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(54) Title: GDF9:BMP15 HETERODIMERS FOR ENHANCING FERTILITY

(57) Abstract: Methods for *in vitro* culture of ovarian follicles, *in vitro* fertilization of oocytes, and/or *in vitro* maturation of cumulus cell-oocyte complexes are described herein. Embodiments of the method relate to growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP 15), oocyte-secreted paralogs of the transforming growth factor β (TGFβ) superfamily, which are shown now to be bioactive as heterodimers and have the ability to mature oocytes surrounded by ovarian granulosa cells. Also described is a method for in vitro maturation of immature oocytes comprising culturing an oocyte surrounded by ovarian granulosa cells in a chemically defined culture medium comprising at least GDF9:BMP15 heterodimers. Embodiments of the invention are useful in any reproductive setting, including the assisted reproduction technology clinic and veterinary/agricultural clinic, for example.

# GDF9:BMP15 HETERODIMERS FOR ENHANCING FERTILITY

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

**[0001]** This application claims the benefit of priority to U.S. Provisional Patent Application Serial No. 61/674,312, filed July 21, 2012, hereby incorporated by reference in its entirety.

**[0002]** This invention was made with government support under HD33438 awarded by National Institutes of Health. The government has certain rights in the invention.

## TECHNICAL FIELD

**[0003]** The field of the invention regards at least cell biology, molecular biology, and medicine. In specific cases, the field of the invention includes mammalian fertility, including oocyte *in vitro* maturation, oocyte growth, and ovarian follicle development.

## BACKGROUND OF THE INVENTION

**[0004]** Assisted reproductive technology (ART) has grown in use since the first *in vitro* fertilization (IVF) was achieved in 1978 and helps thousands of women overcome infertility issues yearly. IVF is a process by which an egg is fertilized by sperm outside the body. Normally, only a single follicle grows to the preovulatory state in a woman's reproductive cycle, and a single oocyte is released by the follicle. Most current IVF treatment protocols require the use of multiple oocytes. Thus, women may be treated with gonadotropin releasing hormone agonists (GnRHa) to suppress pituitary function, followed by treatment with human menopausal gonadotropin (HMG) or purified follicle-stimulating hormone (FSH) to hyperstimulate the ovaries and induce development of multiple follicles. Additional medication with HCG may serve to "prime" the follicle. The oocytes are then collected from the women and undergo *in vitro* processing.

**[0005]** The harvested oocyte may then be further matured, followed by fertilization with sperm. Prior to fertilization, the surrounding cumulus cells may be removed from the oocyte, allowing the sperm easier access. Successful IVF generally requires that the oocyte have reached the metaphase-II (M-II) prior to harvest, because the follicle and its environment

strongly influence the oocyte's ability to complete maturation to M-II and to acquire developmental competence. M-II is characterized by exclusion of one polar body from the cytoplasm. However, not all of the oocytes will have matured to the M-II stage. Currently, these immature oocytes are discarded at most IVF clinics. Thus, further maturation from M-I to M-II phases would result in a greater number of oocytes that could be fertilized. By increasing the yield of mature oocytes, IVM could additionally help to decrease use of exogenous gonadotropins with an associated reduction in side effects and cost to the patient. Current oocyte *in vitro* maturation (IVM) techniques do not support the same rates of embryo development or pregnancy outcome compared with oocytes that mature *in vivo*.

**[0006]** Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), oocyte-secreted paralogs of the transforming growth factor β (TGFβ) superfamily, have been shown genetically to control ovarian physiology. Although GDF9 and BMP15 homodimers can modulate ovarian pathways *in vitro*, the functional species-specific significance of GDF9:BMP15 heterodimers is unresolved.

**[0007]** The TGFβ superfamily of ligands, the largest family of secreted proteins in mammals, are synthesized as dimers and function extracellularly to bind type 1 and type 2 serine-threonine kinase receptors to activate downstream signaling cascades (e.g., the SMADs) in most developmental and physiological processes (*1*). GDF9 and BMP15 are key oocyte-secreted members of the TGFβ superfamily and regulate female fertility in several mammals (*2*). Although GDF9 and BMP15 are closely related paralogs, they have been shown *in vitro* to signal through divergent SMAD2/3 and SMAD1/5 pathways, respectively (*3, 4*). By studying gene knockouts and mutant models, putative roles of the individual proteins in female reproduction have been described in mice, sheep, and humans. Studies previously found that *Gdf9* null female mice are sterile (*5*), and $Gdf9^{+/-}Bmp15^{-/-}$ mice had more severe fertility defects compared to the subfertile $Bmp15^{-/-}$ mice (*6, 7*). BMP15 or GDF9 heterozygous mutant sheep have increased litters, while the homozygous mutants are sterile and phenocopy $Gdf9^{-/-}$ mice (*8, 9*). In human, mutations in *GDF9* and *BMP15* have been associated with premature ovarian failure and dizygotic twinning (*10-12*). While an *in vitro* study has detected GDF9:BMP15 heterodimer by immunoprecipitation (*13*) and cooperative effects of the two homodimers have been studied (*14-16*), the functions of GDF9:BMP15 heterodimers *in vitro* or *in vivo* in any species remain largely unknown.

## BRIEF SUMMARY OF THE INVENTION

**[0008]** The present invention is directed to a system, methods, and/or compositions useful for enhancing human fertility. Embodiments are directed to methods and/or compositions that facilitate *in vitro* (IVM) maturation of oocytes, thereby enhancing the *in vitro* fertilization (IVF) process by providing greater numbers of oocytes for use in the IVF process. In particular embodiments, the methods and compositions of the invention allow a greater number of oocytes that have reached metaphase-II that renders the oocyte more useful for IVF.

**[0009]** Embodiments of the invention provide enhancement of oocyte maturation, including at least oocyte *in vitro* maturation. In particular aspects, the present invention concerns the use of one or more agents that enhance oocyte maturation, including *in vitro* maturation. In particular embodiments, the one or more agents enhance the maturation of one or more oocytes to metaphase-II. In specific embodiments, the one or more agents comprise a GDF9:BMP15 heterodimer; the GDF9:BMP15 heterodimer is useful at least during IVM of oocytes.

**[0010]** Embodiments of the invention include methods of contacting an immature oocyte alone or encased within a follicle with a GDF9:BMP15 heterodimer in an amount effective to mature the oocyte. In particular embodiments, the method is *in vitro*, although in alternative embodiments the method is *in vivo*. In specific embodiments, the GDF9:BMP15 heterodimers act through a signaling complex that includes a type 2 receptor (*e.g.*, BMPR2), an ALK4/5/7 type 1 kinase receptor, and an ALK6 type 1 co-receptor. In particular cases, the GDF9:BMP15 heterodimers directly or indirectly upregulate expression of extracellular matrix genes, such as cumulus expansion-regulated genes (*e.g.*, PTGS2, HAS2, PTX3).

**[0011]** In embodiments of the invention, an individual undergoing IVF or intra-cytoplasmic sperm injection (ICSI ) treatment are provided with an effective amount of the GDF9:BMP15 heterodimer.

**[0012]** In particular aspects of the invention, the methods and compositions of the invention are related to fertility preservation. In specific embodiments, individuals in need of fertility preservation could utilize treatments that include GDF9:BMP15 heterodimer. For example, females that will have a need to preserve oocytes (such as those who will have some treatment that would damage their oocytes (particularly chemotherapy)) may have their ovaries or parts of their ovaries removed and frozen. When oocytes are needed, the strips of ovarian

cortex or the ovaries may be cultured under conditions that utilize treatment with GDF9:BMP15 heterodimer to allow the follicles to progress from the early follicle stages through ovulation and cumulus expansion and fertilization.

**[0013]** In certain cases, the methods and compositions of the invention are utilized for making oocytes *in vitro*. For example using stem cells (such as embryonic stem cells or induced pluripotent stem cells), one can utilize an effective amount of the GDF9:BMP15 heterodimer provided to the stem cells for the generation and maturation of ovarian follicles and oocytes.

**[0014]** In certain embodiments, the oocytes are exposed to the GDF9:BMP15 heterodimer *in vivo* for *in vivo* maturation of the oocytes. For example, an effective amount of the GDF9:BMP15 heterodimer may be provided to the individual or in the ovary, such as by injection. In specific cases, a pharmaceutical carrier is provided with the GDF9:BMP15 heterodimer for the injection.

**[0015]** A general embodiment of the disclosure is a method for *in vitro* maturation of oocytes, comprising the step of culturing an oocyte surrounded by pregranulosa cells, granulosa cells, or a mixture thereof in a culture medium comprising GDF9:BMP15 heterodimer. The GDF9:BMP15 heterodimer may comprise at least one mutated subunit, at least one wild-type subunit, or a wild-type and a mutated subunit. For example, both the GDF9 subunit and the BMP15 subunit may be wild-type. Further, the GDF9 subunit and the BMP15 subunit may both be mutated. In a specific embodiment of the disclosure, the GDF9 protein is wild-type and the BMP15 protein is mutated. In another embodiment of the disclosure, the GDF9 protein is mutated, and the BMP15 protein is wild-type. In another specific embodiment of the disclosure, the mutated subunit is the GDF9 protein with a G72R mutation. In an embodiment of the disclosure, the GDF9 subunit and the BMP15 subunits are from the same species. In another embodiment of the disclosure, the GDF9 subunit and the BMP15 subunit are derived from a different species. For example, the GDF9 subunit may be derived from the mouse GDF9 sequence, and the BMP15 subunit may be derived from the human BMP15 sequence. Additionally, epidermal growth factor (EGF) and/or a related ovarian EGF-like signaling protein (*e.g.*, amphiregulin, epiregulin, or betacellulin) may be added to the medium.

**[0016]** In an embodiment of the disclosure the oocyte comprises an oocyte at any stage of development. The oocyte may also be an immature oocyte. In an embodiment of the disclosure, the oocyte is a mammalian oocyte, such as a human, sheep, mouse, cow, horse, or pig

oocyte. The oocyte may be also be derived from induced pluripotent stem cells (iPSC), or from embryonic stem cells (ESC), for example.

**[0017]** The method may also include additional steps, such as removing the granulosa cells after the oocyte has been cultured, intracytoplasmic sperm injection (ICSI), and/or isolating reproductively competent oocytes from the culture. Additionally, the method may further comprise fertilizing the oocyte with sperm, which may occur before, after or during culturing, for example.

**[0018]** Another general embodiment of the disclosure is a kit for the *in vitro* maturation of oocytes comprising, a GDF9:BMP15 heterodimer; and an *in vitro* maturation or follicle growth medium. The he GDF9:BMP15 heterodimer comprises at least one wild-type subunit. The GDF9:BMP15 heterodimer may comprise at least one mutated subunit, at least one wild-type subunit, or a wild-type and a mutated subunit. For example, both the GDF9 subunit and the BMP15 subunit may be wild-type. Further, the GDF9 subunit and the BMP15 subunit may both be mutated. In a specific embodiment of the disclosure, the GDF9 protein is wild-type and the BMP15 protein is mutated. In another embodiment of the disclosure, the GDF9 protein is mutated, and the BMP15 protein is wild-type. In another specific embodiment of the disclosure, the mutated subunit is the GDF9 protein with a G72R mutation. In an embodiment of the disclosure, the GDF9 subunit and the BMP15 subunits are from the same species. In another embodiment of the disclosure, the GDF9 subunit and the BMP15 subunit are derived from a different species. For example, the GDF9 subunit may be derived from the mouse GDF9 sequence, and the BMP15 subunit may be derived from the human BMP15 sequence. Additionally, epidermal growth factor (EGF) and/or a related ovarian EGF-like signaling protein (e.g., amphiregulin, epiregulin, or betacellulin) may be added to the medium.

**[0019]** The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. Additional features and advantages of the invention will be described hereinafter which form the subject of the claims of the invention. It should be appreciated by those skilled in the art that the conception and specific embodiment disclosed may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present invention. It should also be realized by those skilled in the art that such equivalent constructions do not depart from the spirit and scope of the invention as set forth in the appended claims. The novel features which are believed to be characteristic of the invention, both as to its

organization and method of operation, together with further objects and advantages will be better understood from the following description when considered in connection with the accompanying figures. It is to be expressly understood, however, that each of the figures is provided for the purpose of illustration and description only and is not intended as a definition of the limits of the present invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0020]** For a more complete understanding of the present invention, reference is now made to the following descriptions taken in conjunction with the accompanying drawing, in which:

**[0021]** **FIGs 1A-H -** Fig. 1A illustrates purification of h/mGDF9:BMP15 heterodimers and definition of their activities in the mouse granulose cells (GC) assay. Fig. 1B shows a western blot of h/mBMP15 with anti-FLAG, and h/mGDF9 with anti-MYC. Figs. 1C-H illustrate the results of a mouse GC assay used to quantitate the ability of the ligands to induce *Ptx3, Has2,* and *Ptgs2* mRNAs, which are important for cumulus regulation.

**[0022]** **FIGs 2A-M -** Figs. 2A-F illustrate dose-dependent effects in downstream extracellular matrix (ECM) gene regulation and oocytectomized (OOX; resident oocyte microsurgically removed) cumulus cells expansion. Figs. 2G-K are representative photographs of OOX cumulus cells treated with (G) no ligand, (H) 30 ng/ml hBMP15, (I) 30 ng/ml mGDF9, (J) 0.3 ng/ml hGDF9:BMP15, and (K) 0.3 ng/ml mGDF9:BMP15in the presence of EGF (10 ng/ml). Figs. 2L-M show heterodimer dose-dependent effects tested in OOX cumulus cell expansion.

**[0023]** **FIGs 3A-O -** Figs. 3A-B show identification of the h/mGDF9:BMP15 SMAD-signaling pathway and type 1 receptors in mouse granulosa cells. (A) Wild-type granulosa cells were treated with ligands (100 ng/mL hBMP15, 100 ng/mL mGDF9, 3 ng/mL hGDF9:BMP15, and 16 ng/mL mGDF9:BMP15) for 1 h. Anti–P-SMAD1/5/8 and anti–PSMAD2/3 were used to detect the two SMAD-signaling pathways. Actin was used as the internal control. (B) Alk6−/− granulosa cells were treated with the same ligands to examine the phosphorylation of SMAD1/5/8 and SMAD2/3. Actin was used as the internal control. Figs. 3C-H illustrate the relative value of *Ptx3, Has2,* and *Ptgs2* when inhibitors were used with GDF9 homodimer, BMP15 homodimer, and GDF9:BMP15 heterodimer in order to identify the type 1 receptors.

Figs. 3I-3N illustrate the relative values of *Ptx3, Has2,* and *Ptgs2* with ECD attenuated up-regulation of ECM gene expression by GDF9 homodimer, BMP15 homodimer, and GDF9:BMP15 heterodimer. FIG. 3O shows identification of the h/mGDF9:BMP15 type 2 receptor in mouse granulosa cells. Ligands (100 ng/mL mGDF9 and 3 ng/mL h/mGDF9:BMP15) were incubated with 1 μg/mL BMPR2* (T.B.T), BMPR2, ACVR2A, or ACVR2B ECD. Anti–P-SMAD2/3 was used to compare SMAD2/3 phosphorylation levels among different type 2 receptor ECD treatments. Actin was used as the internal control.

**[0024] Figs 4A-B -** Fig. 4A and B illustrate embodiments of the pathway for sheep, mouse, and human BMP15 and GDF9 homodimers and heterodimers in GCs.

**[0025] Fig 5 -** Fig. 5 illustrates the precursor protein sequence for human and mouse MYC-tagged GDF9 and FLAG-tagged BMP15.

**[0026] Figs 6A-B -** Fig. 6 shows purified protein quantification by western blot. Purified h/m GDF9:BMP15 heterodimers (5 μL) were analyzed under reducing conditions by Western blot. (6A) BMP15 was quantified by FLAG-bacterial alkaline phosphatase (BAP) standards at 5, 10, and 20 ng with anti-FLAG. (6B) GDF9 was quantified by mGDF standards at 1, 2, and 4 ng with anti-GDF9.

**[0027] Figs 7A-C -** Fig. 7 illustrates the effect of a single amino acid change (G72R) in the mature human GDF9 sequence, which results in gain of activity in the in vitro assay for human GDF9 homodimer. Fig. 7A is *Ptx3*, Fig. 7B is *Has2,* and Fig. 7C is *Ptgs2.*

**[0028] Figs 8A-F -** Fig. 8 shows identification of the h/mGDF9:BMP15 SMAD-signaling pathway and type 1 receptors in COV434 cells. (A) COV434 cells were treated with 100 ng/mL hBMP15, 100 ng/mL mGDF9, and 3 ng/mL h/mGDF9:BMP15 for 1 h. Anti–P-SMAD1/5/8 and anti–P-SMAD2/3 were used to detect the two P-SMAD–signaling pathways. Actin was used as the internal control. (B) COV434 cells were treated with 100 ng/mL hBMP15, 100 ng/mL hGDF9, and 3 ng/mL hGDF9:BMP15 and a mix of their homodimers (100 ng/mL) for 1 h. Anti–P-SMAD2/3 was used to define ligand activities. Actin was used as the internal control. (C and D) (Left) COV434 cells were treated with serial dilutions of hBMP15 or mGDF9 (1.0, 10, or 100 ng/mL) or h/mGDF9:BMP15 (0.1, 0.3, 1.0, or 3.0 ng/mL) to test the dosedependent effect on SMAD2/3 phosphorylation. Actin was used as the internal control. (Right) Western blots of three independent experiments were quantified, and the data are shown as the mean ± SEM (n = 10). ***$P < 0.001$ compared with controls not treated with ligand. (E

and F) The ALK2/3/6 inhibitor LDN-193189 (100 nM) or the ALK4/5/7 inhibitor SB-505124 (1 μ M) was coincubated with the ligands to test if the induction of SMAD phosphorylation was abolished compared with controls with no inhibitor treatment. Actin was used as the internal control.

**[0029]** **Fig 9 -** Fig. 9 shows identification of GDF9:BMP15 type 2 receptor in mouse granulosa cells. Ligands (100 ng/mL mGDF9, 3 ng/mL h/mGDF9:BMP15) were coincubated with 1 μg/mL TGFβ receptor type 2 (TGFBR2) extracellular domain (ECD). Anti–P-SMAD2/3 was used to compare SMAD2/3 phosphorylation levels among different type 2 receptor ECD treatments. Actin was used as the internal control.

**[0030]** **Fig 10 -** Fig. 10 shows identification of GDF9:BMP15 type 2 receptor in COV434 cells. Ligands (100 ng/mL mGDF9, 1 ng/mL hGDF9:BMP15) were coincubated with 1 μg/mL BMPR2* (T.B.T), ACVR2A, ACVR2B, or TGFBR2 ECD. Anti–P-SMAD2/3 was used to compare SMAD2/3 phosphorylation levels among different type 2 receptor ECD treatments. Actin was used as the internal control.

## DETAILED DESCRIPTION OF THE INVENTION

**[0031]** The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

**[0032]** Throughout this disclosure, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

**[0033]** As used herein, the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. "Effective amount" means that amount which is sufficient to effect the maturation of an oocyte.

## I. General Embodiments

**[0034]** Of the one million oocytes present at birth, only several hundred will be ovulated, and the rest will die by atresia. The ability to rescue oocytes destined to die and mature them *in vitro* is useful to provide oocytes for infertile women, for example. In vitro maturation (IVM) can be challenging in the human, because folliculogenesis is a complex process encompassing quite a few cellular changes in the oocyte and the surrounding follicle cells. A few live births have resulted from the successful maturation and fertilization of immature human oocytes obtained by aspiration from small antral follicles, for example. In addition, it is possible to grow primordial follicles to pre-antral stages in slices of ovarian tissue, and support antrum formation in isolated pre-antral follicles.

