Aging-related premature luteinization of granulosa cells is avoided by early oocyte retrieval

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Abstract

Why IVF pregnancy rates decline sharply after age 43 is unknown. In this study, we compared granulosa cell (GC) function in young oocyte donors ($n=31$, ages 21–29), middle-aged ($n=64$, ages 30–37) and older infertile patients ($n=41$, ages 43–47). Gene expressions related to gonadotropin activity, steroidogenesis, apoptosis and luteinization were examined by real-time PCR and western blot in GCs collected from follicular fluid. FSH receptor (*FSHR*), aromatase (*CYP19A1*) and 17β-hydroxysteroid dehydrogenase (*HSD17B*) expression were found down regulated with advancing age, while LH receptor (*LHCGR*), P450scc (*CYP11A1*) and progesterone receptor (*PGR*) were up regulated. Upon *in vitro* culture, GCs were found to exhibit lower proliferation and increased apoptosis with aging. While FSH supplementation stimulated GCs growth and prevented luteinization *in vitro*. These observations demonstrate age-related functional declines in GCs, consistent with premature luteinization. To avoid premature luteinization in women above age 43, we advanced oocyte retrieval by administering human chorionic gonadotropin at maximal leading follicle size of 16 mm (routine 19–21 mm). Compared to normal cycles in women of similar age, earlier retrieved patients demonstrated only a marginal increase in oocyte prematurity, yet exhibited improved embryo numbers as well as quality and respectable clinical pregnancy rates. Premature follicular luteinization appears to contribute to rapidly declining IVF pregnancy chances after age 43, and can be avoided by earlier oocyte retrieval.

Key Words
- premature luteinization
- granulosa cell
- oocyte
- *in vitro* fertilization
- early retrieval

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Introduction

Effects of female reproductive aging on assisted reproductive technologies are widely acknowledged. Age-related gradual declines in implantation and pregnancy rates (van Noord-Zaadstra *et al.* 1991) as well as increases in spontaneous miscarriages (Belloc *et al.* 2008, Grande *et al.* 2012) were observed. Declines in oocyte quantity and quality are considered principal driving forces through as yet ill-defined mechanisms (Navot *et al.* 1991).

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Based on annual reports to the Centers for Disease Control and Prevention (CDC), as required under a federal statute from IVF centers in the United States, our center is distinguished from most in serving the oldest IVF patient population. CDC reports further establish that with respect to the oldest age group of patients seeking infertility treatments (>43 years), for which almost no national data are available, our center is unique in accommodating a disproportionate number of patients of advanced age.

While our center's IVF outcomes are reflective of the gradual decline in pregnancy rates normally observed with advancing female age, we have noted that this decline after age 43 sharply increases. Mechanisms leading to accelerated loss of ovarian function are unknown undoubtedly, however, related to the poor reproductive performance in women above age 43.

Accordingly, we hypothesized that fast decreasing IVF pregnancy rates in older patients reflect poor ovarian environments, which adversely impacts oocyte/embryo quality and IVF outcomes (5). Specifically, we predicted that changes in immediate ovaria environments of oocytes, represented by granulosa cells (GCs), should be identifiable by comparing cell function at different maternal ages. Rapid declines in IVF pregnancy rates after age 43 may, therefore, reflect age-related functional decline in ovarian cells. These changes in the ovarian environment should be discoverable by comparing GC function.

Oocytes in primordial follicles remain arrested in meiotic prophase I until recruited for oogenesis. Oocytes and accompanying GCs engage and maintain a symbiotic relationship (Buccione *et al*. 1990). GCs form the follicular microenvironment, which facilitates oocyte development, supplies energy, disposes of waste and participates in molecular signaling. If GC function becomes impaired with advancing age, oocyte growth and competence will be compromised in parallel. For example, GCs synthesize and transport energy substrates, nucleotides and amino acids into oocytes (Buccione *et al*. 1990).

Using an *in vitro* oocyte growth model, Schultz *et al*. demonstrated that oocyte growth positively correlated with the number of adherent cumulus cells (CCs, representing more differentiated GCs) and the extent of metabolic cooperation between them (Brower & Schultz 1982, Herlands & Schultz 1984). Transcription in oocytes depends on the presence of attached CCs (De La Fuente & Eppig 2001). In addition, the maintenance of oocyte arrest before recruitment also relies on the contribution of autocrine and paracrine factors synthesized in GCs, including cAMP/cGMP (Webb *et al*. 2002, Wigglesworth *et al*. 2013, Shuhaibar *et al*. 2015), purine (Downs 1993), kit ligand (Ye *et al*. 2009), and NPR2 (Zhang *et al*. 2010, Tsuji *et al*. 2012, Wigglesworth *et al*. 2013). Oocytes from antral follicles resume and complete meiosis spontaneously after removal of surrounding CCs (Buccione *et al*. 1990, Mehlmann 2005), suggesting that CCs control oocyte nuclear maturation.

Cytoplasmic maturation of oocytes also depends on CCs and GCs. Cumulus-oocyte complexes can mature and support embryo development after *in vitro* maturation (IVM). Removal of CCs before culture, resulting in denuded oocytes (DOs), leads to impaired oocyte and embryo development (Buccione *et al*. 1990). One striking example of metabolic cooperation between oocytes and surrounding CCs pertains to glutathione synthesis. Because glutathione derived from GCs and CCs is required for sperm decondensation and male pronucleus formation, lack of ability to produce glutathione in DOs restrains their development (Perreault *et al*. 1988, Zhou *et al*. 2008, 2010). Hence, when DOs are co-cultured with GC monolayer during IVM, glutathione levels are restored and developmental competence is reestablished (Zhou *et al*. 2008, 2010). Normal growth and maturation of the oocyte is thus a direct reflection of physiological status of the GCs.

Certain defects of gene expression result in loss of GC function, which in turn can lead to reproductive dysfunctions. For example, follicle stimulating hormone receptor (*Fshr*) knockout in mouse GCs results in infertility due to lack of antral follicles (Dierich *et al*. 1998). Similarly, knockout of aromatase (*Cyp19a1*) (Fisher *et al*. 1998), IGF1 (Baker *et al*. 1996), estrogen receptor β (*Esr2*) (Couse *et al*. 2005) and androgen receptor (*Ar*) (Sen & Hammes 2010) in GCs leads to premature ovarian aging (POA) and female subfertility/infertility. To further prove the importance of GCs, Seifer and Sadraie reported significantly higher percentages of apoptotic GCs in infertile women, diagnosed with low functional ovarian reserve (Seifer *et al*. 1996, Sadraie *et al*. 2000). Other investigators reported diminished proliferation (Seifer *et al*. 1993), and high levels of mitochondrial DNA deletions (Seifer *et al*. 2002) in GCs of aged IVF patients. All of these abnormalities in GCs have the potential of contributing to decreased reproductive success in older women.

