising administering an effective amount of the recombinant MIS protein, and wherein the recombinant MIS protein decreases the plasma serum levels of one or more androgens in the subject (a method of decreasing the plasma level of one or more androgens, the method comprising administering to a patient an effective amount of MIS, wherein the amount of MIS is sufficient to decrease the plasma level of the one or more androgens below the normal level for the one or more androgens, Pg. 2, Lns. 23-27).

Further, "Development of an efficiently cleaved, bioactive, highly pure FLAG-tagged recombinant human Mullerian Inhibiting Substance" to Papakostas et al. discloses a recombinant Mullerian Inhibiting Substance (MIS) protein (a recombinant, internally FLAG-tagged form of human Mullerian Inhibiting Substance (hMIS), Abstract) comprising a modification of at least one amino acid between residues 447-451 of SEQ ID NO: 1 to increase cleavage as compared to in the absence of a modification, wherein the recombinant MIS protein has increased cleavage as compared to wild-type MIS protein corresponding to amino acid residues of SEQ ID NO: 1 (we engineered changes to the native human sequence to increase endogenous cleavage, Pg. 33, left-hand column, second full paragraph; construct that is cleaved endogenously in an efficient manner (Q->R427; RARR/SFLAG), Pg. 34, right-hand column, second full paragraph), wherein the modification of at least one amino acid between residues 447-451 of SEQ ID NO: 1 is modification of amino acid 450 of SEQ ID NO: 1 from Q to R (construct had the Q426 mutated to arginine, Pg. 34, right-hand column, second full paragraph; where Q426 corresponds to Q450 of SEQ ID NO:1 of the instant application as amino acid numbering in Papakostas et al. is with signal sequence removed). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the protein of Donahoe et al. to include Q450R mutation as taught by Papakostas et al. as the Q450R mutation resulting in the RARR MIS cleavage sequence was efficiently cleaved, causing complete regression of the Mullerian duct while the wild type RAQR MIS remains uncleaved and inactive (Papakostas et al., see Fig. 3 and caption).

Further still, "Mullerian Inhibiting Substance enhances subclinical doses of chemotherapeutic agents to inhibit human and mouse ovarian cancer" to Pieretti-Vanmarcke et al. discloses a method of decreasing the dose of a chemotherapeutic agent for the treatment of cancer (Mullerian Inhibiting Substance (MIS) and chemotherapeutic drugs can function in rationally selected combinations to achieve better tumor control at decreased doses of either agent, resulting in decreased toxicity, reduced morbidity, and most importantly a wider therapeutic window, Pg. 17427, left-hand column, second full paragraph), the method comprising administering to the subject a therapeutically effective amount of a recombinant MIS protein (MIS is effective against animal models recapitulating human ovarian cancer, with no apparent adverse affects when administered, Pg. 17428, right-hand column, last partial paragraph), wherein the therapeutically effective dose of the chemotherapeutic agent in the presence of the recombinant MIS protein is lower as compared to the therapeutically effective dose of the chemotherapeutic agent alone (MIS and chemotherapeutic drugs can function in rationally selected combinations to achieve better tumor control at decreased doses of either agent, resulting in decreased toxicity, reduced morbidity, and most importantly a wider therapeutic window, Pg. 17427, left-hand column, second full paragraph; allow a lower dose of a cytotoxic agent ...to be used in combination with a nontoxic biological modifier such as MIS, Pg. 17429, left-hand column, second full paragraph). It would have been obvious to one of ordinary skill in the art at the time of the invention to utilize the modified Mullerian Inhibiting Substance (MIS) comprising a non-MIS leader sequence and a modification of at least one amino acid between residues 447-451 of SEQ ID NO: 1 as taught by Donahoe et al. in view of Papakostas et al. in the method of Pieretti-Vanmarcke et al. for decreasing the dose of a chemotherapeutic agent for the treatment of cancer as the modified protein offers increased therapeutic activity over the wild type.

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.

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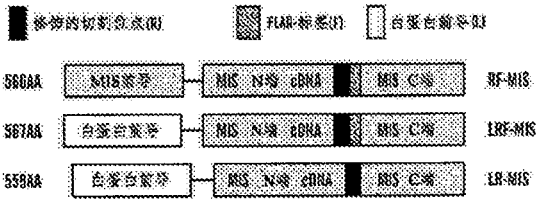
序列表18页 附图6页

(54)发明名称

修饰的缪勒抑制物质(MIS)蛋白及其用于疾病治疗的用途

(57)摘要

本发明涉及修饰的重组人MIS蛋白，所述修饰的重组人MIS蛋白相比野生型人MIS蛋白而言具有改善的切割和增加的生物活性以及增加的效能。本发明的其它方面涉及通过向受试者给予包含重组人MIS蛋白的组合物来预防和治疗癌症(例如，表达II型MIS受体(MISRII)的癌症)的方法。本发明的另一方面涉及降低受试者的血浆雄激素水平的方法和/或用于治疗患有以过多雄激素为特征的疾病的受试者的方法。另一方面提供了含有重组人MIS蛋白的药物组合物和试剂盒以及使用方法。本发明的另一方面涉及通过一起给予化疗剂和所述重组MIS蛋白来减少所述化疗剂的剂量的方法，所述重组MIS蛋白降低了所述化疗剂的有效剂量。



CN 105473154 A

1.一种重组缪勒抑制物质(MIS)蛋白，

所述重组MIS蛋白包含如下方面的组合：代替SEQ ID NO:1的氨基酸1-25的MIS前导序列的非MIS前导序列或其功能片段；以及SEQ ID NO:1的残基447-451之间的至少一个氨基酸的修饰，

从而相比缺乏修饰的情况切割得以增加，

其中，所述重组MIS蛋白相比对应于SEQ ID NO:1的氨基酸残基的野生型MIS蛋白而言具有在体外增加的生产产率和增加的切割。

2.如权利要求1所述的重组MIS蛋白，其中，所述重组MIS蛋白进一步包含标签蛋白。

3.如权利要求1所述的重组MIS蛋白，其中，所述重组MIS蛋白至少含有代替SEQ ID NO:1的氨基酸1-25的MIS前导序列的非MIS前导序列或其功能片段；以及SEQ ID NO:1的残基447-451之间的至少一个氨基酸的修饰，从而相比缺乏修饰的情况切割得以增加。

4.如权利要求1所述的重组MIS蛋白，其中，所述非MIS前导序列为白蛋白前导序列或其功能片段。

5.如权利要求4所述的重组MIS蛋白，其中，所述白蛋白前导序列为人血清白蛋白(HSA)前导序列或其片段。

6.如权利要求5所述的重组MIS蛋白，其中，所述HSA前导序列包含SEQ ID NO:6的氨基酸序列或与其至少80%同源的变体。

7.如权利要求5所述的重组MIS蛋白，其中，所述HSA前导序列的片段包含SEQ ID NO:6的至少10个氨基酸或与其至少80%同源的变体。

8.如权利要求5所述的重组MIS蛋白，其中，所述HSA前导序列包含SEQ ID NO:6的至少15个氨基酸或与其至少80%同源的变体。

9.如权利要求5所述的重组MIS蛋白，其中，所述HSA前导序列包含SEQ ID NO:6的至少11个氨基酸或与其至少80%同源的变体。

10.如权利要求5所述的重组MIS蛋白，其中，所述HSA前导序列的片段选自于由以下序列所组成的组：

MKWVTFISLLFLFSSAYS(SEQ ID NO:13)；

MKWVTFISLLFLFSSAYSRGVFRR(SEQ ID NO:6)；

MKWVSFISLLFLFSSAYS(SEQ ID NO:14)。

11.如权利要求1所述的重组MIS蛋白，其中，所述非MIS前导序列选自于由以下序列所组成的组：

与组织型纤溶酶原激活物前肽融合的免疫球蛋白信号肽(IgSP-tPA)，鼠免疫球蛋白信号肽(IgSP)，MPIF-1信号序列(MKVSVAALSCLMLVTALGSQA(SEQ ID NO:15))；斯钙素信号序列(MLQNSAVLLLLVISASA(SEQ ID NO:16))；转化酶信号序列(MLLQAFLFLLAGFAAKISA(SEQ ID NO:17))；酵母交配因子α信号序列(乳酸克鲁维酵母杀伤毒素前导序列)；杂合信号序列(MKWVSFISLLFLFSSAYSRSLEKR(SEQ ID NO:18))；HSA/MFα-1杂合信号序列(MKWVSFISLLFLFSSAYSRSLDKR(SEQ ID NO:19))；乳酸克鲁维酵母杀伤/MFα-1融合前导序列(MNIFYIFLFLLSFVQGSLDKR(SEQ ID NO:20))；免疫球蛋白Ig信号序列(MGWSCIILFLVATATGVHS(SEQ ID NO:21))；Fibulin B前体信号序列(MERAAPSRRVPLPLLLLGGLALLAAGVDA(SEQ ID NO:22))；簇集蛋白前体信号序列

(MMKTLLLFVGLLLTWESGQVLG(SEQ ID NO:23));以及胰岛素样生长因子结合蛋白4信号序列(MLPLCLVAALLLAAGPGPSLG(SEQ ID NO:24)),或它们的功能片段。

12.如权利要求1所述的重组MIS蛋白,所述重组MIS蛋白包含SEQ ID NO:1的氨基酸449由Q至R的修饰,从而相比缺乏该修饰的情况切割得以增加。

13.如权利要求1所述的重组MIS蛋白,所述重组MIS蛋白进一步包含SEQ ID NO:1的氨基酸451由S至R的修饰,从而相比缺乏该修饰的情况切割得以增加。

14.如权利要求1所述的重组MIS蛋白,其中,所述标签为FLAG标签。

15.如权利要求14所述的重组MIS蛋白,其中,所述FLAG标签包含氨基酸序列DYKDDDDK(SEQ ID NO:8)或其功能衍生物或变体。

16.如权利要求14所述的重组MIS蛋白,其中,所述FLAG标签位于SEQ ID NO:1的氨基酸残基451之后及SEQ ID NO:1的氨基酸残基452之前。

17.如权利要求14所述的重组MIS蛋白,其中,所述FLAG标签位于SEQ ID NO:1的氨基酸残基451和452之间。

18.如权利要求1所述的重组MIS蛋白,所述重组MIS蛋白包含SEQ ID NO:2的氨基酸序列或其功能片段。

19.如权利要求1所述的重组MIS蛋白,所述重组MIS蛋白包含SEQ ID NO:3的氨基酸序列或其功能片段。

20.如权利要求18所述的重组MIS蛋白,所述重组MIS蛋白由SEQ ID NO:4的核酸序列编码。

21.如权利要求19所述的重组MIS蛋白,所述重组MIS蛋白由SEQ ID NO:5的核酸序列编码。

22.一种药物组合物,所述药物组合物含有权利要求1-21中任一项所述的重组MIS蛋白和药学上可接受的载体。

23.编码权利要求1-21中任一项所述的重组MIS蛋白的多核苷酸。

24.如权利要求23所述的多核苷酸,其中,所述核苷酸对应于SEQ ID NO:4或与SEQ ID NO:4的核酸序列具有至少95%的序列一致性的核苷酸。

25.如权利要求23所述的多核苷酸,其中,所述核苷酸对应于SEQ ID NO:5或与SEQ ID NO:5的核酸序列具有至少95%的序列一致性的核苷酸。

26.含有权利要求23-25中任一项所述的多核苷酸的载体。

27.如权利要求26所述的载体,其中,所述载体为病毒载体或表达载体。

28.如权利要求27所述的载体,其中,所述表达载体为用于细菌(例如,大肠杆菌)或噬菌体的pcDNA 3.1或cDNA载体或基因组载体。

29.如权利要求27所述的载体,其中,所述病毒载体选自于由以下病毒载体所组成的组:

腺病毒载体、痘病毒载体以及慢病毒载体。

30.如权利要求26-29中任一项所述的载体,其中,所述核酸序列编码重组MIS蛋白或其片段,所述核酸序列与SEQ ID NO:4或SEQ ID NO:5的核酸序列具有至少95%的序列一致性,其中,所述核酸序列可操作地连接至组织类型特异性启动子或细胞类型特异性启动子。

31.一种由权利要求1所述的重组人MIS蛋白的翻译后加工生产的人MIS蛋白。

32.一种含有权利要求26-30中任一项所述的载体的宿主细胞。

33.一种药物组合物，所述药物组合物含有权利要求26-30中任一项所述的载体和药学上可接受的载体。

34.由权利要求1-21中任一项所述的重组人MIS蛋白生产而来的人MIS蛋白的纯化制剂。

35.一种用于治疗患有癌症的受试者的方法，所述方法包括将含有重组MIS蛋白的组合物进行给药，

其中，所述重组MIS蛋白包含如下方面的组合：代替SEQ ID NO:1的氨基酸1-25的MIS前导序列的非MIS前导序列或其功能片段；以及SEQ ID NO:1的残基447-451之间的至少一个氨基酸的修饰，

从而使得相比缺乏修饰的情况切割有所增加，

其中，相比对应于SEQ ID NO:1的氨基酸残基的野生型MIS蛋白而言，所述重组MIS蛋白具有在体外增加的生产产率以及增加的切割。

36.如权利要求35所述的方法，其中，所述重组MIS蛋白进一步包含标签蛋白。

37.如权利要求35所述的方法，其中，所述重组MIS蛋白至少含有代替SEQ ID NO:1的氨基酸1-25的MIS前导序列的非MIS前导序列或其功能片段；以及SEQ ID NO:1的残基447-451之间的至少一个氨基酸的修饰，从而相比缺乏修饰的情况切割得以增加。

38.如权利要求35所述的方法，其中，所述非MIS前导序列为白蛋白前导序列或其功能片段。

39.如权利要求38所述的方法，其中，所述白蛋白前导序列为人血清白蛋白(HSA)前导序列或其片段。

40.如权利要求35所述的方法，其中，所述重组MIS蛋白包含SEQ ID NO:1的氨基酸449由Q至R的修饰，从而相比缺乏该修饰的情况切割得以增加。

41.如权利要求35所述的方法，其中，所述标签为含有SEQ ID NO:8的氨基酸序列或其功能片段的FLAG标签。

42.如权利要求35所述的方法，其中，所述癌症为MIS响应II型癌症。

43.如权利要求35所述的方法，其中，所述癌症为卵巢癌。

44.如权利要求35所述的方法，其中，所述癌症为化学疗法耐受性癌症或多药物耐药性癌症。

45.如权利要求35所述的方法，其中，所述重组MIS蛋白的给药在给予另外的药剂或癌症疗法之前、期间或之后。

46.如权利要求35所述的方法，其中，所述癌症表达缪勒抑制物质II型受体(MISRII)。

47.如权利要求46所述的方法，其中，在获得自所述受试者的生物样品中对缪勒抑制物质(MIS)受体的表达进行测量。

48.如权利要求47所述的方法，其中，所述生物样品为癌组织样品或肿瘤组织样品，或癌细胞或肿瘤细胞。

49.如权利要求47所述的方法，其中，所述生物样品为活检组织样品。

50.如权利要求35所述的方法，其中，所述癌症为卵巢癌细胞、外阴表皮癌细胞、宫颈癌细胞、子宫内膜腺癌细胞和卵巢腺癌细胞。

51.如权利要求35所述的方法，其中，所述癌症选自于由以下癌症所组成的组：

乳腺癌、肺癌、头颈部癌、膀胱癌、胃癌、神经系统的癌症、骨癌、骨髓癌、脑癌、结肠癌、食管癌、子宫内膜癌、胃肠癌、牙龈癌、肾癌、肝癌、鼻咽癌、卵巢癌、前列腺癌、皮肤癌、胃癌、睾丸癌、舌癌、黑色素瘤、眼黑色素瘤或子宫癌。

52.如权利要求44所述的方法，其中，所述多药物耐药性癌症为紫杉醇耐药性癌症或阿霉素耐药性癌症。

53.如权利要求35所述的方法，其中，所述给药为静脉内给药、真皮内给药、肌内给药、动脉内给药、病灶内给药、经皮给药、或皮下给药、或通过气溶胶给药。

54.如权利要求35所述的方法，其中，所述给药为预防性给药。

55.如权利要求35所述的方法，其中，所述给药为治疗性给药。

56.如权利要求35所述的方法，其中，所述受试者为哺乳动物。

57.如权利要求56所述的方法，其中，所述哺乳动物为人。

58.如权利要求35所述的方法，其中，将至少一种另外的药剂与所述重组人MIS的给药结合(例如，之前、期间或之后)来给予所述受试者。

59.如权利要求58所述的方法，其中，所述另外的药剂为治疗剂或化疗剂。

60.如权利要求59所述的方法，其中，所述化疗剂选自于由以下化疗剂所组成的组：

紫杉醇、顺铂、阿霉素、雷帕霉素、吡唑并蒽酮。

61.如权利要求59所述的方法，其中，所述化疗剂为放疗剂。

62.如权利要求59所述的方法，其中，所述化疗剂为吡唑并蒽酮。

63.如权利要求62所述的方法，其中，所述吡唑并蒽酮为蒽(1,9-cd)吡唑-6(2H)-酮(SP600125)或其功能衍生物或功能类似物。

64.一种减少用于治疗癌症的化疗剂的剂量的方法，所述方法包括给予受试者治疗有效量的重组MIS蛋白，

其中，所述重组MIS蛋白包含如下方面的组合：SEQ ID NO:1的氨基酸449由Q至R的修饰；以及代替SEQ ID NO:1的氨基酸1-25的MIS前导序列的非MIS前导序列或其功能片段，

其中，与单独的所述化疗剂的治疗有效剂量相比，在存在所述重组MIS蛋白的情况下所述化疗剂的治疗有效剂量较低。

65.如权利要求64所述的方法，其中，所述重组MIS蛋白进一步含有标签蛋白。

66.重组MIS蛋白在制备用于治疗癌症的药物中的用途，

其中，所述重组MIS蛋白包含如下方面的组合：SEQ ID NO:1的氨基酸449由Q至R的修饰；以及代替SEQ ID NO:1的氨基酸1-25的MIS前导序列的非MIS前导序列或其功能片段，

并且其中，所述癌症表达缪勒抑制物质(MIS)受体。

67.如权利要求66所述的用途，其中，所述重组MIS蛋白进一步包含标签蛋白。

68.如权利要求66所述的用途，其中，所述缪勒抑制物质(MIS)受体为II型MIS受体或其同源物或功能片段。

69.一种制造品，所述制造品包含包装材料以及药物组合物，所述药物组合物含有权利要求1-21中任一项所述的重组MIS蛋白，其中，所述包装材料包含标示，所述标示表明可将所述药物组合物在足够的期限内以有效的剂量给药，来治疗表达缪勒抑制物质(MIS)受体的癌症或降低其风险。

70.一种治疗患有癌症的受试者的方法，所述方法包括在获得自所述受试者的生物样品中评价缪勒抑制物质II型受体(MISRII)的表达和/或活性，其中，临床医生评审结果，如果结果表明存在MISRII的表达和/或活性，所述临床医生指导所述受试者用权利要求22或权利要求33所述的药物组合物进行治疗。

71.如权利要求70所述的方法，其中，所述生物样品为组织样品。

72.如权利要求71所述的方法，其中，所述组织样品为癌组织样品或肿瘤组织样品，或癌细胞或肿瘤细胞。

73.如权利要求71所述的方法，其中，所述生物样品为活检组织样品。

74.如权利要求70所述的方法，其中，所述癌症为卵巢癌细胞、外阴表皮癌细胞、宫颈癌细胞、子宫内膜腺癌细胞和卵巢腺癌细胞。

75.如权利要求70所述的方法，其中，所述癌症为乳腺癌、肺癌、头颈部癌、膀胱癌、胃癌、神经系统的癌症、骨癌、骨髓癌、脑癌、结肠癌、食管癌、子宫内膜癌、胃肠癌、牙龈癌、肾癌、肝癌、鼻咽癌、卵巢癌、前列腺癌、皮肤癌、胃癌、睾丸癌、舌癌、黑色素瘤、眼黑色素瘤或子宫癌。

76.重组MIS蛋白在减少有需要的受试者中的一种或多种雄激素的血浆血清水平中的用途，

其中，所述重组MIS蛋白包含如下方面的组合：SEQ ID NO:1的氨基酸449由Q至R的修饰；以及代替SEQ ID NO:1的氨基酸1-25的MIS前导序列的非MIS前导序列或其功能片段。

77.如权利要求76所述的用途，其中，所述重组MIS蛋白进一步包含标签蛋白。

78.如权利要求76所述的用途，其中，所述一种或多种雄激素为睾酮。

79.如权利要求76所述的用途，其中，所述有需要的受试者患有良性前列腺肥大。

80.如权利要求76所述的用途，其中，所述有需要的受试者患有前列腺癌。

81.如权利要求76所述的用途，其中，所述有需要的受试者患有多囊卵巢疾病和/或性早熟。

82.如权利要求76所述的用途，其中，所述有需要的受试者患有选自于由以下疾病或紊乱所组成的组中的疾病或紊乱：

良性前列腺增生(BPH)、前列腺癌、睾丸癌、雄激素依赖性痤疮、男性型脱发、性早熟、雄激素过多症、多毛症、男性化、多囊卵巢综合征(POCS)、雄激素过多症(HA)-胰岛素抵抗(IR)-黑棘皮病(AN)(HIAR-AN)综合征、卵巢滤囊泡膜细胞增殖、滤泡成熟停止、闭锁、不排卵、痛经、功能失调性子宫出血、不育症和产雄激素肿瘤。

83.一种治疗以雄激素依赖为特征的疾病或紊乱的方法，所述方法包括给予受试者有效量的权利要求22或权利要求33所述的药物组合物，其中，所述药物组合物使所述受试者的血浆血清中至少一种雄激素的水平降低，并使所述以雄激素依赖为特征的疾病或紊乱的至少一种症状减少。

84.一种减少受试者中一种或多种雄激素的血浆水平的方法，所述方法包括给予有效量的重组MIS蛋白，

其中，所述重组MIS蛋白包含如下方面的组合：SEQ ID NO:1的氨基酸449由Q至R的修饰；以及代替SEQ ID NO:1的氨基酸1-25的MIS前导序列的非MIS前导序列或其功能片段，

以及其中，所述重组MIS蛋白使所述受试者中一种或多种雄激素的血浆血清水平减少。

85.如权利要求84所述的方法，其中，所述重组MIS蛋白进一步包含标签蛋白。

86.如权利要求84所述的方法，其中，所述受试者患有以雄激素依赖为特征的疾病或紊乱。

87.如权利要求84-86中任一项所述的方法，其中，所述疾病或紊乱选自于由以下疾病或紊乱所组成的组：

良性前列腺增生(BPH)、前列腺癌、睾丸癌、雄激素依赖性痤疮、男性型脱发、性早熟、雄激素过多症、多毛症、男性化、多囊卵巢综合征(POCS)、雄激素过多症(HA)-胰岛素抵抗(IR)-黑棘皮病(AN)(HIAR-AN)综合征、卵巢滤囊泡膜细胞增殖、滤泡成熟停止、闭锁、不排卵、痛经、功能失调性子宫出血、不育症和产雄激素肿瘤。

88.含有权利要求1-21中任一项所述的重组MIS蛋白和药学上可接受的载体的试剂盒。

89.如权利要求88所述的试剂盒，所述试剂盒任选进一步含有使用所述重组MIS蛋白来治疗癌症或治疗雄激素依赖性紊乱的说明书。

修饰的缪勒抑制物质(MIS)蛋白及其用于疾病治疗的用途

[0001] 相关申请的交叉引用

[0002] 根据35 U.S.C.§119(e),本申请要求2013年3月12日提交的美国临时申请号61/777,135的优先权,以引用的方式将其内容整体并入本文。

[0003] 序列表

[0004] 本申请包含序列表,所述序列表已以ASCII格式电子提交,在此以引用的方式将其整体并入。所述ASCII副本(2014年3月12日创建)被命名为030258-076964-PCT_SL.txt,大小为28,114字节。

技术领域

[0005] 本发明涉及修饰的重组人MIS蛋白,所述修饰的重组人MIS蛋白相比野生型人MIS蛋白而言具有改善的切割、增加的生物活性以及增加的效能(potency)。在一些方面,所述重组人MIS蛋白包含下列中的至少一种:修饰的Kex切割位点(用于增加的切割)、FLAG标签、以及代替正常的MIS前导序列的非MIS前导序列。本发明的其它方面涉及包含重组人MIS蛋白的方法、用途和试剂盒,用于治疗癌症(例如,表达II型MIS受体(MISRII)的癌症)或者用于治疗以过多雄激素为特征的疾病。

[0006] 政府支持

[0007] 本发明是在美国国立卫生研究院(National Institutes of Health,NIH)授予的基金号CA17393的政府支持下做出的。美国政府对本发明享有一定的权利。

背景技术

[0008] 缪勒抑制物质(Mullerian Inhibiting Substance,MIS)也称为抗缪勒激素(anti-Mullerian hormone,AMH),是糖蛋白的大转化生长因子β(TGFβ)多基因家族的140-kDa的二硫键连接的同二聚体糖蛋白成员。该基因家族的蛋白都被生产为二聚体前体,并经过翻译后加工以活化,需要切割和解离来释放生物活性C端片段。类似地,MIS的140千道尔顿(kDa)的二硫键连接的同二聚体被蛋白水解切割,从而生成它的活性C端片段。

[0009] 人MIS基因位于19号染色体上,该基因的表达是性二型的(sexually dimorphic)。在雄性中,MIS表达始于妊娠9周的胎儿睾丸,并以高水平一直持续到青春期,彼时表达水平大幅下降。在雌性中,MIS仅在出生后在颗粒细胞(granulosa cells)中从青春期前期到更年期以与成年雄性类似的水平生产,此后表达停止。在雄性胎儿中,MIS使缪勒管(输卵管、子宫、宫颈和上三分之一阴道的前体)退化。

[0010] MIS在结合至I型和II型单跨膜丝氨酸苏氨酸激酶受体的异二聚体后发挥其生物效应,引起I型受体的GS盒激酶结构域(GS box kinase domain)被II型受体交叉磷酸化。随后,SMAD 1、SMAD 5和SMAD 8(但主要是SMAD 8)被活化,并与SMAD 4一起调控基因转录。在小鼠、大鼠和兔中仅鉴定出一个II型MIS受体(MISRII)基因,其中,在人中它的基因定位于12号染色体。它为65-kDa蛋白,已在胚胎和成年缪勒结构、乳腺组织、前列腺组织、生殖腺、运动神经元和脑中检测到。在胎儿中,在覆盖尿生殖嵴(urogenital ridge)的体腔上皮

(coelomic epithelium)中表达MISRII的mesoepithelial细胞迁移入并成为缪勒管上皮周围的间充质细胞的一部分。还在生殖腺以及卵巢体腔上皮中检测到表达。已在哺乳动物中鉴定了I型MIS受体,取决于动物种类和所检查的组织,最可能的候选物是激活素(activin)受体样激酶(ALK)2和3。

[0011] 除了在缪勒管退化方面的确定作用外,MIS在体外和体内抑制各种人癌细胞系的增殖。显示出抑制的细胞系来源于卵巢癌、宫颈癌、子宫内膜癌、前列腺癌和乳腺癌。即便是在具有分泌MIS的肿瘤的啮齿动物或人患者中长时间全身性地保持高浓度的MIS,在体内也未观测到毒性。相对受限的受体表达、针对表达MISRI和MISRII的癌细胞的抗增殖活性以及它的明显无毒性,这些发现合起来使MIS成为与现有的用于治疗卵巢癌(已知对这些常规药剂产生抗性)的化疗药物结合的理想试剂。

[0012] MIS通过II型MIS受体细胞起作用,以用作卵巢癌发生的有效肿瘤阻遏物(suppressor)(Teixeira等,未公开)。作为受体介导的事件,MIS还可以靶向卵巢癌细胞系的干细胞/祖细胞群(Meirelles等,2012;Wei等,2010)。MIS可用于治疗例如表达MISRII的癌症。MISRII在大多数上皮性卵巢癌中表达(Masiakos等,1999;Bakkum-Gamez等,2008;Song等,2009)。

[0013] MIS还在体内和体外抑制多种癌症的发展,在体内长期治疗后无明显毒性(Pieretti-Vanmarcke等,2006b)。上皮性卵巢癌概括了胚胎缪勒管及其各种亚型的原始组织学(Scully 1977);例如,浆液性囊腺癌(serous cystadenocarcinoma)类似于胚胎输卵管,子宫内膜样癌类似于子宫内膜,以及粘液癌类似于宫颈。另外,MIS与常用的抗癌药物协同作用或叠加作用,来控制肿瘤生长(Pieretti-Vanmarcke等,2006a)。

[0014] 以前已报道了为卵巢癌干细胞选择化疗药剂,卵巢癌干细胞通常是多药耐药性的和/或耐化疗剂的。特别是,越来越多的研究报道了,卵巢癌和细胞系是异质的,具有耐化疗药物但仍响应于MIS的卵巢癌干细胞群。MIS特别地靶向卵巢癌侧群细胞(side population cells)以及具有干细胞/祖细胞特性的CD44+、CD24+、EpCam+和E钙粘蛋白阴性细胞的群(对目前用于卵巢癌的临床用途的化疗剂响应不佳)(Wei等,2010)。特别是,已证明MIS在体内和体外均抑制卵巢癌细胞,并可特异性地靶向和抑制由CD44+、CD24+、Ep-CAM+和E钙粘蛋白细胞表面标志物富集的卵巢癌祖细胞群的生长。为了实现MIS在卵巢癌患者中的临床测试,必须优化重组人MIS的生产,以增加产率和纯度。

[0015] 然而,由纯化天然MIS或野生型MIS进行制备很复杂,且产率低。此外,生产MIS的活性片段所需的切割也很低效。人MIS蛋白由前原蛋白(pre-proprotein)生产,该前原蛋白含有前导序列。前导序列(SEQ ID NO:1的氨基酸1-25)被切除,剩下的前蛋白(preprotein)(通常被称为"完整人MIS(holo-human MIS)")必须进行翻译后切割,从而产生N端结构域和C端结构域。这些共价连接的N端结构域和C端结构域形成单体,两个相同的单体(含有N端结构域和C端结构域)一起形成并产生同二聚体。完整人MIS最有可能被弗林蛋白酶(furin)或相关激素原转化酶(prohormone convertase)PC5(表达于生殖腺)切割为它的N端结构域和C端结构域。切割主要发生在以+1位点具有丝氨酸的$R^{-4}XXR^{-1}$为特征的kex样位点,这使得MIS切割位点是单碱性的(monobasic)。纯化的C端结构域是生物活性部分,并且切割对生物活性而言是必需的。在残基229-230(对应于SEQ ID NO:1的氨基酸残基254-255)处的第二切割位点(其意义未知)较少被观察到。MIS的非可切割突变体不具有生物活性,人基因中截

短羧基端结构域的突变导致持续性缪勒管综合征(persistent Mullerian duct syndrome)。氨基端结构域在体内的作用可能为协助蛋白质折叠以及促进将C端肽递送至它的受体。在一项研究(Cate,Pepinsky等)中,已证明添加N端肽在体外使C端部分的生物活性增强,但机制还不清楚。由CHO细胞表达的重组MIS的切割不完全,因此需要用外源丝氨酸蛋白酶(例如纤溶酶)进行切割来提高生物活性。

[0016] 因此,需要更有效的方法来产生高浓度的人MIS蛋白以用作治疗性生物药剂。

发明内容

[0017] 本发明涉及修饰的重组人MIS蛋白,所述修饰的重组人MIS蛋白相比野生型人MIS蛋白而言具有改善的切割、增加的生物活性以及增加的效能,其中,所述重组人MIS蛋白包含以下的组合:修饰的Kex切割位点(用于增加的切割)以及代替正常的MIS前导序列的非MIS前导序列,以改善生物活性蛋白的产率,所述生物活性蛋白具有或不具有促进其纯化的内部标记或标签。

[0018] 因此,在本文中,发明人对天然人MIS氨基酸序列进行了工程化改变,从而得出以下的组合:(i)修饰主要切割位点,以增加切割并从而增加MIS(未插入用来促进其纯化的标签)的效能和生物活性;以及(ii)修饰MIS的内源前导序列,以增加生物活性蛋白的产率。令人惊奇的是,前导序列的添加与修饰的主要切割位点相结合使得所生产的蛋白的产率以及来自重组MIS蛋白的主要切割位点的切割的量二者均大幅增加。此外,对于拥有一种标记用于受体和其它结合研究(这对于选择用于治疗的患者以及解决各种受体携带组织中关于MIS的相互作用的分子机制问题二者都非常重要)的生物活性MIS而言,存在尚未满足的需求。另外,标记的配体对于确定在多种组织中是否存在另一受体或其它结合蛋白将是至关重要的。在本文中,发明人在缪勒管退化分析中证明生成了保留完全生物活性的带内部表位标签的MIS。在一个实施方式中,标签为"FLAG"标签,这是由于可得到用于该标签的检测和纯化的高质量试剂。

[0019] 在本文中,发明人证明了,当在CHO细胞中生成时,MIS前导序列置换为人血清白蛋白(HSA)的前导序列并结合将主要内源切割位点由RAQR/S(SEQ ID NO:26)修饰为RARR/S(SEQ ID NO:27)导致更高的表达、增加的C端切割以及不需要的隐秘(cryptic)内部切割的减少。含有这些变化的纯化的MIS保留了它在胎儿大鼠胚胎尿生殖嵴分析中诱导缪勒管退化的能力,并显示出增加的效能。

[0020] 在另一实施方式中,在N端结构域的羰基末端用更有效的切割位点来对重组人MIS进行工程化,由此消除了对外源切割的需要。这种重组MIS蛋白(不具有用于鉴定的标签)可用作治疗性分子和探针分子。

[0021] 重要的是,用另一前导序列(例如,人血清白蛋白(HSA)前导序列)对内源前导序列进行的改变使MIS蛋白的生产增加。令人惊奇的是,发明人证明了前导序列和修饰的切割位点的结合使来自于主要切割位点的切割从37%增加至超过80%,这是出乎预料的,因为蛋白产率的增加一般与降低的翻译后加工(包括切割)关联(这是由于增加的蛋白生产通常使可用的切割酶或内源切割酶饱和)。

[0022] 因此,本发明涉及使用重组人MIS蛋白(例如,多肽和/或编码重组人MIS蛋白的核酸)或其功能片段或衍生物或变体来治疗癌症(例如,表达II型MIS受体(MISRII)的癌症)的

方法。

[0023] 因此，本发明的一个方面涉及重组缪勒抑制物质(MIS)蛋白，所述重组缪勒抑制物质蛋白包含如下方面的组合：代替MIS前导序列(SEQ ID NO:1的氨基酸1-25)的非MIS前导序列或其功能片段；以及SEQ ID NO:1的残基448-452之间的至少一个氨基酸的修饰，从而相比缺乏该修饰的情况切割得以增加，其中，所述重组MIS蛋白相比对应于SEQ ID NO:1的氨基酸残基的野生型MIS蛋白而言具有在体外增加的生产产率和增加的切割。在一些实施方式中，所述重组MIS蛋白缺乏前导序列。在这些实施方式中，所述重组MIS蛋白可由含有代替MIS前导序列(SEQ ID NO:1的氨基酸1-25)的非MIS前导序列或其功能片段的前原蛋白生成，其中，所述前导序列在生产期间被切除。在一些实施方式中，所述重组MIS蛋白进一步包含标签蛋白。

[0024] 在一些实施方式中，非MIS前导序列为白蛋白前导序列或其功能片段，例如，人血清白蛋白(HSA)前导序列或其片段。在一些实施方式中，HSA前导序列包含SEQ ID NO:6的氨基酸序列或与其至少80％同源的变体、或功能片段(例如，包含SEQ ID NO:6的至少10个氨基酸、或至少约11个氨基酸、或至少15个氨基酸的HSA序列的片段)或与其至少80％同源的变体。在一些实施方式中，HSA前导序列的片段选自于由以下序列所组成的组：MKWVTFISLLFLFSSAYS(SEQ ID NO:13)、MKWVTFISLLFLFSSAYSRGVFRR(SEQ ID NO:6)、MKWVSFISLLFLFSSAYS(SEQ ID NO:14)。

[0025] 在一些实施方式中，非MIS前导序列选自于由以下序列所组成的组：与组织型纤溶酶原激活物前肽(propeptide)融合的免疫球蛋白信号肽(IgSP-tPA)，鼠免疫球蛋白信号肽(IgSP)，MPIF-1信号序列(MKVSVAALSCLMLVTALGSQA(SEQ ID NO:15))；斯钙素(stanniocalcin)信号序列(MLQNSAVLLLLVISASA(SEQ ID NO:16))；转化酶(invertase)信号序列(MLLQAFLFLLAGFAAKISA(SEQ ID NO:17))；酵母交配因子α信号序列(乳酸克鲁维酵母杀伤毒素(K.lactis killer toxin)前导序列)；杂合(hybrid)信号序列(MKWVSFISLLFLFSSAYSRSLEKR(SEQ ID NO:18))；HSA/MFα-1杂合信号序列(MKWVSFISLLFLFSSAYSRSLDKR(SEQ ID NO:19))；乳酸克鲁维酵母杀伤/MFα-1融合前导序列(MNIFYIFLFLLSFVQGSLDKR(SEQ ID NO:20))；免疫球蛋白Ig信号序列(MGWSCIILFLVATATGVHS(SEQ ID NO:21))；Fibulin B前体信号序列(MERAAPSRRVPLPLLLLGGLALLAAGVDA(SEQ ID NO:22))；簇集蛋白(clusterin)前体信号序列(MMKTLLLFVGLLLTWESGQVLG(SEQ ID NO:23))；以及胰岛素样生长因子结合蛋白4信号序列(MLPLCLVAALLLAAGPGPSLG(SEQ ID NO:24))，或它们的功能片段。

[0026] 在一些实施方式中，SEQ ID NO:1的氨基酸450由Q至R的修饰使得MIS中来自主要切割位点的切割相比缺乏此类修饰的切割量而言有所增加。在一些实施方式中，重组MIS进一步包含SEQ ID NO:1的氨基酸452由S至R的修饰，从而使得与缺乏此类修饰的情况相比切割增加。

[0027] 在一些实施方式中，本文公开的重组MIS蛋白包含标签，所述标签为FLAG标签(例如，氨基酸序列DYKDDDDK(SEQ ID NO:8))、或其功能衍生物或变体。在一些实施方式中，标签(例如，FLAG标签)位于SEQ ID NO:1的氨基酸残基452之后及SEQ ID NO:1的氨基酸残基453之前。在一些实施方式中，标签(例如，Flag标签)的位置在SEQ ID NO:1的氨基酸残基452和453之间。在一些实施方式中，标签位于MIS C端结构域的N端。在一些实施方式中，标

签的长度不长于50个氨基酸，例如，不长于约50个氨基酸、或约40个氨基酸、或约30个氨基酸、或约20个氨基酸、或约10个氨基酸、或者长度不长于约7个氨基酸。

[0028] 在一些实施方式中，本文所述的重组MIS蛋白包含SEQ ID NO:2或SEQ ID NO:3的氨基酸序列、或它们的功能片段，所述氨基酸序列可分别由核酸序列SEQ ID NO:4和SEQ ID NO:5编码。

[0029] 本发明的另一方面涉及含有本文所述的重组MIS蛋白和药学上可接受的载体的药物组合物。

[0030] 本发明的另一方面涉及编码本文所述的重组MIS蛋白的多核苷酸，例如，其中，所述多核苷酸对应于SEQ ID NO:4或SEQ ID NO:5、或者分别与SEQ ID NO:4或SEQ ID NO:5的核酸序列具有至少95％的序列一致性的核苷酸。本文所述的技术的另一方面涉及含有SEQ ID NO:4或SEQ ID NO:5的多核苷酸、或者分别与SEQ ID NO:4或SEQ ID NO:5的核酸序列具有至少95％的序列一致性的核苷酸的载体。在一些实施方式中，载体为病毒载体或表达载体(例如，pcDNA 3.1)或用于大肠杆菌或噬菌体的其它载体。在一些实施方式中，病毒载体选自于由以下病毒载体所组成的组：腺病毒载体、痘病毒载体以及慢病毒载体。在一些实施方式中，病毒载体为腺相关病毒(AAV)，例如，重组AAV血清型9(rAAV9)。

[0031] 在一些实施方式中，载体含有编码重组MIS蛋白或其片段的核酸序列，所述核酸序列与SEQ ID NO:4或SEQ ID NO:5的核酸序列具有至少95％的序列一致性，其中，所述核酸序列可操作地连接至组织类型或细胞类型特异性启动子。在一些实施方式中，含有此类载体的宿主细胞也涵盖在本发明中。

[0032] 在一些实施方式中，含有本文所述的多核苷酸的载体可在期望的时间段内以恒定的水平表达重组MIS蛋白。

[0033] 本发明的另一方面涉及由本文所述的重组人MIS蛋白的翻译后加工生产的人MIS蛋白。

[0034] 本文所述的技术的另一方面涉及含有本文所述的载体以及药学上可接受的载体的药物组合物。本文所述的技术的另一方面涉及由本文所述的重组人MIS蛋白生产而来的纯化制剂或基本上纯的人MIS蛋白。

[0035] 本文所述的技术的另一方面涉及用于治疗患有癌症的受试者的方法，所述方法包括给予含有重组MIS蛋白的组合物，其中，所述重组MIS蛋白在SEQ ID NO:1的残基448-452之间含有至少一个氨基酸的修饰，从而使得与缺乏修饰的情况相比切割有所增加，所述重组MIS蛋白具有或不具有内部标签蛋白。在一些实施方式中，相比对应于SEQ ID NO:1的氨基酸残基的野生型MIS蛋白而言，所述重组MIS蛋白具有在体外增加的生产产率以及增加的切割。

[0036] 在一些实施方式中，本文所述的重组人MIS蛋白(例如，多肽和/或编码重组人MIS蛋白的核酸)、或者其功能片段或衍生物或变体可用于治疗癌症。在一些实施方式中，可用于癌症治疗的重组人MIS蛋白包含SEQ ID NO:2的氨基酸残基25-559或其功能片段。在一些实施方式中，可用于癌症治疗的重组人MIS蛋白包含SEQ ID NO:3的氨基酸残基25-567或其功能片段。在一些实施方式中，癌症为MIS响应II型(MIS Responsive II，MISRII)癌症，或者，其中癌症表达MISRII(例如，卵巢癌)，或者含有卵巢癌细胞、外阴表皮癌细胞、宫颈癌细胞、子宫内膜腺癌(edenocarcinoma)细胞以及卵巢腺癌。在一些实施方式中，癌症包括但不

限于以下癌症中的任一种:乳腺癌、肺癌、头颈部癌、膀胱癌、胃癌、神经系统的癌症、骨癌、骨髓癌、脑癌、结肠癌、食管癌、子宫内膜癌、胃肠癌、牙龈癌(gum cancer)、肾癌、肝癌、鼻咽癌、卵巢癌、前列腺癌、胰腺癌、皮肤癌、胃癌、睾丸癌、舌癌、黑色素瘤、眼黑色素瘤或子宫癌。

[0037] 在本文所述的方法的一些实施方式中,重组MIS蛋白的给药在给予另外的药剂或癌症疗法之前、期间或之后。

[0038] 在一些实施方式中,在获得自受试者的生物样品中对缪勒抑制物质(MIS)受体的表达进行测量,所述生物样品例如为癌组织或肿瘤组织样品或癌细胞或肿瘤细胞,例如,活检(biopsy)组织样品。

[0039] 在一些实施方式中,癌症为化学疗法耐受性癌症或多药物耐药性癌症,例如,其中所述癌症为紫杉醇耐药性癌症、顺铂耐药性癌症、雷帕霉素耐药性癌症、吡唑并蒽酮(pyrazoloanthrone)耐药性癌症、或阿霉素耐药性癌症。

[0040] 在一些实施方式中,重组MIS可通过任何途径给药,例如,经由静脉内给药、真皮内给药、肌内给药、动脉内给药、病灶内给药、经皮给药、或皮下给药、或通过气溶胶给药。在一些实施方式中,给药为治疗性给药或预防性给药。在如本文所述的所有方面中,受试者为哺乳动物,例如,人。

[0041] 在一些实施方式中,将至少一种另外的药剂与重组人MIS的给药结合(例如,之前、期间或之后)来给予受试者,所述药剂例如为治疗剂或化疗剂,例如,化疗剂选自于由以下药剂所组成的组:紫杉醇、顺铂、阿霉素、雷帕霉素、吡唑并蒽酮[包括但不限于蒽(1,9-cd)吡唑-6(2H)-酮(antra(1,9-cd)pyrazol-6(2H)-one,SP600125)或N1-甲基-1,9-吡唑并蒽酮(M-SP600125)],或它们的功能衍生物或功能类似物。在一些实施方式中,化疗剂为放疗剂。

[0042] 本文所述的技术的另一方面涉及减少用于治疗癌症的化疗剂的剂量的方法,所述方法包括给予受试者治疗有效量的重组MIS蛋白,其中,所述重组MIS蛋白包含SEQ ID NO:1的氨基酸450由Q至R的修饰,其中,与单独的所述化疗剂的治疗有效剂量相比,在存在所述重组MIS蛋白的情况下所述化疗剂的治疗有效剂量较低。在一些实施方式中,重组MIS蛋白任选地包含标签蛋白。

[0043] 本文所述的技术的其它方面涉及重组MIS蛋白在制备用于治疗癌症的药物中的用途,其中,所述重组MIS蛋白包含SEQ ID NO:1的氨基酸450由Q至R的修饰,并且其中,所述癌症表达缪勒抑制物质(MIS)受体。

[0044] 本发明的另一方面涉及制造品,所述制造品包含包装材料以及含有本文所述的重组MIS蛋白的药物组合物,其中,所述包装材料包含标示(label),所述标示表明可将所述药物组合物在足够的期限内以有效的剂量给药,来治疗表达缪勒抑制物质(MIS)受体的癌症或降低其风险。

[0045] 本文所述的技术的其它方面涉及治疗患有癌症的受试者的方法,所述方法包括在获得自所述受试者的生物样品中评价缪勒抑制物质II型受体(MISRII)的表达和/或活性,其中,临床医生评审结果,如果结果表明存在MISRII的表达和/或活性,所述临床医生指导所述受试者用含有本文所述重组MIS蛋白的药物组合物进行治疗。

