The anti-Müllerian hormone (AMH) induces forkhead box L2 (FOXL2) expression in primary culture of human granulosa cells in vitro

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Abstract

Purpose Anti-Müllerian hormone (AMH) and forkhead box L2 (FOXL2) are two pivotal genes expressed in human granulosa cells (hGCs) where both genes share similar inhibitory functions on activation and follicular growth in order to preserve the ovarian follicle reserve. Furthermore, AMH and FOXL2 contribute to inhibit steroidogenesis, decreasing or preventing the activation of gonadotrophin-dependent aromatase CYP19A1 cytochrome P450 family 19 subfamily A member 1 (CYP19A1). The purpose of this study is to evaluate the role of AMH in regulating the expression of FOXL2. **Methods** Primary cultures of hGCs were treated with increasing concentrations of recombinant human AMH (rhAMH; range 10–100 ng/ml) for 3 h. Negative controls were performed using corresponding amounts of AMH vehicle. Total RNA or proteins were purified and quantified by spectrophotometry. *FOXL2* and *CYP19A1* gene expression, normalized by reference gene *ribosomal protein S7 (RpS7)*, was evaluated by RT-qPCR. Each reaction was repeated in triplicate. Statistical analysis was performed. Extracted proteins were analyzed by immunoblot using anti-FOXL2 and anti- β -actin as primary antibodies.

Results rhAMH treatments tested did not modulate the basal expression of aromatase CYP19A1 gene. rhAMH (50 ng/ml) was able to increase *FOXL2* gene expression and its intracellular content.

Conclusions This study demonstrated the existence of an AMH-FOXL2 relationship in hGCs. AMH is capable of

increasing both gene and protein expression of FOXL2. Because FOXL2 induces *AMH* transcription, these ovarian factors could be finely regulated by a positive feedback loop mechanism to preserve the ovarian follicle reserve.

Keywords AMH · FOXL2 · hGCs · CYP19A1

Introduction

An ti-Müllerian hormone (AMH) is a glycoprotein (140 KDa) member of the transforming growth factor-beta (TGF β) superfamily. AMH was first identified in 1947 when it was shown that, during male sexual differentiation, a testicular factor produced by the Sertoli cells of the fetal testis caused the regression of the Müllerian ducts, the embryo structure that will originate the future female genital tract [1–3]. In females, AMH is produced postnatally by human granulosa cells (hGCs) after follicular activation until menopause [4]. AMH is produced by the cohort of growing pre-antral and early antral follicles and acts as a paracrine factor during the early stages of folliculogenesis in which inhibits follicles activation and their initial recruitment, resulting in the maintenance of primordial follicles in a dormant state [5, 6]. Moreover, AMH exerts an inhibitory role in the selection of the dominant follicle [7] and in reducing the responsiveness of growing follicles to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [8].

AMH mediates hGC functions by binding AMH type II receptors that ultimately activate the small mother against decapentaplegic (SMAD) proteins that interact with SMAD-4 and translocate to the nucleus to regulate transcription of target genes.

It has been shown that AMH is able to reduce the FSH receptor (FSHR) expression [9] influencing the

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process of selection through a strong inhibitory effect of AMH on FSH sensitivity of follicles [10]. Follicle growth corresponds to downregulation of AMH expression and to upregulation of aromatase *CYP19A1 cytochrome P450 family 19 subfamily A member 1* (*CYP19A1*) expression which influences the capacity of the follicle to produce estradiol [6]. AMH is capable of reducing FSH-stimulated aromatase mRNA expression and decreasing the number of LH/choriogonadotrophin receptors (LHCGRs) [2, 9, 11]. It is known that estradiol production is associated with high levels of FSH and *CYP19A1* in granulosa cells, but the presence of AMH significantly inhibited FSH-induced estradiol production and caused a reduction in *CYP19A1* mRNA and protein levels [12]. Moreover, although treatments with LH and FSH alone or combined on human granulosa lutein cells showed a strong induction of *CYP19A1* and *cytochrome P450 side chain cleavage* (*P450scc*) mRNA, addition of AMH suppressed this induction [8]. The well-known inhibition of FSH-induced adenylyl cyclase activation as well as the aromatase expression generated by AMH [13] was recently related to a corresponding *miR-181a* and *miR181b* induction [14].

Notably, the aromatase *CYP19A1* promoter contains two forkhead box L2 (FOXL2) binding sites B and one traditional forkhead binding site. FOXL2 is a member of forkhead (FKH) transcription factors, which is highly conserved and contains members that are essential in proliferation, differentiation, embryogenesis, and tumorigenesis [15, 16]. FOXL2 is expressed in vivo in pregranulosa cells of primordial follicles, slowly decreases in granulosa cells of adult follicles from the primordial stage to the pre-antral stage, and it is absent from the corpus luteum [7, 17–20]. Although FOXL2 seems a positive regulator of *CYP19A1* in various species, including goat, mouse, and human [21], in hamster cells (CHO) and mouse primary granulosa cells, the presence of FOXL2 represses the transcriptional activities of human *CYP19A1*. It has been found also that human FOXL2 suppresses ovarian follicle progression in small and medium follicles by avoiding premature differentiation and/or proliferation of hGCs repressing the transcriptional activities of human *P450scc* [16].

