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Growth hormone treatment of premature ovarian failure in a mouse model via stimulation of the Notch-1 signaling pathway

TE LIU[1,2*], SUWEI WANG[2*], LINA ZHANG[1], LIHE GUO[3], ZHIHUA YU[2], CHUAN CHEN[2] and JIN ZHENG[1]

[1]Department of Gynecology; [2]Shanghai Geriatric Institute of Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine; [3]Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, P.R. China

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Abstract. Premature ovarian failure (POF) is a condition affecting 1% of women in the general population, causing amenorrhea, hypergonadotropism and hypoestrogenism before the age of 40. Currently, POF cannot be reversed and, although treatments are available, there is an urgent need for improved treatment strategies. Growth hormone (GH) is a pleiotropic hormone that affects a broad spectrum of physiological functions, from carbohydrate and lipid metabolism to the immune response. GH has previously been used to treat POF in non-transgenic preclinical trials, but the biochemical mechanism underlying these effects are unclear. In the present study, a mouse model of POF was generated using cyclophosphamide. Treatment of POF mice with recombinant mouse growth hormone (rmGH) was revealed to markedly reduce POF histopathology in ovarian tissue, relieve ovarian granulosa cell injury, reduce the number of atretic follicles and significantly increase the number of mature oocytes. Furthermore, an enzyme-linked immunosorbent assay revealed that plasma estradiol levels increased and plasma follicle stimulating hormone levels decreased with time in a group of mice treated with a medium dose of rmGH (0.8 mg/kg) when compared with the POF model group ($P<0.05$). In addition, reverse transcription-quantitative polymerase chain reaction and immunohistochemical analysis demonstrated elevated levels of Notch-1 signaling pathway factors (Notch1, CBF1, and HES1) in wild-type mice and those treated with medium and high doses of rmGH, but not in those treated with low doses of rmGH. In conclusion, GH may promote ovarian tissue repair, estrogen release and oocyte maturation via activation of the Notch-1 signaling pathway in ovarian tissue.

Correspondence to: Professor Jin Zheng, Department of Gynecology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, 725 Wanpin Road, Shanghai 200031, P.R. China
E-mail: jinzheng2013@126.com

Professor Chuan Chen, Shanghai Geriatric Institute of Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, 365 Xiangyang Road, Shanghai 200031, P.R. China
E-mail: ch9453@126.com

*Contributed equally

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Introduction

Premature ovarian failure (POF) is a condition that causes amenorrhea and hypergonadotropic hypoestrogenism before the age of 40 (1-5). POF affects 1% of women in the general population, but its prevalence is steadily increasing (1-5). Patients with POF demonstrate a number of characteristic symptoms, as follows: i) Primary or secondary amenorrhea (2,6-8); ii) intermittent or chronic hypoestrogenism (2,6); and iii) hypergonadotropism (7,8). Furthermore, the age of patients at the time of onset is typically under 40 years of age (3-6). In a number of reports describing patients with POF, laparoscopy has revealed an absence of developing follicles, and ovarian biopsies have demonstrated a network of connective tissue interspersed with fibroblasts. Previous studies have reported that the uterus and vaginal mucosa in patients with POF undergo atrophy due to estrogen understimulation as a result of inactive ovaries (4,9). Currently, POF is irreversible and, although treatments are available, drugs to treat POF are very limited; there are no particularly effective treatments and drugs. Hormonal therapy and *in vitro* fertilization methods are available to help patients with POF conceive, but there is an urgent requirement for improved treatment strategies (5-7,9).

Growth hormone (GH) is a pleiotropic hormone that affects a broad spectrum of physiological functions, from carbohydrate and lipid metabolism to immune response (10-16). Several previous studies have used GH to treat autoimmune diseases and POF. For example, Rojanathammanee *et al* (14) reported that GH alters the glutathione S-transferase and mitochondrial thioredoxin systems in long-living Ames dwarf mice. Villares *et al* (16) used GH to treat type 1 diabetes, demonstrating that it prevented the development of diabetes by a mechanism involving specific GH-mediated effects on islet β-cells, T_h17/T_h1 plasticity, M1/M2 macrophage differentiation and T_{reg} cell function. Visser *et al* (11) reported that serum anti-Müllerian hormone levels correlated with

