Growth hormone during in vitro fertilization in older women modulates the density of receptors in granulosa cells, with improved pregnancy outcomes

Sheena L. P. Regan, Ph.D.,[a] Phil G. Knight, Ph.D.,[b] John L. Yovich, M.D., Ph.D.,[c,d] Frank Arfuso, Ph.D.,[a] and Arun Dharmarajan, Ph.D.[a]

[a] Stem Cell and Cancer Biology Laboratory, School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, Australia; [b] School of Biological Sciences, University of Reading, Whiteknights, Reading, United Kingdom; [c] PIVET Medical Centre, Perth, Australia; and [d] School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, Australia

Objective: To study the effect of aging and granulosa cell growth hormone receptor (GHR) expression, and the effect of growth hormone (GH) co-treatment during IVF on receptor expression.
Design: Laboratory study.
Setting: University.
Patient(s): A total of 445 follicles were collected from 62 women undergoing standard infertility treatment.
Intervention(s): Preovulatory ovarian follicle biopsies of granulosa cells and follicular fluid.
Main Outcome Measure(s): Older women with a poor ovarian reserve were co-treated with GH to determine the effect of the adjuvant during IVF on the granulosal expression density of FSH receptor (FSHR), LH receptor (LHR), bone morphogenetic hormone receptor (BMPR1B), and GHR. Ovarian reserve, granulosa cell receptor density, oocyte quality, and pregnancy and live birth rates were determined.
Result(s): Growth hormone co-treatment increased the receptor density for granulosal FSHR, BMPR1B, LHR, and GHR compared with the non-GH-treated patients of the same age and ovarian reserve. Growth hormone co-treatment increased GHR density, which may increase GHR activity. The GH co-treatment was associated with a significant increase in pregnancy rate.
Conclusion(s): Growth hormone co-treatment restored the preovulatory down-regulation of FSHR, BMPR1B, and LHR density of the largest follicles, which may improve the maturation process of luteinization in older patients with reduced ovarian reserve. The fertility of the GH-treated patients improved. (Fertil Steril® 2018;110:1298–310. ©2018 by American Society for Reproductive Medicine.)
Key Words: Aging, fertility, growth hormone, pregnancy rate, receptor density

Ovarian depletion of primordial follicles is a continual natural process from gestation to adulthood, which culminates in the loss of ovarian function and eventuates in the state of menopause (1, 2). When activated, the primordial follicles grow and develop into small antral follicles, the majority of which succumb to apoptosis (3–5). At puberty, cyclic increases in circulating FSH recruit a cohort of small antral follicles at the start of each menstrual cycle (6, 7). The follicles grow under the influence of FSH and express FSH receptor (FSHR) and LH receptor (LHR). The activation of FSH and the FSHRs stimulates E synthesis, which subsequently stimulates proliferation of the granulosa cells and development of the oocyte (8–11).

Received January 30, 2018; revised and accepted August 6, 2018.
S.L.P.R. has nothing to disclose. P.G.K. has nothing to disclose. J.L.Y. has nothing to disclose. F.A. has nothing to disclose. A.D. has nothing to disclose.
S.L.P.R. was a recipient of an Australian Postgraduate Award. This work was supported by additional private external funding, which was gratefully accepted from Denby Macgregor.
Reprint requests: Sheena L.P. Regan, Ph.D., Curtin University, Curtin Health Innovation Research Institute, School of Pharmacy and Biomedical Sciences, GPO Box U1987, Perth, Western Australia 6845, Australia (E-mail: sheenaregan@aapt.net.au).

Fertility and Sterility® Vol. 110, No. 7, December 2018 0015-0282/$36.00

https://doi.org/10.1016/j.fertnstert.2018.08.018

The ovulation rate is determined by the stage-specific decrease in pituitary secreted FSH and results in follicles with insufficient LHRs that succumb to apoptosis (3, 12–15). The follicle continues to grow until preovulatory maturation, when proliferation ceases and granulosa cell differentiation occurs in preparation for ovulation of the oocyte.

As the ovarian reserve of primordial follicles is depleted over the reproductive lifespan, regulation of folliculogenesis is altered, which results in decreased fertility (16). Ovarian depletion can be indirectly measured by the number of small antral follicles present at the beginning of a cycle and is highly correlated to chronological age (17). During IVF treatment, high doses of recombinant human (r) FSH are administered to recruit more of the small antral follicles and to maintain their growth during pituitary FSH down-regulation (18).

Infertility patients with a poor ovarian reserve have fewer small antral follicles available for recruitment, and higher doses of rFSH are used but with diminishing effectiveness in recruiting more follicles during IVF cycles. In an attempt to improve the pregnancy rate, patients have been offered co-treatment with growth hormone (GH) (18, 19). The patients with a poor response to rFSH treatment represent a large group of patients with critically diminishing ovarian reserve (20, 21). The challenge remains to identify the changes taking place as the ovarian reserve declines and to find alternative stimulation to provide high-quality oocytes for fertilization.

Earlier studies showed that GH treatment in vivo and in vitro, in conjunction with rFSH, increased oocyte survival rate and pregnancy rate (22–25). The granulosa cells, including cumulus cells, as well as the oocyte of antral follicles, express GH receptor (GHR) and are therefore able to react to pituitary-derived or ovarian sources of GH (26, 27). With regard to the latter, granulosa cells and the oocyte, but not cumulus and theca cells, have been shown to express GH messenger RNA (26–30). Growth hormone receptors are activated by GH, which changes the conformation of the receptor, promoting formation of a complex with janus kinase (JAK)2 (31). The GHR–JAK2 complex can elicit numerous cellular responses in the body, such as cell differentiation and oocyte maturation in the ovary (32).

The cellular mechanism underpinning the GH-induced improvement in oocyte quality and reduced miscarriage rate has not been reported in human studies. However, many attempts have been made to delineate the indirect changes taking place to serum and follicular fluid hormone levels. Previously we have presented comprehensive results on the granulosal cell surface receptor density profiles of patients during ovarian aging (33, 34). Ovarian granulosa cell receptor expression was found to fluctuate at the two critical times of dominant follicle selection and again at the terminal end of folliculogenesis in preparation for ovulation. Lower levels of receptor density and a reversal of this regulatory pattern are associated with reduced fertility and ovarian reserve in older patients.

With a view to understanding the cellular mechanism by which GH may improve oocyte quality, we report the granulosal GHR density in different-sized follicles. These were derived from IVF patients undergoing conventional ovarian stimulation, with rFSH alone and with rFSH combined with GH co-treatment, in young compared with older women with a reduced ovarian reserve. In addition, we report the granulosal FSHR, LHR, and bone morphogenetic hormone receptor (BMPR1B) density in older, poor ovarian reserve patients treated with GH.

MATERIALS AND METHODS

Patients

Female patients, selected randomly in a prospective blind regimen, were aged between 23 and 45 years with a range of infertility factors, but limited to exclude endometriosis, unusual medical conditions, endocrine dysfunction, and polycystic ovarian syndrome according to the Rotterdam criteria, initially prepared in 2003 and updated to reflect advances in ultrasound technology (35), specifically, per ovary > 24 follicles, along with other criteria. Infertility issues comprised male factor, low ovarian reserve, donor sperm, or unexplained fertility; and fertilization was via intracytoplasmic sperm injection. A total of 445 follicles were aspirated from 62 women undergoing standard fertility treatment at PIVET Medical Centre Perth, Western Australia (Table 1).

Human IVF: Ovarian Stimulation, Follicular Fluid, and Oocyte

Patient treatment consisted of two types of GnRH–LH suppression (Orgalutran [MSD] and Cetrotide [Merck Serono]) in conjunction with commercially prepared rFSH (Puregon [MSD] and Gonal-f [Merck Serono]), from cycle day 2 for approximately 10 days, as described by Regan et al. (34). The dosages of rFSH were administered according to a well-described and validated algorithm designed to collect 10 ± 2 oocytes and avoid ovarian hyperstimulation syndrome (36). Ovulation was triggered with 10,000 IU hCG (Pregnyl, MSD), and oocyte retrieval was 36 hours later by transvaginal oocyte aspiration (33). Patients classified as poor prognosis due to poor ovarian response or with three or more failed attempts to conceive through IVF treatment with gonadotrophin alone were co-treated with a total of 60 IU GH (Saizen, Serono) over a period of 20–24 days in the lead-up to IVF. Specifically, a total of six injections of GH was administered to 10 patients on day 21 of the preceding cycle, and on days 2, 6, 8, 10, and 12 of the ensuing IVF cycle (10 IU per injection, a total of 60 IU). The women were aged ≥ 39 years and had at least one failed IVF cycle (18).