[0046] 本文所述的技术的其它方面涉及重组MIS蛋白在减少有需要的受试者中的一种或

多种雄激素的血浆血清水平中的用途，其中，所述重组MIS蛋白含有SEQ ID NO:1的氨基酸450由Q至R的修饰以及任选地含有标签(例如，Flag标签)。在一些实施方式中，可用于减少雄激素水平的重组人MIS蛋白包含SEQ ID NO:2的氨基酸残基25-559或其功能片段。

[0047] 在一些实施方式中，一种或多种雄激素为睾酮。在一些实施方式中，有需要的受试者具有良性前列腺肥大(benign prostatic hypertrophy)、或前列腺癌、或多囊卵巢疾病、和/或性早熟。在替代的实施方式中，有需要的受试者具有选自于由以下疾病或紊乱所组成的组中的疾病或紊乱：包括但不限于良性前列腺增生(Benign Prostatic Hyperplasia，BPH)、前列腺癌症(prostate carcinoma)、睾丸癌(testicular cancer)、雄激素依赖性痤疮、男性型脱发、性早熟、雄激素过多症、多毛症、男性化(virilization)、多囊卵巢综合征(POCS)、雄激素过多症(HA)-胰岛素抵抗(IR)-黑棘皮病(AN)(HIAR-AN)综合征、卵巢滤囊泡膜细胞增殖(ovarian hyperthecosis)、滤泡成熟停止(follicular maturation arrest)、闭锁(atresia)、不排卵、痛经、功能失调性子宫出血、不育症以及产雄激素肿瘤(androgen-producing tumors)。

[0048] 本文所述的技术的其它方面涉及治疗以雄激素依赖为特征的疾病或紊乱的方法，所述方法包括给予受试者有效量的含有本文所述的重组MIS蛋白的药物组合物、或含有由本文所述的重组MIS蛋白的切割而来的MIS蛋白的制剂的药物组合物，其中，所述药物组合物降低所述受试者的血浆血清中至少一种雄激素的水平，并使得以雄激素依赖为特征的疾病或紊乱的至少一种症状得以减少。

[0049] 本文所述的技术的其它方面涉及减少受试者中一种或多种雄激素的血浆水平的方法，所述方法包括给予有效量的重组MIS蛋白，其中，所述重组MIS蛋白包含SEQ ID NO:1的氨基酸450由Q至R的修饰，其中，所述重组MIS任选地包含标签，以及其中，所述重组MIS蛋白使所述受试者中一种或多种雄激素的血浆血清水平减少。

[0050] 在一些实施方式中，受试者具有以雄激素依赖为特征的疾病或紊乱，所述疾病或紊乱例如为但不限于选自于以下疾病或紊乱的组中的疾病或紊乱：良性前列腺增生(BPH)、前列腺癌、睾丸癌、雄激素依赖性痤疮、男性型脱发、性早熟、雄激素过多症、多毛症、男性化、多囊卵巢综合征(POCS)、雄激素过多症(HA)-胰岛素抵抗(IR)-黑棘皮病(AN)(HIAR-AN)综合征、卵巢滤囊泡膜细胞增殖、滤泡成熟停止、闭锁、不排卵、痛经、功能失调性子宫出血、不育症以及产雄激素肿瘤。

[0051] 本文所述的技术的其它方面涉及含有本文所述的重组MIS蛋白或由本文所述的重组MIS蛋白的翻译后加工生产的MIS蛋白的制剂、以及药学上可接受的载体的试剂盒。在一些实施方式中，试剂盒可任选地含有使用重组MIS蛋白治疗癌症或治疗雄激素依赖紊乱的说明书。

附图说明

[0052] 图1A-图1B为显示具有白蛋白前导序列的新型重组MIS构建体的设计的示意图。图1A示出了MIS的前导序列(25个氨基酸)与白蛋白的前导序列(24个氨基酸)具有20％的一致性和5个保守氨基酸。图1B为示出了RF(修饰的切割位点加Flag标签)、LRF(前导序列加修饰的切割位点加Flag标签)以及LR(前导序列加修饰的切割位点)构建体的设计的示意图，包括flag标签(F)、修饰的切割位点(R)和白蛋白前导序列(L)的放置。

[0053] 图2示出了用重组人LR-MIS和LRF-MIS构建体稳定转染的CHOK1克隆中的MIS生产和切割。使用针对MIS C端的抗MIS山羊多克隆抗体(1:200)对培养72小时后的培养基上清液进行4%还原性SDS凝胶的蛋白质印迹。纯化的RF-MIS、CHO93培养基和B9培养基作为阳性对照示出。

[0054] 图3A-图3B示出了由还原性SDS凝胶的蛋白质印迹分析的纯化的重组MIS，以评价切割的量。图3A示出了使用针对N端的抗体(该抗体能够识别完整MIS单体、切割的N端、以及含有一部分N端的隐秘切割产物)对纯化的重组RF-MIS、LRF-MIS和WT-MIS进行的比较。图3B示出了使用针对C端的抗体(该抗体能够识别完整MIS单体、切割的C端、以及含有一部分C端的隐秘切割产物)对纯化的重组RF-MIS、LRF-MIS和WT-MIS进行的检测。

[0055] 图4A-图4B示出了缪勒管退化生物分析中5μg/ml(35μM)WT、RF和LRF重组MIS的比较。将重组人MIS产物与胎儿大鼠尿生殖脊孵育72小时。图4A示出了对来自处理过的脊和未经处理的对侧对照脊的代表性部分就缪勒管退化进行的比较。图4B为显示图4A中那些计分的频率分布的柱状图(LRF-MIS N=6,RF-MIS N=39)。W,沃尔弗管(Wolffian duct);M,缪勒管。

[0056] 图5A-图5B示出了野生型MIS蛋白的氨基酸(SEQ ID NO:1)，相应氨基酸残基使用氨基酸标记的传统命名法(其中，第一个编号的氨基酸开始于前导序列之后)。图5A示出了野生型MIS蛋白的氨基酸序列SEQ ID NO:1，突出显示了前导序列(黑体)以及主要切割位点和次要切割位点。使用传统编号作出的相应氨基酸编号显示在括号中。图5B示出了说明SEQ ID NO:1的氨基酸残基上的特征的表格，这些氨基酸残基与使用标准命名法的MIS的氨基酸残基对应(其中，第一个编号的氨基酸开始于前导序列之后)。图5B公开了"RAQR/S"作为SEQ ID NO:26。

具体实施方式

[0057] 本发明涉及修饰的重组人MIS蛋白，所述修饰的重组人MIS蛋白具有以下特征中的至少一种：改善的切割、增加的生物活性、增加的效能，并且，相比野生型人MIS蛋白，所述修饰的重组人MIS蛋白可以高产率地生产，其中，所述重组人MIS蛋白含有以下的组合：修饰的Kex切割位点(用于增加的切割)以及代替正常的MIS前导序列的非MIS前导序列，以改善生物活性蛋白的产率。在一些实施方式中，这一修饰的MIS具有或不具有促进其纯化的内部标记或标签。

[0058] 因此，在本文中，发明人对天然人序列进行了工程化改变来增加内源切割，并由此使得MIS的效能增加。发明人还任选地插入了标签以促进其纯化。

[0059] 发明人还另外将重组人MIS蛋白修饰成包含非MIS前导序列、而不是SEQ ID NO:1的氨基酸1-25的25个氨基酸的MIS前导序列。在一些实施方式中，前导序列包含白蛋白前导序列，例如，人血清白蛋白序列(HSA)或其功能片段或变体。在一些实施方式中，前导序列含有SEQ ID NO:6的24个氨基酸或其功能片段，并替代SEQ ID NO:1的氨基酸残基1-25。令人惊奇的是，这一添加进一步增加了重组MIS蛋白的切割。这一结合产生了较高的产物产率，该产物更均质且由于切割增加而具有增加的效能。这种变化的组合生成了能够满足如下先前尚未满足的需要的重组人MIS变体：拥有一种标记用于受体和其它结合研究(这对于选择用于治疗的患者以及解决各种受体携带组织中关于MIS的相互作用的分子机制问题二者都

非常重要)的生物活性MIS。此外,标记的配体对于确定在多种组织中是否存在另一受体或其它结合蛋白将是至关重要的。在本文中,发明人在缪勒管退化分析中证明生成了保留完全生物活性的带内部表位标签的MIS。在一个实施方式中,标签为"FLAG"标签,这是由于可得到用于该标签的检测和纯化的高质量试剂。

[0060] 如本文中所讨论的,本发明提供了通过向有需要的受试者给予有效量的本文所公开的重组人MIS蛋白及其功能片段和衍生物来治疗多种病症的方法。可由本发明的化合物或含有该化合物的药物组合物治疗的病症包括通过给予人MIS或MIS信号转导的活化或MISRII的活化得到治疗或使症状降低、并由此从给予重组人MIS蛋白及其功能片段和衍生物中受益的任何病症。例如,就这一方面而言,代表性的病症包括但不限于表达MIS受体的癌症,例如表达MISRII的癌症,例如但不限于卵巢癌、宫颈癌和子宫内膜癌。可用MIS或者MIS信号转导的活化来治疗或使症状降低的其它病症是增殖性疾病(如癌症)、或异常高的雄激素时期(如多囊卵巢疾病、性早熟)、以及其它雄激素过多紊乱(如高睾酮血症(testotoxicosis))、或任何雄激素依赖性肿瘤(如前列腺癌)。

[0061] 定义:

[0062] 为了方便起见,这里收集了在整个申请(包括说明书、实施例以及所附的权利要求书)中所使用到的某些术语。除非另有定义,本文使用的所有技术术语和科学术语具有与本发明所属技术领域的普通技术人员通常所理解的含义相同的含义。

[0063] 术语"缪勒抑制物质"和"MIS"在本文中可互换使用,其也被称为抗缪勒激素或AMH,是指在结构上相似于MIS的化合物和物质。"MIS"或"缪勒抑制物质"是指具有与SEQ ID NO:1的氨基酸残基26-560至少约60%、或至少约70%、或至少约80%、或至少约90%、或至少约95%、或至少约96%、或至少约97%、或至少约98%、或至少约99%相同的氨基酸序列的多肽。本发明旨在包括具有与野生型MIS的生物活性基本上相同的生物活性或者更高的生物活性的重组人MIS的突变形式。此类突变的MIS分子的实例为在野生型MIS的氨基酸序列(例如,SEQ ID NO:1的氨基酸残基26-560)中携带删除、插入或改变。所包括的物质的其它形式例如为野生型MIS和重组人MIS的盐、功能衍生物以及糖苷配基(aglycone)形式。此外,人重组MIS蛋白可以使用重组DNA技术来获得,或者可以由MIS蛋白的化学合成来获得。仅供参考,人MIS的野生型核酸对应于RefSeq NO:NM_000479,以引用的方式将其并入本文。

[0064] 术语"缪勒抑制物质II型受体"或"MISRII"在本文中可互换使用,用以指MIS的II型受体。术语MISRII旨在涵盖与MISRII和MISRII的功能衍生物基本上同源的所有MIS受体。MISRII还以AMHR2作为别名,供参考的目的,人MISRII的核酸序列对应于NM_020547和GenBank NO:AF172932,以引用的方式将它们并入本文。

[0065] 术语"野生型"分别是指天然存在的编码蛋白的多核苷酸序列或其部分、或者蛋白序列或其部分,如通常在体内所存在的。因此,如本文所公开的,人MIS的前原蛋白的野生型氨基酸序列对应于SEQ ID NO:1,其中,氨基酸残基1-25对应于前导序列。MIS的前蛋白含有SEQ ID NO:1的氨基酸残基26-560(例如,缺乏1-25的前导序列),然后通过如本文所讨论的切割进行翻译后加工从而形成生物活性MIS同二聚体。

[0066] 本文所用的术语"可溶性MIS多肽"是指不含有使MIS多肽功能性结合至膜的氨基酸的至少一部分或全部的MIS多肽。

[0067] "编码MIS的多核苷酸"意思是指编码多肽的多核苷酸,所述多肽与对应于SEQ ID

NO:1的氨基酸残基26-560的任何氨基酸序列具有至少约60%、或至少约70%、或至少约80%、或至少约90%、或至少约95%、或至少约96%、或至少约97%、或至少约98%、或至少约99%的序列一致性。

[0068] 术语"突变体"是指在有机体的遗传物质中的任何改变,特别是野生型多核苷酸序列中的改变(即,删除、置换、添加或改变)或者野生型蛋白质序列中的任何改变。术语"变体"与"突变体"可互换使用。尽管通常假定遗传物质的改变引起蛋白功能的改变,术语"突变体"和"变体"是指野生型蛋白序列的改变,而不管该改变是否使得蛋白的功能变化(例如增加、减少、赋予新功能),或该改变是否对蛋白的功能没有影响(例如,突变或变异是沉默的)。在本申请中,术语突变与多态性在本文中可互换使用。

[0069] 本文所用的术语"药剂"或"化合物"是指给予受试者以治疗或预防或控制疾病或病症的化学实体或生物产品、或化学实体或生物产品的组合。化学实体或生物产品优选但并非必须是低分子量化合物,但也可为较大的化合物,或任何有机分子或无机分子,包括修饰的核酸和未修饰的核酸,例如反义核酸、RNAi(例如siRNA或shRNA)、肽、肽模拟物(peptidomimetics)、受体、配体和抗体、适体(aptamers)、多肽、核酸类似物,或它们的变体。例如,可为核酸、氨基酸或碳水化合物的寡聚物,包括但不限于:蛋白、寡核苷酸、核酶(ribozymes)、DNA酶、糖蛋白、siRNA、脂蛋白、适体,以及它们的修饰和组合。

[0070] 术语"核酸"是本领域公知的。本文所用的"核酸"通常是指含有核碱基(nucleobase)的DNA、RNA、或它们的衍生物或类似物的分子(即链)。核碱基包括例如DNA中发现的天然存在的嘌呤或嘧啶碱基(例如,腺嘌呤"A"、鸟嘌呤"G"、胸腺嘧啶"T"或胞嘧啶"C")或RNA中发现的天然存在的嘌呤或嘧啶碱基(例如,A、G、尿嘧啶"U"或C)。术语"核酸"涵盖术语"寡核苷酸"和"多核苷酸",其各自为"核酸"的亚属。术语"寡核苷酸"指的是长度为约3至约100个核碱基的分子。术语"多核苷酸"指的是长度大于约100个核碱基的至少一种分子。术语"核酸"也指多核苷酸,例如,脱氧核糖核酸(DNA)和核糖核酸(RNA)(在适当情况下)。该术语也应理解为包括作为等同物的由核苷酸类似物制成的RNA或DNA的类似物、以及适用于所描述的实施方式的单(正义或反义)链和双链多核苷酸。术语"多核苷酸序列"和"核苷酸序列"在本文中也可以互换使用。

[0071] 本文所使用的术语"基因"是指含有编码多肽的开放阅读框的核酸,包括外显子序列以及(任选的)内含子序列。"基因"是指基因产物的编码序列以及该基因产物的非编码区,包括该基因产物的5'UTR区和3'UTR区、内含子以及启动子。这些定义通常是指单链分子,但在具体实施方式中,也将包括与所述单链分子部分互补、基本上互补或完全互补的额外的链。因此,核酸可涵盖双链分子、或包含含有分子的特定序列的一个或多个互补链或"互补物(complement)"的双链分子。本文所用的单链核酸可用前缀"ss"标注,双链核酸用前缀"ds"标注,三链核酸用前缀"is"标注。术语"基因"是指参与产生多肽链的DNA区段(segment),它包含编码区之前和之后的区域以及各编码区段(外显子)之间的间插序列(intervening sequences)(内含子)。"启动子"是控制转录起始和速率的核酸序列区。它可含有调节蛋白和分子(例如RNA聚合酶和其它转录因子)可结合于其上以启动核酸序列的特异性转录的元件。术语"增强子"是指参与核酸序列的转录活化的顺式作用调节序列。增强子可以在任一方向上起作用,并可以在启动子的上游或下游。

[0072] 本文所用的术语"基因产物"通常是指包括由基因转录的RNA(例如mRNA)、或者由

基因编码的或由RNA翻译的多肽。

[0073] 术语“蛋白”与“多肽”可互换使用，指氨基酸残基的聚合物，并且不限制最小长度。肽、寡肽、二聚体和多聚体(multimers)等也是由通过肽键连接的线性排列的氨基酸构成，并且，无论是生物生产、重组生产还是合成生产以及无论是由天然存在的氨基酸构成还是由非天然存在的氨基酸构成，均包括在本定义内。全长蛋白质及其片段均被该定义所涵盖。该术语还包括多肽的共翻译修饰(例如SEQ ID NO:1的氨基酸1-25的前导序列切割)和翻译后修饰，如，例如二硫键形成、糖基化、乙酰化、磷酸化、蛋白水解切割(例如由弗林蛋白酶或金属蛋白酶和激素原转化酶(PC)进行的切割)等。此外，出于本发明的目的，“多肽”涵盖包含对天然序列的修饰(例如删除、添加和置换(本领域技术人员知晓其通常是保守性的))的蛋白，只要所述蛋白维持了所需活性。这些修饰可为有意的(例如通过定点诱变)或者可为偶然的(例如通过产生该蛋白的宿主的突变、或由于PCR扩增或其它重组DNA方法产生的错误)。多肽或蛋白由通过肽键连接的线性排列的氨基酸构成，但与肽相比，具有良好定义的构象。与肽不同，蛋白通常由50个以上的氨基酸的链组成。出于本发明的目的，本文所用的术语“肽”通常是指由通过肽键连接的D-氨基酸或L-氨基酸或者D-氨基酸和L-氨基酸的混合物的单链形成的氨基酸序列。通常，肽包含至少两个氨基酸残基，并且长度小于约50个氨基酸。

[0074] 在某些情况下，非天然氨基酸(包括合成的非天然氨基酸、置换的氨基酸、或者一个或多个D-氨基酸)掺入肽(或组合物的其它组件，除了蛋白酶识别序列)是可取的。相比含有L-氨基酸的形式，含有D-氨基酸的肽在体外或体内表现出增加的稳定性。因此，当想要或需要较大的体内或细胞内稳定性时，构建掺入D-氨基酸的肽可特别有用。更具体而言，D-肽对内源性肽酶和蛋白酶具有耐受性，从而提供了所连接的药物和缀合物的更好的口服跨上皮(trans-epithelial)递送和透皮(transdermal)递送、改善的膜永久复合物(membrane-permanent complexes)的生物利用度(参见下文的进一步讨论)、以及延长的血管内寿命和间质(interstitial)寿命(当想要此类特性时)。使用D-异构体(D-isomer)肽还可增强所连接的药物和其它负载分子的透皮递送和口服跨上皮递送。此外，D-肽不能被有效加工以进行受限于II类主要组织相容性复合物的向辅助T细胞的呈递，并因此不太可能在整个有机体中诱导体液免疫应答。因此，可使用例如细胞穿透肽(penetrating peptide)序列的D-异构体形式、切割位点的L-异构体形式、以及治疗肽的D-异构体形式构建肽缀合物。在一些实施方式中，重组人MIS蛋白由D-氨基酸残基或L-氨基酸残基构成，因为使用天然存在的L-氨基酸残基具有以下优点：任何分解的产物对细胞或有机体来说应该是相对无毒的。

[0075] 在其它进一步的实施方式中，重组人MIS蛋白或其片段或衍生物可为逆反肽(retro-inverso peptides)。“逆反肽”是指在至少一个位置上肽键方向反转(reversal)的肽，即，相对于所述氨基酸的侧链而言氨基末端和羧基末端的反转。因此，逆反类似物具有反转的末端和反转的肽键方向，同时大致保持如同天然肽序列的侧链拓扑学。逆反肽可含有L-氨基酸或D-氨基酸、或者L-氨基酸和D-氨基酸的混合物，至多所有氨基酸都为D-异构体。局部逆反肽类似物为其中仅部分序列反转并用对映体氨基酸残基替换的多肽。由于这样的类似物的逆反部分具有反转的氨基末端和羧基末端，逆反部分侧翼的氨基酸残基分别被侧链相似的α-取代的偕-二氨基甲烷(geminal-diaminomethanes)和丙二酸盐/酯(malonates)替换。已发现细胞穿透肽的逆反形式在易位跨膜方面与天然形式一样有效。逆

反肽类似物的合成在以下文献中有所描述:Bonelli,F.等,Int J Pept Protein Res.24(6):553-6(1984);Verdini,A和Viscomi,G.C.,J.Chem.Soc.Perkin Trans.1:697-701(1985);以及美国专利No.6,261,569,以引用的方式将它们整体并入本文。局部逆反肽类似物的固相合成工艺已有描述(EP 97994-B),也通过引用将其整体并入本文。

[0076] 本文所用的术语肽、多肽或分子的"片段"是指该分子的任何连续的(contiguous)多肽的子集。本文所用的术语"蛋白片段"包括可由天然存在的MIS的氨基酸序列(SEQ ID NO:1)衍生而来的合成氨基酸序列和天然存在的氨基酸序列。如果蛋白可通过将重组人MIS蛋白片段化(fragmenting)而获得、或者如果蛋白可基于天然存在的氨基酸序列或编码该序列的遗传物质(DNA或RNA)的序列的知识而合成,所述蛋白被认为是"可由重组人MIS蛋白的天然存在的氨基酸序列衍生而来"。因此,分子的"片段"意思是指所述分子的任何多肽子集。在一些实施方式中,重组人MIS的功能片段至少包含C端结构域以及至少包含N端结构域。在一些实施方式中,功能片段含有重组人MIS蛋白的C端结构域的部分和/或N端结构域的部分(例如,片段)。所具有的活性至少为本文所公开的SEQ ID NO:1的野生型MIS蛋白的活性或大于本文所公开的SEQ ID NO:1的野生型MIS蛋白的活性且可溶的重组人MIS蛋白的片段也包括在本发明中使用。

[0077] 在本文所公开的方法中有用的重组人MIS蛋白的片段(例如,SEQ ID NO:2或SEQ ID NO:3的功能片段)在体内具有SEQ ID NO:2多肽或SEQ ID NO:3多肽的至少30%的活性,例如,以在缪勒管退化生物分析中引起缪勒管退化(如本文在实施例中所公开的)。换句话说,重组人MIS蛋白的功能片段为SEQ ID NO:2或SEQ ID NO:3中任一种的片段,单独的该片段或作为融合蛋白的该片段可产生与SEQ ID NO:2或SEQ ID NO:3相比至少30%的相同活性,以结合和活化MISRII、或如本文所公开地在缪勒管退化生物分析中引起缪勒管退化(参见图4)。本文所用的片段可为可溶的(即,不是膜结合的)。"片段"可为至少约6个、至少约9个、至少约15个、至少约20个、至少约30个、至少约40个、至少约50个、至少约100个、至少约250个、至少约300个(以及其间的所有整数个)核酸或氨基酸。示例性片段包括C末端截短、N末端截短或C末端和N末端都截短(例如从N末端、C末端、或N末端和C末端二者删除例如至少1个、至少2个、至少3个、至少4个、至少5个、至少8个、至少10个、至少15个、至少20个、至少25个、至少40个、至少50个、至少75个、至少100个或更多个氨基酸)。本领域普通技术人员可通过简单的删除分析来制造此类片段。此类SEQ ID NO:2或SEQ ID NO:3的片段可为例如分别从SEQ ID NO:2或SEQ ID NO:3的N末端和/或C末端删除1、2、3、4、5、6、7、8、9或10个氨基酸或多于10个氨基酸,例如15个、30个、50个、100个或多于100个氨基酸。通过由SEQ ID NO:2或SEQ ID NO:3顺序(sequentially)删除N末端和/或C末端氨基酸或通过由重组人MIS蛋白顺序删除N末端和/或C末端氨基酸,并评估所得肽片段(单独的肽片段或当它被切割时的肽片段)的功能,本领域普通技术人员可以容易地鉴别在本文所公开的方法和组合物中有用或在本文所公开的融合蛋白中有用的SEQ ID NO:2或SEQ ID NO:3的最小肽片段。可以用多个较小片段制造功能片段。这些片段可通过桥接(bridging)肽接头连接。可以容易地选择接头来保持野生型构象。本领域普通技术人员可容易地评估相比对应于SEQ ID NO:2或SEQ ID NO:3的重组人MIS蛋白而言的本文所公开的重组人MIS蛋白活化MISRII或在缪勒管退化生物分析中的功能(如本文中所公开的)。使用此类体内分析,如果重组人MIS蛋白的片段具有如本文所公开的对应于SEQ ID NO:2或SEQ ID NO:3的重组人MIS蛋白的至少30%的生物

活性，那么该片段被认为是有效的(valid)重组人MIS蛋白片段，并可用于本文所公开的组合物和方法中。在一些实施方式中，SEQ ID NO:2或SEQ ID NO:3的片段可为少于200个、或少于150个或少于100个、或少于50个、或少于20个SEQ ID NO:2或SEQ ID NO:3的氨基酸。在一些实施方式中，SEQ ID NO:2或SEQ ID NO:3的片段为长度少于100个氨基酸的肽。然而，如上所述，该片段必须为至少6个氨基酸、至少约9个、至少约15个、至少约20个、至少约30个、至少约40个、至少约50个、至少约100个、至少约250个、至少约500个(或其间的任何整数个)核酸或氨基酸。

[0078] 本文所用的术语“衍生物”是指已通过例如但不限于如泛素化、标记、聚乙二醇化(用聚乙二醇衍生化)或添加其它分子的技术进行化学修饰的肽。当分子含有通常不是所述分子一部分的附加化学部分时，该分子也是另一分子的“衍生物”。此类部分可改善该分子的溶解性、吸收、生物半衰期等。或者，所述部分可以降低分子的毒性、消除或减弱该分子的任何不希望的副作用等。能够介导此类效应的部分公开于Remington's Pharmaceutical Sciences，第18版，A.R.Gennaro著，MackPubl.，Easton，PA(1990)中。

[0079] 当与“衍生物”或“变体”或“片段”结合使用时，术语“功能的”是指具有与某多肽的生物活性基本上相似的生物活性(功能方面或结构方面)的多肽(后一多肽是前一多肽的功能衍生物、变体或功能片段)。术语功能衍生物旨在包括分子的片段、类似物或化学衍生物。在该上下文中，“基本上相似的”意味着生物活性(例如，使MISRII活化)为参比多肽(例如相应的野生型MIS多肽或重组人MIS蛋白)的活性的至少25%、或至少35%、或至少50%，优选为参比多肽活性的至少60%、参比多肽活性的70%、参比多肽活性的80%、参比多肽活性的90%、参比多肽活性的95%、参比多肽活性的100%或甚至更高(即，变体或衍生物的活性比野生型高)，例如为参比多肽活性的110%、参比多肽活性的120%或以上。换句话说，在这一上下文中，重组人MIS蛋白的“基本上相似的”功能片段意思是保留了相应重组人MIS蛋白的相关生物活性或期望生物活性的至少25%、至少35%、至少50%。以本文公开的重组人MIS蛋白(例如，SEQ ID NO:2或SEQ ID NO:3)的功能片段或肽为例，SEQ ID NO:2或SEQ ID NO:3的功能片段将会是保留了活化MISRII的活性或在缪勒管退化生物分析中的活性(如本文实施例中所公开的)的含有SEQ ID NO:2或SEQ ID NO:3的部分的蛋白或肽；优选为在活化MISRII或在缪勒管退化生物分析中引起缪勒管退化方面(如本文所公开的)相比全长SEQ ID NO:2或SEQ ID NO:3而言保留了至少25%、至少35%、至少50%、至少60%、至少70%、至少80%、至少90%、至少95%、至少100%或甚至更高的活性(即，变体或衍生物的活性比SEQ ID NO:1的野生型MIS或者SEQ ID NO:2或SEQ ID NO:3的重组人MIS蛋白高)(例如至少110%、至少120%或以上的活性)的SEQ ID NO:2或SEQ ID NO:3的片段。作为另一实例，以MIS(例如SEQ ID NO:1的氨基酸26-560)的片段为例，MIS的片段将会是保留了关于缪勒管退化的活性的含有SEQ ID NO:1的氨基酸26-560的部分的蛋白或肽；优选在缪勒管退化生物分析中引起缪勒管退化方面(如本文实施例中所述的)相比SEQ ID NO:1的氨基酸26-560的全长而言保留了至少25%、至少35%、至少50%、至少60%、至少70%、至少80%、至少90%、至少95%、至少100%或甚至更高的活性(即，变体或衍生物的活性比野生型的活性高)(例如至少110%、至少120%或以上的活性)的SEQ ID NO:1的氨基酸26-560的片段。作为可选的实例，SEQ ID NO:6的HSA前导序列的片段将会是含有SEQ ID NO:6的部分的蛋白或多肽，由例如US专利5,759,802中公开的分析所测定的(以引用的方式将其整体并入本

文)，相比SEQ ID NO:6的全长HSA前导序列而言，所述片段保留了至少25％、至少35％、至少50％、至少60％、至少70％、至少80％、至少90％、至少95％、至少100％或甚至更高的活性(即，变体或衍生物的活性比野生型HSA序列的活性高)(例如至少110％、至少120％或以上的活性)。

[0080] 术语“功能衍生物”和“模拟物(mimetic)”或“生物活性变体”或“生物活性片段”可互换使用，指具有与某实体或分子(例如，重组人MIS蛋白)的生物活性基本上相似的生物活性(功能方面或结构方面)的化合物(该化合物是所述实体或分子的功能衍生物)。术语功能衍生物意在包括分子的片段、变体、类似物或化学衍生物。

[0081] 术语“功能衍生物”旨在包括分子的“片段”、“变体”、“类似物”或“化学衍生物”。如果两个分子具有基本上相似的结构或者如果两个分子具有相似的生物活性，一个分子被认为与另一分子“基本上相似”。因此，倘若两个分子具有相似的活性，即便是一个分子的结构在另一分子中并未发现、或者即便是氨基酸残基序列并不相同，也认为它们是变体(当该术语在本文中使用时)。重组人MIS蛋白的“类似物”意思是指在功能上与整个分子或其片段基本上相似的分子。当分子含有通常不是所述分子一部分的附加化学部分时，该分子被认为是另一分子的“化学衍生物”(如本文所用的)。此类部分可改善该分子的溶解性、吸收、生物半衰期等。或者，所述部分可以降低分子的毒性、消除或减弱该分子的任何不希望的副作用等。能够介导此类效应的部分公开于Remington's Pharmaceutical Sciences，第18版，A.R.Gennaro著，MackPubl.，Easton，PA(1990)中。

[0082] 重组人MIS蛋白的“变体”意思是指在结构和功能上与整个分子或其片段基本上相似的分子。因此，本文所用的术语“变体”是指通过一个或多个氨基酸或核酸删除、添加、置换或侧链修饰而不同于天然存在的多肽或核酸，但保留了这些天然存在的分子的一种或多种特定功能或生物活性的肽或核酸。氨基酸置换包括用不同的天然存在的氨基酸残基或非常规氨基酸残基替换氨基酸的改变。此类置换可分为“保守的”，在这种情况下，用在极性、侧链功能或尺寸方面具有相似性质的另一天然存在的氨基酸替换多肽中包含的氨基酸残基。本发明所涵盖的置换也可为“非保守的”，其中，用具有不同特性的氨基酸(例如，来自不同组的天然存在的氨基酸)置换存在于肽中的氨基酸残基(例如，用丙氨酸置换带电的氨基酸或疏水性氨基酸)；或者，其中，用非常规氨基酸置换天然存在的氨基酸。在一些实施方式中，氨基酸置换为保守的。当就多核苷酸或多肽使用时，术语“变体”也包含在分别与参比多核苷酸或多肽相比(例如与野生型多核苷酸或多肽相比)时，在一级结构、二级结构、或三级结构中可存在变化的多核苷酸或多肽。重组人MIS蛋白的“变体”意思是指在结构和功能上基本上相似的分子，即，其中所述的功能为活化MISRII的能力。

[0083] 例如，重组人MIS蛋白的变体可含有不同于SEQ ID NO:2或SEQ ID NO:3中的参比氨基酸的突变或修饰。在一些实施方式中，SEQ ID NO:2或SEQ ID NO:3的变体为本文所公开的SEQ ID NO:2或SEQ ID NO:3的片段。在一些实施方式中，变体可为SEQ ID NO:2或SEQ ID NO:3的不同异形体(isoform)，或者可含有不同异构体氨基酸。变体可为使用本领域公知的方法分离或生成的天然存在的、合成的、重组的、或化学修饰的多核苷酸或多肽。如下所述，变体可包含保守氨基酸改变或非保守氨基酸改变。多核苷酸的改变可导致由参比序列编码的多肽中的氨基酸置换、添加、删除、融合和截短。变体还可包含氨基酸的插入、删除或置换，包括通常不出现在作为变体基础的肽序列中的氨基酸和其它分子的插入和置换，

例如但不限于通常不出现在人蛋白中的鸟氨酸的插入。

[0084] 当描述多肽时，术语“保守置换”是指多肽的氨基酸组成改变并不实质上改变多肽的活性。例如，保守置换是指用具有相似化学性质的不同氨基酸残基置换某氨基酸残基。保守氨基酸置换包括将亮氨酸替换为异亮氨酸或缬氨酸、将天冬氨酸替换为谷氨酸、或将苏氨酸替换为丝氨酸。“保守氨基酸置换”由一个氨基酸被具有相似结构和/或化学性质的另一氨基酸替换所导致，例如将亮氨酸替换为异亮氨酸或缬氨酸、将天冬氨酸替换为谷氨酸、或将苏氨酸替换为丝氨酸。因此，特定氨基酸序列的“保守置换”是指将对于多肽活性不关键的那些氨基酸进行置换；或用具有相似性质(例如酸性、碱性、正电性或负电性、极性或非极性等)的其它氨基酸对氨基酸进行置换，从而即使是对关键氨基酸的置换也不降低肽的活性(即，本文所公开的肽减少T-reg细胞和/或减少炎性细胞因子的能力)。提供了功能相似氨基酸的保守置换表在本领域中是公知的。例如，以下六组各自包含彼此为保守置换的氨基酸：1)丙氨酸(A)、丝氨酸(S)、苏氨酸(T)；2)天冬氨酸(D)、谷氨酸(E)；3)天冬酰胺(N)、谷氨酰胺(Q)；4)精氨酸(R)、赖氨酸(K)；5)异亮氨酸(I)、亮氨酸(L)、甲硫氨酸(M)、缬氨酸(V)；以及6)苯丙氨酸(F)、酪氨酸(Y)、色氨酸(W)。(也参见Creighton，Proteins，W.H.Freeman and Company(1984))。在一些实施方式中，改变、添加或删除单个氨基酸或小百分比的氨基酸的各置换、删除或添加也可被认为是“保守置换”。保守置换是并不使MIS蛋白的活性(即，重组人MIS蛋白或变体在体内引起缪勒管退化的能力，这可使用本文所公开的缪勒管退化生物分析进行测定)降低的改变。插入或删除典型地在约1-5个氨基酸的范围内。保守氨基酸的选择可基于肽中待置换的氨基酸的位置进行选择，例如，氨基酸是否在肽的外部且暴露至溶剂、或是否在内部且不暴露至溶剂。

[0085] 在替代的实施方式中，可基于已存在的氨基酸的位置，即，它对于溶剂的暴露(即，相比于不暴露于溶剂的位于内部的氨基酸，该氨基酸是否暴露于溶剂或是否存在于肽或多肽的外表面)，选择将要置换所述已存在的氨基酸的氨基酸。此类保守氨基酸置换的选择在本领域中是公知的，例如，如Dordo等，J.Mol Biol，1999，217，721-739；Taylor等，J.Theor.Biol.119(1986)；205-218；以及S.French和B.Robson，J.Mol.Evol.19(1983)171中所公开的。因此，能够选择适于蛋白或肽外部的氨基酸(即暴露于溶剂的氨基酸)的保守氨基酸置换，可使用例如但不限于以下置换：将Y置换为F，将T置换为S或K，将P置换为A，将E置换为D或Q，将N置换为D或G，将R置换为K，将G置换为N或A，将T置换为S或K，将D置换为N或E，将I置换为L或V，将F置换为Y，将S置换为T或A，将R置换为K，将G置换为N或A，将K置换为R，将A置换为S、K或P。

[0086] 在替代的实施方式中，还能够选择适于蛋白或肽内部氨基酸的保守氨基酸置换，例如，能够使用对蛋白或肽内部氨基酸(即，不暴露于溶剂的氨基酸)而言合适的保守置换，例如但不限于，能够使用以下保守置换：将Y置换为F，将T置换为A或S，将I置换为L或V，将W置换为Y，将M置换为L，将N置换为D，将G置换为A，将T置换为A或S，将D置换为N，将I置换为L或V，将F置换为Y或L，将S置换为A或T以及将A置换为S、G、T或V。在一些实施方式中，非保守氨基酸置换也涵盖在术语变体内。重组人MIS蛋白的变体(例如，SEQ ID NO:2或SEQ ID NO:3的变体)意思是指在结构和功能上与SEQ ID NO:2或SEQ ID NO:3的完整分子或其片段基本上相似的任何分子。

[0087] 术语“同源性(homology)”、“一致性(identity)”和“相似性(similarity)”是指两

个肽之间或两个最佳比对的核酸分子之间序列相似的程度。同源性和一致性各自可通过比较出于比较的目的可进行比对的各序列中的位置来确定。例如，它是基于使用处于缺省位置的标准同源性软件，例如BLAST，2.2.14版本。当比较的序列的等同位置被相同碱基或相同氨基酸占据，那么这些分子在该位置是相同的；当等同位置被相似氨基酸残基(例如，空间和/或电子性质方面相似，如，例如保守氨基酸置换)占据，那么可称这些分子在该位置是同源的(相似的)。作为同源性/相似性或一致性百分比的表达式分别是指在比较的序列共有的位置上相似氨基酸或相同氨基酸的数量的函数。“不相关的”或“非同源的”序列与本文所公开的序列具有低于40％的一致性，但优选具有低于25％的一致性。

[0088] 本文所用的术语“序列一致性”意思是两个多核苷酸或氨基酸序列在整个比较窗口内是相同的(即，以一个核苷酸接一个核苷酸(nucleotide-by-nucleotide)或一个残基接一个残基(residue-by-residue)为基础)。术语“序列一致性的百分比”通过以下方式计算得到：对比较窗口内两个最佳比对的序列进行比较，确定在两个序列中存在相同核酸碱基(如A、T、C、G、U或I)或残基的位置的数量以获得相匹配的位置的数量，用相匹配的位置的数量除以比较窗口中位置的总数(即，窗口大小)，并将结果乘以100，从而获得序列一致性的百分比。

[0089] 本文所用的术语“基本上一致”表示多核苷酸或氨基酸序列的特点，其中，所述多核苷酸或氨基酸含有如下序列：在至少18个核苷酸(6个氨基酸)位置的比较窗口中、通常为在至少24-48个核苷酸(8-16个氨基酸)位置的窗口中，相比参比序列而言具有至少85％的序列一致性、优选至少90％-95％的序列一致性、更通常为至少99％的序列一致性的序列，其中，序列一致性的百分比通过在比较窗口中将参比序列与可含有删除或添加(删除或添加总计为参比序列的20％或以下)的序列进行比较来计算。参比序列可为较大序列的子集。当用于描述多肽时，术语“相似性”通过将一条多肽的氨基酸序列和保守氨基酸置换与第二条多肽的序列进行比较来确定。

[0090] 本文使用的术语“同源的(homologous)”或“同源物(homologues)”可互换使用，并且，在用于描述多核苷酸或多肽时，表示当进行最佳比对和比较(例如使用具有用于比对的默认参数的BLAST，2.2.14版本(参见本文))时，两个多核苷酸或多肽、或其指定序列(具有适当的核苷酸插入或删除或氨基酸插入或删除)在至少70％的核苷酸、通常从约75％-99％、更优选至少约98％-99％的核苷酸中是相同的。本文使用的术语“同系物(homolog)”或“同源的”还指在结构和/或功能方面的同源性。关于序列同源性，如果序列至少50％、至少60％、至少70％、至少80％、至少90％、至少95％相同、至少97％相同、或至少99％相同，则这些序列是同系物。对本发明的基因或肽的同系物的确定可由本领域技术人员容易地确定。

[0091] 术语“基本上同源”是指至少90％、至少95％相同、至少96％相同、至少97％相同、至少98％相同或至少99％相同的序列。同源序列可以是不同物种中的相同功能基因。本发明的基因或肽的同系物的确定可由本领域技术人员容易地确定。

[0092] 如果两个分子具有基本上相似的结构，或者如果两个分子具有相似的生物活性(例如，如果两个分子都能够活化MISRII)，一个分子被认为与另一个分子“基本上相似”。因此，倘若两个分子具有相似的活性(例如，与对应于SEQ ID NO:1的MIS蛋白或对应于SEQ ID NO:2或SEQ ID NO:3的重组人MIS蛋白相似的能够激活MISRII的重组人MIS蛋白的变体)，即

便是一个分子的结构在另一分子中并未发现、或者即便是氨基酸残基序列并不相同，该两个分子也被认为是变体，并且被包含以用于本文所述的用途。因此，倘若两个分子具有相似的生物活性，即便是一个分子的结构在另一分子中并未发现、或者即便是氨基酸残基序列并不相同，它们也被认为是变体(当该术语在本文中使用时)。特别是，当用于定义重组人MIS蛋白(包括相对于由SEQ ID NO:2或SEQ ID NO:3编码的重组人MIS蛋白而言的重组人MIS蛋白的功能变体)，术语“基本上相似”意思是指特定的目标序列(例如，重组人MIS蛋白的变体或衍生物序列)通过一个或多个置换、删除或添加而不同于SEQ ID NO:1的天然(或野生型)MIS序列或重组人MIS蛋白序列(即，由SEQ ID NO:2或SEQ ID NO:3编码)，不过所述特定的目标序列的净效果(net effect)保留了在如本文所公开的重组人MIS蛋白中发现的生物活性的至少一部分。就这一点而论，与重组人MIS蛋白相比具有较低程度的相似性但具有相当的生物活性的核酸序列以及氨基酸序列被认为是等同物。在确定多核苷酸序列时，能够编码基本上相似的氨基酸序列的所有目标多核苷酸序列都被认为是与参比多核苷酸序列基本上相似，而不管密码子序列的差异。如果：(a)核苷酸序列杂交至天然MIS核酸的编码区；或(b)在适度严格(moderately stringent)条件下，核苷酸序列能够杂交至由SEQ ID NO:4或SEQ ID NO:5编码的重组人MIS蛋白的核苷酸序列，并且具有与该重组人MIS蛋白相似的生物活性；或(c)相对于(a)或(b)中所定义的核苷酸序列，核苷酸序列作为遗传密码子的结果是简并的(degenerate)，则该核苷酸序列与本文所公开的SEQ ID NO:4或SEQ ID NO:5的特定核酸序列“基本上相似”。基本上相似的蛋白通常将与天然蛋白的相应序列具有大于约80％的相似性。

[0093] 在多肽序列的情况下，术语“基本相似性(substantial similarity)”表示多肽包含如下序列：在约10-20个氨基酸残基的比较窗口中，该序列与参比序列具有至少60％的序列一致性、或与参比序列具有70％、或80％、或85％的序列一致性、或者最优选具有90％的一致性。在氨基酸序列的情况下，“基本相似性”进一步包括氨基酸的保守置换。因此，例如，在两个肽因一个或多个保守置换不同时，多肽与另一多肽基本相似。

[0094] 在一个实施方式中，就基因转录物而言的术语“人同系物”是指与由人或动物(例如小鼠或转基因动物)的基因组编码的重组人MIS蛋白序列的全长核苷酸序列具有至少约55％的同源性的DNA序列。在一个实施方式中，就鉴定为与重组人MIS蛋白相关的蛋白而言的术语“人同系物”是指与鉴定为与重组人MIS蛋白(由本发明的转基因动物的基因组编码)相关的蛋白的全长氨基酸序列具有40％的同源性的氨基酸序列，更优选至少约50％、还更优选至少约60％的同源性、还更优选至少约70％的同源性、甚至更优选至少约75％的同源性、又更优选至少约80％的同源性、甚至更优选至少约85％的同源性、还更优选至少约90％的同源性、以及更优选至少约95％的同源性。如上所讨论的，同源性为至少约50％至100％和其间的所有间隔值(intervals)(即，55％、60％、70％、75％、80％、85％、90％、95％、98％等)。本发明的基因的人同系物的确定可由本领域技术人员容易地确定。

[0095] 当描述多肽时，术语“保守置换”是指多肽的氨基酸组成改变并不实质上改变多肽的活性。因此，特定氨基酸序列的“保守置换”是指将对于多肽活性不关键的那些氨基酸进行置换；或用具有相似性质(例如酸性、碱性、正电性或负电性、极性或非极性等)的其它氨基酸对氨基酸进行置换，从而即使是对关键氨基酸的置换也不实质上改变活性。提供了功能相似氨基酸的保守置换表是本领域公知的。例如，以下六组各自包含彼此为保守置换的

氨基酸:1)丙氨酸(A)、丝氨酸(S)、苏氨酸(T);2)天冬氨酸(D)、谷氨酸(E);3)天冬酰胺(N)、谷氨酰胺(Q);4)精氨酸(R)、赖氨酸(K);5)异亮氨酸(I)、亮氨酸(L)、甲硫氨酸(M)、缬氨酸(V);以及6)苯丙氨酸(F)、酪氨酸(Y)、色氨酸(W)。也参见Creighton,Proteins,W.H.Freeman and Company(1984)。此外,在编码序列中改变、添加或删除单个氨基酸或小百分比的氨基酸的各置换、删除或添加也是"保守置换"。

[0096] 本文所用的术语"非保守的"指用氨基酸残基来置换具有不同化学特性的不同氨基酸残基。非保守置换包括但不限于用甘氨酸(G)替换天冬氨酸(D)、用赖氨酸(K)替换天冬酰胺(N)、或用精氨酸(R)替换丙氨酸(A)。

[0097] 对于序列比较,通常一个序列作为参比序列,将测试序列与该参比序列进行比较。当使用序列比较算法时,将测试序列和参比序列输入到计算机中,指定子序列坐标(subsequence coordinates)(如果需要的话),并指定序列算法程序参数。然后,序列比较算法基于指定的程序参数来计算测试序列相对于参比序列的序列一致性百分比。

