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Title: A METHOD FOR TREATING ENDOMETRIOSIS BY ADMINISTERING MULLERIAN INHIBITING SUBSTANCE

Abstract: We have discovered that Mullerian Inhibiting Substance (MIS) can be used therapeutically to treat or prevent endometriosis in mammals, particularly humans. In a preferred embodiment a therapeutically effective amount of human MIS or a biologically active fragment or variant thereof is administered to treat a patient having endometriosis, or to prevent endometriosis in a patient who is at risk of developing it or to prevent a reoccurrence of endometriosis. The therapeutically effective amount of MIS is an amount that decreases the proliferation of endometriotic cells, an amount that our results show is significantly higher than the normal circulating levels of endogenous MIS.
A METHOD FOR TREATING ENDOMETRIOSIS BY ADMINISTERING MULLERIAN INHIBITING SUBSTANCE

CROSS-REFERENCE TO RELATED APPLICATIONS
[0001] This application claims benefit of Provisional Appln. 60979863, filed October 14, 2007, the entire contents of which are hereby incorporated by reference as if fully set forth herein, under 35 U.S.C. §119(e).

BACKGROUND OF THE INVENTION
1. Field of the Invention
[0002] The invention is in the field of treatment and prevention of endometriosis.

2. Description of the Related Art
[0003] Endometriosis is a common medical condition characterized by growth beyond or outside the uterus of tissue resembling endometrium, the tissue that normally lines the uterus. Endometriosis is associated with a lowered fertility and is the second leading cause of infertility in females that ovulate normally. Extrauterine endometrial growths typically respond to the varying levels of estrogen associated with the menstrual cycle. Thus, endometrial growths proceed through a cycle of proliferation and breakdown. Unlike the uterine lining, however, the body is unable to shed the extraterine endometrial growths, and breakdown of this tissue results in internal bleeding, inflammation of the surrounding area and formation of scar tissue. A number of complications can also arise, including rupture of growths, which can spread the growths to new regions of the body, and form adhesions.

[0004] Affecting an estimated 89 million women of reproductive age around the world, endometriosis occurs in roughly 5% to 10% of women. Endometriosis can also occur in postmenopausal women, with an estimated 2% to 4% of all endometriosis cases being diagnosed in the postmenopausal phase of a woman's life. Endometriosis in postmenopausal women is an extremely aggressive form of this disease. Endometriosis most commonly exists in the lower region of the female pelvis. The most common site of disease is the ovary (approximately half of the cases); less commonly lesions can be found on the bladder, intestines, ureters, bowel and diaphragm. Very rarely endometriosis is found distant from the pelvis, in sites such as the lung, brain, and kidney.

[0005] Prevention of endometriosis is not currently possible; however, treatment options are available based on the patient's desire for future fertility, symptoms, the stage of disease,
and to some extent, age. Possible treatment options include analgesic treatments, such as nonsteroidal anti-inflammatory agents and prostaglandin synthetase-inhibiting drugs, and hormonal therapy. Common hormonal therapies include oral contraceptive pills; progestational agents, which cause decidualization in the endometriotic tissue; danazol, a weak androgen that is the isoxazole derivative of 17-alpha-ethyl testosterone (ethisterone); and gonadotropin-releasing hormone (GNRH) agonists, which are analogues of the 10-amino-acid polypeptide hormone GnRH and act via the suppression of gonadotropin secretion, resulting in elimination of ovarian steroidogenesis and suppression of endometrial implants. Lastly, surgical treatment, including laparoscopic resection, ablation of minimal or mild endometriosis, presacral neurectomy and uterosacral ligament ablation, may be performed to excise or destroy all endometriotic tissue, remove all adhesions, and restore pelvic anatomy to the best possible condition. Thus there is still a need for an effective therapy to treat or prevent endometriosis.
SUMMARY OF THE INVENTION

[0006] Certain embodiments of the invention are directed to a method for treating endometriosis in a female animal, preferably a human, by administering a therapeutically effective amount of Mullerian inhibiting substance (MIS), preferably human recombinant MIS, or a biologically active fragment or variant thereof. In a preferred embodiment the MIS is administered locally to the site of the endometriosis. In another preferred embodiment the therapeutically effective amount of Mullerian inhibiting substance for treating endometriosis is from about 50 to 1,000 ng/ml, more preferably from about 100 to about 500 ng/ml. In another embodiment the therapeutically effective amount of Mullerian inhibiting substance is an amount that decreases the proliferation of endometriotic cells.

[0007] Another embodiment is directed to a method of preventing a recurrence of endometriosis in a female, preferably a human, by administering a therapeutically effective amount of Mullerian inhibiting substance, or a biologically active fragment or variant thereof, preferably human recombinant MIS.

[0008] Another embodiment is directed to a method of preventing endometriosis in a female, preferably a human, who is at risk of developing endometriosis by administering a therapeutically effective amount of Mullerian inhibiting substance, or a biologically active fragment or variant thereof, preferably recombinant human Mullerian inhibiting substance. Other embodiments are directed to pharmaceutical formulations of MIS, preferably human recombinant Mullerian inhibiting substance, in an amount of from about 50 to 1,000 ng/ml, more preferably from about 100 to about 500 ng/ml.
BRIEF DESCRIPTION OF THE DRAWINGS

[0009] The present invention is illustrated by way of example, and not by way of limitation, in the figures of the accompanying drawings and in which:

[0010] FIG. 1: MIS signalling genes are present in human endometrium. cDNA derived from a representative proliferative endometrium sample was PCR amplified for the following genes and conditions: HPRT (Lane 1), HPRT (no cDNA control (Lane 2), HPRT (no reverse transcriptase control( Lane 3), ALK3 ( Lane 4), MISRII ( Lane 5), SMAD 1 ( Lane 6), MIS ( Lane 7) and SMAD ( Lane 9).

[0011] FIG. 2: MIS and MISRII protein are expressed in human endometrium. Fixed sections of human proliferative (a, d) and secretory (b, e) endometrium stained for MIS (a, d) and MISRII (b, e) revealed strong immunoreactivity for both proteins in the glandular cells. Stromal cell immunoreactivity for these proteins appeared to be increased in the secretory phase (b, e). Utilization of secretory antibody only as a negative control revealed no immunoreactivity (c, f).

[0012] FIG. 3: MIS and MISRII protein are present in mitosing cultured human endometrial cells. Cultured primary human endometrial glandular and stromal cells manifested robust cytoplasmic expression of MIS (a) and MISRII (b) protein in those cells undergoing mitosis (40X magnification.) FIG. 4. Estradiol increases endometrial stromal cell secretion of MIS. Sensitive ELISA reveals that primary human ESCs secrete MIS protein. After 72 hours in culture with estradiol 1 micromolar, there was a two-fold increase in the MIS concentration in the conditioned media. Values expressed as mean plus or minus SEM, p<0.05 compared to unconditioned media; p<0.05 compared to vehicle control.

[0013] FIG. 5 MIS decreases endometrialstromal cell viability in vitro. Treatment of human endometrial cells with 100 ng/mL recombinant human MIS results in a 15% inhibition in cell viability compared to vehicle after 72 hours, p<0.05 compared to vehicle control.