**[0035]** An embodiment of the invention is the *in vitro* maturation (IVM) of human oocytes, an infertility treatment modification of traditional *in vitro* fertilization (IVF) protocols, through the use of GDF9:BMP15 heterodimer. *In vitro* maturation may also include a step of *in vitro* fertilization. The objective of IVM in human assisted reproductive technology (ART) is to avoid side effects of exogenous gonadotropins, increase fetal viability, and reduce the cost of infertility treatments. High expression of cumulus expansion-regulated genes (e.g., *PTGS2*, *HAS2*) is correlated with high quality embryos in ART clinics (*33-35*).

**[0036]** Example 1 illustrates that GDF9:BMP15 heterodimers are the most bioactive ligands to up-regulate these ECM genes. In Example 1, it was found that during mouse granulosa cell and cumulus cell expansion assays, mouse GDF9:BMP15 was ~10-30-fold more biopotent and human GDF9:BMP15 was ~1000-3000-fold more bioactive than the most active species-specific homodimers. Moreover, these heterodimers signal through a signaling pathway that utilizes BMPR2, ALK4/5/7, and SMAD2/3. In an embodiment of the invention, these findings that species-specific GDF9:BMP15 heterodimers are the most bioactive ligands have important clinical implications for improving *in vitro* maturation in human and animal assisted reproductive technology laboratories. Moreover, oocyte developmental competence is enhanced by oocyte-derived paracrine factors during IVM (*36*). Thus, in an embodiment of the disclosure, GDF9:BMP15 heterodimers promote oocyte developmental competence during IVM and provide new opportunities for treatments of human infertility.

**[0037]** As used herein, "subject" or "patient" refers to a female from whom the oocytes have been collected. The subject may be fertile or infertile. The subject may have also undergone traditional IVF protocols, such as the "long protocol" or the "short protocol" in order

to hyperstimulate the ovaries. However, because the immature oocytes may be used in embodiments of the invention, the subject also may not have undergone ovarian hyperstimulation. Since only hBMP15 and mGDF9 homodimers are the biopotent ligands in the in vitro assays, in an embodiment of the invention mGDF9:hBMP has higher activity in the regulation of folliculogenesis and in IVM and IVF assays. Furthermore, a single amino acid change (G72R) in the mature human GDF9 sequence results in gain of activity in the in vitro assay for human GDF9 homodimer (Fig. 7).

**[0038]** As used herein, the term "immature human oocyte" means a human oocyte that has not yet reached metaphase-II (M-II). As discussed previously, metaphase-II is characterized by exclusion of one polar body from the cytoplasm. Immature human oocytes are typically at the germinal vesicle (GV) or metaphase-I (M-I) stage, which may include an immature oocyte in a primordial follicle through ovulation. IVM may also include the process of IVF.

**[0039]** Oocytes may be cultured with their cumulus intact, in a form known as a "cumulus-oocyte-complex" (COC), or oocytes can be encased in granulosa cells from earlier follicles such as primordial, primary, or secondary follicles, or oocytes may be partially or entirely denuded from cumulus cells or granulose cells. COC can be stripped with 85 IU/ml hyaluronidase in HEPES buffered medium and mechanically pipetted until oocytes are denuded, for example. An oocyte that is "essentially free of cumulus cells" is an oocyte that is associated with sufficiently few cumulus cells that the cumulus cells have no detectable physiological effect on the oocyte.

## II. IVF protocols and culture

**[0040]** IVF protocols and culture conditions for human oocytes are known in the art, such as those found in U.S. Patent No. 7,790,459. IVF medium may also be used as culture medium during IVM. Suitable culture conditions include e.g. culturing the oocytes at 37° C in an atmosphere of 95% air and 5% $CO_2$ at high humidity, e.g. 100% humidity. Mineral oil may be overlaid on the medium to control evaporation and/or temperature. Oocytes are typically cultured in a well containing 1 ml of culture medium or more. In embodiments of the invention, oocytes, COCs, or follicles may be cultured in IVF medium comprising GDF9:BMP15 for about 12 to 56 hours or more prior to fertilization, for example. In a specific embodiment, the oocytes and surrounding granulosa cells may be cultured in IVF medium comprising GDF9:BMP15. In an embodiment of the invention, the oocytes are cultured for about 12-48 hours, or for about 24-28 hours. In another embodiment of the invention, the follicles or strips of ovarian tissue could

be cultured for many weeks, for example, 2 weeks, 3 weeks, 4 weeks, or 5 weeks, in the presence of GDF9:BMP15.

**[0041]** IVF media are generally known in the art and may comprise inorganic salts, essential and non-essential amino acids, and energy sources. Inorganic salts are used to buffer the pH of the medium within a range preferably of about 7.2-7.4 and to maintain correct osmolarity of the medium with the oocytes. Inorganic salts include $CaCl_2$, KCl, $MgSO_4$, NaCl, $NaHCO_3$, $NaH_2PO_4H_2O$, FE(NO. $_3)_3 9H_2O$, $KH_2PO_4$, Na acetate, and $Na_2H_2PO_4$, for example. The IVF medium may also include essential amino acids such as isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The IVF medium may additionally include non-essential amino acids, non-naturally occurring amino acids, or amino acid derivatives. In one embodiment of the invention, the IVM medium comprises alanine, arginine, asparagine, aspartic acid, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

**[0042]** The IVF medium also may contain vitamins such as vitamins A1 (retinol), A2 (an alternative form of retinol), B1 (thiamine), B2 (riboflavin), B6 (pyridoxine), B9 (folic acid), B12 (cyanocobalamin), B17, C (ascorbic acid), D, D2 (calciferol), D3 (cholecalciferol), E (tocopherol), H (biotin), K, K1 (phylloquinone), K2, K3 (menadione), P, etc. In an embodiment of the invention, the medium biotin, D-Ca pantothenate, choline chloride, folic acid, i-inositol, nicotinamide, pyroxidal-HCl, riboflavin, and thiamine-HCl.

**[0043]** The IVF medium may also comprise a growth factor (GF). Growth factors result in the activating of cellular proliferation and/or differentiation by binding to cell receptors. Growth factors may positively stimulate oocyte maturation. Useful growth factors in the context of the present invention include those selected from the following growth factor superfamilies: epidermal growth factor (EGF) family; platelet derived growth factor (PDGF) family; insulin-like growth factor (IGF) family; nerve growth factor (NGF) family; transforming growth factor (TGF) family; fibroblast growth factor (FGF) family; hepatocyte growth factor (HGF) family; hematopoietic growth factors; and cytokines, for example.

**[0044]** The IVF medium may also comprise a hormone. Hormones include insulin, estradiol, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), for example. Human menopausal gonadotropin (HMG) may be substituted for FSH, and human chorionic

gonadotropin (HCG) may be substituted for LH. In an embodiment, the IVF medium also contains human transferrin (TF). TF is a 75 kDa glycoprotein containing 679 amino acids and two glycan chains. TF transports iron in extracellular fluid and also stimulates cell growth.

**[0045]** The growth factors, hormones and transferrins discussed above may be naturally occurring, synthetic or recombinant, and encompass biologically active fragments, variants, derivatives and homologs of these substances that retain at least some of the biological activity of the naturally-occurring, synthetic or recombinantly-produced substances. The IVF medium may also include an energy source, such as glucose, sodium pyruvate, lactate, or a mixture of some or all of these energy sources. Further, the IVF medium may also include buffer and antibiotics. Additionally, epidermal growth factor (EGF) and/or a related ovarian EGF-like signaling protein (e.g., amphiregulin, epiregulin, or betacellulin) could be added to the media.

## III. Polypeptide, Peptide, and Protein Compositions

**[0046]** Certain embodiments concern at least one polypeptide, peptide (*e.g.*, a polypeptide segment), protein, or derivative or variant thereof. As used herein, "subunit," in reference to a GDF9:BMP15 heterodimer, refers to either the GDF9 protein or the BMP15 protein within the heterodimer. For example, the GDF9:BMP15 heterodimer comprises one GDF9 subunit and one BMP15 subunit. In an embodiment of the invention, the GDF9:BMP15 heterodimer comprises or consists of one GDF9 subunit and one BMP15 subunit. As used herein, a "protein," "polypeptide," "peptide," "polypeptide or peptide composition," or "polypeptide or peptide compound," generally refers, but is not limited to, a protein or polypeptide of at least five amino acids or amino acid analogs (collectively an amino molecule, see below). All the "polypeptide or peptide" terms described above may be used interchangeably herein.

**[0047]** The GDF9:BMP15 protein heterodimers are comprised of one protein GDF9 protein and one BMP15 protein, in particular embodiments. Specific but merely exemplary sequences and acquisition numbers are recited below. While these sequences are provided as an example, in an embodiment of the invention, any mammalian GDF9:BMP15 protein heterodimer may be used, or a mix of different mammalian heterodimers may also be used. One of skill in the art would have the resources to find and create a mammalian GDF9:BMP15 protein heterodimer given the disclosures here.

**[0048]** An exemplary Human GDF9 (acquisition number ENSG00000164404) (see Ensembl® database) cDNA is provided in SEQ ID NO:1. An exemplary Human GDF9 Protein is provided in SEQ ID NO: 2. An exemplary Human BMP15 cDNA (acquisition number ENSG00000130385) is provided in SEQ ID NO: 3. An exemplary Human BMP15 protein is provided in SEQ ID NO:4. An exemplary Mouse GDF9 cDNA (acquisition number ENSMUSG00000018238) is provided in SEQ ID NO:5. An exemplary Mouse GDF9 Protein is provided in SEQ ID NO:6. An exemplary Mouse BMP15 cDNA (acquisition number ENSMUSG00000023279) is provided in SEQ ID NO:7. An exemplary Mouse BMP15 Protein is provided in SEQ ID NO:8. An exemplary Sheep GDF9 cDNA (Gene ID: 100217402) is provided in SEQ ID NO:9. An exemplary Sheep GDF9 Protein: (from Gene ID: 100217402) is provided in SEQ ID NO:10. An exemplary Sheep BMP15 cDNA (Gene ID: 100141303) is provided in SEQ ID NO:11. An exemplary Sheep BMP15 Protein (from Gene ID: 100141303) is provided in SEQ ID NO:12.

**[0049]** In an embodiment of the invention, a GDF9:BMP15 heterodimer is a heterodimer of GDF9 and BMP15 of any of the above listed sequences. The sequences of the GDF9 proteins in the GDF9:BMP15 heterodimer may also be greater than 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% similar to SEQ ID NO: 1, SEQ ID NO: 3, and/or SEQ ID NO: 5, for example. The sequences of the BMP15 proteins in the GDF9:BMP15 heterodimer may also be greater than 70%, 75%, 80%, 85% , 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% similar to SEQ ID NO: 7, SEQ ID NO: 9, and/or SEQ ID NO: 11, for example.

**[0050]** In certain embodiments the polypeptide or peptide composition comprises at least one protein, polypeptide or peptide. In methods that involve a GDF9:BMP15 heterodimers composition a polypeptide or peptide can have all or part of the amino acid sequence of a polypeptide, including homologous polypeptides. In certain embodiments, protein, polypeptide, or peptide containing compositions will generally be proteins or peptides or synthetic proteins or peptides each essentially free from toxins, pathogens, and harmful immunogens. In certain aspects the polypeptide is a recombinant or synthetic amino acid seqeunce.

**[0051]** In certain embodiments the size of the at least one polypeptide or peptide molecule may comprise, but is not limited to, a molecule having at least, at most, or about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 100, 500, 1000 to about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40,

50, 100, 500, or greater amino molecule residues, and any value or range derivable therein. Embodiments include those lengths of contiguous amino acids or analogs thereof of any sequence discussed herein.

**[0052]** Segments or fragment of a polypeptide or peptide include amino acid 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, 350, 400, 450, to amino acid 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, 350, 400, 450, 500, 550, 600 amino acids of sequences disclosed or referenced herein, including all values and ranges there between. The GDF9:BMP15 heterodimers may include full length protein, or partial GDF9:BMP15 proteins that are still able to form GDF9:BMP15 heterodimers, for example.

**[0053]** As used herein, an "amino molecule" refers to any amino acid, amino acid derivative or amino acid mimic as known to one of ordinary skill in the art. In certain embodiments, the residues of the polypeptide or peptide molecule are sequential, without any non-amino molecule interrupting the sequence of amino molecule residues. In other embodiments, the sequence may comprise one or more non-amino molecule moieties. In certain embodiments, the sequence of residues of the polypeptide or peptide molecule may be interrupted by one or more non-amino molecule moieties.

**[0054]** In embodiments of the invention, the heterodimer may be generated by standard means in the art, such as using recombinant techniques and so forth.

**[0055]** Both GDF9 and BMP15 monomers have three domains: pre-domain, pro-domain and mature domain. The pre-domain is the signal peptide, which leads to the secretion of the protein. The pro-domain is useful for dimerization. Both pre-domain and pro-domain are removed from the mature domain during the protein maturation. In specific embodiments, the respective GDF9 and BMP15 subunits utilize the mature domains, although smaller fragments of GDF9 and BMP15 each that utilize the receptor complex may be employed.

**[0056]** GDF9 and BMP15 are two monomers of the heterodimer and interact with each other *via* noncovalent bonds to form the heterodimer. In specific cases, they are both made as precursors as separate cDNAs, but in specific embodiments a tandem construct comprising both GDF9 and BMP15 on the same molecule. In those cases, the order of the respective components of the GDF9:BMP15 heterodimer fusion protein in a N-terminal to C-terminal orientation, for example, may be of any kind. In specific embodiments, the subunit that is the GDF9 or functional fragment thereof may be N-terminal or C-terminal with respect to the subunit that is

the BMP15 or functional fragment thereof. There may also be intervening sequences to allow the single chain polypeptide to fold correctly into a bioactive GDF9-BMP15 or BMP15-GDF9 sequence.

## IV. Kits

**[0057]** Any of the compositions described herein may be comprised in a kit. In a non-limiting example, GDF9:BMP15 heterodimers and/or reagents for production of GDF9:BMP15 heterodimers are included in a kit. The components of the kits may be packaged either in an aqueous, powdered, or lyophilized form. The container means of the kits will generally include at least one canister, vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit (second agent, *etc.*), the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial, canister, or the like. Kits may also include IVM and/or IVF medium. Kits also may include a container for the described compositions or their variations, and any other reagent containers in close confinement for commercial sale.

**[0058]** When the components of the kit are provided in one and/or more liquid solutions, *e.g.*, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred, but not required. However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder may be reconstituted by the addition of a suitable solvent or medium. It is envisioned that a solvent may also be provided in another container means within the kit.

**[0059]** A kit will also include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented.

**[0060]** It is contemplated that such reagents are embodiments of kits. Such kits, however, are not limited to the particular items identified above and may include any reagent used directly or indirectly in the IVM of oocytes.

**[0061]** In certain aspects, the kit further comprises one or more reagents and/or apparatuses for performing IVM. In some cases, the kit comprises one or more apparatuses

and/or reagents for extracting material from an ovary *in vivo* and/or for delivering material to an ovary *in vivo*.

## EXAMPLES

**[0062]** The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods described herein are presently representative of certain embodiments and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

## EXAMPLE 1

## GDF9 AND BMP15 ACTIVITY AND PATHWAY ANALYSIS

**[0063]** To uncover possible activities of GDF9:BMP15 heterodimers in mammals, subunit specific tags were engineered at the N-terminus of human (h) and mouse (m) GDF9 and BMP15 proteins (Fig. 5). Similar to a previous study (*17*), optimized proteolytic cleavage sites for the mouse and human GDF9 and BMP15 precursors were inserted before the epitope tags (MYC or FLAG) to facilitate production of the mature proteins. Either homodimers (when expressed individually in HEK-293T cells) or heterodimers (when both subunits were produced in the same cell) were purified (Fig. 1A). Western blot analysis was used to detect BMP15 by anti-FLAG and GDF9 by anti-MYC when both subunits were produced together (Fig. 1B and 5). The heterodimers purified by immunoprecipitation were mixture of BMP15 homodimer (~75%) and GDF9:BMP15 heterodimer (~25%), since both dimers can be co-immunoprecipated by the anti-FLAG agarose.

**[0064]** In response to the ovulatory LH surge, cumulus cells become expanded and produce a complex extracellular matrix (ECM), which is essential for ovulation, fertilization, and subsequent embryonic development. This highly coordinated process is known as cumulus expansion (*18*). A number of important cumulus genes have been identified during the process, including hyaluronan synthase 2 (*Has2*) (*19*), pentraxin 3 (*Ptx3*) (*20*), and prostaglandin

synthase 2 (*Ptgs2*) (*21*). To compare the bioactivity of the homodimers and heterodimers in cumulus expansion, the ability of the ligands to induce *Ptx3*, *Has2* and *Ptgs2* mRNAs in mouse granulosa cells (GC) was measured assays after 5 hours of incubation (Fig. 1C-H). hBMP15 stimulated cumulus expansion related gene expression at high concentrations of ligand, while the activity of hGDF9 was very low and 3.6-11.9-fold suppressed compared to hBMP15 at the same concentration of ligand. Surprisingly, hGDF9:BMP15 dramatically induced *Ptx3*, *Has2* and *Ptgs2* mRNA expression at a far lower concentration compared with hBMP15 homodimer (Fig. 1C-E). Besides the no ligand control, another control was set up in which GC was treated with hBMP15 and hGDF9 homodimers. The presence of hGDF9 did not alter the activity of hBMP15 and this control helped rule out synergistic functions between the two homodimers. Similar experiments for mouse homodimers and heterodimers was performed (Fig. 1F-H). In contrast to human ligands, mGDF9 was found to be a potent regulator of cumulus expansion genes, whereas mBMP15 was inactive. Mouse GDF9:BMP15 was found to be ~10-30-fold more biopotent compared with mGDF9 homodimer.

**[0065]** To quantify the heterodimer activities, dose–response experiments were performed with human heterodimers and mouse heterodimers in the GC assays (Fig. 2A-F). Strikingly, 0.03 ng/ml hGDF9:BMP15 had comparable activity with 100 ng/ml hBMP15 homodimer in up-regulating the three cumulus expansion related transcripts, indicating a ~3000-fold increased activity of hGDF9:BMP15 heterodimer compared to active hBMP15 homodimer (Fig. 2A-C). In a parallel experiment for mouse ligands, mGDF9:BMP15 was ~10-30-fold more biopotent compared with mGDF9 homodimer (Fig. 2D-F). These results, in part, explain the previous mouse mutant phenotypes (*5, 6*): *Gdf9* homozygous females are sterile due to the lack of any active homodimer (GDF9:GDF9) or heterodimer (GDF9:BMP15) ligands; since the potent GDF9 homodimer compensates for the absence of GDF9:BMP15 heterodimer in *Bmp15* null females, BMP15 homozygous females have a limited phenotype of slightly reduced fertility; $Gdf9^{+/-}Bmp15^{-/-}$ double mutant mice exhibit sterility defects compared to the subfertile $Bmp15^{-/-}$ mice because of GDF9 dosage effects.