[0098] 可通过如下方式进行用于比较的序列的最佳比对:例如,Smith和Waterman的局部同源性算法(Adv.Appl.Math.2:482(1981),以引用的方式将其并入本文);Needleman和Wunsch的同源性比对算法(J.Mol.Biol.48:443-53(1970),以引用的方式将其并入本文);Pearson和Lipman的相似性检索方法(Proc.Natl.Acad.Sci.USA 85:2444-48(1988),以引用的方式将其并入本文);这些算法的计算机化执行(例如,Wisconsin Genetics软件包中的GAP、BESTFIT、FASTA和TFASTA,Genetics Computer Group,575Science Dr.,Madison,Wis);或目测检查(一般地参见Ausubel等(编),Current Protocols in Molecular Biology,第4版,John Wiley and Sons,New York(1999))。

[0099] 有用算法的一个实例是PILEUP。PILEUP使用渐进的成对比对,由相关序列的组中创建多重序列比对,以显示序列一致性百分比。它还绘制树或系统树图(dendogram)来显示用于创建比对的聚类关系(clustering relationships)。PILEUP使用Feng和Doolittle的渐进比对方法的简化版(J.Mol.Evol.,25:351-360(1987),以引用的方式将其并入本文)。所用方法与Higgins和Sharp所述方法相似(Comput.Appl.Biosci.5:151-153(1989),以引用的方式将其并入本文)。该程序可比对多达300个序列,每个序列的最大长度为5,000个核苷酸或氨基酸。多重比对过程从成对比对两个最相似的序列开始,生成两个比对序列的簇。然后,将该簇与下一个最相关的序列或对比序列的簇进行比对。通过两个单独序列的成对比对的简单延伸来对比两个簇的序列。最终比对通过一系列渐进的成对比对来实现。该程序通过为序列比较区指定特定的序列以及它们的氨基酸或核苷酸坐标并通过指定程序参数来运行。例如,可使用以下参数将参比序列与其它测试序列进行比较,从而确定序列一致性百分比关系:默认空位权重(3.00),默认空位长度权重(0.10)和加权的末端空位。

[0100] 适于确定序列一致性百分比和序列相似性百分比的算法的另一实例是BLAST算法,该算法由Altschul等描述(J.Mol.Biol.215:403-410(1990),以引用的方式将其并入本文)。(还参见Zhang等,Nucleic Acid Res.26:3986-90(1998);Altschul等,Nucleic Acid Res.25:3389-402(1997),以引用的方式将它们并入本文)。用于进行BLAST分析的软件可通过国家生物技术信息中心(National Center for Biotechnology Information)的互联网万维网站公开获得。该算法包括首先通过识别查询序列中的长度W的短字(short words)来识别高得分序列对(HSP),当与数据库序列中相同长度的字进行比对时,该高得分序列对匹

配或满足一些正阈值评分T。T称为相邻字计分阈值(neighborhood word score threshold)(Altschul等(1990),见上文)。这些初始的相邻字命中(hits)用作起始搜索的种子以寻找含有它们的较长的HSP。然后,这些字命中在两个方向沿着每个序列在累积比对得分能够增加的范围内尽可能向远延伸。当发生下列情况时每个方向的字命中的延伸停止:累积比对得分由其最大获得值下降了数量X;由于一个或多个负得分残基比对的累积,累积得分达到零或零以下;或者到达任一序列的末端。BLAST算法参数W、T和X确定比对的灵敏度和速度。BLAST程序使用的默认值为:字长度(W)为11,BLOSUM62评分矩阵(scoring matrix)(参考Henikoff和Henikoff,Proc.Natl.Acad.Sci.USA 89:10915-9,1992,以引用的方式将其并入本文)比对值(alignments)(B)为50,期望值(E)为10,M=5,N=-4,以及两条链的比较。

[0101] 除了计算序列一致性百分比,BLAST算法也进行两个序列之间相似性的统计分析(例如,参考Karlin和Altschul,Proc.Natl.Acad.Sci.USA 90:5873-77(1993),以引用的方式将其并入本文)。由BLAST算法提供的一种相似性计量是最小和概率(smallest sum probability)(P(N)),它提供了两个核苷酸序列或两个氨基酸序列间偶然出现匹配的概率的指示。例如,如果测试核酸与参比核酸的比较中的最小和概率小于约0.1、更典型地小于约0.01、并最典型地小于约0.001,该核酸被认为与该参比序列相似。

[0102] 术语"插入"或"删除"通常在约1-5个氨基酸的范围内。可通过使用重组DNA技术在序列中系统地生成核苷酸的插入、删除或置换并同时合成生产该肽,来实验性确定允许的变异。

[0103] 当涉及肽时,术语"置换"是指将氨基酸变为不同的实体,例如,另一氨基酸或氨基酸部分。置换可为保守置换或非保守置换。

[0104] 分子(诸如重组人MIS蛋白,例如SEQ ID NO:2或SEQ ID NO:3)的"类似物"是指在功能上与完整分子或其片段相似的分子。术语"类似物"还旨在包括等位基因变体、物种变体(species variant)和诱导变体。类似物通常在一个或几个位置上不同于天然存在的肽,这往往是由于保守置换。类似物通常表现出与天然肽至少80%或90%的序列一致性。一些类似物还包含非天然氨基酸、或N端或C端氨基酸的修饰。非天然氨基酸的实例例如但不限于acedisubstituted氨基酸、N-烷基氨基酸、乳酸、4-羟基脯氨酸、γ-羧基谷氨酸、ε-N,N,N-三甲基赖氨酸、ε-N-乙酰赖氨酸、O-磷酸丝氨酸、N-乙酰丝氨酸、N-甲酰甲硫氨酸、3-甲基组氨酸、5-羟基赖氨酸和σ-N-甲基精氨酸。可在如下文所述的转基因动物模型中,就预防效力或治疗效力对片段和类似物进行筛选。

[0105] "共价键合"意味着通过共价化学键直接或间接(例如,通过接头)连接(joined)。

[0106] 本文所用的术语"融合蛋白"是指两个以上蛋白的重组蛋白。融合蛋白例如可通过以下方式生产:将编码一个蛋白的核酸序列与编码另一蛋白的核酸连接,从而它们构成单个开放阅读框,该开放阅读框在细胞中能够翻译成怀有所有目的蛋白的单个多肽。蛋白质的排列顺序可以改变。作为非限制性实例,编码重组人MIS-融合蛋白的核酸序列来源于编码重组人MIS蛋白或其功能衍生物片段或变体的核苷酸序列,该核苷酸序列框内(in frame)融合在编码第一融合伴侣(例如,IgG1Fc片段)的基因的末端(5'末端或3'末端)。以这种方式,通过基因的表达,重组人MIS蛋白或其功能衍生物片段或变体功能性地表达并融合在IgG1Fc的N末端或C末端。在某些实施方式中,对多肽探针进行的修饰使得就生物活性

而言，重组人MIS蛋白或其功能衍生物片段或变体的功能性基本上保持不受与第一融合伴侣(如IgG1Fc)融合的影响。

[0107] “特异性地结合”或“特异的结合”意味着识别和结合所需的多肽但基本上不识别和结合样品(例如生物样品)中的其它分子的化合物或抗体，这自然包括本发明的多肽。

[0108] “基本上纯”是指从与它一起天然存在的组分中分离的核酸、多肽或其它分子。典型地，当从与多肽天然相关的蛋白或天然存在的有机分子中分离的多肽为至少约60wt%、或至少约70wt%、至少约80wt%、至少约90wt%、至少约95wt%、或甚至至少约99wt%时，该多肽是基本上纯的。例如，基本上纯的多肽可通过以下方式获得：通过从天然来源中提取、通过在通常不表达该蛋白的细胞中表达重组核酸、或通过化学合成。

[0109] “增强的蛋白水解稳定性”意思是相比相同条件(例如，体内或体外系统，例如细胞或细胞裂解物中)下的对照序列，肽序列蛋白水解的速率或程度降低至少约2%、至少约5%、至少约10%、至少约20%、至少约30%、至少约40%、至少约50%、至少约60%、至少约70%、至少约80%、至少约85%、至少约90%、至少约95%、至少约98%、或至少约99%。具有增强的蛋白水解稳定性的肽可含有降低或消除遭受特定位点蛋白水解切割的位点的任何修饰，例如，插入、删除或点突变。可根据已知的靶序列或使用计算机软件(例如，Gasteiger等，Protein Identification and Analysis Tools on the ExPASy Server.In John M.Walker编.The Proteomics Protocols Handbook，Humana Press(2005)描述的软件)鉴定蛋白水解切割的位点。或者，蛋白水解位点例如可通过以下方式实验性地确定：在细胞系统或在细胞裂解物中表达或孵育后对蛋白进行蛋白质印迹，接着对所识别的片段进行测序，从而确定切割位点。

[0110] 当用于描述核酸分子时，本文所用的术语“重组体(recombinant)”是指基因组、cDNA、病毒、半合成和/或合成来源的多核苷酸，由于其来源或操作原因，该多核苷酸不与其在自然界中关联的多核苷酸的全部或部分关联。当就蛋白或多肽而使用时，术语重组体是指通过重组多核苷酸的表达而产生的多肽。当就宿主细胞而使用时，术语重组体是指向其中引入了重组多核苷酸的宿主细胞。就某物质(例如细胞、核酸、蛋白或载体)而言，重组体在本文中也用来指所述物质已通过引入异源物质(例如细胞、核酸、蛋白或载体)进行修饰。

[0111] 术语“受试者”和“个体”在本文中可互换使用，是指用根据本发明的药物组合物向其提供处理(包括预防性处理)的动物(例如人)。本文所用的术语“受试者”是指人和非人动物。术语“非人动物”和“非人哺乳动物”在本文中可互换使用，包括所有脊椎动物，例如哺乳动物，如非人灵长类动物(特别是高级灵长类动物)、绵羊、狗、啮齿动物(例如小鼠或大鼠)、豚鼠、山羊、猪、猫、兔和牛；以及非哺乳动物，如鸡、两栖动物、爬行动物等。在一个实施方式中，所述受试者为人。在另一个实施方式中，所述受试者为作为疾病模型的动物替代品(substitute)或实验动物。该术语不表示特定年龄或性别。因此，意味着覆盖不论雄性还是雌性的成年受试者和新生儿受试者以及胎儿。受试者的实例包括人、狗、猫、牛、山羊和小鼠。术语受试者进一步旨在包括转基因物种。术语受试者还包括用含有本文所公开的重组人MIS蛋白的组合物向其提供处理(例如，治疗性处理和/或预防性处理)的哺乳动物(例如人)。

[0112] 术语“组织”旨在包括完整细胞、血、血制品(例如血浆和血清)、骨、关节、肌肉、平滑肌、以及器官。

[0113] 术语“疾病”或“紊乱”在本文中可互换使用，是指中断或干扰功能执行和/或引起折磨人或与人有联系的症状(例如不适(discomfort)、功能障碍(dysfunction)、痛苦(distress)、或甚至死亡)的身体状态或一些器官的状态的任何变化。疾病或紊乱也可涉及精神不佳(distemper)、小病(ailing)、微恙(ailment)、疾患(amlady)、紊乱、患病(sickness)、病态(illness)、身体不适(complaint)、轻病(inderdisposion)、感染(affection)。

[0114] 术语“恶性肿瘤(malignancy)”和“癌症”在本文中可互换使用，指特征为细胞不受控制地异常生长的疾病。癌细胞可局部扩散或通过血流和淋巴系统扩散至身体的其它部分。该术语还旨在包括哺乳动物器官或组织的任何如下疾病：该疾病特征为该组织中的正常或异常细胞扩增控制不良或不受控制及其对整个身体的影响。该定义范围内的癌症疾病包括良性肿瘤、发育异常(dysplasias)、增生以及显示出转移生长或任何其它转化的肿瘤(例如，就像往往出现在癌症爆发前的粘膜白斑(leukoplakias))。

[0115] 本文所用的术语“肿瘤”是指特征至少部分在于含有血管生成脉管系统(angiogenic vasculature)的转化细胞团块。转化细胞的特征在于瘤性的(neoplastic)不受控制的细胞扩增，该扩增快速并甚至在引发新生长的刺激终止后仍继续。术语“肿瘤”用于广义地包括肿瘤实质细胞(tumor parenchymal cells)以及支持基质(supporting stroma)(包括渗入肿瘤实质细胞块的血管生成性血管)。虽然肿瘤通常是恶性肿瘤，即，具有转移能力的癌症(即转移性肿瘤)，肿瘤也可以是非恶性的(即非转移性肿瘤)。肿瘤是癌症的标志(hallmarks)，是一种自然病程致命的瘤性疾病。癌细胞表现出侵袭和转移的特性，并且是高度间变性的(anaplastic)。

[0116] 本文所用的术语“转移”或“转移性肿瘤”是指在不同于原发性肿瘤的身体其它地方独立生长并由脱离的运输的细胞(transported cell)生成的继发性肿瘤，其中，原发性肿瘤为实体瘤。本文所用的原发性肿瘤是指起源于其所存在的位置或器官，而不是从另一位置转移到该位置的肿瘤。本文所用的“恶性肿瘤”是指具有侵袭和转移特性并显示出高间变(anaplasia)程度的肿瘤。间变是细胞向未成熟或较少分化形式的逆转，它发生在大多数恶性肿瘤中。

[0117] 本文所用的术语“疗法耐受性癌症(therapy resistant cancer)”是指存在于受试者中的对至少两种不同的抗癌剂(例如化疗剂)耐受或有抵抗力(refractory)的癌症，这通常意味着受试者已经用至少两种不同的抗癌剂进行了治疗，但未提供有效的治疗(如该术语在本文中所定义的)。

[0118] 术语“致敏/敏化(sensitize/sensitizes)”在本文中可互换使用，指使细胞对其它二级药剂(secondary agents)敏感或使细胞易受其它二级药剂影响，例如，其它前药或其它环境影响(如辐射等)。

[0119] 本文所使用的术语“治疗(treat/treating/treatment)”指的是治疗性处理和预防性(prophylactic)或防护性(preventative)措施，其中，目的在于预防或减慢疾病的发展，例如减慢肿瘤的发展、癌症的扩散、或减少与不适当的增殖或细胞块有关的病症、疾病或紊乱(例如癌症)的至少一种效应或症状。若一种或多种症状或临床标志物降低(如该术语在本文中所定义的)，则治疗一般是“有效的”。或者，若疾病进展减轻或停止，则治疗是“有效的”。也就是说，“治疗”不仅包括症状或标志物改善，还包括疾病或紊乱(例如，癌症)

的一种或多种症状和/或可测量标志物的可测量减小和/或使预期在不治疗的情况下将发生的症状进展或恶化停止或至少减慢。可测量的减小包括可测量标志物或症状的任何统计学显著下降。有益的或期望的临床结果包括但不限于：减轻一种或多种症状、疾病程度缩减、稳定(即不恶化)疾病状态、延迟或减慢疾病进展、改善或缓和疾病状态、以及缓解(无论是部分还是全部)，无论上述结果是否可检测。“治疗”还可以指与未得到治疗时预期的生存期相比而言延长了生存期。需要进行治疗的受试者包括已经诊断患有癌症的受试者、以及由于转移可能发展成继发性肿瘤的受试者。在一些实施方式中，治疗可以是预防性治疗。

[0120] 本文所用的术语“有效量(effective amount)”是指本文所公开的重组人MIS蛋白减轻疾病或紊乱的至少一种或多种症状的量，并涉及足以提供所需效果的药物组合物的量。本文所用的短语“治疗有效量”(例如，包含本文所公开的至少一种重组人MIS蛋白的药物组合物的治疗有效量)指在可适用于任何医药治疗的合理效益/风险比下足以治疗紊乱的组合物的量。因此，术语“治疗有效量”是指足以实现与癌症或癌症介导的病症相关的症状或临床标志物的治疗性显著下降或预防性显著下降的本文所公开的组合物的量。

[0121] 症状的治疗性显著下降或预防性显著下降是指相比对照受试者或未治疗的受试者，测量的参数下降例如至少约10％、至少约20％、至少约30％、至少约40％、至少约50％、至少约60％、至少约70％、至少约80％、至少约90％、至少约100％、至少约125％、至少约150％或更多。测量的参数或可测量的参数包括疾病的临床上可检测的标志物，例如，生物标志物的水平增高或降低；以及涉及疾病或紊乱的症状或标志物的临床上认可的指标的参数。然而，应该理解的是，本文所公开的组合物和制剂的每日总用量(total daily usage)将由主治医师在健全的医学判断范围内来决定。所需的精确量将根据诸如要治疗的疾病类型等因素而变化。

[0122] 关于用含有本文公开的至少一种重组人MIS蛋白的药物组合物对患有癌症的受试者进行的治疗，术语“治疗有效量”是指安全且足以预防或延迟癌症患者中肿瘤的发展及进一步生长或转移扩散的量。因此，该量可治愈癌症或使癌症变得缓解、减慢癌症进展的过程、减慢或抑制肿瘤生长、减慢或抑制肿瘤转移、减慢或抑制继发性肿瘤在转移位点的建立、或抑制新的肿瘤转移的形成。用于治疗癌症的有效量取决于待治疗的肿瘤、肿瘤的严重程度、肿瘤的药物耐受水平、要治疗的物种、受试者的年龄和一般状况、给药方式等。因此，不可能指定精确的“有效量”。然而，对于任何给定情况，适当的“有效量”可由本领域普通技术人员仅使用常规实验来确定。治疗的效力可由本领域普通技术从业者判断，例如，可在癌症和肿瘤的动物模型(例如患有癌症的啮齿动物的治疗)中对效力进行评估，并且，导致癌症的至少一种症状减少(例如，肿瘤的大小减小或肿瘤的生长速率减慢或停止)的任何治疗或组合物给药或制剂给药表示有效的治疗。在组合物被用于治疗癌症的实施方式中，该组合物的效力可以使用癌症的实验动物模型(例如，野生型小鼠或大鼠)或优选使用肿瘤细胞移植来判断。当使用实验动物模型时，当癌症症状的下降(例如，肿瘤大小的下降或肿瘤生长速率的减慢或停止)在处理的动物中比未处理的动物发生得早时，治疗的效力得到证实。“比……早(earlier)”是指减少(例如，肿瘤大小的减少)的发生早至少5％，但优选更多，例如，早1天、早2天、早3天或更多。

[0123] 当就癌症治疗而使用时，本文所用的术语“治疗”用于指减少癌症的症状和/或生化标志物，例如，至少一种癌症生化标志物的显著下降将被认为是有效的治疗。此类癌症生

化标志物的实例包括CD44、端粒酶、TGF-α、TGF-β、erbB-2、erbB-3、MUC1、MUC2、CK20、PSA、CA125和FOBT。癌细胞增殖速率下降至少约10％也将被认为是由本文所公开的方法实现的有效治疗。作为替代的实例，以下情况的癌症症状下降也将被认为是由本文公开的方法实现的有效治疗：例如，癌症的生长速率减慢至少约10％或肿瘤大小的增加停止，或肿瘤大小下降至少约10％，或肿瘤扩散(即，肿瘤转移)下降至少约10％。在一些实施方式中，优选治疗剂实际上杀死肿瘤，但这并非必要。

[0124] 术语“预防有效量(prophylactically effective amount)”指在必要的剂量和时间段上有效实现期望预防结果(例如预防处于发展成癌症的风险中的受试者的癌症发作)的重组人MIS蛋白或其功能片段或变体的量。通常，由于重组人MIS蛋白或其功能片段或变体的预防剂量是在癌症之前或在癌症早期阶段给予受试者，或给予具有患癌症的遗传倾向的受试者(例如但绝不限于给予在基因中具有增加受试者患卵巢癌的可能性的突变的受试者)。在一些实施方式中，预防有效量小于治疗有效量。重组人MIS蛋白或其功能片段或变体的预防有效量也为化合物的有益效果超过任何毒性或有害效果的量。

[0125] 本文所用的术语“预防/防止(prevent/preventing/prevention)”是指避免或延迟疾病或紊乱(例如自身免疫疾病)的一种或多种症状或可测量标志物的显现(manifestation)。症状或标志物显现的延迟是相对于此类症状或标志物在具有发展成所述疾病或紊乱的相似可能性或易感性的对照受试者或未治疗受试者中显现出的时间来说延迟。术语“预防/防止”不仅包括避免或预防疾病的症状或标志物，也包括相比具有发展成所述疾病或紊乱的相似可能性或易感性的对照个体或未治疗个体中的那些症状或标志物、或相比基于受所述疾病或紊乱影响的群体的历史测量或统计测量而可能出现的症状或标志物，所述疾病的症状或标志物中的任何一种的严重性或程度降低。“降低的严重性”是指相比对照或参比，症状或可测量疾病标志物的严重性或程度降低至少10％，例如至少15％、20％、30％、40％、50％、60％、70％、80％、90％、95％、99％或甚至100％(即，无任何症状或可测量标志物)。

[0126] 本文所用的术语“给药/给予”和“引入”在本文可互换使用，指通过使重组人MIS蛋白至少部分定位于所希望的位点的方法或途径，将本发明的代谢调节剂的药剂放置到受试者中。可将本发明的化合物通过在受试者中引起有效治疗的任何适当途径来进行给药。在一些实施方式中，对于癌症的治疗，可将重组人MIS蛋白直接放置在肿瘤位点或放置在肿瘤位点附近，或者可将所述重组人MIS蛋白全身给药。

[0127] “组合物”或“药物组合物”在本文中可互换使用，指通常含有本领域常规的并适于向细胞给予的赋形剂(excipient)(例如药学上可接受的载体)的组合物。所述细胞可为受试者的部分，例如用于治疗目的、诊断目的或预防目的。该细胞也可以是培养的，例如作为用于筛选潜在药物组合物的分析的一部分的细胞，并且该细胞可以是用于研究目的的转基因动物的一部分。组合物也可以是细胞培养物，其中，多肽或编码本发明的代谢调节剂的多核苷酸存在于细胞和/或培养基中。此外，如本领域已知和本文所述的，用于局部(例如口腔粘膜、呼吸道粘膜)给药和/或口服给药的组合物可形成溶液剂、混悬剂、片剂、丸剂、胶囊剂、持续释放制剂、口腔含漱剂(oral rinses)或粉剂。组合物还可包含稳定剂和防腐剂。关于载体、稳定剂和佐剂(adjuvants)的实例参见University of the Sciences in Philadelphia(2005)Remington:The Science and Practice of Pharmacy with Facts

and Comparisons,第21版。

[0128] 本文所用的短语“胃肠外给药(parenteral administration/administered parenterally)”是指除了肠内给药和局部给药以外的给药模式(通常通过注射进行),并且不受限制地包括:静脉内注射和输注、肌内注射和输注、动脉内注射和输注、鞘内注射和输注、心室内注射和输注、囊内注射和输注、眼眶内注射和输注、心内注射和输注、皮内注射和输注、腹膜内注射和输注、经气管注射和输注、皮下注射和输注、表皮下(subcuticular)注射和输注、关节内注射和输注、囊下(sub capsular)注射和输注、蛛网膜下注射和输注、脊柱内(intraspinal)注射和输注、脑脊髓内(intracerebro spinal)注射和输注以及胸骨内注射和输注。本文所用的短语“全身给药/系统给药(systemic administration/administered systemically)”和“外周给药(peripheral administration/administered peripherally)”意味着使得重组人MIS蛋白进入动物的系统(system)内,从而经历代谢及其它相似过程的重组人MIS蛋白给药(例如皮下给药)。

[0129] 短语“药学上可接受的”在本文中用来指在健全的(sound)医学判断范围内,适合用于与人类和动物组织相接触而无过度毒性、刺激、过敏反应或者其它问题或并发症(complication),具有合理的收益/风险比的化合物、材料、组合物和/或剂型。

[0130] 本文所用的短语“药学上可接受的载体”是指参与维持主题试剂的活性或者将主题试剂从身体的一个器官或部分携带或运送至身体的另一器官或部分的药学上可接受的材料、组合物或辅料(例如液态或固态的填充剂、稀释剂、赋形剂、溶剂或封装材料)。除了为“药学上可接受的”(如该术语在本文中所定义的)外,从与剂型的其它成分相容的意义上来说,各载体也必须是“可接受的”。药物制剂含有本发明的化合物和一种或多种药学上可接受的成分。载体可以是固体、半固体或液体稀释剂、霜或胶囊形式。这些药物制剂是本发明的另一个目的。活性化合物的量通常为制剂的0.1wt%-95wt%,在用于胃肠外用途的制剂中优选为0.2wt%-20wt%,在用于口服给药的制剂中优选为1wt%-50wt%。对于本发明方法的临床用途,将本发明的靶向递送组合物配制成用于以下给药方式的药物组合物或药物制剂:胃肠外给药,例如静脉内给药;粘膜给药,例如鼻内给药;肠内给药,例如口服给药;局部给药,例如经皮给药;眼睛给药,例如通过角膜划痕(corneal scarification),或其它给药方式。该药物组合物含有本发明的化合物与一种或多种药学上可接受的成分。载体可以是固体、半固体或液体稀释剂、霜或胶囊形式。

[0131] 本文所用的术语“癌基因(oncogene)”是指编码正常基因的突变版本和/或过表达版本的多肽的核酸序列,正常基因的突变版本和/或过表达版本处于显性方式时可能解除细胞在生长上的正常限制,并由此单独地或与其它改变协同导致细胞的致瘤性(tumorigenicity)。癌基因的实例包括:gp40(v-fms);p21(ras);p55(v-myc);p65(gag-jun);pp60(v-src);v-abl;v-erb;v-erba;v-fos等。原癌基因(proto-oncogene)是指表达癌基因的正常细胞等同物的正常表达的基因,典型地,这些基因通常为涉及信号转导或细胞生长调控的基因。

[0132] 术语“再生”意味着细胞群、器官或组织的再生长,在一些实施方式中为在疾病或创伤之后。

[0133] 术语“载体”指能够运送与其连接的另一核酸的核酸分子;质粒是由“载体”所包含的一个类型。术语“载体”通常是指含有复制起点和对于在宿主细胞中复制和/或维持所必

需的其它实体的核酸序列。能够指导与其可操作地连接的基因和/或核酸序列表达的载体在本文中被称为“表达载体”。通常，具实用性的表达载体通常处于“质粒”形式，这是指环状双链DNA环，处于其载体形式的DNA环不与染色体结合，并且所述质粒通常包含用于稳定表达或瞬时表达编码DNA的实体。可用于本文所公开的方法的其它表达载体包括但不限于质粒、游离体(episomes)、细菌人工染色体(bacterial artificial chromosomes)、酵母人工染色体、噬菌体或病毒载体，并且此类载体可整合到宿主基因组中，或可在特定细胞中自主复制。载体可为DNA载体或RNA载体。也可使用本领域技术人员已知的提供等同功能的其它形式的表达载体，例如自我复制的染色体外载体或整合入宿主基因组中的载体。优选的载体为能够自主复制和/或表达与其连接的核酸的载体。能够指导与其可操作地连接的基因表达的载体在本文被称为“表达载体”。表达载体可导致DNA的稳定表达或瞬时表达。用于本发明的示例性表达载体为pcDNA3.1。

[0134] 术语“病毒载体”是指使用病毒或病毒相关的载体作为核酸构建体进入细胞的载体。构建体可被整合并包装进非复制、缺陷型病毒基因组(像腺病毒、腺相关病毒(AAV)、或单纯疱疹病毒(HSV)或其它，包括逆转录病毒载体和慢病毒载体)，以感染或转导到细胞中。该载体可并入细胞基因组，或者可不并入到细胞基因组。构建体可包含用于转染的病毒序列(如果需要)。或者，可将构建体并入能够附加型复制(episomal replication)的载体，例如EPV载体和EBV载体。

[0135] 本文所使用的“启动子”或“启动子区(promoter region)”或“启动子元件(promoter element)”在本文中可互换使用，指的是控制与其可操作地连接的核酸序列的转录的核酸序列区段，通常为但不限于DNA或RNA或它们的类似物。启动子区包含足以用于RNA聚合酶识别、结合和转录起始的特定序列。启动子区的这一部分被称为启动子。此外，启动子区包含调节RNA聚合酶的这种识别、结合和转录起始活性的序列。这些序列可以是顺式作用的，或可以响应于反式作用因子。启动子可为组成型或调控型，这取决于调控的性质。

[0136] 术语“调控序列”与“调控元件”在本文可互换使用，是指调节与其可操作地连接的核酸序列的转录、并由此作为转录调节剂起作用的核酸区段，通常为但不限于DNA或RNA或它们的类似物。调控序列调节与其可操作地连接的基因和/或核酸序列的表达。调控序列通常包括此类“调控元件”：作为转录结合结构域并由转录蛋白和/或转录因子、阻遏物或增强子等的核酸结合结构域识别的核酸序列。典型的调控序列包括但不限于控制转录的转录启动子、诱导型启动子和转录元件、任选的操作序列，编码合适的mRNA核糖体结合位点的序列以及控制转录和/或翻译终止的序列。调控序列可以是单个调控序列或多个调控序列、或修饰的调控序列、或它们的片段。修饰的调控序列为其中的核酸序列已通过某种手段(例如但不限于突变、甲基化等)改变或修饰的调控序列。

[0137] 本文所用的术语“可操作地连接”是指核酸序列与核苷酸的调控序列(例如启动子、增强子、转录和翻译终止位点、以及其它信号序列)的功能关系。例如，核酸序列(通常为DNA)与调控序列或启动子区的可操作连接是指该DNA与该调控序列或启动子之间的物理和功能关系，从而这样的DNA的转录由该调控序列或启动子通过特异性识别、结合和转录该DNA的RNA聚合酶来起始。为了优化表达和/或体外转录，可能需要修饰调控序列，以用于在表达其的细胞类型中表达该核酸或DNA。此类修饰的期许或需要能够凭经验确定。增强子不必紧邻由它们增强转录的编码序列。此外，通过由第二启动子转录的因子反式调控的启动

子所转录的基因可被认为是可操作地连接至第二启动子。在这样的情况下，第一基因的转录被认为是可操作地连接至第一启动子，同时也被认为是可操作地连接至第二启动子。

[0138] 本文所使用的术语“生物样品”还指的是来自受试者的细胞或细胞群、或一定量的组织或体液。通常来说，样品已从受试者移除，但术语“生物样品”也可以指在体内分析的细胞或组织，即不从受试者中移除。通常，“生物样品”将包含来自受试者的细胞，但该术语也可指非细胞生物材料，例如血、唾液或尿液的非细胞部分(fraction)，这些部分可用于测量蛋白磷酸化水平。在一些实施方式中，“生物样品”或“组织样品”是指由个体分离的组织或流体样品，包括但不限于例如血、血浆、血清、肿瘤活检物、尿液、粪便、痰、脊髓液、胸膜液(pleural fluid)、乳头抽吸物(aspirates)、淋巴液；皮肤、呼吸道、肠道和泌尿生殖道的外部切片(external sections)；眼泪、唾液、乳汁、细胞(包括但不限于血细胞)、肿瘤、器官，且也包括体外细胞培养成分的样品。在一些实施方式中，生物样品来自原发性肿瘤、继发性肿瘤或转移性肿瘤的芯针活检(core needle biopsy)、支气管镜活检或切除术，或为来自胸膜液的细胞块(cellblock)。此外，细针抽吸物生物样品也是有用的。在一些实施方式中，生物样品是原代腹水细胞(primary ascite cells)。样品可以是新鲜的、冷冻的、固定的或任选石蜡包埋的、冷冻或者经受其它组织保存方法，包括例如保存生物样品中多肽的磷酸化状态的方法。生物样品也可以指含有蛋白或细胞的生物组织或流体样品。这样的样品包括但不限于从受试者或动物中分离的组织。生物样品还可以包括组织切片(如活检样品和尸检样品)、用于组织学目的的冷冻切片、血、血浆、血清、痰、粪便、眼泪、粘液、毛发和皮肤。生物样品还包括外植体(explants)和来源于患者组织的原代细胞培养物和/或转化的细胞培养物。生物样品可以通过从受试者中移除细胞样品来提供，但也可以通过使用先前分离的细胞(例如，在其它时间和/或用于其它目的地由另一人分离)或通过在体内执行本发明的方法来完成。也可以使用存档组织(archival tissues)(例如具有治疗或结果历史的组织)。生物样品包括但不限于组织活检、刮取物(例如口腔刮取物)、全血、血浆、血清、尿液、唾液、细胞培养物、或脑脊髓液。生物样品还包括组织活检、细胞培养物。生物样品可以通过从受试者中移除细胞样品来获得，但也可以通过使用先前分离的细胞(例如，由另一人分离)或通过在体内执行本发明的方法来完成。这样的样品包括但不限于全血、培养的细胞、原代细胞制剂、痰、羊膜液、组织或细针活检样品、腹膜液以及胸膜液等。在一些实施方式中，生物样品取自人类患者；在替代的实施方式中，生物样品取自任何哺乳动物，如啮齿动物、疾病的动物模型、商业动物、伴侣动物、狗、猫、绵羊、牛和猪等。可以根据存储或保存的需要，通过稀释于适当的缓冲液中或浓缩对生物样品进行预处理(如果需要的话)。可使用处于生理pH下的多种标准水性缓冲液中的任一种，所述标准水性缓冲液采用各种缓冲剂之一，例如磷酸盐、Tris等。在某些情况下，在用于本文所公开的分析之前，可将生物样品储存备用。这样的储存可在+4℃或冷冻(例如-20℃或-80℃)下进行，只要一旦将细胞解冻，合适的冷冻保存剂用于维持细胞活力。

[0139] 本文所用的术语“降低/下降(reduced/reduce)”或“减少(decrease或lower)”通常都意味着相对于参比而言减少统计学显著的量。然而，为避免疑义，如本文中对该术语的定义，“降低/下降”意味着相比参比水平而言至少10%的统计学显著性减少，例如相比参比水平而言减少至少20%、至少30%、至少40%、至少50%、或至少60%、或至少70%、或至少80%、至少90%以上，上至并包括减少100%(即，相比参比样品而言的缺失水平)，或减少

10%-100%之间的任意量。在基因的表达或活性水平的上下文中使用的术语“减少”或“抑制”是指细胞、细胞提取物或细胞上清液中蛋白或核酸水平的下降。例如，此类降低可由下降的RNA稳定性、转录或翻译，增加的蛋白降解或RNA干扰所致。优选地，该降低为对照条件下的表达或活性水平的至少约5%、至少约10%、至少约25%、至少约50%、至少约75%、至少约80%、或甚至至少约90%。

[0140] 本文所用的术语“低(low)”通常意味着低了统计学显著的量；为了避免疑义，“低”意味着比参比水平低至少10%的统计学显著性值，例如，比参比水平低至少20%、比参比水平低至少30%、比参比水平低至少40%、比参比水平低至少50%、比参比水平低至少60%、比参比水平低至少70%、比参比水平低至少80%、比参比水平低至少90%、上至并包括比参比水平低100%(即，相比参比样品而言的缺失水平)的值。

[0141] 本文所用的术语“增加/提高(increased/increase)”通常都意味着增加了统计学显著的量；为了避免疑义，如本文中对该术语的定义，“增加/提高”意味着相比参比水平而言至少10%的统计学显著性增加，包括相比参比水平而言增加至少20%、至少30%、至少40%、至少50%、至少60%、至少70%、至少80%、至少90%、至少100%以上；例如包括至少2倍、至少3倍、至少4倍、至少5倍、至少10倍的增加或更大量的增加。在基因或蛋白的表达或活性的上下文中使用的术语“增加/提高”意思是指细胞、细胞提取物或细胞上清液中蛋白或核酸的水平或活性的正向改变。例如，此类增加可由增加的RNA稳定性、转录或翻译，或者减少的蛋白降解所致。优选地，该增加为超过对照条件下的表达或活性水平至少5%、至少约10%、至少约25%、至少约50%、至少约75%、至少约80%、至少约100%、至少约200%、或甚至约500%或更多。

[0142] 本文所用的术语“高(high)”通常意味着相对于参比而言高了统计学显著的量；为了避免疑义，“高”意味着比参比水平高至少10%的统计学显著性值，例如相比参比水平而言高至少20%、高至少30%、高至少40%、高至少50%、高至少60%、高至少70%、高至少80%、高至少90%、高至少100%、高至少2倍、高至少3倍、高至少4倍、高至少5倍、高至少10倍或更高。

[0143] 冠词“一(a/an)”在本文中用于指一个或多于一个(即，至少一个)的该冠词的语法对象(grammatical object)。通过实例的方式，“元件(an element)”意味着一个元件或多于一个的元件。

[0144] 除非在操作实施例中或另有说明，本文所用的表示成分的量或反应条件的所有数字在所有情况下均应被理解为由术语“约”修饰。当与百分比相连使用时，术语“约”可以是指±1%。将通过下面的实施例进一步对本发明进行详细阐述，但本发明的范围不应限于此。

[0145] 应当理解的是，本发明并不限于本文所述的具体方法学、方案和试剂等，并且本文所述的具体方法学、方案和试剂等可以发生变化。本文所使用的术语仅出于描述具体实施方式的目的，而并不意图限制本发明的范围，本发明的范围只通过权利要求进行定义。本发明的其它特征和优势由以下详细描述、附图和以及权利要求将变得显而易见。

[0146] 缪勒抑制物质(MIS)

[0147] 不希望受理论束缚，缪勒抑制物质(MIS)为糖蛋白的TGFβ多基因家族的成员。该基因家族的蛋白均被生产为二聚体前体，并经历翻译后加工来活化，需要切割和解离来释放

生物活性C端片段。MIS为由相同的70kDa的二硫键连接的单体构成的140-kDa二聚体，每个单体由57kDa的N端结构域和12.5kDa的羧基端(C端)组成。因此，MIS含有2个相同的单体(因此被叫做“同二聚体”)，每个单体含有以非共价结合保持的两个结构域(N端结构域和C端结构域)。纯化的C端结构域为生物活性部分，且切割是活性所必需的。N端结构域可辅助体内蛋白折叠，并促进将C端肽递送至它的受体(例如，MISRI和MISRII)。MIS的不可切割突变体是无生物活性的。

[0148] 羧基端活性结构域与其它TGFb家族成员(例如，TGF-B 1、TGF-B 2和TGF-B 3、抑制素、激活素和骨形态发生蛋白)以及生长分化因子(GDF)的成员享有氨基酸同源性。如使用分子建模得到的与TGFb的三维结构的同源性所揭示的，MIS羧基端结构域的结构由参与赋予其结构稳定性的分子内二硫桥和分子间二硫桥的七个半胱氨酸支持(Lorenzo，Donahoe等，未公开的数据)。

[0149] 类似于其它TGFb家族成员，MIS可被纤溶酶切割，从而生成其氨基端结构域和羧基端结构域。该蛋白水解过程对其生理活性是必需的，并发生在与TGFb序列中发现的双碱性切割位点(dibasic cleavage site)相似的位置中的位点处。所生成的产物在非共价复合物中紧密结合，该复合物在低pH下解离；因此，从技术上讲，需要使用纤溶酶处理和分子尺寸排阻色谱的复杂且耗时的方案来增强或完成羧基端与氨基端的分离。

[0150] MIS含有对纤溶酶敏感的两个重要切割位点；主要单碱性位点位于人野生型MIS的氨基酸位置426-427(对应于本文SEQ ID NO:1的氨基酸451-452)。在这一位点的切割(释放MIS的活性羧基端结构域)像是共有的(consensus)弗林蛋白酶切割位点。次要切割位点(被称为“R/S”)(由MIS片段的氨基端测序鉴定出)位于野生型MIS的氨基端结构域中的残基229-230(对应于SEQ ID NO:1的氨基酸254-255)。这一位点含有R/S，但并不遵从用于弗林蛋白酶切割的共有Arg-X-(Arg/Lys)-Arg。在先用外源纤溶酶消化后，纯化的羧基端与MIS氨基端的分离在酸性条件下使用分子尺寸排阻色谱进行。这一技术需要极其注意控制MIS消化，因为在纤溶酶中长时间孵育MIS生成羧基端MIS结构域以及22kDa和34kDa的其它片段(这是由于在主要位点和次要位点均发生切割)，而通过尺寸排阻将它们彼此分开极其困难。由于纤溶酶消化后生成的所有片段均为糖基化的(羧基端结构域例外)，可将麦胚凝集素亲和用作尺寸色谱分离的替代方式，从而纯化MIS的羧基端结构域。纤溶酶切割后，可将生成的片段加载至处于pH 3.5的麦胚凝集素柱上，从而使氨基端结构域与羧基端结构域解离(如Lorenzo等，J.Chromatography，(2001)，776；89-98中所述，以引用的方式将其整体并入本文)。

[0151] 因此，为了克服关于避免MIS的片段生成(例如，由于在主要位点和次要位点均发生切割，同时存在羧基端MIS结构域以及22kDa和34kDa片段)的数个问题，发明人将位于人野生型MIS的氨基酸位置426-427(对应于本文SEQ ID NO:1的氨基酸451-452)的主要切割位点进行了修饰。为了在不需要使用麦胚凝集素亲和或尺寸色谱柱的复杂方法的情况下有助于C端结构域(C端最具柔性的结构域)的纯化，发明人使所述C端结构域的N端含有标签。

[0152] 此外，将野生型MIS蛋白生产为含有N端前导序列的激素原，所述N端前导序列对应于SEQ ID NO:1的氨基酸残基1-25。成熟激素MIS蛋白的加工可涉及蛋白水解切割和前导序列(例如，SEQ ID NO:1的氨基酸1-25)的移除，MIS蛋白主要位点处的切割生成N端结构域和C端结构域，并且，这些结构域形成单体，所述单体通过链间二硫键和链内二硫键二硫连接

至相同的单体，从而形成生物活性同二聚体MIS蛋白。

[0153] 前导序列

[0154] 不希望受理论束缚，前导序列改善宿主细胞中感兴趣的多肽的表达和/或分泌，并且对蛋白的重组生产有用。通常，作为用于通过基因工程程序分泌期待的蛋白的有效方法，以下方法是已知的：其中，含有期待的蛋白(例如MIS)和前原肽(信号肽+前肽)的融合蛋白在宿主细胞中表达，然后在细胞内被宿主的酶切割(加工)，然后被分泌到细胞外。然而，根据这一过程，融合蛋白必须被宿主的酶切割两次，才成为成熟蛋白，这使得成熟蛋白的产率较低，并且成熟蛋白被残留的融合蛋白污染。

[0155] 因此，分泌的蛋白最初以含有前导序列的前体形式在细胞内部表达，以确保能够进入分泌途径。此类前导序列(也称为信号肽)指导表达的产物穿过内质网(ER)的膜。在转移至ER的过程中，信号肽通常被信号肽酶切除。一旦进入分泌途径，蛋白便被运输至高尔基体。来自高尔基体的蛋白可遵循通往区室(例如细胞液泡)或细胞膜的不同路径，或者所述蛋白可被移出细胞，从而分泌至外部培养基(Pfeffer和Rothman(1987)，Ann.Rev.Biochem.56:829-852)。

[0156] 对于分泌蛋白的工业生产，待生产的蛋白需要从宿主细胞或宿主有机体中高效地分泌。信号肽可为例如待生产的蛋白的天然信号肽、异源信号肽、或天然信号肽和异源信号肽的杂合体。然而，使用目前已知的信号肽遇到了几个问题。由非人宿主细胞或有机体生产人蛋白时经常遇到的一个问题是天然信号肽不能确保信号肽的有效转移和/或切割。这导致低比例的蛋白分泌和/或导致由于信号肽的错误切割而呈现N端延长的成熟蛋白的分泌。因此，信号肽的选择对蛋白的工业生产而言非常重要。

[0157] 除了指导蛋白分泌的前导序列外，前体形式还可含有在成熟过程中被切割的补充(supplemental)前导序列。这些补充前导序列(命名为前肽)通常接着信号肽。事实上，所有肽激素、许多生物活性蛋白(例如，生长因子、受体和细胞粘附分子，并包括MIS)以及很多细菌毒素和病毒包膜糖蛋白都含有前肽，所述前肽在翻译后被切掉，从而生成成熟的生物活性蛋白(Seidah和Chretien(1999)，Brain Res.848:45-62)。

[0158] 肽进一步被名为前蛋白转化酶的酶切割。哺乳动物前蛋白转化酶包括例如枯草杆菌蛋白酶(subtilisin)转化酶PCSK1、PCSK2和弗林蛋白酶。弗林蛋白酶广泛表达，并位于反面高尔基体网络(trans-Golgi network)中。弗林蛋白酶在分泌途径区室中通过蛋白水解活化大量前蛋白底物(Thomas(2002)，Nat Rev Mol Cell Biol.3:753-766)。更具体而言，弗林蛋白酶定位于反面高尔基体网络(晚期高尔基体结构，负责将分泌途径蛋白分选至它们最终的目的地，包括细胞表面、内体、溶酶体和分泌颗粒)。已对弗林蛋白酶切割的位点进行了广泛研究。切割位点位于共有序列R-X-L/R-R的羧基端精氨酸之后，其中，X可表示任何氨基酸(Nakayama(1997)，Biochem.J 327:625-635)。当X为赖氨酸、缬氨酸、异亮氨酸或丙氨酸时，切割效率增加(Watanab等(1992)，J Biol.Chem.267:8270-8274)。

[0159] 在一些实施方式中，重组人MIS蛋白含有替代SEQ ID NO:1的MIS蛋白的野生型前导序列的修饰的前导序列。在一些实施方式中，将天然前导序列(SEQ ID NO:1的氨基酸残基1-25)用非MIS前导序列替代，所述非MIS前导序列例如但不限于白蛋白前导序列或其功能片段。在一些实施方式中，非MIS前导序列为人血清白蛋白序列(HSA)，例如，对应于SEQ ID NO:6的前导序列，其由对应于SEQ ID NO:7的核酸编码。

[0160] 在一些实施方式中，HSA序列为SEQ ID NO:6的功能片段，例如，SEQ ID NO:6的至少23个、或至少22个、或至少21个、或至少20个、或至少19个、或至少18个、或至少17个、或至少16个、或至少15个、或至少14个、或至少13个、或至少12个、或至少11个、或至少10个、或少于10个连续氨基酸或非连续氨基酸。HSA前导序列的修饰版本也包含用于本发明，并在美国专利5,759,802中公开，以引用的方式将其整体并入本文。在一些实施方式中，HSA前导序列的功能片段为MKWVTFISLLFLFSSAYS(SEQ ID NO:13)或其变体，这公开在EP专利EP2277889中(将其整体并入本文)。HSA信号序列的前原区(pre-pro region)(例如，MKWVTFISLLFLFSSAYSRGVFRR,SEQ ID NO:6)的变体包括片段，例如HSA信号序列的前区(pre region)(例如，MKWVTFISLLFLFSSAYS，SEQ ID NO:13)或其变体(例如，MKWVSFISLLFLFSSAYS,SEQ ID NO:14)。