[0014] FIG. 6 Effect of increasing the endogenous production of MIS in human ESCs. Transient transfection of human ESCs with an MIS expression plasmid (100 ng/well) induced a 30-fold increase in the amount of MIS secreted into the culture media. No such increase was seen after transfection with similar amounts of empty vector or MISRII plasmids (a). Transient transfection of human ESCs plated in a 96 well tray with 100 ng/well of an MIS or MISRII expression plasmid results in a significantly decreased (60% and 25% respectively) viable cell number compared to transfection with the empty vector as assessed by metabolic conversion of resazurin to resorufin (b). Transient transfection of human ESCs plated in a 96
well tray with 100 ng/well of an MIS or MISRII expression plasmid increased the rate of apoptosis compared to the transfection of empty vector as assessed by a caspase 3/7 assay. Transient transfection of both plasmids (50 ng of each plasmid for a combined total of 100 ng/well) further increased the caspase 3/7 activity (c). p<0.05 compared to pCMV (empty vector) control.
DETAILED DESCRIPTION

[0015] We have discovered that Mullerian Inhibiting Substance (MIS) can be used therapeutically to treat or prevent endometriosis in mammals, particularly humans. In a preferred embodiment a therapeutically effective amount of human MIS or a biologically active fragment or variant thereof is administered to a patient having endometriosis, or at risk of developing or having a reoccurrence of endometriosis. The therapeutically effective amount of MIS is an amount that decreases the proliferation of endometriotic cells, an amount that our results show is significantly higher than the normal circulating levels of endogenous MIS. In a preferred embodiment the subject is human and MIS is recombinant human MIS or a biologically active fragment or variant thereof, that is preferably administered locally to one or more of the sites of the endometriosis. In certain embodiments the therapeutically effective amount of MIS ranges from about 50-1000 ng/ml, more preferably from about 100-500 ng/ml. Certain other embodiments are directed to a pharmaceutical composition for treating endometriosis that contains from about 50-1,000 ng/ml, more preferably from 100-500 ng/ml MIS, preferably recombinant human MIS.

[0016] The embryonic reproductive system of both sexes is initially indistinguishable, consisting of indifferent gonads and the anlagen of the male and female reproductive tract. In female embryos, the Mullerian duct forms from the coelomic epithelium and develops into the fallopian tubes, uterus, cervix, upper vagina, and ovarian surface epithelium. In male embryos, regression of the Mullerian duct is essential for correct sexual differentiation. This process is induced by Mullerian Inhibiting Substance (MIS), a 140 kDa homodimeric glycoprotein which belongs to the TGF-β superfamily, is secreted by Sertoli cells in the embryonic testes. In female embryos, the absence of testicular tissue and hence MIS, permits the development and differentiation of the Mullerian duct into its adult derivatives.

[0017] Mullerian Inhibiting Substance (MIS) is also known as Anti-Mullerian hormone (AMH), Mullerian inhibiting factor (MIF), and Mullerian inhibiting hormone (M1H). The GenBank number for the cDNA for human MIS is K03474, and the amino acid sequence is AC005263.1. The significance of MIS in the postnatal period has not been firmly established. In males, Sertoli cells continue to secrete MIS, the levels of which remain elevated until puberty when they diminish to the 2-5 ng/ml range [1, 2], suggesting a suppressive effect of MIS on Leydig cell testosterone synthesis [3, 4]. Further exploration of this association using transgenic mouse models has demonstrated that MIS overexpression induces Leydig cell hypoplasia, decreased expression of several steroidogenic enzymes, and lower serum
testosterone levels [5, 6]. Conversely, mice with null mutations in either MIS or MISRII have Leydig cell hyperplasia and higher expression levels of cytochrome P450C17-20 hydroxylase/lyase which catalyzes the committed step in testosterone synthesis [6- 8].

In the female, ovarian granulosa cells begin to secrete low levels of MIS postnatally. Levels surge at the time of puberty to approximately 5-8 ng/ml, but then gradually decline throughout reproductive life until they become undetectable by menopause [2, 9]. Analogous to the suppressive effect MIS exerts on Leydig cell differentiation and testosterone synthesis in the testes, in vitro experiments suggest that MIS partially inhibits the initial recruitment of primordial follicles [10] and antagonizes subsequent FSH-dependent follicular growth [H]. Supporting these observations, female MIS knockout mice exhibit increased recruitment of primordial follicles during the prepubertal period, resulting in the premature exhaustion of the follicle pool and earlier cessation of ovulation [12]. Despite the fact that MIS appears to modulate ovarian follicular recruitment in ovulatory women, its role in the physiology of adult endometrium, another key Müllerian duct derivative, has never been explored. Although the expression of MISRII has been shown in human endometrium [13] and rat cervical tissue [14] by immunohistochemistry, functional analyses to investigate the effect of MIS on these tissues have not been undertaken. We therefore performed a series of experiments to determine whether adult human endometrium possesses an intact MIS signaling system, and moreover, whether activation of this system could influence endometrial cell growth. We hypothesized that MIS would negatively inhibit endometrial cell viability given its proapoptotic effects in the Müllerian ducts as well as in several cancer cell lines in vitro [15, 16].

In the studies described below we show that:

1) Quantitative real-time PCR revealed that adult human endometrium produces MIS, MISRII, ALK3, and Smadl;
2) Immunohistochemistry reveals that both MIS and MISRII protein are expressed in human endometrium primarily in the glandular epithelium;
3) Immunocytochemistry of cultured human endometrial stromal cells (ESCs) reveals that MIS and MISRII protein production is essentially restricted to cells undergoing mitosis;
4) ELISA reveals that MIS is actively secreted by human endometrial stromal cells in vitro, and this process is significantly increased by estradiol treatment; and
5) Increasing local MIS concentration in cultured human endometrial stromal cells either by exogenous administration or transient transfection significantly decreases the number of viable cells and increases their rate of apoptosis.

[0020] Genes necessary for MIS signaling are expressed by human endometrium and cultured human ESCs. Like other members of the TGF-β superfamily, MIS signals through a two receptor system. MIS type II receptor (MISRII), a transmembrane serine-threonine kinase, binds specifically to MIS and only shares 30% overall homology with other type II receptors from the TGF-β family. Upon ligand binding, MISRII recruits and phosphorylates a type I receptor. Among the postulated type I MIS receptors are ALK2 (ACTRIA), ALK3 (BMPRIA), and ALK6, which are also utilized in the bone morphogenetic protein (BMP) signaling pathway [17-19]. This group of BMP type I receptors preferentially phosphorylates the regulated Smad1, Smad5, or Smad9, which then forms a complex with the common Smad4 and translocates to the nucleus where it regulates gene expression [20].