**[0066]** Although the above results show that GDF9:BMP15 heterodimers are more potent than their active homodimers in up-regulating cumulus expansion related transcripts, it was also investigated whether heterodimers are sufficient to promote the full process of cumulus expansion *in vitro* using previously described methods (*22*). In the presence of epidermal growth factor (EGF), cumulus expansion was induced when mouse oocytectomized (OOX;

resident oocyte microsurgically removed) cumulus cells were treated with hBMP15, mGDF9, or either heterodimer using serial dilutions (Fig. 2G- M). Results of the OOX complex expansion assay matched the previous mouse GC assay: hGDF9:BMP15 showed about 1000-fold increased activity compared with hBMP15, while mGDF9:BMP15 showed more than 10-fold enhanced biopotency compared with mGDF9.

**[0067]** GDF9 and BMP15 are closely related paralogs in the TGFβ superfamily, but species-specific homodimers signal via different SMAD pathways: SMAD2/3 for mGDF9 and SMAD1/5 for hBMP15 (*3, 4*). To define the downstream signaling cascades of the heterodimers in GCs, SMAD1/5/8 and SMAD2/3 phosphorylation levels were examined one hour after treatment with hBMP15, mGDF9 and GDF9:BMP15 heterodimers (Fig. 3A, B). Compared with hBMP15 homodimer, human and mouse GDF9:BMP15 heterodimers and mGDF9 showed minimal SMAD1/5/8 phosphorylation. In contrast, mGDF9:BMP15 and hGDF9:BMP15 dramatically stimulated SMAD2/3 phosphorylation, indicating that SMAD2/3 is the major signaling pathway for GDF9:BMP15 heterodimers. hBMP15 also induces SMAD2/3 phosphorylation at a low level, indicating crosstalk between the two signaling pathways by hBMP15 homodimer . These findings corroborate the cumulus cell SMAD pathways identified genetically; SMAD2/3 double knockout mice have severely impaired fertility secondary to a defect in cumulus expansion (*23*), whereas SMAD1/5/8 triple knockouts do not display such defects in cumulus cells (*24*).

**[0068]** To investigate the receptor signaling pathway for the heterodimers, inhibitors were used to identify the type 1 receptors (Fig. 3C-H). LDN-193189 (a dorsomorphin derivative) is a potent inhibitor of ALK2/3/6 (*25, 26*), and SB-505124 is an inhibitor of ALK4/5/7 (*27*). In the assay, LDN-193189 showed only a subtle effect on heterodimer action in one of the qPCR assays (Fig. 3C-E). In contrast, SB-505124 abolished heterodimer activities (Fig. 3F-H), similar to its actions on mGDF9. Thus, ALK4, ALK5, and/or ALK7 are the type 1 receptor for the most potent ligands, GDF9:BMP15 heterodimers. Although rat GDF9 homodimers have been reported to signal through ALK5 in an *in vitro* assay (*3*), it was discovered that *Alk5* conditional knockout (cKO) mice have no defects in follicular development and cumulus expansion, indicating that ALK5 is not the sole type 1 receptor through which GDF9 signals in the mouse ovary (*28*). Since the heterodimers are most active and the *Alk5* cKO mice have no cumulus expansion defect, unlike the $Gdf9^{+/-}Bmp15^{-/-}$ double mutant mice (*5, 6*), ALK5 may be excluded as the sole receptor for GDF9:BMP15 heterodimer. Since ALK7 has relatively low expression

in the ovary (*29*), ALK4 is the most likely type 1 receptor for both GDF9 and GDF9:BMP15 heterodimers, although this may be further tested. It is possible that all three receptors (ALK4, ALK5, and ALK7) act redundantly to permit signaling by GDF9:BMP15 heterodimer.

**[0069]** *In vitro*, BMPR2 is a type 2 receptor for both GDF9 and BMP15 (*4, 30*). To test the roles of BMPR2 as a candidate type 2 receptor for GDF9:BMP15 heterodimers, it was determined if the BMPR2 extracellular domain (ECD) could abolish activities of GDF9:BMP15 heterodimers. Confirming this, BMPR2 ECD attenuated the up-regulation of ECM gene expression by GDF9:BMP15 heterodimers (human > mouse) as well as hBMP15 and mGDF9 (Fig. 3I-O).

**[0070]** Previous studies have identified a number of heterodimeric TGFβ ligands that have novel functions or enhanced potency compared to the homodimers. For example, inhibins (α:βA and α:βB) antagonize activins (βA:βA, βB:βB, and βA:βB) through their binding to specific and unique receptors (*1, 31*), and BMP2:BMP7 heterodimers showed a specific activity about 20-fold higher than their homodimers in an *in vitro* assay (*32*). In this example, an even more pronounced bioactivity of human oocyte-secreted heterodimers is seen. In humans, GDF9:BMP15 heterodimer is the most bioactive ligand in cumulus expansion, while BMP15 homodimer has an over 1000-fold lower activity, signaling *via* both SMAD2/3 and SMAD1/5/8 pathways (Fig. 4A). In mouse, the >10-fold less active GDF9 homodimers cooperate with GDF9:BMP15 heterodimers to regulate GC function exclusively through a SMAD2/3 pathway (Fig. 4B). GDF9:BMP15 heterodimers likely bind to a BMPR2 (type 2) and ALK4 (type 1) receptor complex to transmit a signal through phosphorylation of SMAD2/3 in mice (Fig. 4). Since BMP15 null and GDF9 null sheep have infertility phenotypes (*8, 9*) that resemble *Gdf9*$^{-/-}$ mice (*i.e.*, a block at the primary follicle stage) (*5*), in an embodiment of the invention GDF9:BMP15 heterodimers in sheep may be more active than either of the homodimers (Fig. 4A). Thus, co-evolutionary changes in the GDF9 and BMP15 coding sequences have allowed GDF9:BMP15 heterodimers to form, be more active than homodimers, and function as the essential oocyte-secreted TGFβ dimers, especially in women, sheep, and likely most other mammals.

**[0071]** Confirmation of the SMAD Signaling Pathway by the h/mGDF9:BMP15 heterodimer in human granulosa cells was achieved. To validate the conclusions in human granulosa cells, the inventors tested GDF9:BMP15 heterodimer activities in COV434 cells, an

immortalized human granulosa cell line. Similar to the results in mouse granulosa cells, activehBMP15 and mGDF9 homodimers signal *via* SMAD1/5/8 and SMAD2/3, respectively (Fig. 8A). The h/mGDF9:BMP15 heterodimers use SMAD2/3 as the major signaling pathway (Fig. 8A). Furthermore, assay results in COV434 cells confirmed that the high activity of hGDF9:BMP15 is not the result of a synergistic effect of hGDF9 and hBMP15 homodimers (Fig. 8B).

**[0072]** In dose–response experiments, h/mGDF9:BMP15 heterodimers show dramatically higher activities in SMAD2/3 phosphorylation than their corresponding homodimers (Fig. 8 C and D). There was SMAD2/3 phosphorylation at the lowest tested dose (0.1 ng/mL) of the hGDF9:BMP15 heterodimer, but there was no apparent SMAD2/3 phosphorylation by the hGDF9 and hBMP15 homodimers when tested at a dose of 100 ng/mL (Fig. 8C). This result confirms the >1,000-fold higher activity of hGDF9:BMP15 compared with hBMP15 that was observed in the mouse granulosa cell assays. When the inventors tested mGDF9 homodimer in the COV434 cells, there was low phosphorylation of SMAD2/3 at 10 ng/mL of GDF9, approximating the SMAD2/3 phosphorylation by mGDF9:BMP15 heterodimer (0.1–0.3 ng/mL) (Fig. 8D). This result also confirms the ~30-fold higher activity of mGDF9:BMP15 compared with GDF9 that was observed in mouse granulosa cell assays.

**[0073]** Last, the inventors examined the effects of the type 1 inhibitors and type 2 ECDs on the ligand-signaling pathways in COV434 cells. The heterodimer activities in SMAD2/3 phosphorylation assays were abolished specifically by SB-505124 but not by LDN-193189 (Fig. 8 E and F), confirming that ALK4/5/7 is the type 1 receptor kinase that phosphorylates SMAD2/3 in human as well as mouse. To evaluate the GDF9:BMP15 heterodimer type 2 receptor in COV434 cells, the inventors compared SMAD2/3 phosphorylation levels among treatments with four different type 2 receptor ECDs and found that only BMPR2 ECD decreased activities of mGDF9 and hGDF9:BMP15 in SMAD2/3 phosphorylation (Fig. 10).

## EXAMPLE 2

## EXEMPLARY MATERIALS AND METHODS

**[0074]** Exemplary materials and/or methods of the invention are described herein, although the skilled artisan recognizes that modifications and optimizations to these materials and methods are routine and encompassed by the invention(s).

### Construction of Expression Plasmids

**[0075]** Plasmids containing the native hGDF9:hBMP15 and mGDF9:mBMP15. cDNA were used as templates, and overlap extension PCR was performed to engineer optimized cleave sites (including surrounding amino acids) and FLAG/MYC-tag before the ligand mature domains as well as fuse pre-pro and modified mature domains of human GDF9 and BMP15(*37*). For mouse GDF9 and BMP15, human pre-pro domain was fused to the modified mouse mature domain to increase its expression in HEK-293T cells. To enhance the expression of mGDF9/BMP15 in HEK-293T cells, the original mouse pre-pro domain was replaced by the human pre-pro domain (Fig. 5). For homodimer expression plasmids, FLAG-tagged h/mGDF9 and h/mBMP15 were cloned into pEFIRES-P, respectively. For heterodimer expression plasmids, both MYC-tagged h/mGDF9 and FLAG-tagged h/mBMP15 were cloned into pCEBud4.1 (Invitrogten). MYC-h/mGDF9 is under the control of the EF-1α promoter, while FLAG-h/mBMP15 is under the control of the CMV promoter in pCEBud4.1. All plasmids encoding the precursor sequence were confirmed by DNA sequencing.

### Transfection and Selection of Stable Cell Clones

**[0076]** HEK-293T cells were cultured in DMEM (Invitrogen) containing 10% FBS (Sigma-Aldrich) and 100 μg/ml penicillin–streptomycin (Invitrogen). All plasmids were transfected into HEK-293T cells using FuGENE6 (Roche) transfection reagent according to the manufacturer's instructions. Two days after transfection, cells containing the homodimer expression plasmids (pEFIRES-P) were selected with 5 μg/ml of puromycin (Invitrogen), while cells containing the heterodimer expression plasmids (pCEBud4.1) were selected under 0.5 μg/ml of Zeocin (Invitrogen). Puromycin/Zeocin resistant cell colonies were selected two weeks after transfection, and their protein expression was confirmed by western blot. HEK-293 hBMP15 stable cells were generated as described in a prevous study (*37*).

### Protein Purification, Quantification and Immunoprecipitation

**[0077]** When the stable cells reached confluency, DMEM containing 2% FBS and 100 μg/ml penicillin–streptomycin was used for the production of proteins. Roller bottle cultures were harvested 5 days after seeding, and dishes were harvested 3 days after seeding. Purification of FLAG-tagged homodimers was conducted using anti-FLAG M2 affinity gel (Sigma-Aldrich) according to the manufacturer's protocol. The FLAG-tagged homodimers were eluted with 3× FLAG (Sigma-Aldrich) elution buffer (25μg/μl in TBS pH8.0), and 1 mg/ml BSA (Sigma-Aldrich) was added to the proteins before storage at -80°C. The purified FLAG-tagged ligands were quantified by western blot using FLAG-bacterial alkaline phosphatase (BAP) standards (Sigma-Aldrich) and mouse anti-FLAG M2 antibody (Sigma-Aldrich) (Fig. 6A). MYC-GDF9:FLAG-BMP15 heterodimers were Immunoprecipitated by the same method as above. The purified MYC-m/hGDF9 was quantified using FLAG-mGDF9 standards with a GDF-9 monoclonal antibody as described in our previous study (*38*) (Fig. 6B).

**Mouse Granulosa Cell Isolation**

**[0078]** Mice used in this example were maintained on a mixed C57BL/6/129S6/SvEv genetic background and handled according to NIH Guide for the Care and Use of Laboratory Animals. Female mice 21–24 days of age were injected with 5 IU PMSG (Calbiochem). Ovaries were harvested 44–46 h after injection. Granulosa cells (GC) were released by puncturing large antral follicles. The collection media was DMEM/F12 (Invitrogen) containing 0.3% BSA (Sigma-Aldrich), 100 μg/ml penicillin–streptomycin, and 10 mM HEPES (Invitrogen). To remove oocytes, the GC suspension was filtered through a 40μm nylon cell strainer and washed twice with the collection media. For further treatment, CGs were maintained in DMEM/F12 containing insulin-transferrin-sodium selenite (ITS) supplement (Sigma-Aldrich), 0.5% heat-inactivated FBS, and 100 μg/ml penicillin–streptomycin.

**Mouse Granulosa Cell Assay**

**[0079]** Mouse and Human Granulosa Cell Assays. Mouse granulosa cells and COV434 cells were maintained in DMEM/F12 containing insulin-transferrin-sodium selenite supplement (Sigma-Aldrich), 0.5% heat-inactivated FBS, and 100 μg/mL penicillin–streptomycin. In the gene-induction assays, mouse granulosa cells were treated with ligands for 5 h, and total RNA was extracted. Realtime PCR was conducted to test the fold changes of Ptx3, Has2, and Ptgs2.

**[0080]** For SMAD activation analysis, mouse granulosa cells and COV434 cells were treated with ligands for 1 h and lysed with RIPA buffer in the presence of proteinase and phosphatase inhibitors (Roche). SMAD phosphorylation was detected by Western blot with

anti–P-SMAD1/5/8 or anti–P-SMAD2/3 (Cell Signaling). Actin was used as the internal control detected by monoclonal anti-actin (Sigma-Aldrich).

**[0081]** In the gene induction assays, GCs were treated with no ligand, 100 ng h/mGDF9, 100 ng h/mBMP15, 3 ng hGDF9:BMP15 or 16 ng mGDF9:BMP15. In the dose-response experiments, mGCs were treated with no ligand, 100 ng hBMP15, 10 ng mGDF9, or a 1 to 3 serial dilution of h/mGDF9:BMP15. GCs were collected after 5 hours treatments, and subjected to RNA extraction. Real-time PCR was conducted to test the fold changes of *Ptx3*, *Has2*, and *Ptgs2*.

**[0082]** For SMAD activation analysis, GCs were treated with no ligand, 100 ng hBMP15, 100 ng mGDF9, 3 ng hGDF9:BMP15 or 16 ng mGDF9:BMP15. GCs were collected after 1 hour treatments, and lysed with lysis buffer (50 mM Tris-HCl pH7.5, 50 mM NaCl, 1% Triton X-100) in the presence of proteinase inhibitor (Roche). SMAD phosphorylation was detected by western blot with anti-Phospho-SMAD1/5/8 or anti- Phospho-SMAD2/3 (Cell Signaling). Actin was used as the internal control detected by monoclonal anti-actin (Sigma-Aldrich).

**[0083]** Fig. 1A illustrates purification of h/mGDF9:BMP15 heterodimers and definition of their activities in the mouse GC assay. A plasmid containing MYC-tagged GDF9 and FLAG-tagged BMP15 was transfected into HEK-293T cells to yield GDF9 homodimers, BMP15 homodimers, and GDF9:BMP15 heterodimers. Use of anti-FLAG agarose allowed for immunoprecipitation of BMP15 homodimers and GDF9:BMP15 heterodimers. By western blot analyses, h/mBMP15 was detected by anti-FLAG, and h/mGDF9 was detected by anti-MYC (Fig. 1B). In the mouse GC assay, GCs were treated with no ligand (control), h/mBMP15 (100ng), h/mGDF9 (100ng), h/mGDF9:BMP15 (3ng) and mix of their homodimers (100ng+100ng) for 5 hours. Total RNA was extracted from those GCs, and downstream ECM genes *Ptx3* (Figs. 1C, 1F), *Has2* (Figs. 1D, 1G) and *Ptgs2* (Figs. 1E, 1H) were quantified by qPCR. Figs. 1C-H represent the mean ±SEM (n=3), $*P<0.05$; $**P<0.01$; $***P<0.001$.

**[0084]** Fig. 7 shows a mouse granulosa cell (GC) assay with mutated mGDF9, GCs were collected and treated with no ligand (control), 100ng/ml mGDF9, 100ng/ml mGDF9 R72G, 100ng/ml hGDF9, and 100ng/ml hGDF9 G72R for 5 hours. Total RNA was extracted from those GCs, and downstream ECM genes *Ptx3* (A), *Has2* (B) and *Ptgs2* (C) were quantified by qPCR. (A) to (C) represent the mean ±SEM (n=3), $*P<0.05$; $**P<0.01$; $***P<0.001$. Thus,

demonstrating that in some embodiments, R72 in the mGDF9 pre-helix loop is important for GDF9 biopotency.

**Mouse Cumulus Expansion Assay**

**[0085]** *In vitro* mouse cumulus expansion assays were performed as previously (*39*). Briefly, oocytes were removed from oocyte-cumulus cell complexes (OCC) using a microsurgical apparatus. The resulting oocytectomized (OOX) complexes consisted of the spherical zona pellucida surrounded by the cumulus cell mass without the oocyte. The culture media used in the assay was MEM alpha media (Invitrogen) containing 5% FBS and 10mM milrinone (Sigma-Aldrich). Ten OOX cumulus cells were treated with no ligand, 30ng h/mGDF9, 30ng h/mBMP15, or a 1 to 10 serial dilution of h/mGDF9:BMP15 in the presence of 10 ng/ml EGF (BD Biosciences). After 15 hour incubation, the cumulus expansion index (CEI) was scored based on the degree of OOX cumulus cell expansion using a scale from 0 (no expansion) to 4 (complete expansion) (*39*).

**[0086]** Fig. 2 shows the human GDF9:BMP15 and mouse GDF9:BMP15 dose-dependent effects in downstream ECM gene regulation and OOX cumulus cells expansion. In the mouse GC assay, mural GCs were treated with a 1 to 3 serial dilution of h/mGDF9:BMP15 heterodimers. hBMP15 and mGDF9 were used as positive controls. *Ptx3* (Figs. 2A, 2D), *Has2* (Figs. 2B, 2E) and *Ptgs2* (Figs. 2C, 2F) gene expression were measured to quantify ligand activities. Representative photographs of OOX cumulus cells treated with no ligand (Fig. 2G), 30 ng/ml hBMP15 (Fig. 2H), 30 ng/ml mGDF9 (Fig. 2I), 0.3 ng/ml hGDF9:BMP15 (Fig. 2J), and 0.3 ng/ml mGDF9:BMP15 (Fig. 2K) in the presence of EGF (10 ng/ml). Heterodimer dose-dependent effect was also tested in OOX cumulus cell expansion (Figs. 2L, 2M). Figs. 2A-F represent the mean ±SEM (n=3); (L) and (M) represent the mean ±SEM (n=10). $*P<0.05$; $**P<0.01$; $***P<0.001$.

**Receptor Selectivity Assay**

**[0087]** GCs were treated with no ligand, 100 ng hBMP15 or mGDF9, 3 ng hGDF9:BMP15, or 16 ng mGDF9:BMP15, and co-incubated with/without 100 nM LDN-193189, 1 μM SB-505124 (Sigma-Aldrich), or 1 μg BMPR2 extracellular domain (ECD) with immunoglobulin Fc tag. GCs were collected and subjected to RNA extraction after 5 hours treatment. Real-time PCR was conducted to test the fold changes of *Ptx3*, *Has2*, and *Ptgs2*. The extracellular domain of hBMPR2 (27-150) was cloned into the pFUSE-Fc vector (Invivogen).

