Leptin suppresses anti-Mullerian hormone gene expression through the JAK2/STAT3 pathway in luteinized granulosa cells of women undergoing IVF

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STUDY QUESTION: Do the adipocytokines, leptin and adiponectin affect the granulosa cell expression of anti-Mullerian hormone (AMH) and its receptor (AMHR-II)?

SUMMARY ANSWER: Leptin suppresses AMH mRNA levels in human luteinized granulosa cells through the JAK2/STAT3 pathway, while adiponectin has no such effect.

WHAT IS KNOWN ALREADY: AMH is one of the most reliable markers of ovarian reserve. Serum AMH levels decline with obesity. Obesity is associated with elevated leptin and reduced adiponectin levels.

STUDY DESIGN, SIZE AND DURATION: This prospective study included 60 infertile women undergoing fresh IVF and ICSI cycles utilizing autologous oocytes at Montefiore’s Institute for Reproductive Medicine and Health between July 2010 and April 2012.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Follicular fluid was collected from small (SFs; < 14 mm) and large follicles (LFS; ≥ 14 mm) from 38 participants. Total RNA was extracted separately from mural and cumulus granulosa cells and mRNA levels were measured by RT–PCR. In an additional group of participants (N = 22), primary cumulus and mural granulosa cells (pooled SFs and LFs) were cultured in media alone or with addition of either leptin (N = 7), adiponectin (N = 8) or JAK2/STAT3 inhibitor + leptin (N = 7), and AMH and AMHR-II mRNA levels measured. Levels of AMH, leptin and adiponectin protein were measured in follicular fluid.

MAIN RESULTS AND THE ROLE OF CHANCE: AMH and AMHR-II mRNA and follicular fluid AMH protein levels were inversely correlated with age. AMH mRNA expression was six times higher in cumulus compared with mural granulosa cells in SFs (P < 0.05) and eight times higher in cumulus compared with mural granulosa cells in LFs (P < 0.001). In follicular fluid, leptin protein level positively correlated (r = 0.7, P = 0.03), while adiponectin protein level inversely correlated (r = −0.46, P = 0.02) with BMI. Leptin treatment suppressed AMH and AMHR-II mRNA in both cumulus and mural granulosa cells (all P < 0.05). In the presence of JAK2/STAT3 inhibitor, leptin treatment did not alter AMH but continued to suppress AMHR-II mRNA in cumulus cells (P = 0.02). Adiponectin treatment did not alter AMH or AMHR-II mRNA levels.

LIMITATIONS, REASONS FOR CAUTION: This study included a luteinized granulosa cell model as these cells were collected from women who were hyperstimulated with gonadotrophins. The results obtained may not fully extrapolate to non-luteinized granulosa cells.

WIDER IMPLICATIONS OF THE FINDINGS: Leptin may program abnormal AMH signaling, thereby resulting in ovarian dysfunction. This study opens a new perspective for understanding the low ovarian reserve seen in obese women and provides new insights into potential mechanisms that explain the lower AMH seen in obese women. Whether our findings explain the worse response to ovulation induction observed in obese women needs to be further elucidated.
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**Key words:** anti-Mullerian hormone / granulosa cell / leptin / adiponectin / JAK2/STAT3

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**Introduction**

Obesity continues to be an epidemic in the USA and throughout the world with approximately two-thirds of adults being either overweight (BMI ≥ 25 kg/m²) or obese (BMI ≥ 30 kg/m²) (Hedley et al., 2004). Contributions of obesity to infertility and to poor IVF outcomes are well recognized (Fedorcsak et al., 2004). There is strong evidence that obesity is associated with a higher IVF cycle cancelation rate ( despite administration of higher doses of exogenous gonadotrophins), a lower mature oocyte yield and lower number of cryopreservation. Obesity is associated with a higher IVF cycle cancelation rate ( Hedley et al., 2004). Furthermore, obese women have been shown to have impaired response to ovarian stimulation and significantly lower live births after IVF (Lintsen et al., 2005). Indeed, obesity is associated with ovarian intrafollicular alterations at multiple cellular levels including steroidogenic, metabolic and inflammatory pathways (Robker et al., 2009).