Antral Follicle Count

Patients received daily rFSH according to a long-established algorithm based on the patient's profile of age and ovarian reserve to determine the rFSH dose required to stimulate 8–12 preovulatory follicles (37). Ovarian reserve was measured indirectly by the antral follicle count and was defined as the number of follicles between 2 and 10 mm in diameter, combining the number measured from both ovaries, that were present on approximately day 5 of a preliminary

TABLE 1

Patient ovarian reserve, based on antral follicle count.

Age (y)	IVF patient	Total follicle	Ovarian reserve[a]	Ovarian reserve group,[b] no. of follicles collected per group					Oocyte quality, n			Fertility, n (%)[c]		
				A+	A	B	C	D	E	n[d]	CCF[e]	ET	Not pregnant	Pregnant	Miscarriage	Live birth
21–30[f]	10	68	20–39	26	42	–	–	–	–	10	0	12	4 (33)	8 (67)	3 (25)	5 (41)
31–34	12	96	13–29	–	48	23	16	9	–	8	0	16	9 (56)	7 (44)	1 (6)	6 (37.5)
35–38	12	103	9–19	–	6	46	17	34	–	9	0	16	5 (31)	11 (69)	5 (31)	6 (37.5)
39–45	18	130	3–8	–	–	42	5	64	19	7	3	27	24 (89)	3 (11)[g]	2 (7)	1 (3.7)
+GH 39–45	11	63	3–8	–	–	15	–	26	22	5.7	1	16	9 (56)	7 (44)[h]	5 (31)	2 (12.5)

[a] Typical ovarian reserve for age group.
[b] Ovarian reserve measured indirectly by the antral follicle count (AFC), which is the number of follicles between 2 and 10 mm on day 2–5 of a cycle: group A+ = 30–39 follicles; group A = 20–29 follicles; group B = 13–19 follicles; group C = 9–12 follicles; group D = 5–8 follicles; group E = ≤4 follicles. Follicle count is based on the combined total from both ovaries to determine AFC. The number of follicles aspirated from patients from the specified ovarian reserve group.
[c] Percentage per total number of embryos transferred.
[d] The average number of oocytes collected at transvaginal ovarian aspiration for the age group.
[e] Number of patients with complete failed fertilization compared with same age group without GH.
[f] One patient with an ectopic pregnancy (classed as miscarriage).
[g] Pregnancy rate, 39+ years, all patient AFC groups (B, C, D and E); compared to "h" below.
[h] Pregnancy rate, +GH 39+ y, B, D, and E only: χ^2 $P = .0143$.

Regan. GH modulates fertility receptors. Fertil Steril 2018.

assessment cycle, without rFSH (17). The patients were divided by age and ovarian reserve into groups based on the algorithm, as described previously by Regan et al. (33, 34) and a well-established clinical practice of patient treatment (36, 37): group A+ = 30–39 small follicles; group A = 20–29 small follicles; group B = 13–19 small follicles; group C = 9–12 small follicles; group D = 5–8 small follicles; and group E = ≤4 small follicles.

Immunolabeling of Granulosa Cells

The ovarian follicles studied ranged in diameter from 4 to 27 mm, and an average of approximately 8,000 granulosa cells per individual follicle were analyzed as described previously (34). Cell surface–expressed mature GHR protein density was measured by immunofluorescent labeling and flow cytometry. The diameter of the follicle was calculated using ultrasonography as described previously (33, 34, 38). Flushing of the follicle (Quinn's Advantage with Hepes, Sage Media) removed the loosely attached layers of granulosa cells. Aliquots of suspended granulosa cells (1×10^6 cells in 100 μL) were immunolabeled and incubated separately with an optimized concentration of 4 μg/mL affinity purified polyclonal antibody to BMPR1B, FSHR, LHR, or GHR for 25 minutes at 5°C.

Three-dimensional image analysis using immunofluorescence detection has established the specificity of the antibodies in sheep, polyclonal goat anti-BMPR1B (sc-5679), goat anti-FSHR (sc-7798), and goat anti-LHR (sc-26341) (Santa Cruz Biotechnology) (39); and GHR (AF1210; Life Technologies) (40). In addition, use of these antibodies has been previously reported in human studies (41–48) and for use in flow cytometry (42). The cells were washed with phosphate-buffered saline and centrifuged at 300 × *g* at 5°C for 5 minutes. To render a homogeneous population of granulosa cells, the monoclonal antibody CD45 was added to BMPR1B, GHR, and LHR tubes to enable the subtraction of the positive leukocyte common antigen (approximately 3%) not removed during isolation of the granulosa cells with the Ficoll gradient (555485; BD Biosciences) (Supplemental Fig. 1A and B).

Unstained samples or the substitution of a primary antibody with preimmune goat IgG (Millennium Science) at the same concentration as the primary antibody served as a negative control for autofluorescence (Supplemental Fig. 1A). A blocking peptide for FSHR and BMPR1B indicated nonspecific binding applied to human granulosa cells (sc-7798P, sc-5679P; Millennium Science) (Supplemental Fig. 1B) and as previously reported (41, 42). Preabsorbed LH (Lutropin, Merck Serono) and GH (Saizen, Merck Serono) also confirmed binding specificity. In the present study, the "normal" goat IgG and unstained control cells emitted an average mean fluorescent intensity that was classified as nonspecific autofluorescence. The autofluorescence and the nonspecific binding determined by the unstained control for each follicle was subtracted from each follicle (Supplemental Fig. 1B), as described previously (33, 34).

Resuspended 10-μL aliquots of GHR immunolabeled live granulosa cells were placed on slides and visualized using an

Olympus DP 70 camera fitted to an Olympus BX-51 upright fluorescent microscope with a 40× UPlan N 0.4 N.A. objective (Olympus Imaging Australia) (Supplemental Fig. 1C). Fluorescent microscopy revealed a positive staining of the cell membrane–bound GHR as an intermittent, bright, ring-like pattern around the cells (Supplemental Fig. 1). Preabsorbed GH was used as a negative control. A pure granulosa cell population was identified by graphing forward scatter to remove doublets (FSC-H vs. FSC-A), as previously described (33, 34). The uniform granulosa cell population revealed positive staining for FSHR, which is unique to granulosa cells (49). The data were analyzed using FlowJo software (Tree Star).

Serum and Follicular Fluid Assessment

The peak estrogen (E) concentration in serum was used to predict the follicular health of the follicle, as opposed to the serum levels collected at the time of follicle aspiration. Serum was analyzed using biochemical analysis on the days leading up to collection and on the day of collection. In vitro fertilization patients undergoing treatment were examined in a natural cycle and during exogenous rFSH-stimulated cycles. Follicular fluid collected from follicles 17–23 mm were analyzed for T, FSH, and LH using a random access immunoassay system (Siemens Medical Solutions). Follicular fluid, T, FSH, and LH were analyzed undiluted, whereas E and P were diluted manually 1:1,000 with a multi-diluent and, when required, a further manual dilution of P 10× and E 5×. Percentage coefficient of variance for a concentration range 137.4 pmol/L to 3,257 pmol/L was E = 5.2; LH = 3.9; FSH = 2.9; T = 5.9; and P=9.4.

