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;102:847–55. ©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

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AMH expression increases until the large preantral and small antral 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

electric pulses were applied 8 times at 50 V, each pulse with a constant duration of 50 ms. Mice were divided into the following four groups: [1] control group, scramble RNA and vehicle DNA injection; [2] FOXL2 group, scramble RNA and FOXL2 DNA injection; [3] shAmh group, shAmh and vehicle DNA injection; [4] shAmh and FOXL2 group, shAmh and FOXL2 DNA injection. After 3 days, mice were euthanized to examine the short-term effect of gene modulation. Ovaries were collected and stored either in a deep freezer or in 10% formaldehyde until further analysis.

Preparation of Ovarian Tissue Sections

Ovarian tissue sections were prepared according to our previous report (24). Briefly, ovarian tissue samples stored in 10% formaldehyde were sequentially transferred into 70%, 80%, 90%, 95%, and 100% ethanol (Merck) for 1 hour each. Dehydrated tissues were then transferred to a xylene (Duksan) tank for clearing. Tissues were embedded in paraffin, cut into 6 μm-slices using a FINESSEE microtome (Thermo Scientific), and placed onto slides.

Immunoblot Analysis

After homogenization, lysates were prepared from ovaries using PRO-PREP solution (Intron). Equal amounts of total protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to membranes. Membranes were immunoblotted with anti-AMH antibodies (Abgent) or anti-FOXL2 antibodies (23), and the same membranes were incubated with anti-β-actin antibodies (Santa Cruz Biotechnology). After washing, membranes were incubated at room temperature for 2 hours with horseradish peroxidase–conjugated anti-rabbit IgG secondary antibodies (Santa Cruz Biotechnology) at a 1:5,000 dilution. Immunoreactive bands were visualized with enhanced chemiluminescence solution (Animal Genetics) and detected using a ChemiDoc XRS+ System Imager (Bio-Rad Laboratories).

Counting of Ovarian Follicles

Ovarian slides were deparaffinized and stained with hematoxylin and eosin. After washing in distilled water, slides were dehydrated and mounted for microscopic observation (Olympus). The numbers of follicles were counted from four ovaries per group. The number of total follicles in five sections from every sixth section of a distance of approximately 30 μm was counted. Follicle classification and counting were performed according to Pedersen and Peters (25).

PCNA (Proliferation Cell Nuclear Antigen) Staining

Immunohistochemical staining was performed as described elsewhere (24). Slides were incubated with mouse monoclonal anti-PCNA antibodies (DAKO) for 22 hours at 4°C at a 1:400 dilution.

Statistical Analysis

Data analysis was performed using SAS version 9.2 (SAS Institute). Statistically significant differences were identified with either the Student-Newman-Keuls multiple range test or Fisher's least significant difference test, at the 5% level of significance.

RESULTS

Identification of AMH as a Target Gene of FOXL2

To examine the relationship between two critical ovarian factors, FOXL2 and AMH, the human *AMH* promoter was cloned and tested for transcriptional activation by FOXL2. Ectopic expression of FOXL2 significantly activated the *AMH* promoter, and the basal level of *AMH* transactivation was decreased in FOXL2-knockdown KGN cells accompanying reduced endogenous AMH protein level (Fig. 1A). Based on a previous report (26), we predicted two putative binding elements for FOXL2 in the human *AMH* promoter sequence, at -206 to -200 (TGTTGAC) and -55 to -49 (ACAAACA), which we designated forkhead binding element (FBE) -1 and -2, respectively (Fig. 1B). We generated two mutant versions of the *AMH* promoter, harboring a mutation at either FBE1 or -2 and performed reporter assays to examine their transcriptional activation. As shown in Figure 1C, FOXL2 failed to fully transactivate versions of the *AMH* promoter with mutations in either the FBE1 or FBE2 sequence, compared with the wild-type (WT) *AMH* promoter (Fig. 1C). EMSAs performed with recombinant human FOXL2 confirmed that the regions from -206 to -200 and -55 to -49 in the *AMH* promoter are FOXL2-binding elements (Fig. 1D).