[0161] 在一些实施方式中，前导序列是与SEQ ID NO:6的氨基酸残基至少约60%、或至少约70%、或至少约80%、或至少约90%、或至少约95%、或至少约96%、或至少约97%、或至少约98%、或至少约99%相同的前导序列。

[0162] 本文所用的HSA前导序列导致重组人MIS蛋白的产量如预期增加(浓度较高且产量也较高)(参见图2和图3)。然而，HSA前导序列的存在还使来自主要切割位点的切割(对应于SEQ ID NO:1的451/452(或野生型人MIS蛋白的传统氨基酸命名法的426/427)处的切割)令人惊讶且预料不到地增加(参见图2和图3)。这种增加的产率和增加的切割是令人惊讶的，因为伴随着增加的产率(因此由细胞生产了较多的蛋白)，将预期的是减少的切割(因为可用的切割酶的活性变的饱和且承担过多)。然而，并非如此——事实上完全相反，其中，伴随着增加的蛋白生产，来自主要切割位点的切割增加。

[0163] 将其它前导序列涵盖在内以用于本文公开的重组人MIS蛋白中，例如，以用于替代SEQ ID NO:1的氨基酸1-25。此类前导序列在本领域中是众所周知的，并包括US 2007/0141666中公开的含有融合至组织型纤溶酶原激活物前肽的免疫球蛋白信号肽(IgSP-tPA)的前导序列(以引用的方式将其整体并入本文)。许多其它信号肽被用于分泌蛋白的生产。它们中的一种为鼠免疫球蛋白信号肽(IgSP,EMBL登录号M13331)。IgSP由Loh等在1983年首次鉴别(Cell.33:85-93)。已知IgSP在哺乳细胞中提供很好的表达。例如，EP专利号0382762公开了通过在IgSP和辣根过氧化物酶之间构建融合多肽来生产辣根过氧化物酶的方法。

[0164] 例如，其它前导序列包括但不限于：MPIF-1信号序列(例如，GenBank登录号AAB51134的氨基酸1-21，MKVSVAALSCLMLVTALGSQA(SEQ ID NO:15))；斯钙素信号序列(MLQNSAVLLLLVISASA,SEQ ID NO:16)；转化酶信号序列(例如，MLLQAFLFLLAGFAAKISA,SEQ ID NO:17)；酵母交配因子α信号序列(例如，乳酸克鲁维酵母杀伤毒素前导序列)；杂合信号序列(例如，MKWVSFISLLFLFSSAYSRSLEKR,SEQ ID NO:18)；HSA/MFα-1杂合信号序列(也称为HSA/kex2)(例如，MKWVSFISLLFLFSSAYSRSLDKR,SEQ ID NO:19)；乳酸克鲁维酵母杀伤/MFα-1融合前导序列(例如，MNIFYIFLFLLSFVQGSLDKR,SEQ ID NO:20)；免疫球蛋白Ig信号序列(例如，MGWSCIILFLVATATGVHS,SEQ ID NO:21)；Fibulin B前体信号序列(例如，MERAAPSRRVPLPLLLLGGLALLAAGVDA,SEQ ID NO:22)；簇集蛋白前体信号序列(例如，MMKTLLLFVGLLLTWESGQVLG,SEQ ID NO:23)；以及胰岛素样生长因子结合蛋白4信号序列(例如，MLPLCLVAALLLAAGPGPSLG,SEQ ID NO:24)。

[0165] 当需要在细菌系统中生产重组MIS，前导序列可包括如美国申请2011/0020868中

所述的细菌前导序列。已公开了用于表达重组多肽或蛋白的很多其它分泌信号。例如，参见美国专利No.5,914,254；美国专利No.4,963,495；欧洲专利No.0177343；美国专利No.5,082,783；PCT公开No.WO 89/10971；美国专利No.6,156,552；美国专利No.6,495,357、6,509,181、6,524,827、6,528,298、6,558,939、6,608,018、6,617,143；美国专利No.5,595,898、5,698,435和6,204,023；美国专利No.6,258,560；PCT公开No.WO 01/21662、WO 02/068660；以及美国申请公开2003/0044906；美国专利No.5,641,671；以及欧洲专利No.EP 0 121 352，以引用的方式将它们整体并入本文。

[0166] 修饰的切割位点

[0167] 如本文所讨论的，用于临床前使用的MIS蛋白的制备是复杂且低效的。人MIS蛋白由前原蛋白生产，该前原蛋白含有前导序列。前导序列(SEQ ID NO:1的氨基酸1-25)被切除，剩余的蛋白(通常被称为"完整人MIS"，对应于SEQ ID NO:1的氨基酸残基26-560)必须进行额外的翻译后切割，以生成N端结构域和C端结构域。这些N端结构域和C端结构域形成单体，两个相同的单体(含有N端结构域和C端结构域)一起形成并产生同二聚体。完整人MIS最有可能被弗林蛋白酶或相关激素原转化酶PC5(表达于生殖腺)切割为它的N端结构域和C端结构域。切割主要发生在以+1位点具有丝氨酸的$R^{-4}XXR^{-1}$为特征的kex样位点，这使得MIS切割位点是单碱性的，但更多是弗林蛋白酶/hex共有区。纯化的C端结构域是生物活性部分，并且切割对生物活性而言是必需的。在残基229-230(对应于SEQ ID NO:1的氨基酸254-255)处的次要切割位点(其意义未知)不太常被观察到。MIS的非可切割突变体不具有生物活性，人基因中截短羧基端结构域的突变导致持续性缪勒管综合征。由CHO细胞重组表达的MIS蛋白的切割是不完全且低效的，因此需要用外源丝氨酸蛋白酶(例如纤溶酶)进行切割来提高生物活性。

[0168] 本文中，发明人已经修饰了以在-2位点具有R的$R^{-4}XXR^{-1}$为特征的kex样位点，这使得单碱性MIS切割位点更像Kex/弗林蛋白酶共有识别位点。特别是，在一个实施方式中，重组人MIS由前蛋白产生，其中SEQ ID NO:1的位置450处的氨基酸残基已从Q(谷氨酰胺，或Gln)变为R(精氨酸，或Arg)。这一突变可被称为SEQ ID NO:1的Q450R。这对应于用传统蛋白质编号所编号的MIS的氨基酸残基425(Q425R)上的变化，其中，第一个编号的氨基酸开始于前导序列之后。

[0169] SEQ ID NO:1的Q450R的氨基酸序列中的这一变化允许生产具有完全生物活性的人MIS蛋白的高度纯化的、经切割的制剂。

[0170] 在替代的实施方式中，可将MIS蛋白中的主要切割位点(例如，位于人野生型MIS的氨基酸位置426-427(对应于本文中SEQ ID NO:1的氨基酸451-452)处的单碱性位点)修饰成被不同切割酶识别的氨基酸识别位点。例如，可将MIS蛋白中的主要切割位点(例如，位于氨基酸位置426-427处的单碱性位点)修饰成被蛋白酶或肽酶(例如，激素原转化酶(PC))或者由细胞表达并在周围组织中发现的、或由能够在哺乳动物中建立感染的微生物产生的其它切割剂所识别的氨基酸序列。除了氨基酸识别序列外，可由酶切割的肽还可含有一个或多个氨基酸，但对此不作要求；额外的氨基酸可添加到识别序列的氨基末端、羧基末端、或氨基末端和羧基末端两端。鉴于本发明的教导，将氨基酸添加至氨基酸序列的手段(例如，在自动肽合成仪中)以及对肽的切割进行检测的手段(例如，通过用于此类切割的氨基酸产物的色谱分析来进行)对本领域技术人员而言是公知的。

[0171] 激素原蛋白转化酶构成了在结构上与细菌枯草杆菌蛋白酶和酵母kexin相关的丝氨酸蛋白酶家族。这一家族的几个真核成员目前是已知的。激素原转化酶(PC)在特定的碱性残基处(最常在选择的成对碱性残基之后)切割前体多肽,从而生成生物活性肽和蛋白。蛋白的胰岛素家族的很多成员(例如,胰岛素、Igf-1)是PC的底物。

[0172] 增强纯化的标签

[0173] 在一些实施方式中,重组MIS蛋白含有至少一个内部标记或"标签"。在一些实施方式中,标签可为例如c-myc标签、多组氨酸标签或FLAG标签。在一些实施方式中,标签为FLAG标签,例如,SEQ ID NO:8的FLAG标签。FLAG标签可由SEQ ID NO:9的核酸编码。

[0174] 在一些实施方式中,重组人MIS蛋白上的标签是内部的,处于紧邻切割位点下游的羧基端。因为它是C端最具柔性的部分并且不涉及结合受体以及呈现特异性,和C端的"指尖(fingertips)"一样(Papakostas等,2010;Lorenzo等,2002)。在一些实施方式中,该位点的标记最有可能保留生物活性。在一些实施方式中,标签(例如,FLAG标签)位于主要切割位点之后,例如,SEQ ID NO:1的氨基酸450(对应于传统蛋白质命名法的氨基酸残基425)之后。在一些实施方式中,标签位于SEQ ID NO:1的氨基酸残基452和氨基酸残基453(对应于基于MIS蛋白的标准氨基酸命名法的氨基酸残基427和氨基酸残基428)之间。

[0175] 在替代的实施方式中,标签或标记位于SEQ ID NO:1的序列450-560之间的任何位置。在一些实施方式中,将标签插入在SEQ ID NO:1的位置450处的修饰氨基酸之后2个氨基酸残基处。然而,优选标签位置处于MIS的C端结构域的N端,因为它位于C端结构域的C端致使C端结构域完全无活性,从而使MIS蛋白的生物活性显著降低。

[0176] 在一些实施方式中,重组MIS蛋白含有多于一个标签,例如,至少2个标签或至少3个标签、或至少4个标签或多于4个标签。在重组人MIS蛋白中,在一些实施方式中,标签是相继的(例如,一个接着另一个);在一些实施方式中,它们是分散的(例如,间隔的)。优选地,标签不干扰或基本上不影响在结合和活化MISRII中重组MIS蛋白功能的生物活性。在一些实施方式中,当重组MIS蛋白含有多于一个标签时,所述标签是相同的标签。在替代的实施方式中,当重组MIS蛋白含有多于一个标签时,所述标签是不同的标签,例如,重组MIS蛋白可包含FLAG标签和组氨酸标签。Flag标签的小尺寸使它能够被包含在C端的柔性非结合N端结构域中。因此,在一些实施方式中,本领域普通技术人员已知的任何标签可用来替代Flag标签,例如,在约5-10个氨基酸之间的标签、或在约10-15个氨基酸之间的标签、或在约15-20个氨基酸之间的标签、或在20-30个氨基酸之间的标签、或在约30-50个氨基酸之间的标签。在一些实施方式中,不推荐长度大于50个氨基酸的标签,因为标签可能在空间上阻碍C端结构域的柔性N端,由此抑制重组MIS蛋白的生物活性。

[0177] 在一些实施方式中,标签标记的重组人MIS蛋白(例如,带FLAG标签的重组人MIS蛋白)(例如,本文公开的LRF重组人MIS蛋白)(参见图1)可通过单一步骤进行洗脱,来产生具有完全生物活性的高度纯化且高效切割的制剂。当规模扩大时,重组人MIS蛋白的这种纯化将适于临床应用;此外,它对于临床设置和实验设置中的各种结合分析将是有用的。已证明,翻译过程中的蛋白内部标记比蛋白纯化后的标记更有效,因为碘化或生物素化大大降低MIS生物活性。令人惊讶的是,发明人发现,LRF重组人MIS蛋白构建体比野生型MIS更具生物活性。插入FLAG标签序列具有几个其它的明显优势。第一,它独特的氨基酸结构域并不存在于任何其它基因(除了小鼠脑磷酸酶)中,从而使抗FLAG抗体非常特异。第二,用3×FLAG

肽进行的蛋白洗脱对于FLAG MIS是特异性的，而对于非特异性结合至琼脂糖珠上的其它蛋白并不特异。

[0178] 令人惊讶地，当与天然人MIS或与先前制备的未带标签的RAQR/R(SEQ ID NO:28)MIS相比时，带FLAG标签、切割优化的重组人MIS(例如，RF重组人MIS或RARR/S(SEQ ID NO:27)FLAG MIS)具有生物活性，而带FLAG标签、非切割优化的重组人MIS(例如，RAQR/R(SEQ ID NO:28)FLAG MIS)并没有。因为酸性FLAG标签存在于如此接近切割位点的地方可能会损害切割的程度，从而导致活性丧失。因此，发明人没有预料到FLAG标签的添加伴随有增强的切割。此外，CHO(或HEK)细胞中制备的完整RAQR/R FLAG MIS(如SEQ ID NO:28公开的"RAQR/R")是无生物活性的，因为在RAQR/R(SEQ ID NO:28)切割位点上并未发生内源加工，这与Kurian的报道相反(Cancer Res.，1995.1；343-349)(此时构建体缺乏FLAG标签)。另一方面，位置428处丝氨酸的保留和单碱性位点向双碱性位点的转化(对应于使用传统蛋白质命名法的氨基酸位置425处的Q>R，或SEQ ID NO:1的位置450处的Q>R)使内源切割更有效且非常特异。此外，标签(如FLAG)MIS是结合研究的有力工具，并且可用于在无交联的情况下对内源MISRII进行免疫沉淀。因此，在一些实施方式中，标记的重组人MIS蛋白(例如，具有内部FLAG的MIS)在用于生产高纯度且具有生物活性的内部标记形式的MIS的有效方法中有用，可使其规模扩大以用于临床前用途和临床用途、用于MIS结合蛋白的研究、以及用于药代动力学研究中的示踪。

[0179] 人重组MIS蛋白的变体

[0180] 在一些实施方式中，所公开的重组人MIS蛋白可在核心MIS蛋白序列(例如，SEQ ID NO:1的氨基酸残基26-560)中具有修饰(包括SEQ ID NO:1的氨基酸残基450从Q至R的修饰、和/或标签在C端结构域的开始部分的插入)。这样的变体被认为是与野生型MIS蛋白同源的。

[0181] 本文所使用的术语"多肽"是指氨基酸的聚合物及其等同物，而不涉及产物的具体长度；因此，肽、寡肽和蛋白均包括在多肽的定义内。衍生物是相比另一序列而言具有保守氨基酸置换的多肽。衍生物进一步包含蛋白质的其它修饰，包括例如糖基化、乙酰化、磷酸化等修饰。

[0182] 在一些实施方式中，重组人MIS蛋白至少75％、至少80％、至少85％、至少90％、或至少95％相似于同源重组人MIS蛋白。在两个以上多肽序列的情况下，本文所用的"相似性(similarity)"或"相似性百分比(percent similarity)"是指如使用下列序列比较算法之一或通过目测观察所测定的，当就最大对应(maximum correspondence)进行比较和比对时，相同的两个以上的序列或子序列、或者具有相同的指定百分比的氨基酸残基或其保守置换的两个以上的序列或子序列。通过实例的方式，如下面所讨论的，当与第一序列所含的数量同等的氨基酸数量相比时，或者当与已通过本领域已知的计算机相似性程序比对的多肽比对结果相比时，第一氨基酸序列与第二氨基酸序列至少50％、60％、70％、75％、80％、90％或甚至95％相同或基于第二氨基酸序列进行了保守置换时，可认为所述第一氨基酸序列与所述第二氨基酸序列相似。

[0183] SEQ ID NO:1的MIS的同源物和功能衍生物以及功能片段也被涵盖用于本发明，并且还可通过例如由表达文库表达MIS来进行鉴定。(例如，参见Sambrook等(2001)，Molecular cloning:a laboratory manual，第三版，(Cold Spring Harbor,N.Y.,Cold

Spring Harbor Laboratory Press);Ausubel等,同上)。突变的内源基因序列可被称为异源转基因:例如,编码在天然存在的基因组中未知的MIS突变的转基因相对于鼠和非鼠(例如,人)物种是异源转基因。例如,美国专利公开号5,427,780、5,359,033和5,661,126中公开的MIS蛋白(以引用的方式将它们的公开内容并入本文)。

[0184] 核心人MIS蛋白序列(例如,SEQ ID NO:1的氨基酸残基26-560)的一级结构中的变异(包括SEQ ID NO:1的氨基酸残基450从Q至R的修饰、和/或标签在C端结构域的N端结构域的开始部分的插入)、或功能片段或同源物的一级结构中的变异也被涵盖用于本发明,例如,所述变异可包括删除、添加以及置换。置换可为保守的或非保守的。重组人MIS蛋白和变体之间的差异通常保留所期望的性质、减轻或消除不期望的性质、以及增添所期望的性质或新的性质。例如,相比野生型MIS蛋白,重组人MIS蛋白的变体可具有优异的活性。

[0185] 本领域技术人员将理解的是,能够容易地对如本文所公开的重组人MIS蛋白的核心人MIS蛋白序列(例如,SEQ ID NO:1的氨基酸残基26-560)进行操作来改变蛋白的氨基酸序列。可通过用于体外诱变等的各种众所周知的技术对编码MIS蛋白或其功能片段、同源物或变体的基因进行操作,来生产天然存在的人蛋白或其片段的变体(在本文中称为变体或突变体),可根据本发明使用所述变体。

[0186] 对重组人MIS蛋白的其它修饰

[0187] 在本发明中有用的重组人MIS蛋白还可在其氨基端处进行修饰,例如以增加它们的亲水性。增加的疏水性增强基于脂质的载体的表面上肽的暴露,母体肽-脂质缀合物已掺入所述载体中。适于连结至肽以增加它们的亲水性的极性基团是已知的,并包括例如但不限于:乙酰基("Ac")、3-环己基丙氨酰("Cha")、乙酰基-丝氨酸("Ac Ser")、乙酰基-丝氨酰-丝氨酸("Ac-Ser-Ser-")、琥珀酰基("Suc")、琥珀酰基-丝氨酸("Suc-Ser")、琥珀酰基-丝氨酰-丝氨酸("Suc-Ser-Ser")、甲氧基琥珀酰基("MeO-Suc")、甲氧基琥珀酰基-丝氨酸("MeO-Suc-Ser")、甲氧基琥珀酰基-丝氨酰-丝氨酸("MeO-Suc-Ser-Ser")和丝氨酰-丝氨酸("Ser-Ser-")基团、聚乙二醇("PEG")、聚丙烯酰胺、polyacrylomorpholine、聚乙烯吡咯烷酮、多羟基基团和羧基糖(例如,乳糖酸、N-乙酰基神经氨酸和唾液酸)基团。这些糖的羧基基团将经由酰胺键被连接到肽的N端。目前,优选的N端修饰是甲氧基-琥珀酰基修饰。

[0188] 在一些实施方式中,重组人MIS蛋白可融合至一个以上融合伴侣。在某些实施方式中,融合伴侣之一是Fc蛋白(例如,小鼠Fc或人Fc)。融合蛋白可进一步包含第二融合伴侣,例如纯化标签或检测标签,例如可直接或间接检测到的蛋白(如绿色荧光蛋白、血凝素或碱性磷酸酶)、DNA结合结构域(例如,GAL4或LexA)、基因活化结构域(例如,GAL4或VP16)、纯化标签或分泌信号肽(例如,preprotyrypsin信号序列)。

[0189] 在一个实施方式中,在本文所公开的方法和组合物中有用的重组人MIS蛋白融合蛋白可含有人Fc蛋白或其功能片段。因此,在一个实施方式中,在本文所公开的方法和组合物中有用的重组人MIS蛋白融合蛋白可含有人Fc分子作为第一融合伴侣,其中,所述Fc片段可为SEQ ID NO:10或其功能变体或功能衍生物,其中,SEQ ID NO:10如下:

[0190]

LELVPRGSGDPIEGRGGGGGDPKSCDKPHTCPLCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY
TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKATPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS

VMHEALHNHYTQKSLSLSPGK

[0191] 重组人MIS蛋白和载体的变异和修饰可用于增加或减少重组人MIS蛋白的表达，以及提供用于靶向的手段。例如，重组人MIS蛋白可与用于靶向癌细胞或卵巢细胞的靶向分子连接，从而使得重组人MIS蛋白分别对癌组织或卵巢具有特异性。

[0192] 在一个实施方式中，将重组人MIS蛋白融合至第二融合伴侣(例如载体分子)以增强其生物利用度。此类载体在本领域中是已知的，包括聚(烷基)二醇，例如聚乙二醇(PEG)。与血清白蛋白的融合也可以增加治疗性多肽的血清半衰期。

[0193] 在一些实施方式中，也可将重组人MIS蛋白融合至第二融合伴侣，例如，融合至将产物靶向至期待位置的多肽、或者例如促进它的纯化的标签(如果需要的话)。在一些实施方式中，可将标签和融合伴侣设计成可切割的(如果需要的话)。特别考虑的另一修饰是连结(例如，共价连结)至聚合物。在一个方面中，聚合物(如聚乙二醇(PEG)或甲氧基聚乙二醇(mPEG))可增加与它们缀合的蛋白的体内半衰期。多肽药剂的PEG化方法是本领域技术人员公知的，例如使用多大的PEG聚合物的注意事项也一样。

[0194] 在一些实施方式中，对重组人MIS蛋白或其功能片段进行修饰以实现充足的循环半衰期，这影响用量、药物给药以及效力。为了增加生物疗法的半衰期，已进行了很多方法。低于60kD的小蛋白被肾脏快速清除，因此无法达到它们的靶标。这意味着需要高剂量来达到效力。本发明的方法中所涵盖的用来增加循环中蛋白的半衰期的重组人MIS蛋白及片段的修饰包括：PEG化；与蛋白缀合或基因融合，所述蛋白例如为转铁蛋白(WO06096515A2)、白蛋白、生长激素(US2003104578AA)；与纤维素缀合(Levy和Shoseyov，2002)；与Fc片段缀合或融合；糖基化处理和诱变处理(Carter，2006)，以引用的方式将它们并入本文。

[0195] 在PEG化的情况中，将聚乙二醇(PEG)与重组人MIS蛋白或片段缀合，可为例如血浆蛋白、抗体或抗体片段。在20世纪80年代进行了关于抗体的PEG化效果的首次研究。缀合可用酶法或化学方法完成，并且在本领域中已充分建立(Chapman，2002；Veronese和Pasut，2005)。PEG化可增加总大小，从而降低了肾滤过的机会。PEG化进一步防止蛋白水解降解并使得从血液中的清除减慢。此外，据报道，PEG化可降低免疫原性并使溶解性增加。通过加入PEG而得到的改善的药代动力学是由于几种不同的机制：分子大小增加、防止蛋白水解、抗原性降低、以及向细胞受体掩蔽了特定序列。在抗体片段(Fab)的情况下，通过PEG化实现了血浆半衰期的20倍增加(Chapman，2002)。

[0196] 迄今为止，有几个经批准的PEG化药物，例如，2000年上市的PEG-干扰素α2b(PEG-INTRON)和2002年上市的α2a(Pegasys)。针对TNFα的PEG化抗体片段(名为Cimzia或Certolizumab Pegol)在2007年申请FDA批准用于克罗恩病的治疗，并已在2008年4月22日获得批准。PEG化的局限在于难以合成长的单分散物质，特别是当需要超过1000kD的PEG链时。对于很多应用，使用链长超过10000kD的多分散PEG，生成具有不同长度PEG链的缀合物群，这需要广泛的分析来确保各生产之间为等效批次。不同长度的PEG链可能会导致不同的生物活性，由此导致不同的药代动力学。PEG化的另一局限是亲和力或活性减少，如已经用α-干扰素Pegasys观测到的，它只有天然蛋白7％的抗病毒活性，但是由于具有增强的血浆半衰期而具有改善的药代动力学。

[0197] 在一些实施方式中，将重组人MIS蛋白或其片段与长寿命蛋白缀合，所述长寿命蛋白例如为白蛋白，其为67kD并在人中具有19天的血浆半衰期(Dennis等，2002)。白蛋白是血

浆中最丰富的蛋白，参与血浆的pH调节，但也在血浆中用作物质的载体。在CD4的情况下，在将它融合至人血清白蛋白后获得了增加的血浆半衰期(Yeh等，1992)。关于融合蛋白的其它实例是胰岛素、人生长激素、转铁蛋白以及细胞因子(Ali等，1999；Duttaroy等，2005；Melder等，2005；Osborn等，2002a；Osborn等，2002b；Sung等，2003；并参见US2003104578A1、WO06096515A2和WO07047504A2，以引用的方式将它们整体并入本文)。

[0198] 糖基化对血浆半衰期和蛋白活性的影响也已得到广泛的研究。在组织纤溶酶原激活物(tPA)的情况下，新糖基化位点的添加使血浆清除减少，并使效能得到了改善(Keyt等，1994)。糖工程已成功地应用于多种重组蛋白和免疫球蛋白(Elliott等，2003；Raju和Scallon，2007；Sinclair和Elliott，2005；Umana等，1999)。此外，糖基化影响免疫球蛋白的稳定性(Mimura等，2000；Raju和Scallon，2006)。

[0199] 在一些实施方式中，可将重组人MIS蛋白或其片段融合至IgG的Fc片段(Ashkenazi和Chamow，1997)。Fc融合方法已用于例如由Regeneron开发的陷阱技术(例如，IL1陷阱和VEGF陷阱)。使用白蛋白来延长肽的半衰期已在US2004001827A1中公开。对于Fab片段和scFv-HSA融合蛋白，白蛋白的积极效果也已有报道(Smith等，2001)。已证明白蛋白延长的血清半衰期是由于由FcRn介导的再循环过程(Anderson等，2006；Chaudhury等，2003；Smith等，2001)。

[0200] 在一些实施方式中，将重组人MIS蛋白缀合至美国申请2010/0209424中公开的生物素化Fc蛋白，以引用的方式将其整体并入本文。

[0201] 本文所用的术语“缀合物”或“缀合”是指两个以上实体连结以形成一个实体。例如，本发明的方法提供了连接有另一实体的重组人MIS蛋白(即，SEQ ID NO:2或SEQ ID NO:3、或其片段或衍生物或变体)的缀合物，所述另一实体例如为部分(例如，使重组人MIS蛋白稳定的第一融合伴侣，例如Ig载体颗粒，如IgG1Fc)。连结可借助接头、化学修饰、肽接头、化学接头、共价键或非共价键、或蛋白融合或者通过本领域技术人员已知的任何手段。连接可以是永久的或可逆的。在一些实施方式中，可包括数种接头，以便利用缀合物中各接头和各蛋白的期望性质。考虑将柔性接头以及增加缀合物溶解性的接头单独使用或者与本文所述的其它接头一起使用。可通过表达编码接头的DNA将肽接头连接至缀合物中的一个以上蛋白。接头可为酸可切割接头、光可切割接头和热敏感接头。缀合方法是本领域技术人员公知的，并被包含以用于本发明。

[0202] 根据本发明，重组人MIS蛋白(即，SEQ ID NO:2或SEQ ID NO:3，或其片段、衍生物或变体)可经由本领域所知的任何合适的手段连接到第一融合伴侣，参见例如美国专利号4,625,014、5,057,301和5,514,363，以引用的方式将它们整体并入本文。例如，重组人MIS蛋白可直接或通过一个或多个接头共价缀合至IgG1Fc。在一个实施方式中，将本文所公开的重组人MIS蛋白直接缀合至第一融合伴侣(例如Fc)；在替代的实施方式中，可通过接头(例如，运输增强接头)将本文所公开的重组人MIS蛋白缀合至第一融合伴侣(例如，IgG1Fc)。

[0203] 用于将本文所公开的重组人MIS蛋白与第一融合伴侣(例如Fc)缀合的各种各样的方法在本领域中是已知的。此类方法例如由Hermanson公开(1996，Bioconjugate Techniques，Academic Press)和在US 6,180,084和US 6,264,914中公开(以引用的方式将它们整体并入本文)，并且包括例如用于将半抗原连接到通常用于应用免疫学的载体蛋白

的方法(参见Harlow和Lane,1988,"Antibodies:A laboratory manual",Cold Spring Harbor Laboratory Press,Cold Spring Harbor,NY)。已认识到,在一些情况下,重组人MIS蛋白经过缀合可能失去效力或功能,这取决于例如其中使用的缀合方法或化学基团。然而,考虑到存在种类繁多的缀合方法,本领域技术人员能够找到不影响或最低程度影响待缀合的实体(例如,重组人MIS蛋白)的效力或功能的缀合方法。

[0204] 用于将本文所公开的重组人MIS蛋白与第一融合伴侣(例如,Fc)缀合的合适方法包括例如碳二亚胺缀合(Bauminger和Wilchek,1980,Meth.Enzymol.70:151-159)。或者,可将部分(moiety)偶联至如Nagy等,Proc.Natl.Acad.Sci.USA 93:7269-7273(1996)和Nagy等,Proc.Natl.Acad.Sci.USA 95:1794-1799(1998)所述的靶向药剂,以引用的方式将它们各自并入本文。可使用的另一缀合方法是例如高碘酸钠氧化继以合适反应物的还原性烷基化和戊二醛交联。

[0205] 可使用各种不同的接头来将本文所公开的重组人MIS蛋白与第一融合伴侣(例如Fc)缀合,所述接头例如但不限于氨基己基辣根过氧化物酶(HRP)或杂双功能(heterobiofunctional)交联接头(如羰基反应性和巯基反应性交联接头)。杂双功能交联试剂通常含有可在两步骤或三步骤过程中偶联至蛋白和其它大分子上的两个不同的功能靶标的两种反应性基团,这会限制通常与使用同双功能交联接头相关的聚合的程度。此类多步骤方案可对缀合物大小以及组分的摩尔比提供出色控制。

[0206] 术语"接头"是指用于连接两个以上实体(例如,本文所公开的重组人MIS蛋白和第一融合伴侣(例如,Fc))的任何工具(means)。接头可为共价接头或非共价接头。共价接头的实例包括共价键或共价连结至待连接的一个或多个蛋白的接头部分。接头也可为非共价键,例如通过金属中心(例如铂原子)的有机金属键。对于共价连接,可使用各种官能团,例如酰胺基团(包括碳酸衍生物)、醚、酯(包括有机酯和无机酯)、氨基、氨基甲酸乙酯(urethane)和尿素等。为了提供连接,可以通过氧化、羟基化、取代、还原等对效应分子和/或探针进行修饰,以提供用于偶联的位点。应该理解的是,不显著降低如本文所公开的重组人MIS蛋白或第一融合伴侣(例如,Fc)的功能的修饰是优选的。

[0207] 靶向(targeting)。在一些实施方式中,可经由靶向配体使本文所公开的方法和组合物中使用的重组人MIS蛋白、或功能片段、或同源物靶向癌症或卵巢细胞。靶向配体是以高亲和力与靶标(例如,预先选定的细胞上的细胞表面标志物,如表面蛋白(例如,相比任何其它身体组织而言更大程度地存在于预先选定的细胞靶标上的受体))特异性结合的分子(例如,小分子、蛋白或其片段)。因此,在一些实施方式中,可将用于本文所公开的组合物和方法中的重组人MIS蛋白融合至Fc和/或还任选地融合至靶向分子。在一些实施方式中,可将编码靶向配体的核酸融合到编码重组人MIS蛋白、或其片段或同源物或变体的核苷酸。靶向配体的另一实例是来自于人钙粘蛋白的钙粘蛋白结构域的组。与重组人MIS蛋白连结的靶向配体组分可包括能够结合预先选定的靶细胞的天然存在的配体或重组配体或工程化配体、或它们的片段。

[0208] 靶向配体的进一步实例还包括但不限于以高亲和力与预先选定的细胞表面蛋白特异性结合的抗体及其部分。"高亲和力"的意思是据本领域已知的分析方法(例如BiaCore分析)确定的至少摩尔级的平衡解离常数。在一个实施方式中,靶向配体也可包含一个或多个免疫球蛋白结合结构域,所述免疫球蛋白结合结构域分离自针对选择的组织特异性表面

蛋白或靶组织特异性受体而生成的抗体。本文使用的术语“免疫球蛋白或抗体”是指特异性结合并识别抗原的哺乳动物(包括人)多肽,所述多肽含有来自免疫球蛋白基因或其片段的框架区域(framework region),其中,在本发明的情况下,所述抗原为组织特异性表面蛋白、靶组织特异性受体、或它们的部分。如果目的靶向融合多肽将用作哺乳动物治疗剂,免疫球蛋白结合区应来源于相应哺乳动物的免疫球蛋白。如果打算将靶向融合多肽用于非治疗用途(例如,用于诊断和ELISA),免疫球蛋白结合区可来源于人或非人哺乳动物(例如小鼠)。人免疫球蛋白基因或基因片段包括κ、λ、α、γ、δ、ε和μ恒定区基因,以及大量免疫球蛋白可变区基因。轻链被分类成κ或λ。重链被分类成γ、μ、α、δ或ε,从而分别定义了免疫球蛋白类别IgG、IgM、IgA、IgD和IgE。在每个IgG种类中,有不同的同种型(如IgG1、IgG2等)。通常,抗体的抗原结合区在确定结合特异性和亲和力方面是最关键的。

[0209] 人IgG的示例性免疫球蛋白(抗体)结构单元包含四聚体。各四聚体由相同的两对多肽链组成,每对多肽链具有一条轻链(约25kD)和一条重链(约50kD-70kD)。各链的N端定义了约100-110个或更多个氨基酸的可变区,主要负责抗原识别。术语“可变轻链”(VL)和可变重链(VH)分别指这些轻链和重链。抗体以完整免疫球蛋白的形式、或者以通过用各种肽酶消化所生产的许多良好表征的片段的形式存在。例如,胃蛋白酶在铰链区的二硫键下消化抗体,从而产生F(ab)'2(Fab的二聚体,Fab本身是通过二硫键与VH-CH连接的轻链)。可在温和条件下将F(ab)'2还原,以打断铰链区中的二硫键,从而将F(ab)'2二聚体转化为Fab'单体。Fab'单体本质上是带有铰链区一部分的Fab。尽管根据对完整抗体的消化定义了多种抗体片段,本领域技术人员将理解的是,此类片段可以通过化学方法或通过使用重组DNA方法从头合成。因此,本文所用的术语免疫球蛋白或抗体还包括通过完整抗体的修饰所产生的抗体片段、或使用重组DNA方法从头合成的抗体片段(例如,单链Fv(scFv))、或使用噬菌体展示文库鉴定的抗体片段(参见例如McCafferty等,(1990),Nature 348:552-554)。此外,本发明的融合多肽包含免疫球蛋白重链(VH)或轻链(VL)的可变区;以及组织特异性表面蛋白及其靶受体结合部分。此类可变区的生产方法描述于Reiter等,(1999)J.Mol.Biol.290:685-698。

[0210] 制备抗体的方法是本领域已知的。参见例如Kohler&Milstein(1975)Nature 256:495-497;Harlow&Lane(1988)Antibodies:a Laboratory Manual,Cold Spring Harbor Lab.,Cold Spring Harbor,NY。编码感兴趣的抗体的重链和轻链的基因可克隆自细胞,例如,编码单克隆抗体的基因可克隆自杂交瘤并被用于生产重组单克隆抗体。编码单克隆抗体的重链和轻链的基因文库也可由杂交瘤或浆细胞制成。重链和轻链基因产物的随机组合生成具有不同抗原特异性的大抗体池。用于生产单链抗体或重组抗体的技术(美国专利号4,946,778、美国专利号4,816,567)能适于产生本发明的融合多肽和方法中使用的抗体。此外,转基因小鼠或其它生物(如其它哺乳动物)可用于表达人抗体或人源化抗体。或者,噬菌体展示技术可用于鉴定与选定抗原特异性结合的抗体、抗体片段(如可变结构域)以及异聚Fab片段。

[0211] 优选的免疫球蛋白(例如,抗体)的筛选和选择可通过本领域已知的多种方法来进行:例如,可通过使用基于ELISA的方法或噬菌体展示来进行对组织特异性受体或靶受体具有特异性的单克隆抗体的存在的初步筛选。优选进行二次筛选来鉴定和选择期待的单克隆抗体,以用于构建本发明的组织特异性融合多肽。二次筛选可用本领域已知的任何合适方

法进行。称为“Biosensor Modification-Assisted Profiling”(“BiaMAP”)的一种方法(美国专利公开2004/101920)允许快速鉴定产生具有所期望特性的单克隆抗体的杂交瘤克隆。更具体而言,基于对抗体:抗原相互作用的评价,将单克隆抗体分选成不同的表位相关组。

[0212] 重组人MIS蛋白的生产

[0213] 本文所公开的重组人MIS蛋白及其功能片段和衍生物可通过任何合适的方法得到。例如,多肽可使用常规重组核酸技术(如DNA或RNA,优选DNA)来生产。关于使用重组DNA技术生产多肽的方法和材料的指导和信息可在很多论文和参考手册中找到。参见例如Sambrook等,1989,Molecular Cloning-A Laboratory Manual,第2版,Cold Spring Harbor Press;Ausubel等(编),1994,Current Protocols in Molecular Biology,John Wiley&Sons,Inc.;Innis等(编),1990PCR Protocols,Academic Press。

[0214] 或者,可通过化学合成(例如根据供应商的说明书使用商业化肽合成仪)直接获得重组人MIS蛋白或其功能片段。用于多肽化学合成的方法和材料是本领域公知的。参见例如Merrifield,1963,“Solid Phase Synthesis”,J.Am.Chem.Soc.83:2149-2154。

[0215] 在一些实施方式中,在引入编码蛋白的DNA(例如,编码重组人MIS蛋白、或其同源物或功能衍生物的核酸)(例如,在本文所公开的常规表达载体中、或通过导管(catheter)、或通过用经核酸离体转化并移植进受试者中的细胞)后,重组人MIS蛋白、或其功能片段或衍生物或变体可在细胞中表达。

[0216] 确定重组人MIS蛋白的活性的分析

[0217] 在一个实施方式中,器官培养分析系统可用于分析本文所公开的人重组MIS蛋白的生物活性。所使用的分析系统描述于Donahoe等,J.Surg.Res.,23,141-148,1977(缪勒退化器官培养分析)中。从14天雌性大鼠胚胎中切出尿生殖脊,并转移到器官培养皿(Falcon,3010)中。将样本放置在涂有2%琼脂薄层的不锈钢网格上,并在37℃、5%CO_2和95%空气中,在2ml培养基[CMRL 1066,含有10%胎牛血清、1%青霉素(10,000单位/ml)]中或在培养基与待测试的上清液或梯度部分(gradient fraction)的1:1混合物中培养72小时。然后在44℃用2%琼脂和白蛋白的混合物涂覆培养的组织,在缓冲甲醛中固定,在乙醇中脱水,在二甲苯中清洗,并包埋在石蜡中。用苏木精和曙红对八微米连续切片进行染色,以用于通过光学显微镜进行观察。给来自缪勒管头端(cephalic end)的切片分配编号,并基于等级0-V对退化进行分级(Donahoe等,Biol.Reprod.,15,329-334,1976)。每个分析读取5个载玻片,每个载玻片上有6-8个切片。活性的等级以最接近平均值的整数列出。用于分级分馏(fractionation)过程的测试组代表至少10个分析。如果平均值落在两个数字中间,则这两个数字都列出。0级是指没有退化。缪勒管具有广泛的开放内腔(widely patent lumen),缪勒管内衬有细胞核为基底取向(basilar orientation)的柱状上皮细胞。I级是最小退化。缪勒管稍微变小,并且周围间充质围绕着管凝聚(如在塑料切片中所见)或者管的周围存在透明区域(如在石蜡切片中所见)。II级指轻度退化。缪勒管较小,并且间充质凝聚或管周围的透明区域更加明显。较短的上皮细胞的细胞核摆脱了它们的基底取向。III级是中度退化。管非常小且是无组织的(disorganized)。在沃尔弗管的远端,尿生殖脊的顶部发育不佳。IV级为重度退化。管被环生的细胞代替。V级是指完全退化。未检测到管的剩余。各实验中均包括阳性组织对照(使用胎儿睾丸)和阴性组织对照(其中,将缪勒管单独孵育或与肌肉一起孵育)。将暴露于来自非睾丸组织的提取物、暴露于非活性睾丸部分、或暴露于盐水

的缪勒管用作生化对照。用蒸馏水对所有部分的等分试样进行透析并将其冷冻干燥，并测定蛋白质含量。

[0218] 重组人MIS蛋白的递送

[0219] 本领域中已知的用于重组人MIS蛋白和/或编码该蛋白的核酸的治疗性递送的方法可用于在受试者中治疗疾病或紊乱(例如癌症)，例如，细胞转染、基因疗法、使用递送载体或药学上可接受的载体直接给药、通过提供含有编码本发明的靶向融合多肽的核酸的重组细胞间接递送。

[0220] 在一些实施方式中，在体外对重组人MIS蛋白进行切割以形成MIS的生物活性halo-二聚体，然后给予受试者，所述二聚体含有两个相同的单体，各单体由N端结构域和C端结构域组成。

[0221] 多种递送系统是已知的，并且可以用于向受试者给予重组人MIS蛋白(将它切割成它的生物活性形式之前或之后)，例如，包封在脂质体中、微颗粒、微胶囊、能够表达该化合物的重组细胞、受体介导的内吞作用(参见例如Wu和Wu，1987，J.Biol.Chem.262:4429-4432)、将核酸构建为逆转录病毒载体或其它载体的一部分等。引入方法可以是肠内的或胃肠外的，并包括但不限于皮内途径、肌内途径、腹膜内途径、静脉内途径、皮下途径、肺途径、鼻内途径、眼内途径、硬膜外途径以及口服途径。重组人MIS蛋白可通过任何方便的途径(例如，通过输注或快速浓注(bolus injection)，通过经由上皮内层或粘膜皮肤内层(例如，口腔粘膜、直肠和肠粘膜等)吸收)进行给药，并且可以与其它生物活性剂一起给药。给药可为全身的或局部的。此外，理想的是，在切割成生物活性形式之前或之后，通过任何合适的途径(包括心室内和鞘内注射)将含有重组人MIS蛋白的药物组合物引入中枢神经系统；心室内注射可借助于例如连结到储存器(如Ommaya储存器)的心室内导管。也可采用肺给药，例如，通过使用吸入器或雾化器以及具有雾化剂的剂型。

[0222] 治疗增殖性疾病和癌症的方法

[0223] 本发明的一个方面提供了用于在受试者中治疗癌症(例如，表达MISRII的癌症)的方法。因此，本发明的一个方面大体上涉及在受试者中治疗增殖性疾病或紊乱的方法，其中，所述增殖性疾病或紊乱与表达MIS受体的细胞(例如表达MISRII的细胞)有关。在一些实施方式中，增殖性疾病或紊乱为癌症，其中，所述癌症或癌细胞表达至少一种MIS受体，例如，表达MISRII的癌症或癌细胞。本发明的方法包括向患有增殖性紊乱的受试者给予有效量的本文所公开的重组人MIS蛋白或其功能片段或衍生物，其中，与所述增殖性紊乱有关的细胞表达至少一种MIS受体，例如，所述细胞表达MISRII。例如，将有效量的本文所公开的重组人MIS蛋白或功能片段给予患有表达至少一种MIS受体(例如，表达MISRII)的癌症的受试者。因此，通过使用本发明的方法，可干预增殖性疾病(例如癌症)、改善症状，在某些情况下可治愈疾病。在一些实施方式中，可用于治疗增殖性疾病和癌症的重组人MIS蛋白含有SEQ ID NO:2的氨基酸序列残基25-559或其功能片段。

[0224] 病因是表达至少一种MIS受体(例如表达MISRII)的细胞的增殖的此类疾病的实例为癌症，例如宫颈癌和卵巢癌。在一些实施方式中，表达至少一种MIS受体(例如MISRII)的癌症是癌细胞。在一些实施方式中，表达至少一种MIS受体(例如表达MISRII)的此类癌细胞是例如但不限于卵巢癌细胞、外阴表皮癌细胞、宫颈癌细胞、子宫内膜腺癌细胞、卵巢腺癌细胞。

[0225] 在替代的实施方式中，表达至少一种MIS受体的癌症(例如表达MISRII的癌症)例如但不限于：乳腺癌、肺癌、头颈部癌、膀胱癌、胃癌、神经系统的癌症、骨癌、骨髓癌、脑癌、结肠癌、食管癌、子宫内膜癌、胃肠癌、生殖泌尿系统癌症、胃癌、淋巴瘤、黑色素瘤、神经胶质瘤、膀胱癌、胰腺癌、牙龈癌、肾癌、视网膜癌、肝癌、鼻咽癌、卵巢癌、口腔癌、膀胱癌、血液肿瘤(hematological neoplasms)、滤泡性淋巴瘤、宫颈癌、多发性骨髓瘤、骨肉瘤、甲状腺癌、前列腺癌、结肠癌、前列腺癌、皮肤癌、胃癌、睾丸癌、舌癌或子宫癌。

[0226] 在替代的实施方式中，本发明涉及本文所公开的重组人MIS蛋白、或其功能片段或衍生物或变体用于治疗任何紊乱的用途，其中，给予所述MIS蛋白或编码所述MIS蛋白的核酸或者使MISRII活化是治疗方案的全部或部分。

[0227] 在一些实施方式中，癌症为MIS响应型癌症，例如但不限于卵巢癌和宫颈癌。在一些实施方式中，癌症表达MISRII，例如但不限于卵巢癌和宫颈癌。在一些实施方式中，紊乱是与雄激素过多的状态有关的紊乱，例如如美国专利号6,673,352中所公开的紊乱，以引用的方式将其整体并入本文。在一些实施方式中，本发明的方法用于治疗前列腺癌、多囊卵巢疾病、良性前列腺肥大以及性早熟。

[0228] 在一些实施方式中，癌症为化学疗法耐受性癌症或多药物耐药性癌症，例如，其中所述癌症为紫杉醇耐药性癌症、顺铂耐药性癌症、雷帕霉素耐药性癌症、吡唑并蒽酮耐药性癌症、或阿霉素耐药性癌症。

[0229] 在相关实施方式中，待治疗的组织为受试者的表达至少一种MIS受体(例如表达MISRII)的肿瘤组织，例如，所述肿瘤组织为但不限于实体瘤、转移瘤、皮肤癌、乳腺癌、卵巢癌、宫颈癌、血管瘤或血管纤维瘤或相似的癌症。可由本发明的药物组合物治疗的典型实体瘤组织包括例如但不限于肺、胰腺、乳腺、结肠、喉、卵巢及相似组织的肿瘤。在一些实施方式中，可由本方法治疗的实体瘤组织包括甲状腺，且癌症的类型是甲状腺髓样癌。