**[0088]** Recombinant hBMPR2-Fc fusion protein was generated by transient expression in HEK-293 Freestyle cells (Invitrogen) and purified by affinity chromatography using Protein G affinity resin.

**[0089]** Fig. 3 shows the identification of h/mGDF9:BMP15 heterodimer SMAD signaling pathway and potential type 1 and type 2 receptors. After 1 hour treatment with ligands, α-P-SMAD1/5/8 (Fig. 3A) and α-P-SMAD2/3 (Fig. 3B) were used in western blot to detect the two phospho-SMAD signaling pathways in GCs. Actin was used as the internal control. To identify type 1 receptor, ligands were co-incubated with 100 nM LDN-193189 (ALK2/3/6 inhibitor) or 1 μM SB-505124 (ALK4/5/7 inhibitor) to test if the induction of downstream ECM genes *Ptx3* (Figs. 3C, 3F), *Has2* (Figs. 3D, 3G) and *Ptgs2* (Figs. 3E, 3H) were abated compared to controls without inhibitors. To identify type 2 receptor, ligands were co-incubated with 1 μg BMPR2 ECD (with Fc tag) to test if the induction of downstream ECM genes *Ptx3* (Figs. 3I, 3L), *Has2* (Figs. 3J, 3M) and *Ptgs2* (Figs. 3K, 3O) were abated compared to controls without BMPR2 ECD. Figs. 3C-O represent the mean ±SEM (n=3), $*P<0.05$; $**P<0.01$; $***P<0.001$.

**[0090]** Figure 4 is an embodiment of a pathway analysis for mouse and human BMP15 and GDF9 homodimers and heterodimers in GCs. In human (and sheep), GDF9:BMP15 heterodimer likely binds to an ALK4 and BMPR2 receptor complex to transmit a signal through phosphorylation of SMAD2/3, while BMP15 homodimer binds to ALK6 and BMPR2 to slightly increase ECM genes via SMAD2/3 and SMAD1/5/8 pathways (Fig. 4A). In mouse, GDF9 homodimer cooperates with GDF9:BMP15 heterodimer to regulate GC function via SMAD2/3 pathway (Fig. 4B).

**Real-time PCR**

**[0091]** Total RNA of GCs was extracted using RNeasy Micro Kit (Qiagen) according to the manufacturer's protocols. 200 ng total RNA was first converted to cDNA using Superscript III reverse transcriptase (Invitrogen), RNaseOUT (Invitrogen), and Oligo$(dT)_{12-18}$ primers. Gene expression was analyzed by real-time PCR using 7500 Fast Real-time System (Applied Biosystems). Taqman gene expression probes (*Ptx3* Mm00477267_g1, *Has2* Mm00515089_m1, *Ptgs2* mM00478374_m1) were used to test the fold changes of the three transcripts. All real-time PCR analyses were performed in duplicates, and the results were from at least three independent experiments. The relative fold change of transcript was calculated by

the $2^{-\Delta\Delta CT}$ method as described previously (*40*), and normalized to *Gapdh* as an endogenous reference.

**Statistical Analysis**

**[0092]** All experiments presented in this study were repeated at least 3 times independently. Differences among groups were analyzed for statistical significance by using Student's *t* test or one-way ANOVA. The data represent the mean ±SEM, and a *p* value of <0.05 was considered to be statistically significant.

**[0093]** Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the present invention, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the present invention. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

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# CLAIMS

What is claimed is:

1. A method for *in vitro* maturation of oocytes, comprising the step of culturing an oocyte surrounded by pregranulosa cells, granulosa cells, or a mixture thereof in a culture medium comprising GDF9:BMP15 heterodimer.

2. The method of claim 1, wherein the GDF9:BMP15 heterodimer comprises at least one mutated subunit.

3. The method of claim 1, wherein the GDF9:BMP15 heterodimer comprises at least one wild-type subunit.

4. The method of claim 1, wherein the GDF9:BMP15 heterodimer comprises both a wild-type and a mutated subunit.

5. The method of claim 1, wherein the GDF9 subunit and the BMP15 subunits are derived from the same species.

6. The method of claim 1, wherein the GDF9 subunit and the BMP15 subunit are derived from a different species.

7. The method according to claim 1, wherein said oocyte comprises an oocyte at any stage of development.

8. The method of claim 1, wherein the oocyte is at a stage of development prior to metaphase-II.

9. The method according to claim 1, wherein said oocyte is derived from induced pluripotent stem cells (iPSC).

10. The method according to claim 1, wherein said oocyte is derived from embryonic stem cells (ESC).

11. The method according to claim 1, comprising removing said granulosa cells after the oocyte has been cultured.

12. The method according to claim 1, further comprising isolating reproductively competent oocytes from the culture.

13. The method according to claim 1, further comprising fertilizing the oocyte with sperm, to produce a fertilized oocyte.

14. The method according to claim 13, wherein the fertilization occurs before, after, or during culturing.

15. The method according to claim 1, wherein the oocyte is a mammalian oocyte.

16. The method according to claim 15, wherein the oocyte is a human oocyte.

17. The method according to claim 1, wherein the oocyte is an immature oocyte.

18. The method of claim 2, wherein the mutated subunit is the GDF9 protein with a G72R mutation.

19. The method of claim 1, further comprising intracytoplasmic sperm injection.

20. The method of claim 13, wherein the fertilized oocyte is delivered to an individual in need thereof.

21. The method of claim 20, wherein the individual is a mammal.

22. The method of claim 20, wherein the individual is a human, cow, horse, sheep, pig, goat, dog, cat, mouse, or rat.

23. A kit for the *in vitro* maturation of oocytes comprising, a GDF9:BMP15 heterodimer.

24. The kit of claim 23, further comprising an *in vitro* maturation or follicle growth medium.

25. The kit of claim 23, wherein the GDF9:BMP15 heterodimer comprises at least one wild-type subunit.

26. The kit of claim 23, wherein the GDF9:BMP15 heterodimer comprises at least one mutated subunit.

27. The kit of claim 23, wherein the GDF9:BMP15 heterodimer is comprised of subunits from different species.

28. The kit of claim 23, wherein the GDF9:BMP15 heterodimer is comprised of subunits from the same species.

29. The kit of claim 23, wherein the GDF9:BMP15 heterodimer is comprised of one wild-type subunit and one mutated subunit.

30. The kit of claim 23, wherein the mutated subunit is the GDF9 protein with a G72R mutation.

31. The kit of claim 24, wherein the medium comprises epidermal growth factor (EGF), an EGF-like signaling protein, or a mixture thereof.

A

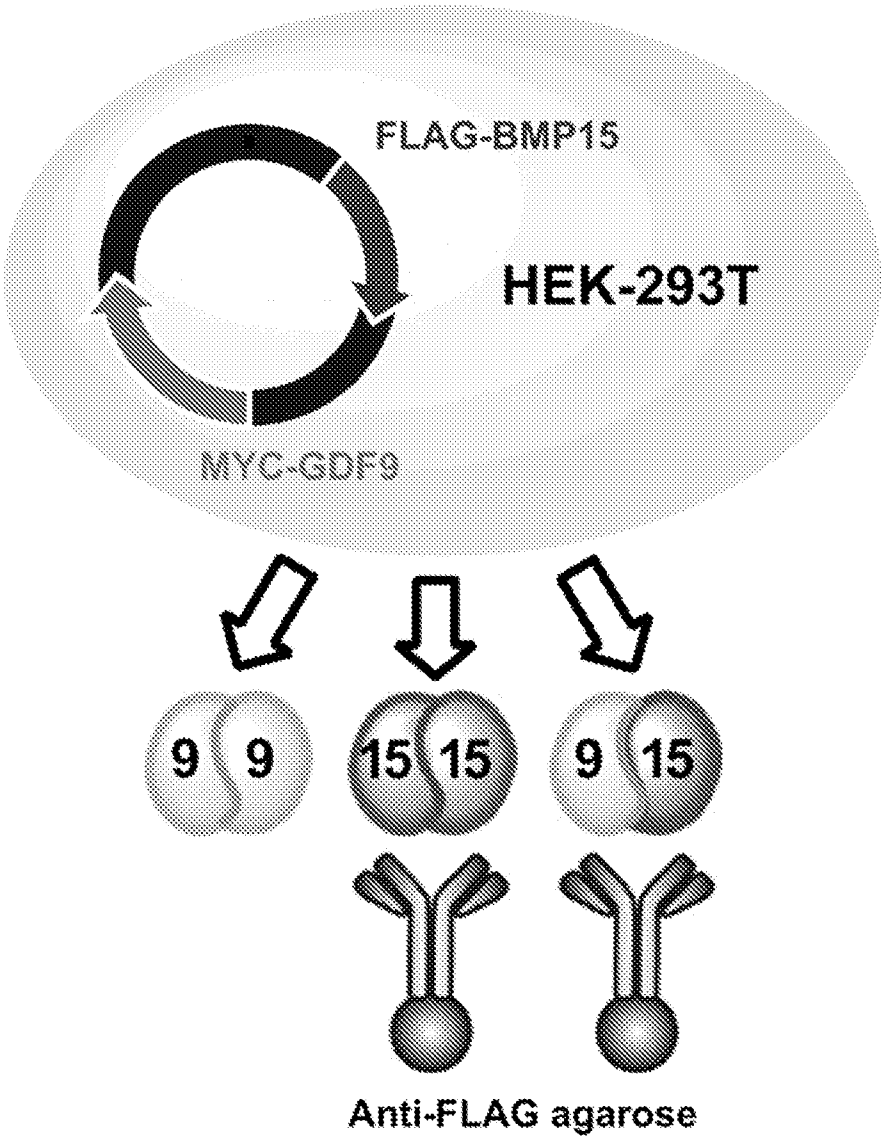
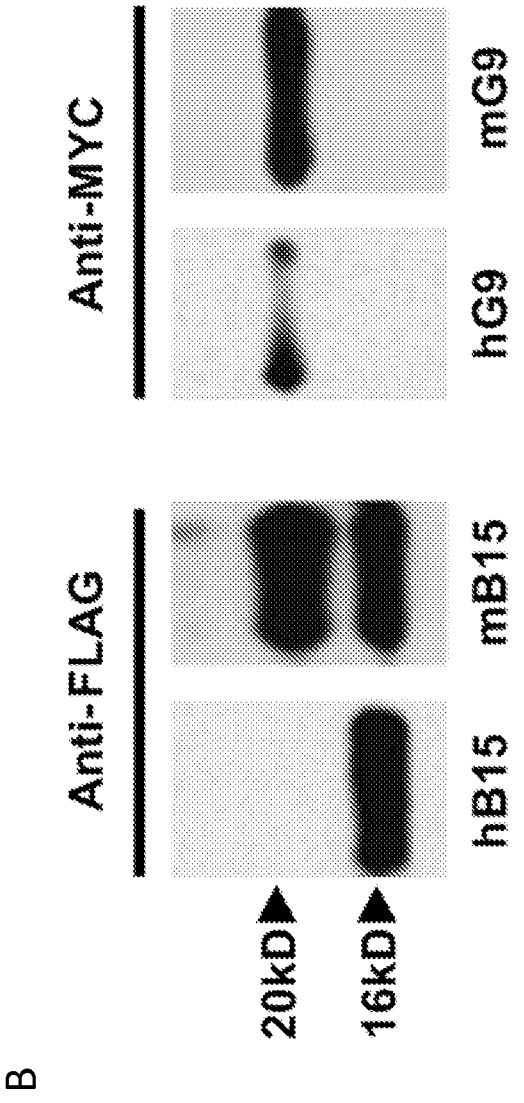



FIG. 1

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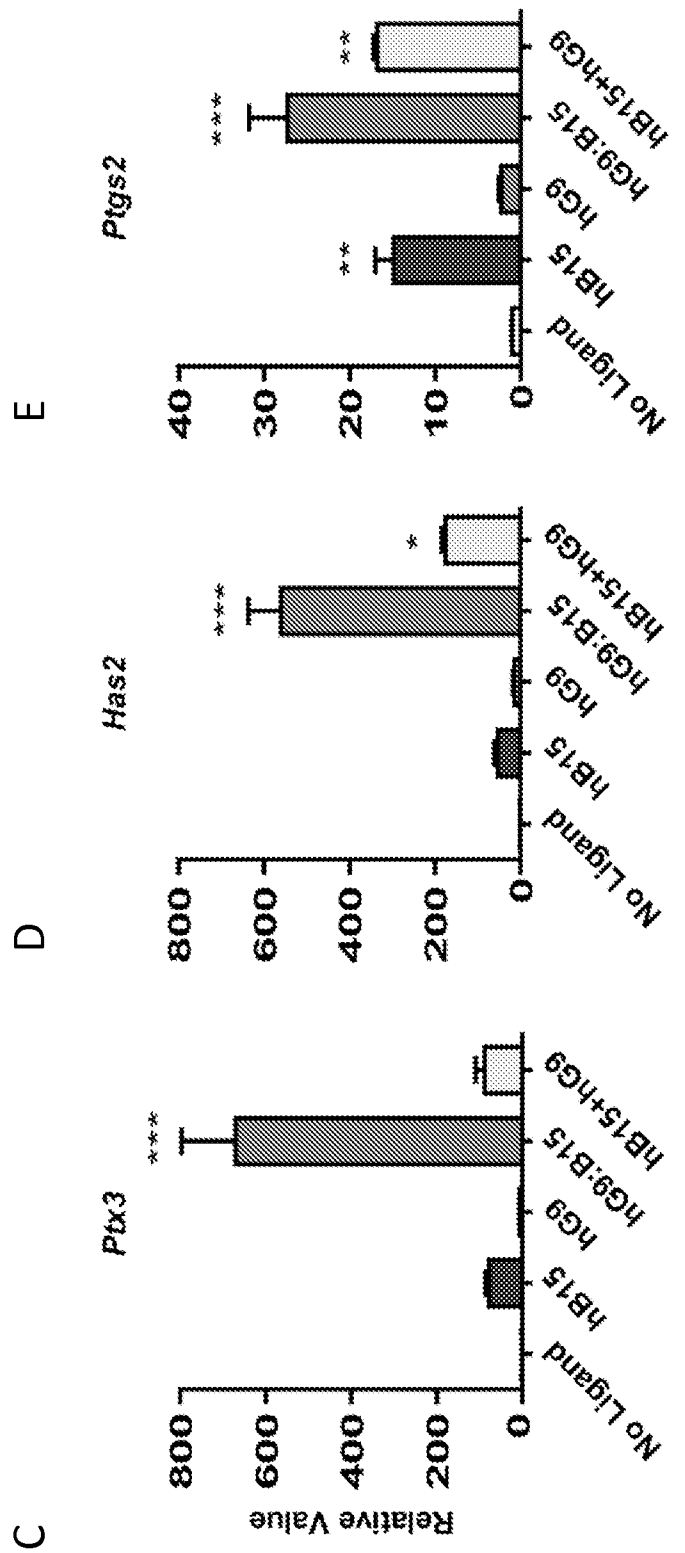


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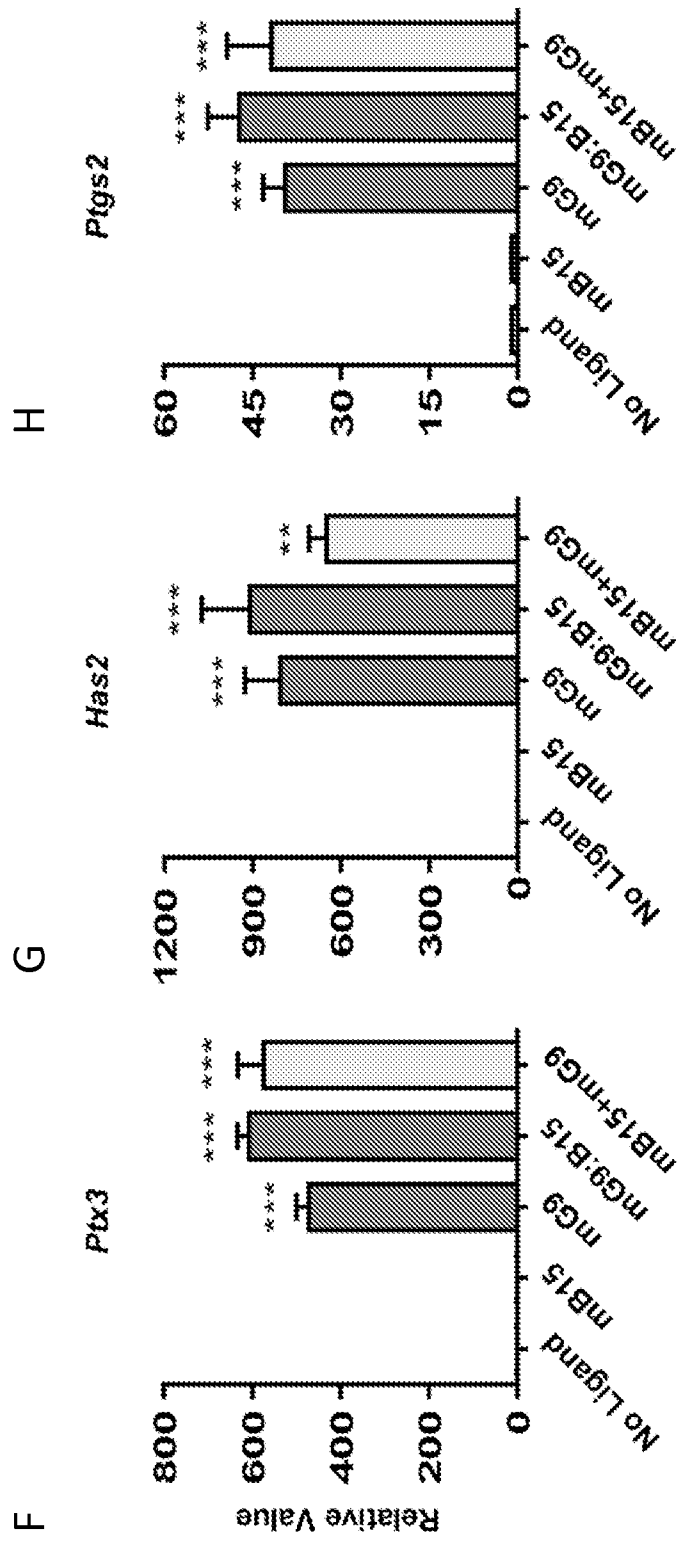