In our study, the effect of AMH on FOXL2 gene expression was investigated in hGCs recovered from pre-ovulatory follicles of patients that underwent to IVF, which constitutes a well-established experimental model to study the role of hormones such as gonadotrophins and AMH on hGCs [8, 22–24].

Since AMH and FOXL2 share a similar inhibitory role on the expression of key genes for steroidogenesis (i.e., *CYP19A1*), the main topic of our study was to clarify if AMH is able to regulate the ovarian response by modulating the expression of the transcription factor *FOXL2* gene.

Material and methods

Patients' selection

For this, study were enrolled a total of 15 patients (mean age of 33 ± 9) undergoing in vitro fertilization (IVF) because of male infertility. Patients selected were tested negative for either *chlamydia* antibody testing (CAT) or pelvic inflammatory disease (PID) or any other known metabolic or endocrinological disease; patients had regular menstrual cycles and did not have previous ovarian surgery or presence of ovarian cysts, had normal body weight and were not smoker.

Patients underwent IVF cycles according to the gonadotrophin-releasing hormone (GnRH) antagonist protocol here briefly described. Patients received a minimum of 150 IU/d subcutaneously from day 2 or 3 of a spontaneous menstrual cycle of recombinant human FSH (rhFSH, Gonal F®, Merck Serono, Italy or Puregon®, MSD Organon, Italy). Follicular growth was evaluated, and when the first follicle reached 14 mm of diameter in size, patients were then treated with subcutaneous injection of 0.25 mg/day daily with the GnRH antagonist ganirelix (Orgalutran, Schering-Plough, Kenilworth, NJ, USA) or cetrorelix (Cetrotide, Merck Serono, Italy). When follicles reached size of 18 mm or more, 10,000 IU of human corionic gonadotrophin (hCG) were administered intramuscularly. Follicles were aspirated 35 ± 1 h later and processed for oocyte retrieval. Study approval was obtained from the local ethics committee and informed, written consent was obtained from each patient.

hGC preparation and purification

hGCs were isolated from pre-ovulatory ovarian follicles of women undergoing IVF protocol after oocyte retrieval. The medium aspirated from ovaries (50 ml) in which oocytes were removed was processed by centrifugation through a discontinuous gradient of Percoll (Amersham, Sweden) as indicated in Nordhoff et al. 2011 [25], and the purified granulosa cells were plated (10^6 cells/well) in six well plates.

hGC primary cell culture and treatments

Primary hGC cultures were maintained at 37 °C under a controlled atmosphere of 5% CO₂ for 6 days to avoid side effect due to previous IVF hormone stimulatory treatment and subjected to daily medium changes with fresh culture medium (McCoy 5A medium (Carlo Erba, Italy) supplemented with 5% fetal bovine serum (FBS) South America (EU Approved, Carlo Erba, Italy), 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (Sigma Aldrich, St. Louis, MO, USA)).

hGC primary cultures were then incubated overnight in starvation buffer (McCoy 5A medium supplemented with

0.1% of FBS without antibiotic) to synchronize the metabolism of granulosa cells before treatments. hGCs were finally incubated with either 10, 50, or 100 ng/ml of recombinant human AMH (rhAMH, R&D Systems, Minneapolis, MN, USA) reconstituted in sterile 4 mM HCl and 0.1% bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO, USA) dissolved into starvation medium supplemented with 0.1% FBS South America for 3 h. Three single wells containing 10^6 cells/well from each single patient plated independently were treated with the same AMH dose and employed in the experiments. Negative controls using corresponding amount of vehicle control for rhAMH treatment were performed. Comparison between negative controls performed and untreated cells showed no differences in terms of cells vitality, toxic effects, or impaired gene expression caused directly by the vehicle in which substances were dissolved.

Trypan blue exclusion test of cell viability

The viability of rhAMH-treated hGC primary cultures was tested incubating hGCs corresponding to each treatment and controls with 0.4% Trypan blue in 1X Dulbecco's phosphate buffered saline (DPBS, Sigma Aldrich, St. Louis, MO, USA) solution added directly in the wells then immediately counted ($n = 200$) under inverted microscope Primo-Vert (Carl Zeiss Microscopy GmbH, Germany). Percentage of viable cells were calculated and expressed as ratio between blue-stained dead cells and not colored viable cells.