[0230] 在相关实施方式中，本发明考虑了将包含本文所公开的重组人MIS蛋白或功能片段的组合物与其它疗法(例如针对实体瘤以及用于控制转移建立的常规化疗)联合给予的方法的实践。例如，用于化疗的化疗剂包括但不限于紫杉醇、顺铂、阿霉素、雷帕霉素、吡唑并蒽酮(包括但不限于蒽(1,9-cd)吡唑-6(2H)-酮(SP600125)或N1-甲基-1,9-吡唑并蒽酮(M-SP600125))，或它们的功能衍生物或功能类似物。在一些实施方式中，化疗剂是放疗剂。本文所述的化合物的给药通常在化疗之前和/或同时和/或之后进行，不过有时在化疗方案后抑制细胞增殖也涵盖在本发明内，其中，肿瘤组织将通过诱导血管生成来响应毒性攻击，从而通过向肿瘤提供血液和营养物供给而得以恢复。另外，本发明的用于治疗增殖性紊乱(例如，癌症)的药物组合物可以预防性给药和/或在肿瘤发展之前给药(如果受试者被鉴定为具有发展为癌症的风险，例如癌细胞或肿瘤的生物标志物呈阳性的受试者)。在本发明的方法适用于抑制细胞增殖的情况下，这些方法也能够适用于抑制肿瘤组织生长、抑制肿瘤转移的形成以及使已建立的肿瘤退化。

[0231] 在一些实施方式中，对获得自受试者的生物样品(例如，癌症或肿瘤组织样品、或癌细胞、或肿瘤细胞，例如活检组织样品)中的缪勒抑制物质(MIS)受体的表达进行测量。

[0232] 流体(例如血液)的细胞中存在MISRII可指示存在癌症。不邻近肿瘤的部位或流体中存在MISRII可指示转移。在一些此类实施方式中，将本发明的化合物给予受试者；在某些实施方式中，在药物组合物中将本发明的化合物给予受试者，所述药物组合物含有一种或

多种其它疗法。

[0233] 本文公开的创新性方法提供了向需要此类治疗的受试者胃肠外给予和口服给予本文所公开的重组人MIS蛋白或其功能片段或衍生物连同其它药物组合物。胃肠外给药包括但不限于静脉内(IV)途径、肌内(IM)途径、皮下(SC)途径、腹膜内(IP)途径、鼻内途径和吸入途径。在本发明的方法中,本文所公开的重组人MIS蛋白或其功能片段或类似物优选口服给药。IV给药、IM给药、SC给药和IP给药可以通过推注(bolus)或输注进行,并且也可以通过缓释植入装置进行,所述缓释植入装置包括但不限于泵、缓释剂型和机械设备。剂型、给药途径和方法、以及剂量将取决于待治疗的紊乱以及受试者的病史。通常,通过皮下注射给予的剂量将大于静脉内给予或肌内给予的治疗等效剂量。优选地,本发明的化合物的剂量将以约0.1mg至约250mg的剂量给药。在一些实施方式中,本发明的化合物的剂量将为约1mg至约60mg。

[0234] 用于治疗表达至少一种MIS受体(例如表达MISRII)的癌症的本发明的方法对于增殖相关的疾病或癌症的治疗有用,所述增殖相关的疾病或癌症与表达至少一种MIS受体(例如MISRII)的细胞有关,所述方法包括将其中正在发生增殖或处于发生风险中的组织与含有治疗有效量的本文公开的重组人MIS蛋白或其功能片段或功能衍生物的组合物接触。

[0235] 在一些实施方式中,在本发明的很多实施方式中用本发明的方法进行治疗的受试者是人受试者,然而可以理解的是,本发明的原理表明本发明对于所有哺乳动物均有效。在这种情况下,将哺乳动物理解为包括在其中期望对与癌症或增殖相关紊乱相关的疾病进行治疗的任何哺乳动物种类,特别是农业哺乳动物种类和家养哺乳动物种类以及转基因动物。

[0236] 用途

[0237] 在另一实施方式中,本发明提供了通过向有需要的受试者给予有效量的本发明的重组人MIS蛋白或其功能衍生物来治疗多种病症的方法。可由本发明的化合物或含有本发明的化合物的药物组合物治疗的病症包括如下病症:通过给予MIS或MIS信号转导的活化或MISRII的活化得到治疗或使症状降低、并由此从给予重组人MIS蛋白或其功能衍生物中受益的任何病症。例如,就这一方面而言,代表性的病症包括但不限于表达MIS受体的癌症,例如表达MISRII的癌症,例如但不限于卵巢癌、宫颈癌和子宫内膜癌。可用MIS或者MIS信号转导的活化来治疗以使症状下降的其它病症是例如类风湿性关节炎、增殖性疾病(如癌症)、前列腺癌、多囊卵巢疾病、良性前列腺肥大和性早熟、以及其它雄激素过多紊乱(如高睾酮血症)。

[0238] 因此,本发明涉及重组人MIS蛋白或其功能衍生物用于治疗任何紊乱的用途,其中,给予所述MIS蛋白或编码MIS蛋白的核酸或MIS的功能衍生物、或使MISRII活化是治疗方案的全部或部分。在一些实施方式中,可使用的重组人MIS蛋白含有SEQ ID NO:2的氨基酸残基25-559或其功能片段。

[0239] 在一些实施方式中,本发明的方法针对重组人MIS蛋白或其功能衍生物连同其它治疗剂(例如,化疗剂)一起的用途,其中,化疗剂(例如紫杉醇)或MIS可在产生减小的副作用的较低剂量下使用。

[0240] 重组人MIS蛋白或其功能衍生物或类似物用于治疗雄激素过多的状态的用途

[0241] 在另一实施方式中,重组人MIS蛋白或其功能衍生物或类似物可用于治疗与受试

者中过多的雄激素产生有关的疾病。本发明人以前已证明，MIS蛋白和/或MIS核酸给药使受试者的雄激素水平降低，并使受试者的雄激素血清水平降低(如美国专利6,673,352和美国专利申请10/683,346中所公开的，以引用的方式将它们整体并入本文)。还证明了过表达MIS的转基因小鼠具有降低的血清睾酮浓度以及MIS给药导致降低的血清睾酮水平(Sriraman等，J Androl.2001，22(5):750-8以及Trbovich等，PNAS，2001Mar 13;98(6):3393-7)。还证明了MIS抑制雄激素刺激的细胞生长和雄激素非依赖性的细胞存活，并且，MIS通过抑制睾丸睾酮合成调控前列腺生长，还在前列腺中在细胞水平上直接调控雄激素诱导的基因表达和生长(Trann等，Mol Endocrinol.2006，20(10):2382-91)。

[0242] 雄激素通过结合至雄激素受体来刺激或控制脊椎动物中雄性特征的发育和维持。雄激素也被称为雄性激素或睾丸激素(testoids)，并且还是所有雌激素(雌性的性激素)的前体。主要且最广为人知的雄激素是睾酮。

[0243] 不希望受理论束缚，肾上腺和/或卵巢过多的雄激素生产使得雄激素过多，这也可由局部组织对循环雄激素的敏感性增加所致。雄激素过多影响不同的组织和器官系统，引起从痤疮到多毛症到弗兰克男性化(frank virilization)的临床症状。

[0244] 雄激素过多症，指雄激素及前体的过多生产和分泌，对于育龄妇女是常见的、有时甚至是严重的内分泌病。过多的雄激素及其前体以不同比例起源于肾上腺和卵巢，取决于过多雄激素的量，体现为不同的影响。临床表现的范围从多毛症(雄性型毛发过度生长，有时伴有痤疮)到男性化(阴蒂增大(clitorimegaly)、暂时秃头、声音低沉、或增强的肌肉)。

[0245] 雄激素过多症作为广泛疾病表现的一部分而发生(包括多囊卵巢综合征(PCOS))，它是以下疾病表现的可变组合：多毛症、不育症、肥胖、胰岛素抵抗和多囊卵巢、HAIR-AN综合征(雄激素过多症、胰岛素抵抗和黑棘皮病)、卵巢滤囊泡膜细胞增殖(HAIR-AN与卵巢间质中黄素化卵泡膜细胞(luteinized theca cells)的增生)、以及高卵巢内雄激素浓度的其它表现(例如，卵泡成熟停止、闭锁、不排卵、痛经、功能失调性子宫出血、不育症)、产雄激素肿瘤(男性化卵巢或肾上腺肿瘤)。

[0246] 多毛症是可识别的毛发过度生长，特征为雄激素敏感区域的终毛(terminal hairs)的数量和长度增加。种族、家族、遗传和人种差异都影响多毛症的发生。多毛症难以量化。需要对整个身体进行检查，并且必须仔细记录调查结果。应特别注意的是下巴、嘴唇、鬓角、乳房、胸骨、肚脐与耻骨和大腿之间的中线。

[0247] Ferriman和Gallwey发表了用于多毛症分级的评定量表，这是本领域普通技术人员公知的。该量表允许医生客观地测量对治疗的响应。该系统使用最广泛，分别用得分0至4来评价身体区域的无多毛症至重度多毛症。8及更高的分数与多毛症的诊断一致。该量表并不测量毛发厚度，这是客观评估过多毛发的另一方式。评分系统有助于量化多毛症，并有助于评估治疗响应。即使得分超过8，患者提供定义。从临床观点看，患者可决定他或她是否注意到差异。照片对于文档编制和跟踪治疗进展有帮助。

[0248] 男性化相对少见；它与极端的雄激素过多症一起发生。男性化的特征是暂时秃头、乳腺萎缩、雄激素性肌肉发育、阴蒂肥大、闭经、声音低沉和极端的多毛症。

[0249] 目前用于女性的医学疗法针对肾上腺、卵巢或雄激素受体。糖皮质激素疗法针对肾上腺，但在某些情况中受到不希望的皮质醇合成抑制的限制。GnRH疗法针对卵巢，但很昂贵，而且它的长期影响未知。此外，使用口服避孕药的疗法可能不合适，因为大多数口服避

孕药都含有具有雄激素活性的孕激素(progestins)。

[0250] 因为雄激素的异常生产牵涉到没有可接受的治疗措施的很多疾病和/或紊乱的通路中,需要找到抑制哺乳动物的促性腺激素和/或雄激素的生产的小分子,以用于它们的治疗和/或预防。

[0251] 因此,在一个实施方式中,重组人MIS蛋白或其功能衍生物或类似物可用于治疗与受试者中过多的雄激素产生有关的疾病。在一些实施方式中,可使用的重组人MIS蛋白含有SEQ ID NO:2的氨基酸残基25-559或其功能片段。

[0252] 术语"雄激素"在本文中用于指激发雄性性征发育的类固醇,包括雄甾烷的类固醇衍生物,包括睾酮、雄烯二酮和类似物。

[0253] 本文所用的以"雄激素依赖性"为特征的疾病状态或紊乱是由不足的、过多的、不适当的或不受调控的雄激素产生加重或引起的疾病状态。在男性中,此类疾病的实例包括但不限于BPH、转移性前列腺癌、睾丸癌、雄激素依赖性痤疮、男性型脱发和男孩的性早熟。在女性中,此类疾病的实例包括但不限于雄激素过多症、多毛症、男性化、POCS、HAIR-AN综合征、卵巢滤囊泡膜细胞增殖、滤泡成熟停止、闭锁、不排卵、痛经、功能失调性子宫出血、不育症、产雄激素肿瘤。

[0254] 本文所用的"雄激素抑制"是指当将如本文所定义的有效量的吡唑并蒽酮或其功能衍生物或类似物(如SP600125)给予受试者以用于预防或治疗由过多或不受调控的雄激素生产加重或引起的疾病状态时,其将使得体内雄激素水平降至正常或亚正常(sub-normal)水平。

[0255] 在一些实施方式中,本文公开的重组人MIS蛋白或其功能衍生物或类似物可用于治疗前列腺癌。雄激素对前列腺癌的影响是已知的,如同通过雄激素脱除来治疗前列腺癌,包括雄激素阻断和抑制雄激素合成(Huggins等,Archs.Surg.,Vol.43,pp.209-223(1941);J.Steroid Biochem.Molec.Biol.,Vol.37,pp.349-362(1990))。此外,类固醇激素被广泛地用作避孕药。抗生精剂(Anti-spermatogenic agent)是抑制精子生成(产生成熟精子的过程)的男用避孕药。干扰这一过程的药物包括雄激素和抗雄激素。由于本文公开的重组人MIS蛋白或其功能衍生物或类似物的抗雄激素作用是可逆的,重组人MIS蛋白也可用作男性避孕剂。Korolkovas,A.,Essentials Of Medicinal Chemistry,第二版,pp.1032(1988)。

[0256] 在一些实施方式中,其它药剂可与本文公开的含有重组人MIS蛋白或其功能衍生物或类似物的药物组合物联用来治疗受试者中过多的雄激素。在一些实施方式中,该药剂作用为降低非血清雄激素水平和阻断外周雄激素作用。此类药剂的实例包括但不限于:通过给予雌激素和/或孕激素(即,避孕药丸)或GnRH激动剂和反加雌激素疗法(add-back estrogen therapy)来抑制卵巢雄激素;通过给予糖皮质激素(如地塞米松、泼尼松龙)、抗雄激素(如螺内酯(spironolactone)、氟他胺(flutamide)、醋酸环丙孕酮)、5α还原酶抑制剂(如非那雄胺)、溴隐亭和胰岛素增敏剂(如二甲双胍、噻唑烷二酮类)来抑制肾上腺雄激素。

[0257] 适于用重组人MIS蛋白或其功能衍生物或类似物通过本文公开的方法治疗的受试者是已被鉴定为患有与过多雄激素水平相关的疾病或紊乱的受试者,如,例如紊乱,例如但不限于BPH、前列腺癌、良性前列腺肥大、睾丸癌、雄激素依赖性痤疮、男性型脱发、性早熟、雄激素过多症、多毛症、男性化、POCS、HIAR-AN综合征、卵巢滤囊泡膜细胞增殖、滤泡成熟停

止、闭锁、不排卵、痛经、功能失调性子宫出血、不育症和产雄激素肿瘤。

[0258] 在一些实施方式中，适于用重组人MIS蛋白或其功能衍生物或类似物通过本文公开的方法治疗的受试者为患有先天性肾上腺增生(congenical adrenal hyperplasma，CAH)的受试者，这通常可由本领域普通技术人员确定。CAH是最典型的常染色体隐性紊乱，其中，酶21-水解酶缺失或功能缺陷。或者，患有CAH的受试者可出现11α-羟化酶和/或3α-羟基-类固醇脱氢酶的功能丧失和/或下降。当这些酶缺失或以低水平起作用时，身体不能生成足够量的肾上腺类固醇激素皮质醇和醛固酮。高水平的ACTH接着发生，刺激肾上腺增生以及用于皮质醇和醛固酮合成的雄激素前体分泌过多。CAH可在子宫内出现，或者可在出生后发展而来。假两性畸形(pseudohermaphroditism)可在出生时呈现。

[0259] 21-羟化酶缺陷是最常见的常染色体隐性紊乱(比囊性纤维化更常见)，其本身表现为升高的17-羟孕酮水平。11a-羟化酶缺陷的特征为升高的11-脱氧-皮质醇(化合物S)水平，并导致升高的去氧皮质酮(DOC)(一种盐皮质激素)水平。高血压和低钾血症可能是11a-羟化酶缺陷的显著特征。CAH的另一种形式，3a-羟基-类固醇脱氢酶缺陷，导致升高的孕烯醇酮、17-羟基-孕烯醇酮和DHEA水平。由于没有皮质类固醇合成，该病症如果没被检查出则是致命的。

[0260] 青春期后表现出的上述酶的部分缺陷通过相同机制导致升高的肾上腺类固醇水平。该升高不如先天性病症中的升高那样显著，该病症被称为非典型(成年型(maturity-onset)或迟发型)CAH。因此，在一些实施方式中，适于用重组人MIS蛋白或其功能衍生物或类似物通过本文公开的方法治疗的受试者为患有非典型(成年型或迟发型)CAH的受试者。

[0261] 在一些实施方式中，适于用重组人MIS蛋白或其功能衍生物或类似物通过本文公开的方法治疗的受试者是睾酮水平约为或超过2.0ng/mL(200ng/dL，8.92nmol/L)或为参考范围上限的至少约2.5倍的雌性受试者。在一些实施方式中，此类受试者具有支持-间质细胞肿瘤(Sertoli-Leydig cell tumors)、门细胞肿瘤(hilus cell tumors)和类脂细胞(肾上腺剩余(adrenal rest))肿瘤，这些是最常见的。在临床诊断时，支持-间质细胞肿瘤达到可触知(palpable)大小，而门细胞肿瘤和类脂细胞肿瘤难以通过任何手段来检测，因为它们尺寸小。

[0262] 在一些实施方式中，适于用重组人MIS蛋白或其功能衍生物或类似物通过本文所公开的方法治疗的受试者为具有肾上腺肿瘤(腺瘤、癌)的受试者，所述受试者分泌升高水平的雄激素。在此类实施方式中，适于通过本文所公开的方法治疗的此类受试者可由具有约7μg/mL(18μmol/L)或超过7μg/mL(18μmol/L)的DHEAS水平来鉴别。

[0263] 适于本文所公开的治疗过多雄激素的状态的方法的其它受试者包括例如典型和非典型(迟发型)CAH，库欣综合征(cushing syndrome)(其中，患有库欣综合征的受试者分泌升高的雄激素)，雄激素过多、胰岛素抵抗和黑棘皮病(HAIR-AN)综合征。在一些实施方式中，适于本文所公开的治疗过多雄激素的状态的方法的其它受试者包括例如患有轻度雄激素紊乱的受试者，例如但不限于排卵PCOS(在超声检查中具有多囊卵巢的排卵雄激素过多症(Ovulatory hyperandrogenic)受试者)、特发性雄激素过多症(排卵雄激素过多症受试者，但在超声检查时具有正常卵巢)；特发性多毛症(具有正常雄激素并具有雄激素表型的受试者)。

[0264] 参比睾酮水平和DHEAS水平是本领域技术人员公知的，并公开于Guay等，

International Journal of Impotence Research(2004)16,112-120中,以引用的方式将其整体并入本文。简要来说,年龄20至49岁之间的女性的正常雄激素水平为:DHEAS约195.6-140.4μg/dl;血清睾酮约51.5-33.7ng/dl;以及游离睾酮1.51-1.03pg/ml。因此,相比DHEAS(195.6μg/dl)、血清睾酮(51.5ng/dl)、游离睾酮(1.51pg/ml)的正常值的最高范围值而言,适于通过本文所公开的方法利用吡唑并蒽酮或其功能衍生物或类似物治疗的受试者的DHEAS或血清睾酮或游离睾酮水平具有至少约20%、或至少约30%、或至少约40%、或至少约50%、或至少约60%、或至少约70%、或至少约80%、或至少约90%、或至少约100%或更大的增加。在一些实施方式中,相比DHEAS(195.6μg/dl)、血清睾酮(51.5ng/dl)、游离睾酮(1.51pg/ml)的正常值的最高范围值而言,适于通过本文所公开的方法利用吡唑并蒽酮或其功能衍生物或类似物治疗的受试者的DHEAS或血清睾酮或游离睾酮水平具有至少约2倍、或至少约3倍、或至少约4倍、或至少5倍、或至少约10倍或更大的增加。

[0265] DHEAS可以由本领域普通技术人员使用来自Diagnostic Products Corporation of Los Angeles,California,USA的试剂盒进行测定。先前已对交叉反应性进行了确定,DHEAS为100%,雄烯二酮为0.121%,9-羟基雄烯二酮为15%,雌酮-硫酸盐为0.046%,雄酮硫酸盐为0.55%,DHEA为0.5%,所测试的所有其它类固醇可忽略不计。游离睾酮可由本领域普通技术人员使用Diagnostic Products Corporation,Los Angeles,California,USA的Coat a Count试剂盒进行测量。先前已对交叉反应性进行了确定,对于二氢睾酮为0.41%,对于雄烯二酮为0.01%,对于甲基睾酮为0.10%,以及对于所测试的所有其它类固醇为0.01%。总血清睾酮水平可由本领域普通技术人员使用ICN Biomedicals Inc.,Diagnostic Division of Costa Mesa,California,USA的Immunochem血清睾酮试剂盒进行测量。

[0266] 确定血清孕烯醇酮和17-羟基孕烯醇酮的分析可由本领域普通技术人员利用来自美国加里福吉亚的Tarzana的Quest Laboratory的试剂盒进行。游离雄激素指数(FAI)可使用下式进行计算:(总睾酮ng/dl×0.0347)/(SHBG nmol/l)×100=FAI。

[0267] 药物组合物的给药

[0268] 重组人MIS蛋白或其衍生物或功能片段可通过本领域已知的或本文所述的任何途径给药,例如口服给药、胃肠外给药(例如静脉内给药或肌内给药)、腹膜内给药、直肠给药、皮肤给药、鼻给药、阴道给药、吸入给药、皮肤给药(贴剂)或眼睛给药。重组人MIS蛋白或其衍生物或功能片段可采用任何剂量或给药方案给药。

[0269] 对于本发明的治疗方法,并不意味着重组人MIS蛋白或编码此类重组人MIS蛋白的多核苷酸或它们的功能片段的给药限于特定的给药模式、剂量或给药频率;本发明考虑所有给药模式,包括肌内给药、静脉内给药、腹膜内给药、囊内(intravesicular)给药、关节内给药、病灶内给药、皮下给药、或足以提供足够治疗如本文所公开的自身免疫疾病或免疫相关紊乱的剂量的任何其它途径。可将重组人MIS蛋白的有效量(例如治疗有效剂量)以单剂量或以多剂量给予患者。当给予多剂量时,所述剂量可彼此相隔例如1小时、3小时、6小时、8小时、1天、2天、1周、2周或1个月。例如,可将包含重组人MIS蛋白药剂的组合物给予例如2周、3周、4周、5周、6周、7周、8周、10周、15周、20周或更多周。应该理解的是,对于任何具体的受试者而言,特定剂量方案应根据个体需要和管理或指导组合物给药的人的专业判断随时间调整。例如,如果较低剂量未提供足够的治疗活性,治疗剂的剂量可以增加。

[0270] 虽然主治医师最终将决定适当的量和剂量方案，有效量的重组人MIS蛋白或其衍生物或功能片段可以以0.0001mg/kg、0.01mg/kg、0.01mg/kg、0.1mg/kg、1mg/kg、5mg/kg、10mg/kg、25mg/kg、50mg/kg、100mg/kg、500mg/kg或1000mg/kg的剂量提供。有效剂量可从由体外或动物模型测试生物分析或系统得到的剂量应答曲线中推测出。在一些实施方式中，重组人MIS蛋白的剂量为约1pg/kg至10pg/kg(患者的体重)，不过也可给予较低和较高的剂量。

[0271] 在一些实施方式中，重组人MIS剂量的参考范围由美国的参比组估计出，并公开于Antimullerian Hormone(AMH),Serum from Mayo Medical Laboratories.Retrieved April 2012。在一些实施方式中，可给予雌性受试者以下剂量的重组人MIS：年龄小于24个月的雌性，小于5ng/mL；24个月至12岁的雌性，少于10ng/mL；13-45岁的雌性，1-10ng/mL；大于45岁的雌性，小于1ng/mL。在一些实施方式中，可给予雄性受试者以下剂量的重组人MIS：年龄小于24个月的雄性，15-500ng/mL；24个月至12岁的雄性，7-240ng/mL；大于12岁的雄性，0.7-20ng/mL。值得注意的是，如果被测量的人为维生素D缺乏，MIS测量结果可能准确度较低。

[0272] 此外，由于已证明MIS与雷帕霉素、AzadC、阿霉素、顺铂和紫杉醇的叠加、协同或竞争，可将本文公开的重组人MIS与选择性靶向疗法联合给药，例如，以获得相比单独使用该重组人MIS或该化疗剂而言更大的对抗卵巢癌的活性。

[0273] 用于具体患者或受试者的剂量可由本领域普通技术人员使用常规注意事项(例如借助适当的常规药理学方案)确定。医生例如可首先开出相对低剂量的药方，随后增加剂量直至获得恰当响应。取决于应用，给予患者的剂量足以随着时间推移在患者中产生有益的治疗响应；或者例如足以减轻症状；或者足以产生其它适当活性。剂量由以下因素确定：具体制剂的功效；以及本文公开的重组人MIS蛋白或其功能衍生物或功能片段的活性、稳定性或血清半衰期；以及患者的状况；待治疗的自身免疫疾病；以及待治疗的患者的体重或表面积。剂量大小还由如下因素确定：伴随在具体受试者中给予具体载体或剂型等发生的任何不利副作用的存在、性质和程度。任选根据本领域公知的方法在一个或多个合适的体外和/或体内疾病动物模型(例如本文在实施例中公开的缪勒管退化生物分析和本领域普通技术人员已知的模型)中对含有重组人MIS蛋白或其功能衍生物或功能片段的治疗组合物进行测试，以确认功效、组织代谢，并估计剂量。特别地，剂量最开始可由相关分析中的治疗对比非治疗(例如经治疗的细胞或动物模型与未经治疗的细胞或动物模型的比较)的活性、稳定性或者其它合适的测量结果来确定。将制剂按例如适用于患者的质量和整体健康的速率/比率(由相关制剂的LD50和/或在各种浓度下对重组人MIS蛋白或其功能衍生物或功能片段的任何副作用的观察而确定)给予。给药可通过单次剂量或分次剂量完成。

[0274] 确定在疾病的治疗或预防中要给予的重组人MIS蛋白或其功能衍生物或功能片段的有效量时，医生对循环血浆水平、制剂毒性以及疾病进展进行评价。所选剂量水平也将取决于多种因素，包括所采用的本发明具体化合物或其酯、盐或酰胺的活性；给药途径；给药次数；所采用的具体化合物的排泄率(rate of excretion)；治疗持续时间；与所采用的具体化合物联用的其它药物、化合物和/或材料；治疗的患者的年龄、性别、重量、状态、一般健康状况以及既往病史；以及医学领域公知的相似因素。

[0275] 在一些实施方式中，本文公开的重组人MIS蛋白可按照良好医疗实践的剂量给药，

考虑个体患者的临床状况;给药位点和方法;给药时间安排;患者年龄、性别、体重;以及医疗从业人员所知晓的其它因素。

[0276] 可对本文所公开的含有重组人MIS蛋白或其功能片段或变体的组合物的给药方案进行调整,以提供最佳的期望响应(例如治疗响应或预防响应)。例如,根据治疗情况的迫切需求所指明的,可单次大剂量(single bolus)给予,可在一段时间内给予数个分次剂量,或者可将剂量按比例减少或增加。特别有利的是将胃肠外组合物配制成单位剂量形式(dosage unit form),从而易于给药并使剂量均一。

[0277] 此外,药物组合物中重组人MIS蛋白的实际剂量水平可以变化,以获得在具体受试者、组合物和给药模式中有效地实现期望的治疗响应且对所述受试者无毒的活性成分的量。本文所公开的含有重组人MIS蛋白或其功能片段或变体的药物组合物可为"治疗有效量"和/或"预防有效量"。通常,本文所公开的含有重组人MIS蛋白或其功能片段或变体的组合物的合适的每日剂量将是作为重组人MIS蛋白有效地产生治疗效果(例如本文所公开的增殖性紊乱或癌症的症状减少)的最低剂量的量。此类有效剂量通常会取决于上文所述的因素。

[0278] 如果需要,可将含有重组人MIS蛋白或其功能片段或变体的组合物的有效每日剂量任选地以单位剂量形式在全天中以适当的时间间隔分为两个、三个、四个、五个、六个或更多个子剂量(sub-doses)分别给予。

[0279] 给予受试者的剂量水平可在所需时间段内(例如,至少1周、至少2周、至少3周、至少1个月、至少2个月、至少3个月、至少6个月、至少1年、或至少5年)是恒定的。或者,给予受试者的剂量水平可根据所治疗的病症的进展而变化。

[0280] 应当注意的是,剂量值可随待减轻的癌症的类型及严重性而有所变化。应当进一步理解的是,对于任何具体受试者而言,具体的剂量方案应根据个体需要和管理或指导组合物给药的人的专业判断而随时间进行调整,并且本文阐述的剂量范围仅为示例性的,并不意味着限制所请求保护的组合物的范围或实践。

[0281] 化合物的功效和毒性可通过标准药学程序在细胞培养物或实验动物中确定,例如ED50(在50%的群体中有效的剂量)和LD50(对50%的群体是致死的的剂量)。毒性效果与治疗效果的剂量比是治疗指数,它可表示为比值LD50/ED50。优选表现出高治疗指数的药物组合物。可使用的适当实验模型包括:确定剂量时可使用缪勒管退化生物分析(如本文在实施例中所公开的),或本领域普通技术人员公知的体内癌症模型。体内癌症模型在以下文献中有所讨论:Frese等,"Maximizing mouse cancer models"Nat Rev Cancer.2007Sep;7(9):645-58;和Santos等,Genetically modified mouse models in cancer studies.Clin Transl Oncol.2008Dec;10(12):794-803;以及"Cancer stem cells in mouse models of cancer",6th Annual MDI Stem Cell Symposium,MDI Biological Lab,Salisbury Cove,ME,2007年8月10-11,以引用的方式将它们整体并入本文。

[0282] 例如,治疗有效量起初可在细胞培养分析或在动物模型(通常是小鼠、兔、狗或猪)中进行估计。动物模型还用于获得理想的浓度范围和给药途径。然后,可将这类信息用于确定对在其它受试者中给药有用的剂量和途径。通常,治疗有效量取决于所期望的治疗效果。例如,重组人MIS蛋白的治疗有效量可在癌症小鼠模型中或使用如本文在实施例和图4中所公开的缪勒管退化生物分析进行评定。

[0283] 具有本领域普通技能的医师或兽医可容易地确定和开出所需药物组合物的有效量。例如，医师或兽医可以以低于达到期望治疗效果所需的水平开始在药物组合物中使用的本发明的化合物的给药，并逐渐增加剂量直至达到所期望的效果。还注意到，相比本文所例举的小鼠或其它实验动物，对人的治疗通常较长，该治疗的长度与疾病进程长度和药效成比例。在数天的时间内，剂量可为单剂量或多剂量，但优选单剂量。

[0284] 在一些实施方式中，重组人MIS蛋白(例如，蛋白或编码重组人MIS蛋白的核酸或它们的片段)可通过任何合适的给药途径给予人和其它动物以用于治疗，包括口服给药、鼻腔给药(通过例如喷雾)、直肠给药、阴道内给药、胃肠外给药、脑池内(intracisternally)给药和局部给药(如通过粉剂、软膏剂或滴剂(drops))，包括经颊给药和舌下给药。

[0285] 在以所需剂量与合适的药学上可接受的载体进行配制后，可向受试者给予本文中所公开的含有重组人MIS蛋白或其功能片段或变体的药物组合物。含有重组人MIS蛋白或其功能片段或变体的药物组合物可使用任何合适的手段给予受试者。通常，合适的给药手段包括但不限于局部途径、口服途径、胃肠外途径(例如静脉内途径、皮下途径或肌内途径)、直肠途径、脑池内途径、阴道内途径、腹膜内途径、眼睛途径或鼻腔途径。

[0286] 在具体实施方式中，将包含重组人MIS蛋白的药物组合物局部给予至需要治疗的区域是可取的；这可不受限地通过例如手术期间的局部输注；局部应用，例如通过注射、借助导管、或借助植入物(植入物为多孔材料、非多孔材料或凝胶状材料，包括膜(如硅橡胶(sialastic)膜)、纤维或商业皮肤替代品)来实现。在一些实施方式中，可使用局部霜剂、贴剂、肌内注射剂等将如本文所公开的重组人MIS蛋白施用至肌肉。

[0287] 在一些实施方式中，重组人MIS蛋白可口服给予(例如在胶囊剂、混悬剂或片剂中)受试者或通过胃肠外给药给予受试者。常规口服给药方法包括将重组人MIS蛋白作为片剂、混悬剂、溶液剂、乳剂、胶囊剂、粉剂、糖浆剂等可用形式中的任一种给予。优选口服或静脉内递送重组人MIS蛋白并保留生物活性的已知技术。胃肠外给药可包括例如肌内给药、静脉内给药、关节内给药、动脉内给药、鞘内给药、皮下给药或腹膜内给药。重组人MIS蛋白也可口服给药、透皮给药、局部给药、吸入给药(例如支气管内、鼻内、口腔吸入或鼻内滴剂)或直肠给药。如所说明的，给药可为局部的或全身的。药剂(例如编码重组人MIS蛋白或其功能片段的核酸药剂)也可通过本领域技术人员公知的方法使用载体(例如病毒载体)来进行递送。

[0288] 当胃肠外给予如本文所公开的含有重组人MIS蛋白或其功能片段或变体的组合物时，通常将其配制成单位剂量的可注射形式(例如溶液剂、混悬剂、乳剂)。适于注射的药物制剂包括无菌水溶液剂或分散剂和用于复溶为无菌可注射溶液剂或分散剂的无菌粉剂。载体可为溶剂或者含有如下成分的分散介质：例如水、乙醇、多元醇(例如甘油、丙二醇、液态聚乙二醇)、上述物质合适的混合物、以及植物油。

[0289] 本文所用的术语“单位剂量(dosage unit)”形式是指用于待治疗的哺乳动物受试者的适合作为单位剂量的物理上离散的单位；每个单位含有经计算与所需的药物载体组合产生所需治疗效果的预定量的活性化合物。本发明对单位剂量形式的规定由以下方面决定并直接取决于这些方面：(a)本文所公开的重组人MIS蛋白或其功能片段或变体的独特特征和要实现的特定治疗效果或预防效果；以及(b)本领域中将重组人MIS蛋白配制为用于在个体中进行敏感性治疗的活性剂的固有限制。

[0290] 包含本文所公开的重组人MIS蛋白或其功能片段或变体的药学上可接受的组合物可悬浮于水性媒介(vehicles)中,并通过常规的皮下注射针或使用输注泵引入。

[0291] 药物组合物

[0292] 在一些实施方式中,包含本文所公开的重组人MIS蛋白或其功能片段或变体的组合物可配制成任何合适的形式,例如作为无菌可注射溶液剂,例如,所述无菌可注射溶液剂可通过将重组人MIS蛋白加入所需量的适当溶剂(根据需要具有各种其它成分)中而制备。

[0293] 包含本文所公开的重组人MIS蛋白或其功能片段或变体的组合物的药物制剂可在可注射制剂中给予患者,所述可注射制剂含有任何相容的载体,例如各种媒介、佐剂、添加剂和稀释剂;或者,本发明中使用的化合物可以如下形式胃肠外给予患者:缓释(slow-release)皮下植入物或靶向递送系统(例如单克隆抗体)、载体递送(vectored delivery)、离子导入(iontophoretic)、聚合物基质、脂质体和微球。在本发明中有用的递送系统的实例包括以下专利文献中记载的递送系统:美国专利号5,225,182、5,169,383、5,167,616、4,959,217、4,925,678、4,487,603、4,486,194、4,447,233、4,447,224、4,439,196和4,475,196。其它这样的植入物、递送系统和模块为本领域技术人员所熟知。

[0294] 例如可通过如下方式保持适当的流动性:使用包衣(例如卵磷脂);在分散剂的情况下通过维持所需粒径;以及通过使用表面活性剂。非水性媒介(如棉籽油、芝麻油、橄榄油、大豆油、玉米油、葵花籽油(sunflower oil)或花生油以及酯(例如肉豆蔻酸异丙酯))也可用作用于化合物组合物的溶剂系统。此外,可添加增强组合物的稳定性、无菌性和等渗性的各种添加剂,包括抗微生物防腐剂、抗氧化剂、螯合剂和缓冲液。防止微生物活动可通过各种抗细菌剂和抗真菌剂(例如对羟基苯甲酸酯、氯丁醇、苯酚和山梨酸)来确保。在许多情况下,理想的是包含等渗剂(例如糖、氯化钠等)。可注射药物形式的延长吸收可通过使用延迟吸收的试剂(例如单硬脂酸铝和明胶)来实现。但是,根据本发明,所用的任何媒介、稀释剂或添加剂都必须与所述化合物相容。

[0295] 在另一实施方式中,包含本文所公开的重组人MIS蛋白或其功能片段或变体的组合物可包含基于脂质的制剂。任何已知的基于脂质的药物递送系统都可用于本发明的实践中。例如,多囊脂质体(multivesicular liposomes)、多层脂质体(multilamellar liposomes)和单层脂质体(unilamellar liposomes)都可以使用,只要可确立所包封的活性化合物的持续释放速率。制造受控释放多囊脂质体药物递送系统的方法描述于PCT申请公开号WO 9703652、WO 9513796和WO 9423697中,以引用的方式将它们的内容并入本文。

[0296] 合成的膜囊泡的组分通常为磷脂的组合,所述磷脂通常与类固醇(特别是胆固醇)联用。也可以使用其它磷脂或其它脂质。在合成的膜囊泡生产中有用的脂质的实例包括磷脂酰甘油、磷脂酰胆碱、磷脂酰丝氨酸、磷脂酰乙醇胺、鞘脂、脑苷脂和神经节苷脂,并在优选的实施方式中包括蛋磷脂酰胆碱、二棕榈酰磷脂酰胆碱、二硬脂酰磷脂酰胆碱、二油酰磷脂酰胆碱、二棕榈酰磷脂酰甘油和二油酰磷脂酰甘油。

[0297] 在制备含有重组人MIS蛋白或其功能片段或变体的基于脂质的囊泡中,此类变量应予以考虑:活性化合物的包封效率、活性化合物的labiality、所得囊泡群的均质性和大小、活性化合物与脂质的比、渗透性、制品的不稳定性以及制剂的药学可接受性。

[0298] 在另一实施方式中,重组人MIS蛋白可在囊泡、特别是脂质体中递送(参见Langer(1990)Science 249:1527-1533)。在又一实施方式中,重组人MIS蛋白可在受控释放系统中

递送。在一个实施方式中，可使用泵(参见Langer(1990)，同上)。在另一实施方式中，可使用聚合材料(参见Howard等(1989)J.Neurosurg.71:105)。在本发明的活性剂是编码重组人MIS蛋白的核酸的另一实施方式中，可通过以下方式体内给予所述核酸以促进其编码的蛋白的表达：将所述核酸构建为合适核酸表达载体的一部分并给予所述表达载体，使其成为细胞内的，例如，通过使用逆转录病毒载体(参见例如美国专利号4,980,286)；或通过直接注射；或通过使用微粒轰击(例如基因枪；Biolistic，Dupont)；或用脂质或细胞表面受体或转染剂包被；或通过给予连接至已知进入核的同源异型盒样(homeobox-like)肽的所述核酸(参见例如Joliot等，1991，Proc.Natl.Acad.Sci.USA 88:1864-1868)等。或者，可通过同源重组将核酸引入细胞内并并入宿主细胞DNA，从而表达。

[0299] 在引入前，可通过本领域的众多可用技术中的任何技术来对包含本文所公开的重组人MIS蛋白或其功能片段或变体的组合物进行杀菌，例如，用γ辐射或电子束灭菌。

[0300] 在本发明的另一实施方式中，包含本文公开的重组人MIS蛋白或其功能片段或变体的组合物可与任何其它治疗剂结合(例如联合)给药和/或配制。为了给药目的，优选将本文公开的重组人MIS蛋白或其功能片段或变体配制成药物组合物。本发明的药物组合物包含本发明的化合物和药学上可接受的载体，其中，所述化合物以对治疗感兴趣的病症有效的量存在于所述组合物中。合适的浓度和剂量可由本领域技术人员容易地确定。

[0301] 药学上可接受的载体是本领域技术人员所熟悉的。对于配制成液体溶液的组合物，可接受的载体包括盐水和无菌水，并且可任选地包含抗氧化剂、缓冲剂、抑菌剂(bacteriostats)以及其它常用添加剂。所述组合物也可配制成丸剂、胶囊剂、颗粒剂或片剂，除本发明的化合物外，所述组合物还含有稀释剂、分散剂和表面活性剂、粘合剂(binders)和润滑剂。本领域技术人员可进一步以适当的方式并根据公认的操作(例如Remington's Pharmaceutical Sciences，Gennaro著，Mack Publishing Co.，Easton，Pa.1990中所公开的操作)对本发明的化合物进行配制。

[0302] 本发明的组合物可为任何形式。这些形式包括但不限于：溶液剂、混悬剂、分散剂、软膏剂(包括口服软膏剂)、霜剂、糊剂(pastes)、凝胶剂、粉剂(包括牙粉)、牙膏、锭剂(lozenges)、油膏剂(salve)、咀嚼胶剂(chewing gum)、口腔喷雾剂、软锭剂(pastilles)、冲剂(sachets)、漱口剂、气溶胶、片剂、胶囊剂、透皮贴剂，上述形式均包含本发明的一种或多种消退素(resolvins)和/或保护素(protectins)或它们的类似物。

[0303] 包含本文所公开的重组人MIS蛋白或其功能片段或变体的组合物的制剂可通过本领域技术人员已知的多种手段来制备。在一些实施方式中，例如可通过如下方式制备作为气溶胶制剂给药的制剂：混合(combining)(i)本文所公开的重组人MIS蛋白或其功能片段或变体，足以提供多个治疗有效剂量的量；(ii)水，以有效稳定各制剂的量添加；(iii)推进剂(propellant)，足以从气溶胶桶中推动出多个剂量的量；以及(iv)任何进一步的任选组分(例如作为共溶剂(cosolvent)的乙醇)；以及分散上述组分。可使用常规混合器或均质器通过震动、或通过超声波能量使各组分分散。可通过使用阀至阀转移方法(valve to valve transfer methods)、压力填充或者通过使用常规冷填充方法来将散装制剂(bulk formulation)转移到较小的单个气溶胶小瓶中。不需要悬浮气溶胶制剂中所使用的稳定剂可溶于推进剂。可将没有足够溶解性的稳定剂以合适的量涂覆在药物颗粒上，然后可如上所述将涂覆的颗粒掺入制剂中。

[0304] 在某些实施方式中，包含本文所公开的重组人MIS蛋白的组合物可与药学上可接受的载体一起作为药物组合物给予受试者。在某些实施方式中，这些药物组合物任选地进一步包含一种或多种其它治疗剂。在某些实施方式中，所述其它治疗剂为自身免疫疾病药物，例如免疫阻遏剂(immune suppressant)等。在一些实施方式中，其它治疗剂是皮质类固醇。在一些实施方式中，其它治疗剂选自于由以下治疗剂所组成的组：泼尼松(Prednisone)、甲泼尼龙(methylprednisolone)、Kenalog、Medrol Oral、Medrol(Pak) Oral、Depo-Medrol Inj、泼尼松龙Oral、Solu-Medrol Inj、氢化可的松(hydrocortisone) Oral、Cortef Oral、Solu-Medrol IV、可的松Oral、Celestone Soluspan Inj、Orapred ODT Oral、Orapred Oral、Prelone Oral、甲泼尼龙醋酸盐Inj、泼尼松Intensol Oral、倍他米松(betamethasone)acet&sod phos Inj、Veripred、Celestone Oral、甲泼尼龙琥珀酸钠(methylprednisolone sodium succ)IV、甲泼尼龙琥珀酸钠Inj、Millipred Oral、Solu-Medrol(PF)Inj、Solu-Cortef Inj、Aristospan Intra-Articular Inj、氢化可的松sod琥珀酸盐Inj、泼尼松龙磷酸钠Oral、甲泼尼龙sod suc(PF)IV、Solu-Medrol(PF)IV、己曲安奈德(triamcinolone hexacetonide)Inj、A-Hydrocort Inj、A-Methapred Inj、Millipred DP Oral、Flo-Pred Oral、Aristospan病灶内Inj、倍他米松Oral、甲泼尼龙sod suc(PF) Inj、氢化可的松sod succ(PF)Inj、Solu-Cortef(PF)Inj、泼尼松龙醋酸盐Oral、处于0.9% NaCl中的地塞米松IV、Rayos、左旋甲状腺素(levothyroxine)。当然，本领域普通技术人员已知的这类治疗剂是能容易地被替换的，因为该列表不应被认为是穷举或限制性的。

[0305] 润湿剂、乳化剂和润滑剂(例如十二烷基硫酸钠和硬脂酸镁)以及着色剂、脱模剂(release agent)、包衣剂、甜味剂、调味剂和芳香剂、防腐剂和抗氧化剂也可存在于组合物中。药学上可接受的抗氧化剂的实例包括：水溶性抗氧化剂，如抗坏血酸、半胱氨酸盐酸盐、硫酸氢钠、焦亚硫酸钠(sodium metabisulfate)、亚硫酸钠等；油溶性抗氧化剂，如抗坏血酸棕榈酸酯、丁基羟基茴香醚(BHA)、丁基羟基甲苯(BHT)、卵磷脂、没食子酸丙酯、α-生育酚等；以及金属螯合剂，如柠檬酸、乙二胺四乙酸(EDTA)、山梨醇、酒石酸、磷酸等。

[0306] 本发明的制剂包括适合于静脉内给予、口服给予、鼻腔给予、局部给予、透皮给予、口腔给予、舌下给予、直肠给予、阴道给予和/或胃肠外给予的制剂。制剂可以方便地处于单位剂量形式，并且可以通过药学领域所公知的任何方法制备。可与载体材料相联合以生产单一剂量形式的活性成分的量通常是产生治疗效果的化合物的量。通常，在100%之中，该量的范围将为约1%至约99%的活性成分、优选约5%至约70%、最优选约10%至约30%。

[0307] 适于口服给予的本发明的制剂可以处于以下形式：胶囊剂、扁囊剂(cachets)、丸剂、片剂、锭剂(使用调味基质，通常为蔗糖和阿拉伯胶或西黄蓍胶)、粉剂、颗粒剂；或作为处于水性或非水性液体中的溶液剂或混悬剂；或作为水包油或油包水液态乳剂；或作为酏剂(elixir)或糖浆剂；或作为软锭剂(使用惰性基质，如明胶和甘油，或蔗糖和阿拉伯胶)和/或漱口剂等；各形式都包含预定量的本发明的化合物作为活性成分。本发明的化合物也可作为大丸剂、药糖剂(electuary)或糊剂给予。