Statistics

Mean fluorescent intensity was obtained using approximately 8,000 granulosa cells per individual follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way analysis of variance (ANOVA), P values shown in figures. An uncorrected Fisher's least significant difference (LSD) was used for follicular size as repeated measures were infrequent, using GraphPad Prism 7. The receptor variation between the same size follicles were consistently different within the same patient (34), and most patients were represented only once or sometimes twice within a follicle size group. Values in graphs are means ± SEM, and significant differences are indicated as follows: *P<.05, **P<.01, ***P<.005, and ****P<.001. In the graphs, the letter, such as "a," signifies a statistically significant difference to the matching letter (e.g. "a*"). The attached asterisk (a*) indicates the significance level for the size follicle. A two-tailed, Student t-test and χ^2 test were also used.

Human Ethics

Patients undergoing standard fertility treatment at PIVET Medical Centre, Perth, Australia provided informed consent according to Curtin University Human Research Committee (HR RD26-10:2010 and 2016); and all methods were performed in accordance with the relevant institutional review board (Curtin University) guidelines and regulations under state legislation and national accreditation processes.

RESULTS

GHR Density without GH Co-treatment and Ovarian Reserve Depletion

In the youngest patients with good ovarian reserve, a constant level of granulosal GHR was expressed during follicular growth in both the A+ and A groups, both of which are typical for a patient in this age group (Fig. 1A).

Growth hormone receptor density was significantly decreased as the ovarian reserve was depleted in all of the three older age groups. In the 31–34-year patient group, GHR density on the granulosa cells from follicles of the same size was significantly reduced in the patients with a reduced ovarian reserve for the age group (P=.039, 14-mm follicles; .0037, 16-mm follicles; Fig. 1B). This trend was also found in the 35–38-year patient group (P=.029, 4-mm follicles; Fig. 1C) and in the 39+-year patient group (P=.0001, 4-mm follicles; P=.0012, 14-mm follicles; Fig. 1D). In the older patients (39+ years) with a comparatively better ovarian reserve of B or C, the level of GHR was significantly reduced in the larger follicles to the level observed in the poorer D and E ovarian reserve group (P<.001; Fig. 1D).

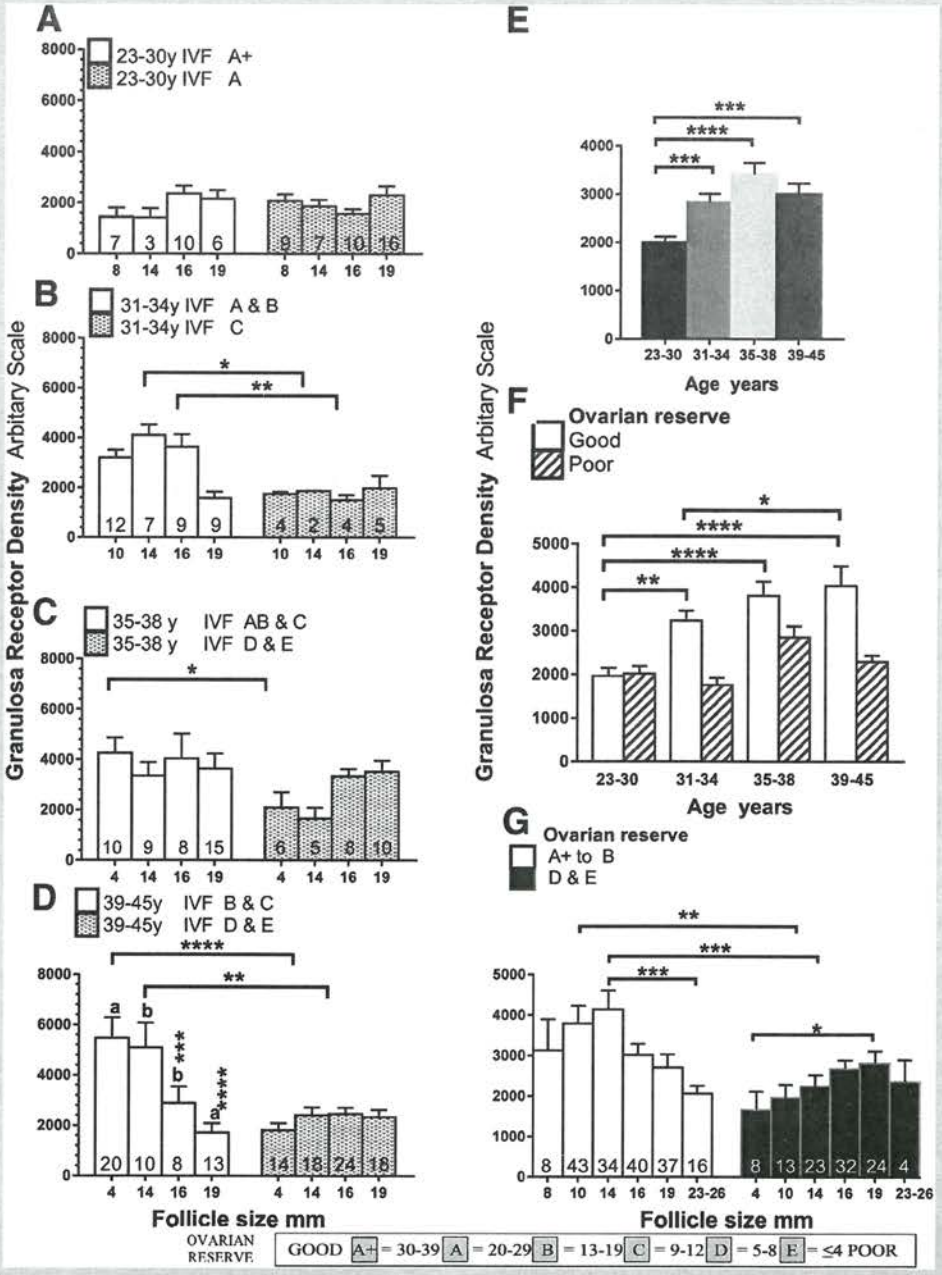
GHR Receptor Density Profile Independent of Patient Age

Growth hormone receptor expression based on chronological age increased in the older patients, masking the confounding influence of ovarian reserve (Fig. 1E). Clearly, when ovarian reserve rated as high (good) or low (poor) for the age group was defined, GHR expression increased with age when the reserve was "good for age" (Fig. 1F); whereas, if the patient data were analyzed purely on the basis of ovarian follicle reserve, independent of chronological age (Fig. 1G), an initial high level of GHR in the smaller follicles was followed by a decline as the follicles increased in size (14–23-mm follicles, P=.0005). This pattern was reversed in the poorer ovarian reserve patient groups of D and E (P<.05). Granulosal GHR receptor density was greater in the 10-mm (P<.01) and 14-mm (P<.005) follicles in the higher ovarian reserve patient group compared with patients with the poorest ovarian reserve.

GH Co-treatment Restores Preovulatory Down-Regulation of FSHR, BMPR1B, and LHR

In the study of GH-treated women, the patients were selected randomly from the same age group and had a similar ovarian reserve (D and E), antimüllerian hormone level, body mass index, and recruitment of ovarian follicles (Table 2). In this homogenous group, the level of GHR was significantly increased in IVF patients receiving GH co-treatment in follicles from 10 to 23 mm compared with the same age patients of 39+ years with an ovarian reserve of D and E (P<.01 to P<.001; Fig. 2A and Fig. 2A inset). However, in patients treated with GH, the level of GHR expression in different sized follicles did not reach significance.

FIGURE 1



Granulosal GHR density and ovarian reserve depletion. Growth hormone receptor expression density on granulosa cells collected from patients during IVF treatment with a range of ovarian reserves of follicles. (**A**) 23–30-year patient group ANOVA $P=.3369$. (**B**) 31–34-year patient group ANOVA $P<.0001$. (**C**) 35–38-year patient group ANOVA $P=.1851$. (**D**) 39+-year patient group ANOVA $P<.0001$. (**E**) Growth hormone receptor expression as a function of age ANOVA $P=.0002$. (**F**) Age and the influence of ovarian reserve on GHR expression (antral follicle count equivalent to "good for age group" or "poor for age group") ANOVA $P<.0001$. (**G**) Growth hormone receptor protein expression purely on the basis of ovaian reserve and not age ANOVA $P<.0004$. Ovarian reserve was measured indirectly by the antral follicle count. Antral follicle count is the number of follicles between 2 and 10 mm on day 2–5 of a cycle. Mean fluorescent intensity was obtained using an average of approximately 8,000 granulosa cells per follicle for the direct measurement of receptor protein expression. The number within the column represents the number of follicles analyzed for that group. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± SEM, and differences were considered significant as follows: $*P<.05$; $**P<.01$; $***P<.005$ ANOVA $P<.0001$.