karyotype, pubertal development, luteinizing hormone and follicle-stimulating hormone (FSH), and is detected at higher levels in patients with Turner syndrome undergoing GH therapy. Furthermore, Hartmann *et al* (12) evaluated the effects of oral hormone replacement therapy (HRT) on body weight, insulin-like growth factor 1 (IGF-1) levels and GH response to exogenous GnRH in women with POF. This previous study revealed that women with POF who were not treated with HRT had significantly higher IGF-1 levels, compared with women with POF treated with HRT, which reduced during HRT; however, the body weight of these patients remained stable (12). Numerous studies are beginning to generate and characterize a number of GH-encoding transgenes in mice, expressing factors such as ovine GH, human GH and bovine GH (11-13,16). Although GH has been used in preclinical trials to treat POF, the mechanisms underlying its activity in the alleviation of POF are poorly understood.

The Notch signaling pathway has critical roles in the development and homeostasis of tissues by regulating cell fate, proliferation, differentiation and apoptosis, and in stem cell self-renewal (17,18). The Notch proteins are a family of evolutionarily conserved receptors that regulate cell fate (17-22). These Notch receptors are activated following direct contact with their ligands, which are expressed on adjacent cells (18,19). In mammals, there are four Notch receptors (NOTCH1-NOTCH4) and five ligands (Jagged-1 and -2 and delta-like protein 1, 3, and 4) (18,20). NOTCH receptors have extracellular, transmembrane and intracellular domains (18,19). Upon ligand binding, the NOTCH intracellular domain (NICD) of the receptor is cleaved by γ-secretase and translocates to the nucleus, where it associates with the recombination signal-binding protein jκ (RBPjκ) (16,17,20). RBPjκ is a key transcription factor in the canonical Notch signaling pathway, and acts downstream of all four NOTCH receptors (19-21). Within the nucleus, NICD forms a large transcriptional activator complex with RBPjκ/CBF1 and Mastermind (19-21). This transcriptional complex then activates the transcription of target genes such as hairy/enhancer of split (Hes) and Hes-associated with YRPW motif, two proteins of the basic helix-loop-helix gene families (21,22). A previous study has demonstrated that the Notch/Hes-1 signaling pathway controls the proliferation of intestinal immature progenitor cells (21). A number of previous studies have indicated that Notch also works with other transcription factors to regulate the expression of its target genes such as cyclin D1, B cell lymphoma 2 and Survivin (16-19,21,22). Furthermore, Notch-1 overexpression has been reported to inhibit apoptosis in numerous types of human cancer, suggesting that it has potential as a therapeutic target (19-22).

The current study aimed to determine whether recombinant GH can be used as a treatment of POF, and to investigate whether the therapeutic activity of GH is associated with the activation of the Notch-1 signaling pathway.

Materials and methods

Generation of a mouse model of POF and treatment with recombinant mouse growth hormone (rmGH). Female C57BL/6 mice (n=72) at 4-5 weeks of age, ~20 g weight, were obtained from the Animal Research Center, Longhua Hospital (Shanghai, China). The present study received ethical approval from the Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine, in compliance with the Experimental Animal Regulations of the National Science and Technology Commission, China. A total of 3-4 mice per cage were maintained for 14 days in a temperature-controlled environment under 12 h light-dark cycles with *ad libitum* access to food and water as previously described (23). To induce POF, mice were administered a single intraperitoneal injection of 70 mg/kg cyclophosphamide (Sigma-Aldrich, St. Louis, MO, USA) at 7 weeks of age. The animals were divided into control and experimental groups as follows: i) An untreated control group (wild-type; WT) of 12 mice; ii) a negative control group of 12 POF mice receiving a saline injection (100 μl); four experimental groups of 12 POF mice, injected daily with 100 μl of either iii) 0.4 (low-dose); iv) 0.8 (medium-dose); or v) 1.6 (high-dose) mg/kg rmGH (Sigma-Aldrich), dissolved in saline; vi) 12 POF mice were not administered any treatment. Injections of saline or rmGH were provided a week after induction of POF, and additional experiments were conducted 21 days after this treatment. At this point, mice were sacrificed by cervical dislocation.