Our study, therefore, reports on functional attributes of GCs derived from three groups of women: young oocyte donors (Group 1), middle-aged infertile women (Group 2) and old infertile women above age 43 (Group 3). As we demonstrate, in Group 3, a significant decline in GC

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function is detected that exhibits characteristics of premature luteinization. Based on these findings, we also report on results of a clinical pilot study of early oocyte retrieval in Group 3, which appear to ameliorate the negative impact of premature luteinization. This discovery provides insights into ovarian aging, and offers alternative strategies for improving pregnancy chances in older women.

Materials and methods

Patient populations and institutional review board

The Institutional Review Board of the Center for Human Reproduction (CHR) approved this study in expedited review.

Based on age, three distinct age groups were investigated (Table 1): oocyte donors represented the youngest (Group 1; $n=31$). By definition, they are young and carefully selected, meeting age-specific ovarian reserve parameters. Group 2 represented young to middle-aged infertility patients within an age range of 28–38 years ($n=64$) while Group 3 included the oldest infertility patients at 43–47 years ($n=41$).

Patient and IVF cycle characteristics are shown in Table 1. All subjects underwent controlled ovarian hyperstimulation and oocyte maturation by human chorionic gonadotropin (hCG) according to previously described protocols (Gleicher & Barad 2011, Gleicher *et al.* 2013), followed by transvaginal ultrasound-guided oocyte retrieval. hCG was administered when leading follicles reached 19–21 mm. Oocyte donors were stimulated in a long gonadotropin releasing hormone agonist cycle (GnRHa, Lupron, leuprolide acetate, Takeda Pharmaceutical USA, Inc., Deerfield, IL, USA) with daily dosages of 150–300 IU of human menopausal gonadotropin (hMG) from various manufacturers. In contrast, infertility patients were stimulated in microdose agonist cycles (Lupron) with daily dosages of 450–600 IU of gonadotropins, typically in a majority (300–450 IU) administered as FSH, and in a minority (150 IU) as hMG.

Follicular fluid was collected at time of oocyte retrieval only from follicles 15 mm or larger.

Oocyte/embryo assessment and fertilization

All media and reagents for IVF were purchased from LifeGlobal (Guilford, CT, USA). Oocytes collected at retrievals were cultured in HTF medium containing 10% human serum albumin (HSA) for 2 h before insemination. After removal of cumulus by hyaluronidase treatment, oocytes were assessed according to morphology. Oocytes with obvious first polar body (1st Pb) were identified as mature (MII); oocytes without 1st Pb were identified as immature (MI & GV); oocytes with brown dark color, cytoplasmic fragments and/or broken membranes were identified as atretic. Only MII oocytes were used for insemination.

Fertilized embryos were cultured *in vitro* in Blastocyst medium (LifeGlobal) for 3 days, then were assessed according to their morphology. Embryos with 4–12 blastomeres of equal size and minimal cytoplasmic fragmentation were identified as good embryos, and designated as suitable for transfer or cryopreservation.

Hormone measurement

All serum hormone concentrations of patients were examined with AIA 900 Automated Immunoassay Analyzer (Tosoh, Minato, Japan) by following the instruction of user manual except AMH. Basic concentrations of FSH and

Table 1 Patient populations and cycle characteristics

	Group 1 Donors ($n=31$)	Group 2 Intermediate age infertility patients ($n=64$)	Group 3 Older infertility patients ($n=41$)
Average age (years)	24.4 ± 0.52^{a}	34.1 ± 0.38^{b}	44.3 ± 0.23^{c}
FSH (mIU/ml)	6.3 ± 0.23^{a}	7.6 ± 0.57^{a}	10.3 ± 0.34^{b}
AMH (ng/ml)	3.1 ± 0.23^{a}	2.8 ± 0.26^{a}	0.28 ± 0.08^{b}
Number of follicles/cycle	22.5 ± 8.3^{a}	10.5 ± 7.1^{b}	6.8 ± 5.1^{c}
Number of oocytes retrieved/cycle	20.6 ± 1.2^{a}	9.8 ± 2.5^{b}	5.2 ± 1.3^{c}
Number of MII oocytes retrieved/cycle	15.5 ± 5.2^{a}	7.1 ± 2.3^{b}	3.6 ± 0.8^{c}
Number of artretic oocytes retrieved/cycle	1.3 ± 0.4^{a}	0.9 ± 0.2^{ab}	0.6 ± 0.1^{b}
Number of embryo $\geq$4 cells	15.5 ± 4.6^{a}	7.1 ± 0.89^{b}	3.6 ± 20^{c}
Pregnant rate/cycle	16 (51.6%)^{a}	22 (34.4%)^{b}	3 (7.3%)^{c}
Progesterone/estradiol ratio	0.26 ± 0.08^{a}	0.5 ± 0.15^{b}	1.96 ± 0.47^{c}

Values with same letters in their superscripts in same row were not different significantly ($P>0.05$). *n*, number of patients.

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AMH were determined on day 2 or day 3 of menstrual cycle. Progesterone (P_4) and estradiol (E_2) were measured on the hCG administration day. Regarding the user manual and installment instruction from the technique supports, coefficient of variation (CV) was performed by running one sample 20 times, then was calculated using the following equation: CV = (S.D.) (100)/mean. The results were analyzed by Tosoh technique support and listed as follows: CV (FSH) = 1.1%; CV (E_2) = 2.1; CV (P_4) = 2.2. All CV values were verified as normal by Tosoh. The P_4/E_2 ratio was calculated as P_4 (in ng/ml) × 1000/E_2 (in pg/ml). Serum AMH was measured commercially (LabCorp., Ramsey, NJ, USA).

GC isolation

Following retrieval, clumps of GCs were removed from follicular fluid. To avoid blood contamination, collected GCs were washed twice in D-PBS (Zenith Biotech, Guilford, CT, USA) by centrifugation (326 ***g***, 5 min), and following PBS removal GCs pellets were either frozen at −80 ° for future use or prepared for *in vitro* culture.