[0308] 在用于口服给予的本发明的固体剂型(胶囊剂、片剂、丸剂、糖衣剂(dragees)、粉剂和颗粒剂等)中，将活性成分与一种或多种药学上可接受的载体(如柠檬酸钠或磷酸二钙)和/或任何以下物质混合：填充剂或增量剂(extenders)，如淀粉、乳糖、蔗糖、葡萄糖、甘露醇和/或硅酸；粘合剂，如，例如羧甲基纤维素、海藻酸盐/酯、明胶、聚乙烯吡咯烷酮、蔗糖

和/或阿拉伯胶；湿润剂，例如甘油；崩解剂，如琼脂-琼脂、碳酸钙、马铃薯淀粉或木薯(tapioca)淀粉、海藻酸、某些硅酸盐/酯和碳酸钠；溶液阻滞剂(retarding agents)，如石蜡；吸收加速剂，如季铵化合物；润湿剂，如，例如鲸蜡醇和甘油单硬脂酸酯；吸收剂，如高岭土和膨润土(bentonite clay)；润滑剂，如滑石、硬脂酸钙、硬脂酸镁、固态聚乙二醇、十二烷基硫酸钠和它们的混合物；以及着色剂。在胶囊剂、片剂和丸剂的情况下，该药物组合物还可包含缓冲剂。相似类型的固态组分也可以在软填充明胶胶囊和硬填充明胶胶囊(使用如乳糖或奶糖以及高分子量聚乙二醇等的赋形剂)中用作填充剂。

[0309] 可任选地与一种或多种辅助成分一起通过压制或模制来制备片剂。可使用粘合剂(例如明胶或羟丙基甲基纤维素)、润滑剂、惰性稀释剂、防腐剂、崩解剂(例如羟乙酸淀粉钠或交联羧甲基纤维素钠)、表面活性剂或分散剂来制备压制片剂。可以在合适的机器中对用惰性液态稀释剂润湿的粉状化合物的混合物进行模制来制备模制片剂。

[0310] 本发明药物组合物的片剂和其它固体剂型(如糖衣剂、胶囊剂、丸剂和颗粒剂)可任选地进行压痕(scored)或制备为具有包衣和壳(例如肠溶包衣和在药物制剂领域中公知的其它包衣)。可使用例如不同比例的羟丙基甲基纤维素(以获得所需的释放曲线)、其它聚合物基质、脂质体和/或微球对所述片剂和其它固体剂型进行配制，以使得其中的活性成分缓释或受控释放。所述片剂和其它固体剂型可以通过以下方式灭菌：例如通过细菌滞留过滤器过滤，或通过在临近使用时加入处于无菌固体组合物形式的灭菌剂(所述灭菌剂可溶于无菌水或一些其它无菌可注射介质)。这些组合物还可以任选地含有乳浊剂(opacifying agents)，并且可以是仅在或优先在胃肠道的某一部分任选地以延迟方式释放活性成分的组合物。可使用的包埋(embedding)组合物的实例包括聚合物质和蜡。如果合适，所述活性成分也可与一种或多种上述赋形剂处于微囊化形式。

[0311] 用于口服给予本发明化合物的液态剂型包括药学上可接受的乳剂、微乳剂、溶液剂、混悬剂、糖浆剂和酏剂。

[0312] 除了活性成分以外，液态剂型还可含有本领域中常用的惰性稀释剂，如，例如水或其它溶剂、增溶剂和乳化剂，如乙醇、异丙醇、碳酸乙酯、乙酸乙酯、苄醇、苯甲酸苄酯、丙二醇、1,3-丁二醇、油(特别是棉籽油、落花生油、玉米油、胚芽油、橄榄油、蓖麻油和芝麻油)、甘油、四氢呋喃醇、聚乙二醇和脱水山梨糖醇的脂肪酸酯，以及上述物质的混合物。除惰性稀释剂外，口服组合物还可以包含佐剂，如润湿剂、乳化剂和悬浮剂(suspending agents)、甜味剂、调味剂、着色剂、芳香剂和防腐剂。

[0313] 除了活性化合物以外，混悬剂(suspensions)还可含有悬浮剂，例如乙氧基化异硬脂醇(ethoxylated isostearyl alcohol)、聚氧乙烯山梨醇和脱水山梨醇酯、微晶纤维素、偏氢氧化铝(aluminum metahydroxide)、膨润土、琼脂-琼脂和西黄蓍胶，以及上述悬浮剂的混合物。

[0314] 在一些情况下，包含本文所公开的重组人MIS蛋白或其功能片段或变体的组合物可处于适于直肠给药或阴道给药的制剂中(例如作为栓剂)，栓剂可通过将一种或多种本发明的化合物与一种或多种适当的无刺激性赋形剂或载体混合而制备，所述赋形剂或载体包括例如可可脂、聚乙二醇、栓剂蜡或水杨酸盐/酯；并且所述栓剂在室温下是固体，而在体温下是液体，从而释放出活性化合物。适合用于此类给药的载体和制剂是本领域已知的。

[0315] 用于本发明的重组人MIS蛋白的局部给药或透皮给药(例如用于肌肉给药)的剂型

包括:粉剂、喷雾剂、软膏剂、糊剂、霜剂、洗剂(lotions)、凝胶剂、溶液剂、贴剂和吸入剂。本文公开的重组人MIS蛋白或其功能片段或变体可以在无菌条件下与药学上可接受的载体以及与可能需要的任何防腐剂、缓冲剂或推进剂混合。

[0316] 除本发明的活性化合物以外,软膏剂、糊剂、霜剂和凝胶剂还可以含有赋形剂,如动物和植物脂肪、油、蜡、石蜡、淀粉、西黄蓍胶、纤维素衍生物、聚乙二醇、硅酮、膨润土、硅酸、滑石和氧化锌,或上述赋形剂的混合物。除本发明的化合物以外,粉剂和喷雾剂还可含有赋形剂,如乳糖、滑石、硅酸、氢氧化铝、硅酸钙和聚酰胺粉末,或这些物质的混合物。喷雾剂可以额外含有常用的推进剂,如氯氟烃和挥发性未取代的烃类,如丁烷和丙烷。

[0317] 透皮贴剂具有将本发明的重组人MIS蛋白受控地递送至身体的额外优点。将化合物溶解或分散于适当介质中可以生产此类剂型。也可以使用吸收促进剂增加化合物穿过皮肤的通量。无论是提供速率控制膜、还是将活性化合物分散在聚合物基质或凝胶中,均可以控制该通量的速率。

[0318] 适于胃肠外给药的本发明的药物组合物包含一种或多种本发明的化合物以及一种或多种药学上可接受的无菌等渗水性或非水性溶液、分散液、混悬液或乳剂、或无菌粉末(所述无菌粉末可在临使用时在无菌可注射溶液或分散液中复溶)的组合,所述药学上可接受的无菌等渗水性或非水性溶液、分散液、混悬液或乳剂、或无菌粉末可包含抗氧化剂、缓冲剂、抑菌剂、使得制剂与目标接受者(intended recipient)的血液等渗的溶质、或悬浮剂、或增稠剂。

[0319] 可用于本发明的药物组合物中的合适的水性和非水性载体的实例包括水、乙醇、多元醇(如甘油、丙二醇、聚乙二醇等)及它们适当的混合物、植物油(例如橄榄油)和可注射的有机酯(如油酸乙酯)。例如可以通过以下方式保持适当流动性:使用包衣材料(如卵磷脂);在分散剂的情况下维持所需粒径;以及使用表面活性剂。

[0320] 这些组合物还可以包含佐剂,如防腐剂、润湿剂、乳化剂和分散剂。可通过包含各种抗细菌剂和抗真菌剂来确保防止微生物活动,例如对羟基苯甲酸酯(paraben)、氯丁醇、苯酚山梨酸(phenol sorbic acid)等。可期望在组合物中包含等渗剂,如糖和氯化钠等。此外,可注射药物形式的延长吸收可通过包含延迟吸收的试剂(如单硬脂酸铝和明胶)来实现。

[0321] 在一些情况下,为了延长药物的作用,期望减缓来自皮下注射或肌内注射的药物的吸收。这可以通过使用水溶性差的结晶材料或无定形材料的液体悬浮液来实现。这样,药物的吸收速率取决于其溶解速率,而其溶解速率转而又可取决于晶体大小和晶型。或者,通过将药物溶解或悬浮在油性媒介中来实现胃肠外给予的药物形式的延迟吸收。

[0322] 通过在可生物降解聚合物(如聚丙交酯-聚乙交酯)中形成主题化合物的微囊化基质来制备可注射储库(depot)形式。根据药物与聚合物的比例及所用具体聚合物的性质,可控制药物释放速率。其它可生物降解聚合物的实例包括聚(原酸酯)和聚(酸酐)。还通过将药物封装(entrapping)在与身体组织相容的脂质体或微乳剂中来制备储库可注射制剂。

[0323] 在某些实施方式中,可通过本文描述的或本领域技术人员已知的一种或多种纯化方法对重组人MIS蛋白或其功能片段或变体进行分离和/或纯化或基本上(substantially)纯化。通常,纯度为至少90%、特别是95%、并通常高于99%。在某些实施方式中,天然存在的化合物被排除在较广的属的一般描述(general description of the broader genus)

之外。

[0324] 在一些实施方式中，所述组合物包含至少一种重组人MIS蛋白与药学上可接受的载体的组合。可充当药学上可接受的载体的材料的一些实例不受限制地包括：糖类，如乳糖、葡萄糖和蔗糖；淀粉类，如玉米淀粉和马铃薯淀粉；纤维素及其衍生物，如羧甲基纤维素钠、乙基纤维素和乙酸纤维素；西黄蓍胶粉；麦芽；明胶；滑石；赋形剂，如可可脂和栓剂蜡；油类，如花生油、棉籽油、红花油、芝麻油、橄榄油、玉米油和大豆油；二醇类(glycols)，如丙二醇；多元醇类，如甘油、山梨醇、甘露醇和聚乙二醇；酯类，如油酸乙酯和月桂酸乙酯；琼脂；缓冲剂，如氢氧化镁和氢氧化铝；海藻酸；无热原水(pyrogen-free water)；等渗盐水；林格氏溶液；乙醇；磷酸盐缓冲溶液；以及在药物制剂中使用的其它无毒相容性物质。

[0325] 在某些实施方式中，包含本文所公开的重组人MIS蛋白或其功能片段或变体的组合物可含有一个或多个酸性官能团，因此，该组合物能够与药学上可接受的碱形成药学上可接受的盐。本文所用的术语“药学上可接受的盐、酯、酰胺和前药”是指在健全的医学判断范围内，适用于与患者的组织相接触而无过度的毒性、刺激性、过敏反应等，具有合理的利益/风险比；并且对于本发明化合物的预期用途而言有效的本发明化合物的羧酸盐、氨基酸加成盐、酯、酰胺和前药。术语“盐”是指本发明化合物的相对无毒的无机酸加成盐和有机酸加成盐。

[0326] 这些盐可在化合物的最终分离和纯化期间原位(in situ)制备，或者可通过将纯化的化合物以其游离碱的形式与合适的有机酸或无机酸单独反应并分离由此形成的盐来制备。这可以包括基于碱金属和碱土金属(如钠、锂、钾、钙、镁等)的阳离子；以及无毒的铵、季铵和胺阳离子，包括但不限于铵、四甲铵、四乙铵、甲胺、二甲胺、三甲胺、三乙胺、乙胺等(参见例如Berge S.M.等，“Pharmaceutical Salts,”J.Pharm.Sci.,1977;66:1-19，以引用的方式将其并入本文)。

[0327] 术语“药学上可接受的酯”是指本发明化合物的相对无毒的酯化产物。这些酯可在化合物的最终分离和纯化期间原位制备，或者可通过将纯化的化合物以其游离酸的形式或羟基与合适的酯化试剂单独反应来制备。经由在催化剂的存在下用醇处理，可将羧酸转化为酯。该术语进一步意在涵盖能够在生理条件下被溶剂化(solvated)的低级烃基，例如烷基酯，甲基酯、乙基酯和丙基酯。

[0328] 本文所用的“药学上可接受的盐或前药”是在健全的医学判断范围内，适用于与患者的组织相接触而无过度的毒性、刺激性、过敏反应等，具有合理的利益/风险比；并且对于其预期用途而言有效的盐或前药。在可能的情况下，这些化合物包括本发明化合物的两性离子形式。

[0329] 术语“盐”是指本发明化合物的相对无毒的无机酸加成盐和有机酸加成盐。这些盐可在化合物的最终分离和纯化期间原位制备，或者可通过将纯化的化合物以其游离碱的形式与合适的有机酸或无机酸单独反应并分离由此形成的盐来制备。这可以包括基于碱金属和碱土金属(如钠、锂、钾、钙、镁等)的阳离子；以及无毒的铵、季铵和胺阳离子，包括但不限于铵、四甲铵、四乙铵、甲胺、二甲胺、三甲胺、三乙胺、乙胺等(参见例如Berge S.M.等(1977)J.Pharm.Sci.66,1，以引用的方式将其并入本文)。

[0330] 术语“前药”是指在体内快速转化以产生活性重组人MIS蛋白的化合物或药剂，例如生物活性或功能活性MIS蛋白或编码功能活性MIS蛋白的核酸(例如，mRNA、DNA、MOD-

RNA)。在一些实施方式中，重组人MIS蛋白前药可通过在血液中水解而活化，例如，通过前导序列的切割以及在主要切割位点切割来生成N端结构域和C端结构域，以用于生产生物活性MIS蛋白，这类似于胰岛素从其前蛋白活化为活性胰岛素蛋白的方式。如下文献中提供了彻底讨论：T.Higachi和V.Stella，“Pro-drugs as Novel Delivery Systems”，the A.C.S.Symposium Series，第14卷；以及Bioreversible Carriers in:Drug Design，Edward B.Roche著，American Pharmaceutical Association and Pergamon Press，1987，以引用的方式将二者并入本文。本文所用的前药是当体内给药后，代谢为或以其它方式转化为化合物的生物活性形式、药学活性形式或治疗活性形式的化合物。前药可被设计为改变重组人MIS蛋白的代谢稳定性或转运特性、掩蔽副作用或毒性、或改变重组人MIS蛋白的其它特性或性质。

[0331] 凭借药效动力学过程和药物代谢或MIS在体内的翻译后蛋白加工的知识，一旦鉴定出药物活性化合物，药物领域中的技术人员通常可以设计出能够在体内被活化以增加受试者中的生物活性MIS蛋白水平的重组人MIS蛋白前药(参见例如Nogrady(1985)Medicinal Chemistry A Biochemical Approach,Oxford University Press,N.Y.,第388-392页)。用于选择和制备合适前药的常规方法公开于例如“Design of Prodrugs,”H.Bundgaard著，Elsevier,1985中。前药的合适实例包括相应酸的甲基酯、乙基酯和甘油酯。

[0332] 如本文所讨论的，在一些实施方式中，包含本文所公开的重组人MIS蛋白或其功能片段或变体的组合物可缀合或共价连接至靶向剂，以增加它们的组织特异性和对细胞(例如肌肉细胞)的靶向性。靶向剂可包括例如但不限于抗体、细胞因子和受体配体，如标题为“靶向”的部分中所讨论的。在一些实施方式中，靶向剂在所靶向的细胞(例如，相比非肌肉细胞而言的肌肉细胞)上过表达。

[0333] 不管所选择的给药途径，通过本领域普通技术人员所已知的常规方法将本发明的化合物(可以适当的水合(hydrated)形式使用)和/或本发明的药物组合物配制成药学上可接受的剂型。

[0334] 基因疗法

[0335] 在一些实施方式中，可将编码本文所公开的重组人MIS蛋白或其功能片段的核酸作为载体(例如，病毒载体)合适地给予。

[0336] 在一些实施方式中，编码重组人MIS蛋白的核酸可有效地用于通过基因疗法进行的治疗中。一般地参见例如美国专利号5,399,346，以引用的方式将其并入本文。一般原则是将多核苷酸引入患者的靶细胞中，在该靶细胞中该多核苷酸转录并成为蛋白。

[0337] 可通过本领域已知的合适技术(例如，提供处于合适载体形式的多核苷酸，或将多核苷酸封装在脂质体中)来帮助进入细胞。

[0338] 基因疗法的期待模式是通过使多核苷酸在细胞内部复制、增强和延长所需效果这样的方式来提供该多核苷酸。因此，将多核苷酸可操作地连接到合适的启动子，例如，相应基因的天然启动子；在肝细胞、神经元细胞、骨骼细胞、肌肉细胞、皮肤细胞、关节细胞或软骨细胞中固有有效的异源启动子；或可被合适的药剂诱导的异源启动子。

[0339] 可将与真核细胞相容的表达载体、优选与脊椎动物细胞相容的表达载体用于生产表达本文所公开的重组人MIS蛋白或其功能衍生物或功能变体或功能片段的重组构建体。真核细胞表达载体是本领域公知的，并可从多个商业来源获得。典型地，提供了含有方便所

需DNA区段插入的限制性位点这样的载体。此类载体可为病毒载体，例如，腺病毒、腺相关病毒、痘病毒(如正痘(orthopox)(牛痘和减毒牛痘)、禽痘病毒(avipox)、慢病毒、鼠莫洛尼白血病病毒(moloney leukemia virus)等。

[0340] 或者，在一些实施方式中，可使用质粒表达载体。质粒表达载体包括但不限于：pcDNA3.1、pET载体(Novagen®)、pGEX载体(GE Life Sciences)和pMAL载体(New England labs.Inc.)，用于大肠杆菌宿主细胞(如BL21、BL21(DE3)和AD494(DE3)pLysS、Rosetta(DE3)和Origami(DE3)(Novagen®))中的蛋白表达；基于强CMV启动子的pcDNA3.1(Invitrogen™Inc.)和pCIneo载体(Promega)，用于哺乳动物细胞系(如CHO、COS、HEK-293、Jurkat和MCF-7)中的表达；复制缺陷型腺病毒载体pAdeno X、pAd5F35、PLP-Adeno-X-CMV(Clontech®)、pAd/CMV/V5-DEST、pAd-DEST载体(Invitrogen™Inc.)，用于哺乳动物细胞中腺病毒介导的基因转移和表达；供来自Clontech的Retro-X™系统使用的pLNCX2、pLXSN和pLAPSN逆转录病毒载体，用于哺乳动物细胞中逆转录病毒介导的基因转移和表达；pLenti4/V5-DEST™、pLenti6/V5-DEST™和pLenti6.2/V5-GW/lacZ(INVITROGEN™Inc.)，用于哺乳动物细胞中慢病毒介导的基因转移和表达；腺病毒相关病毒表达载体，如pAAV-MCS和pAAV-IRES-hrGFP，用于哺乳动物细胞中腺相关病毒介导的基因转移和表达；BACpak6杆状病毒(Clontech®)和pFastBac™HT(Invitrogen™Inc.)，用于草地贪夜蛾9(Spodopera frugiperda 9,Sf9)和Sf11昆虫细胞系中的表达；pMT/BiP/V5-His(Invitrogen™Inc.)，用于施耐德果蝇S2(Drosophila Schneider S2)细胞中的表达；毕赤酵母表达载体pPICZα、pPICZ、pFLDα和pFLD(Invitrogen™Inc.)，用于巴斯德毕赤酵母(Pichia pastoris)中的表达，以及载体pMETα和PMET，用于甲醇毕赤酵母(P.methanolica)中的表达；pYES2/GS和pYD1(Invitrogen™Inc.)载体，用于酵母酿酒酵母(Saccharomyces cerevisiae)中的表达。在莱茵衣藻(Chlamydomonas reinhardtii)中大规模表达异源蛋白的最新进展由Griesbeck C.等，2006Mol.Biotechnol.34:213-33和Fuhrmann M.2004，Methods Mol Med.94:191-5描述。通过同源重组将外来异源编码序列插入细胞核、叶绿体和线粒体的基因组中。携带有最通用的叶绿体可选择标记氨基糖苷腺嘌呤转移酶(aadA)的叶绿体表达载体p64(其给予壮观霉素(spectinomycin)或链霉素抗性)可用于在叶绿体中表达外来蛋白。Biolistic基因枪方法用于将载体引入藻类。当它进入叶绿体后，外来DNA从基因枪颗粒释放，并通过同源重组整合到叶绿体基因组中。

[0341] 可在本发明中使用的病毒载体系统包括但不限于：(a)腺病毒载体；(b)逆转录病毒载体；(c)腺相关病毒载体；(d)单纯疱疹病毒载体；(e)SV40载体；(f)多瘤病毒载体；(g)乳头状瘤病毒载体；(h)小核糖核酸病毒载体；(i)痘病毒载体，例如正痘，如牛痘病毒载体或禽痘病毒(例如，金丝雀痘或鸡痘(fowl pox))；以及(j)辅助病毒依赖型腺病毒(helper-dependent adenovirus)或裸腺病毒(gutless adenovirus)。在优选实施方式中，载体为腺病毒。复制缺陷型病毒也可能是有利的。

[0342] 载体可以掺入细胞基因组中，或者可不掺入细胞基因组中。构建体可包含用于转染的病毒序列(如果需要的话)。或者，构建体可以掺入能够附加复制的载体，例如，EPV和EBV载体。

[0343] 用于表达编码本文所公开的重组人MIS蛋白的核酸(例如，DNA、MOD-RNA或RNAa)的

构建体通常可以可操作地连接到调控元件(例如,启动子、增强子等),以确保该构建体在靶细胞中的表达。关于载体和构建体的其它说明在下文中进一步详细描述。

[0344] 典型的调控序列包括但不限于转录启动子、可诱导的启动子和转录元件、控制转录的可选操作序列、编码合适的mRNA核糖体结合位点的序列以及控制转录和/或翻译终止的序列。诱导或控制与其可操作地连接的蛋白编码序列的转录的核酸序列(例如起始信号、增强子和启动子)包括在术语"调控元件"中。在一些实例中,重组基因的转录处于启动子序列(或其它转录调控序列)的控制之下,启动子序列(或其它转录调控序列)控制重组基因在想要在其中进行表达的细胞类型中的表达。还应当理解的是,重组基因可处于与对天然存在形式的蛋白的转录进行控制的转录调控序列相同或不同的转录调控序列的控制之下。在一些情况下,启动子序列被起始特定基因的转录所需的细胞合成机制或引入的合成机制识别。

[0345] 调控序列可以是单个调控序列或多个控调序列、或修饰的调控序列、或它们的片段。修饰的调控序列为其中的核酸序列已通过某种手段改变或修饰(例如但不限于突变、甲基化等)的调控序列。在如本文所公开的方法中有用的调控序列为足以使启动子依赖性基因表达在细胞类型特异性、组织特异性方面是可控的(controllable)、或通过外部信号或药剂(例如,增强子或抑制子)是可诱导的的启动子元件;这样的元件可以位于天然基因的5'或3'区或内含子内。

[0346] 本文所用的术语"组织特异性启动子"是指用作启动子(即调控可操作地连接到该启动子的选定核酸序列的表达),并且在特定组织细胞(如卵巢来源的细胞)中选择性地影响选定核酸序列的表达的核酸序列。

[0347] 术语"组成型活性启动子"是指在给定细胞内所有时间中均表达的基因的启动子。用于在哺乳动物细胞中使用的示例性启动子包括巨细胞病毒(CMV)启动子;用于在原核细胞中使用的示例性启动子包括噬菌体T7启动子和T3启动子等。术语"诱导型启动子"是指能够响应于给定信号(例如试剂的增加或减少)而表达的基因的启动子。诱导型启动子的非限制性实例是"tet-on"和"tet-off"启动子、或者在特定组织类型中受调控的启动子。

[0348] 在具体实施方式中,可使用包含编码本文所公开的重组人MIS蛋白或其功能片段的核酸序列(例如,DNA、MOD-RNA或RNAa)的病毒载体。例如,可使用逆转录病毒载体(参见Miller等,Meth.Enzymol.217:581-599(1993))。这些逆转录病毒载体含有正确包装病毒基因组和整合入宿主细胞DNA所需的组件。将编码重组人MIS蛋白的核酸序列克隆到一个或多个载体中,这有助于将基因递送到患者中。关于逆转录病毒载体的更多详细信息存在于Boesen等,Biotherapy 6:291-302(1994)中,其描述了使用逆转录病毒载体来将mdr1基因递送到造血干细胞,以使干细胞更耐化疗。说明逆转录病毒载体在基因疗法中的使用的其它参考文献为:Clowes等,J.Clin.Invest.93:644-651(1994);Kiem等,Blood 83:1467-1473(1994);Salmons和Gunzberg,Human Gene Therapy 4:129-141(1993);以及Grossman和Wilson,Curr.Opin.in Genetics and Devel.3:110-114(1993)。

[0349] 携带感兴趣的基因的重组逆转录病毒载体的制造通常以两步骤实现。首先,可将编码单独的或与-Fc融合的重组人MIS蛋白或其功能衍生物或功能变体或功能片段的序列插入逆转录病毒载体中,所述逆转录病毒载体包含有效表达代谢调节剂所需的序列(包括可通过病毒长末端重复(LTR)或者通过内部启动子/增强子及相关剪接信号提供的启动子

元件和/或增强子元件)；以及将病毒RNA有效地包装成感染性病毒粒子(infectious virions)所需的序列(例如，包装信号(Psi)、tRNA引物结合位点(-PBS)、反转录所需的3'调控序列(+PBS))；以及病毒的LTR。LTR含有为病毒基因组RNA结合、逆转录酶和整合酶功能所需的序列；以及涉及指导待包装在病毒颗粒中的基因组RNA表达的序列。

[0350] 在构建重组逆转录病毒载体后，将载体DNA引入包装细胞系中。包装细胞系提供以反式(in trans)将病毒基因组RNA包装成具有所需宿主范围的病毒颗粒所需的病毒蛋白(例如，病毒编码核心(gag)、聚合酶(pol)和包膜(env)蛋白)。通过病毒颗粒表面上表达的包膜基因产物的类型来部分控制宿主范围。包装细胞系可表达亲嗜性(ecotrophic)、兼嗜性(amphotropic)或异嗜性(xenotropic)包膜基因产物。或者，包装细胞系可以缺乏编码病毒包膜(env)蛋白的序列。在这种情况下，包装细胞系可将病毒基因组包装成缺乏膜结合蛋白(membrane-associated protein)(例如，env蛋白)的颗粒。为了生产含有允许病毒进入细胞的膜结合蛋白的病毒颗粒，可用编码膜结合蛋白(例如，水泡性口炎病毒(vesicular stomatitis virus，VSV)的G蛋白)的序列转染含有逆转录病毒序列的包装细胞系。然后，转染的包装细胞可产生含有由被转染的包装细胞系表达的膜结合蛋白的病毒颗粒；含有来源于由另一病毒的包膜蛋白包壳化的一种病毒的病毒基因组RNA的这些病毒颗粒被认为是假型病毒颗粒。

[0351] 腺病毒是可用于基因疗法的其它病毒载体。对于将基因递送到呼吸道上皮细胞，腺病毒是特别引人注目的媒介。腺病毒天生感染呼吸道上皮细胞，并在此引起轻微的疾病。基于腺病毒的递送系统的其它靶标是肝脏、中枢神经系统、内皮细胞和肌肉。腺病毒具有能够感染非分裂细胞的优点。Kozarsky和Wilson，Current Opinion in Genetics and Development 3:499-503(1993)给出了基于腺病毒的基因疗法的综述。Bout等，Human Gene Therapy 5:3-10(1994)证明了使用腺病毒载体将基因转移到恒河猴的呼吸道上皮细胞。另一优选的病毒载体是痘病毒，如牛痘病毒，例如减毒牛痘(如改良安卡拉痘苗病毒(MVA)或NYVAC)；禽痘病毒，如鸡痘或金丝雀痘。在基因疗法中使用腺病毒的其它实例可发现于：Rosenfeld等，Science 252:431-434(1991)；Rosenfeld等，Cell 68:143-155(1992)；Mastrangeli等，J.Clin.Invest.91:225-234(1993)；PCT公开WO94/12649；以及Wang等，Gene Therapy 2:775-783(1995)。在另一实施方式中，使用慢病毒载体，例如美国专利号6,143,520、5,665,557和5,981,276中描述的基于HIV的载体，以引用的方式将它们并入本文。在一些实施方式中，使用诸如腺相关病毒(AAV)载体的病毒载体。示例性的AAV载体公开于：Walsh等，Proc.Soc.Exp.Biol.Med.204:289-300(1993)；美国专利号5,436,146中，以引用的方式将其并入本文；Gao等，Gene Therapy 2005,5,285-297；Vandenberghe等，Gene Therapy 2009,16,311-319；Gao等，PNAS 2002,99,11854-11859；Gao等，PNAS 2003,100,6081-6086；Gao等，J.of Virology 2004,78,6381-6388；由M.Green和J.Sambrook编的Molecular Cloning:A Laboratory Manual(第4版)。在一些实施方式中，所述AAV载体是AAV1、AAV2、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAVrh.10、AAV2.5。应当指出的是，特定类型的AAV载体的选择可取决于靶组织。

[0352] 在一些实施方式中，当由病毒载体编码的重组人MIS蛋白在受试者中内源表达时，本文所公开的重组人MIS蛋白的表达水平可在期望的时间段内是恒定的，例如，至少1周、至少2周、至少3周、至少1个月、至少2个月、至少3个月、至少6个月、至少1年、或至少5年。在一

些实施方式中，本文所公开的重组人MIS蛋白的表达可在期望的时间段内维持或高于治疗有效剂量水平。

[0353] 基因疗法的另一方法涉及通过诸如电穿孔、脂转染(lipofection)、磷酸钙介导的转染、或病毒感染的方法来将基因转移到组织培养中的细胞。通常，转移方法包括将可筛选标记转移至细胞。然后，将细胞置于筛选下，以分离出摄取并表达该转移基因的细胞。然后，将这些细胞递送给患者。

[0354] 美国专利号5,676,954(以引用的方式将其并入本文)报道了将与阳离子脂质体载体复合的遗传物质注射到小鼠中。美国专利号4,897,355、4,946,787、5,049,386、5,459,127、5,589,466、5,693,622、5,580,859、5,703,055和国际公开号WO94/9469(以引用的方式将它们并入本文)提供了用于将DNA转染进细胞和哺乳动物的阳离子脂质。美国专利号5,589,466、5,693,622、5,580,859、5,703,055和国际公开号WO 94/9469(以引用的方式将它们并入本文)提供了将DNA-阳离子脂质复合体递送进哺乳动物的方法。这样的阳离子脂质复合体或纳米颗粒也可用于递送蛋白。

[0355] 可通过任何合适的方法将基因或核酸序列引入靶细胞中。例如，可通过转染(例如，磷酸钙或DEAE-葡聚糖介导的转染)、脂转染、电穿孔、显微注射(例如，通过直接注射裸DNA)、biolistics、用含有肌肉相关转基因的病毒载体感染、细胞融合、染色体介导的基因转移、微细胞介导的基因转移、核转移等，将重组人MIS蛋白构建体引入到细胞中。可通过电穿孔(参见例如，Wong和Neumann，Biochem.Biophys.Res.Commun.107:584-87(1982))和biolistics(例如，基因枪；Johnston和Tang，Methods Cell Biol.43Pt A:353-65(1994)；Fynan等，Proc.Natl.Acad.Sci.USA 90:11478-82(1993))将编码重组人MIS蛋白的核酸引入到细胞中。

[0356] 在某些实施方式中，可通过转染或脂转染将编码重组人MIS蛋白的核酸序列或基因引入靶细胞中。用于转染或脂转染的合适试剂包括例如磷酸钙、DEAE葡聚糖、lipofectin、lipfectamine、DIMRIE C、Superfect和Effectin(Qiagen)、unifectin、maxifectin、DOTMA、DOGS(Transfectam；双十八烷基酰胺甘氨酰基精胺(dioctadecylamidoglycylspermine))、DOPE(1,2-二油酰基-sn-甘油-3-磷酸乙醇胺)、DOTAP(1,2-二油酰基-3-三甲基铵丙烷)、DDAB(二甲基双十八烷基溴化铵)、DHDEAB(N,N-二-n-十六烷基-N,N-二羟乙基溴化铵)、HDEAB(N-n-十六烷基-N,N-二羟乙基溴化铵)、聚凝胺、聚(乙烯亚胺)(PEI)等。参见例如Banerjee等，Med.Chem.42:4292-99(1999)；Godbey等，Gene Ther.6:1380-88(1999)；Kichler等，Gene Ther.5:855-60(1998)；Birchaa等，J.Pharm.183:195-207(1999)。

[0357] 本领域已知的用于药剂(例如，蛋白和/或核酸)的治疗性递送的方法可用于将多肽或编码重组人MIS蛋白的核酸递送给受试者，例如，细胞转染、基因疗法、用递送媒介或药学上可接受的载体直接给药、通过提供含有编码本发明的靶向融合多肽的核酸的重组细胞进行间接递送。

[0358] 各种递送系统是已知的，并且可用于直接给予治疗性多肽(例如重组人MIS蛋白)和/或编码如本文所公开的重组人MIS蛋白的核酸，例如，包封于脂质体中、微颗粒、微胶囊、能够表达该化合物的重组细胞、以及受体介导的内吞作用(参见例如Wu和Wu，1987，J.Biol.Chem.262:4429-4432)。引入方法可为肠内的或胃肠外的，包括但不限于皮内途径、

肌内途径、腹膜内途径、静脉内途径、皮下途径、肺途径、鼻内途径、眼内途径、硬膜外途径以及口服途径。药剂可以通过任何方便的途径(例如,通过输注或快速浓注,通过经由上皮内层或粘膜皮肤内层(例如,口腔粘膜、直肠和肠粘膜等)吸收)进行给药,并且可以与其它生物活性剂一起给药。给药可为全身的或局部的。

[0359] 在具体实施方式中,将本发明的药物组合物局部给予至需要治疗的区域是可取的;这可不受限地通过例如手术期间的局部输注;局部应用,例如通过注射、借助导管、或借助植入物(植入物为多孔材料、非多孔材料或凝胶状材料,包括膜(如硅橡胶膜)、纤维或商业皮肤替代品)来实现。

[0360] 在另一实施方式中,活性剂可在囊泡、特别是脂质体中递送(参见Langer(1990) Science 249:1527-1533)。在又一实施方式中,活性剂可在受控释放系统中递送。在一个实施方式中,可使用泵(参见Langer(1990),同上)。在另一实施方式中,可使用聚合材料(参见Howard等(1989)J.Neurosurg.71:105)。

[0361] 因此,广泛而多样的基因转移/基因疗法载体和构建体是本领域公知的。这些载体容易适用于本发明的方法。通过使用重组DNA/分子生物学技术的适当操作将可操作地连接的重组人MIS蛋白编码核酸区段插入所选的表达/递送载体,可以生成很多用于实践本文所述方法的等效载体。

[0362] 本领域技术人员可以理解的是,可容易地对克隆的基因进行操作,以改变蛋白的氨基酸序列。可通过用于体外诱变等的各种众所周知的技术对用于重组人MIS蛋白的克隆的基因进行操作,来生产天然存在的人蛋白的变体(本文中称为重组人MIS蛋白的突变蛋白或变体或突变体),可根据本文所述的方法和组合物使用所述变体。

[0363] 在本发明中有用的重组人MIS蛋白的突变蛋白的一级结构中的变化例如可包括删除、添加和置换。置换可为保守的或非保守的。天然蛋白和突变蛋白之间的差异通常保留所期望的性质、减轻或消除不期望的性质、以及增添所期望的性质或新的性质。

[0364] Remington's Pharmaceutical sciences Ed.Germany,Merk Publishing, Easton,PA,1995(以引用的方式将其内容并入本文)公开了用于配制药物组合物的各种载体以及用于其制备的已知技术。可用作药学上可接受的载体的材料的一些实例包括但不限于:糖类,如乳糖、葡萄糖和蔗糖;淀粉类,如玉米淀粉和马铃薯淀粉;纤维素及其衍生物,如羧甲基纤维素钠、乙基纤维素和醋酸纤维素;麦芽;明胶;滑石;赋形剂,如可可脂和栓蜡;油类,如花生油、棉籽油、红花油、芝麻油、橄榄油、玉米油和大豆油;二醇类,如丙二醇;酯类,如油酸乙酯和月桂酸乙酯;琼脂;缓冲剂,如氢氧化镁和氢氧化铝;水;等渗盐水;林格氏溶液;乙醇;和磷酸缓冲液;以及其它无毒的相容性润滑剂,如十二烷基硫酸钠和硬脂酸镁;根据配方设计师的判断,着色剂、脱模剂、包衣剂、甜味剂、调味剂和芳香剂、防腐剂以及抗氧化剂也可出现在组合物中。

[0365] 试剂盒

[0366] 本发明还提供了含有重组人MIS蛋白或其功能变体或功能片段或融合蛋白的试剂盒或药物包装,用于预防和/或治疗增殖性疾病或紊乱,例如,本文所公开的癌症或过多雄激素的疾病。该试剂盒可含有处于例如片剂、胶囊剂、或冻干粉剂形式的重组人MIS蛋白组合物,并可任选包含使用重组人MIS蛋白来治疗与雄激素依赖相关的疾病或癌症的说明书。包含重组人MIS蛋白或其功能变体或功能片段或融合蛋白的组合物可在瓶或另一适当的形

式(例如,泡罩包装)中提供于试剂盒或包装中。任选地,试剂盒或药物包装还可包含其它药学活性剂(参见例如上面列出的药剂,如用于治疗自身免疫疾病和紊乱的其它药剂);和/或用于药物给药的材料(例如稀释剂、针头、注射器和施用器等)。

[0367] 本公开的各种实施方式也可包括权利要求中列举的各种要素的排列变化(permutations),如同每个从属权利要求为并入在先从属权利要求以及独立权利要求中的每一个的限定的多项从属权利要求。此类排列变化明确处于本公开的范围内。

[0368] 尽管已参考许多实施方式具体示出和描述了本发明,本领域技术人员将理解的是,可在不脱离本发明的精神和范围的情况下对本文中公开的各种实施方式在形式和细节上做出改变,并且本文所公开的各种实施例并不旨在作为对权利要求的范围的限制。以引用的方式将本文所引用的所有参考文献整体并入本文。

[0369] 在此,以引用的方式将本文中所引用的每个申请和专利、以及各申请和专利中引用的每个文件或参考文献(包括每个授权专利的审查过程中的文件或参考文献;"申请引用的文件")、以及对应于这些申请和专利的任一个和/或请求这些申请和专利的任一个的优先权的每个PCT和国外申请或专利、以及每个申请引用的文件中引用或参考的每个文件明确并入本文,并且这些文件可用于本发明的实践。更一般而言,在权利要求前的参考文献列表中或在本文本中对文件或参考文献进行了引用;并且,在此将这些文件或参考文献("本文所引用的参考文献")中的每一个、以及本文所引用的参考文献中的每一个所引用的每一个文件或参考文献(包括任何制造商的规范、说明书等)以引用的方式明确并入本文。

[0370] 本发明的一些实施方式在下列段落中列出:

[0371] 1.一种重组缪勒抑制物质(MIS)蛋白,所述重组MIS蛋白包含SEQ ID NO:1的残基448-452之间的至少一个氨基酸的修饰,从而相比缺乏修饰的情况切割得以增加。

[0372] 2.如段落1所述的重组MIS蛋白,所述重组MIS蛋白进一步包含代替SEQ ID NO:1的氨基酸1-25的MIS前导序列的非MIS前导序列或其功能片段,其中,所述重组MIS蛋白相比对应于SEQ ID NO:1的氨基酸残基的野生型MIS蛋白而言具有在体外增加的生产产率和增加的切割。

[0373] 3.如段落1或2所述的重组MIS蛋白,其中,所述重组MIS蛋白进一步包含标签蛋白。

[0374] 4.如段落2所述的重组MIS蛋白,其中,所述非MIS前导序列为白蛋白前导序列或其功能片段。

[0375] 5.如段落4所述的重组MIS蛋白,其中,所述白蛋白前导序列为人血清白蛋白(HSA)前导序列或其片段。

[0376] 6.如段落5所述的重组MIS蛋白,其中,所述HSA前导序列包含SEQ ID NO:6的氨基酸序列或与其至少80%同源的变体。

[0377] 7.如段落5所述的重组MIS蛋白,其中,所述HSA前导序列的片段包含SEQ ID NO:6的至少10个氨基酸或与其至少80%同源的变体。

[0378] 8.如段落5所述的重组MIS蛋白,其中,所述HSA前导序列包含SEQ ID NO:6的至少15个氨基酸或与其至少80%同源的变体。

[0379] 9.如段落5所述的重组MIS蛋白,其中,所述HSA前导序列包含SEQ ID NO:6的至少11个氨基酸或与其至少80%同源的变体。

[0380] 10.如段落5所述的重组MIS蛋白,其中,所述HSA前导序列的片段选自于由以下序

列所组成的组：

[0381] MKWVTFISLLFLFSSAYS(SEQ ID NO:13)；

[0382] MKWVTFISLLFLFSSAYSRGVFRR(SEQ ID NO:6)；

[0383] MKWVSFISLLFLFSSAYS(SEQ ID NO:14)。

[0384] 11.如段落2所述的重组MIS蛋白，其中，所述非MIS前导序列选自于由以下序列所组成的组：

[0385] 与组织型纤溶酶原激活物前肽融合的免疫球蛋白信号肽(IgSP-tPA)，鼠免疫球蛋白信号肽(IgSP)，MPIF-1信号序列(MKVSVAALSCLMLVTALGSQA(SEQ ID NO:15))；斯钙素信号序列(MLQNSAVLLLLVISASA(SEQ ID NO:16))；转化酶信号序列(MLLQAFLFLLAGFAAKISA(SEQ ID NO:17))；酵母交配因子α信号序列(乳酸克鲁维酵母杀伤毒素前导序列)；杂合信号序列(MKWVSFISLLFLFSSAYSRSLEKR(SEQ ID NO:18))；HSA/MFα-1杂合信号序列(MKWVSFISLLFLFSSAYSRSLDKR(SEQ ID NO:19))；乳酸克鲁维酵母杀伤/MFα-1融合前导序列(MNIFYIFLFLLSFVQGSLDKR(SEQ ID NO:20))；免疫球蛋白Ig信号序列(MGWSCIILFLVATATGVHS(SEQ ID NO:21))；Fibulin B前体信号序列(MERAAPSRRVPLPLLLLGGLALLAAGVDA(SEQ ID NO:22))；簇集蛋白前体信号序列(MMKTLLLFVGLLLTWESGQVLG(SEQ ID NO:23))；以及胰岛素样生长因子结合蛋白4信号序列(MLPLCLVAALLLAAGPGPSLG(SEQ ID NO:24))，或它们的功能片段。

[0386] 12.如段落1所述的重组MIS蛋白，所述重组MIS蛋白包含SEQ ID NO:1的氨基酸450由Q至R的修饰，从而相比缺乏该修饰的情况切割得以增加。

[0387] 13.如段落1所述的重组MIS蛋白，所述重组MIS蛋白进一步包含SEQ ID NO:1的氨基酸452由S至R的修饰，从而相比缺乏该修饰的情况切割得以增加。

[0388] 14.如段落3所述的重组MIS蛋白，其中，所述标签为FLAG标签。

[0389] 15.如段落14所述的重组MIS蛋白，其中，所述FLAG标签包含氨基酸序列DYKDDDDK(SEQ ID NO:8)或其功能衍生物或变体。

[0390] 16.如段落14所述的重组MIS蛋白，其中，所述FLAG标签位于SEQ ID NO:1的氨基酸残基452之后及SEQ ID NO:1的氨基酸残基453之前。

[0391] 17.如段落14所述的重组MIS蛋白，其中，所述FLAG标签位于SEQ ID NO:1的氨基酸残基452和453之间。

[0392] 18.如段落1所述的重组MIS蛋白，所述重组MIS蛋白包含SEQ ID NO:2的氨基酸序列或其功能片段。

[0393] 19.如段落1所述的重组MIS蛋白，所述重组MIS蛋白包含SEQ ID NO:3的氨基酸序列或其功能片段。

[0394] 20.如段落18所述的重组MIS蛋白，所述重组MIS蛋白由SEQ ID NO:4的核酸序列编码。

[0395] 21.如段落19所述的重组MIS蛋白，所述重组MIS蛋白由SEQ ID NO:5的核酸序列编码。

[0396] 22.一种药物组合物，所述药物组合物含有段落1-21中任一项所述的重组MIS蛋白和药学上可接受的载体。

[0397] 23.编码段落1-21中任一项所述的重组MIS蛋白的多核苷酸。

[0398] 24.如段落23所述的多核苷酸，其中，所述核苷酸对应于SEQ ID NO:4或与SEQ ID NO:4的核酸序列具有至少95％的序列一致性的核苷酸。

[0399] 25.如段落23所述的多核苷酸，其中，所述核苷酸对应于SEQ ID NO:5或与SEQ ID NO:5的核酸序列具有至少95％的序列一致性的核苷酸。

[0400] 26.含有段落23-25中任一项所述的多核苷酸的载体。

[0401] 27.如段落26所述的载体，其中，所述载体为病毒载体或表达载体。

[0402] 28.如段落27所述的载体，其中，所述表达载体为用于细菌(例如，大肠杆菌)或噬菌体的pcDNA 3.1或cDNA载体或基因组载体。

[0403] 29.如段落27所述的载体，其中，所述病毒载体选自于由以下病毒载体所组成的组：

[0404] 腺病毒载体、痘病毒载体以及慢病毒载体。

[0405] 30.如段落27所述的载体，其中，所述病毒载体为腺相关病毒载体(AAV)。

[0406] 31.如段落30所述的载体，其中，所述AAV为AAV9。

[0407] 32.如段落26-31中任一项所述的载体，其中，所述核酸序列编码重组MIS蛋白或其片段，所述核酸序列与SEQ ID NO:4或SEQ ID NO:5的核酸序列具有至少95％的序列一致性，其中，所述核酸序列可操作地连接至组织类型特异性启动子或细胞类型特异性启动子。

[0408] 33.一种由段落1所述的重组人MIS蛋白的翻译后加工生产的人MIS蛋白。

[0409] 34.一种含有段落26-32中任一项所述的载体的宿主细胞。

[0410] 35.一种药物组合物，所述药物组合物含有段落26-32中任一项所述的载体和药学上可接受的载体。

[0411] 36.由段落1-21中任一项所述的重组人MIS蛋白生产而来的人MIS蛋白的纯化制剂。

[0412] 37.一种用于治疗患有癌症的受试者的方法，所述方法包括将含有重组MIS蛋白的组合物进行给药，其中，所述重组MIS蛋白包含SEQ ID NO:1的残基448-452之间的至少一个氨基酸的修饰，从而使得相比缺乏修饰的情况切割有所增加，