Enzyme-linked immunosorbent assay (ELISA). Mouse blood plasma (100 μl) was obtained by mouse retro-orbital blood collection (23), centrifuged at 453 x g at 4˚C for 10 min, and the supernatant was collected. The mouse estradiol (E_2) and FSH ELISA kit (cat. nos. 29764 and 29755; Westang Bio, Shanghai, China) was used according to the manufacturer's protocol, in order to determine the levels of E_2 or FSH in the mouse blood plasma. Briefly, 100 μl of mouse E_2 or FSH antigens standardized to 125-8,000 pg/ml or 0.156-10 ng/ml, or diluted mouse plasma, were added to the anti-E_2 or FSH antibody-precoated microwells (as appropriate, in the case of the antigens) and incubated for 60 min. Following 3 washes, horseradish peroxidase-conjugated detection antibodies were added, followed by the substrate solution (Westang Bio). The absorbance of each well was measured at 450 nm using a microplate reader (BioTek Synergy Mx; BioTek Instruments, Inc., Winooski, VT, USA).

Hematoxylin and eosin staining. Briefly, all ovarian tissue samples were washed 3 times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min, dehydrated using a graded series of ethanol, vitrified in xylene and embedded in paraffin (both purchased from Sigma-Aldrich). Following this, serial 6-μm thick sections (Leica RM2235 microtome; Leica Microsystems, Wetzlar, Germany) were made and stained with hematoxylin and eosin (Sigma-Aldrich).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA (200 ng/μl) was isolated from mouse ovary tissue from each cell type using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. RNA samples were treated with DNase I (2.0 mU/μl; Sigma-Aldrich), quantified and reverse-transcribed into cDNA (20 μl) using the ReverTra Ace-α reverse transcription kit (cat. no. FKS-101; Toyobo Co., Ltd., Osaka,

Japan). RT-qPCR was conducted using a realplex4 real-time PCR detection system (Eppendorf, Hamburg, Germany) with SYBR Green PCR Master mix (Toyobo Co., Ltd.). Primers used for cDNA amplification are listed in Table I. RT-qPCR amplification was performed over 40 cycles of denaturation at 95˚C for 15 sec, annealing at 58˚C for 45 sec and final elongation at 72˚C for 42 sec (initial denaturation at 95˚C for 5 min for 1 cycle, and final elongation at 72˚C for 10 min for 1 cycle). Target cDNA levels were then measured using the $2^{-\Delta\Delta Cq}$ relative quantification method (24). Comparative quantification cycle (C_q) values were used to determine relative gene expression, normalized to 18S rRNA. For each sample, C_q values were normalized using the following formula: $\Delta C_q = C_q$ experimental genes - C_q 18S rRNA. Relative expression levels were calculated using the formula: $\Delta\Delta C_q = \Delta C_q$ all groups - ΔC_q untreated control group.

Immunohistochemistry. Immunohistochemical analysis was performed as previously described (23,25). Briefly, all ovarian tissue samples were washed 3 times with PBS, fixed with 4% paraformaldehyde for 30 min, dehydrated through a graded series of ethanol, vitrified in xylene and embedded in paraffin. Subsequently, serial 6-μm thick sections were made, rinsed with 3% phosphate buffer (Sigma-Aldrich), and subjected to microwave heat repairing. The samples were then incubated with primary antibodies for 45 min at 37˚C, as follows: Anti-NOTCH-1 (1:1,000 dilution; mouse polyclonal; cat. no. sc-6014); anti-CBF-1 (1:1,000 dilution; mouse polyclonal; cat. no. sc-9417); anti-Hes-1 (1:100 dilution; mouse polyclonal; cat. no. sc-13844); and anti-p53 (1:100 dilution; goat polyclonal; sc-1313) (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Horseradish-peroxidase conjugated goat anti-rabbit immunoglobulin G (1:1,000 dilution; sc-2768; Santa Cruz Biotechnology Inc.) was then incubated with samples for 45 min at 37˚C. Finally, ABC chromogenic reagent (Sigma-Aldrich) was added to aid the visualization of the secondary antibody, in addition to color detection. PBS (pH 7.4) was used as a negative control in the place of primary antibody. Five randomly selected fields of view (magnification, x200; Olympus BX43; Olympus Corporation, Tokyo, Japan) were observed for each tissue section and analyzed using IPP software (version 4.0; Intel Corporation, Santa Clara, CA, USA).