RNA extraction and real-time PCR

Total RNA was extracted using TRIzol-reagent (Invitrogen) and 1 μg of RNA was reverse transcribed using RT enzyme (Invitrogen). cDNA amplification and quantification of PCR products was done with the StepOne real-time PCR system (Applied Biosystems) according to the manufacturer's instructions using Sybr Green (Invitrogen). Standard PCR settings (95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min, then dissociation stage for 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C, and 15 s at 60 °C) were used. PCR primers and product information of all tested genes are listed in Supplementary Table 1, see section on supplementary data given at the end of this article. To avoid DNA contamination in PCR, primers pair must be separated by at least one intron (at least 5000 bp) and the corresponding genomic DNA. The specific PCR amplifications were validated by running melting curve analysis and gel analysis. The primers that have only one PCR product with correct size were chosen for the study. The efficiency of amplification was determined by running standard curve (efficiency of assay: 90–105%; R2 > 0.98; all Cq values were similar). Each sample was run in duplicate. For each target gene, the number of mRNA molecules was calculated and expressed relative to ribosomal protein L19 (*RPL19*) reference mRNA. To compare and calculate results from different PCR running, the normalization was performed as follows: each PCR run included a reference cDNA sample as control, which was made by mixing ten different patients' GCs. The results from different PCR runs were calculated according to these reference samples and final average gene expressions were then analyzed statistically.

Western blot analysis

All antibodies were purchased from Santa Cruz. GCs were homogenized in Ripa buffer (Sigma), and protein was purified as described by instructions. Protein concentrations were determined by using the Pierce BCA protein kit (Thermo Fisher Scientific, Rockford, IL, USA). Gel runs separated 20 μg of total protein and Ripa buffer was used as negative control. After electric transfer, membranes were blocked for 2 h with 5% nonfat dry milk. Then membranes were incubated overnight at 4 °C with anti-CYP19A1 (sc-130733, 50 kDa) (1:500), anti-FSHR (sc-13935, 75 kDa) (1:500), anti-LHCGR (sc-25828, 85 kDa) (1:250), anti-BCL2 (sc-492, 26 kDa) (1:500) or anti-ACTB (sc-47778, 34 kDa) (1:500) antibody. After wash, secondary antibodies, conjugated to HRP (sc-2004) (1:10 000) were incubated for 2 h with membranes. Protein bands were visualized by incubating the membranes with Immoblot (Millipore Corp., Billerica, MA, USA). The specific bands were recognized regarding the expected sizes on the blot. Band densities were determined and normalized against the beta actin (ACTB) signal using Image J software (NIH, Bethesda, MD, USA).

GC culture

Isolated GCs were seeded into six-well plates (BD Bioscience, San Jose, CA, USA) at density of 10×10^5/ml in DMEM/F12 containing 10% FBS, followed by incubation for 8 h at 37 ° with 5% CO_2 to allow GC attachment. To remove cell debris and serum factors, cultures were washed twice and incubated in serum-free DMEM/F12, containing 2 mg/ml of HSA (LifeGlobal), 2 mM glutamine (Life Technologies) and 1× Insulin-Transferin-Selenium X (Life Technologies). After overnight culture, medium was replaced once more. GCs were cultured for 1, 3 or 5 days, and medium was changed every 48 h.

Cell proliferation and apoptosis assays

Cell proliferation analyses were performed by using Vybrant MTT Cell Proliferation Assay Kit (Life Technologies). Briefly, GCs were seeded at densities of

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5000 cells/well in 96-well plates. Following a medium change, 10 μl of 12 mM MTT stock solution was added to each well and the plate was incubated at 37 °C for 4 h. Then 100 μl of SDS–HCl was added, and following another 4 h of incubation absorbance of each well was read at 570 nm, using a micro-plate reader (Tecan, Mannedorf, Switzerland). Apoptosis was determined in GCs plated at the density previously described in four-well chamber slides (Thermo Fisher Scientific). Cultured GCs were fixed by adding in 1 ml D-PBS containing 4% paraformaldehyde for 15 min. Slides were then labeled with 10 μg/ml 4,6-diamidino-2-phenylindole-2-HCL (DAPI) (Sigma) for 10 min, and nuclear morphology was assessed using fluorescence microscopy. Apoptotic GCs, exhibiting distinct fragmented nuclei, were counted, and the apoptotic ratio was calculated for each treatment group based on number of apoptotic cells out of a total of 200 cells/slide.

Pilot study of early oocyte retrieval

Given the results of the previously described experiments, we hypothesized that the oldest patients (Group 3) might benefit clinically if their risk of premature luteinization could be curtailed or completely avoided. Therefore, we reasoned that development of premature luteinization could be pre-empted by scheduling oocyte retrieval earlier. We report here a preliminary summary of such an early retrieval group (ERG), that included 71 consecutive IVF cycles in women above age 43 (mean age 44.8±0.3 years), for which cycle outcomes could be compared to a reference group of 91 women above age 43, who in the preceding year had been treated with normal retrieval timing (NRG, mean age 44.3±0.15 years).

The ERG received identical stimulation as previously described for the infertile patients (Groups 2 and 3) and, therefore, identical stimulation to the NRG control group. What distinguished these groups, however, was the timing of hCG administration. While NRG patients had been triggered with hCG at leading follicle size of 19–21 mm, the ERG group was triggered at 16 mm. Otherwise, IVF cycles were identical.

Statistical analysis

All statistical analyses were performed using Prism software (GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA, followed by the Tukey test was used for the statistical analysis of real-time PCR, western blot, MTT assay and cell number calculations. Unpaired *t*-tests with Welch's correction was used for statistical comparison of clinical data between ERG and NRG patients. The data in all tables and figures are shown as value±S.E.M. Values were considered statistically significant at $P<0.05$.

Results

Patient populations and cycle characteristics

Patient and IVF cycle characteristics are summarized in Table 1. Mean ages were 24.4±0.52 years for Group 1, 34.1±0.38 years Group 2 and 44.3±0.23 years for Group 3. The table also demonstrates expected increases in FSH and decreases in AMH values with advancing age as well as declining oocyte/embryo numbers and pregnancy rates. Thus, Group 3 clearly reflected the lowest reproductive potential.

The table also reports serum P_4 to E_2 ratios (P_4/E_2) in all three groups. The P_4/E_2 was significantly higher in Group 3, while there was no significant difference between Groups 1 and 2. An elevated P_4/E_2 is a well known marker of premature luteinization (Ozcakir *et al.* 2004) and, therefore, suggested that older patients might be at higher risk for premature luteinization than the other two groups.

Impact of maternal aging on gene expression in human GCs

To determine the impact of maternal aging, we quantified expressions of gonadotropin and sex hormone receptors in GCs. *FSHR* expression was significantly lower in Group 3 patients than Group 1 and Group 2 (Fig. 1A), while, in contrast, LH receptor (*LHCGR*) expression was significantly higher in Group 3 than Groups 1 and 2 (Fig. 1B). Expressions of estrogen receptor β (ESR2) (Fig. 1C) and androgen receptor (AR) (Fig. 1D) did not differ between the three groups. Down-regulation of *FSHR* and up-regulation of *LHCGR* mRNA levels in older patients were then confirmed by western blot (Fig. 2 A, C and D).