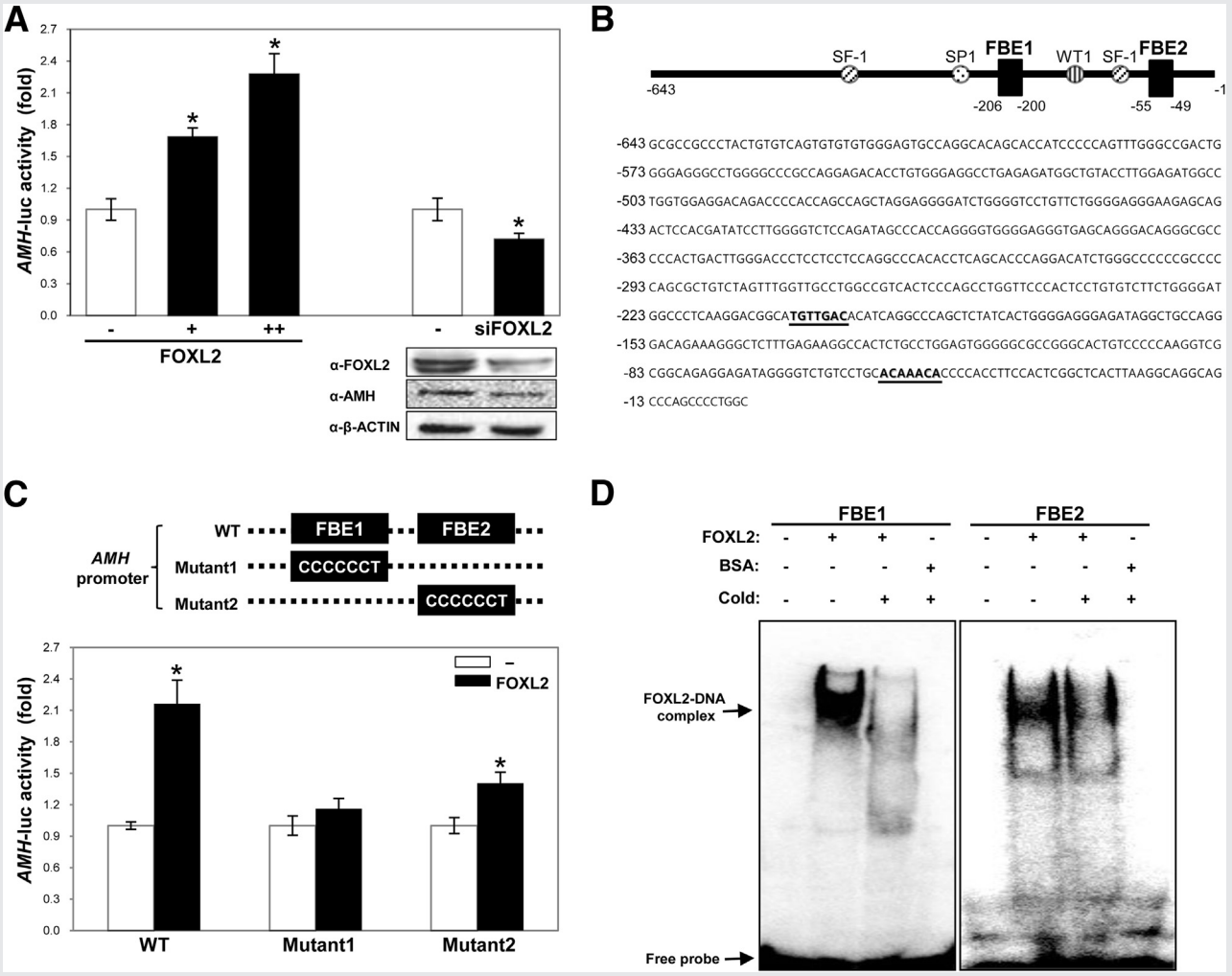
Defective Transcriptional Regulation of AMH by FOXL2 Mutants

Various versions of FOXL2 found in patients with BPES type I and POF, including versions harboring I80T, I84S, 1-94, and Q219X point mutations, were generated to determine their transcriptional activities on *AMH* (Fig. 2A). In contrast to WT FOXL2, all mutants failed to activate the *AMH* promoter (Fig. 2A). EMSAs performed using nuclear extracts of KGN cells transfected with either WT FOXL2 or mutant versions thereof demonstrated that the versions of FOXL2 found in patients with BPES type I (I80T and Q219X) exhibited no detectable binding to the *AMH* probe (Fig. 2B). In agreement with these data, ChIP-qPCR experiments showed that WT FOXL2 protein recruited significant amounts of the *AMH* promoter when it contained either FBE1 or -2 sequences; however, no enrichment of *AMH* by the mutant FOXL2 proteins was observed (Fig. 2C).