Statistical analysis. All data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, CA, USA). The data in each experiment are reported as the mean ± standard error, where applicable, and the differences were evaluated for statistical significance with one-way analysis of variance. P<0.05 was considered to represent a statistically significant difference.

Results

Medium doses of rmGH rescue ovarian weight, hormone secretion and the number of normal follicles in POF mice. At 14 days after injection, the weight of the ovaries in each group was determined (Fig. 1A). A statistically significant difference was not observed in ovarian weight between POF mice (2.934±0.257 mg), POF mice treated with a low dose of rmGH (3.939±0.321 mg) and POF mice treated with saline (3.014±0.365 mg) 14 days after injection. However, ovarian weight in medium-dose (4.268±0.405 mg) and high-dose groups (6.545±0.551 mg) was significantly increased when compared with that of the POF model group (P<0.05; n=12 mice; Fig. 1A). An ELISA revealed no observably statistically significant difference in plasma E_2 levels between the POF model group (64.58±7.93 pg/ml), the low-dose group (112.41±10.15 pg/ml) and the negative control group treated with saline (72.94±9.32 pg/ml). Conversely, the plasma E_2 levels increased with time in medium-dose (242.12±15.39 pg/ml) and high-dose (463.32±29.91 pg/ml) groups compared with that of the POF model group (P<0.05; n=12 mice; Fig. 1B). An ELISA also indicated that plasma FSH levels did not appear to vary significantly between the POF model group (1,351.01±68.61 pg/ml), the low-dose group (778.41±97.48 pg/ml) and the saline-treated group (1,308.02±81.14 pg/ml), but decreased significantly with time in the medium-dose group (618.22±95.11 pg/ml) compared with the POF model group (P<0.05; n=12 mice; Fig. 1B). Conversely, the plasma FSH levels increased with time in the high-dose group (3,357.04±407.81 pg/ml) when compared with the POF model group (P<0.01; n=12 mice; Fig. 1B).

Following this, the number of normal and atretic ovarian follicles were counted in each group. A statistically significant difference was not identified in the number of normal follicles and atretic follicles between the POF model group (3±1 and 11±2, respectively), the low-dose group (5±2 and 9±1, respectively) and the saline-treated group (3±2 and 11±2, respectively). Conversely, the medium-dose (10±2 normal and 4±1 atretic follicles) and high-dose (6±2 normal and 6±2 atretic follicles) groups had a lower number of atretic follicles than that observed in the POF model group (P<0.05; n=12 mice; Fig. 1C).

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer (5'-3')
NOTCH1	
F	CTCCAACTGTGACACCAACCCTG
R	TGTAGCCCTGTAGACACTGGCACTC
CBF1	
F	GTGGCACTGTTCAATCGCCTTCG
R	CAGTCTGCCCGTAATGGATGTAG
Hes1	
F	GAGAAGAGGCGAAGGGCAAGAATAAA
R	CAACACGCTCGGGTCTGTGCTGA
Ccnd1	
F	TGTGAGGAGCAGAAGTGCGAAGA
R	GCCGGATAGAGTTGTCAGTGTAGATGC
18S rRNA	
F	AGGGGAGAGCGGGTAAGAGA
R	GGACAGGACTAGGCGGAACA

F, forward; R, reverse; Hes1, hairy/enhancer of split; Ccnd1, cyclin D1; rRNA, ribosomal RNA.