**MIS is expressed in both proliferative and secretory human endometrium**

[0021] We were able to demonstrate by RTPCR analysis that both proliferative and secretory human endometrium express mRNA for MIS, MISRII, ALK3, Smad 1, and Smad9 (FIG. 1). As expected, no amplification was seen from PCR reactions in which no cDNA was added (lane 2) or where the template came from a reverse transcriptase-free synthesis reaction (lane 3). The fact that we noted expression of both the MIS and MISRII genes shows that an intact MIS signal transduction system of an autocrine/paracrine nature exists in adult human endometrium. Human endometrium and cultured human endometrial cells produce both MIS and MISRII protein. Thus, cultured human endometrial cells are a good model for studying endometriosis, particularly abnormal proliferation. Fixed cross-sections of proliferative and secretory human endometrium demonstrated strong immunoreactivity for both MIS and MISRII in the glandular epithelium.

[0022] The expression of MIS and MISRII in the stromal cells of secretory endometrium was noticeably increased compared to the stromal cells of proliferative endometrium (FIG. 2 a, b, d, e). Hybridizing these slides to secondary antibody only yielded no immunoreactivity (FIG. 2 c, f). Immunocytochemistry of cultured human endometrial stromal and glandular cells revealed that MIS (FIG. 3 a, c) and MISRII (FIG. 3b, d) were consistently and nearly exclusively expressed by cells undergoing or having recently completed mitosis. This finding is consistent with a previous study demonstrating a similar positive correlation between MIS mRNA levels and mitotic activity in rat ovarian Sertoli and granulose cells [21].
temporally restricted expression of MIS/MISRII during mitosis shows that this signaling pathway is an important autocrine/paracrine regulator of endometrial cell proliferation. [0023] MIS protein expression in ESCs is increased by the administration of estradiol. Estradiol has been reported to activate the MIS promoter in vitro [22]. Based on this and our immunocytochemistry findings, we hypothesized that estradiol would affect the in vitro expression of MIS in our ESCs (ESCs). Consistent with our hypothesis, estradiol treatment of ESCs for 72 hours significantly increased MIS secretion by 57% (P< 0.0001) compared to that of the vehicle control group using Students t-test (FIG. 4). This observation shows that endometrial cell function may be modulated by an interplay between estradiol and MIS.

Recombinant Human MIS (rhMIS) Induces Growth Inhibition in ESCs derived from human endometrium. [0024] Given the results of the above experiments, we next exposed human endometrial cells to recombinant human MIS (rhMIS) to investigate the effect of the MIS/MISRII pathway on endometrial stromal cell growth. Treatment of ESCs with 100ng/mL rhMIS for 72 hours resulted in a 15% decrease (P<0.01) in viable cells when compared to the vehicle control group (FIG. 5). This proves that human ESCs have a functional MIS signal transduction system, and this system when activated diminishes cell viability. CellTiter-Blue® cell viability reagent (Promega) was used to quantify the number of live cells per well.

Transient transfection of MIS Expression Plasmid into ESCs Significantly Decreases the Number of Viable Cells and Increases the Rate of Apoptosis. [0025] As expected, transient transfection with the MIS expression plasmid, but not the empty vector or the MISRII expression plasmid, significantly increased production of MIS in human ESCs in vitro, with a 30-fold increase in the media concentration of MIS as determined by ELISA after 24 hours in culture (FIG. 6a). Furthermore, when these cells were transfected with a plasmid expressing either MIS or MISRII, a significant decrease in cell viability was seen (FIG. 6b). Transfection with MIS alone reduced viability by nearly 60% (from 0.4 absorbance to about 1.75). A significant increase in cellular caspase 3/7 activity (FIG. 6c) was noted, showing that MIS signaling diminished cell number by inducing apoptosis in these cells. A small additive effect on these parameters was noted when the two plasmids were co-transfected.

[0026] Based on the results of the experiments described above, we have discovered that endometriosis can be treated or prevented by administering a therapeutically effective amount
of MIS or a biologically active fragment or variant thereof. The therapeutically effective amount is an amount that reduces or ameliorates one or more symptoms of endometriosis, which is typically an amount that decreases the proliferation of abnormal endometriotic cells. In a preferred embodiment human MIS is administered to human subjects, preferably as recombinant human MIS.

[0027] Even peak serum levels of endogenous MIS are likely too low to exert an endocrine effect upon the endometrium. In terms of pharmacodynamics, the half-maximal inhibitory concentration (IC50) for the inhibition of endometrial cell growth has not been reported for MIS. However, saturation of the MISRII receptor occurs at a concentration of 75-100 nM, and the dissociation constant, Kd, has been estimated to be in the range of 6 to 12 nM [32-34] which is 1000-fold greater than the average serum MIS level in human adult females (7.1 x 10-3 nM). Moreover, the fact that serum MIS levels in the short-term minimally fluctuate over the course of the menstrual cycle [35] further shows that a dynamic influence of circulating MIS upon endometrial remodeling is unlikely. Our results showed that rhMIS in an amount of 100ng/mL rhMIS for 72 hours resulted in a 15% decrease (P<0.01) in viable ESCs. Because this amount is so high compared to circulating levels of MIS, a preferred embodiment for treating endometriosis is by administering MIS locally to the site of the endometriosis in order to achieve a high enough therapeutic concentration at a reasonable cost. In certain embodiments MIS is administered in an amount of between 75 ng/ml to about 1,000 ng/ml, most preferably from about 50-100 ng/ml. In one example, MIS is administered locally to areas with endometriosis at time of surgery such as the bed of an ovarian endometriosis cyst after it has been removal to kill any remaining abnormal cells and to prevent recurrence. Any method known in the art for delivering MIS to the endometrial site can be used. As is explained below, the therapeutically effective amounts of MIS will vary depending on the severity of the condition, route of administration, pharmaceutical formulation, frequency of administration and other factors known to a person of skill in the art. Where the patient does not require surgery, MIS can be administered locally by injection, or can be delivered by implanting a pump or slow release preparation such as a hydrogel containing MIS.

[0028] While local administration is a preferred embodiment for treating existing endometriosis, maintenance doses of MIS to prevent recurrence could be considerably lower; routine experimentation will determine if these maintenance doses can be administered systemically. Prevention of endometriosis in a high risk individual may also be achieved with lower doses that are needed to treat existing endometriosis. Routine experimentation will
determine the optimal dose and the best route of administration for all levels of treatment and prevention of endometriosis.

[0029] Certain other embodiments are directed to pharmaceutical compositions that include MIS, preferably human recombinant MIS, or a biologically active fragment or variant thereof, preferably in an amount of from about 75 ng/ml to about 1,000 ng/ml. In some embodiments, MIS is administered by vaginal suppository.