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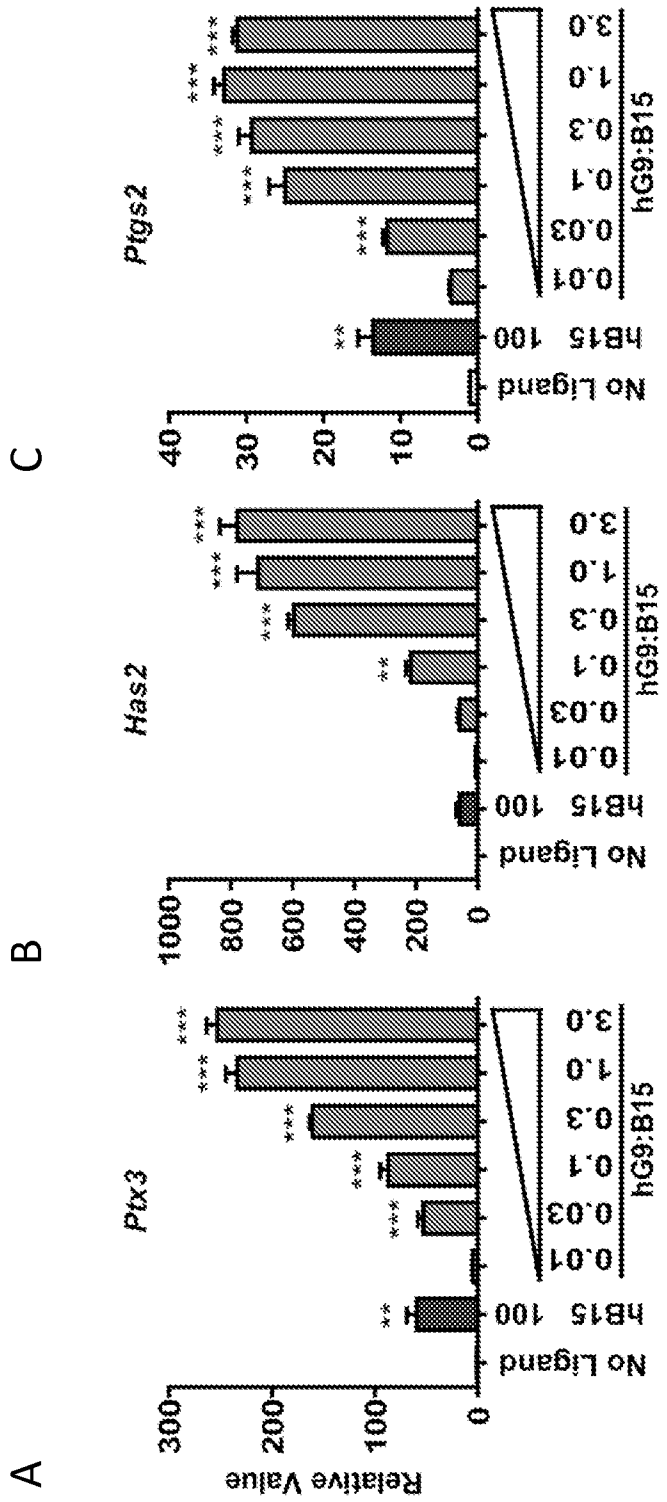
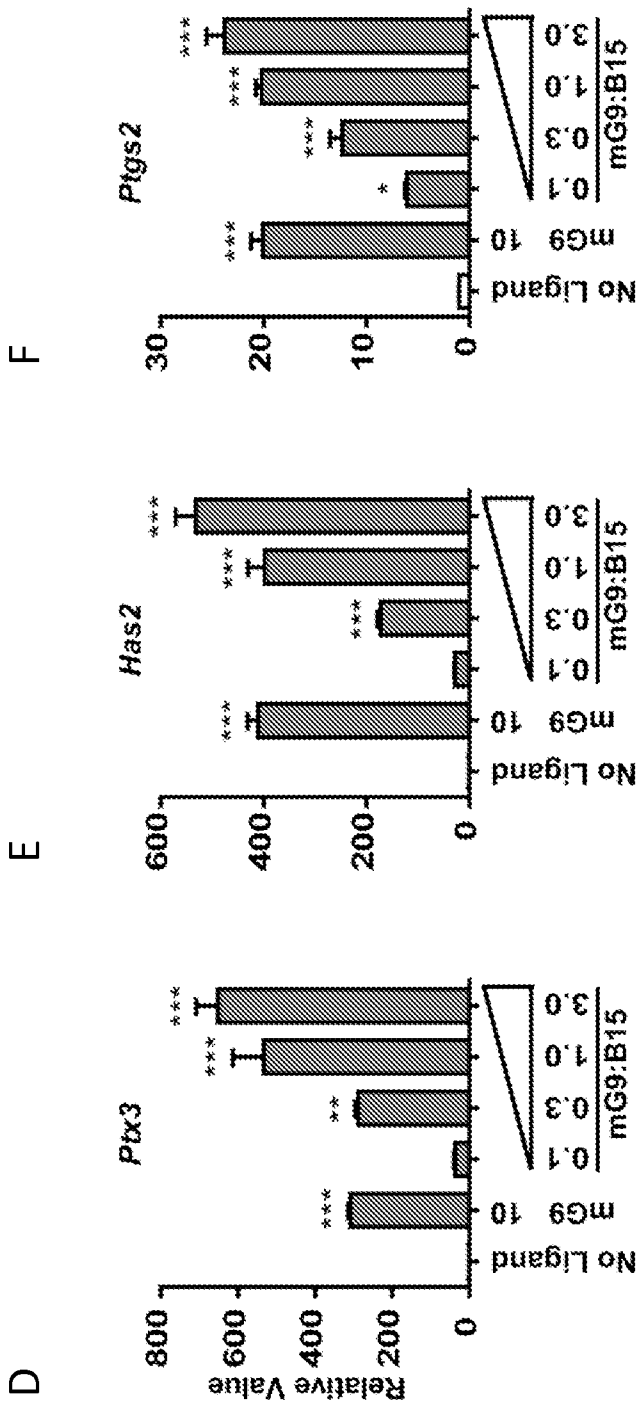

A
B
C
Ptx3
Has2
Ptgs2
Relative Value
300
200
100
0
1000
800
600
400
200
40
30
20
10
No Ligand
hB15 100
hG9:B15
0.01
0.03
0.1
0.3
1.0
3.0
**
***

FIG. 2

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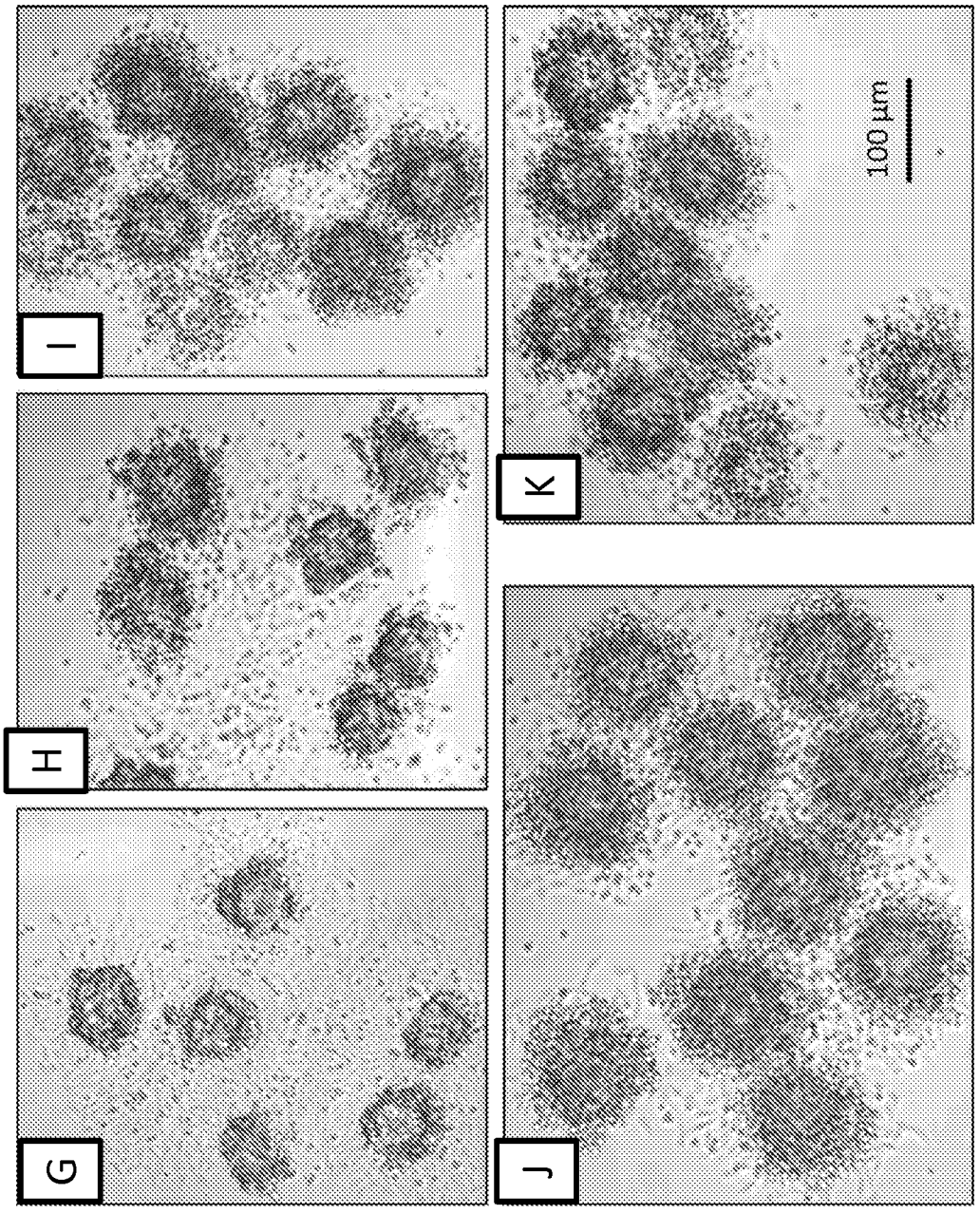


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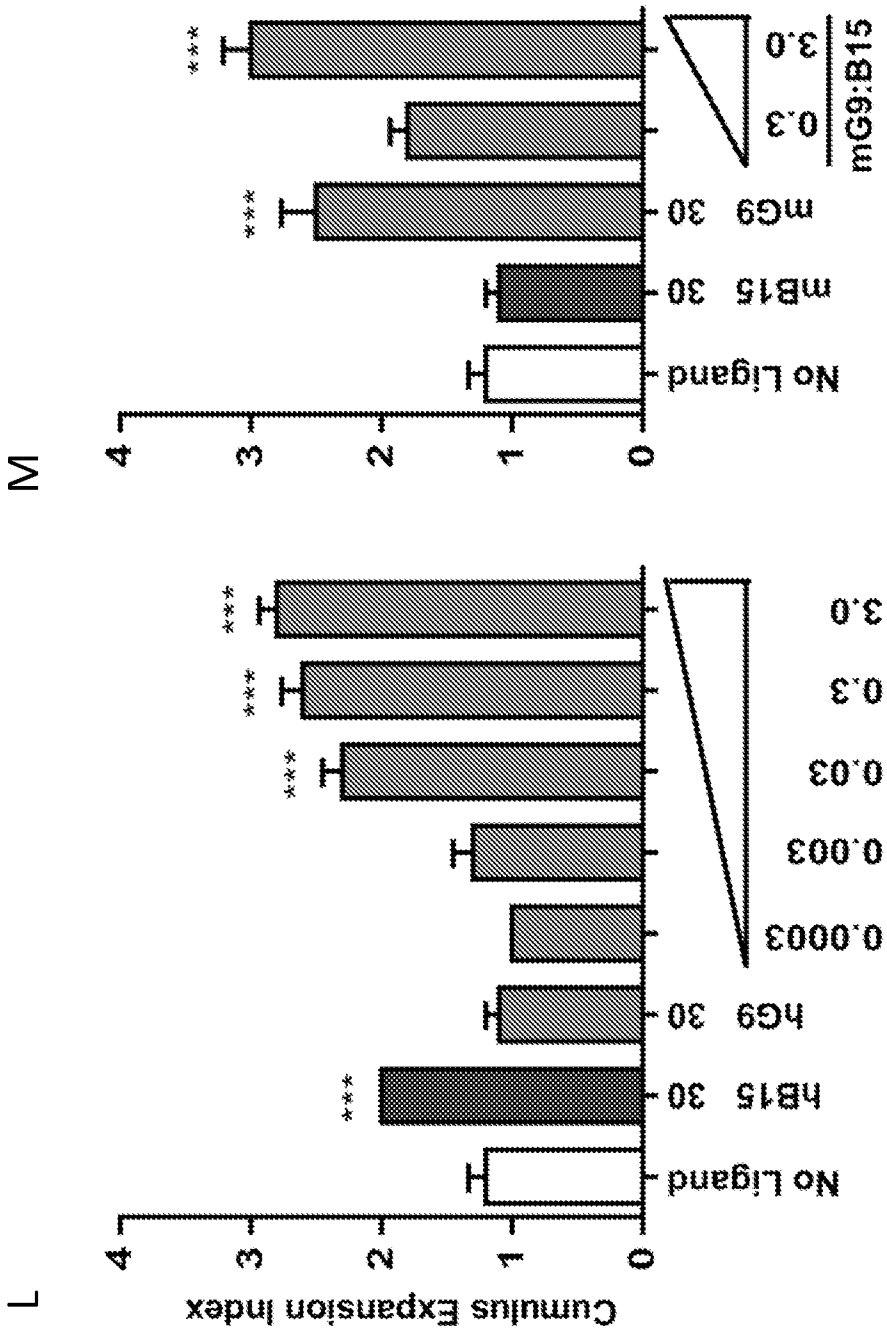


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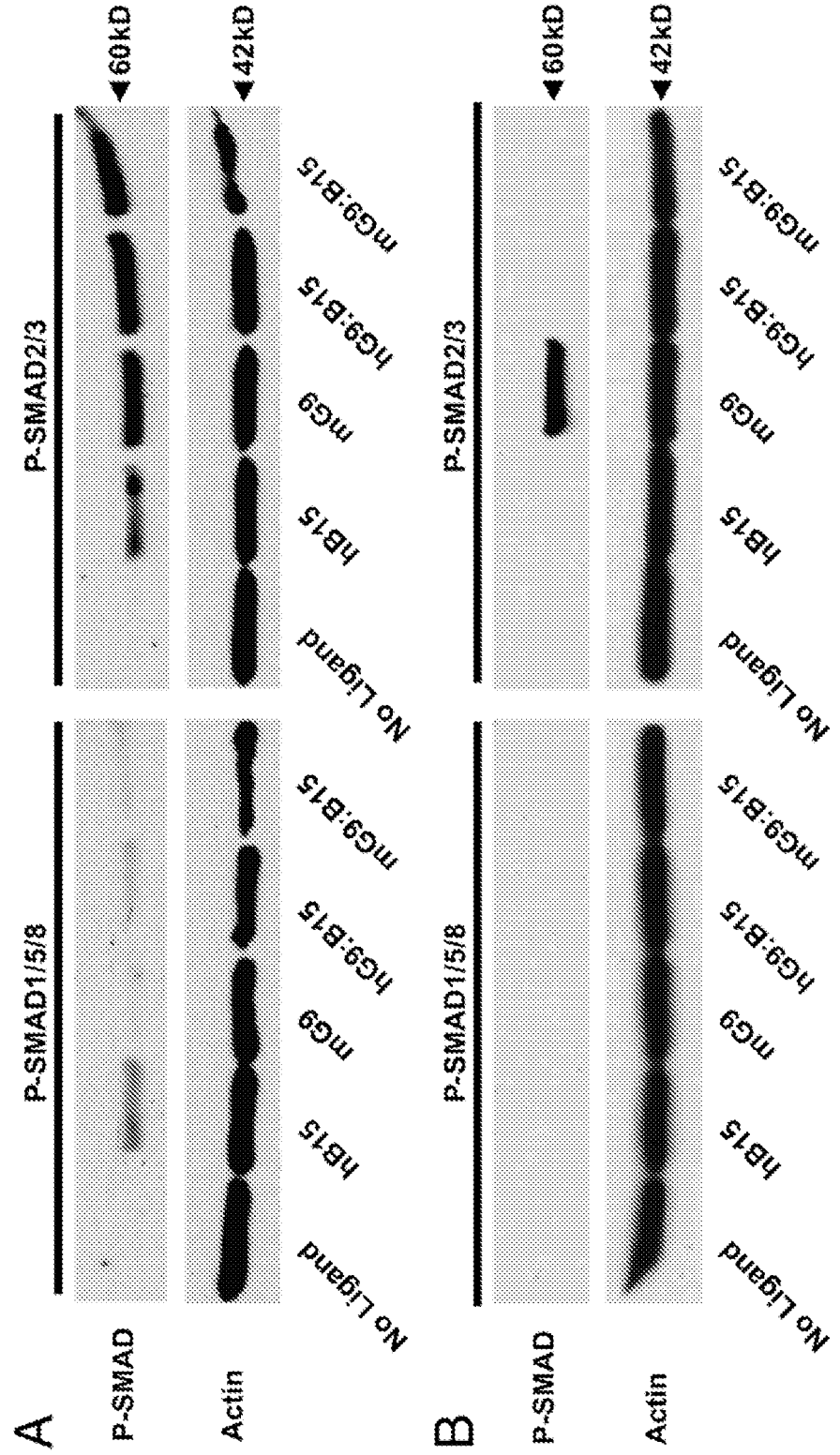
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P-SMAD
Actin
P-SMAD1/5/8
P-SMAD2/3
60kD
42kD
No Ligand
hBMP15
mGDF9
hGDF9:BMP15
mGDF9:BMP15
B
P-SMAD
Actin
P-SMAD1/5/8
P-SMAD2/3
60kD
42kD
No Ligand
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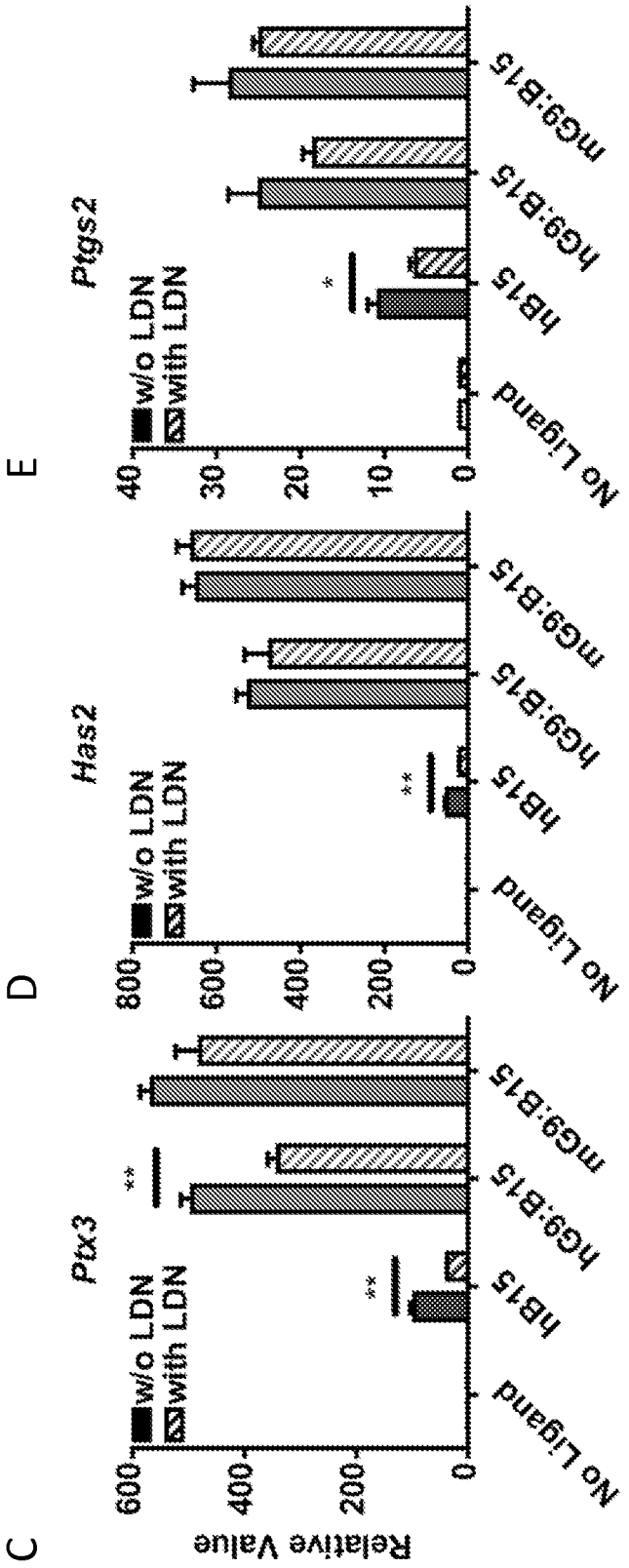
mGDF9
hGDF9:BMP15
mGDF9:BMP15