To define the steroidogenic activity of GCs with advancing age, mRNA expression of steroidogenic enzymes was analyzed. Aromatase (*CYP19A1*) (Fig. 1E) and 17β-HSD (*HSD17B*) (Fig. 1H) were expressed significantly lower in Group 3 women, while P450scc (*CYP11A1*) (Fig. 1F) was expressed higher. In contrast, expression of the steroidogenic acute regulatory protein (*StAR*) was similar in all three groups (Fig. 1G). Interestingly, despite small patient numbers, *CYP19A1* expression in donors (Group 1) was higher than in both infertility

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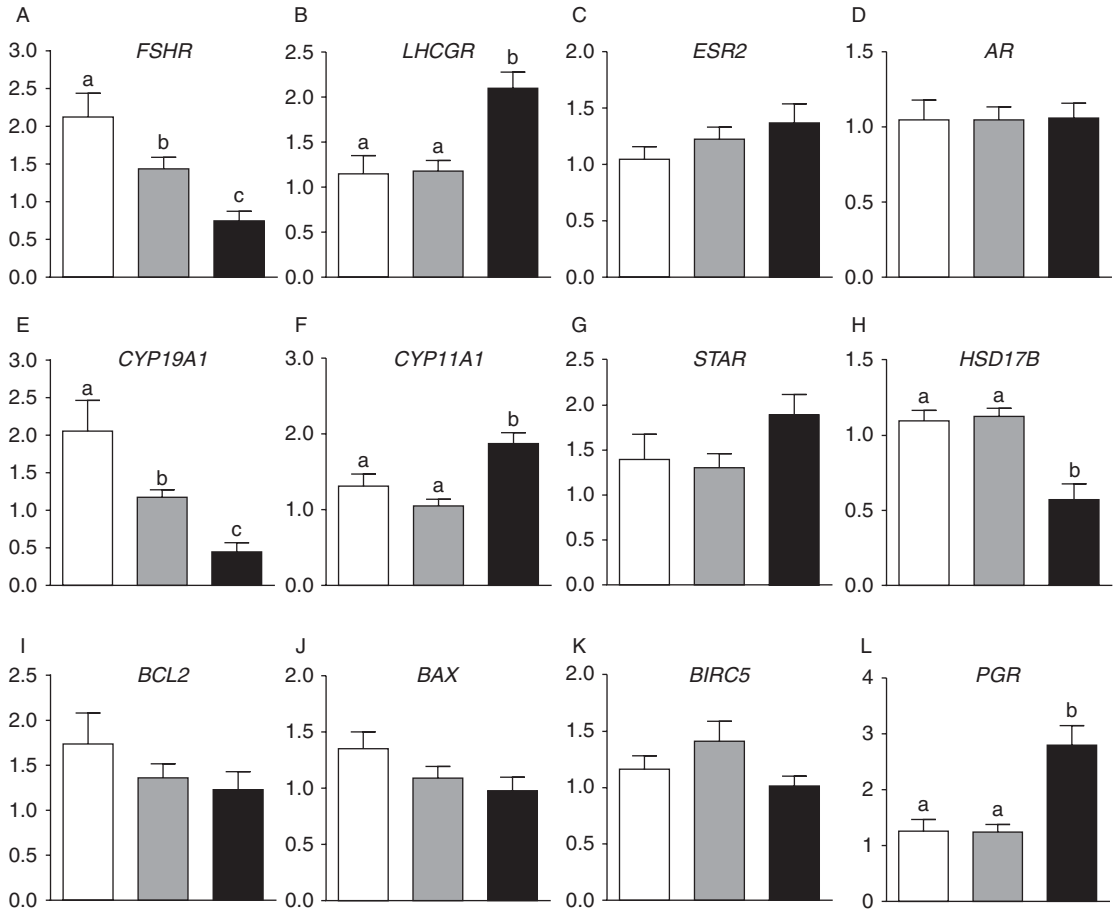

Figure 1
mRNA expression of GC genes was determined by real-time PCR. Values with same letters or without letters above the columns within each unit figure were not different significantly ($P>0.05$). White columns: Group 1 (oocyte donors), $n=7$; Grey columns: Group 2 (middle-aged infertile patients), $n=10$; Black columns: Group 3 (older infertile patients), $n=10$.

groups (Group 2 and Group 3, $P<0.05$), a finding confirmed then by western blot (Fig. 2A and B).

Because apoptosis is generally increased in older women (Seifer *et al.* 1996, Sadraie *et al.* 2000), we also investigated expressions of apoptosis-related genes in GCs (Fig. 1I, J and K). We found no differences in expression of B-cell lymphoma 2 (*BCL2*), bcl-2-associated X protein (*BAX*) and survivin (*BIRC5*) in all groups. These PCR results were then confirmed by western blot (Fig. 2A and E).

Combined with increased *LHCGR* expression, reduced *FSHR* and *CYP19A1* expression in older infertile women further suggests that their GCs undergo earlier luteinization. To obtain further evidence, we also measured expression of progesterone receptor (*PGR*), another GC differentiation marker. Q-PCR results demonstrated that GCs from older women (Group 3) expressed higher *PGR* than the other groups (Fig. 1L). Luteinization of GCs, therefore, appears to happen earlier and faster in older women.

Impact of maternal aging on proliferation and apoptosis of GCs during *in vitro* culture

To investigate the effect of maternal aging on GC proliferation, we cultured GCs of all three groups *in vitro* with or without FSH. As Fig. 3A demonstrates, in absence of FSH, cell proliferation between days 1–5 of GCs in Groups 1 and 2 did not change, while in Group 3 patients proliferation declined fast, and to an extremely low level. Distinctively different growing patterns are apparent in Fig. 3C.

Even though we did not observe changed apoptosis-related gene expression in freshly obtained GCs (Fig. 1), we still considered the possibility that the poor cell