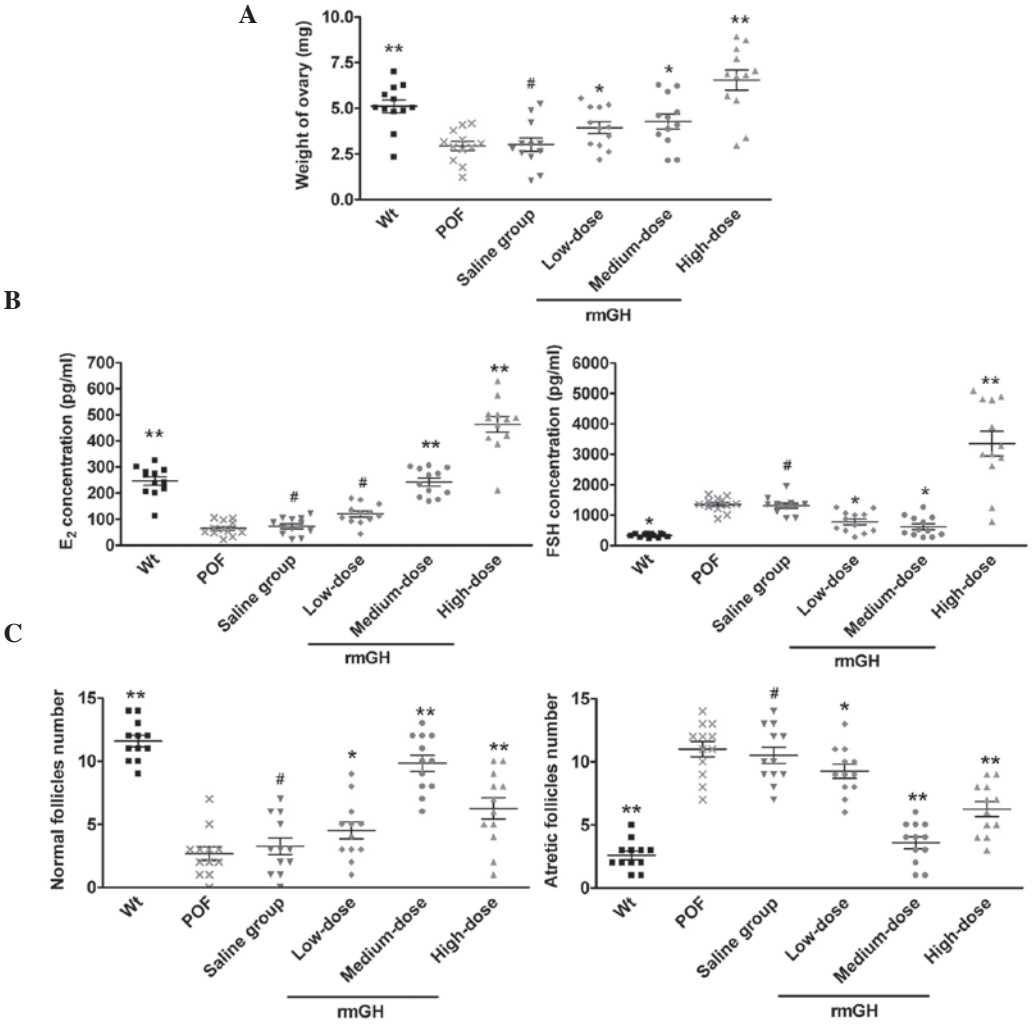

Figure 1. Ovarian weight, plasma E_2 and FSH levels and follicle count following treatment with rmGH or saline, reported as means ± standard error. (A) Ovarian weight in Wt mice was significantly higher compared with the POF model and saline-treated mice, but no significant difference was identified between medium- and high-dose rmGH-treated POF mice. (B) Plasma E_2 levels increased with time in medium-dose and high-dose groups compared with the POF model group. Meanwhile, plasma FSH levels did not appear to vary significantly between the POF model group, the low-dose group and the saline-treated group, but decreased significantly with time in the medium-dose group compared with the POF model group. (C) Follicle counts revealed no statistically significant difference in normal and atretic follicles counts between the POF model and saline-treated groups. Conversely, medium- and high-dose groups had a lower number of atretic follicles than the POF model group. **P<0.01, vs. the POF group; *P<0.05, vs. the POF group; $^{\#}$P>0.05, vs. the POF group; n=12 mice/group. Wt, wild-type; POF, premature ovarian failure model (untreated group); rmGH, recombinant mouse growth hormone; E_2, estradiol; FSH, follicle stimulating hormone.

Hematoxylin and eosin staining revealed that the ovaries of WT mice contained a large number of follicles at all stages of development, ranging from immature to mature (Fig. 2). Conversely, the atrophied ovaries of POF mice consisted primarily of interstitial cells in a fibrous matrix, with a reduced number of follicles at each stage. Furthermore, the ovaries of the POF mice contained an increased number of collapsed oocytes and ovaries were smaller than those of WT mice (Fig. 2). However, following treatment with rmGH, mice exhibited a significant reduction in POF pathology within their ovaries, attenuation of ovarian granulosa cell injury, reduction in the number of atretic follicles and a significant increase in the number of mature oocytes (Fig. 2).