Leptin is synthesized predominantly by fat cells and is also expressed in the stomach, placenta and mammary gland (Bohler et al., 2010). Leptin circulates in concentrations that are directly correlated with absolute fat mass (Rosenbaum et al., 1996). Adiponectin is expressed exclusively by adipocytes (Bohler et al., 2010). In contrast to leptin, adiponectin circulates in concentrations that are inversely correlated with obesity (Meilleur et al., 2010). In addition to their metabolic actions, leptin and adiponectin regulate the female reproductive system at all levels of the hypothalamic-pituitary-ovarian axis. Granulosa cells (GCs) in the ovaries have receptors for both adiponectin and leptin (Mitchell et al., 2005; Chabrolle et al., 2009). Recent data have revealed that adiponectin causes an increase in insulin-like growth factor-1 induced steroid release by human GCs while leptin inhibits this effect (Agarwal et al., 1999; Chabrolle et al., 2009). Therefore, alterations in these adipokines in the context of obesity may counteract the sensitizing effect of locally produced growth factors on GCs. Additionally, there is in vitro evidence that leptin may have a function in steriodogenesis (Bohler et al., 2010). Further, Ledoux et al. (2006) have recently demonstrated that in porcine GCs, adiponectin at physiologically relevant levels provokes expression of genes associated with periovulatory remodeling of the ovarian follicle: cyclo-oxygenase-2, prostaglandin E synthase and vascular endothelial growth factor.

Anti-Mullerian hormone (AMH) is a protein produced by GCs in the ovaries, which is then secreted into the blood (Seifer and Maclaughlin, 2007). AMH regulates follicular recruitment and development within the ovary. In women, the highest values of AMH are attained after puberty and subsequently decrease with age, likely reflecting the age-related decline in ovarian reserve (Seifer and Maclaughlin, 2007). AMH is one of the most reliable markers for ovarian reserve and is commonly used in assisted reproduction as a predictor of ovarian response to controlled ovarian hyperstimulation (COH) for IVF (Seifer et al., 1999).

We (Buyuk et al., 2011) and others (Freeman et al., 2007; Su et al., 2008) have shown that obese women have a lower ovarian reserve than normal weight women; this is reflected by their lower serum AMH levels (up to 77% lower), suggesting that obesity may impair GC hormone production. Additionally, obese women have elevated serum and follicular fluid (FF) leptin levels and suppressed serum and follicular fluid adiponectin (Mitchell et al., 2005) levels. We, therefore, hypothesize that leptin down-regulates while adiponectin up-regulates AMH and/or AMH-receptor (R-II) gene expression in human luteinized GCs. To test this hypothesis, we performed a prospective study analyzing the effects of leptin and adiponectin treatment on the expression of AMH and its receptor genes during the final stages of follicular maturation in cultured GCs from patients undergoing COH during IVF.

**Materials and Methods**

**Subjects**

Thirty-eight infertile women undergoing fresh IVF and ICSI cycles utilizing autologous oocytes at Montefiore’s Institute for Reproductive Medicine and Health, NY, USA, between July 2010 and April 2012 were prospectively enrolled for the first experiment. The inclusion criteria consisted of women with normal ovarian reserve defined as Day 3 FSH < 10 mIU/ml and Day 3 estradiol (E₂) < 80 pg/ml. Reasons for infertility were male, tubal, unexplained, endometriosis, and uterine factors. Women with polycystic ovary syndrome (PCOS) were excluded from the study. The diagnosis of PCOS was made according to the Rotterdam criteria (Franks, 2006) on the presence of at least two of the three following criteria: (i) ovulatory disturbance (oligomenorrhea or amenorrhea); (ii) hyperandrogenism, as defined clinically by hirsutism and severe acne/seborrhea and/or biologically by elevated testosterone serum levels and (iii) more than 12 follicles in the 2- to 9-mm range in each ovary at ultrasonography and/or an ovarian volume of >10 ml. All the patients gave informed consent and the study was approved by the Institutional Review Board of Montefiore Medical Center.

**Ovarian stimulation**

Pituitary desensitization was performed using GnRH agonists (started in the mid-luteal phase of the previous menstrual cycle) or GnRH antagonists (added daily, starting when the leading follicle reached a diameter of 14 mm or serum E₂ reached 400 pg/ml). Follicular growth was achieved with injectable gonadotrophins and was monitored with transvaginal ultrasound and serum E₂ levels. hCG injection (10 000 U hCG (Novarel; Ferring Pharmaceuticals) or recombinant hCG 250 µg (Ovidrel; EMD Serono]) was administered when at least two follicles had a mean diameter of >17 mm. Oocyte retrieval was performed 34 h after hCG injection under transvaginal ultrasound guidance.