Discussion

[0030] The endometrium is a highly specialized tissue capable of dramatically remodeling itself on a monthly basis. This process requires cellular proliferation, differentiation, breakdown and repair. Although this cyclic activity is broadly orchestrated by the ovarian sex steroids estrogen and progesterone, autocrine/paracrine mechanisms at the level of the endometrium are crucial for fine-tuning these processes. Indeed, a role for the local modulation of endometrial physiology has already been described for a myriad of growth factors, cytokines, proteases, and immunomodulatory agents [23-28]. Members of the TGF-ß superfamily such as TGFß1, TGFß2, TGFß3, activin A, macrophage inhibitory cytokine (MIC)-I, BMP-2, BMP-7, BMP-4, and BMP-8 are dynamically expressed during different phases of the menstrual cycle, consistent with their critical roles in cell proliferation, differentiation, immunomodulation, apoptosis, and tissue remodeling [29]. In addition, cyclic fluctuations in the expression of their receptors also have been demonstrated. For example, TGF-ßR1 and -R2 expression are increased 3-4 fold in the endometrial stroma during the secretory phase of the menstrual cycle, coinciding with the upregulation of TGF-ß1 expression [25]. Similarly, the expression of activin and its type Ia and Ha receptors is increased 2-4 fold during the secretory phase of the menstrual cycle [30]. We have discovered that MIS, another member of the TGF-ß superfamily, also plays a role in the modulation of cellular functions involved in endometrial tissue remodeling. MIS production in the adult female has been heretofore presumed to be primarily restricted to ovarian granulose cells, a concept largely attributable to the finding that a woman's serum MIS level gradually diminishes over the course of her reproductive lifespan until it becomes undetectable at menopause [31].

[0031] As we explained above, even peak serum MIS levels are likely too low to exert an endocrine effect upon the endometrium. Serum hormone levels, however, frequently do not reflect the existence of an autocrine or paracrine modality. As our data reveal, treating ESC cells with 100 ng/mL of recombinant human MIS produced an approximately 15% reduction
in endometrial stromal cell viability. However, when these same cells were transiently transfected with an MIS expression plasmid, a situation more closely approximating an MIS autocrine/paracrine mechanism, their cell viability was reduced by nearly 60% despite a concomitant MIS media concentration of only 3 ng/mL. These results are reminiscent of those obtained using another TGF-β superfamily member, activin, in which only endogenously produced activin (from transient transfection) was able to decrease the proliferation of PAI cells [36]. An autocrine/paracrine mode for MIS endometrial regulation would be consistent with the modus operandi of Sertoli cell produced MIS in genetically male embryos; early experiments revealed that the MIS secreted by each testis induces regression of only the ipsilateral Müllerian duct, confirming a paracrine effect [37].

We have shown that increasing local MIS concentrations, either by exogenous administration of recombinant MIS or by transiently transfecting an MIS expression plasmid, diminished the viable ESC number. As expected, cell viability was significantly decreased by both of these techniques. As evidenced by the increase in caspase 3/7 activity induced by MIS transient transfection, this decrease in viability is at least partially attributable to an increase in the rate of apoptosis. However, the fact that both MIS and MISRII protein production appeared visually limited to ESCs undergoing mitosis shows that endogenous MIS signaling may also play an important role in modulating endometrial cellular proliferation. Given the fact that both MIS and MISRII appear contemporaneously and nearly exclusively expressed by dividing endometrial stromal and glandular cells in vitro, one possible scenario would involve an autocrine mechanism whereby dividing cells increase their production of MIS and its main receptor in an effort to limit further proliferation. In this scenario, enhanced MIS production would be a response to cellular division rather than a catalyst for it. The suppressive role of MIS in cellular proliferation is well documented. In addition to its antiproliferative effect on Leydig cells in the testes and granulosa cells within primordial follicles in the ovaries, MIS has also been shown to inhibit the proliferation of several human cancer cell lines, including those originating from the cervix [14], endometrium [13], ovarian epithelium [32, 34], and breast [38]. MIS-mediated inhibition of cancer cell proliferation correlates with the upregulation of p16INK4a protein, an inhibitor of the cyclin/CDK complex kinase activity [32]. In cell cycle regulation, decreased cyclin/CDK complex phosphorylation of the retinoblastoma family of pocket proteins (Rb, pLO7, and p330) results in increased inhibition of E2F which is critical in mediating S phase entry from G0/G1 [39]. Indeed cancer cell cultures treated with MIS demonstrated a 10-15% increase in
the G1 phase fraction and a concomitant 70-80% inhibition of colony growth compared with untreated cells [13, 14, 32].

[0033] Before this study the role of MIS in the physiology of adult endometrium, another key Müllerian duct derivative, had never been explored. Although the expression of MISRII has been shown in human endometrium [13] and rat cervical tissue [14] by immunohistochemistry, functional analyses to investigate the effect of MIS on these tissues had not been undertaken. The experiments described above show for the first time that an intact MIS/MISRII pathway exists in human endometrial cells; that this system appears to be of an autocrine/paracrine nature; and that activation of this system serves to negatively modulate cell viability, at least in part by increasing apoptosis and providing a new treatment for endometriosis.

Pharmaceutical Compositions

[0034] Certain embodiments of the invention are directed to pharmaceutical compositions that include MIS, preferably the human recombinant form, or a biologically active fragment or variant thereof, in an amount that decreases the proliferation of abnormal endometriotic cells. The compounds of this invention can be formulated and administered to prevent or treat endometriosis by any means that produces contact of the active ingredient with the agent's site of action. In a preferred embodiment the human MIS is administered locally to the site of the endometriotic cells, however, MIS can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as an individual therapeutic active ingredient or in a combination of therapeutic active ingredients. MIS can be administered alone, but it is generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice. The dosage administered will be a therapeutically effective amount of the compound sufficient to reduce or ameliorate one or more symptoms of the endometriosis.

[0035] In the case of endometriosis the therapeutically effective amount of MIS is an amount that significantly reduces endometrial cell proliferation. Significantly lower or significantly higher means that the difference is statistically significant.

[0036] Our results show that the amount of MIS needed to reduce ESC proliferation was significantly higher than the typical circulating levels of endogenous MIS. Therefore in a preferred embodiment, MIS is administered locally to the site of endometriosis instead of systemically to treat endometriosis in order to achieve a high enough local concentration. In the preferred embodiment, the therapeutically effective amount of MIS for local
administration ranges from about 50-1000 ng/ml, more preferably from about 100-500 ng/ml. In another preferred embodiment MIS is administered locally as a hydrogel. 0.000001 mg/kg body weight to about 200 mg/kg body weight. Local delivery can be accomplished by applying or installing the formulation into body cavities, by parenteral introduction, peritoneal, subcutaneous, or intradermal administration. One skilled in the art will recognize that although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route, depending on the circumstances. Local delivery can be accomplished by any method known in the art.

[0037] The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient or the reduction of symptoms. Persons of ordinary skill can determine optimum dosages, dosing methodologies and repetition rates. Persons of ordinary skill in the art can estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. The actual effective amount of a peptide also varies according to its size, biodegradability, bioactivity and bioavailability. Optimum dosages can generally be estimated based on EC50s found to be effective in in vitro and in vivo animal models. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease, preferably using lower amounts than were required to treat the initial endometriosis.

[0038] Factors that may influence the dosage required to effectively treat a subject, including but not limited to the severity of the condition, previous treatments, the general health and/or age of the subject, and other disorders or diseases present. Treatment of a subject with a therapeutically effective amount of a protein or polypeptide can include a single treatment or, preferably, can include a series of treatments. Appropriate doses also depend upon the potency of the therapeutic agent with respect to the expression or activity to be modulated. A physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained.