Medium doses of rmGH activate the Notch-1 signaling pathway in the ovarian granulosa cells of POF mice. At 14 days after injection, the expression levels of the genes involved in the Notch-1 signaling pathway in mouse ovarian granulosa cells were analyzed by RT-qPCR and immunohistochemistry. The RT-qPCR results revealed that the expression levels of Notch1 signaling pathway genes (*NOTCH1*, *Cbf1* and *Hes1*) and cell proliferation factor (*Ccnd1*; cyclin D1) had decreased in the POF model group compared with the WT group (P<0.05; n=12 mice; Fig. 3). Furthermore, a statistically significant difference was not identified in the expression levels of the Notch-1 signaling pathway genes and the *Ccnd1* between the POF model group, the low-dose group, high-dose group and the saline-treated group (P>0.05; n=12 mice; Fig. 3). However, the expression levels of Notch-1 signaling pathway genes and *Ccnd1* were significantly elevated in the medium-dose group compared with the POF model group (P<0.01; n=12 mice; Fig. 3). Immunohistochemical staining also revealed positive staining for Notch1 signaling pathway factors (Notch1, CBF1, and HES1) in the WT group, medium-dose group and high-dose group, but not in the low-dose or saline-treated groups (Fig. 4). However, p53 staining was positive in the

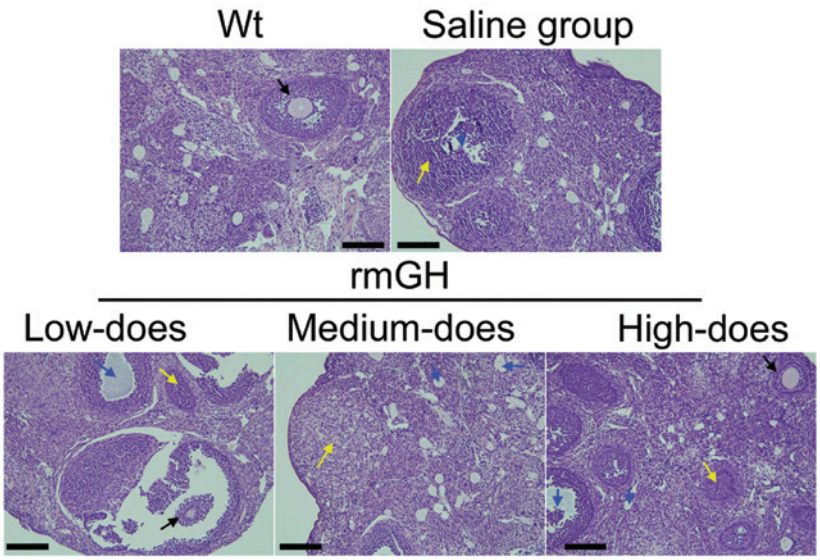

Figure 2. Pathology of mouse ovarian tissue samples following treatment with rmGH or saline, observed using hematoxylin and eosin staining. rmGH treatment significantly improved ovarian tissue pathology, attenuated ovarian granulosa cell injury, reduced the number of atretic follicles and increased the number of mature oocytes in a dose-dependent manner. Magnification, x200. Scale bar = 200 μm. Wt, wild-type; rmGH, recombinant mouse growth hormone.