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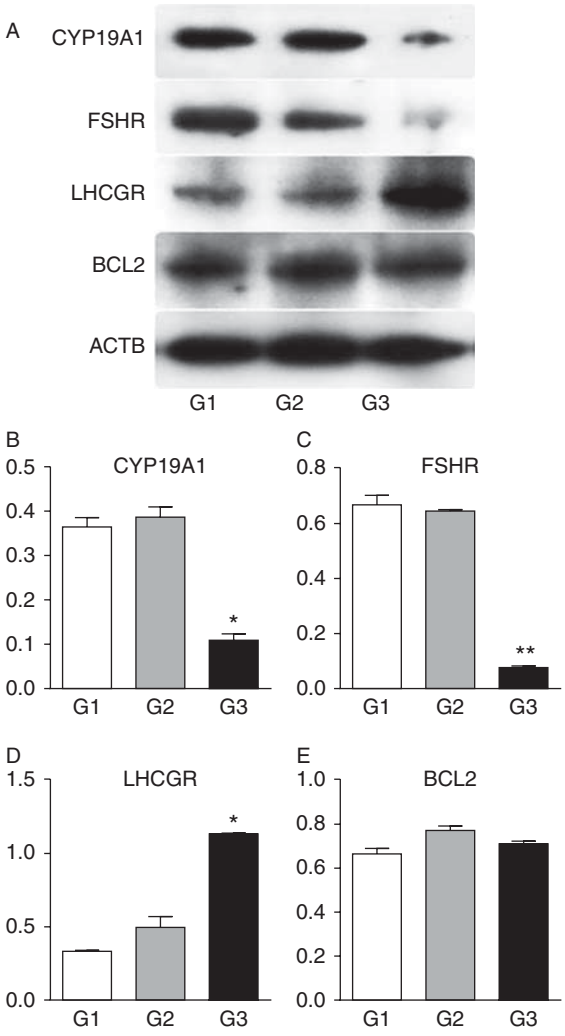

Figure 2
Protein expression of GC genes was determined by western blot. (A) Protein levels of aromatase, FHSR, LHR, BCL-2 and β-actin were evaluated by western blot analysis. G1: Group 1; G2: Group 2; G3: Group 3; (B, C, D, and E) relative quantitative protein levels of aromatase (B), FHSR (C), LHCGR (D), BCL2 (E) by western blot. The experiment was performed four times by using different samples of each age group. White columns: Group 1 (oocyte donors); grey columns: Group 2 (younger infertile patients); black columns: Group 3 (older infertile patients). *$P<0.05$; **$P<0.01$.

proliferation we observed in cultured GCs in Group 3 may be caused by increasing apoptosis. As Fig. 3B demonstrates, apoptotic cells increased during culture in all three groups but the increase in apoptotic cells occurred much faster in Group 3. As suggested by others (Tapanainen *et al.* 1987, Langhout *et al.* 1991, Rouillier *et al.* 1998), our results also showed that FSH in all three groups demonstrated positive effects on proliferation and apoptosis of cultured GCs (Fig. 3A and B). In the presence of FSH, GCs from older patients, however, still demonstrate lower proliferation and higher apoptosis after culture.

Impact of maternal aging on gene expression of GCs during *in vitro* culture

To determine the effect of age on gene expression of cultured GCs, expression of *FSHR*, *LHCGR*, *CYP19A1* and *BCL2* were investigated by Q-PCR and western blot. As shown in Fig. 4A and C, during culture *FSHR* and *CYP19A1* mRNA expression increased in Groups 1 and 2, but not in Group 3. Presence of FSH in medium enhanced this upregulated expression even in Group 3.

PCR results were then confirmed by protein level examination (Fig. 4E). As shown in Fig. 4B, *LHCGR* expression also increased during culture, though differently from *FSHR* in that the increase was much faster in Group 3 (Fig. 4B and E). *BCL2* expression decreased in all three groups (Fig. 4D), though the fastest in Group 3, with BCL2 protein expression by western blot concurring with PCR results (Fig. 4E). FSH inhibited this decline, suggesting inhibition of apoptosis by FSH in concurrence with previously noted results in Fig. 3B.

As demonstrated in Fig. 3B, compared to other groups, we noted higher cell apoptosis in Group 3 after culture in presence of FSH, which did not concur with the observed *BCL2* expression in Fig. 4D and E. We, therefore, investigated in addition expressions of two other molecular markers for apoptosis, *BAX* and *BIRC5* by real-time PCR (Supplementary Figure 1, see section on supplementary data given at the end of this article). Since we did not find differences in expression of both of these genes, jointly with our *BCL2* findings, this suggests that, though FSH apparently can regulate expression of apoptosis-related genes, it cannot completely reverse apoptosis.

Effects of early oocyte retrieval in women of very advanced age (>43 years)

A preliminary assessment of early hCG administration in women of advanced age is presented in Table 2. As the table demonstrates, in comparison to 91 historical control cycles in women of very advanced age, who were retrieved with standard timing (the normal retrieval group, NRG), 71 women in this ERG were actually older (44.8±0.3 vs 44.3±0.15 years; $P=0.001$); Their atretic oocytes were significantly reduced (0.31±0.07 vs 0.78±0.14, $P=0.02$). Immature oocytes were significantly increased (1.98±0.98 vs 1.1±0.17; $P=0.01$) but good quality embryos per cycle still significantly increased (3.6±0.36 vs 2.8±0.24; $P=0.04$). Moreover, clear trends in favor of the ERG were also seen in clinical pregnancy rate per cycle start, clinical pregnancy rate per embryo transfer and in embryo

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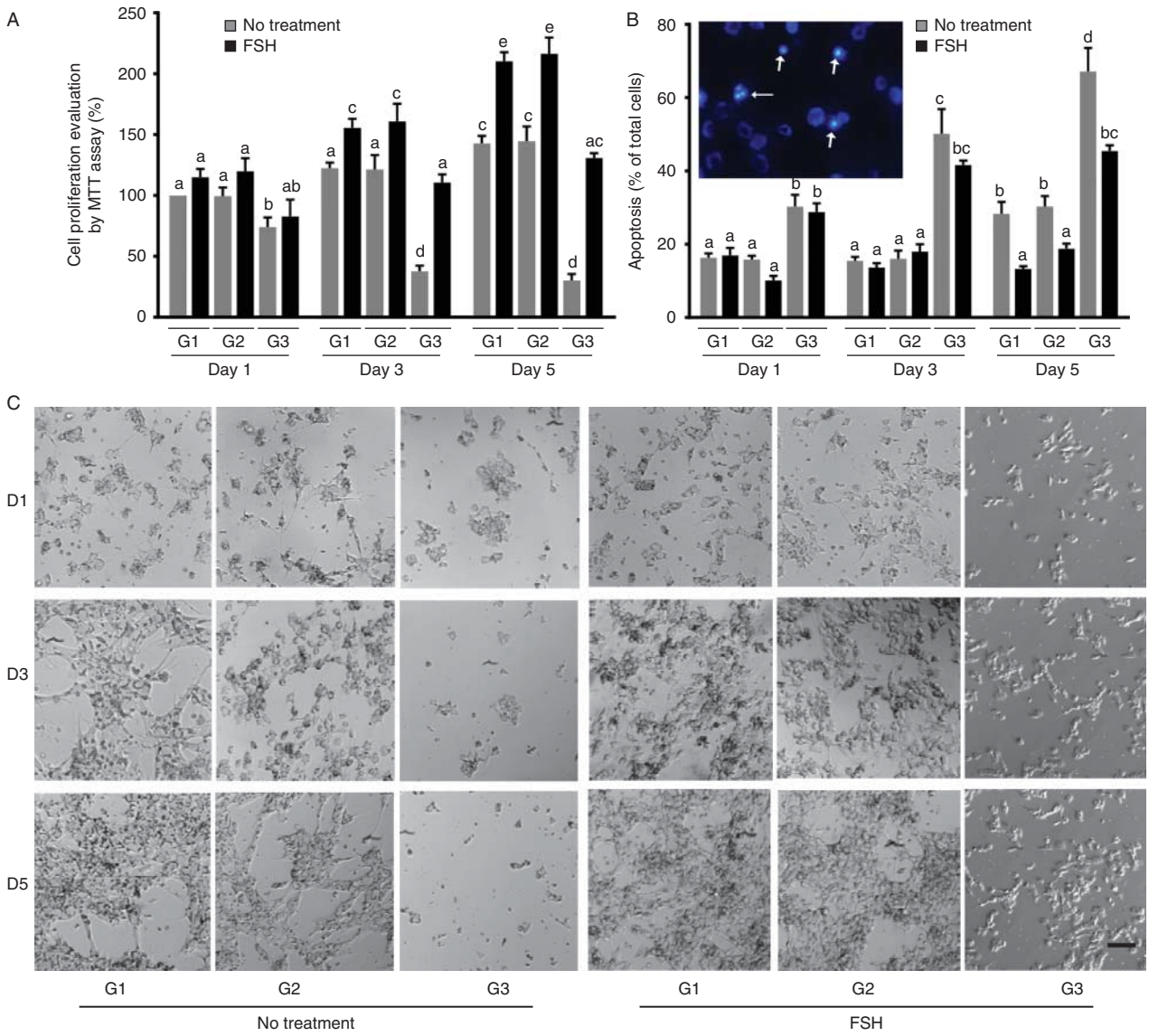