**Collection of GCs**

Follicle size was estimated immediately at the time of retrieval under ultrasound, and samples of follicular fluid from small follicles (SFs (<14 mm))
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and large follicles [LFs (≥ 14 mm)] were collected in separate tubes. SFs and LFs were examined separately to pinpoint differences between immature and mature follicles, respectively. The size cutoff for SFs and LFs was chosen based on a previous study (Catteau-Jonard et al., 2008). To avoid mixing the fluid from SFs and that from LFs from one ovary in the same aspirating needle, follicular fluid of LFs was aspirated first. Then two SFs were aspirated but their follicular fluid was discarded and not used for the study in order to avoid contamination. Lastly, follicular fluid of all the other remaining SFs was aspirated and reserved. Follicular aspirates included oocytes and both mural and cumulus GCs. The fluid from the first aspirated follicle was used for hormonal protein measurement but it was not used for gene expression evaluation because of possible contamination from vaginal mucosa cells. After removal of the oocyte, fluid from each SF or LF was pooled from the same woman, and cells were concentrated by centrifugation at 300g and 600g for 5 min each. The superficial layer of the pellet was collected and layered on 40% Pureception (Cooper Surgical, Trumbull, CT, USA) and centrifuged at 400g for 12 min to remove red and white blood cells. GCs were collected from the layer interface and subjected to 5 mg/ml collagenase for 2 min at room temperature to disperse cells. Cells were then washed with phosphate-buffered saline (PBS) and incubated with CD 45+ tagged magnetic beads (Invitrogen, Carlsbad, CA, USA) for 20 min at +4°C to remove the remaining white blood cells. The beads were then separated, and the remaining fluid was centrifuged for 5 min at 600g to collect the mural GCs of each SF and LF.

After identification of the cumulus–oocyte complex in the aspirate, cumulus GCs were mechanically collected by cutting the cumulus cell layer from each oocyte and washed with PBS. For each patient, mural and cumulus GCs from either SFs or LFs were pooled in order to extract sufficient RNA for quantification and RT–PCR analyses.

RNA extraction and reverse transcription

For each patient and for SFs and LFs, the RNA was isolated from the collected GCs, using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The cells were incubated with Trizol for ~15 min at room temperature and then mixed with chloroform, followed by centrifugation at +4°C and 12,000g for 15 min. The aqueous phase containing RNA was separated. Total RNA was precipitated with propanol and centrifugation at +4°C and 12,000g for 10 min, washed with 75% ethanol followed by centrifugation at +4°C and 7500 g for 5 min, air-dried and reconstituted in diethylpyrocarbonate (DEPC)-treated water. The DNA was stored at −20°C until further analysis. The RNA was cleaned using the RNeasy mini kit (Qiagen, Valencia, CA, USA), and reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). RNA quality analysis was performed using the Nanodrop Spectrophotometer. Samples with a minimum concentration of 10 ng/µl and with an OD 260/280 ratio of 1.8–2.0 were used. Hence, sample sizes for gene expression analyses are smaller than the number of participants.

Quantitative RT–PCR

Evaluation of mRNA levels was achieved by RT–PCR kinetics using the SYBR Green I chemistry. The complementary DNA (cDNA) was then amplified in triplicate using LightCycler 480 SYBR Green PCR Master Mix. Real-time PCR was performed with 5 µl of diluted cDNA in a total volume of 20 µl. 500 nM of forward and reverse specific primers for human AMH (forward 5′-GCG CTG GTG GTC CTA CAC-3′ and reverse 5′-GAA CCT CAG CGA GGG TGT T-3′) and AMHR-II (forward 5′-TGT GTT TCT CCC AGG TAA TCC G-3′ and reverse 5′-AAT GTG GTG GTG GTG TAG GC3′), and 1x LightCycler 480 SYBR Green I Master mix (Roche Diagnostics, Indianapolis, IN, USA) in a LightCycler (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s protocol. The primers were synthesized by Fisher (Pittsburg, PA, USA). The PCR protocol used an initial denaturing step at 95°C for 10 min followed by 45 cycles at 95°C for 10 s and 72°C for 2–10 s with a transition rate of 20°C per second. Crossing point values were acquired using the second derivative maximum method of the LightCycler software 3.3 (Roche). The specificity of the desired product was documented with the analysis of the melting curve. The melting curve was achieved by first cooling samples to 60°C at a transition rate of 20°C per second after 30 s of incubation and by a slow-heating step at a rate of 0.1°C per second until a maximum temperature of 95°C. The mix was then cooled at 40°C for 1 min, at a transition rate of 20°C per second. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as a loading control (forward: 5′-ACCCACTCTCCACCTTTGA-3′; reverse: 5′-TGTTGC TGTAGCCA AATTCGTTT-3′). The level of mRNA for each gene relative to GAPDH was calculated using the ΔΔCT method (Livak and Schmittgen, 2001).