Total RNA extraction and reverse transcription

hGCs retrieved from 11 different patients were plated separately. For each patient, three wells containing 10^6 cells each were singularly treated with either 10, 50, or 100 ng/ml then processed as follows.

hGCs were lysed in 1 ml of commercial product Tri-Reagent (Sigma Aldrich, St Louis, MO, USA) and immediately processed for total RNA extraction following the manufacturer's protocol. Briefly, the total RNA in the colorless upper aqueous phase was precipitated with isopropanol, washed with 75% ethanol, and finally suspended in 20 μ l of RNase free water. After an optional DNase I (Promega, Madison, WI, USA) digestion of 30 min at 37 °C, the extracted total RNA was evaluated by spectrophotometry using Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) to determine its quality and concentration and 1 μ g of RNA for each sample was reverse-transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), according to the protocol. The thermal profile applied was 5 min at 25 °C, 30 min at 42 °C, and final 5 min at 85 °C using 2720 Thermal cycler (Applied Biosystem, Foster City, CA, USA).

RT-quantitative PCR (RT-qPCR)

The gene expression was evaluated by RT-qPCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) following the conditions suggested by the manufacturer's protocol. Same amount of template (2 μ l of cDNA) corresponding to each sample was analyzed in triplicate. Negative control reactions omitted templates.

Primers used in RT-qPCR reaction were designed, where possible, across the intron to avoid gDNA contaminants amplification, and they share the same melting temperature, so the general thermal profile applied (StepOne, Applied Biosystem Foster City, CA, USA) was the same for each gene tested: 30 s at 95 °C for initial activation, followed by 40 cycles of 5 s denaturation at 95 °C, and annealing/extension at 60 °C for 20 s. The sequences of the primers employed in RT-qPCR were the following: RpS7 (NM_001011.3) forward: 5'-AATC TTTGTTCCCGTTCCTCA-3'; RpS7 reverse: 5'-CGAG TTGGCTTAGGCAGAA-3'; CYP19A1 (NM_000103.3) forward: 5'-CCCTTCTGCGTCGTGTCAT-3'; CYP19A1 reverse: 5'-GATTTTAACCACGATAGCACTTTCG-3'; FOXL2 (NM_023067.3) forward 5'-GAGA AGAGGCTCACGCTGTC-3'; FOXL2 reverse 5'-TGAT GAAGCACTCGTTGAGG-3'. Results were normalized by using constitutively expressed *ribosomal protein S7 (RpS7)* as reference gene.

The specificity of each assay was validated by dissociation curve analysis, and amplicons were separated by gel electrophoresis and imaged. Assay performance was validated by evaluating amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount versus DCq (also known as DCT) has a slope. Each reaction was repeated in triplicate. Negative control reactions omitted templates.

FOXL2 immunoblot analysis

hGCs retrieved from four different patients were plated separately. For each patient, six wells containing 10^6 cells each were either untreated (control) or singularly treated with 50 ng/ml of rhAMH then pooled.

Proteins were extracted by direct lysis of rhAMH-treated hGCs in 4 °C lysis buffer containing 1X protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) and 5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Aldrich, St. Louis, MO, USA), then boiled.

Same amount of samples (30 μ g), 3 μ l of ColorBurst Electrophoresis Marker (Sigma Aldrich, St. Louis, MO, USA), were resolved through 12% polyacrylamide/bis-polyacrylamide gel, under denaturing conditions (SDS-PAGE). After 90 min at 120 V run, the resulting gel was transferred using Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA, USA) to a polyvinylidene fluoride

(PVDF, Thermo scientific, Rockford, IL, USA) membrane then blocked overnight in 5% skim milk powder (Sigma Aldrich, St. Louis, MO, USA) reconstituted in Tris-buffered saline (TBS, pH 7.4) solution. Filters with equal amount of samples were finally incubated for 1 h either with primary antibody anti-FOXL2 (Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:200 or anti- β actin (Abcam, Cambridge, UK) diluted 1:500. After three washes with 0.05% Tween 20 (Sigma Aldrich, St. Louis, MO, USA) in TBS, filters were furtherly incubated with 1:10,000 secondary antibody rabbit anti-Goat horseradish peroxidase (HRP) conjugated (Bethyl, Montgomery, TX, USA). Filters were covered with ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA), and bands were revealed by chemiluminescence using ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA). The signals were acquired and semi-quantitatively evaluated by an image analysis system (VersaDoc Imaging System and QuantityOne software, Bio-Rad Laboratories Inc.).

Statistical analysis

Statistical analysis was performed using Software GraphPad Prism applying student *t* test, as appropriate ($p < 0.05$) set for statistical significance. The relative expression of each gene has been evaluated using the $2^{-\Delta\Delta C_t}$ method [26] and was calculated as the relative ratio in comparison to the first control sample, set arbitrarily to 1.