[0413] 38.如段落37所述的方法，其中，相比对应于SEQ ID NO:1的氨基酸残基的野生型MIS蛋白而言，所述重组MIS蛋白具有在体外增加的生产产率以及增加的切割。

[0414] 39.如段落37所述的方法，其中，所述重组MIS蛋白由含有代替SEQ ID NO:1的氨基酸1-25的MIS前导序列的非MIS前导序列或其功能片段的前原蛋白生产而来。

[0415] 40.如段落37所述的方法，其中，所述重组MIS蛋白进一步含有标签蛋白。

[0416] 41.如段落39所述的方法，其中，所述非MIS前导序列为白蛋白前导序列或其功能片段。

[0417] 42.如段落41所述的方法，其中，所述白蛋白前导序列为人血清白蛋白(HSA)前导序列或其片段。

[0418] 43.如段落37所述的方法，其中，所述重组MIS蛋白包含SEQ ID NO:1的氨基酸450由Q至R的修饰，从而相比缺乏该修饰的情况切割得以增加。

[0419] 44.如段落40所述的方法，其中，所述标签为含有SEQ ID NO:8的氨基酸序列或其功能片段的FLAG标签。

[0420] 45.如段落37所述的方法，其中，所述重组MIS蛋白包含SEQ ID NO:2的氨基酸残基

25-559或其功能片段。

[0421] 46.如段落37所述的方法，其中，所述重组MIS蛋白包含SEQ ID NO:3的氨基酸残基25-567或其功能片段。

[0422] 47.如段落37所述的方法，其中，所述癌症为MIS响应II型癌症。

[0423] 48.如段落37所述的方法，其中，所述癌症为卵巢癌。

[0424] 49.如段落37所述的方法，其中，所述癌症为化学疗法耐受性癌症或多药物耐药性癌症。

[0425] 50.如段落37所述的方法，其中，所述重组MIS蛋白的给药在给予另外的药剂或癌症疗法之前、期间或之后。

[0426] 51.如段落37所述的方法，其中，所述癌症表达缪勒抑制物质II型受体(MISRII)。

[0427] 52.如段落51所述的方法，其中，在获得自所述受试者的生物样品中对缪勒抑制物质(MIS)受体的表达进行测量。

[0428] 53.如段落52所述的方法，其中，所述生物样品为癌组织样品或肿瘤组织样品，或癌细胞或肿瘤细胞。

[0429] 54.如段落52所述的方法，其中，所述生物样品为活检组织样品。

[0430] 55.如段落37所述的方法，其中，所述癌症为卵巢癌细胞、外阴表皮癌细胞、宫颈癌细胞、子宫内膜腺癌细胞和卵巢腺癌细胞。

[0431] 56.如段落37所述的方法，其中，所述癌症选自于由以下癌症所组成的组：

[0432] 乳腺癌、肺癌、头颈部癌、膀胱癌、胃癌、神经系统的癌症、骨癌、骨髓癌、脑癌、结肠癌、食管癌、子宫内膜癌、胃肠癌、牙龈癌、肾癌、肝癌、鼻咽癌、卵巢癌、前列腺癌、皮肤癌、胃癌、睾丸癌、舌癌、黑色素瘤、眼黑色素瘤或子宫癌。

[0433] 57.如段落49所述的方法，其中，所述多药物耐药性癌症为紫杉醇耐药性癌症或阿霉素耐药性癌症。

[0434] 58.如段落37所述的方法，其中，所述给药为静脉内给药、真皮内给药、肌内给药、动脉内给药、病灶内给药、经皮给药、或皮下给药、或通过气溶胶给药。

[0435] 59.如段落37所述的方法，其中，所述给药为预防性给药。

[0436] 60.如段落37所述的方法，其中，所述给药为治疗性给药。

[0437] 61.如段落37所述的方法，其中，所述受试者为哺乳动物。

[0438] 62.如段落61所述的方法，其中，所述哺乳动物为人。

[0439] 63.如段落37所述的方法，其中，将至少一种另外的药剂与所述重组人MIS的给药结合(例如，之前、期间或之后)来给予所述受试者。

[0440] 64.如段落63所述的方法，其中，所述另外的药剂为治疗剂或化疗剂。

[0441] 65.如段落64所述的方法，其中，所述化疗剂选自于由以下化疗剂所组成的组：

[0442] 紫杉醇、顺铂、阿霉素、雷帕霉素、吡唑并蒽酮。

[0443] 66.如段落64所述的方法，其中，所述化疗剂为放疗剂。

[0444] 67.如段落64所述的方法，其中，所述化疗剂为吡唑并蒽酮。

[0445] 68.如段落67所述的方法，其中，所述吡唑并蒽酮为蒽(1,9-cd)吡唑-6(2H)-酮(SP600125)或其功能衍生物或功能类似物。

[0446] 69.一种减少用于治疗癌症的化疗剂的剂量的方法，所述方法包括给予受试者治

疗有效量的重组MIS蛋白，其中，所述重组MIS蛋白包含SEQ ID NO:1的氨基酸450由Q至R的修饰；以及其中，与单独的所述化疗剂的治疗有效剂量相比，在存在所述重组MIS蛋白的情况下所述化疗剂的治疗有效剂量较低。

[0447] 70.如段落69所述的方法，其中，所述重组MIS蛋白进一步含有标签蛋白。

[0448] 71.重组MIS蛋白在制备用于治疗癌症的药物中的用途，其中，所述重组MIS蛋白包含SEQ ID NO:1的氨基酸450由Q至R的修饰；并且其中，所述癌症表达缪勒抑制物质(MIS)受体。

[0449] 72.如段落71所述的用途，其中，所述重组MIS蛋白进一步包含标签蛋白。

[0450] 73.如段落71所述的用途，其中，所述缪勒抑制物质(MIS)受体为II型MIS受体或其同源物或功能片段。

[0451] 74.一种制造品，所述制造品包含包装材料以及药物组合物，所述药物组合物含有段落1-21中任一项所述的重组MIS蛋白，其中，所述包装材料包含标示，所述标示表明可将所述药物组合物在足够的期限内以有效的剂量给药，来治疗表达缪勒抑制物质(MIS)受体的癌症或降低其风险。

[0452] 75.一种治疗患有癌症的受试者的方法，所述方法包括在获得自所述受试者的生物样品中评价缪勒抑制物质II型受体(MISRII)的表达和/或活性，其中，临床医生评审结果，如果结果表明存在MISRII的表达和/或活性，所述临床医生指导所述受试者用段落22或段落35所述的药物组合物进行治疗。

[0453] 76.如段落75所述的方法，其中，所述生物样品为组织样品。

[0454] 77.如段落76所述的方法，其中，所述组织样品为癌组织样品或肿瘤组织样品，或癌细胞或肿瘤细胞。

[0455] 78.如段落76所述的方法，其中，所述生物样品为活检组织样品。

[0456] 79.如段落75所述的方法，其中，所述癌症为卵巢癌细胞、外阴表皮癌细胞、宫颈癌细胞、子宫内膜腺癌细胞和卵巢腺癌细胞。

[0457] 80.如段落75所述的方法，其中，所述癌症为乳腺癌、肺癌、头颈部癌、膀胱癌、胃癌、神经系统的癌症、骨癌、骨髓癌、脑癌、结肠癌、食管癌、子宫内膜癌、胃肠癌、牙龈癌、肾癌、肝癌、鼻咽癌、卵巢癌、前列腺癌、皮肤癌、胃癌、睾丸癌、舌癌、黑色素瘤、眼黑色素瘤或子宫癌。

[0458] 81.重组MIS蛋白在减少有需要的受试者中的一种或多种雄激素的血浆血清水平中的用途，其中，所述重组MIS蛋白包含SEQ ID NO:1的氨基酸450由Q至R的修饰。

[0459] 82.如段落81所述的用途，其中，所述重组MIS蛋白进一步包含标签蛋白。

[0460] 83.如段落81所述的用途，其中，所述一种或多种雄激素为睾酮。

[0461] 84.如段落81所述的用途，其中，所述有需要的受试者患有良性前列腺肥大。

[0462] 85.如段落81所述的用途，其中，所述有需要的受试者患有前列腺癌。

[0463] 86.如段落81所述的用途，其中，所述有需要的受试者患有多囊卵巢疾病和/或性早熟。

[0464] 87.如段落81所述的用途，其中，所述有需要的受试者患有选自于由以下疾病或紊乱所组成的组中的疾病或紊乱：

[0465] 良性前列腺增生(BPH)、前列腺癌、睾丸癌、雄激素依赖性痤疮、男性型脱发、性早

熟、雄激素过多症、多毛症、男性化、多囊卵巢综合征(POCS)、雄激素过多症(HA)-胰岛素抵抗(IR)-黑棘皮病(AN)(HIAR-AN)综合征、卵巢滤囊泡膜细胞增殖、滤泡成熟停止、闭锁、不排卵、痛经、功能失调性子宫出血、不育症和产雄激素肿瘤。

[0466] 88.一种治疗以雄激素依赖为特征的疾病或紊乱的方法，所述方法包括给予受试者有效量的段落22或段落35所述的药物组合物，其中，所述药物组合物使所述受试者的血浆血清中至少一种雄激素的水平降低，并使所述以雄激素依赖为特征的疾病或紊乱的至少一种症状减少。

[0467] 89.一种减少受试者中一种或多种雄激素的血浆水平的方法，所述方法包括给予有效量的重组MIS蛋白，其中，所述重组MIS蛋白包含SEQ ID NO:1的氨基酸450由Q至R的修饰；以及其中，所述重组MIS蛋白使所述受试者中一种或多种雄激素的血浆血清水平减少。

[0468] 90.如段落89所述的方法，其中，所述重组MIS蛋白进一步包含标签蛋白。

[0469] 91.如段落89所述的方法，其中，所述受试者患有以雄激素依赖为特征的疾病或紊乱。

[0470] 92.如段落89-91中任一项所述的方法，其中，所述疾病或紊乱选自于由以下疾病或紊乱所组成的组：

[0471] 良性前列腺增生(BPH)、前列腺癌、睾丸癌、雄激素依赖性痤疮、男性型脱发、性早熟、雄激素过多症、多毛症、男性化、多囊卵巢综合征(POCS)、雄激素过多症(HA)-胰岛素抵抗(IR)-黑棘皮病(AN)(HIAR-AN)综合征、卵巢滤囊泡膜细胞增殖、滤泡成熟停止、闭锁、不排卵、痛经、功能失调性子宫出血、不育症和产雄激素肿瘤。

[0472] 93.含有段落1-21中任一项所述的重组MIS蛋白和药学上可接受的载体的试剂盒。

[0473] 94.如段落93所述的试剂盒，所述试剂盒任选进一步含有使用所述重组MIS蛋白来治疗癌症或治疗雄激素依赖性紊乱的说明书。

[0474] 参考下列详细描述及说明性实施例可更全面地了解本发明，它们意在例示出本发明的非限制性实施方式。

[0475] 实施例

[0476] 以下实施例仅出于说明目的而提供，而并不意图限制本发明的范围。

[0477] 本发明的描述出于说明和描述的目的而呈现，但并不打算穷举或将本发明限制在所公开的形式。本发明的范围仅由所附权利要求的范围限定。很多修改和变化对于本领域技术人员而言将是显而易见的。选择和描述在附图中描述和示出的实施方式从而最好地阐释本发明的原理和实际应用，并使本领域技术人员理解适用于所预期的特定用途的本发明的具有各种修改的各种实施方式。

[0478] 材料和方法

[0479] 构建体和质粒克隆。

[0480] WT-MIS：具有MIS的基因组序列的pBG311载体。根据以前所公开的方法来构建该载体(Cate等，1986)。简要而言，将人MIS的基因组序列从chMIS33亚克隆进pBG311表达载体，该chMIS33使用牛cDNA探针从人粘粒文库中分离得到(Cate等，1986)。

[0481] RF-MIS：含有MIS cDNA(具有天然MIS前导序列、修饰的切割位点和flag标签)的pcDNA 3.1载体和pAAV-IRES-NEO载体。将存在于以前所述的含有FLAG标记的全长人MIS cDNA序列的pcDNA3.1载体(Papakostas等，2010)中的MIS的编码序列在ECORV位点处亚克隆

进pAAV-IRES-Neo表达载体。该克隆序列含有插入在位置428处的修饰的切割位点(RARR/S)(SEQ ID NO:27)后的FLAG表位(Papakostas等,2010)。

[0482] LR-MIS:含有MIS cDNA(具有人血清白蛋白前导序列和修饰的切割位点)的pcDNA 3.1载体。使用以前所述的包含含有修饰的切割位点的全长人MIS cDNA序列的pcDNA3.1载体(Papakostas等,2010)来引入白蛋白前导序列。使用含有EcoRV位点的正向引物CGAGATAC**ATGAAGTGGGTGAGCTTCATCAGCCTGCTGTTCCTGTTCAGCAGCGCTTA**

CTCCCGCGGTGTGTTCCGGCGCAGAGCAGAGGAGCCAGCTGTG(SEQ ID NO:11)(其中编码前导序列的核酸以粗体强调)和位于MIS的位置451-432的反向引物GCTCCTGGAACCTCAGCGAG(SEQ ID NO:12)克隆该白蛋白前导来代替MIS前导。

[0483] LRF-MIS:含有MIS cDNA(具有人血清白蛋白前导序列、修饰的切割位点和Flag标签)的pcDNA 3.1载体。如上所述,使用以前所述的包含含有修饰的切割位点和flag标签的全长人MIS cDNA序列的pcDNA3.1载体(Papakostas等,2010)来引入白蛋白前导序列。

[0484] 转染和克隆:

[0485] 野生型MIS(WT-MIS)。如以前所述,先将WT-MIS构建体(pBG311)与pSV2DHFR一起转染DHFR-CHO细胞,并选择作为最高表达者(the highest expresser)的B9克隆(Cate等,1986)。

[0486] RARR/S-Flag MIS(RF-MIS)(作为SEQ ID NO:27公开的"RARR/S"):使用Fugene 6(Roche),根据制造商的方案将RF-MIS构建体(处于pAAV-IRES-NEO中)转染进CHO-S细胞,并在遗传霉素(geneticin)选择(550μg/ml)下选择由蛋白质印迹确定的作为最高表达者的CHO93稳定表达克隆。

[0487] LR-MIS。使用lipofectamine 2000(invitrogen),根据制造商的方案将LR-MIS构建体(处于pcDNA3.1中)转染进CHO-K1细胞。在800μg/ml的遗传霉素中选择克隆,并选择由蛋白质印迹确定的最高表达者(LR8、LR11和LR22)用于进一步研究。

[0488] LRF-MIS。使用lipofectamine 2000(invitrogen),根据制造商的方案将LRF-MIS构建体(处于pcDNA3.1中)转染进CHO-K1细胞。在800μg/ml的遗传霉素(G418)中选择克隆,并选择由蛋白质印迹确定的最高表达者(LRF8、LRF18和LRF22)用于进一步研究。

[0489] 培养基和培养条件:

[0490] B9克隆。在37℃、5%CO_2中,使B9在具有250ml αMEM的滚瓶(roller bottles)(1700cm^2)中生长并维持汇合数月,该αMEM补充有以下物质:5%雌性胎牛血清(FFCS)(Biologos)、0.24μM甲氨蝶呤、2nM谷氨酰胺、100U/ml青霉素和100μg/ml链霉素(Invitrogen);同时每3-4天收集培养基。通过Western和MIS ELISA对培养基进行筛选,以对生产进行监控和测量。

[0491] CHO93克隆。在37℃、5%CO_2中,使CHO93在具有250ml DMEM:F12的滚瓶(1700cm^2)中生长并维持汇合数月,该DMEM:F12补充有以下物质:10%FFCS、550μg/ml遗传霉素、2nM谷氨酰胺、100U/ml青霉素和100μg/ml链霉素(Invitrogen);同时每3-4天收集培养基。通过Western和MIS ELISA对培养基进行筛选,以对生产进行监控和测量。

[0492] LR8、LR11、LR22和LRF8、LRF18、LRF22克隆。在37℃、5%CO_2中,使LR和LRF克隆均在具有250ml DMEM的滚瓶(1700cm^2)中生长并保持汇合数月,该DMEM补充有以下物质:10%FFCS、800μg/ml遗传霉素、2nM谷氨酰胺、100U/ml青霉素和100μg/ml链霉素(Invitrogen);

同时每7天收集培养基。通过Western和MIS ELISA对培养基进行筛选，以对生产进行监控和测量。

[0493] MIS的纯化。

[0494] 使用免疫亲和抗Flag珠进行的纯化。从由如上所述的CHO稳定表达克隆(CHO93、LRF8、LRF18、LRF22)的滚瓶中收集的含血清培养基中分离含有flag标签的RF-MIS和LRF-MIS。旋转沉降(spun down)所收集的培养基以除去死细胞，将上清液收集在500ml容器中并储存在-20℃直至纯化。关于纯化，将培养基在4℃过夜解冻，然后与抗FLAG琼脂糖珠(SIGMA，500μl填装的珠/500ml培养基)孵育，在4℃下过夜旋转混合。随后，将珠以13000rpm沉降10秒，并用冷的1×Tris缓冲盐水(TBS)(SIGMA)充分洗涤(7×)。将所有试剂在冰上保存。用50μg 3×FLAG肽(SIGMA)/500μl珠(处于1×TBS中)将RF-MIS和LRF-MIS在25℃下洗脱45分钟，伴有旋转。将珠在室温下以13000rpm旋转沉降10秒，收集含有FLAG MIS的上清液，将其等分，并于-80℃储存在低蛋白结合Eppendorf管(VWR)中，以供后续使用。

[0495] 使用抗MIS 6E11免疫亲和柱进行的纯化。

[0496] 根据以前所述，制备6E11MIS单克隆抗体柱(Ragin等，1992)。简要地说，根据制造商的说明书，使用共价连接至5ml装填Affigel-10琼脂糖树脂(Biorad Laboratories，Richmont，CA)的约50mg蛋白A-sepharose(Sigma Chemical Co.，St Louis，MO)纯化的小鼠单克隆抗人rhMIS抗体(如以前所述，[Ragin 1992，Hudson 1990])构建5毫升免疫亲和柱(约80％的偶联效率)。用乙醇胺(ethonalamine)对柱进行封闭，并用以1柱vol/h加载的50ml的20mM 4-(2-羟乙基)-1-哌嗪乙磺酸(Hepes)(pH 7.4)和200ml浓缩的(10×，无血清)条件培养基在4℃下进行平衡。装载后，将柱用10柱体积的20mM Hepes(pH 7.4)洗涤。预洗脱步骤采用1柱体积，含有处于20mM Hepes中的0.5M NaCl(pH 7.4)。结合的rhMIS的洗脱使用处于20mM Hepes中的1M乙酸(pH 3.0)来实现。大多数rhMIS在2-5ml部分(fraction)中洗脱，后2ml在空体积部分(void volume fraction)。将洗脱的rhMIS立即用NaOH中和至pH 7.0-7.4。将酸洗脱部分相对于0.02M Hepes(pH 7.4)透析过夜。所得的rhMIS通过Bradford方法(Bradford，1976)分析总蛋白，通过酶联免疫分析(Hudson，1990)分析rhMIS浓度；并通过聚丙烯酰胺凝胶电泳(Weber，1969)、蛋白质印迹分析(Towbin，1979)、体外缪勒管退化生物分析以及肿瘤抗增殖分析(Chin，1991)进行检查。

[0497] 电泳和蛋白质印迹

[0498] 将样品用处于1×Laemmli缓冲液(0.0625mM Tris pH 6.8、2％(w/v)SDS储液、10％(v/v)甘油、0.002％(w/v)溴酚蓝)中的100mM二硫苏糖醇还原，并在加热块(thermoblock)上以70℃热变性10分钟。用1×MES运行缓冲液(Invitrogen)将样品在4-12％Tris-Bis NuPage Novex“小型”凝胶(Invitrogen)上以130V运行。将凝胶用考马斯染色剂(0.3％亮蓝R-250、45％甲醇、10％乙酸，处于H_2O中)在室温下染色15分钟，伴随振荡。接着，将它们在脱色液(20％甲醇、10％乙酸，处于H_2O中)中室温摇动过夜，伴随振荡。

[0499] 关于蛋白质印迹分析，以25V进行45分钟和35V进行另外45分钟，将凝胶转移到PVDF(Millipore)膜上，该PVDF膜预先在含有12％(v/v)甲醇的1×NuPage转移缓冲液(Invitrogen)中平衡。将膜在室温用含5％脱脂奶粉的1×PBS(0.1％Tween-20)封闭30分钟，并用辣根过氧化物酶缀合的小鼠抗FLAG M2单克隆抗体(SIGMA)(1:1000)、山羊C20抗MIS C端抗体(Santa Cruz)(1:200)或兔MGH4抗MIS N端MIS抗体(定制)(1:1000)进行探测。

将印迹在室温下用1×PBS(Tween-200.1%)洗涤两次,每次5min,并与适当的二级抗体孵育(如有必要),并洗涤三次(5min)。用ECL试剂盒检测系统(Perkin-Elmer)在Kodak Biomax MR胶片上使蛋白条带可视化。

[0500] 动物和器官培养:

[0501] 根据以前所述(Donahoe,1977),进行针对缪勒抑制物质(MIS)的标准器官培养生物分析。简要地说,将来自定时怀孕(timed pregnant)大鼠(处于E14.5)(Harlan)的雌性尿生殖脊切开,并在包覆有琼脂的不锈钢网格上培养,所述不锈钢网格安装在补充有以下物质的强化的Cambridge Medical Research Laboratories(CMRL)1066培养基(Life Technologies)之上:10%FFCS(以避免雄性血清中牛MIS的影响)、1%青霉素/链霉素、1% L-谷氨酰胺、1%两性霉素(Fungizone,Invitrogen)和1nM睾酮(Sigma)。在37℃下于湿润的5%CO_2中孵育72小时后,将样本在Zamboni缓冲液(15%甲醛溶液和5%苦味酸)进行固定,包埋于石蜡中,并将头端的8-μm切片用苏木精和曙红进行染色。然后由两名经验丰富的观测者对切片进行从0(无退化)到5(完全退化)的评分。用纯化的RF-MIS、LRF-MIS或WT-MIS以终浓度5μg/ml以及较低的剂量3μg/ml、2μg/ml和1μg/ml来进行培养。

[0502] 实施例1

[0503] 用于临床前效力的缪勒抑制物质(MIS)蛋白的纯化(Pieretti-Vanmarcke等,2006)主要由来自用基因组克隆转染的CHO细胞的条件培养基完成(Cate等,1986)。然后,使用小鼠单克隆抗体(Hudson等,1990)对培养基进行免疫亲和纯化(Ragin等,1992),或者通过连续色谱进行纯化(Lorenzo等,2002)。在胚胎器官培养缪勒管退化分析中检测生物活性(Donahoe等,1977),并使用针对人MIS产生的单克隆抗体和多克隆抗体通过ELISA对免疫活性进行检测(Hudson等,1990)。随后,使转染的CHO细胞适应无血清条件和悬浮培养(MacLaughlin/Stafford/Dean,Donahoe,未发表),克隆筛选,扩大规模(scaled)并如上进行纯化。Western分析证实了25%-30%的切割,得到同二聚体化的C端生物活性部分,该C端生物活性部分与同二聚化的N端保持非共价结合,并且切割发生在位于氨基酸残基426-427处的Kex样主要切割位点和位于氨基酸位置229-230处的次要切割位点。还原性电泳凝胶上位于70kDa、55kDa、34kDa、24kDa和12.5kDa处的条带均是MIS片段,这通过氨基酸测序确定(Ragin等,1992;Lorenzo等,2002),并且这些条带表示预测的Kex切割产物和双碱性切割产物。

[0504] 为了优化切割和氨基酸位置427处的主要切割位点,对识别序列进行诱变以创造双碱性切割位点;RAQR/R(SEQ ID NO:28)变体是生物活性的(Kurian等,1994)。然后,对位置425(对应于SEQ ID NO:1的氨基酸残基450)进行诱变以创造更共有的Kex切割位点(Nachtigal&Ingraham 1996)(Hosaka等,1991),RARR/S(SEQ ID NO:27);在紧靠C端的第一个丝氨酸的下游添加8个氨基酸的Flag(DYKDDDDK)(SEQ ID NO:8)标签以帮助检测和纯化。这个变体的表达引起改善的切割和增加的生物活性。通过比较,当C端精氨酸(Kurian等,1994)后接着Flag时,由该构建体产生的蛋白是无生物活性的(Papakostas等,2010);因此,该丝氨酸似乎对保留生物活性非常重要。将RARR/S(SEQ ID NO:27)Flag构建体(Papakostas等,2010)转染到CHO细胞中,并证实了改善的切割和生物活性的保留(Papakostas等,2010)。切割位点的修饰使切割增加至超过50%-60%(Papakostas等,2010)。

[0505] 为了规模表达，对MIS RARR/S(SEQ ID NO:27)Flag构建体进行进一步修饰，用人血清白蛋白(HSA)的前导序列替代内源MIS前导序列。HSA是血浆中最丰富的蛋白，并由肝脏以非常高的水平生产，血液浓度达到3.4-5.4g/dL(Farrugia 2010)。HSA的生产和加工受到精细调控，以允许高效的蛋白成熟和分泌。HSA像MIS一样被合成为前原蛋白，该前原蛋白包含随后在成熟过程中被切掉的前导序列。该HSA前导序列由仅24个AA组成，在人中不具有免疫原性，并在蛋白加工过程中被除去。此处，发明人证明了，用HSA前导序列替代MIS的前导序列使生产增加并预料之外地使切割增加，该切割与重组人MIS产物的效能增加有关。

[0506] 实施例2

[0507] 以前扩大重组人MIS生产规模的努力使我们开发出了新构建体，该构建体的特征在于在位置427/428处具有修饰的切割位点的hMIS的cDNA被插入pcDNA3.1(Papakostas等，2010)。通过用修饰的RARR/S(SEQ ID NO:27)替代内源RAQR/S(SEQID NO:26)(在构建体中记为R)，并紧靠切割位点下游插入Flag标签(在构建体中记为F)(表1)(图1)，发明人证明了，带标签的C端的切割增加(Papakostas等，2010)。此外，重组RARR/S-Flag MIS("RARR/S"作为SEQ ID NO:27公开)(在本文中称为"RF-MIS")蛋白在胎儿大鼠尿生殖脊分析中保留了生物活性(Papakostas等，2010)。为了克服低表达产率，将RF-MIS的骨架载体转变为pAAV-IRES-Neo，克隆到CHO-S细胞中，并在高遗传霉素浓度下进行筛选。所得到的表达载体是多顺反子的，并包含驱动MIS下游的新霉素抗性盒表达的内部核糖体进入位点(IRES)，从而允许更好地筛选高表达者。然后使用滚瓶将最高表达克隆CHO93扩大生产，并使用抗flag M2免疫亲和珠纯化重组RF-MIS(表2)。然而，虽然RF-MIS具有增加的活性C端切割，并重要地具有较少的内部隐秘切割(图2)(图3)，cDNA克隆CHO93的产率和产量(0.16pg/细胞/天)仍比基因组克隆B9的产率和产量(10.59pg/细胞/天)低得多(表3)，不过还不清楚这一结果是否是由表达载体、CHO细胞、药物选择的性质、或产生的信息的类型(cDNA相较于基因组MIS)所致。

[0508] 为了改善生产，通过用24AA的HSA前导序列(前原肽)(本文在构建体中记为"L")替代25AA的MIS前导序列，对pcDNA3.1载体中的原始R-MIS构建体和RF-MIS构建体进行了修饰，从而创造了"LR"构建体和"LRF"构建体(表1)(图1)。

[0509] 表1：对MIS野生型序列的修饰及相应命名法的列表。

[0510]

记号	天然	修饰(以粗体示出)	位置(AA)(标准蛋白命名法)	SEQ ID NO: 1上的位置	目的
R	RAQR/S (SEQ ID NO: 26)	RARR/S (SEQ ID NO: 27)	423-427	448-452	弗林蛋白酶/Kex2共有位点，用于改善的切割
F	n/a	FLAG标签 (DYKDDDDK) (SEQ ID NO: 8)	位于427-428之间	位于SEQ ID NO: 1的452-453之间	C端FLAG标签，易于纯化和追踪
L	MIS前导序列	白蛋白前导序列	1-25	1-25	增加的产量、分泌和切割

[0511] 已证明HSA前导序列融合使重组白细胞介素(Carter等，2010)和TNF-α(Maeda Y等，1997)的产量增加，并已被建议作为生产原本难以表达和扩大规模的蛋白的方法。此外，

已知HSA也提高融合蛋白(例如使用巴斯德毕赤酵母的酵母表达系统中的人溶菌酶)的分泌(Xiong等,2008)。将在CHOK1中最高稳定表达的三个克隆选择用于进一步分析:LR8/11/18和LRF8/18/22(图2)。LR克隆的克隆效率和表达水平都比LRF克隆高,这表明Flag标签可能使表达效率较低。类似于CHO93,当与由B9生产的野生型(WT-MIS)蛋白相比时,所有LR和LRF克隆由位置229处的内部隐秘切割得到的肽片段都减少了。出乎意料的是,它们似乎也具有较高比例的切割的C端(图2和图3)。这种增加的切割可由白蛋白前导在反面高尔基体网络中的有效加工和转运到分泌小泡方面的强进化压力来解释,因为白蛋白以比MIS高得多的速率进行内源分泌(Rothschil等,1988)。选择LRF18来进行表征,因为它是最高表达的LRF克隆,并且可使用Flag标签纯化和追踪(表2)。

[0512] 表2:构建体和生产MIS的细胞系克隆以及相应的纯化方法的列表。

[0513]

构建体	克隆	载体	细胞系	纯化
MIS	B9	处于 pBG311 质粒中的 MIS WT 基因组序列	缺乏 DHFR 基因的 CHO 细胞	使用针对 MIS 的 6E11 单克隆抗体的免疫亲和或连续色谱。
RF-MIS	CHO93	插入 pAAV-IRES-Neo 质粒的 MIS cDNA 序列	CHO-S	使用针对 FLAG 标签的 M2 单克隆抗体的免疫亲和。
LR-MIS	LR8 LR11 LR18	插入 pcDNA3.1 质粒的 MIS cDNA 序列	CHO-K1	使用针对 MIS 的 6E11 单克隆抗体的免疫亲和或连续色谱。
LRF-MIS	LRF8 LRF18 LRF22	插入 pcDNA3.1 质粒的 MIS cDNA 序列	CHO-K1	使用针对 FLAG 标签的 M2 单克隆抗体的免疫亲和。

[0514] 当在烧瓶中培养24小时时,由ELISA检测的MIS的浓度,在B9(WT-MIS)的培养基(含15μg/ml)中高于以下克隆的培养基的浓度:LR8:,3.04μg/ml;LR11:,11.66μg/ml;LR22:,6.28μg/ml(见表3)。LR的最高产生了生产克隆LR11分泌了3.24pg/细胞/天的MIS,而WT克隆B9产生了10.58pg/细胞/天的MIS,但是不过LR11细胞生长更加紧凑,;相反地,LRF的最高表达克隆LRF18相比RF-MIS(CHO93,,具有0.67μg/ml和0.15pg/细胞/天)而言既具有较高的浓度(1.1μg/ml)还又具有较高的产量(0.26pg/细胞/天)(表3)。因此,HSA前导的添加增加了带flag标签化的MIS产物的产量而非未不带标签化的产物的产量。然而,由于带flag标签化的构建体的生产明显与没有未不带标签化的构建体不一样的生产多,该flag标签可能会干扰蛋白质稳定性或表达。考马斯染色和蛋白印迹证明,与使用抗MIS亲和纯化由WT-MIS(B9)纯化的MIS相比,通过抗flag免疫亲和纯化从由LRF18中纯化出的产品产物与使用抗MIS亲和纯化从WT-MIS(B9)中纯化出的MIS相比而言,具有较少的代表内部切割的条带(Ragin,1992)(图3)。

[0515] 表3:来自各种构建体的MIS的纯化产率。

[0516]

	WT-MIS	RF-MIS	LRF-MIS	LR-MIS
24小时时的MIS浓度(μg/ml)	15	0.67	1.10	11.67
产量(pg/细胞/天)	10.59	0.15	0.26	3.24
纯化产率(%w/w)	15%	20%	20%	15%

切割百分比	20%	50%	90%	90%

[0517] 因为以前已证明MIS的C端是活性部分(Pepinski,1988;Maclaughlin等,1992),增加的切割应该与大鼠UGR分析中较高的生物活性相关。在此,本发明人证明了,5μg/ml(35μM)的LRF-MIS能够使缪勒管完全退化,并且相比处于该浓度的RF-MIS和WT-MIS(仅展示出部分退化)而言显示出更高的活性(图4)。此外,LRF-MIS甚至在较低的剂量(降至2μg/ml)继续展示出完全退化,处于该剂量的WT-MIS不再显示任何活性(数据未示出)。因此,相比RF-MIS构建体,LRF-MIS重组人MIS蛋白中前导序列(L)的存在使缪勒管的退化呈剂量依赖性的减少,这表明该构建体具有较高效能并且比RF-MIS构建体更具活性。

[0518] 合起来讲,发明人在本文中证明了,LR产物导致较大的生产产率,并具有增加的切割和较高的生物活性或效能。

[0519] 因此,本发明证明了,HSA前导序列令人惊讶地引起重组人MIS蛋白产率增加(较高的浓度和较高的产量)(参见图2和图3)。此外,HSA前导序列的存在也导致来自主要切割位点的切割(对应于SEQ ID NO:1的451/452(或野生型人MIS蛋白的常规氨基酸命名法的426/427)处的切割)出乎意料地增加(参见图2和3)。这种产率增加和切割增加是令人吃惊的,因为随着产率增加(并由此由细胞产生了更多蛋白),将预期的是减少的切割(由于可用的切割酶的活性变得饱和且承担过多)。然而,并非如此——事实上完全相反,其中,伴随着增加的蛋白生产,来自主要切割位点的切割增加。

[0520] 这是特别出乎意料的,因为并未预期到前导序列(其并不位于接近MIS的主要切割位点这一切割位点的任何地方)会具有增加切割的作用,因为该前导序列通常在前蛋白MIS的翻译后切割之前首先就被切除。

[0521] 此外,前导序列还导致较少的来自次要切割位点(位于正常野生型MIS编号的氨基酸残基229/230或对应于SEQ ID NO:1的残基254/255之间)的切割。考虑到并未修饰该次要切割位点,这也是令人惊奇的。

[0522] 此外,前导序列的存在还增加了产量和产率,甚至在FLAG标签存在于重组人MIS蛋白中时。(如表3中所示,FLAG标签显著降低产率)。这又是一个令人惊讶的发现,因为前导序列不位于接近FLAG标签的任何地方;并且,未曾预料到对前导序列的此类修饰会增加含FLAG标签的蛋白的生产产率。

[0523] 实施例3

[0524] 在37℃、5%CO_2中,使LR11在具有250ml DMEM的5层烧瓶或具有500ml培养基的10层烧瓶(1700cm^2)中生长并维持汇合数月,上述培养基补充有10%FFCS、800μg/ml遗传霉素、2nM谷氨酰胺、100U/ml青霉素和100μg/ml链霉素(Invitrogen)。每周一次,用其中省略了FFCS并用非必需氨基酸(NEAA)和ITS(胰岛素、转铁蛋白、硒)补充物替代的无血清培养将培养基替换72h。然后,使用切向流渗透膜将培养基10×浓缩。使用这些方法,将4-5μg/ml的培养基浓缩成25-50μg/ml,LR-MIS的有效纯化产率上升至约30%。

[0525] 表4:使用新无血清培养基纯化方案的来自各种构建体的MIS的纯化产率。

[0526]

	WT-MIS	RF-MIS	LRF-MIS	LR-MIS
24小时时的MIS浓度(μg/ml)	16.821	1.236	2.149	4.866
产量(pg/细胞/天)	7.597	0.254	0.430	1.142

24h时无血清培养基中的浓度	1.528	0.223	0.457	1.411
纯化产率(%w/w)	15%	20%	20%	30%
无血清培养基中的切割百分比	25%	50%	37%	79%

[0527] 参考文献

[0528] 在此,以引用的方式将本文中所引用的每个申请和专利、以及各申请和专利中引用的每个文件或参考文献(包括每个授权专利的审查过程中的文件或参考文献;"申请引用的文件")、以及对应于这些申请和专利的任一个和/或请求这些申请和专利的任一个的优先权的每个PCT和国外申请或专利、以及每个申请引用的文件中引用或参考的每个文件明确并入本文,并且这些文件可用于本发明的实践。更一般而言,在权利要求前的参考文献列表中或在本文本中对文件或参考文献进行了引用;并且,在此将这些文件或参考文献("本文所引用的参考文献")中的每一个、以及本文所引用的参考文献中的每一个所引用的每一个文件或参考文献(包括任何制造商的规范、说明书等)以引用的方式明确并入本文。

[0529] 因此,通过引用将这些参考文献各自整体并入本文。

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[0541] 序列表：

[0542] SEQ ID NO:1 MIS(560AA)-氨基酸序列(下划线确定天然MIS前导序列)

[0543]
mrdlpltsla lvlsalgall qtealraeep avgtsglifr edldwppgsp qeplclvalg
gdsngsseel rvvgalsaye qaflgavqra rwgprdlatf gvcntgdrqa alpslrrlga
wlrdpggqrl vvlhleevtw eptpslrfqe pppggagppe lallvlypgp gpevtvtrag
lpgaqelcps rdtrylvlav drpagawrge glaltlqprg edsrlstarl qallfgddhr
cftrmtpall llprsepapl pahgqldtvp fppprpsael eesppsadpf lstltrlvra
lrvpparasa prlaldpdal agfpqqlvnl sdpaalerll dgeeplllll rptaattgdp
aplhdptsap watalarrva aelqaaaael rslpglppat apllarllal cpggpgglgd
plrallllka lqglrvewrg rdprggpgra**q** rsagataadg pcalrelsvd lraersvlip
etyqannccg vcgwpqsdrn prygnhvvll lkmqvrgaal arppccvpta yagkllislS
eerisahhvp nmvatecgcr

[0544] SEQ ID NO:2 LR(559AA)粗体表示-白蛋白前导序列;下划线确定修饰的切割位点

[0545]
mkwvtfisll flfssaysrg vfrr raeep avgtsglifr edldwppgsp qeplclvalg
gdsngsseel rvvgalsaye qaflgavqra rwgprdlatf gvcntgdrqa alpslrrlga
wlrdpggqrl vvlhleevtw eptpslrfqe pppggagppe lallvlypgp gpevtvtrag
lpgaqelcps rdtrylvlav drpagawrge glaltlqprg edsrlstarl qallfgddhr
cftrmtpall llprsepapl pahgqldtvp fppprpsael eesppsadpf lstltrlvra
lrvpparasa prlaldpdal agfpqqlvnl sdpaalerll dgeeplllll rptaattgdp
aplhdptsap watalarrva aelqaaaael rslpglppat apllarllal cpggpgglgd
plralllka lqglrvewrg rdprggpgra**R** rsagataadg pcalrelsvd lraersvlip
etyqannccg vcgwpqsdrn prygnhvvll lkmqvrgaal arppccvpta yagkllislS
eerisahhvp nmvatecgcr

[0546] SEQ ID NO:3 LRF(567AA)斜体表示Flag标签(DYKDDDDK(SEQ ID NO:8))

[0547]
mkwvtfisll flfssaysrg vfrr raeep avgtsglifr edldwppgsp qeplclvalg
gdsngsseel rvvgalsaye qaflgavqra rwgprdlatf gvcntgdrqa alpslrrlga
wlrdpggqrl vvlhleevtw eptpslrfqe pppggagppe lallvlypgp gpevtvtrag
lpgaqelcps rdtrylvlav drpagawrge glaltlqprg edsrlstarl qallfgddhr
cftrmtpall llprsepapl pahgqldtvp fppprpsael eesppsadpf lstltrlvra
lrvpparasa prlaldpdal agfpqqlvnl sdpaalerll dgeeplllll rptaattgdp
aplhdptsap watalarrva aelqaaaael rslpglppat apllarllal cpggpgglgd
plralllka lqglrvewrg rdprggpgra**R** rs***DYKDDDDK*** agataadg pcalrelsvd
lraersvlip etyqannccg vcgwpqsdrn prygnhvvll lkmqvrgaal arppccvpta
yagkllislS eerisahhvp nmvatecgcr

[0548] SEQ ID NO:4 LR-核酸序列

[0549]
ATGAAGTGGGTGAGCTTCATCAGCCTGCTGTTCCTGTTCAGCAGCGCTTACTCCCGCGGTGTGTTCCGC CGCAGAGCA

GAGGAGCCAGCTGTGGGCACCAGTGGCCTCATCTTCCGAGAAGACTTGGACTGGCCTCCAGGCAGCCCACPAGAGCC
TCTGTGCCTGGTGGCACTGGGCGGGGACAGCAATGGCAGGAGCTCCCCCCTGCGGGTGGTGGGGGCTCTAAGCGCCT
ATGAGCAGGCCTTCCTGGGGGCCGTGCAGAGGGCCCGCTGGGGCCCCCGAGACCTGGCCACCTTCGGGGTCTGCAAC
ACCGGTGACAGGCAGGCTGCCTTGCCCTCTCTACGGCGGCTGGGGGCCTGGCTGCGGGACCCTGGGGGGCAGCGCCT
GGTGGTCCTACACCTGGAGGAAGTGACCTGGGAGCCAACACCCTCGCTGAGGTTCCAGGAGCCCCCGCCTGGAGGAG
CTGGCCCCCCAGAGCTGGCGCTGCTGGTGCTGTACCCTGGGCCTGGCCCTGAGGTCACTGTCACGAGGGCTGGGCTG
CCGGGTGCCCAGAGCCTCTGCCCCTCCCGAGACACCCGCTACCTGGTGTTAGCGGTGGACCGCCCTGcGGGGGCCTG
GCGCGGCTCCGGGCTGGCCTTGACCCTGCAGCCCCGCGGAGAGGACTCCCGGCTGAGTACCGCCCGGCTGCAGGCAC
TGCTGTTCGGCGACGACCACCGCTGCTTCACACGGATGACCCCGGCCCTGCTCCTGCTGCCGCGGTCCGAGCCCGCG
CCGCTGCCTGCGCACGGCCAGCTGGACACCGTGCCCTTCCCGCCGCCCAGGCCATCCGCGGAACTCGAGGAGTCGCC
ACCCAGCGCAGACCCCTTCCTGGAGACGCTCACGCGCCTGGTGCGGGCGCTGCGGGTCCCCCCGGCCCGGGCCTCCG
CGCCGCGCCTGGCCCTGGATCCGGACGCGCTGGCCGGCTTCCCGCAGGGCCTAGTCAACCTGTCGGACCCCGCGGCG
CTGGAGCGCCTACTCGACGGCGAGGAGCCGCTGCTGCTGCTGCTGAGGCCCACTGCGGCCACCACCGGGGATCCTGC
GCCCCTGCACGACCCCACGTCGGCGCCGTGGGCCACGGCCCTGGCGCGCCGCGTGGCTGCTGAACTGCAAGCGGCGG
CTGCCGAGCTGCGAAGCCTCCCGGGTCTGCCTCCGGCCACAGCCCCGCTGCTGGCGCGCCTGCTCGCGCTCTGCCCA
GGTGGCCCCGGCGGCCTCGGCGATCCCCTGCGAGCGCTGCTGCTCCTGAAGGCGCTGCAGGGCCTGCGCGTGGAGTG
GCGCGGGCGGGATCCGCGCGGGCCGGGTCGGGCACGGCGCAGCGCGGGGGCCACCGCCGCCGACGGGCCGTGCGCGC
TGCGCGAGCTCAGCGTAGACCTCCGCGCCGAGCGCTCCGTACTCATCCCCGAGACCTACCAGGCCAACAATTGCCAG
GGCGTGTGCGGCTGGCCTCAGTCCGACCGCAACCCGCGCTACGGCAACCACGTGGTGCTGCTGCTGAAGATGCAGGC
CCGTGGGGCCGCCCTGGCGCGCCCACCCTGCTGCGTGCCCACCGCCTACGCGGGCAAGCTGCTCATCAGCCTGTCGG
AGGAGCGCATCAGCGCGCACCACGTGCCCAACATGGTGGCCACCGAGTGTGGCTGCCGGTGA

[0550] SEQ ID NO:5 LRF-核酸序列

[0551]

ATGAAGTGGGTGAGCTTCATCAGCCTGCTGTTCCTGTTCAGCAGCGCTTACTCCCGCGGTGTGTTCCGC
CGCAGAGCA

GAGGAGCCAGCTGTGGGCACCAGTGGCCTCATCTTCCGAGAAGACTTGGACTGGCCTCCAGGCAGCCCACAAGAGCC
TCTGTGCCTGGTGGCACTGGGCGGGGACAGCAATGGCAGCAGCTCCCCCCTGCGGGTGGTGGGGGCTCTAAGCGCCT
ATGAGCAGGCCTTCCTGGGGGCCGTGCAGAGGGCCCGCTGGGGCCCCCGAGACCTGGCCACCTTCGGGGTCTGCAAC
ACCGGTGACAGGCAGGCTGCCTTGCCCTCTCTACGGCGGCTGGGGGCCTGGCTGCGGGACCCTGGGGGGCAGCGCCT
GGTGGTCCTACACCTGGAGGAAGTGACCTGGGAGCCAACACCCTCGCTGAGGTTCCAGGAGCCCCCGCCTGGAGGAG
CTGGCCCCCCAGAGCTGGCGCTGCTGGTGCTGTACCCTGGGCCTGGCCCTGAGGTCACTGTGACGAGGGCTGGGCTG
CCGGGTGCCCAGAGCCTCTGCCCCTCCCGAGACACCCGCTACCTGGTGTTAGCGGTGGACCGCCCTGCGGGGGCCTG
GCGCGGCTCCGGGCTGGCCTTGACCCTGCAGCCCCGCGGAGAGGACTCCCGGCTGAGTACCGCCCGGCTGCAGGCAC
TGCTGTTCGGCGACGACCACCGCTGCTTCACACGGATGACCCCGGCCCTGCTCCTGCTGCCGCGGTCCGAGCCCGCG
CCGCTGCCTGCGCACGGCCAGCTGGACACCGTGCCCTTCCCGCCGCCCAGGCCATCCGCGGAACTCGAGGAGTCGCC
ACCCAGCGCAGACCCCTTCCTGGAGACGCTCACGCGCCTGGTGCGGGCGCTGCGGGTCCCCCCGGCCCGGGCCTCCG
CGCCGCGCCTGGCCCTGGATCCGGACGCGCTGGCCGGCTTCCCGCAGGGCCTAGTCAACCTGTCGGACCCCGCGGCG
CTGGAGCGCCTACTCGACGGCGAGGAGCCGCTGCTGCTGCTGCTGAGGCCCACTGCGGCCACCACCGGGGATCCTGC
GCCCCTGCACGACCCCACGTCGGCGCCGTGGGCCACGGCCCTGGCGCGCCGCGTGGCTGCTGAACTGCAAGCGGCGG