Culture of GCs

A further twenty-two women undergoing fresh IVF and ICSI cycles were prospectively enrolled and GCs were collected as described above. However, because we found no differences in mRNA levels for AMH or AMHR-II in SFs versus LFs in the first experiment, we pooled all follicles rather than separating them into the SF and LF groups. For each patient, we were successful in obtaining both mural GC and cumulus GC samples after cell processing. The resultant mural GC pellet was incubated in 5 mg/ml collagenase (Sigma, MO, USA) for 2 min at room temperature until cell clumps separated. Cumulus GCs were incubated with 0.614 U/ml hyaluronidase (Sigma, MO), followed by 2× washes with PBS and centrifugation. The pellet of each mural and cumulus GCs was washed with fresh medium (Dulbecco’s modified Eagle’s medium-F12 and 10% fetal bovine serum), counted in a hemocytometer and cell viability was determined using trypan blue dye exclusion. Cumulus and mural GCs from each patient were divided into two samples and cultured in 24-well culture plates (pretreated with poly-L-lysine for 5 min) for 24 h with no other treatment in order for the cells to attach to the plates, followed by incubation in fresh culture medium with or without human recombinant leptin (100 ng/ml; n = 7) (Sigma, MO, USA) or human recombinant adiponectin (5 µg/ml; n = 8) (Sigma, MO, USA) for another 24 h. Therefore, for each patient one sample of cells was cultured only in media throughout the experiment (serving as control) and a second sample of cells was cultured only with recombinant hormones (treatment arm). The doses of recombinant leptin (Agarwal et al., 1999) and adiponectin (Chabrolle et al., 2009) were chosen based on previous studies showing an effect of these doses on steroid release by GCs. Plates were incubated under a humidified atmosphere of 95% air and 5% CO2 at 37°C. At the end of the experiment, RNA extraction and RT–PCR for AMH and AMHR-II gene expression were performed as described above.

Next, we assessed whether leptin alters AMH and/or AMHR-II mRNA expression through the JAK2/STAT3 signaling pathway. Mural and cumulus GCs were collected as described above for seven participants and were cultured with medium only or were pretreated with JAK2/STAT3 inhibitor (AG490; 50 µM) (Fisher, Pittsburg, PA, USA) for 30 min followed by treatment with leptin (100 ng/ml) for 24 h. RNA extraction and RT–PCR for AMH and AMHR-II gene expression were performed as described above.

Follicular fluid AMH, leptin and adiponectin protein levels

Levels of AMH protein were measured in follicular fluid with an enzyme-linked immunosorbent assay (ELISA) kit (DSL-10-14400; Diagnostic Systems Laboratories, Webster, TX, USA) according to the
manufacturer’s recommendations. Samples were run in duplicate by a single operator without knowledge of group assignment. The lower limit of sensitivity was 0.1 ng/ml, and inter-assay coefficients of variation were 6, 7 and 10% at concentrations of 1.5, 2.2 and 8.1 ng/ml, respectively. Adiponectin and leptin protein levels were measured by human ELISA kits according to the manufacturer’s protocol (Quantakine kit from R&D Systems, Inc., Minneapolis, MN, USA); the intra-assay and inter-assay coefficients of variation were <15%.