Results

To investigate a possible role of rhAMH as modulator of gene expression, human granulosa cells retrieved from pre-ovulatory follicles of patients who underwent IVF were purified, cultured, and incubated for 3 h with increasing concentration of rhAMH. As presented in Fig. 1, both rhAMH concentrations tested (50 and 100 ng/ml) do not affect the basal

Fig. 1 Effect of 3 h incubation with increasing dosage of rhAMH (50 and 100 ng/ml) on aromatase *CYP19A1* gene expression normalized by the reference *Rps7* gene in primary culture of hGCs in vitro by RT-qPCR

expression of *CYP19A1* gene expression, normalized by reference gene *Rps7* on RT-qPCR assay.

To understand the role of rhAMH in regulating *FOXL2* gene expression, dose/response experiments were performed. hGCs were separately incubated with increasing dosage of rhAMH ranging from 0 to 100 ng/ml for 3 h. Results of *FOXL2* gene expression normalized by the reference gene are presented in Fig. 2. The different dosages of rhAMH tested were able to increase the *FOXL2* gene expression although due to their high standard deviations, the 10 ng/ml and 100 ng/ml treatments were not significantly different from control, while the 50 ng/ml treatment showed a consistent activation of *FOXL2* gene expression.

Based on the evidences previously obtained, we chose to analyze the effect of rhAMH (50 ng/ml for 3 h) on *FOXL2* protein production by immunoblot. Fig. 3a shows the image of one representative immunoblotted membrane. As shown in Fig. 3b, the mean of the semi-quantified amount of *FOXL2* generated by rhAMH treatment is increased in comparison to controls.

The percentage of viable granulosa cells at the end of each AMH treatment, tested using the Trypan blue exclusion assay, was consistently higher than 97% excluding the possibility of false negatives (data not shown).

Discussion

In the present study, we showed that rhAMH acts as a positive regulator of *FOXL2* gene in the primary cultures of hGCs. *AMH* and *FOXL2* are two pivotal genes expressed in hGCs, and both exert a general inhibitory function on activation and follicular growth, in order to preserve the ovarian follicle reserve [20].

The existence of a common regulatory pathways that involve *FOXL2* and *AMH* has already been demonstrated. In a study of Park et al. in 2014, it has been hypothesized the existence of a positive regulatory network involving *AMH* and *FOXL2* that controls follicular reserve. In this system, *FOXL2* may be the transcriptional activator of *AMH* and they could limit together early follicular growth. This relationship was confirmed in human granulosa-like cell lines (KGN cells) *FOXL2* knockout in which the transactivation level of *AMH* and its production were decreased and in *AMH* null mice where ovaries showed a decreased *FOXL2* expression and the accelerated follicular growth generated by the *AMH* absence, was reverted by intraovarian injection of *FOXL2* transfected vector. Finally, when KGN cells and murine granulosa cells were incubated with *AMH*, the *FOXL2* promoter activation and protein level increased [17].

The present study is based on mature granulosa lutein cells, and consequently to ovarian stimulation therapy, this may not reflect the physiological condition. Moreover, our cultural

Fig. 2 Effect of rhAMH (range 10–100 ng/ml) on *FOXL2* expression after 3 h of incubation in primary culture of hGCs in vitro. Ratio of expression was normalized using the reference gene *RpS7*. Asterisk indicates significantly different values compared with untreated cell controls (* $p < 0.05$, Student's *t* test)

system is not able to separate mural granulosa cells from cumulus cells. Anyway, the data obtained showed that both *FOXL2* gene and its protein expression were increased after treatment with rhAMH, demonstrating the existence of the relationship between AMH and *FOXL2* in primary culture of hGCs as already demonstrated in different experimental models. However, due to the absence of FSH in culture, in the current study, neither the AMH treatment nor the increased

FOXL2 mRNA and protein levels generated by AMH treatment affected *aromatase CYP19A1* gene expression.

Since *FOXL2* is a positive activator of AMH transcription, it is possible to speculate that these ovarian factors interact during folliculogenesis through a finely regulated positive feedback loop.

Future studies using siRNA and knockout mutant murine for *FOXL2* and *AMH* genes will expand further our knowledge on the events that influence early phases of folliculogenesis.

Fig. 3 **a** Image of one representative immunoblot of *FOXL2* intracellular production in primary culture of hGCs 3 h incubated with 50 ng/ml of rhAMH normalized by β -actin. **b** Mean of *FOXL2* relative expression ($n = 4$) elicited by 3 h incubation with 50 ng/ml of rhAMH in primary cultures of hGCs. Untreated controls were set arbitrarily to 1. Significant differences versus the respective controls were marked by * $p < 0.05$, Student's *t* test

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study. (Stated also in “Patients’ selection” section of Materials and Methods in the manuscript)

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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