FIG. 3

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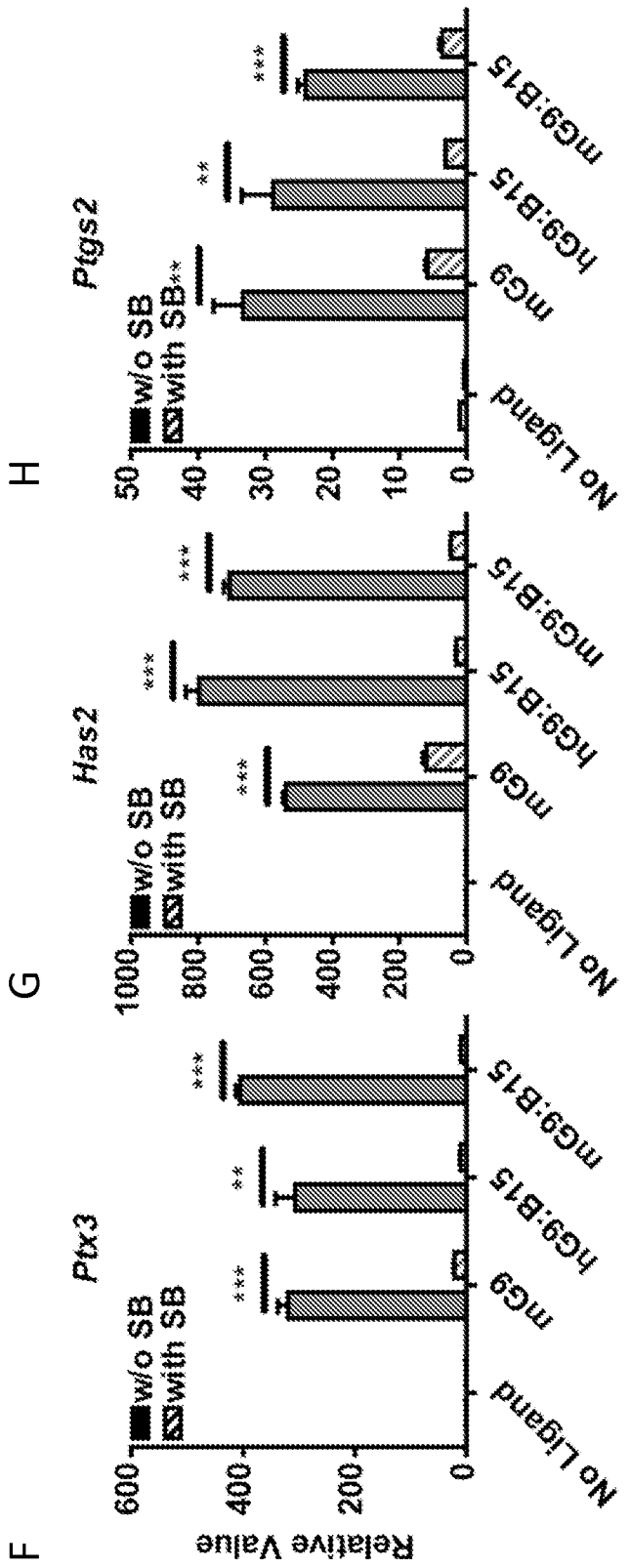


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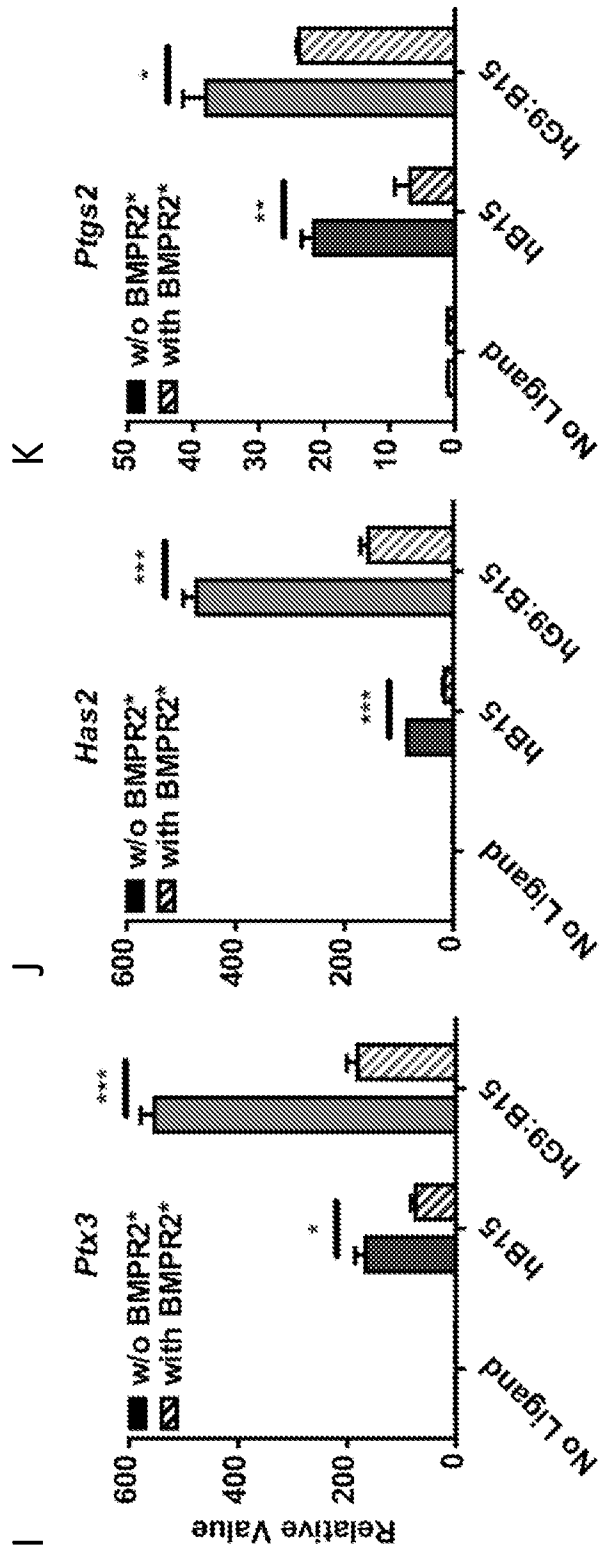


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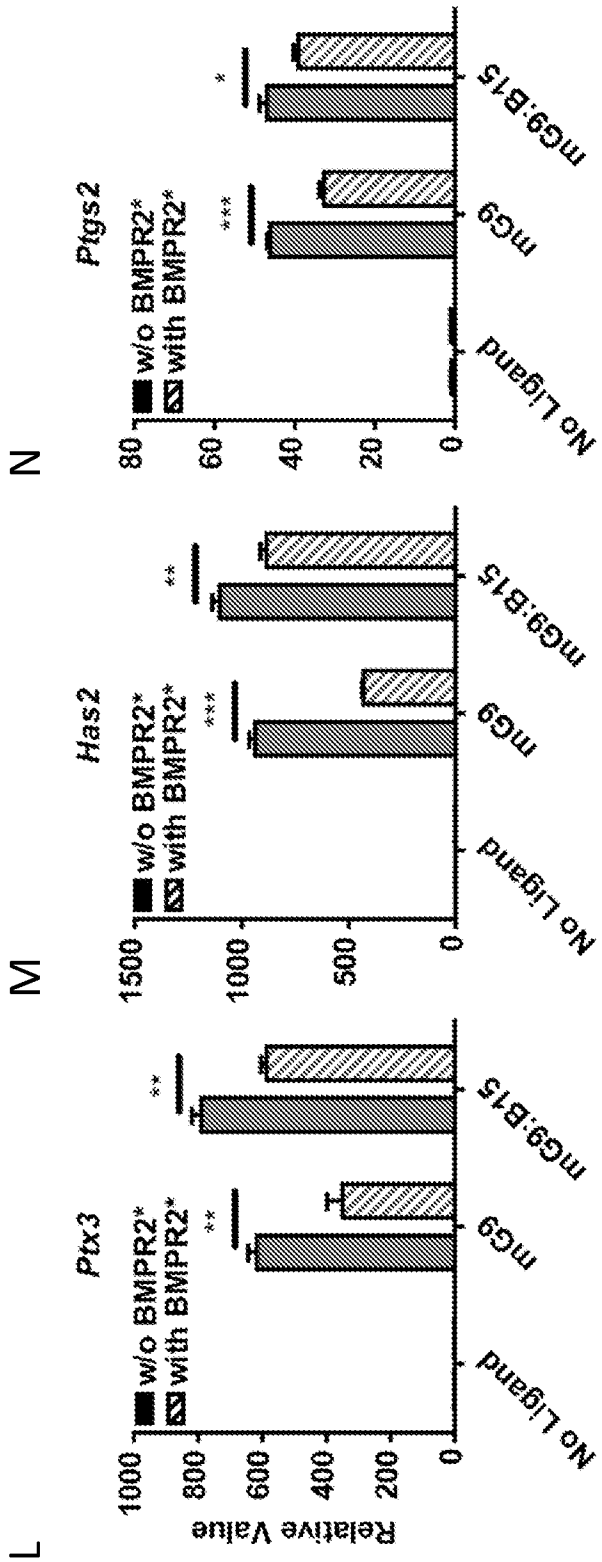


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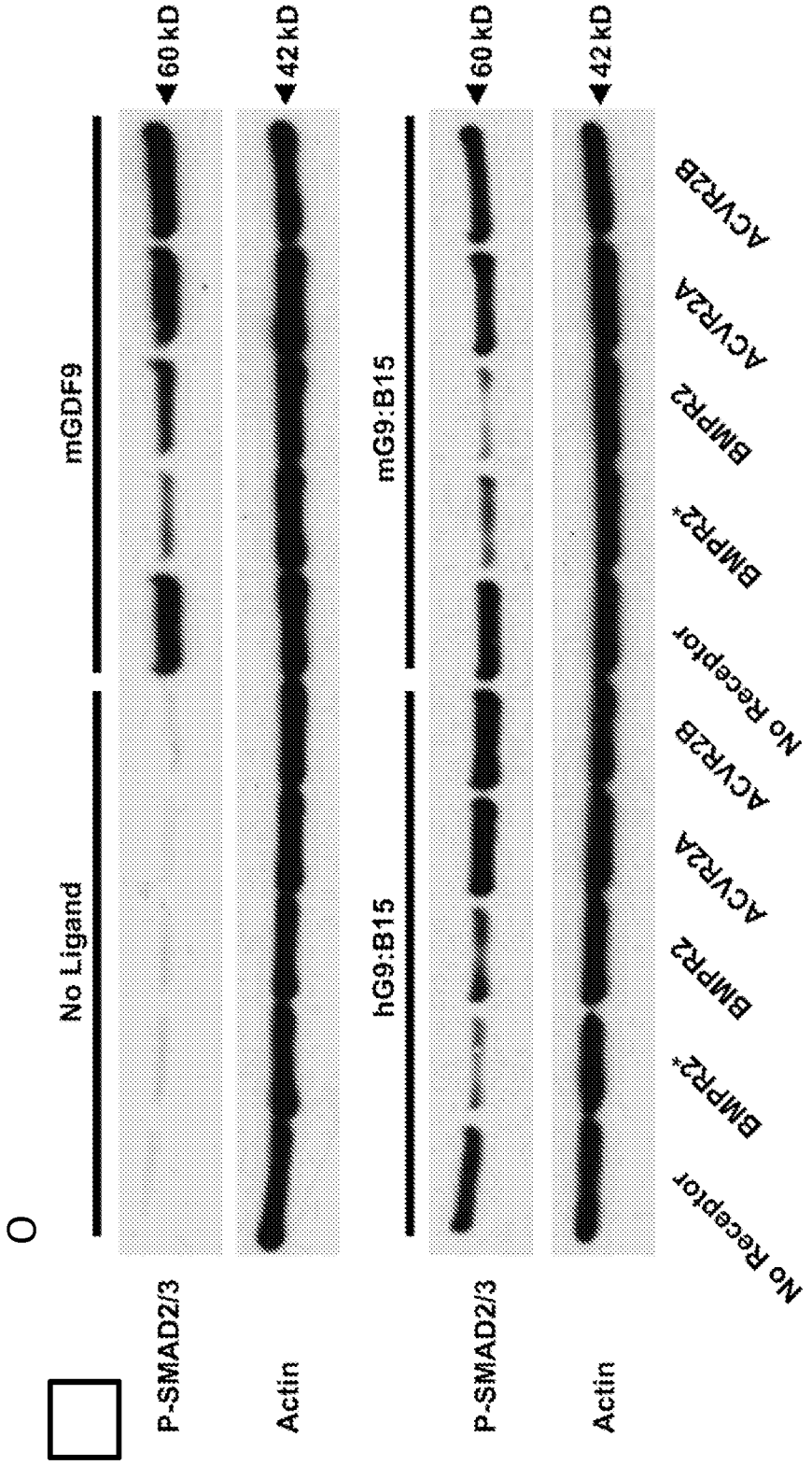


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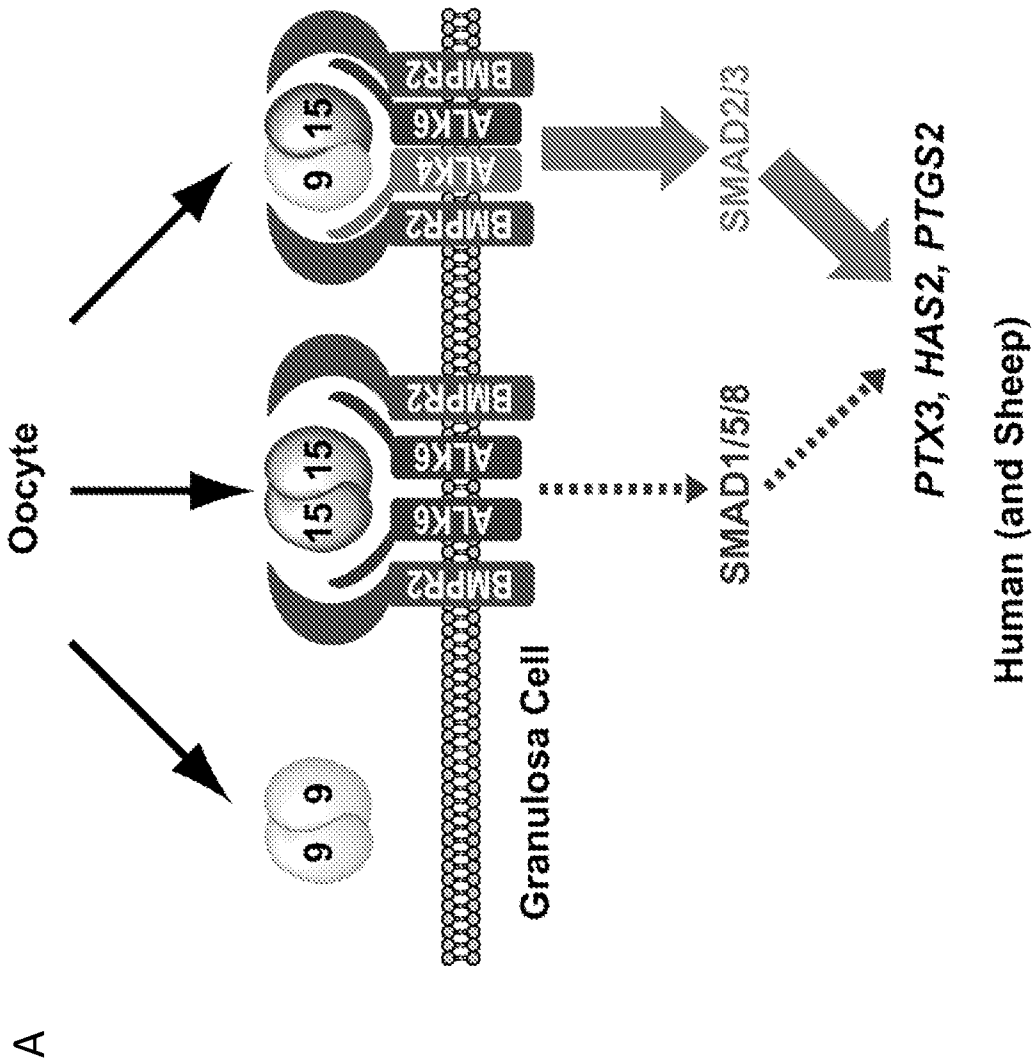

A
Oocyte
9
9
15
15
9
15
BMPR2
ALK6
ALK4
BMPR2
BMPR2
ALK6
ALK6
BMPR2
Granulosa Cell
SMAD1/5/8
SMAD2/3
PTX3, HAS2, PTGS2
Human (and Sheep)