Regan. GH modulates fertility receptors. Fertil Steril 2018.

TABLE 2

Comparative analysis of the effect of growth hormone co-treatment on fertility.

Variable	GH−, 39–45 y, AFC D and E only	GH+, 39–45 y, AFC D and E only	*P* value
No. of women	13	10	n.s.
Age at cycle (y)	42.1 ± 2.1	41.7 ± 2.8	.615
AFC group	D and E with <6	D and E with <6	n.s.
Body mass index (kg/m^2)	24 ± 3.8	27 ± 4.6	.073
Oocytes retrieved	7.0 ± 4.7	5.3 ± 2.8	.309
Embryos transferred (double)	5 (2× ET)	5 (2× ET)	n.s.
Blastocysts	2	0	n.s.
Complete failed fertilization	2	1	n.s.
Day 3	14	14	n.s.
Frozen embryo transfers	2	0	n.s.
Total embryo transfers, fresh or frozen	16	14	n.s.
Pregnancy/ET[a]	1 (6.2)	5 (36)*	.044*
Live birth rate/ET[a]	1 (6.2)	2 (14)	.464

Note: Values are number (percentage) or mean ± SEM. n.s. = nonsignificant.
[a] Live birth rate and pregnancy rate are based on number of embryos transferred, fresh and frozen. All subsequent frozen embryo (FET) cycles were included in the analysis; therefore, the data were based on number of embryos transferred.
* $P<.05$ significant.

Regan. GH modulates fertility receptors. Fertil Steril 2018.

The level of FSHR was significantly increased in IVF patients receiving GH in 16-mm follicles compared with the same age patients of 39+ years with an ovarian reserve of D and E without GH ($P<.001$; Fig. 2B and 2B inset). The level of FSHR in GH-treated patients was also increased in the larger follicles from 4 mm to 16 mm (Fig. 2B; $P<.005$). This was followed by a significant down-regulation of the largest preovulatory follicles ($P<.01$, 19 mm).

The level of LHR was significantly increased in IVF patients receiving GH in 16-mm follicles ($P<.005$; Figs. 2C, 3C inset). The LHR density of the granulosa cells collected from patients who received GH co-treatment during an IVF cycle was also significantly elevated in the 10–16-mm follicles ($P<.01$; Fig. 2C). In contrast to the untreated group, GH co-treated patients showed down-regulation of granulosal LHR density in follicles between 16 and 19 mm in diameter ($P<.005$; Fig. 2C).

The level of BMPR1B was significantly increased in IVF patients receiving GH in 10-mm, 14-mm, and 16-mm follicles compared with the same age patients of 39+ years with an ovarian reserve of D and E without GH ($P<.001$, $P<.005$, $P<.05$, respectively; Figs. 2D, 3D inset). Granulosal BMPR1B density was significantly higher in 10-mm follicles from the GH co-treated patients compared with the larger preovulatory follicles of either 16 mm or 19 mm ($P<.05$; Fig. 2D). In contrast to the untreated group, GH co-treated patients showed down-regulation of granulosal BMPR1B density in the largest follicles of 16–19 mm ($P<.05$, $P<.05$, respectively; Fig. 2D).

When the follicles sizes are combined, the average granulosal density for GHR, FSHR, LHR, and BMPR1B was significantly higher in the GH treated group with the same ovarian reserve and age (Fig. 2A–2D inset; $P<.005$).

GH Co-treatment and Fertility in IVF Patients

The number of pregnancies was calculated according to the number of embryos that were transferred to the patients, which included subsequent frozen embryo transfer (FET) cycles of cryopreserved embryos. The number of FET cycles was low and not significantly different between the patient groups. This study was not powered to detect an improvement in live birth rate; as such it would require a larger number of women compared with the 62 women recruited for the present granulosa cell study.

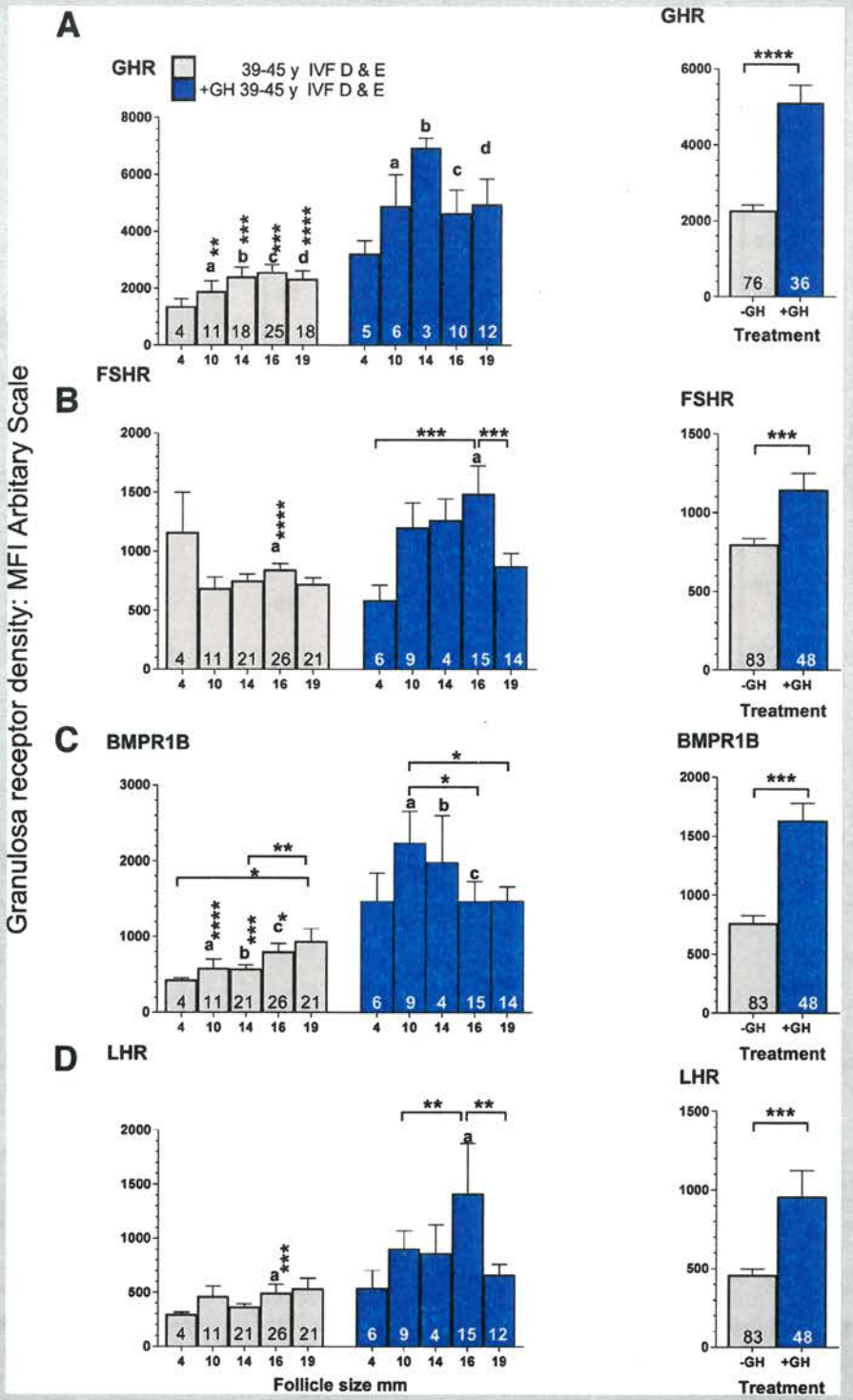
Nonetheless, some findings proved significant. Only on the basis of age, the pregnancy rate in GH-treated patients was significantly higher compared with those without GH ($P=.0143$; Table 1, Fig. 3A). The live birth rate per number of embryos transferred, fresh or frozen, for the GH group was 12.5%, compared with 3.7% without GH (Table 1, Fig. 3B). Two cryopreserved, high-quality blastocysts remain in long-term storage from the GH-treated patients (Table 1).