Statistics

Demographic and clinical data were expressed as median (range). Because AMH and AMHR-II mRNA expression was highly variable among women, results were expressed either as relative number of copies ± SEM or as percentages ± SEM in the split culture experiments (controls being set at 100%). All comparisons were performed using the nonparametric Mann–Whitney U-test for unpaired comparisons and the Wilcoxon matched-pairs signed rank test was used for paired comparisons. BMI was sub-categorized into normal (18.5–24.9 kg/m²), overweight (25–29.9 kg/m²) and obese (≥30 kg/m²). The Kruskal–Wallis test was used for comparisons among the three subgroups of BMI. Univariate correlation analyses of age, follicular fluid leptin protein levels or follicular fluid adiponectin protein levels with logAMH or logAMHR-II gene expression were performed using linear regression. Multivariate linear regression was used to compare among the three subgroups of BMI. Univariate correlation analyses of age, follicular fluid leptin protein levels or follicular fluid adiponectin protein levels with logAMH or logAMHR-II gene expression were performed using linear regression. Multivariate linear regression was then performed to test the correlation between gonadotrophin dose and logAMH and logAMHR-II after adjusting for age and BMI. All statistical procedures were run on the STATA software (StataCorp). P ≤ 0.05 was considered statistically significant.

Results

AMH mRNA expression is higher in cumulus compared with mural GCs

The clinical parameters of the participants in the initial experiment are summarized in Table I. We measured AMH and AMHR-II mRNA in cumulus and mural GCs from both SFs and LFs. The sample sizes for RT–PCR analyses are smaller than the number of participants (n = 13–18), because we used only high-quality samples and those with sufficient RNA for RT–PCR. AMH mRNA expression was approximately six times higher in cumulus GCs compared with mural GCs in SFs (P = 0.02, n = 18 RNA samples) and eight times higher in cumulus GCs compared with mural GCs in LFs (P = 0.007, n = 13 RNA samples) (Fig. 1). Differences in AMH-Ⅱ mRNA expression did not reach statistical significance when compared between cumulus and mural compartments (P = 0.1; data not shown). There was no difference in either AMH or AMHR-Ⅱ mRNA expression between SFs and LFs.

AMH and AMHR-Ⅱ mRNA, and follicular fluid AMH protein levels inversely correlate with age

We measured follicular fluid AMH protein levels in most participants (n = 33). We observed a statistically significant inverse correlation between age and follicular fluid AMH protein levels (r = −0.45, P = 0.008). As seen in Table II, AMH mRNA level was inversely correlated with age in mural and cumulus GCs from both SFs and LFs. AMHR-Ⅱ mRNA was also inversely correlated with age in both cumulus GCs of both SFs and LFs but did not reach significance in the mural compartment.

AMH mRNA levels inversely correlate with gonadotrophin dose

In a linear regression model adjusting for age and BMI, we measured total gonadotrophin dose received during controlled ovulation induction as a predictor and logAMH as the outcome variable of interest. We found that the higher the dose of gonadotrophin used, the lower the AMH mRNA levels (Table III) in all cell compartments except for the mural GCs of LFs. No correlation was found between gonadotrophin dose used and AMHR-Ⅱ mRNA levels (data not shown).

Human recombinant leptin down-regulates AMH and AMHR-Ⅱ mRNA, while adiponectin has no effect

Follicular fluid leptin protein level positively correlated (r = 0.7, P = 0.03), while follicular fluid adiponectin protein level inversely correlated (r = −0.46, P = 0.02) with BMI (Table IV). Recombinant leptin treatment of GCs in vitro [from n = 7 women, age = 39 (26–47) years, BMI = 27 (21–32) kg/m²] significantly suppressed AMH (P = 0.02) and AMHR-Ⅱ (P = 0.02) mRNA levels in cumulus and also suppressed AMH (P = 0.02) and AMHR-Ⅱ (P = 0.03) mRNA levels in mural GCs (Fig. 2A and B). Recombinant adiponectin treatment of GCs in vitro [from n = 8 women, age = 32 (24–38) years, BMI = 28.5 (19–42) kg/m²] did not alter either AMH (P = 0.8 for cumulus and P = 1.0 for mural GCs) or AMHR-Ⅱ (P = 0.8 for cumulus and P = 0.2 for mural GCs) mRNA levels (Fig. 3).