FIG. 4

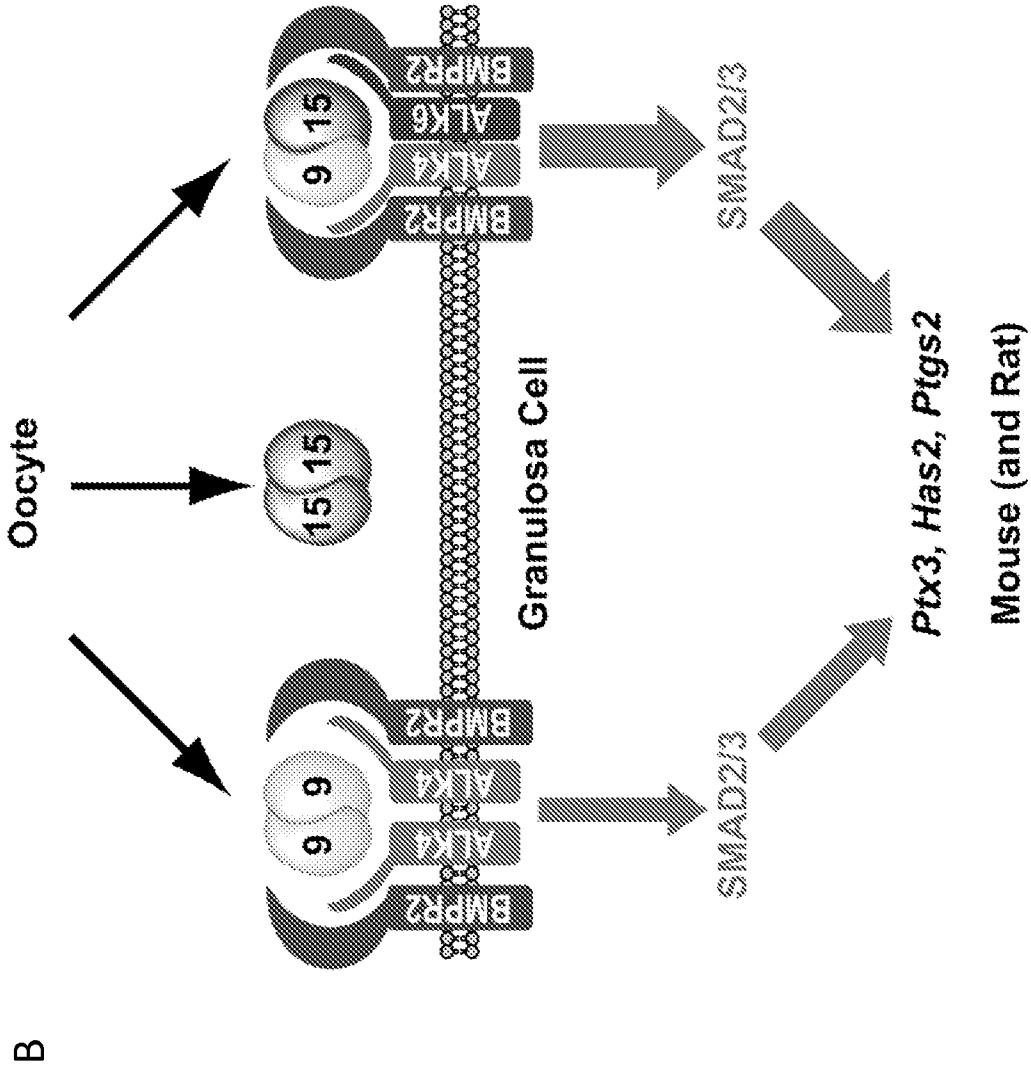
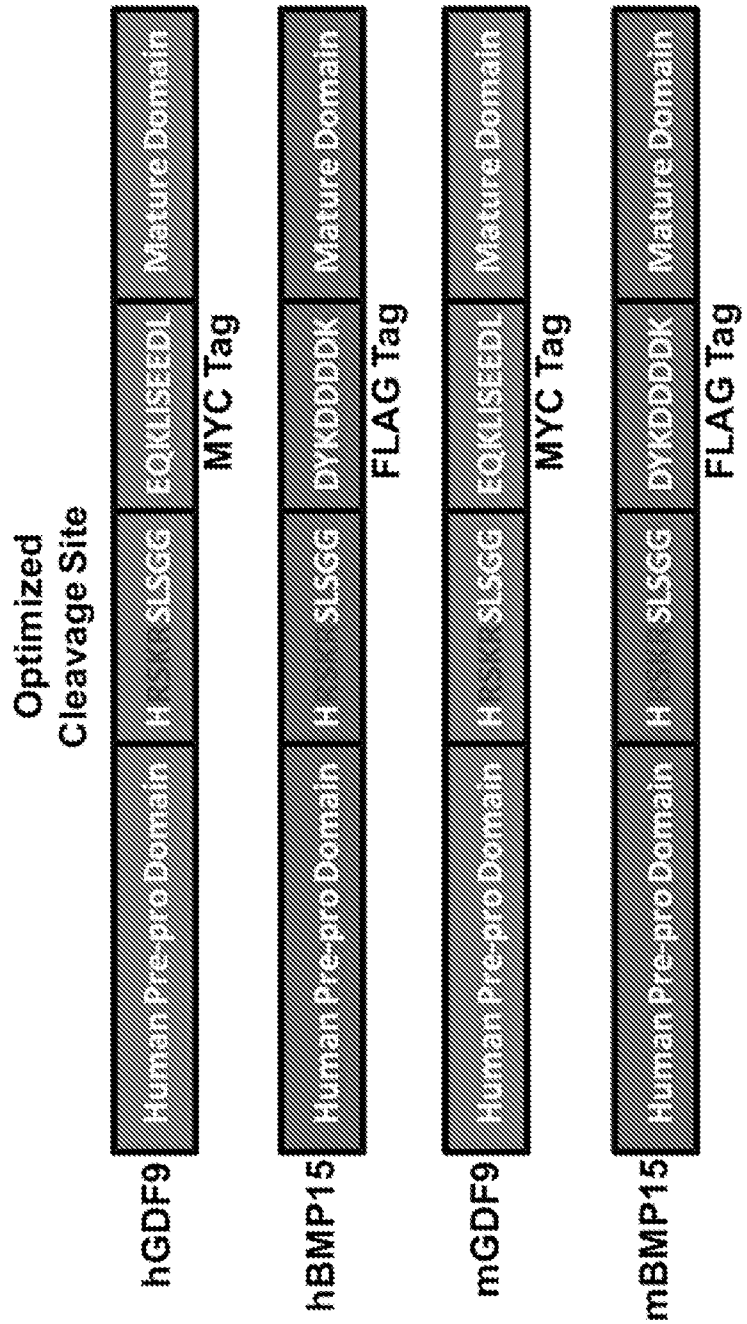



FIG. 4 (continued)


Optimized Cleavage Site
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Human Pre-pro Domain
H
SLSGG
EQKLISEEDL
Mature Domain
MYC Tag
hBMP15
Human Pre-pro Domain
H
SLSGG
DYKDDDDK
Mature Domain
FLAG Tag
mGDF9
Human Pre-pro Domain
H
SLSGG
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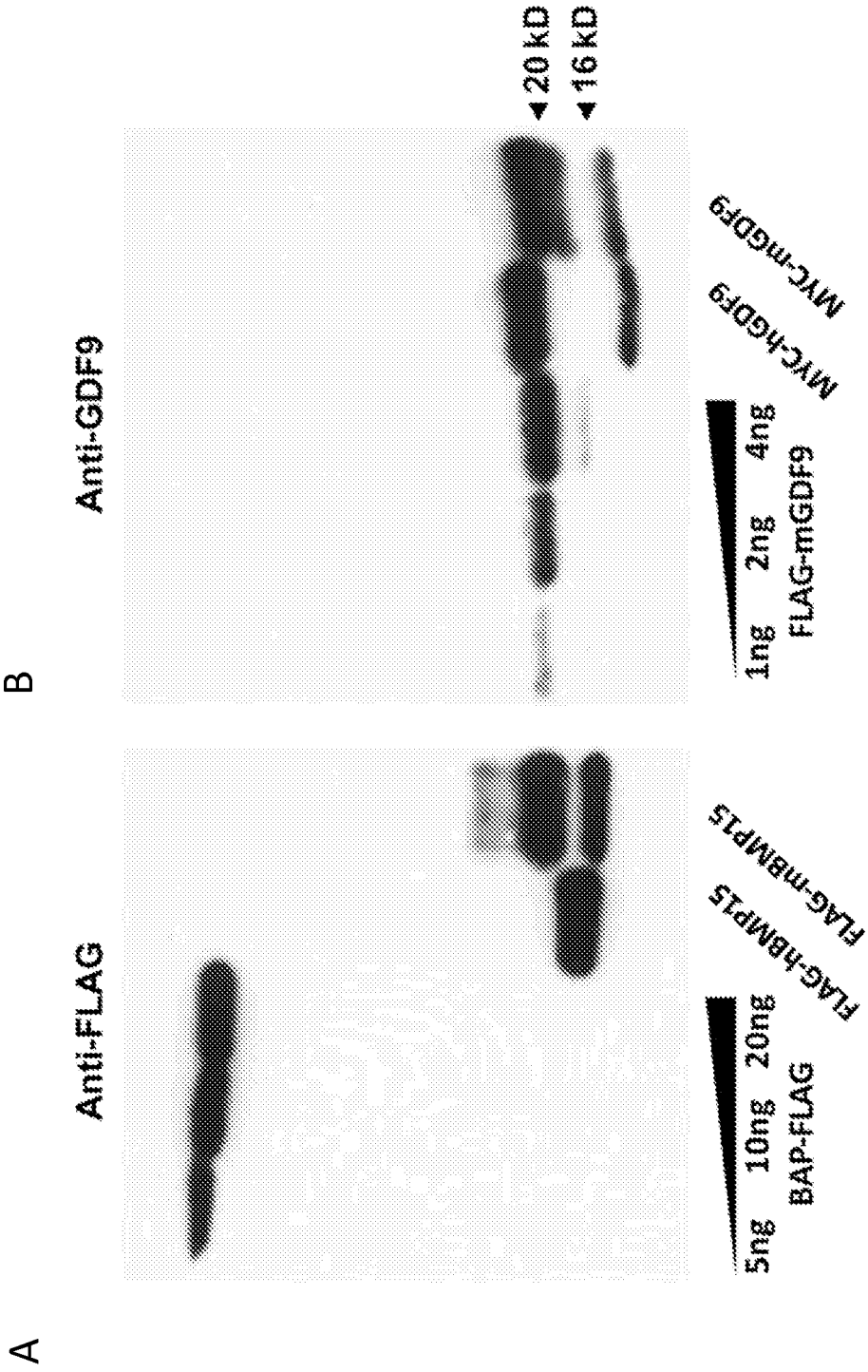
Mature Domain
FLAG Tag

FIG. 5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Anti-FLAG
5ng 10ng 20ng
BAP-FLAG
FLAG-hBMP15
FLAG-mBMP15
B
Anti-GDF9
1ng 2ng 4ng
FLAG-mGDF9
MYC-hGDF9
MYC-mGDF9
20 kD
16 kD

FIG. 6

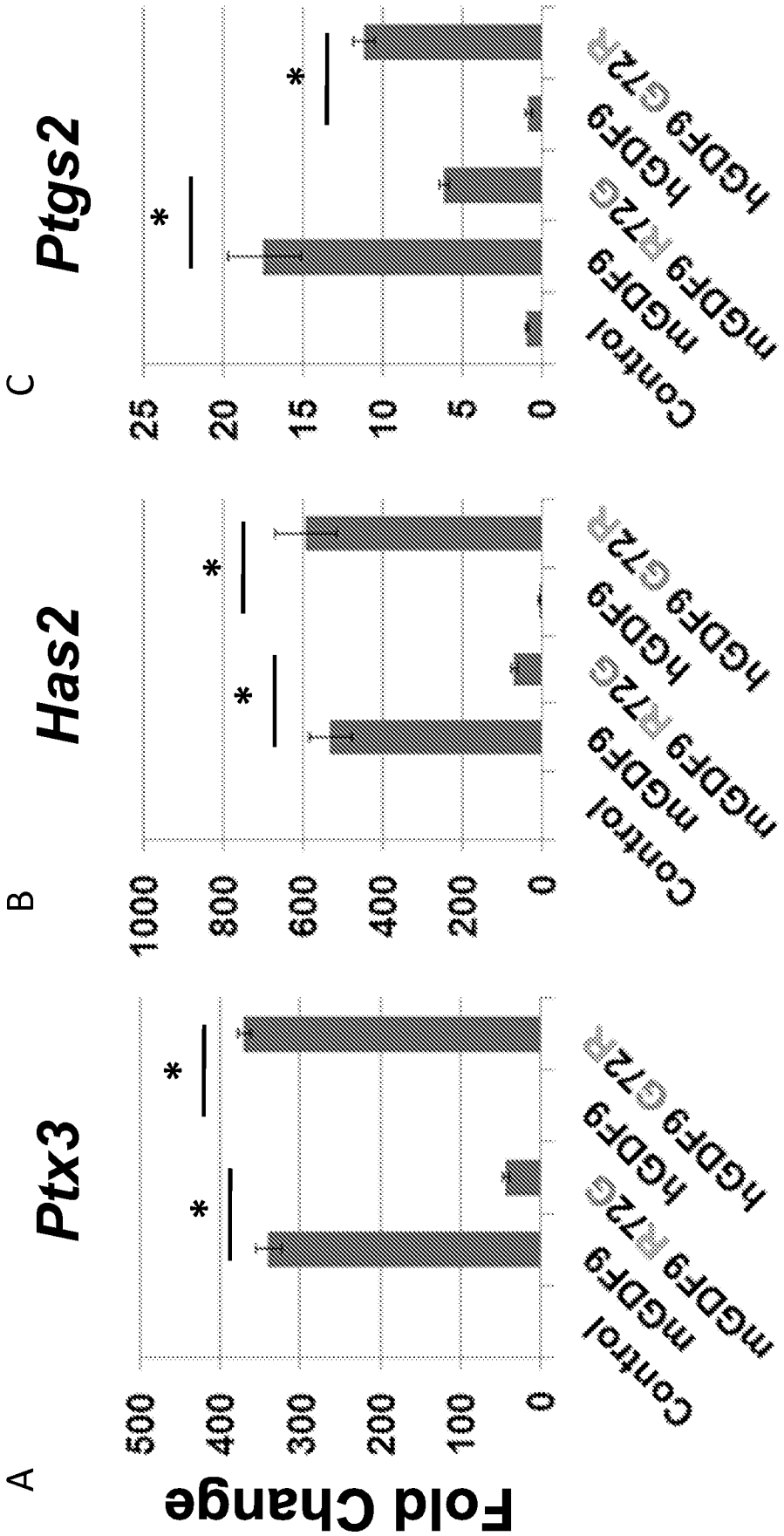
A
Ptx3
Fold Change
500
400
300
200
100
0
*
*
B
Has2
1000
800
600
400
200
0
*
*
C
Ptgs2
25
20
15
10
5
0
*
*
Control
mGDF9
mGDF9 R72G
hGDF9
hGDF9 G72R

Figure 7

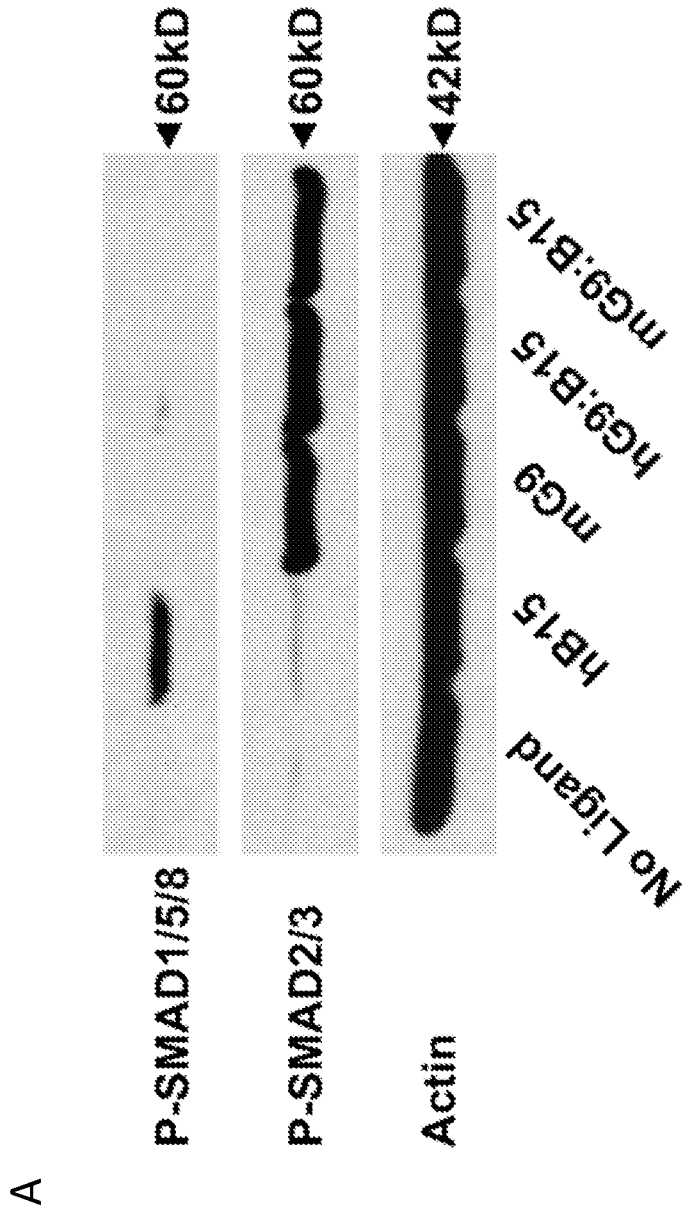
A
P-SMAD1/5/8
P-SMAD2/3
Actin
60kD
60kD
42kD
No Ligand
hB15
mG9
hG9:B15
mG9:B15

FIG. 8

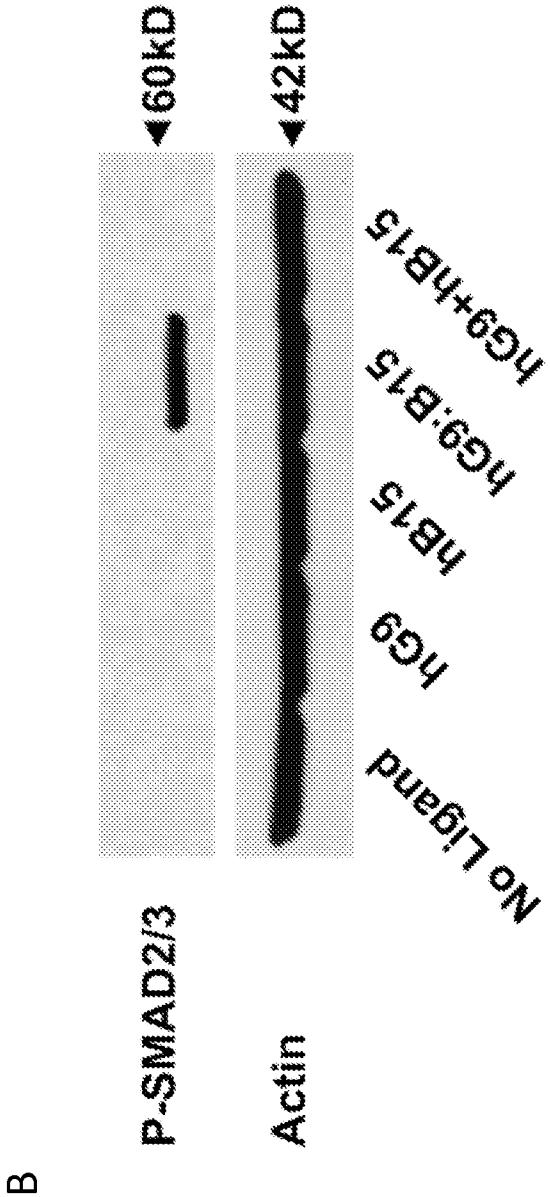


FIG. 8 (continued)