Figure 3

GC proliferation was determined by MTT assay. PI staining after cell culture determined GC apoptosis. (A) Cell proliferation assay was performed after 1–5 days culture. GCs were collected from three different patients in each group and cultured separately. (B) PI staining after 1–5 days culture evaluated GC apoptosis. (C) Distinctively different growing patterns of cultured GCs on days 1–5, with and without FSH supplementation, are apparent in all three age groups. GCs were collected from three different patients in each age group. In the inserted photograph in B, arrows indicate apoptotic cells after PI staining. Values with common letters above the columns within each unit figure were not different significantly ($P>0.05$). G1: Group 1 (oocyte donors); G2: Group 2 (younger infertile patients); G3: Group 3 (older infertile patients). D1–5: day 1–5 of cell culture; Bar = 20 μm.

implantation rate, though so far limited patient numbers did not offer the statistical power to reach statistical significance.

The table, however, also demonstrates that early retrievals significantly decreased P_4/E_2 ratios in the ERG in comparison to the NRG (1.46 ± 0.16 vs 1.94 ± 0.12, $P=0.002$). Additionally, Fig. 5 presents gene expression studies, including 12 ERG, 12 NRG and six donor women, demonstrating significant improvements in *LHCGR*, *PGR* and *CYP19A1* expression of ERG patients, while *FSHR* expression was not affected significantly ($P>0.05$) by early retrievals.

These preliminary data suggested that early ovulation induction in women of very advanced age demonstrates no adverse outcome effects on IVF, improves a number of well-established outcome parameters of IVF and, likely,

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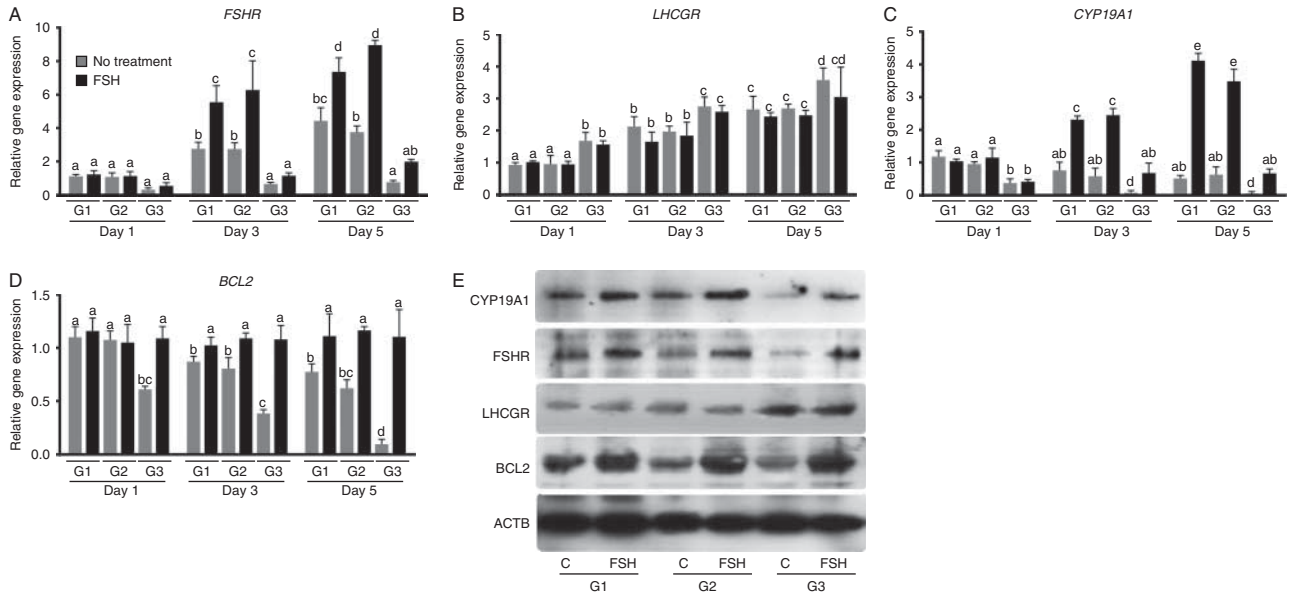

Figure 4

mRNA expression of GC genes was determined by real-time PCR (A, B, C, and D). Protein expression was determined by western blot. GCs were collected from three different patients in each group and separately cultured. Values with common letters above the columns within each unit figure did not differ significantly ($P>0.05$). G1, Group 1 (oocyte donors); G2, Group 2 (younger infertile patients); G3, Group 3 (older infertile patients). C, control; FSH, follicle-stimulating hormone.

ultimately may also improve clinical pregnancy rates in women above age 43, though confirmation of the latter point awaits larger patient numbers.