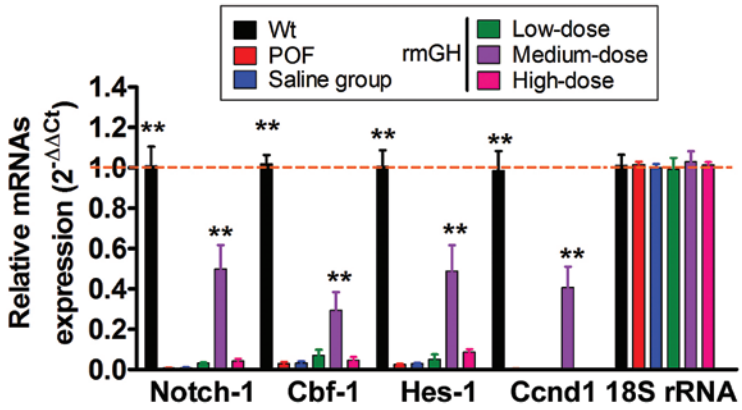

Figure 3. mRNA expression analysis of the Notch1 signaling pathway and cell proliferation factors using reverse transcription-quantitative polymerase chain reaction. Expression levels of Notch1 signaling pathway genes (*Notch1*, *Cbf1*, and *Hes1*) and cell proliferation factor (*Ccnd1*) were significantly elevated in the medium-dose group compared with the POF model group. **P<0.01, vs. the POF (untreated) group; n=12 mice/group. Wt, wild-type; POF, premature ovarian failure model; rmGH, recombinant mouse growth hormone; *Hes1*, hairy/enhancer of split; *Ccnd1*, cyclin D1.

low-dose and saline-treated groups, but not in the WT, medium-dose or high-dose groups (Fig. 4).

Discussion

POF is an impactful disease and, at present, there is no cure or effective treatment available for POF. Women diagnosed with POF are faced with significant physical and emotional challenges, as this disease can lead to loss of fertility, in addition to osteoporosis and other complications (1-5). GH is a pleiotropic hormone that affects a broad spectrum of physiological functions, from carbohydrate and lipid metabolism to the immune response (10-16). Several previous studies have reported the effective use of GH to treat autoimmune diseases and POF (14-16). However, the mechanism underlying GH activity in the treatment of these diseases remains unclear. In the present study, three main questions are addressed: i) Does rmGH improve hormone release and oocyte maturation in mice exhibiting POF? ii) Is the effect of rmGH on the regulation of hormone levels and oocyte maturation dose-dependent? and iii) What therapeutic mechanism does rmGH employ to control hormone release and oocyte maturation in POF mice?

Although E_2 and FSH hormone levels in POF mice were significantly improved following treatment with medium and high doses of rmGH treatment, a medium dose of rmGH was the most effective, leading to significantly increased E_2 levels and decreased FSH levels in the peripheral blood of POF mice. High doses of rmGH also elevated E_2 levels in peripheral blood, although FSH levels significantly increased from that observed in the medium-dose group. It may be speculated that high doses of GH stimulate the body to release hormones in a non-specific manner, causing this increase in FSH. GH was also reported to stimulate oocyte maturation and reduce the number of atretic follicles in a dose-dependent manner; these effects were only observed in