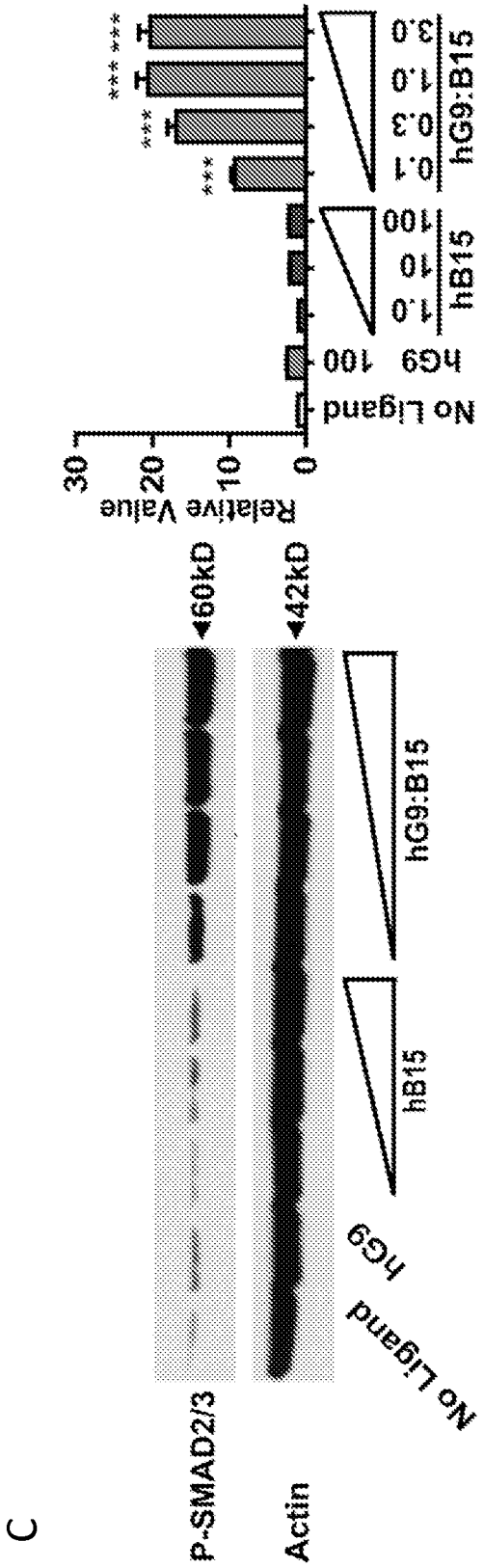


FIG. 8 (continued)

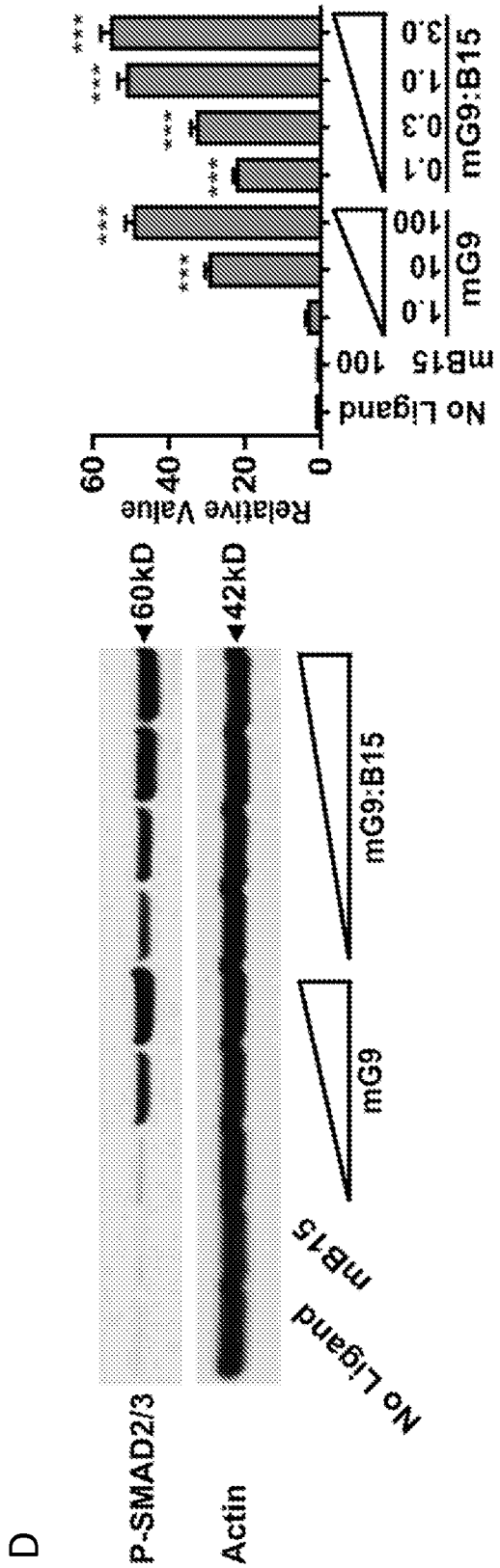


FIG. 8 (continued)

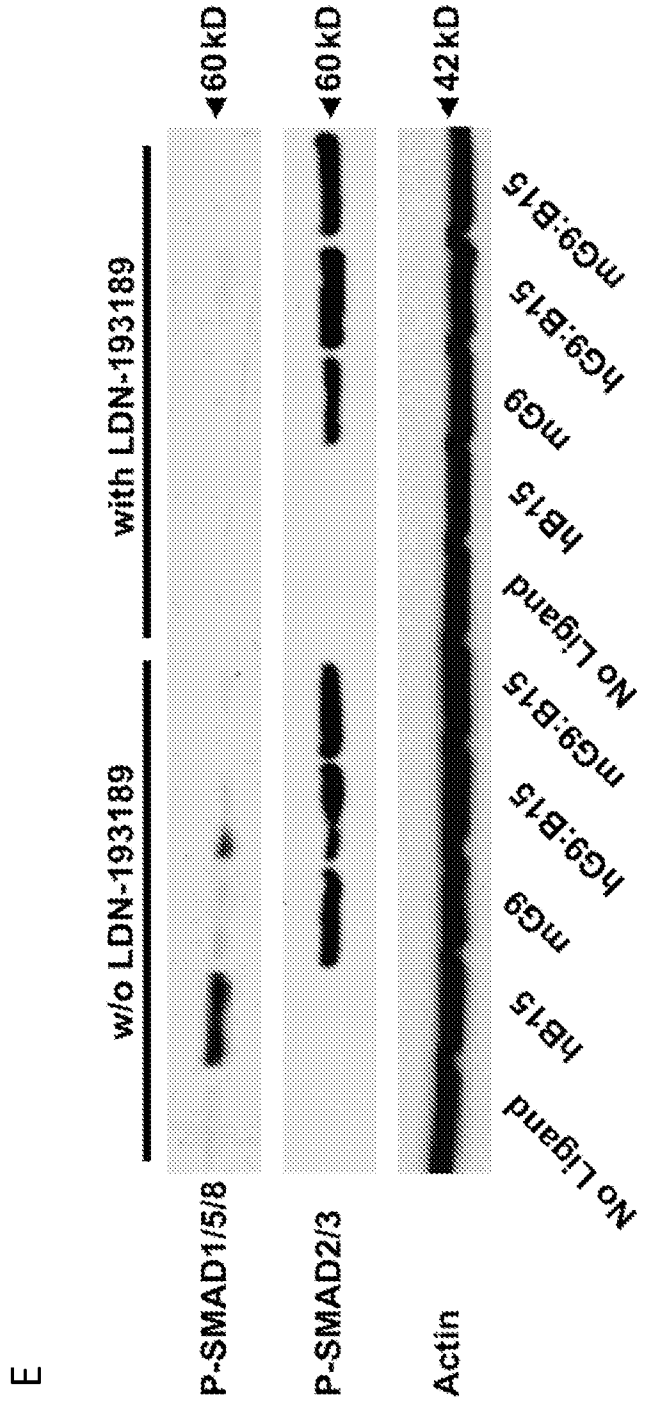


FIG. 8 (continued)

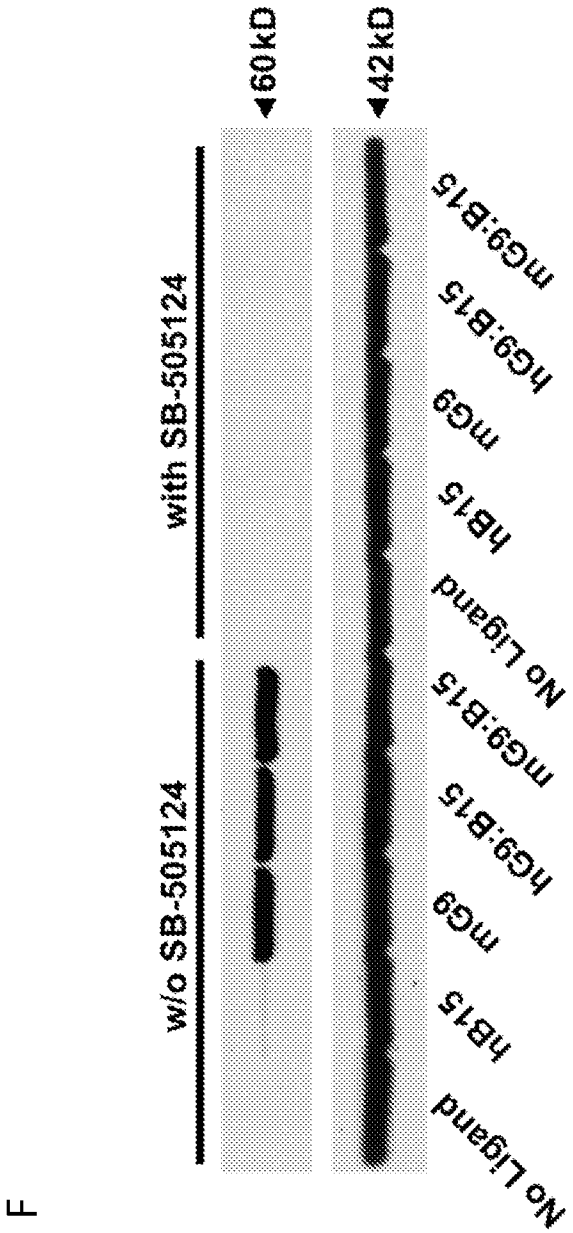


FIG. 8 (continued)

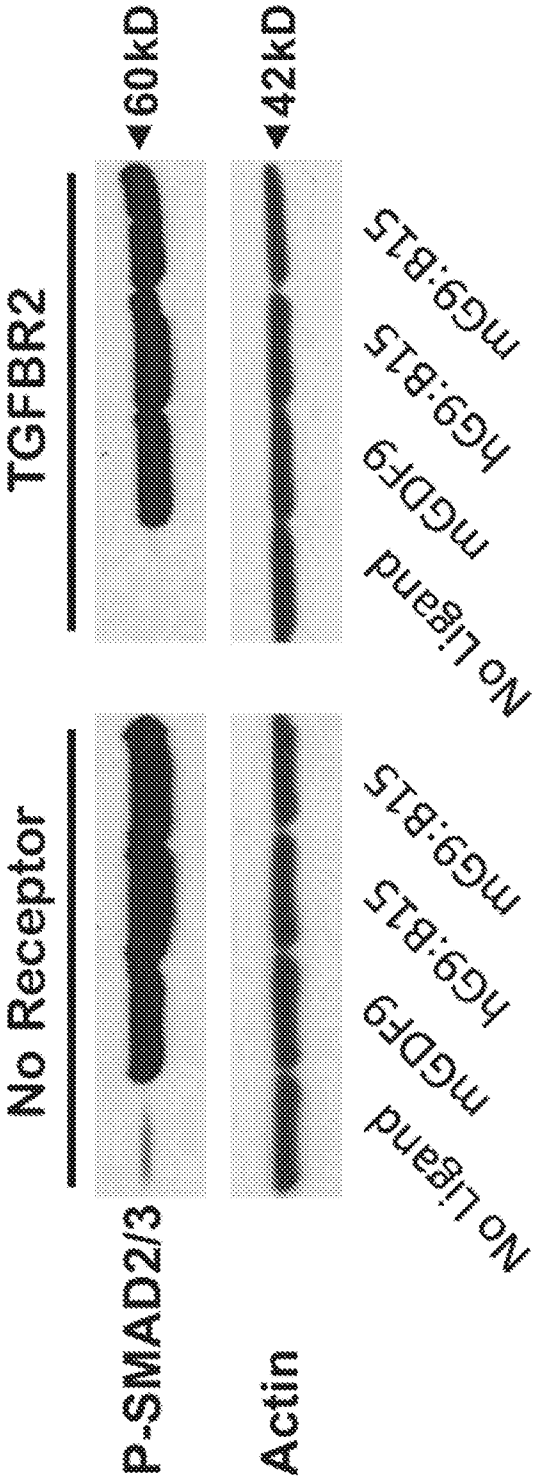
TGFBR2
No Receptor
60kD
42kD
P-SMAD2/3
Actin
No Ligand
mGDF9
hGg:BI5
mGg:BI5

FIG. 9

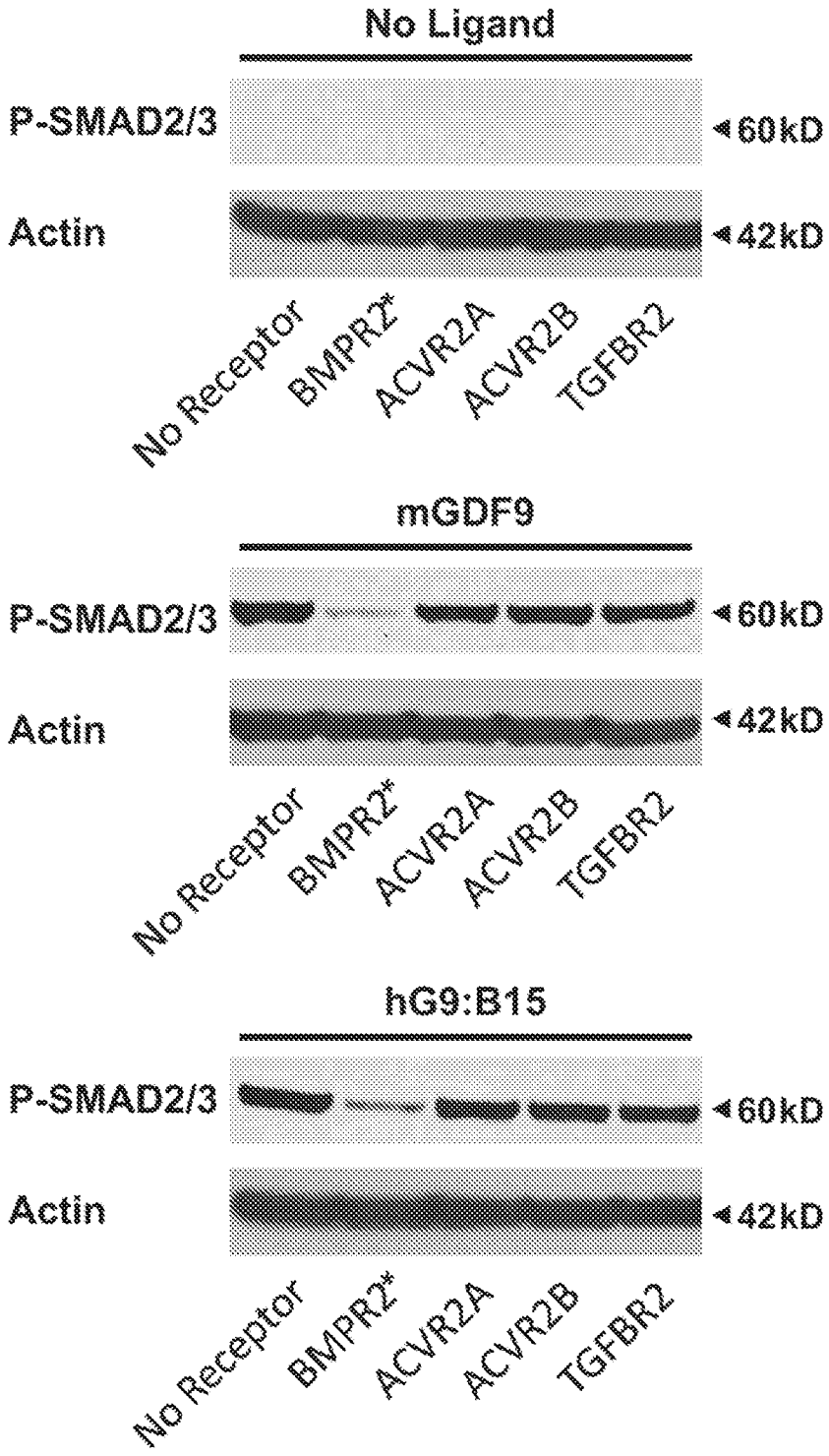
No Ligand
P-SMAD2/3
60kD
Actin
42kD
No Receptor
BMPR2*
ACVR2A
ACVR2B
TGFBR2
mGDF9
P-SMAD2/3
60kD
Actin
42kD
No Receptor
BMPR2*
ACVR2A
ACVR2B
TGFBR2
hG9:B15
P-SMAD2/3
60kD
Actin
42kD
No Receptor
BMPR2*
ACVR2A
ACVR2B
TGFBR2

FIG. 10

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/51314

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/17, C12N 5/02, C12N 5/075 (2013.01)
USPC - 435/375, 435/387
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 38/17, C12N 5/02, C12N 5/075 (2013.01)
USPC:435/375, 435/387

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/375, 435/387; 435/1.1, 435/325, 435/377, 435/406

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, Google Web, search terms: GDF9:BMP15 heterodimer, in vitro oocyte maturaton, GDF9 or BMP15, human stem cell into oocyte kit, oocyte from ESC, GDF9 mutant subunit, GDF9 G72R mutant, metaphase-II, interspecies GDF9 BMP15, iPSC into oocyte, embryoid bodies, pregranulosa, granulosa, in vitro oocyte maturaton

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>Y<br>---<br>A | US 2008/0274963 A1 (GILCHRIST et al.) 6 November 2008 (06.11.2008) para [0004], [0021], [0065], [0067], [0147], [0149], [0176], [0182], [0199], [0218], [0230], [0273], [0275], [0326]-[0328], [0330]-[0332], [0339], [0489], [0490], [0499], [0562], [0660], [0790], [0791], [0828], [0829], [0876], [0909], [0924], [0926], [0927 | 1-8,11-17,19-29, 31<br>---<br>9, 10<br>---<br>18, 30 |
| Y | WO 2011/050251 A1 (KEE et al.) 28 April 2011 (28.04.2011) para [0004], [0056], [0057], [0085], [0086] | 9,10 |
| A | WO 2003/102199 A1 (DAVIS et al.) 11 December 2003 (11.12.2003) Figure 1 | 18, 30 |
| A | LAISSUE et al., Mutations and sequence variants in GDF9 and BMP15 in patients with premature ovarian failure. Eur J Endocrinol, 1 May 2006, vol 154, no 5, pp 739-744. Abstract | 18, 30 |

☒ 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 |
|---|---|
| 28 October 2013 (28.10.2013) | 22 NOV 2013 |
| Name and mailing address of the ISA/US<br>Mail Stop PCT, Attn: ISA/US, Commissioner for Patents<br>P.O. Box 1450, Alexandria, Virginia 22313-1450<br>Facsimile No. 571-273-3201 | Authorized officer:<br>Lee W. Young<br>PCT Helpdesk: 571-272-4300<br>PCT OSP: 571-272-7774 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/51314

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | Hanrahan et al. 'Mutations in the Genes for Oocyte-Derived Growth Factors GDF9 and BMP15 Are Associated with Both Increased Ovulation Rate and Sterility in Cambridge and Belclare Sheep (Ovis aries)' BIOLOGY OF REPRODUCTION 70, 900-909 (2004); abstract, Table 1 | 18, 30 |
| A | Palmer et al. 'Novel Variants in Growth Differentiation Factor 9 in Mothers of Dizygotic Twins' J Clin Endocrinol Metab 91: 4713-4716, (2006) abstract, Table 1 | 18, 30 |
| A | Zhao et al. 'GDF9 Mutation Analyses in 100 Chinese Women with Premature Ovarian Failure (POF)' Fertil Steril. 2007 November ; 88(5): 1474-1476; abstract, Table 1 | 18, 30 |