The pregnancy rate in GH-treated patients with both the same ovarian reserve (antral follicle count group D and E) and age group (39–45 years) was greater ($P=.044$; Table 2, Fig. 3C). The live birth rate per number of embryos transferred, fresh or frozen, for the GH group was 14%, compared with 6.2% without GH (Table 2, and Fig. 3D).

Serum and Follicular Fluid and GH Co-treatment

The level of E and P in serum and follicular fluid was not significantly different when comparing GH treatment in the equivalent older patient group of 39+ years. The results from the present study indicate that the GH co-treatment did not alter the E level of the 39+ year group cohort with an ovarian reserve of D or E during an IVF cycle (Supplemental Fig. 2A). Furthermore, neither the ratio of E was altered nor the levels of E secreted, according to either the total number of follicles or the number of follicles >14 mm present in the ovary at the time of collection, which were not significantly different. In addition, the follicular fluid concentrations of E, P, FSH, or T were not significantly different from those in

FIGURE 2

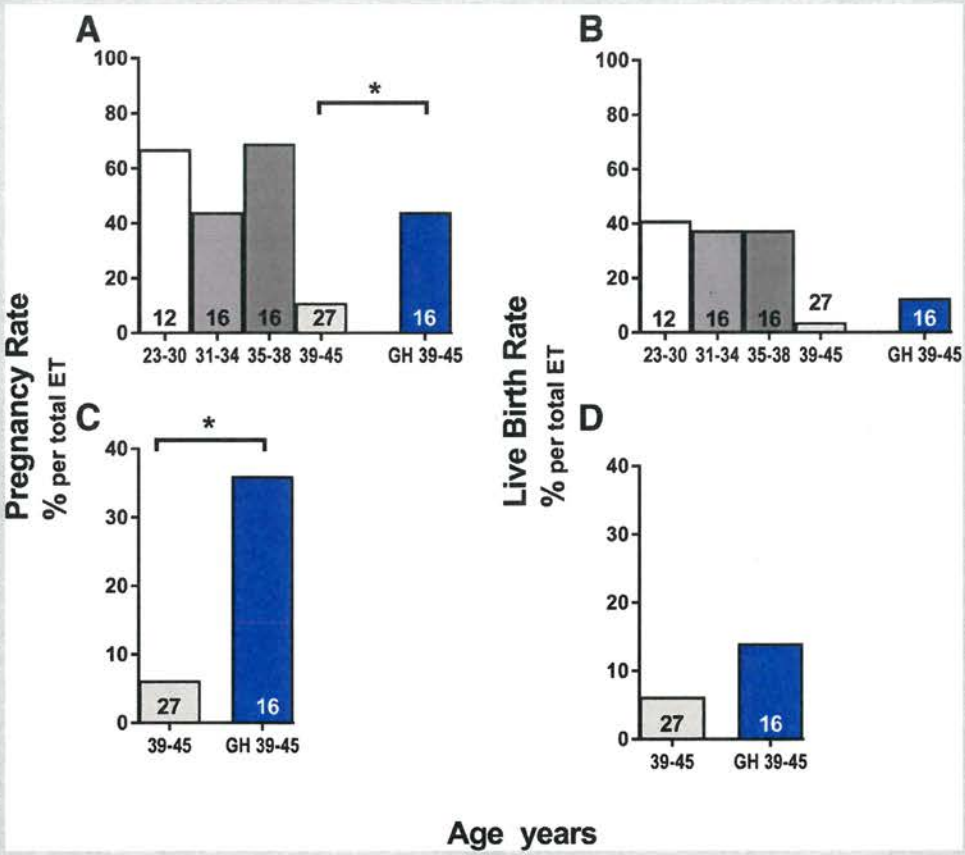


Follicle size and the granulosa cell density of GHR, FSHR, BMPR1B, and LHR in poor response 39+-year-old patients co-treated with GH. Follicles of different sizes were individually collected and analyzed. Granulosa receptor density during an IVF cycle with or without GH co-treatment was measured by flow cytometry. (**A**) Growth hormone receptor $P<.0001$. (**B**) FSHR ANOVA $P<.0001$. (**C**) BMPR1B ANOVA $P<.0001$. (**D**) LHR ANOVA $P<.0038$. The number within the column represents the number of follicles analyzed for that follicle size. **Insets**: Combined follicles of different sizes: GHR (**A**); FSHR (**B**); BMPR1B (**C**); LHR (**D**). The number within the column represents the number of follicles analyzed. Ovarian reserve measured indirectly by the antral follicle count. Antral follicle count is the number of follicles between 2 and 10 mm on day 2–5 of a cycle. Mean fluorescent intensity was obtained using an average of approximately 8,000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± SEM, and differences were considered significant as follows: $*P<.05$; $**P<.01$; $***P<.005$; $****P<.001$.

Regan. GH modulates fertility receptors. Fertil Steril 2018.

print & web 4C/FPO

FIGURE 3



Growth hormone–associated pregnancy and live birth outcome. The effect of GH co-treatment on embryo utilization rate (total embryos transferred) associated with age and growth hormone co-treatment. The data were based on the number of embryos transferred per patient age group or treatment, including subsequent frozen embryo cycles (FET). A woman with an ectopic pregnancy (classed as miscarriage) was present in the 23–30-year and the 35–38-year groups. The number in each column represents the total number of embryo transfers, fresh and frozen combined. (**A**) The effect of GH co-treatment on pregnancy rate, according to age. The χ^2 statistic *P* value is *P*=.0143 (Table 1). (**B**) The effect of GH treatment on live birth rate, according to age. The χ^2 statistic *P* value is *P*=.273 (Table 1). (**C**) The effect of GH treatment on pregnancy rate per total embryos transferred in a fresh or frozen cycle within a discrete poor prognosis group of the same age and with a similar ovarian reserve. The χ^2 statistic *P* value is *P*=.044 (Table 2). (**D**) The effect of GH treatment on the live birth rate per total embryos transferred in a fresh or frozen cycle within a discrete poor prognosis group of the same age and with a similar ovarian reserve. The χ^2 statistic *P* value is *P*=.464 (Table 2).

Regan. GH modulates fertility receptors. Fertil Steril 2018.

age-matched patients with a similar ovarian reserve who were co-treated with GH (Supplemental Fig. 2B).

DISCUSSION

Growth hormone receptors are predominantly found on the granulosa cell membrane surface and in the endoplasmic reticulum and to a lesser degree, but commonly, in the nuclear membrane of highly prolific cells (50, 51). The GHR is regulated by GH-binding proteins and indirectly by other growth factors, such as FSH, BMPs, and somatostatin (52, 53). In the present study, GH treatment induced a direct change to the receptor expression of GHR itself and indirectly to FSHR, LHR, and BMPR1B.

In support of the clinical data on aging, human granulosa receptor density and dysregulation of FSHR, LHR, and BMPR1B has been associated with ovarian depletion and reduced fertility (33, 34). We now provide additional data in support of a reversal of the dysregulated receptor expression observed in older women that occurs when they are treated with GH. In addition, depletion of the ovarian reserve was accompanied by a reduction in GHR density, whereas GH co-treatment during an IVF stimulated cycle increased the receptor density in older women who had a reduced ovarian reserve. These findings provide a possible cellular regulatory mechanism involved in the poor pregnancy and live birth rate in the older 39–45-year patients, as reported by others (18, 19, 54–56) and reviewed by others (57–59). The intention of the present prospective study was to focus on the granulosa cell receptor expression, which is novel. In a published, much larger retrospective study investigating 161 GH co-treated women over a period of approximately 8 years at the same IVF center, we reported a significant difference in the pregnancy and live birth rate of the GH-treated patients (60). This finding is despite the GH-treated group

comprising a significantly older group of patients with a poorer ovarian reserve. Because the patient sample size of 11 was small for the detection of a significant clinical outcome, the increased pregnancy rate observed was not expected, albeit consistent with our larger studies.