Inhibition of JAK2/STAT3 prevented the effect of leptin on AMH mRNA

To assess whether leptin down-regulates AMH and AMHR-Ⅱ mRNA through the JAK2/STAT3 pathway, we pretreated GCs with AG490 (JAK2/STAT3 inhibitor) before treatment with leptin, and compared results with control GCs (cultured without AG490) from the same participant. Treatment with AG490 prevented the leptin-induced down-regulation in AMH (P = 0.8 for both cumulus and mural GCs) but not AMHR-Ⅱ mRNA in cumulus GCs (P = 0.02) and there was

| Table I Demographic and cycle characteristics of infertile women in the initial study of gene expression in small and large ovarian follicles. |
|---------------------|---------------------|
| All participants (n = 38) |               |
| Age (years)         | 36.2 (24–46) |
| BMI (kg/m²)         | 26.5 (19–41) |
| Day 3 FSH (mIU/ml)  | 6.7 (2.6–9.7) |
| Day 3 E2 (pg/ml)    | 38.4 (26–73) |
| Follicular fluid AMH (ng/ml) | 1.5 (0.2–15.7) |
| Total IUs of gonadotrophins | 3536 (825–7425) |
| Number of oocytes   | 12.4 (3–29) |

Values are expressed as median (range). E2, estradiol; AMH, anti-Mullerian hormone; IU, international unit.
a non-significant trend toward lack of effect in mural GCs ($P = 0.06$) (Fig. 2C and D).

**Discussion**

In this prospective study, we report for the first time that leptin down-regulates mRNA for AMH and its receptor in primary cultures of human GCs. Leptin down-regulates AMH but not AMHR-II gene expression in a JAK2/STAT3-dependent manner. Secondly, gonadotrophin use during ovulation induction in IVF is associated with lower AMH gene expression (in both cell compartments except for the mural GCs in LFs). Thirdly, AMH gene expression in GCs is compartmentalized, being more pronounced in the cumulus compared with the mural compartment. Finally, age is inversely correlated with AMH and its receptor gene expression, as well as AMH protein levels in follicular fluid.

Few studies have evaluated the effect of obesity on ovarian reserve in patients who do not have PCOS (Freeman et al., 2007; Merhi et al., 2008; Su et al., 2008; Merhi, 2009). Su et al. (2008) examined the association between obesity and measures of ovarian reserve in women in their late reproductive years (age 40–52 years). In that study, the authors found that in 36 healthy women (obese and normal BMI) early follicular phase serum AMH was significantly lower (by 77%) in obese women compared with normal weight women, despite having similar antral follicle count (a good indicator of ovarian reserve) and ovarian volume. These findings indicate that obesity alters AMH production despite no change in antral follicle count. Another study by Freeman et al. (2007) described the relationship between obesity and AMH levels in healthy women in their late reproductive years (age 35–47 years). They found that AMH levels were 65% lower in obese women compared with normal BMI women. BMI remained significantly associated with AMH levels in multivariable models after adjusting for menopausal status, age, race and day of the menstrual cycle. These findings suggest evidence of the negative effect of obesity on ovarian function. In order to study a causal relationship between obesity and genes related to ovarian reserve (AMH and AMHR-II), we treated primary cultures of GCs with either leptin (elevated levels in obesity) or adiponectin (reduced levels in obesity) and measured gene expression following treatment. We found that leptin suppressed AMH and AMHR-II mRNA expression in both cell compartments (mural and cumulus) but adiponectin had no effect on these genes. Our results also indicate that leptin suppresses AMH mRNA expression via the JAK2/STAT3 pathway, as treatment with
Table III  Gonadotrophin dose inversely correlates with AMH mRNA levels.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Follicle size</th>
<th>Granulosa cell</th>
<th>n</th>
<th>$R^2$</th>
<th>Estimate</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>Large</td>
<td>Cumulus</td>
<td>15</td>
<td>0.77</td>
<td>−0.5</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mural</td>
<td>16</td>
<td>0.48</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>AMH</td>
<td>Small</td>
<td>Cumulus</td>
<td>18</td>
<td>0.61</td>
<td>−0.4</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mural</td>
<td>13</td>
<td>0.84</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Linear regression between gonadotrophin dose and logAMH gene after adjusting for age and BMI. NS, not significant.

Table IV  Leptin and adiponectin levels in follicular fluid from normal weight, overweight and obese women.

<table>
<thead>
<tr>
<th></th>
<th>Normal weight (n = 16)</th>
<th>Overweight (n = 13)</th>
<th>Obese (n = 9)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin (ng/ml)**</td>
<td>16 (9.9−44)$^a$</td>
<td>22.2 (12.7−74)$^b$</td>
<td>48.5 (37−52)$^c$</td>
<td>0.003</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)**</td>
<td>1.9 (0.8−2.8)</td>
<td>2.1 (1.0−5.2)</td>
<td>1.0 (0.7−2.2)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Values are expressed as medians (range). The Kruskal–Wallis test was used for comparisons among the subgroups of BMI: normal (18.5−24.9 kg/m²), overweight (25−29.9 kg/m²) and obese (≥ 30 kg/m²).

