ORIGINAL ARTICLE: REPRODUCTIVE BIOLOGY

Positive cross talk between FOXL2 and antimüllerian hormone regulates ovarian reserve

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Objective: To demonstrate interregulation between FOXL2 and antimüllerian hormone (AMH) in ovarian folliculogenesis.
Design: Cell culture and animal study.
Setting: University research laboratory.
Animal(s): Five-week-old B6C3F1 mice.
Interventions(s): Molecular analysis and in vivo mouse experiment were performed to demonstrate that *AMH* is a target gene of FOXL2 in the ovary.
Main Outcome Measure(s): To determine whether FOXL2 transactivates AMH, luciferase reporter assay, electrophoretic mobility shift assay, and chromatin immunoprecipitation were conducted. Using an in vivo nucleic acid delivery system, the expression of AMH and/or FOXL2 was modulated in the mouse, and the ovaries were histologically analyzed.
Result(s): *AMH* is an endogenous target gene of FOXL2. In contrast, mutated FOXL2s found in premature ovarian failure patients were defective in their ability to activate *AMH* transcription in human granulosa cells. In vivo mouse gene delivery experiments revealed that *Amh*-knockdown accelerated follicle growth; however, the acceleration was prevented by ectopic expression of FOXL2.
Conclusion(s): AMH and FOXL2 collaboratively work to reserve ovarian follicles. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Folliculogenesis, ovarian reserve, transactivation

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FOXL2 is a member of the forkhead transcription factor family, whose members share a forkhead DNA binding domain (1). Mutations in FOXL2 cause blepharophimosis-ptosis-epicanthus inversus syndrome (BPES; OMIM #110100), an autosomal dominant familiar disease manifested by malformations of the eyelid and/or premature ovarian failure (POF) (2). Although the etiology of POF is largely unknown, failure to acquire a sufficient follicle pool or early exhaustion of the follicle pool can result in premature depletion of the ovarian follicles (3). Ovarian folliculogenesis is a complex process involving follicular maturation, from the primordial to the Graafian follicles, in preparation for ovulation. FOXL2 is highly expressed in oocyte-nurturing granulosa cells, especially in small ovarian follicles (4). FOXL2 deletion reprograms adult ovarian follicles into testicular cells, suggesting that FOXL2 is required to maintain ovarian properties (5). Furthermore, $FoxL2^{lacZ}$ homozygous mutant female mice are infertile owing to early ovarian follicle depletion (6, 7). These reports indicate that FOXL2 is an essential molecule in the regulation of folliculogenesis.

Antimüllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a member of the transforming growth factor-ß (TGF-ß) signaling family. AMH was initially identified as an embryonic testicular growth factor involved in the regression of Müllerian ducts in male sex differentiation (8, 9). AMH is produced by granulosa cells in the ovary and Sertoli cells in the testis (10–13). In the ovary, AMH is expressed in the cuboidal granulosa cells of the primary follicles as soon as primordial follicles are recruited from the dormant pool, and AMH expression increases until the large preantral and small antral

Received March 10, 2014; revised May 15, 2014; accepted May 19, 2014.
M.P. has nothing to disclose. D.-S.S. has nothing to disclose. K.L. has nothing to disclose. J.B. has nothing to disclose.
This work was supported by a grant from the National R&D Program for Cancer Control, Ministry for Health and Welfare, Republic of Korea (1220090). It was also partially supported by the Next-Generation BioGreen 21 Program (SSAC, grant no. PJ009025), Rural Development Administration, Republic of Korea.
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Fertility and Sterility® Vol. ■, No. ■, ■ 2014 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2014.05.031

follicular stages are reached (14, 15). AMH knockout mice show enhanced initial and cyclic recruitment, followed by early depletion of primordial follicles (16, 17). Recently, AMH has been identified as a promising biomarker that accurately reflects ovarian reserve (15). The serum AMH levels in normal cycling women have been shown to decrease with age, eventually becoming undetectable in women undergoing menopause (18). In addition, changes in serum AMH levels occur early, as a result of events associated with ovarian aging (19), and serum AMH levels in patients with POF are extremely low or undetectable (20, 21). These studies indicate that proper expression of ovarian AMH is critical for normal folliculogenesis. However, the regulatory network controlling AMH production in the ovary is poorly understood.

Since the defects of both FOXL2 and AMH are associated with POF, we hypothesized that FOXL2 transactivates *AMH* to prevent early follicle recruitment. Here we identify a positive regulatory network, involving FOXL2 and AMH, which controls ovarian follicular reserve.

MATERIALS AND METHODS

Chemicals

Chemicals used were purchased from Sigma-Aldrich unless otherwise indicated.