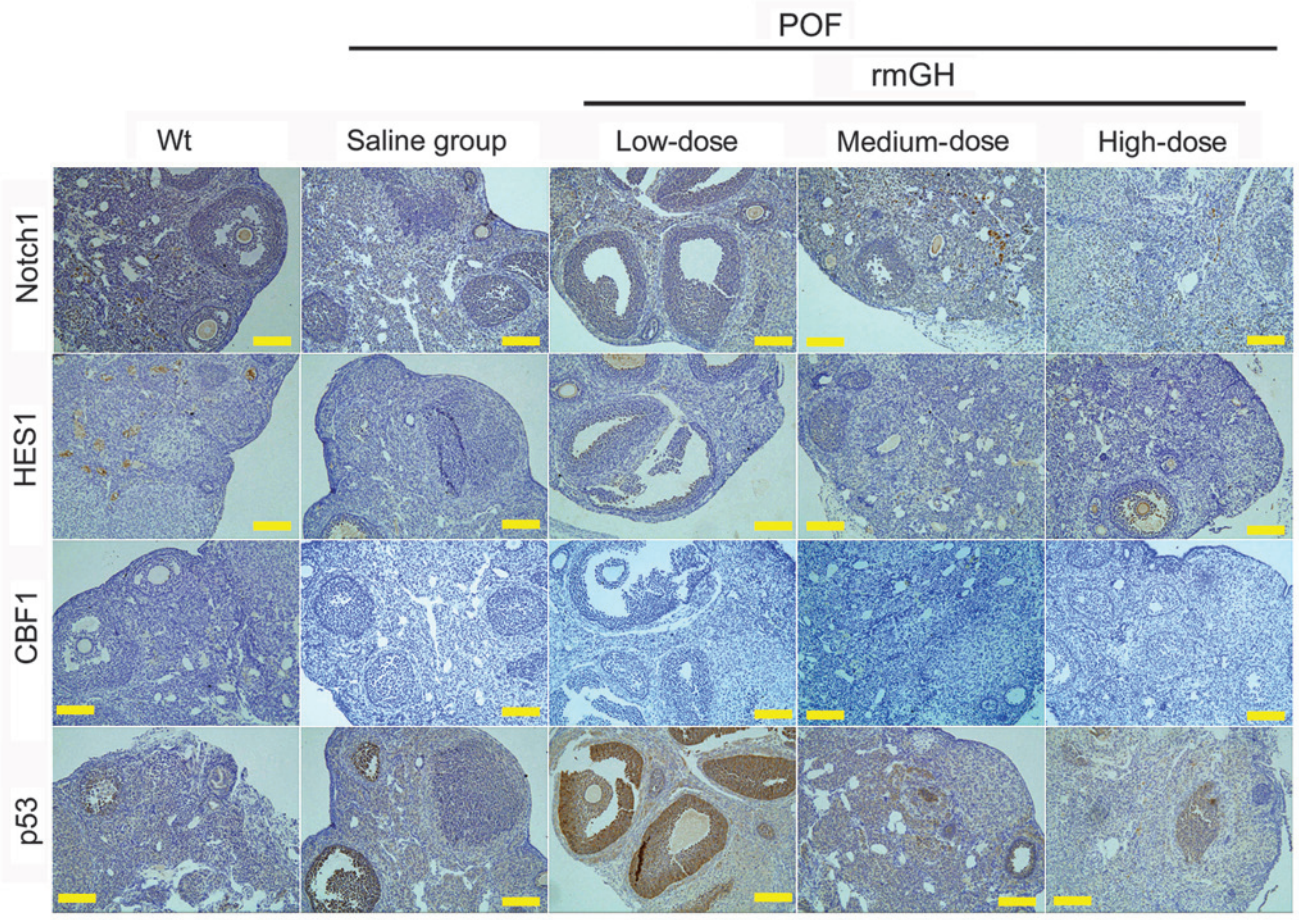

Figure 4. Protein expression of Notch1 signaling pathway factors and p53 using immunohistochemistry. Staining indicated positive or markedly positive staining for Notch1 signaling pathway factors (Notch1, CBF1, and HES1), but not for p53 in the Wt, medium-dose, and high-dose groups, as compared with the low-dose and saline-treated groups. Magnification, x200. Scale bar=200 μm. POF, premature ovarian failure model; rmGH, recombinant mouse growth hormone; Wt, wild-type; HES1, hairy/enhancer of split.

POF mice treated with medium and high doses of rmGH. Oocyte maturation is closely associated with several factors, including the ovarian microenvironment, the extent of development of the ovarian corpus luteum, the activity of ovarian granulosa cells, hormone release and endocrine regulation (1-5). The results of the current study confirm that GH may effectively treat multiple features of POF in mice displaying disease symptoms.

The novel evidence that GH regulates the expression of Notch-1 signaling pathway genes in ovarian tissue from POF mice in order to induce regeneration and repair was also presented. The Notch-1 signaling pathway is known to regulate cell proliferation and tumorigenesis but, to the best of our knowledge, it has not previously been associated with POF. In the current study, Notch-1 signaling pathway genes were expressed at different levels when POF mice were treated with varying concentrations of GH. For example, a medium dose of rmGH caused the greatest activation of Notch-1 signaling pathway genes in the ovarian tissue of POF mice. The hypothesized role of the Notch pathway genes based on the current model suggests that the direct effect of Notch-1 signaling pathway activation is the proliferation of ovarian cells to restore function, and thus, repair and replace damaged ovarian cells in the POF mouse model. It is therefore hypothesized that GH was activated through the expression of the Notch-1 pathway in ovarian cells to repair ovarian function.

In conclusion, the results of the present study suggest that GH promotes ovarian tissue repair and regeneration, estrogen release and oocyte maturation by activating the expression of Notch-1 signaling pathway factors in the ovarian tissue of mice exhibiting POF.

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