CTGCCGAGCTGCGAAGCCTCCCGGGTCTGCCTCCGGCCACAGCCCCGCTGCTGGCGCGCCTGCTCGCGCTCTGCCCA
GGTGGCCCCGGCGGCCTCGGCGATCCCCTGCGAGCGCTGCTGCTCCTGAAGGCGCTGCAGGGCCTGCGCGTGGAGTG
GCGCGGGCGGGATCCGCGCGGGCCGGGTCGGGCACGGCGCAGCgactacaaggatgacgacgacaag
GCGGGGGCCACCGCCGCCGACGGGCCGTGCGCGCTGCGCGAGCTCAGCGTAGACCTCCGCGCCGAGCGCTCCGTACT
CATCCCCGAGACCTACCAGGCCAACAATTGCCAGGGCGTGTGCGGCTGGCCTCAGTCCGACCGCAACCCGCGCTACG
GCAACCACGTGGTGCTGCTGCTGAAGATGCAGGCCCGTGGGGCCGCCCTGGCGCGCCCACCCTGCTGCGTGCCCACC
GCCTACGCGGGCAAGCTGCTCATCAGCCTGTCGGAGGAGCGCATCAGCGCGCACCACGTGCCCAACATGGTGGCCAC
CGAGTGTGGCTGCCGGTGA

[0552] SEQ ID NO:6 HSA前导序列(氨基酸序列):

[0553] mkwvtfisll flfssaysrg vfrr

[0554] SEQ ID NO:7-HSA前导序列(核酸序列):

[0555]

ATGAAGTGGGTGAGCTTCATCAGCCTGCTGTTCCTGTTCAGCAGCGCTTACTCCCGCGGTGTGTTCCGC
CGCAGAGCA

[0556] SEQ ID NO:8-FLAG标签(氨基酸序列):

[0557] DYKDDDDK

[0558] SEQ ID NO:9-FLAG标签(核酸序列):

[0559] gactacaaggatgacgacgacaag

序列表

<110> 通用医疗公司

<120> 修饰的缪勒抑制物质 (MIS) 蛋白及其用于疾病治疗的用途

<130> 030258-076964-PCT

<140>
<141>

<150> 61/777,135
<151> 2013-03-12

<160> 28

<170> PatentIn version 3.5

<210> 1
<211> 560
<212> PRT
<213> 智人

<400> 1
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1 5 10 15

[0001] Gly Ala Leu Leu Gly Thr Glu Ala Leu Arg Ala Glu Glu Pro Ala Val
20 25 30

Gly Thr Ser Gly Leu Ile Phe Arg Glu Asp Leu Asp Trp Pro Pro Gly
35 40 45

Ser Pro Gln Glu Pro Leu Cys Leu Val Ala Leu Gly Gly Asp Ser Asn
50 55 60

Gly Ser Ser Ser Pro Leu Arg Val Val Gly Ala Leu Ser Ala Tyr Glu
65 70 75 80

Gln Ala Phe Leu Gly Ala Val Gln Arg Ala Arg Trp Gly Pro Arg Asp
85 90 95

Leu Ala Thr Phe Gly Val Cys Asn Thr Gly Asp Arg Gln Ala Ala Leu
100 105 110

Pro Ser Leu Arg Arg Leu Gly Ala Trp Leu Arg Asp Pro Gly Gly Gln
115 120 125

Arg Leu Val Val Leu His Leu Glu Glu Val Thr Trp Glu Pro Thr Pro

130 135 140

Ser Leu Arg Phe Gln Glu Pro Pro Pro Gly Gly Ala Gly Pro Pro Glu
145 150 155 160

Leu Ala Leu Leu Val Leu Tyr Pro Gly Pro Gly Pro Glu Val Thr Val
165 170 175

Thr Arg Ala Gly Leu Pro Gly Ala Gln Ser Leu Cys Pro Ser Arg Asp
180 185 190

Thr Arg Tyr Leu Val Leu Ala Val Asp Arg Pro Ala Gly Ala Trp Arg
195 200 205

Gly Ser Gly Leu Ala Leu Thr Leu Gln Pro Arg Gly Glu Asp Ser Arg
210 215 220

Leu Ser Thr Ala Arg Leu Gln Ala Leu Leu Phe Gly Asp Asp His Arg
225 230 235 240

Cys Phe Thr Arg Met Thr Pro Ala Leu Leu Leu Leu Pro Arg Ser Glu
[0002] 245 250 255

Pro Ala Pro Leu Pro Ala His Gly Gln Leu Asp Thr Val Pro Phe Pro
260 265 270

Pro Pro Arg Pro Ser Ala Glu Leu Glu Glu Ser Pro Pro Ser Ala Asp
275 280 285

Pro Phe Leu Glu Thr Leu Thr Arg Leu Val Arg Ala Leu Arg Val Pro
290 295 300

Pro Ala Arg Ala Ser Ala Pro Arg Leu Ala Leu Asp Pro Asp Ala Leu
305 310 315 320

Ala Gly Phe Pro Gln Gly Leu Val Asn Leu Ser Asp Pro Ala Ala Leu
325 330 335

Glu Arg Leu Leu Asp Gly Glu Glu Pro Leu Leu Leu Leu Leu Arg Pro
340 345 350

Thr Ala Ala Thr Thr Gly Asp Pro Ala Pro Leu His Asp Pro Thr Ser
355 360 365

Ala Pro Trp Ala Thr Ala Leu Ala Arg Arg Val Ala Ala Glu Leu Gln
370 375 380

Ala Ala Ala Ala Glu Leu Arg Ser Leu Pro Gly Leu Pro Pro Ala Thr
385 390 395 400

Ala Pro Leu Leu Ala Arg Leu Leu Ala Leu Cys Pro Gly Gly Pro Gly
405 410 415

Gly Leu Gly Asp Pro Leu Arg Ala Leu Leu Leu Leu Lys Ala Leu Gln
420 425 430

Gly Leu Arg Val Glu Trp Arg Gly Arg Asp Pro Arg Gly Pro Gly Arg
435 440 445

Ala Gln Arg Ser Ala Gly Ala Thr Ala Ala Asp Gly Pro Cys Ala Leu
450 455 460

Arg Glu Leu Ser Val Asp Leu Arg Ala Glu Arg Ser Val Leu Ile Pro
465 470 475 480

[0003]

Glu Thr Tyr Gln Ala Asn Asn Cys Gln Gly Val Cys Gly Trp Pro Gln
485 490 495

Ser Asp Arg Asn Pro Arg Tyr Gly Asn His Val Val Leu Leu Leu Lys
500 505 510

Met Gln Val Arg Gly Ala Ala Leu Ala Arg Pro Pro Cys Cys Val Pro
515 520 525

Thr Ala Tyr Ala Gly Lys Leu Leu Ile Ser Leu Ser Glu Glu Arg Ile
530 535 540

Ser Ala His His Val Pro Asn Met Val Ala Thr Glu Cys Gly Cys Arg
545 550 555 560

<210> 2
<211> 559
<212> PRT
<213> 人工序列

<220>
<223> 人工序列的描述：合成的多肽

<400> 2

Met Lys Trp Val Thr Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ala
1 5 10 15

Tyr Ser Arg Gly Val Phe Arg Arg Arg Ala Glu Glu Pro Ala Val Gly
20 25 30

Thr Ser Gly Leu Ile Phe Arg Glu Asp Leu Asp Trp Pro Pro Gly Ser
35 40 45

Pro Gln Glu Pro Leu Cys Leu Val Ala Leu Gly Gly Asp Ser Asn Gly
50 53 60

Ser Ser Ser Pro Leu Arg Val Val Gly Ala Leu Ser Ala Tyr Glu Gln
65 70 75 80

Ala Phe Leu Gly Ala Val Gln Arg Ala Arg Trp Gly Pro Arg Asp Leu
85 90 95

Ala Thr Phe Gly Val Cys Asn Thr Gly Asp Arg Gln Ala Ala Leu Pro
100 105 110

[0004]

Ser Leu Arg Arg Leu Gly Ala Trp Leu Arg Asp Pro Gly Gly Gln Arg
115 120 125

Leu Val Val Leu His Leu Glu Glu Val Thr Trp Glu Pro Thr Pro Ser
130 135 140

Leu Arg Phe Gln Glu Pro Pro Pro Gly Gly Ala Gly Pro Pro Glu Leu
145 150 155 160

Ala Leu Leu Val Leu Tyr Pro Gly Pro Gly Pro Glu Val Thr Val Thr
165 170 175

Arg Ala Gly Leu Pro Gly Ala Gln Ser Leu Cys Pro Ser Arg Asp Thr
180 185 190

Arg Tyr Leu Val Leu Ala Val Asp Arg Pro Ala Gly Ala Trp Arg Gly
195 200 205

Ser Gly Leu Ala Leu Thr Leu Gln Pro Arg Gly Glu Asp Ser Arg Leu
210 215 220

Ser Thr Ala Arg Leu Gln Ala Leu Leu Phe Gly Asp Asp His Arg Cys
225 230 235 240

Phe Thr Arg Met Thr Pro Ala Leu Leu Leu Leu Pro Arg Ser Glu Pro
245 250 255

Ala Pro Leu Pro Ala His Gly Gln Leu Asp Thr Val Pro Phe Pro Pro
260 265 270

Pro Arg Pro Ser Ala Glu Leu Glu Glu Ser Pro Pro Ser Ala Asp Pro
275 280 285

Phe Leu Glu Thr Leu Thr Arg Leu Val Arg Ala Leu Arg Val Pro Pro
290 295 300

Ala Arg Ala Ser Ala Pro Arg Leu Ala Leu Asp Pro Asp Ala Leu Ala
305 310 315 320

Gly Phe Pro Gln Gly Leu Val Asn Leu Ser Asp Pro Ala Ala Leu Glu
325 330 335

[0005]

Arg Leu Leu Asp Gly Glu Glu Pro Leu Leu Leu Leu Leu Arg Pro Thr
340 345 350

Ala Ala Thr Thr Gly Asp Pro Ala Pro Leu His Asp Pro Thr Ser Ala
355 360 365

Pro Trp Ala Thr Ala Leu Ala Arg Arg Val Ala Ala Glu Leu Gln Ala
370 375 380

Ala Ala Ala Glu Leu Arg Ser Leu Pro Gly Leu Pro Pro Ala Thr Ala
385 390 395 400

Pro Leu Leu Ala Arg Leu Leu Ala Leu Cys Pro Gly Gly Pro Gly Gly
405 410 415

Leu Gly Asp Pro Leu Arg Ala Leu Leu Leu Leu Leu Lys Ala Leu Gln Gly
420 425 430

Leu Arg Val Glu Trp Arg Gly Arg Asp Pro Arg Gly Pro Gly Arg Ala
435 440 445

Arg Arg Ser Ala Gly Ala Thr Ala Ala Asp Gly Pro Cys Ala Leu Arg
450 455 460

Glu Leu Ser Val Asp Leu Arg Ala Glu Arg Ser Val Leu Ile Pro Glu
465 470 475 480

Thr Tyr Gln Ala Asn Asn Cys Gln Gly Val Cys Gly Trp Pro Gln Ser
485 490 495

Asp Arg Asn Pro Arg Tyr Gly Asn His Val Val Leu Leu Leu Lys Met
500 505 510

Gln Val Arg Gly Ala Ala Leu Ala Arg Pro Pro Cys Cys Val Pro Thr
515 520 525

Ala Tyr Ala Gly Lys Leu Leu Ile Ser Leu Ser Glu Glu Arg Ile Ser
530 535 540

Ala His His Val Pro Asn Met Val Ala Thr Glu Cys Gly Cys Arg
545 550 555

[0006]

<210> 3
<211> 567
<212> PRT
<213> 人工序列

<220>
<223> 人工序列的描述：合成的多肽

<400> 3
Met Lys Trp Val Thr Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ala
1 5 10 15

Tyr Ser Arg Gly Val Phe Arg Arg Arg Ala Glu Glu Pro Ala Val Gly
20 25 30

Thr Ser Gly Leu Ile Phe Arg Glu Asp Leu Asp Trp Pro Pro Gly Ser
35 40 45

Pro Gln Glu Pro Leu Cys Leu Val Ala Leu Gly Gly Asp Ser Asn Gly
50 55 60

Ser Ser Ser Pro Leu Arg Val Val Gly Ala Leu Ser Ala Tyr Glu Gln
65 70 75 80

Ala Phe Leu Gly Ala Val Gln Arg Ala Arg Trp Gly Pro Arg Asp Leu
85 90 95

Ala Thr Phe Gly Val Cys Asn Thr Gly Asp Arg Gln Ala Ala Leu Pro
100 105 110

Ser Leu Arg Arg Leu Gly Ala Trp Leu Arg Asp Pro Gly Gly Gln Arg
115 120 125

Leu Val Val Leu His Leu Glu Glu Val Thr Trp Glu Pro Thr Pro Ser
130 135 140

Leu Arg Phe Gln Glu Pro Pro Pro Gly Gly Ala Gly Pro Pro Glu Leu
145 150 155 160

Ala Leu Leu Val Leu Tyr Pro Gly Pro Gly Pro Glu Val Thr Val Thr
165 170 175

Arg Ala Gly Leu Pro Gly Ala Gln Ser Leu Cys Pro Ser Arg Asp Thr
180 185 190

[0007] Arg Tyr Leu Val Leu Ala Val Asp Arg Pro Ala Gly Ala Trp Arg Gly
195 200 205

Ser Gly Leu Ala Leu Thr Leu Gln Pro Arg Gly Glu Asp Ser Arg Leu
210 215 220

Ser Thr Ala Arg Leu Gln Ala Leu Leu Phe Gly Asp Asp His Arg Cys
225 230 235 240

Phe Thr Arg Met Thr Pro Ala Leu Leu Leu Leu Pro Arg Ser Glu Pro
245 250 255

Ala Pro Leu Pro Ala His Gly Gln Leu Asp Thr Val Pro Phe Pro Pro
260 265 270

Pro Arg Pro Ser Ala Glu Leu Glu Glu Ser Pro Pro Ser Ala Asp Pro
275 280 285

Phe Leu Glu Thr Leu Thr Arg Leu Val Arg Ala Leu Arg Val Pro Pro
290 295 300

Ala Arg Ala Ser Ala Pro Arg Leu Ala Leu Asp Pro Asp Ala Leu Ala

305 310 315 320

Gly Phe Pro Gln Gly Leu Val Asn Leu Ser Asp Pro Ala Ala Leu Glu
325 330 335

Arg Leu Leu Asp Gly Glu Glu Pro Leu Leu Leu Leu Leu Arg Pro Thr
340 345 350

Ala Ala Thr Thr Gly Asp Pro Ala Pro Leu His Asp Pro Thr Ser Ala
355 360 365

Pro Trp Ala Thr Ala Leu Ala Arg Arg Val Ala Ala Glu Leu Gln Ala
370 375 380

Ala Ala Ala Glu Leu Arg Ser Leu Pro Gly Leu Pro Pro Ala Thr Ala
385 390 395 400

Pro Leu Leu Ala Arg Leu Leu Ala Leu Cys Pro Gly Gly Pro Gly Gly
405 410 415

[0008] Leu Gly Asp Pro Leu Arg Ala Leu Leu Leu Leu Lys Ala Leu Gln Gly
420 425 430

Leu Arg Val Glu Trp Arg Gly Arg Asp Pro Arg Gly Pro Gly Arg Ala
435 440 445

Arg Arg Ser Asp Tyr Lys Asp Asp Asp Asp Lys Ala Gly Ala Thr Ala
450 455 460

Ala Asp Gly Pro Cys Ala Leu Arg Glu Leu Ser Val Asp Leu Arg Ala
465 470 475 480

Glu Arg Ser Val Leu Ile Pro Glu Thr Tyr Gln Ala Asn Asn Cys Gln
485 490 495

Gly Val Cys Gly Trp Pro Gln Ser Asp Arg Asn Pro Arg Tyr Gly Asn
500 505 510

His Val Val Leu Leu Leu Lys Met Gln Val Arg Gly Ala Ala Leu Ala
515 520 525

Arg Pro Pro Cys Cys Val Pro Thr Ala Tyr Ala Gly Lys Leu Leu Ile
530 535 540

[0009]

Ser Leu Ser Glu Glu Arg Ile Ser Ala His His Val Pro Asn Met Val
545 550 555 560

Ala Thr Glu Cys Gly Cys Arg
565

<210> 4
<211> 1680
<212> DNA
<213> 人工序列

<220>
<223> 人工序列的描述：合成的多核苷酸

<400> 4
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gtgttccgcc gcagagcaga ggagccagct gtgggcacca gtggcctcat cttccgagaa 120
gacttggact ggcctccagg cagcccacaa gagcctctgt gcctggtggc actgggcggg 180
gacagcaatg gcagcagctc ccccctgcgg gtggtggggg ctctaagcgc ctatgagcag 240
gccttcctgg gggccgtgca gagggcccgc tggggccccc gagacctggc caccttcggg 300
gtctgcaacg ccggtgacag gcaggctgcc ttgccctctc tacggcggct ggggggcctgg 360
ctgcgggacc ctgggggggca gcgcctggtg gtcctacacc tggaggaagt gacctgggag 420
ccaacaccct cgctgaggtt ccaggagccc ccgcctggag gagctggccc cccagagctg 480
gcgctgctgg tgctgtaccc tgggcctggc cctgaggtca ctgtgacgag ggctgggctg 540
ccgggtgccc agagcctctg ccccttcccga gacaccccct acctggtgtt agcggtggac 600
cgccctgcgg gggcctggcg cggctccggg ctggccttga ccctgcagcc ccgcggagag 660
gactcccggc tgagtaccgc ccggctgcag gcactgctgt tcggcgacga ccaccgctgc 720
ttcacacgga tgaccccggc cctgctcctg ctgccgcggt ccgagccccgc gccgctgcct 780
gcgcacggcc agctggacac cgtgccccttc ccgccgcccca ggccatccgc ggaactcgag 840
gagtcgccac ccagcgcaga ccccttcctg gagacgctca cgcgcctggt gcgggcgctg 900
cgggtccccc cggcccgggc ctccgcgccg cgcctggccc tggatccgga cgcgctggcc 960
ggcttcccgc agggcctagt caacctgtcg gacccccgcgg cgctggagcg cctactgcac 1020
agcgaggagc cgctgctgct gctgctgagg cccactgcgg ccaccaccgg ggatcctgcg 1080
ccccctgcacg acccccacgtc ggcgcccgtgg gccacggccc tggcgcgccg cgtggctgct 1140

[0010]

gaactgcaag cggcggctgc cgagctgcga agcctcccgg gtctgcctcc ggccacagcc 1200

ccgctgctgg cgcgcctgct cgccgctctgc ccaggtggcc ccggcggcct cggcgatccc 1260

ctgcgagcgc tgctgctcct gaaggcgctg cagggcctgc gcgtggagtg gcgcgggcgg 1320

gatccgcgcg ggccgggtcg ggcacggcgc agcgcggggg ccaccgccgc cgacgggccg 1380

tgcggctgc gcgagctcag cgtagacctc cgcgccgagc gctccgtact catccccgag 1440

acctaccagg ccaacaattg ccagggcgtg tgcggctggc ctcagtccga ccgcaacccg 1500

cgctacggca accacgtggt gctgctgctg aagatgcagg cccgtggggc cgccctggcg 1560

cgccccaccct gctgcgtgcc caccgcctac gcgggcaagc tgctcatcag cctgtcggag 1620

gagcgcatca gcgcgcacca cgtgcccaac atggtggcca ccgagtgtgg ctgccggtga 1680

<210> 5
<211> 1704
<212> DNA
<213> 人工序列

<220>
<223> 人工序列的描述：合成的多核苷酸

<400> 5
atgaagtggg tgagcttcat cagcctgctg ttcctgttca gcagcgctta ctccccgcggt 60

gtgttccgcc gcagagcaga ggagccagct gtgggcacca gtggcctcat cttccgagaa 120

gacttggact ggcctccagg cagcccacaa gagcctctgt gcctggtggc actgggcggg 180

gacagcaatg gcagcagctc ccccctgcgg gtggtggggg ctctaagcgc ctatgagcag 240

gccttcctgg gggccgtgca gagggcccgc tggggccccc gagacctggc cacccttcggg 300

gtctgcaaca ccggtgacag gcaggctgcc ttgccctctc tacggcggct ggggggcctgg 360

ctgcgggacc ctgggggggca gcgcctggtg gtcctacacc tggaggaagt gacctgggag 420

ccaacaccct cgctgaggtt ccaggagccc ccgcctgggag gagctggccc cccagagctg 480

gcgctgctgg tgctgtaccc tgggcctggc cctgaggtca ctgtgacgag ggctgggctg 540

ccgggtgccc agagcctctg ccccctcccga gacaccccgct accctggtgtt agcggtggac 600

cgccctgcgg gggcctggcg cggctccggg ctggccttga ccctgcagcc ccgcggagag 660

gactcccggc tgagtaccgc ccggctgcag gcactgctgt tcgggcgacga ccaccgctgc 720

ttcacacgga tgaccccggc cctgctcctg ctgccgcggt ccgagccccgc gccgctgcct 780

gcgcacggcc agctggacac cgtgcccttc ccgccgccca ggccatccgc ggaactcgag 840

gagtcgccac ccagcgcaga ccccttcctg gagacgctca cgcgcctggt gcgggcgctg 900

[0011]

cgggtccccc cggcccgggc ctccgcgccg cgcctggccc tggatccgga cgccgctggcc 960

ggcttcccgc aggggctagt caacctgtcg gaccccgcgg cgctggagcg cctactcgac 1020

ggcgaggagc cgctgctgct gctgctgagg cccactgcgg ccaccaccgg ggatcctgcg 1080

cccctgcacg accccacgtc ggcgccgtgg gccacggccc tggcgcggcg cgtggctgct 1140

gaactgcaag cggcggctgc cgagctgcga agcctcccgg gtctgcctcc ggccacagcc 1200

ccgctgctgg cgcgcctgct cgcgctctgc ccaggtggcc ccggcggcct cggcgatccc 1260

ctgcgagcgc tgctgctcct gaaggcgctg cagggcctgc gcgtggagtg gcgcgggcgg 1320

gatccgcgcg ggccgggtcg ggcacggcgc agcgactaca aggatgacga cgacaaggcg 1380

ggggccaccg ccgccgacgg gccgtgcgcg ctgcgcgagc tcagccgtaga cctccgcgcc 1440

gagcgctccg tactcatccc cgagacctac caggccaaca attgccaggg cgtgtgcggc 1500

tggcctcagt ccgaccgcaa cccgcgctac ggcaaccacg tggtgctgct gctgaagatg 1560

caggcccgtg gggccgcccc ggcgcgcccа ccctgctgcg tgcccaccgc ctacgcgggc 1620

aagctgctca tcagcctgtc ggaggagcgc atcagcgcgc accacgtgcc caacatggtg 1680

gccaccgagt gtggctgccg gtga 1704

<210> 6
<211> 24
<212> PRT
<213> 智人

<400> 6
Met Lys Trp Val Thr Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ala
1 5 10 15

Tyr Ser Arg Gly Val Phe Arg Arg
20

<210> 7
<211> 78
<212> DNA
<213> 智人

<400> 7
atgaagtggg tgagcttcat cagcctgctg ttcctgttca gcagcgctta ctcccgcggt 60

gtgttccgcc gcagagca 78

<210> 8
<211> 8

[0012]

<212> PRT
<213> 人工序列

<220>
<223> 人工序列的描述：合成的肽

<400> 8
Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

<210> 9
<211> 24
<212> DNA
<213> 人工序列

<220>
<223> 人工序列的描述：合成的寡核苷酸

<400> 9
gactacaagg atgacgacga caag 24

<210> 10
<211> 252
<212> PRT
<213> 人工序列

<220>
<223> 人工序列的描述：合成的多肽

<400> 10
Leu Glu Leu Val Pro Arg Gly Ser Gly Asp Pro Ile Glu Gly Arg Gly
1 5 10 15

Gly Gly Gly Gly Asp Pro Lys Ser Cys Asp Lys Pro His Thr Cys Pro
20 25 30

Leu Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe
35 40 45

Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val
50 55 60

Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe
65 70 75 80

Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro
85 90 95

Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr

[0013]

100 105 110

Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
115 120 125

Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala
130 135 140

Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg
145 150 155 160

Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly
165 170 175

Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
180 185 190

Glu Asn Asn Tyr Lys Ala Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
195 200 205

Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln
210 215 220

Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
225 230 235 240

Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
245 250

<210> 11
<211> 101
<212> DNA
<213> 人工序列

<220>
<223> 人工序列的描述：合成的多核苷酸

<400> 11
cgagatacat gaagtggagtg agcttcatca gcctgctgtt cctgttcagc agcgcttact 60

cccgcggtgt gttccggcgc agagcagagg agccagctgt g 101

<210> 12
<211> 20
<212> DNA

[0014]

<213> 人工序列

<220>
<223> 人工序列的描述：合成的引物

<400> 12
gctcctggaa ccteagegag 20

<210> 13
<211> 18
<212> PRT
<213> 智人

<400> 13
Met Lys Trp Val Thr Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ala
1 5 10 15

Tyr Ser

<210> 14
<211> 18
<212> PRT
<213> 智人

<400> 14
Met Lys Trp Val Ser Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ala
1 5 10 15

Tyr Ser

<210> 15
<211> 21
<212> PRT
<213> 未知

<220>
<223> 未知的描述：MPIF-1信号序列

<400> 15
Met Lys Val Ser Val Ala Ala Leu Ser Cys Leu Met Leu Val Thr Ala
1 5 10 15

Leu Gly Ser Gln Ala
20

<210> 16
<211> 17
<212> PRT

<213> 未知

<220>
<223> 未知的描述：斯钙素信号序列

<400> 16
Met Leu Gln Asn Ser Ala Val Leu Leu Leu Leu Val Ile Ser Ala Ser
1 5 10 15

Ala

<210> 17
<211> 19
<212> PRT
<213> 未知

<220>
<223> 未知的描述：转化酶信号序列

<400> 17
Met Leu Leu Gln Ala Phe Leu Phe Leu Leu Ala Gly Phe Ala Ala Lys
1 5 10 15

[0015] Ile Ser Ala

<210> 18
<211> 24
<212> PRT
<213> 人工序列

<220>
<223> 人工序列的描述：合成的肽

<400> 18
Met Lys Trp Val Ser Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ala
1 5 10 15

Tyr Ser Arg Ser Leu Glu Lys Arg
20

<210> 19
<211> 24
<212> PRT
<213> 人工序列

<220>
<223> 人工序列的描述：合成的肽

<400> 19
Met Lys Trp Val Ser Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ala
1 5 10 15

Tyr Ser Arg Ser Leu Asp Lys Arg
20

<210> 20
<211> 21
<212> PRT
<213> 人工序列

<220>
<223> 人工序列的描述：合成的肽

<400> 20
Met Asn Ile Phe Tyr Ile Phe Leu Phe Leu Leu Ser Phe Val Gln Gly
1 5 10 15

Ser Leu Asp Lys Arg
20

[0016]

<210> 21
<211> 19
<212> PRT
<213> 未知

<220>
<223> 未知的描述：免疫球蛋白Ig信号序列

<400> 21
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
1 5 10 15

Val His Ser

<210> 22
<211> 29
<212> PRT
<213> 未知

<220>
<223> 未知的描述：Fibulin B前体信号序列

<400> 22
Met Glu Arg Ala Ala Pro Ser Arg Arg Val Pro Leu Pro Leu Leu Leu
1 5 10 15

Leu Gly Gly Leu Ala Leu Leu Ala Ala Gly Val Asp Ala
20 25

<210> 23
<211> 22
<212> PRT
<213> 未知

<220>
<223> 未知的描述：簇集蛋白前体信号序列

<400> 23
Met Met Lys Thr Leu Leu Leu Phe Val Gly Leu Leu Leu Thr Trp Glu
1 5 10 15

Ser Gly Gln Val Leu Gly
20

<210> 24
<211> 21
<212> PRT
<213> 未知

[0017] <220>
<223> 未知的描述：胰岛素样生长因子结合蛋白4信号序列

<400> 24
Met Leu Pro Leu Cys Leu Val Ala Ala Leu Leu Leu Ala Ala Gly Pro
1 5 10 15

Gly Pro Ser Leu Gly
20

<210> 25
<211> 25
<212> PRT
<213> 智人

<400> 25
Met Arg Asp Leu Pro Leu Thr Ser Leu Ala Leu Val Leu Ser Ala Leu
1 5 10 15

Gly Ala Leu Leu Gly Thr Glu Ala Leu
20 25

<210> 26
<211> 5
<212> PRT

<213> 智人

<400> 26
Arg Ala Gln Arg Ser
1 5

<210> 27
<211> 5
<212> PRT
<213> 人工序列

<220>
<223> 人工序列的描述：合成的肽

[0018] <400> 27
Arg Ala Arg Arg Ser
1 5

<210> 28
<211> 5
<212> PRT
<213> 人工序列

<220>
<223> 人工序列的描述：合成的肽

<400> 28
Arg Ala Gln Arg Arg
1 5

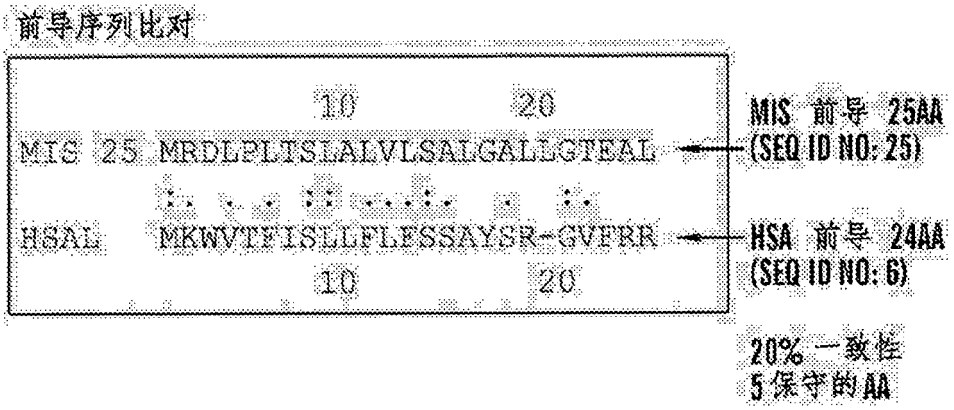
前导序列比对
10 20
MIS 25 MRDLPLISLALVLSALGALLGTEAL
HSAL MKWVTFISLLFLFSSAYSR-GVFRR
10 20
MIS 前导 25AA
(SEQ ID NO: 25)
HSA 前导 24AA
(SEQ ID NO: 6)
20% 一致性
5 保守的 AA

图1A

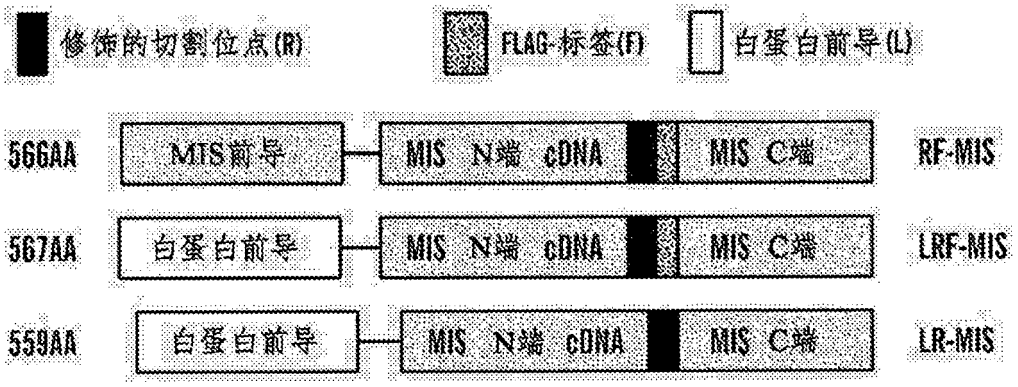
修饰的切割位点(R)
FLAG-标签(F)
白蛋白前导(L)
566AA
MTS前导
MIS N端 cDNA
MIS C端
RF-MIS
567AA
白蛋白前导
MIS N端 cDNA
MIS C端
LRF-MIS
559AA
白蛋白前导
MIS N端 cDNA
MIS C端
LR-MIS

图1B

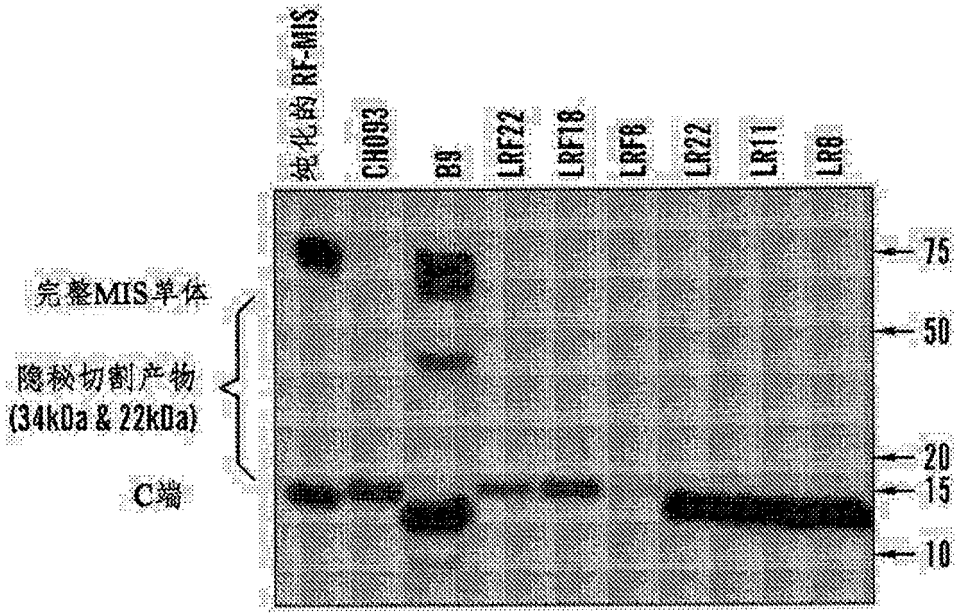
纯化的 RF-MIS
CHO93
B9
LRF22
LRF18
LRF8
LR22
LR11
LR8
完整MIS单体
隐秘切割产物
(34kDa & 22kDa)
C端
75
50
20
15
10

图2

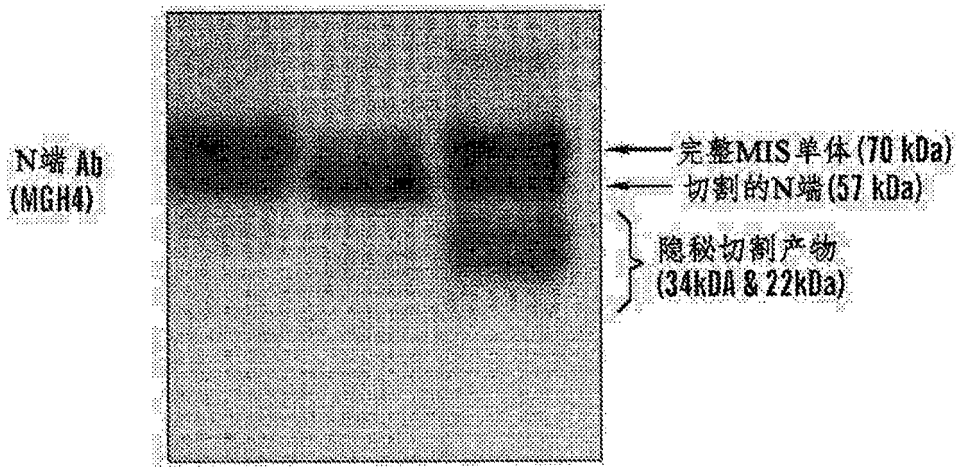
N端 Ab
(MGH4)
完整MIS单体(70 kDa)
切割的N端(57 kDa)
隐秘切割产物
(34kDA & 22kDa)

图3A

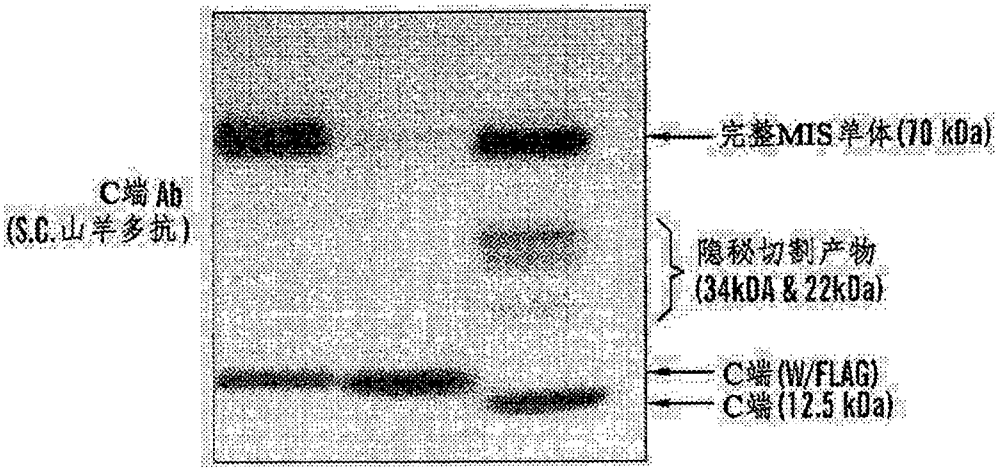
C端 Ab
(S.C.山羊多抗)
完整MIS单体(70 kDa)
隐秘切割产物
(34kDA & 22kDa)
C端(W/FLAG)
C端(12.5 kDa)

图3B

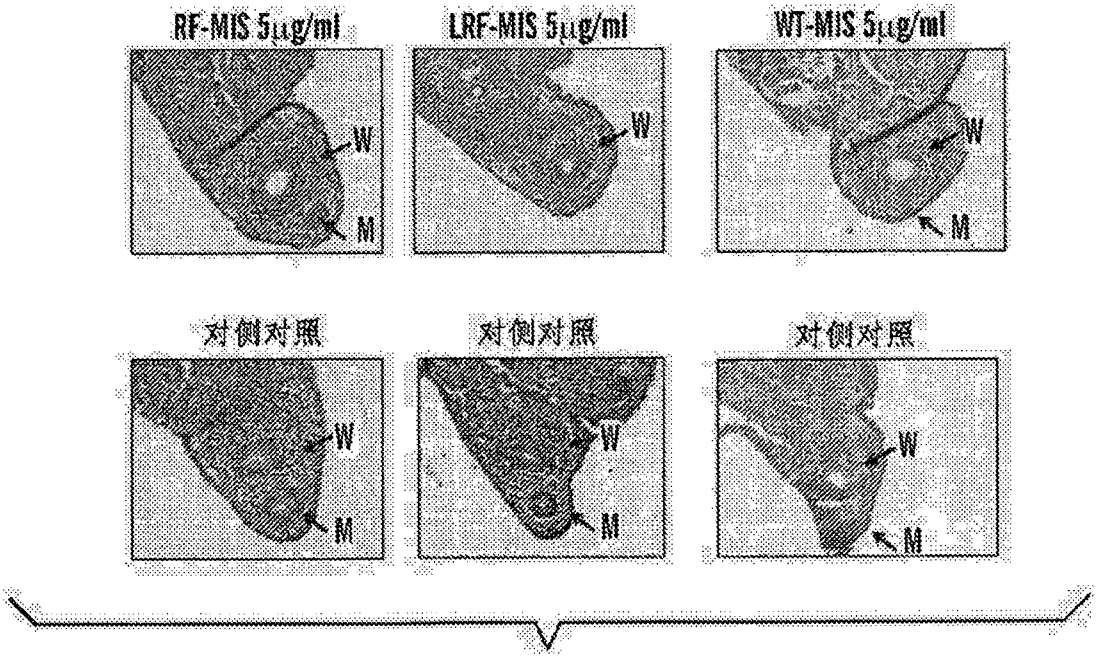
RF-MIS 5μg/ml
LRF-MIS 5μg/ml
WT-MIS 5μg/ml
W
M
对侧对照
对侧对照
对侧对照

图4A

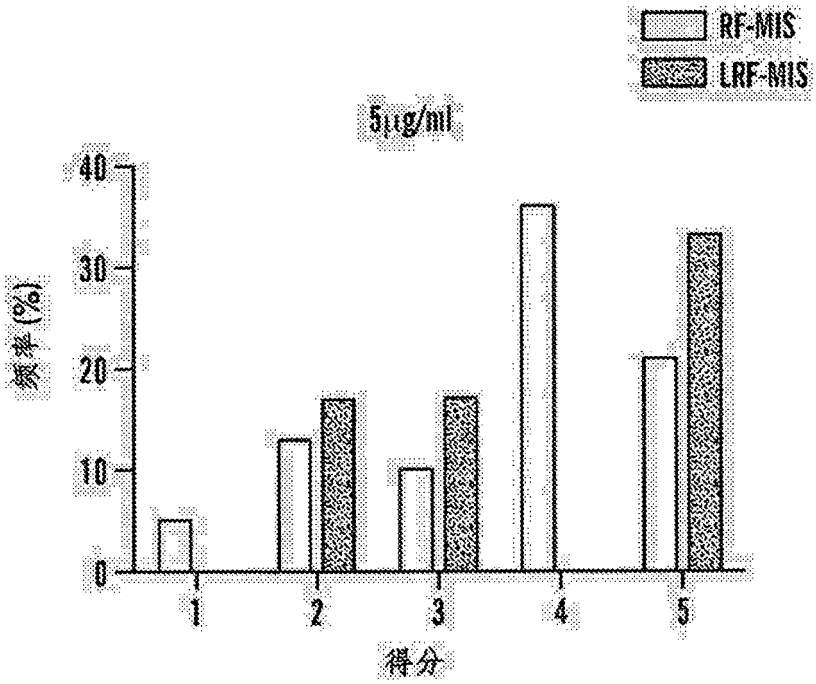

图4B

（传统编号）

mrdlpltsla lvlsalgall gtealraeep avgtsglifr edldwppgsp qeplclvalg 60 (35)
前导序列（SEQ ID NO：1的1-25）

gdsngssspl rvvgalsaye qaflgavqra rwgprdlatf gvcntgdrqa alpslrrlga 120 (95)

wlrdpggqrl vvlhleevtw eptpslrfqe pppggagppe lallvlypgp gpevtvtrag 180 (155)

lpgaqslcps rdtrylvlav drpagawrgs glaltlqprg edsrlstarl qallfgddhr 240 (215)

cftrmtpall llprsepapl pahgqldtvp fppprpsael eesppsadpf letltrlvra 300 (275)
次要切割位点（SEQ ID NO：1的254/255）

lrvpparasa prlaldpdal agfpqglvnl sdpaalerll dgeeplllll rptaattgdp 360 (335)

aplhdptsap watalarrva aelqaaaael rslpglppat apllarllal cpggpgglgd 420 (395)

plrallllka lqglrvewrg rdprqpg**raq rs**agataadg pcalrelsvd lraersvlip 480 (455)
主要切割识别序列（SEQ ID NO：1的448-452）

etyqanncgg vcgwpqsdrn prygnhvvll lkmqvrgaal arppccvpta yagkllisls 540 (515)

eerisahhvp nmvatecgcr 560 (535) (SEQ ID NO:1)

图5A

	在SEQ ID NO：1上的位置	在标准命名的MIS上的位置（第一个氨基酸在前导序列之后）
前导序列：	1-25	氨基酸残基-24 0
主要切割识别序列	448-452 (RAQR/S)	423-427
主要切割位点	在451和452之间（451/452）	在426和427之间（426/427）
主要切割识别序列的改变	氨基酸450从Q变为R（Q450R）	氨基酸425从Q变为R（Q425R）
次要切割识别位点	在254和255之间（254/255）	在229和230之间（229/230）

图5B

Our Ref：16HKI055-101

說明書摘要

發明名稱：修飾的繆勒抑制物質(MIS)蛋白及其用於疾病治療的用途

本發明涉及修飾的重組人 MIS 蛋白，所述修飾的重組人 MIS 蛋白相比野生型人 MIS 蛋白而言具有改善的切割和增加的生物活性以及增加的效能。本發明的其它方面涉及通過向受試者給予包含重組人 MIS 蛋白的組合物來預防和治療癌症(例如，表達 II 型 MIS 受體(MISRII)的癌症)的方法。本發明的另一方面涉及降低受試者的血漿雄激素水平的方法和/或用於治療患有以過多雄激素為特徵的疾病的受試者的方法。另一方面提供了含有重組人 MIS 蛋白的藥物組合物和試劑盒以及使用方法。本發明的另一方面涉及通過一起給予化療劑和所述重組 MIS 蛋白來減少所述化療劑的劑量的方法，所述重組 MIS 蛋白降低了所述化療劑的有效劑量。

ABSTRACT

Title of Invention : MODIFIED MULLERIAN INHIBITING SUBSTANCE (MIS) PROTEINS AND USES THEREOF FOR THE TREATMENT OF DISEASES

The present invention relates to modified recombinant human MIS protein which has improved cleavage and increased bioactivity and increased potency as compared to wild-type human MIS protein. Other aspects of the invention relate to methods to prevent and treat cancers, such as cancers that express the MIS receptor type II (MISRII) by administering to a subject a composition comprising a recombinant human MIS protein. Another aspect of the present invention relates to methods to lower plasma androgen levels in a subject, and/or for the treatment of a subject with a disease characterized by excess androgen. Another aspect provides pharmaceutical compositions and kits and methods for use comprising a recombinant human MIS protein. Another aspect of the present invention relates to methods to decrease the dose of a chemotherapeutic agent by administering the chemotherapeutic agent with the recombinant MIS protein that lowers the effective dose of the chemotherapeutic agent.