Evidence from our previously published work on receptor expression and the present study suggest that ovarian reserve and age are associated with reduced and dysregulated levels of receptor expression on granulosa cells. Therefore, the influence of age and ovarian reserve of subjects or animals needs to be considered as a confounding variable in historic studies (61). For example, in heifers, GH may not have resulted in any change to FSH and LH receptor binding because the cows were young, with an uncompromised ovarian reserve and a sufficient receptor density (62). The effect of GH co-treatment on receptor density in patients with a good ovarian reserve for age is unknown.

Although GH increased the receptor expression on granulosa cells from the larger follicles, it had no effect on the FSHR and LHR density at the critical time of dominant follicle selection. Previously, a poor ovarian response to rFSH stimulation has been associated with reduced granulosal FSHR expression in women (41). However, GH co-treatment was found not to alter the FSHR density in small bovine follicles, which is consistent with our findings for small human follicles (63). The lack of effect on FSHR and LHR expression of small pre-ovulatory follicles may explain why the number of oocytes collected was not increased in the present human model and by others (19, 57, 58).

Conversely, animal studies have reported an increase in oocyte number (62, 63). For example, even though more small bovine antral follicles were produced after 45 days of GH treatment in a natural cycle, the granulosal FSHRs and LHRs from pre-ovulatory bovine follicles were not affected (62, 63). This is surprising; however, the receptor-binding studies were determined only for the three largest follicles from each cow. Therefore, the expected pre-ovulatory down-regulation of these large follicles would have reduced FSHR expression, which would confound these results. Added to this, the receptor binding was not measured in any of the smaller follicles.

In other studies, GH treatment increased the receptors in the rat (64); however, in the pig, the receptor expression was reduced (65). In our human model, small antral follicles had high levels of FSHR followed by down-regulation, which coincides with dominant follicle selection. The high level of FSHRs would induce the LHR expression in a natural cycle to ensure recruitment to the dominant cohort of follicles (71). In a natural cycle, pituitary secreted FSH is reduced at this critical time, whereas in an IVF cycle, rFSH is abundant; therefore, the densities of the gonadotrophin receptors (FSHR and LHR) are pivotal in regulating follicle growth and dominance.

Patients with a reduced ovarian reserve have a poorer response to rFSH in an IVF stimulated cycle and produce fewer oocytes of poorer quality (41). The poor responder group of patients also has an associated higher risk of fetal aneuploidy that has been correlated to ovarian reserve and age (72). Recently published data have shown that GH co-treatment increases the pregnancy rate by a suggested improvement in oocyte quality, rather than an improvement to the quantity of follicles recruited (18, 19). In the present study, the women were of the same age and ovarian reserve group and recruited a similar number of oocytes. Therefore, the effect of GH was an associated increase in granulosa cell receptor expression, which resulted in an increase in pregnancy rate and live birth rate. Regulation of cell proliferation, steroid production, luteinization, ovulation, and recommencement of meiosis fundamentally resides with the functional expression of receptors in the follicle cells and oocyte. If the recruited oocyte quantity is not increased in the GH-treated group, then the focus shifts to the effect of GH on the improved quality of the oocyte and the potential to reduce detrimental changes to the DNA during follicle growth and maturation. In addition, improved receptivity of the endometrium in GH-treated women may favor successful implantation of the embryo (68). Similarly, the quality of the endometrium is pivotal to early establishment of the placenta and survival of the embryo. Growth hormone and insulin-like growth factor both signal via the JAK-STAT axis and are involved in the decidualization of the endometrium cells and proliferation and invasion of the trophoblasts (69–71). The effect of GH on the endometrium remains an under-investigated area of interest (68, 72).

A decline in granulosal BMPR1B and FSHR density occurred at the time of cyclic dominant follicle selection and again during the terminal stage of folliculogenesis, in young (23–30 years) IVF patients with good ovarian reserve (33, 34). Older patients (39+ years) with poor ovarian reserve experienced a reversal of this pattern (33, 34). In addition, the LHR density failed to be down-regulated during pre-ovulatory maturation in the 39+-year group and was reduced with ovarian reserve (34). In the present study we report increased granulosa cell GHR density in different sized follicles from IVF patients undergoing conventional ovarian stimulation in young compared with older women with a reduced ovarian reserve. In addition, we report increased granulosal GHR, FSHR, LHR, and BMPR1B receptor density in older, poor ovarian reserve patients treated with GH. Importantly, the women treated with GH demonstrated receptor expression down-regulation in the largest follicles. The down-regulation would be essential for maturation of the ovulatory follicles, luteinization, and a shift to the luteal phase.

In addition, the increased granulosal LHR density observed in the GH co-treated patients would have the potential to increase the sensitivity during the hCG/LH surge to trigger final maturation and ovulation of the oocyte (73, 74). The improved sensitivity may give rise to improved oocyte/embryo quality and live birth rate, as observed in the previously published larger retrospective study (75). In support of the link between receptor density and maturation, a previous electron microscopy study revealed that oocytes that did not fertilize had reduced levels of granulosal luteinization and were less responsive to hCG, which binds to the LHR (76).

In conclusion, the complexity and limitations of a largely observational in vivo study in humans makes it difficult to define the cellular mechanism through which numerous growth factors and pathways contribute to the regulation of follicular growth and differentiation. However, the present study has generated evidence suggesting several cellular

mechanisms that could contribute to the improved oocyte quality observed in GH co-treated IVF patients with a poor ovarian reserve.

Growth hormone co-treatment increased granulosal GHR density that would increase GHR-JAK-STAT activity and result in an increase in the intermediate products of transcription. This, in turn, could be mechanistically linked to the corresponding increase in gonadotrophin receptors and BMPR1B density observed in GH co-treated patients. Growth hormone co-treatment did not alter the gonadotrophin receptor density of the small follicles and would therefore account for the lack of improvement in the number of follicles recruited during dominant follicle selection for some women.

In contrast, GH co-treatment also restored the preovulatory down-regulation of FSHR, BMPR1B, and LHR density, which may improve the maturation process of luteinization in GH co-treated patients with reduced ovarian reserve. Combined with the latter, an increase in LHR density may improve follicle development and provide another possible cellular mechanism responsible for the improved pregnancy and live birth rate. Objectively, we remain uncertain whether the beneficial action is mediated via improved oocyte quality or other responses, such as endometrial receptivity [72, 76].

Acknowledgments: The authors thank all the participants who generously donated their samples to this study, as well as the clinical doctors, embryologists, and nursing staff.