Discussion

The decline of female fertility with advancing age is well documented (Tatone 2008, Tatone *et al*. 2008, Weeg *et al*. 2012, Younis 2012). It is usually attributed to declining oocyte numbers (Nasseri & Grifo 1998, Out *et al*. 2000) and quality (Nasseri & Grifo 1998, Slovis & Check 2013). If the assumption of poorer oocyte quality is correct, then even resting follicles and their enclosed oocytes should exhibit the detrimental consequences of 'aging'. We have questioned this 'oocentric' viewpoint on theoretical as well as practical grounds. Since primordial follicles are progenitor structures, widely held to have limited energy needs and metabolic activity, one could alternatively propose that their predisposition toward 'aging' is, likely, only minimal. Had they been subject to cumulative damage during natural aging, they only unlikely would have retained the ability to yield pregnancies and normal offspring. Even women of very advanced age and/or with very low functional ovarian reserve, if treated appropriately,

Table 2 Comparison of IVF cycle outcomes between ERG and NRG

	ERG ($n=71$)	**NRG** ($n=91$)	***P* value**
Average age (years)	44.8±0.3	44.3±0.15	0.001
Number of follicles/cycle	7.2±0.58	7.3±0.56	0.93
Number of oocytes/cycle	6.7±0.63	5.9±0.49	0.31
Number of immature oocytes	1.98±0.29	1.1±0.17	0.01
Number of atretic oocytes from retrieval/cycle	0.31±0.07	0.78±0.14	0.02
Number of good embryos/cycle	3.6±0.36	2.7±0.24	0.04
Percentage of cycles resulting in pregnancies	15.5 (11/71)	7.7 (7/91)	0.14
Percentage of transferred cycles resulting in pregnancies	19.3 (11/57)	8.9 (7/78)	0.12
Implantation rate (%)	5.3	3.3	0.34
Progesterone/estradiol ratio	1.46±0.16	1.94±0.12	0.002

ERG, early retrieval group; NRG, normal retrieval group. *n*, patient number.

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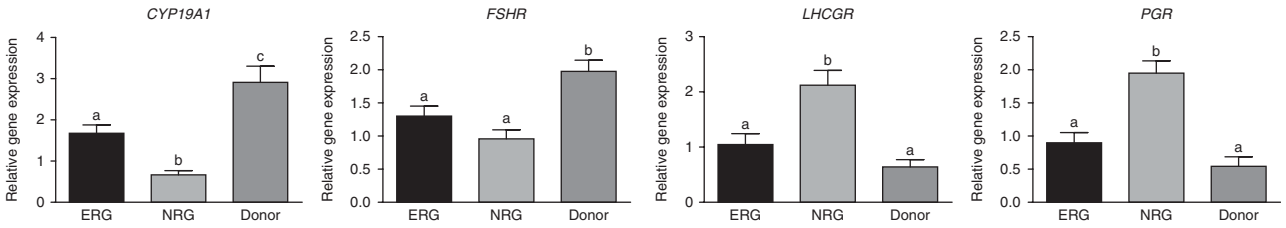

Figure 5
mRNA expression of GC genes was determined by real-time PCR. Values with common letters above the columns within each unit figure were not different significantly ($P > 0.05$). Black columns: older patients with early retrieval, $n = 3$; light grey columns: older patients with normal retrieval $n = 3$; dark grey columns: oocyte donor with normal retrieval (older infertile patients), $n = 12$.

however, still conceive and give birth to normal offspring. We, therefore, suggested that the concept of the 'aging oocyte' might have to be replaced by a concept of 'aging ovarian environments' in which follicles after recruitment undergo growth and maturation (Gleicher *et al.* 2011*a*). The difference between these two concepts is fundamental since likelihood of reversing intrinsic aging damage in an 'aged oocyte' is practically zero. If the culprit behind ovarian aging is, however, of somatic origin, therapeutic strategies directed towards reconstituting and/or rejuvenating 'aged ovarian environments', from which developmentally competent oocytes could be obtained, would offer promise for treatment of age-related infertility in older women.

This prospect is supported by androgen-related observations: older women exhibit relative low androgen levels (Gleicher *et al.* 2013). Moreover, androgens are known to be essential for normal follicle development during early growing follicle stages (Gleicher *et al.* 2011*b*). Indeed, androgen supplementation of the ovarian environment by raising testosterone levels improves egg/embryo numbers and quality as well as pregnancy rates (Gleicher & Barad 2011), offering evidence for a novel approach toward therapeutically reversing to a degree selected effects of ovarian 'aging'.

Since GCs surrounding the oocyte define the immediate ovarian microenvironment, their critical role in supporting oocyte development might be the underlying target for endocrine perturbations associated with aging (Buccione *et al.* 1990). Although numerous studies have documented the relationship between poor oocyte quality and GC abnormalities in human and animals (Buccione *et al.* 1990, Senbon *et al.* 2003, Assou *et al.* 2012, Huang & Wells 2012, Matsuda *et al.* 2012), effects of age on physiological function and molecular signature of GCs have so far been only sparsely investigated. Indeed, to our knowledge, they have never before been performed in such distinct age groupings (Hurwitz *et al.* 2010, McReynolds *et al.* 2012).

Our study comprehensively investigated the effects of ovarian aging in humans, and suggests that gene expression, proliferation, apoptosis and ability to respond to FSH stimulation in human GCs are all significantly affected by female age. Notably, Group 3 GCs demonstrated significantly increased *LHCGR*, *PGR* and *CYP11A1* but reduced *FSHR* and *CYP19A1* expression in comparison to other groups. Similar results have been observed in primates and other species. Luborsky *et al.* (2002) reported up-regulated *LHCGR* expression in human luteinized GCs. Increased *PGR* (Natraj & Richards 1993) and *CYP11A1* (Rao *et al.* 1978) expression has been reported in rat luteinized GCs. Studies in humans and in the bovine suggest that LH surge-induced declines of *FSHR* represent the initiation of GC luteinization (Nimz *et al.* 2009, Jeppesen *et al.* 2012). Likewise, disappearance of *CYP19A1* is another marker of luteinization in GCs (Campbell *et al.* 1998). Our results in older infertility patients (Group 3), therefore, closely parallel previously findings in, and suggest that premature luteinization of GCs is more likely to occur in older than in younger women.

High FSH initiates in natural cycles follicular development, leading to rising serum E_2 concentrations by *CYP19A1*. This, in turn, causes a negative feedback on FSH release, and arrests the development of small growing follicles. High concentrations of E_2 result in the preovulatory LH surge, which is responsible for final oocyte maturation and ovulation (Laven & Fauser 2006). In here, reported IVF patients, FSH is, however, because of controlled ovarian hyperstimulation, maintained at higher levels. The consequence in some patients can be the triggering of a premature LH surge before follicles/oocytes have fully matured, a phenomenon given the acronym 'premature luteinization' (60). Our results are, therefore, consistent with the fact that women above age

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43 are at significantly increased risk to develop premature luteinization.