[0039] The therapeutic agent MIS and biologically active fragments or variants thereof (also referred to as "active compounds") can be incorporated into pharmaceutical compositions suitable for administration to a subject, e.g., a human. It is understood however,
that administration can also be to cells in vitro as well as to in vivo model systems such as non-human transgenic animals.

[0040] Formulations of MIS proteins or peptides may contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

[0041] It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" as used herein refers to physically discrete units suited as unitary dosages for the subject 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 the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals. MIS can be modified as described below to increase efficacy or stability or other desirable properties.

[0042] As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention.

Supplementary active compounds or therapeutic agents can also be incorporated into the compositions. A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration.

[0043] Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous. A person of skill in the art will know which methods will optimize delivery of the active therapeutic agents to the targeted site of endometriosis.

Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylene diamante tetra acetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such
as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. Sterile injectable solutions can be prepared by incorporating the active compound (e.g., protein, peptide) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems.

MIS may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection or using a pump. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Standard pharmaceutical methods can be employed to control the duration of action. Such sustained and/or timed release formulations may be made by sustained release means or delivery devices that are well known to those of ordinary skill in the art, such as those described in U.S. Pat. Nos. 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 4,710,384; 5,674,533; 5,059,595; 5,591,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; and 5,733,566, the disclosures of which are each incorporated herein by reference. These methods are well known in the art and include control release preparations and can include appropriate macromolecules, for example polymers, polyesters, polyamino acids, polyvinyl, pyrolidone, ethylenevinylacetate, methyl cellulose, carboxymethyl cellulose, hydrogels, poly (lactic acid) or protamine sulfate. The concentration of macromolecules as well as the methods of incorporation can be adjusted in order to control release. In addition to being
incorporated, these agents can also be used to trap the compound in microcapsules. The pharmaceutical compositions of the present invention can be formulated using, for example, hydropropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems, multilayer coatings, microparticles, liposomes, microspheres, or the like, or a combination thereof to provide the desired release profile in varying proportions. Suitable sustained release formulations known to those of ordinary skill in the art, including those described herein may be readily selected for use with the pharmaceutical compositions of the invention.

**Protein variants.**

[0048] Variants of MIS or biologically active fragments thereof, include proteins and peptides that are substantially homologous to MIS that are produced by chemical synthesis or by recombinant methods.

[0049] As used herein, two proteins (or a region of the proteins or peptides) are substantially homologous when the amino acid sequences are at least about 70-75%, typically at least about 80-85%, and most typically at least about 90-95%, 97%, 98% or 99% or more homologous. Variants include conservative Amino Acid Substitutions: Aromatic Phenylalanine Tryptophan Tyrosine Hydrophobic Leucine Isoleucine Valine Polar Glutamine Asparagine Basic Arginine Lysine Histidine Acidic Aspartic Acid Glutamic Acid Small Alanine Serine Threonine Methionine Glycine.

[0050] A variant polypeptide can differ in amino acid sequence by one or more substitutions, deletions, insertions, inversions, fusions, and truncations or a combination of any of these. Variant polypeptides can be fully functional or can lack function in one or more activities.

[0051] Fully functional variants typically contain only conservative variation or variation in non-critical residues or in non-critical regions. Functional variants can also contain substitution of similar amino acids, which results in no change or an insignificant change in function. Variants of MIS include those that reduce endometrial cell proliferation.

[0052] As indicated, variants can be naturally-occurring or can be made by recombinant means of chemical synthesis to provide useful and novel characteristics of the desired protein.

[0053] Substantial homology can be to the entire amino acid sequence or to fragments of these sequences. Biologically active fragments can be derived from the full naturally occurring amino acid sequence. However, the invention also encompasses fragments of the
variants of MIS as described herein. Accordingly, a fragment can comprise any length that
retains one or more of the desired biological activities of the protein. Fragments can be
discrete (not fused to other amino acids or polypeptides) or can be within a larger
polypeptide. Further, several fragments can be comprised within a single larger polypeptide.
[0054] Polypeptides often contain amino acids other than the 20 amino acids
commonly referred to as the 20 naturally-occurring amino acids. Further, many amino acids,
including the terminal amino acids, may be modified by natural processes, such as processing
and other post-translational modifications, or by chemical modification techniques well
known in the art. Common modifications that occur naturally in polypeptides are described
below.
[0055] Accordingly, the polypeptides also encompass derivatives or analogs in which a
substituted amino acid residue is not one encoded by the genetic code, in which a substituent
group is included, in which the mature polypeptide is fused with another compound, such as a
compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or
in which the additional amino acids are fused to the mature polypeptide, such as a leader or
secretory sequence or a sequence for purification of the mature polypeptide or a pro-protein
sequence.

Protein Modifications
[0056] MIS and biologically active analogs, derivatives, fragments and variants for use in
the present invention can be modified according to known methods in medicinal chemistry to
increase its stability, half-life, uptake or efficacy. Certain known modifications are described
below.
[0057] As is also well known, polypeptides are not always entirely linear. For instance,
polypeptides may be branched as a result of ubiquitination, and they may be circular, with or
without branching, generally as a result of post-translation events, including natural
processing events and events brought about by human manipulation which do not occur
naturally. Circular, branched and branched circular polypeptides may be synthesized by non-
translational natural processes and by synthetic methods.
[0058] Modifications can occur anywhere in a polypeptide, including the peptide
backbone, the amino acid side-chains and the amino or carboxyl termini. Blockage of the
amino or carboxyl group in a polypeptide, or both, by a covalent modification, is common in
naturally-occurring and synthetic polypeptides. For instance, the amino terminal residue of
polypeptides made in E. coli, prior to proteolytic processing, almost invariably will be N-formylmethionine.

[0059] The modifications can be a function of how the protein is made. For recombinant polypeptides, for example, the modifications will be determined by the host cell posttranslational modification capacity and the modification signals in the polypeptide amino acid sequence. Accordingly, when glycosylation is desired, a polypeptide should be expressed in a glycosylating host, generally a eukaryotic cell. Insect cells often carry out the same posttranslational glycosylations as mammalian cells, and, for this reason, insect cell expression systems have been developed to efficiently express mammalian proteins having native patterns of glycosylation. Similar considerations apply to other modifications. The same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain more than one type of modification.