Plasmids

Primers were purchased from Bioneer. The nucleotide sequences of all primers used are shown in Supplemental Table 1. The human *AMH* and *FOXL2* promoter were polymerase chain reaction (PCR) amplified using KGN genomic DNA as a template. The PCR product was digested with *Mlu*I and *Xho*I (Takara Bio) and ligated into pGL3 (Clontech). Constructs driving the expression of myc-tagged mutated FOXL2s were generated by PCR. The resultant PCR products were digested with *Eco*RI and *Xho*I (Takara Bio) and ligated into the pCMV-Myc vector (Clontech). For recombinant protein purification, AMH and FOXL2 were amplified by PCR. The PCR products were digested with *Nco*I/*Not*I and *BamH*I/*Xho*I (Takara Bio), respectively, and ligated into the pET28a(+) (Millipore).

Small Interfering RNAs

The target sequences of short interfering RNAs (Bioneer) used are as follow: siFOXL2, 5′-GCUCCUGUCGCUCCUCUUU. The sense and antisense oligonucleotides were annealed in the presence of annealing buffer (Bioneer). For short-hairpin RNA (shRNA)-mediated knockdown of mouse Amh, a shRNA plasmid was generated. RNA-interference oligos were purchased from Bioneer, with the following sequences: 5′-GATCCCCCTAGTCCTACATCTGGCTGTTCAAGAGACAGCCAGATGTAGGACTAGTTTTTA and 5′-AGCTTAAAAACTAGTCCTACATCTGGCTGTCTCTTGAACAGCCAGATGTAGGACTAGGGG. The pSUPER vector system (OligoEngine), designed for the expression of short interfering RNA, was then used to generate the shRNA plasmid.

Human Granulosa Cell Culture and Transfection

Human granulosa cell tumor–derived KGN cells (Yoshihiro Nishi and Toshihiko Yanase) were cultured in Dulbecco's modified Eagle medium/F12 medium (Caisson). Medium contained 10% fetal bovine serum (Caisson) and 1% penicillin-streptomycin (Caisson). KGN cells (4×10^5) were resuspended in resuspension R Buffer (Invitrogen), electroporated with 170 ng of pCMV ß-galactosidase plasmid DNA (Clontech), 300 ng of a luciferase reporter plasmid, and plasmids encoding either FOXL2 WT or a mutant version thereof using a Neon system (Invitrogen) and incubated on plates containing fresh media.

Luciferase Assay

Luciferase assay was performed as described by Kim et al. (22). Absorbances were measured with a FlexStation3 Microplate Reader (Molecular Devices).

Recombinant Protein Purification

His-tagged human FOXL2 and AMH proteins were purified based on our previous study (23).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as we reported elsewhere (23). Double-stranded oligonucleotides with the following human AMH sequences: 5′-CCTGCACAAACACCCC or 5′-GGGGTGT TTG TGCAGG, and 5′-ACGGCATGTTGACACATC or 5′-GATGTGT CAACATGCCGT, were used.

Chromatin Immunoprecipitation-quantitative Polymerase Chain Reaction (ChIP-qPCR) Analysis

KGN cells (2×10^7) were electroporated with plasmids using a Neon system and then incubated in 100-mm dishes for 24 hours. ChIP assays were performed as described elsewhere (23). DNA was amplified using primer sets flanking the putative FOXL2 binding motifs in the *AMH* promoter: FBE1, 5′-AGCGCTGTCTAGTTTGGTTGC and 5′-TCTCCCTCCCCAGTG ATAGAG; FBE2, 5′-AAAGGGCTCTTTGAGAAGGCC and 5′-GCCTTAAGTGAGCCGAGTGGA. PCR products were analyzed by qPCR.

In Vivo Intraovarian Injection and Animals

Five-week-old B6C3F1 mice (a hybrid between C57BL6/N and C3H/HeN) were obtained from SLC Inc. The animal room was maintained at 30%–40% humidity, and a temperature of 22 ± 1°C. Lighting in the room followed a 12 hour light/dark cycle. All animals were treated humanely, with every attempt made to ease suffering. Additionally, the experimental protocol was approved by the Chung-Ang University Institutional Animal Care and Use Committee. Injections were performed on postnatal day 35. For injection of the shAmh (1 μg) and FOXL2 (1 μg) plasmids, nucleic acid was slowly injected with a microsyringe (Hamilton). In vivo electroporation was performed immediately after nucleic acid injection with an Electrosquare Porator ECM830 electroporator (Harvard Apparatus). Mouse ovaries were held in tweezer-type electrodes, and square