FOXL2 Expression Rescues the Accelerated Follicle Recruitment Induced by AMH Knockdown

To investigate the physiological significance of the FOXL2-AMH regulatory network in the ovary, we performed in vivo mouse experiments employing gene delivery of *FOXL2* and silencing of *Amh*. An shRNA vector specific for mouse *Amh* and/or plasmid DNA encoding human FOXL2 were delivered to female mice by intraovarian injection (Fig. 3A). Three days after transfection, ovaries were removed and examined. Efficient knockdown of Amh and overexpression of FOXL2 were confirmed by immunoblot analysis of

FIGURE 1



Transcriptional activation of *AMH* by FOXL2. (A) Promoter activation of human *AMH* by FOXL2 was determined by the luciferase reporter assay after transfection of increasing amounts of a FOXL2-encoding plasmid (100 and 150 ng) into KGN cells. Reduced transcriptional activity of *AMH* in FOXL2-knockdown KGN cells is also presented. Western blot analyses of FOXL2 and AMH are shown. Data (means ± SEM) from all promoter assays were obtained from at least three independent experiments, each conducted in triplicate, and are presented as fold increases of relative luciferase units compared with controls. *Asterisks* indicate significant differences compared with the control ($P<.05$). (B) Two putative FOXL2-binding elements (FBEs), FBE1 (-206/-200) and FBE2 (-55/-49), in human *AMH* promoter sequences are shown. Other binding elements for known transcription factors are also indicated. (C) FOXL2-induced reporter activation was determined using WT and mutated (FBE1 and -2 mutants) versions of *AMH* promoters. Luciferase assay was performed as indicated in panel A. (D) Results of EMSAs conducted using purified FOXL2 protein (3 μg) and ^{32}P -labeled probes (20 fmol), corresponding to the FBE1 and FBE2 sequences in the *AMH* promoter are shown. BSA (3 μg) and ×200 cold were used.

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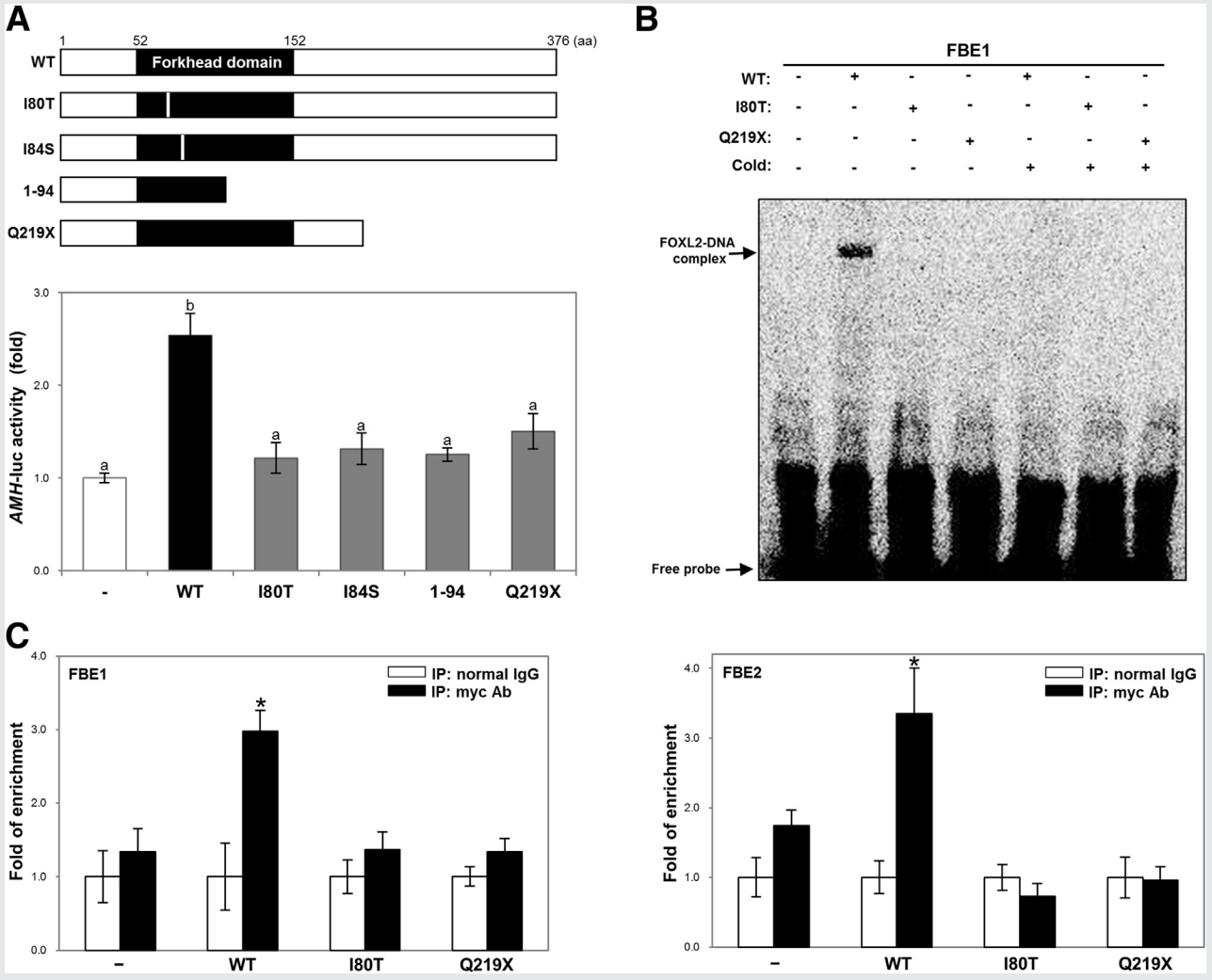
ovarian extracts (Fig. 3B). Interestingly, ectopic FOXL2 expression significantly upregulated the level of endogenous Amh, thus providing in vivo evidence that supports Amh being an endogenous downstream target of FOXL2 (Fig. 3B, left graph). In addition, the level of FOXL2 was moderately decreased in *Amh*-knockdown ovaries (Fig. 3B, right graph).