<table>
<thead>
<tr>
<th>Protein Modification</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Acetylation</td>
<td>Acetylation of N-terminus or e-lysines. Introducing an acetyl group into a protein, specifically, the substitution of an acetyl group for an active hydrogen atom. A reaction involving the replacement of the hydrogen atom of a hydroxyl group with an acetyl group (CH$_3$CO) yields a specific ester, the acetate. Acetic anhydride is commonly used as an acetylating agent, which reacts with free hydroxyl groups. Acylation may facilitate addition of other functional groups. A common reaction is acylation of e.g., conserved lysine residues with a biotin appendage.</td>
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<tr>
<td>Protein Modification</td>
<td>Description</td>
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</tr>
<tr>
<td>ADP-ribosylation</td>
<td>Covalently linking proteins or other compounds via an arginine-specific reaction.</td>
</tr>
<tr>
<td>Alkylation</td>
<td>Alkylation is the transfer of an alkyl group from one molecule to another. The alkyl group may be transferred as an alkyl carbocation, a free radical or a carbanion (or their equivalents). Alkylation is accomplished by using certain functional groups such as alkyl electrophiles, alkyl nucleophiles or sometimes alkyl radicals or carbene acceptors. A common example is methylation (usually at a lysine or arginine residue).</td>
</tr>
<tr>
<td>Amidation</td>
<td>Reductive animation of the N-terminus. Methods for amidation of insulin are described in U.S. 4,489,159.</td>
</tr>
<tr>
<td>Carbamylolation</td>
<td>Nigen et al. describes a method of carbamylating hemoglobin.</td>
</tr>
<tr>
<td>Carboxylation</td>
<td>Carboxylation typically occurs at the glutamate residues of a protein, which may be catalyzed by a carboxylase enzyme (in the presence of Vitamin K – a cofactor).</td>
</tr>
<tr>
<td>Citrullination</td>
<td>Citrullination involves the addition of citrulline amino acids</td>
</tr>
<tr>
<td>Protein Modification</td>
<td>Description</td>
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</tr>
<tr>
<td><strong>Citrullination</strong></td>
<td>to the arginine residues of a protein, which is catalyzed by peptidylarginine deaminase enzymes (PADs). This generally converts a positively charged arginine into a neutral citrulline residue, which may affect the hydrophobicity of the protein (and can lead to unfolding).</td>
</tr>
<tr>
<td><strong>Condensation of amines with aspartate or glutamate</strong></td>
<td>Such reactions, may be used, e.g., to attach a peptide to other proteins labels.</td>
</tr>
<tr>
<td><strong>Covalent attachment of flavin</strong></td>
<td>Flavin mononucleotide (FAD) may be covalently attached to serine and/or threonine residues. May be used, e.g., as a light-activated tag.</td>
</tr>
<tr>
<td><strong>Covalent attachment of heme moiety</strong></td>
<td>A heme moiety is generally a prosthetic group that consists of an iron atom contained in the center of a large heterocyclic organic ring, which is referred to as a porphyrin. The heme moiety may be used, e.g., as a tag for the peptide.</td>
</tr>
<tr>
<td><strong>Attachment of a nucleotide or nucleotide derivative</strong></td>
<td>May be used as a tag or as a basis for further derivatising a peptide.</td>
</tr>
<tr>
<td><strong>Cross-linking</strong></td>
<td>Cross-linking is a method of covalently joining two proteins. Cross-linkers contain reactive ends to specific functional groups (primary amines, sulphhydryls, etc.) on proteins or other molecules. Several chemical groups may be targets for reactions in proteins and peptides. For example, Ethylene glycol bis[succinimidylsuccinate, Bis[2-(succinimidoxy carbonyloxy)ethyl]sulfone, and</td>
</tr>
<tr>
<td>Protein Modification</td>
<td>Description</td>
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</tr>
<tr>
<td><strong>Cyclization</strong></td>
<td>Bis[sulfosuccinimidyl] suberate link amines to amines.</td>
</tr>
<tr>
<td><strong>Disulfide bond formation</strong></td>
<td>For example, cyclization of amino acids to create optimized delivery forms that are resistant to, e.g., aminopeptidases (e.g., formation of pyroglutamate, a cyclized form of glutamic acid).</td>
</tr>
<tr>
<td><strong>Demethylation</strong></td>
<td>Disulfide bonds in proteins are formed by thiol-disulfide exchange reactions, particularly between cysteine residues (e.g., formation of cystine).</td>
</tr>
<tr>
<td><strong>Formylation</strong></td>
<td>See, e.g., U.S. 4,250,088 (Process for demethylating lignin).</td>
</tr>
<tr>
<td><strong>Glycylation</strong></td>
<td>The addition of a formyl group to, e.g., the N-terminus of a protein. See, e.g., U.S. Patent Nos. 4,059,589, 4,801,742, and 6,350,902.</td>
</tr>
<tr>
<td><strong>Glycosylation</strong></td>
<td>The covalent linkage of one to more than 40 glycine residues to the tubulin C-terminal tail.</td>
</tr>
<tr>
<td></td>
<td>Glycosylation may be used to add saccharides (or polysaccharides) to the hydroxy oxygen atoms of serine and threonine side chains (which is also known as O-linked Glycosylation). Glycosylation may also be used to add saccharides (or polysaccharides) to the amide nitrogen of asparagine side chains (which is also known as N-linked</td>
</tr>
<tr>
<td>Protein Modification</td>
<td>Description</td>
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</tr>
<tr>
<td>Glycosylation</td>
<td>Glycosylation, e.g., via oligosaccharyl transferase.</td>
</tr>
<tr>
<td>GPI anchor formation</td>
<td>The addition of glycosylphosphatidylinositol to the C-terminus of a protein. GPI anchor formation involves the addition of a hydrophobic phosphatidylinositol group - linked through a carbohydrate containing linker (e.g., glucosamine and mannose linked to phosphoryl ethanolamine residue) - to the C-terminal amino acid of a protein.</td>
</tr>
<tr>
<td>Hydroxylation</td>
<td>Chemical process that introduces one or more hydroxyl groups (-OH) into a protein (or radical). Hydroxylation reactions are typically catalyzed by hydroxylases. Proline is the principal residue to be hydroxylated in proteins, which occurs at the C\textsuperscript{\text{\textgamma}} atom, forming hydroxyproline (Hyp). In some cases, proline may be hydroxylated at its C\textsuperscript{\text{\textbeta}} atom. Lysine may also be hydroxylated on its C\textsuperscript{\text{\textgamma}} atom, forming hydroxyllysine (Hyl). These three reactions are catalyzed by large, multi-subunit enzymes known as prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl 5-hydroxylase, respectively. These reactions require iron (as well as molecular oxygen and \alpha-ketoglutarate) to carry out the oxidation, and use ascorbic acid to return the iron to its reduced state.</td>
</tr>
<tr>
<td>Iodination</td>
<td>See, e.g., U.S. 6,303,326 for a disclosure of an enzyme that is capable of iodinating proteins. U.S. 4,448,764 discloses,</td>
</tr>
<tr>
<td>Protein Modification</td>
<td>Description</td>
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</tr>
<tr>
<td>ISGylation</td>
<td>Covalently linking a peptide to the ISG15 (Interferon-Stimulated Gene 15) protein, for, e.g., modulating immune response.</td>
</tr>
<tr>
<td>Methylation</td>
<td>Reductive methylation of protein amino acids with formaldehyde and sodium cyanoborohydride has been shown to provide up to 25% yield of N-cyanomethyl (-CH₂CN) product. The addition of metal ions, such as Ni²⁺, which complex with free cyanide ions, improves reductive methylation yields by suppressing by-product formation. The N-cyanomethyl group itself, produced in good yield when cyanide ion replaces cyanoborohydride, may have some value as a reversible modifier of amino groups in proteins. (Gidley et al.) Methylation may occur at the arginine and lysine residues of a protein, as well as the N- and C-terminus thereof.</td>
</tr>
<tr>
<td>Myristoylation</td>
<td>Myristoylation involves the covalent attachment of a myristoyl group (a derivative of myristic acid), via an amide bond, to the alpha-amino group of an N-terminal glycine residue. This addition is catalyzed by the N-myristoyltransferase enzyme.</td>
</tr>
</tbody>
</table>
| Oxidation            | - Oxidation of cysteines.  
<pre><code>                   | - Oxidation of N-terminal Serine or Thrreonine residues |
</code></pre>
<table>
<thead>
<tr>
<th>Protein Modification</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(followed by hydrazine or aminooxy condensations). -Oxidation of glycosylations (followed by hydrazine or aminooxy condensations).</td>
</tr>
<tr>
<td><strong>Palmitoylation</strong></td>
<td>Palmitoylation is the attachment of fatty acids, such as palmitic acid, to cysteine residues of proteins. Palmitoylation increases the hydrophobicity of a protein.</td>
</tr>
<tr>
<td><strong>(Poly)glutamylation</strong></td>
<td>Polyglutamylation occurs at the glutamate residues of a protein. Specifically, the gamma-carboxy group of a glutamate will form a peptide-like bond with the amino group of a free glutamate whose alpha-carboxy group may be extended into a polyglutamate chain. The glutamylation reaction is catalyzed by a glutamylase enzyme (or removed by a deglutamylase enzyme). Polyglutamylation has been carried out at the C-terminus of proteins to add up to about six glutamate residues. Using such a reaction, Tubulin and other proteins can be covalently linked to glutamic acid residues.</td>
</tr>
<tr>
<td><strong>Phosphopantetheinylation</strong></td>
<td>The addition of a 4’-phosphopantetheinyl group.</td>
</tr>
<tr>
<td><strong>Phosphorylation</strong></td>
<td>A process for phosphorylation of a protein or peptide by contacting a protein or peptide with phosphoric acid in the presence of a non-aqueous apolar organic solvent and contacting the resultant solution with a dehydrating agent is disclosed e.g., in U.S. 4,534,894. Insulin products are</td>
</tr>
<tr>
<td>Protein Modification</td>
<td>Description</td>
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</tr>
<tr>
<td><strong>Prenylation</strong></td>
<td>Prenylation (or isoprenylation or lipidation) is the addition of hydrophobic molecules to a protein. Protein prenylation involves the transfer of either a farnesyl (linear grouping of three isoprene units) or a geranyl-geranyl moiety to C-terminal cysteine(s) of the target protein.</td>
</tr>
<tr>
<td><strong>Proteolytic Processing</strong></td>
<td>Processing, e.g., cleavage of a protein at a peptide bond.</td>
</tr>
<tr>
<td><strong>Selenoylation</strong></td>
<td>The exchange of, e.g., a sulfur atom in the peptide for selenium, using a selenium donor, such as selenophosphate.</td>
</tr>
<tr>
<td><strong>Sulfation</strong></td>
<td>Processes for sulfating hydroxyl moieties, particularly tertiary amines, are described in, e.g., U.S. 6,452,035. A process for sulphation of a protein or peptide by contacting the protein or peptide with sulphuric acid in the presence of a non-aqueous apolar organic solvent and contacting the resultant solution with a dehydrating agent is disclosed. Insulin products are described to be amenable to this process. See, e.g., U.S. 4,534,894.</td>
</tr>
<tr>
<td><strong>SUMOylation</strong></td>
<td>Covalently linking a peptide a SUMO (small ubiquitin-related Modifier) protein, for, e.g., stabilizing the peptide.</td>
</tr>
<tr>
<td><strong>Transglutamination</strong></td>
<td>Covalently linking other protein(s) or chemical groups (e.g., PEG) via a bridge at glutamine residues</td>
</tr>
<tr>
<td>Protein Modification</td>
<td>Description</td>
</tr>
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<td>----------------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>tRNA-mediated addition of amino acids (e.g., arginylation)</td>
<td>For example, the site-specific modification (insertion) of an amino acid analog into a peptide.</td>
</tr>
</tbody>
</table>
EXAMPLES