That premature luteinization negatively impacts oocyte quality, fertilization and implantation is supported by Skiadas *et al*. (2012) who demonstrated an association between low functional ovarian reserve in older women and premature luteinization, clinically characterized by higher peripheral LH and lower AMH levels. Also, oocyte numbers and top quality embryo numbers have been reported to be significantly higher in normal patients than in women with premature luteinization (Bosch *et al*. 2003, Elnashar 2010). In conjunction with here reported molecular data of older women, all of this points to premature luteinization as a principal cause in the age-related decline of female fertility.

Higher exogenous FSH exposure may be a contributing factor to the increased risk toward premature luteinization (Elnashar 2010). Women with premature luteinization, indeed, may have higher day 3 FSH levels (Younis *et al*. 1998, 2001), though there are no data to support higher intracycle levels in the literature. In this study, FSH levels during ovarian hyperstimulation were not higher in Group 3 patients. Their elevated P_4/E_2 (>1) still suggests an increased risk for premature luteinization (Ozcakir *et al*. 2004).

In light of the widely held notion that physiological luteinization involves GC cell cycle exit and terminal differentiation, our results also suggest that premature luteinization may be linked to GC proliferation arrest and apoptosis. In support, Christenson & Stouffer (1996) reported in primates and rats that an ovulatory luteinizing stimulus causes proliferation arrest and luteinization in cell differentiation (Rao *et al*. 1978, Oonk *et al*. 1989, Christenson & Stouffer 1996), findings further supported by the down-regulation of cell cycle proteins, such as $p27^{Kips}$ and cyclin D2 (Fero *et al*. 1996, Cheng *et al*. 1999). In cancer cells, cell cycle regulators such as these are well-known mediators, which initiate apoptosis in response to cell cycle arrest (Murphy 2000, Gutierrez *et al*. 1997). Whether a similar cell cycle checkpoint is operative in GCs of aged women remains to be determined but would be consistent with our observation that GCs of Group 3, indeed, demonstrated a higher level of apoptosis during culture (Fig. 3B).

Some caution is, nevertheless, warranted, especially in the presence of serum. GCs in culture spontaneously undergo structural and functional luteinization based upon changes in cell morphology, steroidogenesis and metabolism (Murphy 2000). Although GCs were cultured with serum-free medium here, it is impossible to completely prevent luteinization. FSH supplementation in medium can, inhibit GC luteinization *in vitro*, as demonstrated in the cow where GC morphology and estrogen production indicate maintenance of a pre-luteinized state (Gutierrez *et al*. 1997). Similar positive effects of FSH on GC growth and prevention of luteinization were also observed in human and rat (Lambert *et al*. 2000, Kwintkiewicz *et al*. 2010, Zhou *et al*. 2013).

The effects of FSH in this study are particularly noteworthy: it significantly enhanced cell proliferation (Fig. 3A and C), reduced apoptosis (Fig. 3B), and up-regulated *FSHR* (Fig. 4A) and *CYP19A1* (Fig. 4C) expression, suggesting at least partial inhibition of luteinization by FSH. Interestingly, although it caused significant improvements, GC function after FSH treatment was still far weaker in GCs of older women (Group 3), when compared to the other two groups, suggesting insufficient FSHR expression. Poor follicular response to FSH in older women is well recognized (Gleicher & Barad 2006). Our observations heightens the significance, which provide compelling evidence that GCs of older women respond less effectively to FSH stimulation during *in vitro* culture, suggesting an underlying pathophysiology for declining female fertility.

Finally, our observation that FSH did not induce *LHCGR* expression in cultured GCs (Fig. 4B) requires further study because it is generally held that FSH induces *LHCGR* expression *in vivo* (Hirakawa *et al*. 1999, Orisaka *et al*. 2006, Cannon *et al*. 2009). A likely explanation for this result is that here investigated GCs were exposed to hCG *in vivo*. As shown by us and others (Murphy 2000, Hurwitz *et al*. 2010, Jeppesen *et al*. 2012, McReynolds *et al*. 2012), hCG administration causes luteinization of GCs, and a changing physiological and molecular signatures. Therefore, it is possible that hCG administration changes cell sensitivity of the FSH response. This hypothesis is supported by evidence from non-luteinizing GCs, where FSH activates the protein kinase A (PKA) pathway and then induces LHCGR transcription (Oury *et al*. 1992). But luteinization increases the stability of PKA subunit, which inhibited PKA activation by FSH (Gonzalez-Robayna *et al*. 1999). That the FSH/PKA-driven transcriptional protein, CREB, undergoes inhibitory phosphorylation in luteinized GCs (78), also supports this hypothesis.

Recognizing this pathophysiology in aged GCs then raised the question how such premature luteinization could be prevented. We hypothesized that the likelihood was early oocyte retrieval, which should release oocytes earlier from the hyper-luteinized follicular environments.

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Preliminary outcome analysis of 71 early retrieval IVF cycles in women above age 43, in comparison to 91 normal routine retrieval cycles, is highly encouraging (Table 2), though much larger patient numbers will be required to unequivocally demonstrate in this patient population that this new management scheme, ultimately, improves IVF pregnancy and delivery rates.

This study, however, with considerable certainty established non-inferiority for this new treatment and, with a reasonable level of likelihood suggest that early oocyte retrieval may improve IVF outcomes. The observation that earlier retrieval increased the number of high quality embryos available for transfer by reducing atretic oocyte numbers is reassuring because pregnancy and delivery success in IVF usually follows high quality embryo numbers. Optimism is also warranted since every outcome parameter, which did not significantly improve, without exception, strongly trended in favor of the ERG. It will take at least 150 IVF cycles in this patient population to reach adequate power for final statistical evidence that clinical pregnancy and live birth rates are, indeed, improved by a minimum of 20 percent.

In summary, we present here convincing *in vivo* and *in vitro* evidence that premature luteinization in infertile women of advanced age was associated with rapidly declining IVF pregnancy rates. We also present preliminary evidence, suggesting that, if such premature luteinization is avoided by earlier oocyte retrieval, IVF outcomes will be improved. Final confirmation of improved pregnancy and delivery outcomes will, however, require a larger patient pool than is available at time of this publication.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-15-0246.

Declaration of interest
D H B and N G are listed as inventors on a number of US patents, among them, peripherally relevant to this study, patents claiming therapeutic benefits from androgen supplementation of women with LFOR. Both receive royalties from Fertility Nutraceuticals, LLC for licensing of some of these patents. N G holds shares in this company. None of the other authors report any potential conflicts in respect to the study.

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