REFERENCES

1. Gougeon A. Dynamics of follicular growth in the human: a model from preliminary results. Hum Reprod 1986;1:81–7.
2. Richards JS. Hormonal control of gene expression in the ovary. Endocr Rev 1994;15:725–51.
3. Regan SLP, Knight PG, Yovich JL, Leung Y, Arfuso F, Dharmarajan A. Granulosa cell apoptosis in the ovarian follicle—a changing view. Front Endocrinol 2018;9:1–10.
4. Hillier SG. Gonadotropic control of ovarian follicular growth and development. Mol Cell Endocrinol 2001;179:39–46.
5. Ginther OJ, Gastal EL, Gastal MO, Beg MA. Regulation of circulating gonadotropins by the negative effects of ovarian hormones in mares. Biol Reprod 2005;73:315–23.
6. Ginther OJ, Beg MA, Gastal EL, Gastal MO, Baerwald AR, Pierson RA. Systemic concentrations of hormones during the development of follicular waves in mares and women: a comparative study. Reproduction 2005; 130:379–88.
7. Austin EJ, Mihm M, Evans ACO, Knight PG, Ireland JLH, Ireland JJ, et al. Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. Biol Reprod 2001;64:839–48.
8. Campbell BK, Dobson H, Baird DT, Scaramuzzi RJ. Examination of the relative role of FSH and LH in the mechanism of ovulatory follicle selection in sheep. J Reprod Fertil 1999;117:355–67.
9. Ginther OJ, Khan FA, Hannan MA, Rodriguez MB, Pugliesi G, Beg MA. Role of LH in luteolysis and growth of the ovulatory follicle and estradiol regulation of LH secretion in heifers. Theriogenology 2012;77:1442–52.
10. Luo W, Gumen A, Haughian J, Wiltbank M. The role of luteinizing hormone in regulating gene expression during selection of a dominant follicle in cattle. Biol Reprod 2011;84:369–78.
11. Picton HM, McNeilly AS. Evidence to support a follicle-stimulating hormone threshold theory for follicle selection in ewes chronically treated with gonadotrophin-releasing hormone agonist. J Reprod Fertil 1991;93:43–51.
12. Minegishi T, Tano M, Abe Y, Nakamura K, Ibuki Y, Miyamoto K. Expression of luteinizing hormone/human chorionic gonadotrophin (LH/HCG) receptor mRNA in the human ovary. Mol Hum Reprod 1997;3:101–7.
13. Yung Y, Aviel-Ronen S, Maman E, Rubinstein N, Avivi C, Orvieto R, et al. Localization of luteinizing hormone receptor protein in the human ovary. Mol Hum Reprod 2014;20:844–9.
14. Mihm M, Baker PJ, Ireland JLH, Smith GW, Coussens PM, Evans ACO, et al. Molecular evidence that growth of dominant follicles involves a deduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. Biol Reprod 2006;74:1051–9.
15. Regan S, Knight PG, Yovich JL, Yeung Y, Arfuso F, Dharmarajan A. Involvement of bone morphogenetic proteins (BMP) in the regulation of ovarian function. Vitam Horm 2018;107:227–61.
16. Klein NA, Battaglia DE, Fujimoto VY, Davis GS, Bremner WJ, Soules MR. Reproductive aging: accelerated ovarian follicular development associated with a monotropic follicle-stimulating hormone rise in normal older women. J Clin Endocrinol Metab 1996;81:1038–45.
17. Hansen KR, Hodnett GM, Knowlton N, Craig LB. Correlation of ovarian reserve tests with histologically determined primordial follicle number. Fertil Steril 2011;95:170–5.
18. Yovich JL, Stanger JD. Growth hormone supplementation improves implantation and pregnancy productivity rates for poor-prognosis patients undertaking IVF. Reprod Biomed Online 2010;21:37–49.
19. Tesarik J, Hazout A, Mendoza C. Improvement of delivery and live birth rates after ICSI in women aged >40 years by ovarian co-stimulation with growth hormone. Hum Reprod 2005;20:2536–41.
20. de Ziegler D, Streuli I, Meldrum D, Chapron C. The value of growth hormone supplements in ART for poor ovarian responders. Fertil Steril 2011;96:1069–76.
21. Kyrou D, Kolibianakis E, Venetis C, Papanikolaou E, Bontis J, Tarlatzis B. How to improve the probability of pregnancy in poor responders undergoing in vitro fertilization: a systematic review and meta-analysis. Fertil Steril 2009;91:749–66.
22. Folch J, Ramon JP, Cocero MJ, Alabart JL, Beckers JF. Exogenous growth hormone improves the number of transferable embryos in superovulated ewes. Theriogenology 2001;55:1777–85.
23. Barreca A, Artini PG, Del Monte P, Ponzani P, Pasquini P, Cariola G, et al. In vivo and in vitro effect of growth hormone on estradiol secretion by human granulosa cells. J Clin Endocrinol Metab 1993;77:61–7.
24. Izadyar F, Colenbrander B, Bevers MM. In vitro maturation of bovine oocytes in the presence of growth hormone accelerates nuclear maturation and promotes subsequent embryonic development. Mol Reprod Dev 1996;45:372–7.
25. Izadyar F, Zeinstra E, Bevers MM. Follicle-stimulating hormone and growth hormone act differently on nuclear maturation while both enhance developmental competence of in vitro matured bovine oocytes. Mol Reprod Dev 1998;51:339–45.
26. Izadyar F, Zhao J, Van Tol HT, Colenbrander B, Bevers MM. Messenger RNA expression and protein localization of growth hormone in bovine ovarian tissue and in cumulus oocyte complexes (COCs) during in vitro maturation. Mol Reprod Dev 1999;53:398–406.
27. Abir R, Garor R, Felz C, Nitke S, Krissi H, Fisch B. Growth hormone and its receptor in human ovaries from fetuses and adults. Fertil Steril 2008;90: 1333–9.
28. Izadyar F, Hage WJ, Colenbrander B, Bevers MM. The promotory effect of growth hormone on the developmental competence of in vitro matured bovine oocytes is due to improved cytoplasmic maturation. Mol Reprod Dev 1998;49:444–53.
29. Izadyar F, Van Tol HT, Colenbrander B, Bevers MM. Stimulatory effect of growth hormone on in vitro maturation of bovine oocytes is exerted through cumulus cells and not mediated by IGF-I. Mol Reprod Dev 1997;47:175–80.
30. Bevers MM, Izadyar F. Role of growth hormone and growth hormone receptor in oocyte maturation. Mol Cell Endocrinol 2002;197:173–8.
31. Lan H, Li W, Fu Z, Yang Y, Wu T, Liu Y, et al. Differential intracellular signalling properties of the growth hormone receptor induced by the activation of an anti-GHR antibody. Mol Cell Endocrinol 2014;390:54–64.
32. Waters MJ, Hoang HN, Fairlie DP, Pelekanos RA, Brown RJ. New insights into growth hormone action. J Mol Endocrinol 2006;36:1–7.