Statistical significance: $^a$ versus $^b$, $^b$ versus $^c$.

$^a_{r} = 0.7$, $P = 0.03$.

$^b_{r} = 0.46$, $P = 0.03$.

JAK2/STAT3 inhibitor prevented leptin-induced changes in AMH mRNA in all compartments but not AMHR-II mRNA expression in cumulus GCs. The pathway through which leptin suppresses AMHR-II gene in mural GCs remains to be determined. We, therefore, hypothesize that as women gain more fat cells, elevated leptin levels circulate to the ovaries, where it suppresses the expression of AMH and its receptor. Whether weight loss, and hence reduced levels of leptin, improves AMH production remains to be determined.

There is strong evidence for a role of adiponectin in the ovarian physiology (Michalakis and Segars, 2010). This adipokine has a direct effect on the late stages of folliculogenesis, and additive interactions of adiponectin with insulin and gonadotrophins in inducing periovulatory changes in ovarian follicles have been demonstrated (Ledoux et al., 2006; Campos et al., 2008). Ledoux et al. (2006) found that adiponectin receptor (AdipoR1 and AdipoR2) mRNAs are present in theca and GCs. Additionally, Chabrolle et al. (2009) showed that these receptors are detected in human GCs and that adiponectin increases IGF-I-induced E₂ and progesterone secretion in primary human GCs. In this study, we hypothesized that adiponectin treatment in vitro would up-regulate AMH and/or AMHR-II gene expression in human luteinized GCs. However, our results showed no changes in these two genes upon treatment with adiponectin. Although we used the same adiponectin dose and duration of culture that showed an effect on steroid release by GCs (Chabrolle et al., 2009), it could be that this dose and duration might not be appropriate for alterations in AMH and AMHR-II gene expression.

Our results also indicate that gonadotrophins received during ovulation induction for IVF could have a negative impact on AMH gene expression in human GCs as we found an inverse correlation between the gonadotrophin dose and AMH mRNA levels in most cell compartments after adjusting for age and BMI. Indeed, Pellat et al. (2007) have shown that FSH treatment significantly reduced AMH expression in cultured GCs of women with PCOS. Additionally, Baarends et al. (1995) reported that recombinant FSH treatment suppressed the expression of AMH mRNA in adult rat ovaries. Not only AMH mRNA levels but also serum and follicular fluid AMH protein levels might be altered by gonadotrophins. For instance, Lee et al. (2010) recently studied the dynamics of serum AMH levels in 74 women undergoing COH for IVF. They measured serum AMH at baseline, on Day 5 of stimulation and on the day of hCG administration. They found that serum AMH declined progressively and significantly during COH until the day of hCG. They have also shown a significant inverse correlation between gonadotrophin dose used and serum and follicular fluid AMH levels at all times. Serum and follicular fluid AMH levels and dynamic changes were not different between the GnRH agonist and antagonist cycles in that study. As for serum and follicular fluid AMH, we report that AMH mRNA expression is inversely correlated with the cumulative dose of gonadotrophins used during COH in women undergoing IVF. We also found no difference between GnRH agonist and antagonist cycles in AMH or AMHR-II mRNA levels (data not shown). Taken together, these results suggest that the changes in hormonal milieu may affect AMH production at gene level.

We also found that there is higher AMH gene expression in cumulus compared with mural GCs. Similar to our results, others (Grondahl et al., 2011) have evaluated using the microarray technique whether AMH mRNA was differentially expressed in cumulus and mural GCs from large antral and pre-ovulatory follicles collected from individual follicles in women undergoing in vitro maturation or IVF treatment. They found that AMH gene expression was significantly higher in cumulus than mural GCs from both mature and immature follicles. Unlike mural GCs, cumulus GCs surround the oocyte, and therefore, we hypothesize that there is a cross talk (such as release of factors from the oocyte) between cumulus GCs and the oocyte.
that might be influencing this pronounced AMH gene expression. This hypothesis will be further evaluated.