Example 1: Materials and Methods

1. Source of human endometrial tissue: All study participants gave written informed consent prior to participating in this study, which was approved by the IRB of Columbia University. Approximately 1 cm² pieces of endometrial tissue were obtained from adult women in differing phases of their menstrual cycle at the time of their medically indicated endometrial biopsy, endometrial curettage, or hysteroscopic surgery. These samples were then divided into equal portions for gene expression studies, cell culture, immunohistochemistry, and routine pathological evaluation.

2. Gene expression studies: A portion of freshly obtained endometrium was placed into RNA later® (Ambion, Austin, TX) and then frozen at -80°C. When a sufficient number of samples were obtained, they were thawed, removed from the RNA later®, and then dounced in Trizol® reagent (Invitrogen, Carlsbad, CA). RNA was then extracted according to manufacturer's instructions. Approximately 500ng of total RNA as assessed by spectrophotometry were then reversed transcribed into cDNA using the Sensiscript RT kit (Qiagen, Valencia, CA) according to manufacturer's instructions. cDNA was then purified using QIAEX II kit (Qiagen). PCR was then carried out on a Bio-Rad thermocycler (Hercules, CA) using LA-Taq (Takara Bio USA, Madison WI) and functionally prevalidated primers for MIS, MISRII, ALK3 (BMPRIA), SMAD1, SMAD9, and HPRT (Qiagen). PCR conditions were 95°C for 10 minutes, followed by 40 cycles of 30 seconds each of 94°C, 55°C, 72°C.

3. Endometrial Cell Culture: At the time of specimen acquisition, a small portion of endometrium was placed into DMEM/F12 media. This tissue was then mechanically cleared of clots and debris and minced into small pieces (~1 mm³) under a dissecting microscope. The minced tissue was then incubated in culture media with 0.5% collagenase and deoxyribonuclease. Endometrial cells were collected by centrifugation (500 x g, 10 min), resuspended in "complete" DMEM/F12 culture media (10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin, 1mM sodium pyruvate) and plated in culture flasks at 37°C in a 5% CO2 atmosphere. Endometrial stromal cells (ESCs), which were not adherent after 30 minutes, were aspirated from the flask and replated, leaving the adherent epithelial (glandular) cells. These cultured primary human endometrial cells were confirmed to be either predominantly of stromal or epithelial origin by virtue of mutually exclusive positive immunoreactivity for vimentin or cytokeratin, respectively. The
ESCs also demonstrated positive immunoreactivity for estrogen receptor-α and progesterone receptor.