Histological examination of ovary sections from each group showed altered folliculogenesis, especially in *Amh*-knockdown ovaries, in which increased numbers of small growing follicles were present (Fig. 3C, III). Interestingly, the follicular changes induced by shRNA-mediated *Amh* knockdown were no longer observed when a FOXL2-expressing plasmid was coinjected (Fig. 3C, IV). Ectopic expression of FOXL2 itself did not induce any dramatic changes in the ovaries compared with in the control ovaries (Fig. 3C, I vs. II). To quantitatively analyze ovarian changes, follicles were counted. Compared with the control ovaries, *Amh*-knockdown ovaries exhibited decreased primordial follicle counts, whereas the numbers of primary and preantral follicles were increased by more than 200% (Fig. 3D). These observations indicate that *Amh* depletion leads to accelerated follicle recruitment. Surprisingly, the changes in follicular development induced by *Amh* knockdown were prevented when ovaries were cotransfected with a FOXL2-encoding

FIGURE 2



Differential transcriptional activation of *AMH* by WT FOXL2 and mutant versions of FOXL2 found in patients with BPES. (A) Mutant versions of FOXL2 generated for this study are presented. In addition, activities of FOXL2 proteins on *AMH* transactivation, as assessed by luciferase assays with KGN cells, are shown. Different letters denote statistically significant differences between groups ($P<.05$). (B) EMSA was performed using nuclear extracts (5 μg) isolated from KGN cells overexpressing either WT or mutated FOXL2 (I80T and Q219X). Biotin-labeled probes (20 fmol), corresponding to the -206/-200 (FBE1) sequences in the *AMH* promoter, were used. (C) KGN cells were transfected with plasmids encoding either WT or mutated FOXL2, and ChIP assays were performed as indicated in Materials and Methods. The FBE1 or -2 regions amplified by PCR in the assay are indicated on the left or right graph, respectively. As a negative control, normal IgG was used.