33. Regan SLP, Knight PG, Yovich J, Stanger J, Leung Y, Arfuso F, et al. Dysregulation of granulosal bone morphogenetic protein receptor 1B density is associated with reduced ovarian reserve and the age-related decline in human fertility. Mol Cell Endocrinol 2016;425:84–93.
34. Regan SLP, Knight PG, Yovich JL, Stanger JD, Leung Y, Arfuso F, et al. Infertility and ovarian follicle reserve depletion are associated with dysregulation of the FSH and LH receptor density in human antral follicles. Mol Cell Endocrinol 2017;446:40–51.
35. Lujan ME, Jarrett BY, Brooks ED, Reines JK, Peppin AK, Muhn N, Haider E, Pierson RA, Chizen DR. Updated ultrasound criteria for polycystic ovary syndrome: reliable thresholds for elevated follicle population and ovarian volume. Human Reproduction 2013;28:1361–8.
36. Yovich JL, Alsbjerg B, Conceicao JL, Hinchliffe PM, Keane KN. PIVET rFSH dosing algorithms for individualized controlled ovarian stimulation enables optimized pregnancy productivity rates and avoidance of ovarian hyperstimulation syndrome. Drug Des Devel Ther 2016;10:2561–73.
37. Yovich J, Stanger J, Hinchliffe P. Targeted gonadotrophin stimulation using the PIVET algorithm markedly reduces the risk of OHSS. Reprod Biomed Online 2012;24:281–92.
38. Regan SLP, Knight PG, Yovich JL, Stanger JD, Leung Y, Arfuso F, et al. The effect of ovarian reserve and receptor signalling on granulosa cell apoptosis during human follicle development. Mol Cell Endocrinol 2018; 470:219–27.
39. Al-Samerria S, Almahbobi G. Three-dimensional image analysis to quantify the temproro-smacial expression of cellular receptors. J Med Bioeng 2014;3:179–82.
40. Weall BM, Al-Samerria S, Conceicao J, Yovich JL, Almahbobi G. A direct action for GH in improvement of oocyte quality in poor-responder patients. Reproduction 2015;149:147–54.
41. Cai J, Lou H, Dong M, Lu X, Zhu Y, Gao H, et al. Poor ovarian response to gonadotropin stimulation is associated with low expression of follicle-stimulating hormone receptor in granulosa cells. Fertil Steril 2007;87:1350–6.
42. Gao S, De Geyter C, Kossowska K, Zhang H. FSH stimulates the expression of the ADAMTS-16 protease in mature human ovarian follicles. Mol Hum Reprod 2007;13:465–71.
43. Pidoux G, Gerbaud P, Tsatsaris V, Marpeau O, Ferreira F, Meduri G, et al. Biochemical characterization and modulation of LH/CG—receptor during human trophoblast differentiation. J Cell Physiol 2007;212:26–35.
44. Abir R, Ben-Haroush A, Melamed N, Felz C, Krissi H, Fisch B. Expression of bone morphogenetic proteins 4 and 7 and their receptors IA, IB, and II in human ovaries from fetuses and adults. Fertil Steril 2008;89:1430–40.
45. Haÿ E, Lemonnier J, Fromigué O, Guénou H, Marie PJ. Bone morphogenetic protein receptor IB signaling mediates apoptosis independently of differentiation in osteoblastic cells. J Biol Chem 2004;279:1650–8.
46. Bozzola M, Zecca M, Locatelli F, Radetti G, Pagani S, Autelli M, et al. Evaluation of growth hormone bioactivity using the Nb2 cell bioassay in children with growth disorders. J Endocrinol Invest 1998;21:765–70.
47. ~~Weall BM, Al-Samerria S, Conceicao J, Yovich JL, Almahbobi G. A direct action for growth hormone in improving oocyte quality in poor responder patients, Reproduction; 2014.~~
48. Regan SLP, McFarlane JR, O'Shea T, Andronicos N, Arfuso F, Dharmarajan A, et al. Flow cytometric analysis of FSHR, BMPR1B, LHR and apoptosis in granulosa cells and ovulation rate in merino sheep. Reproduction 2015;150:151–63.
49. Hermann BP, Heckert LL. Transcriptional regulation of the FSH receptor: new perspectives. Mol Cell Endocrinol 2007;260–262:100–8.
50. Brooks AJ, Wooh JW, Tunny KA, Waters MJ. Growth hormone receptor; mechanism of action. Int J Biochem Cell Biol 2008;40:1984–9.
51. Zhu T, Goh EL, Graichen R, Ling L, Lobie PE. Signal transduction via the growth hormone receptor. Cell Signal 2001;13:599–616.
52. LeRoith D, Yakar S. Mechanisms of disease: metabolic effects of growth hormone and insulin-like growth factor 1. Nat Clin Pract Endocrinol Metabol 2007;3:302–10.
53. Nakamura E, Otsuka F, Inagaki K, Miyoshi T, Matsumoto Y, Ogura K, et al. Mutual regulation of growth hormone and bone morphogenetic protein system in steroidogenesis by rat granulosa cells. Endocrinology 2012;153: 469–80.
54. Volpe A, Coukos G, Barreca A, Artini PG, Minuto F, Giordano G, et al. Ovarian response to combined growth hormone-gonadotropin treatment in patients resistant to induction of superovulation. Gynecol Endocrinol 1989;3:125–33.
55. Eftekhar M, Aflatoonian A, Mohammadian F, Eftekhar T. Adjuvant growth hormone therapy in antagonist protocol in poor responders undergoing assisted reproductive technology. Arch Gynecol Obstet 2013;287:1017–21.
56. Levy T, Limor R, Villa Y, Eshel A, Eckstein N, Vagman I, et al. Another look at co-treatment with growth hormone and human menopausal gonadotrophins in poor ovarian responders. Hum Reprod 1993;8:834–9.
57. Homburg R, Singh A, Bhide P, Shah A, Gudi A. The re-growth of growth hormone in fertility treatment: a critical review. Hum Fertil 2012;15:190–3.
58. Kolibianakis EM, Venetis CA, Diedrich K, Tarlatzis BC, Griesinger G. Addition of growth hormone to gonadotrophins in ovarian stimulation of poor responders treated by in-vitro fertilization: a systematic review and meta-analysis. Hum Reprod Update 2009;15:613–22.
59. Kyrou D, Kolibianakis EM, Venetis CA, Papanikolaou EG, Bontis J, Tarlatzis BC. How to improve the probability of pregnancy in poor responders undergoing in vitro fertilization: a systematic review and meta-analysis. Fertility and Sterility 2009;91:749–66.
60. Keane KN, Yovich JL, Hamidi A, Hinchliffe PM, Dhaliwal SS. Single-centre retrospective analysis of growth hormone supplementation in IVF patients classified as poor-prognosis. BMJ Open 2017;7.
61. Suikkari A, MacLachlan V, Koistinen R, Seppälä M, Healy D. Double-blind placebo controlled study: human biosynthetic growth hormone for assisted reproductive technology. Fertil Steril 1996;Apr 65:800–5.
62. Gong JG, Bramley T, Webb R. The effect of recombinant bovine somatotropin on ovarian function in heifers: follicular populations and peripheral hormones. Biol Reprod 1991;45:941–9.
63. Garverick H, Baxter G, Gong J, Armstrong D, Campbell B, Gutierrez C, et al. Regulation of expression of ovarian mRNA encoding steroidogenic enzymes and gonadotrophin receptors by FSH and GH in hypogonadotrophic cattle. Reproduction 2002;123:651–61.
64. Jia XC, Kalmijn J, Hsueh AJW. Growth hormone enhances follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. Endocrinology 1986;118:1401–9.
65. Spicer LJ, Klindt J, Buonomo FC, Maurer R, Yen JT, Echternkamp SE. Effect of porcine somatotropin on number of granulosa cell luteinizing hormone/human chorionic gonadotropin receptors, oocyte viability, and concentrations of steroids and insulin-like growth factors I and II in follicular fluid of lean and obese gilts. J Anim Sci 1992;70: 3149–57.
66. Rice S, Ojha K, Whitehead S, Mason H. Stage-specific expression of androgen receptor, follicle-stimulating hormone receptor, and anti-müllerian hormone type ii receptor in single, isolated, human preantral follicles: relevance to polycystic ovaries. J Clin Endocrinol Metab 2007;92: 1034–40.
67. Grande M, Borobio V, Jimenez JM, Bennasar M, Stergiotou I, Peñarrubia J, et al. Antral follicle count as a marker of ovarian biological age to reflect the background risk of fetal aneuploidy. Hum Reprod 2014;29:1337–43.
68. Wang XM, Jiang H, Zhang WX, Li Y. The effects of growth hormone on clinical outcomes after frozen-thawed embryo transfer. Int J Gynecol Obstet 2016;133:347–50.
69. Borg AJ, Yong HE, Lappas M, Degrelle SA, Keogh RJ, Da Silva-Costa F, et al. Decreased STAT3 in human idiopathic fetal growth restriction contributes to trophoblast dysfunction. Reproduction 2015;149:523–32.
70. Kajimura S, Aida K, Duan C. Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation. Proc Natl Acad Sci U S A 2005;102:1240–5.
71. Pollheimer J, Haslinger P, Fock V, Prast J, Saleh L, Biadasiewicz K, et al. Endostatin suppresses IGF-II-mediated signaling and invasion of human extravillous trophoblasts. Endocrinology 2011;152:4431–42.

72. Altmäe S, Mendoza-Tesarik R, Mendoza C, Mendoza N, Cucinelli F, Tesarik J. Effect of growth hormone on uterine receptivity in women with repeated implantation failure in an oocyte donation program: a randomized controlled trial. J Endocr Soc 2018;2:96–105.
73. Greisen S, Ledet T, Ovesen P. Effects of androstenedione, insulin and luteinizing hormone on steroidogenesis in human granulosa luteal cells. Hum Reprod 2001;16:2061–5.
74. Donadeu F, Ascoli M. The differential effects of the gonadotropin receptors on aromatase expression in primary cultures of immature rat granulosa cells are highly dependent on the density of receptors expressed and the activation of the inositol phosphate cascade. Endocrinology 2005;146: 3907–16.
75. Rotmensch S, Dor J, Furman A, Rudak E, Mashiach S, Amsterdam A. Ultrastructural characterization of human granulosa cells in stimulated cycles: correlation with oocyte fertilizability. Fertil Steril 1986; 45:671–9.
76. Horcajadas JA, Riesewijk A, Polman J, van Os R, Pellicer A, Mosselman S, et al. Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. Mol Hum Reprod 2004; 11:195–205.