Age is the most important negative predictor of serum AMH and we found that there is significant inverse correlation between age and AMH gene expression in all cell compartments (i.e. mural and cumulus) in all follicles (i.e. small and large). The correlation between age and AMHR-II gene expression reached significance only in cumulus GCs in small and large follicles. We also found that age is inversely correlated with follicular fluid AMH protein levels. These results indicate that as women get older, there is both a decrease in AMH production at mRNA level by GCs and a decline in the release of AMH protein into the follicular fluid environment.

In this study, sample sizes for gene expression analyses are lower than the number of participants because we used only high-quality samples and those samples with sufficient RNA for RT–PCR. Limitations to this study include a luteinized GC model, as these cells were collected from women who were hyperstimulated with gonadotrophins. On the other hand, the stimulation protocols were similar for all women, and the differential gene expression between the cumulus and the mural compartments seen under these supraphysiologic conditions may point to a bigger difference in the physiologic state. Another limitation is that due to paucity of GCs, we were unable to divide the GCs from the same patient into more than two samples in order to investigate a dose–response relationship for leptin and adiponectin in vitro treatment.

The reason for this is that the RNA quantity and quality was not good enough for RT–PCR when samples were further divided.

In conclusion, we have shown that leptin, a hormone elevated in the serum and follicular fluid of obese women, directly suppresses mRNAs for AMH and its receptor. AMH is one of the most accurate markers

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**Figure 2** Effects of leptin and AG490 (JAK2/STAT3 inhibitor) on AMH and AMHR-II mRNA in human GCs. Recombinant leptin treatment (100 ng/ml) in vitro down-regulates AMH (A) and its receptor AMHR-II (B) mRNA levels in primary cell culture of human GCs (n = 7). We pre-treated cumulus and mural GCs from seven women [age = 31 (29–44) years, BMI = 27 (19–37) kg/m²] with AG490 (JAK2/STAT3 inhibitor) before treatment with leptin, and compared AMH and AMHR-II mRNA levels with the control GCs (cultured without AG490) from the same woman. Treatment with AG490 (50 μM) prevented leptin (100 ng/ml)-induced changes in AMH (n = 7 in cumulus, n = 4 in mural) (C) but not AMHR-II (n = 7 in cumulus, n = 4 in mural) (D) mRNA expression. Results were calculated using the ΔΔCT method relative to GAPDH. AMH level was variable among women, and therefore, results were expressed as percentages ± SEM and compared with the effect in the control medium (arbitrarily fixed at 100%). Results were analyzed using the Wilcoxon matched-pairs signed rank test between the control and the treated group. *P < 0.05, **P = 0.06.
used in ovarian reserve testing to date (Seifer et al., 2002; Hazout et al., 2004; Seifer and Maclaughlin, 2007; Nakhuda, 2008). These data may provide new insights into potential mechanisms that explain the lower AMH seen in obese women and might explain the poor response to ovulation induction observed in this population. Our data also suggest that leptin may program abnormal AMH signaling, thereby resulting in ovarian dysfunction. Understanding the impact of obesity on the ovary may offer opportunities for innovative therapeutic options for the treatment of ovulatory dysfunction in obese women. Future studies that target and block leptin receptors in GCs might be insightful in ascertaining whether such a therapy might restore AMH levels in the population of obese women, potentially resulting in a better response to ovulation induction.

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Authors’ roles

Z.M. performed the experiments, analyses and interpretation of the data and wrote the manuscript; E.B. contributed to performing the experiments and data interpretation and participated in writing the manuscript; D.S.B. and A.Z. performed the granulosa cell collection experiments and analyses; D.I. contributed to RT–PCR and data interpretation and participated in writing the manuscript; S.C. and S.J. participated in the study design, writing the manuscript and final approval.

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Conflict of interest

None declared.

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Figure 3 Effect of adiponectin on AMH and AMHR-II mRNA in human GCs. Recombinant adiponectin (5 μg/ml) in vitro does not alter AMH (A) or AMHR-II (B) mRNA levels in primary cell culture of human GCs (n = 8 for cumulus, n = 3 for mural). Results were calculated using the ∆∆CT method relative to GAPDH. AMH varied among women, and therefore results were expressed as percentages ± SEM and compared with the effect in the control medium (arbitrarily fixed at 100%). Results were analyzed using the Wilcoxon matched-pairs signed rank test between the control and the treated group.