4. **Immunohistochemistry:** Endometrial cells were grown to confluence in culture flasks and then trypsinized, resuspended in complete DMEM/F12 culture media, and replated onto glass cover slips. Once the cells reached 80% confluence, they were washed with PBS and fixed in a zinc-containing formaldehyde solution (Z-Fix, Anatech Ltd, Battle Creek, MI) in preparation for immunocytochemistry. Immunostaining was performed by incubating coverslips with the following antibodies overnight at 4°C: 1) rabbit anti-human MISRII (Abgent, San Diego, CA), 2) goat anti-human MIS (R&D Systems, Minneapolis, MN), 3) mouse antihuman ER-α (ABR-Affinity BioReagents, Golden CO), 4) mouse anti-human progesterone receptor (R&D Systems), 5) rabbit anti-human vimentin (ABR-Affinity BioReagents), and 6) goat anti-human cytokeratin (ABR-Affinity BioReagents). Diaminobenzidine tetrachloride (Vector Laboratories, Burlingame, CA) was used to colorize the tissue after incubation with appropriate HRP-conjugated secondary antibodies. Some freshly obtained endometrial tissue was placed directly into Z-fix for immunohistochemistry using the MIS and MISRII antibodies. Slides hybridized only to secondary antibodies were also prepared to serve as negative controls.

5. **Transient transfection:** The full-length coding sequence of the human MIS gene (GenBank No. KQ147474) and the MISRII genes were individually ligated between two Notl recognition sites within the multiple cloning region of the mammalian expression vector pCMV-XL6 (Origene, Rockville, MD). Purified plasmid preparations were made (WizardPlus SV minipreps, Promega, Madison, WI) from overnight LB-ampicillin broths containing a single transformed colony of competent E. coli cells (Top10 cells, Invitrogen, Carlsbad, CA). Presence and orientation of inserted sequences were validated by PCR and restriction digestion with Notl. Human endometrial cells plated in 96 well trays were transfected with pCMV-MIS, pCMV-MISRII, or both using Effectene reagent (Qiagen) per manufacturer’s recommendations. The empty pCMV-XL vector was also transfected as a negative control. A total of 100ng/well of plasmid DNA was used.

6. **MIS ELISA:** Prior to all MIS assays, human endometrial cells were twice washed in PBS and then cultured in serum-free media in order to prevent potential confounding from any MIS present in the fetal calf serum. Media conditioned for 72 hours was then assayed for MIS using a highly specific two site ELISA (Diagnostic Systems Laboratories, Webster, TX). This assay has a dynamic range of 0.05-10 ng/mL and sensitivity of 0.017 ng/mL. In a separate experiment to assess the effect of estradiol upon MIS
production, ESCs were plated onto 6-well plates and then incubated in 2ml of serum-free DMEM containing either vehicle or 10 µM estradiol (Steraloids, Newport, RI). Culture media was aspirated after 72 hours of treatment and then assayed for MIS levels.

7. Multiplexed Cell-Viability/Apoptosis Assays: ESCs were grown to 80% confluence, trypsinized, and then transferred into 96-well plates at a density of 2000 cells in 100 µL of serum-free DMEM per well. After 16 hours, the media was aspirated, the cells were washed in PBS, and 100µL of fresh serum-free, phenol red-free DMEM media containing either vehicle or 100 ng/mL recombinant human MIS (R&D Systems, Minneapolis, MN) was placed into each well. After 72 hours in culture, 20 µL of a resazurin containing reagent (CellTiter-Blue, Promega) was then added directly to each well. After a further incubation at 37°C for 4 hours, the fluorescence per well (560Ex/590Em) produced by the conversion of resazurin to resorufin, which is directly proportional to viable cell number, was quantified. The caspase activity within these same wells was then measured by adding 120µL of Apo-ONE Homogeneous Caspase-3/7 Assay reagent (Promega) to each well. Cells were incubated for an additional hour at ambient temperature prior to recording fluorescence (485Ex/527Em).

These methods described herein are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan. The invention is illustrated herein by the experiments described above and by the examples, which should not be construed as limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference as if set forth herein in their entirety, except where terminology is not consistent with the definitions herein.
REFERENCES


What is claimed is:

1. A method for treating endometriosis in a female animal, comprising administering a therapeutically effective amount of Mullerian inhibiting substance, or a biologically active fragment or variant thereof.

2. The method of claim 1, wherein the Mullerian inhibiting substance is administered locally to the site of the endometriosis.

3. The method of claim 2, wherein the site of the endometriosis includes a cyst.

4. The method of claim 2, wherein the MIS is administered by injection.

5. The method of claim 2, wherein the Mullerian inhibiting substance is administered by a pump.

6. The method of claim 2, wherein the Mullerian inhibiting substance is administered by implantation near the site of the endometriosis.

7. The method of claim 1, wherein the therapeutically effective amount of Mullerian inhibiting substance is from about 50 to 1,000 ng/ml, more preferably from about 100 to about 500 ng/ml.

8. The method of claim 1, wherein the animal is a human.

9. The method of claim 8, wherein the Mullerian inhibiting substance is recombinant human Mullerian inhibiting substance.

10. The method of claim 1, wherein the therapeutically effective amount of Mullerian inhibiting substance is an amount that decreases the proliferation of endometrial cells.

11. A method of preventing a recurrence of endometriosis in a female animal, comprising administering a therapeutically effective amount of Mullerian inhibiting substance, or a biologically active fragment or variant thereof.

12. The method of claim 11, wherein the animal is a human.

13. The method of claim 12, wherein the Mullerian inhibiting substance is recombinant human Mullerian inhibiting substance.
14. A method of preventing endometriosis in a female human who is at risk of developing endometriosis, comprising administering a therapeutically effective amount of Mullerian inhibiting substance, or a biologically active fragment or variant thereof.

15. The method of claim 14, wherein the Mullerian inhibiting substance is recombinant human Mullerian inhibiting substance.

16. A pharmaceutical formulation, comprising human recombinant Mullerian inhibiting substance in an amount of from about 50 to 1,000 ng/ml, more preferably from about 100 to about 500 ng/ml.

17. The pharmaceutical formulation of claim 14, wherein the Mullerian inhibiting substance is formulated as a slow release preparation.
**INTERNATIONAL SEARCH REPORT**

**International application No**
PCT/US 08/79890

**CLASSIFICATION OF SUBJECT MATTER**

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<td>424/93.2</td>
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According to International Patent Classification (IPC) or to both national classification and IPC

**FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

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

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

- USPTO, PubWest, Google Scholar, and DialogPro (Engineering)
- Muliti-an Inhibiting Substance (MIS), endometriosis, injection, pump, slow release, prevent, reocurrence, recombinant

**DOCUMENTS CONSIDERED TO BE RELEVANT**

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<td>US 2004/0258685 A1 (Brunnetta et al.) 23 December 2004 (23 12 2004) para [0016] [0017], [0071], [0085], [0229], [0238], [0239], [0241], [0242], [0290], [0291]</td>
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<td>US 2007/0128203 A1 (Giles-Komar et al.) 7 June 2007 (07 06 2007) para [0201], [207], and [0250]</td>
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Date of the actual completion of the international search

28 November 2008 (28 11 2008)

Date of mailing of the international search report

2 2 DEC 2008

Name and mailing address of the ISA/US

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