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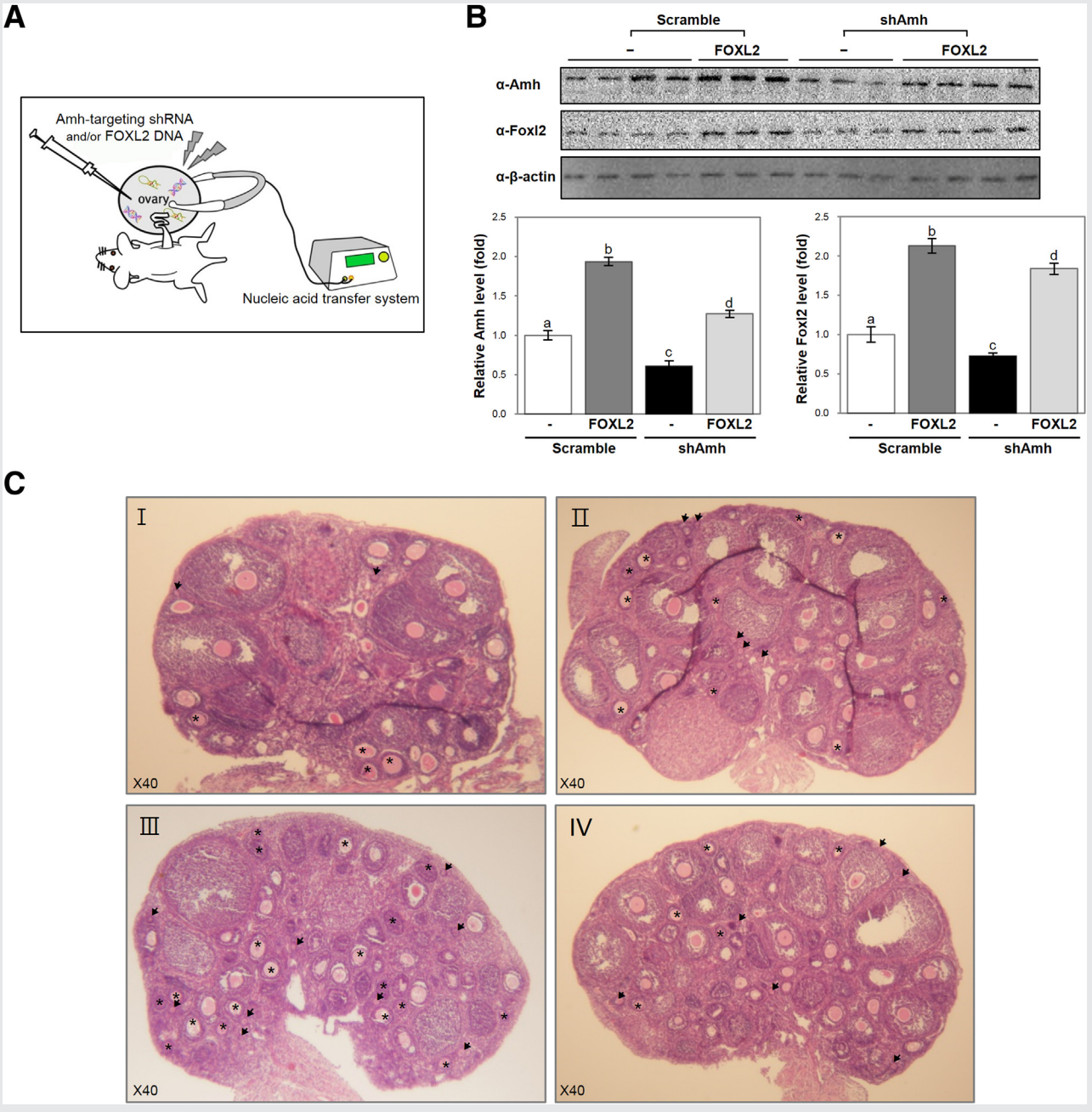
plasmid (Fig. 3D), which was consistently observed by analysis of absolute follicle counts (data not shown). These results suggest that forced FOXL2 expression can rescue the follicular defects resulting from *Amh* depletion. As a complementary technique, immunohistochemical analysis of PCNA, a biomarker of growing ovarian follicles (27), was performed. PCNA-positive granulosa cells were significantly increased in *Amh*-knockdown ovaries, especially in small (primary and preantral) follicles, indicating increased numbers of proliferating early-stage follicles (Fig. 3E). In contrast, this increase was greatly diminished by ectopic expression of FOXL2 in *Amh*-deficient ovaries (Fig. 3E), indicating that FOXL2 prevents the small follicle growth induced by Amh silencing.

Positive Feedback Regulation between AMH and FOXL2

Based on the observation that FOXL2 expression was decreased in Amh-knockdown ovaries (Fig. 3B, right graph), we investigated whether AMH plays any role in the regulation of FOXL2 expression. Incubation of KGN cells with recombinant AMH enhanced *FOXL2* promoter activation (Fig. 4A); furthermore, AMH-induced *FOXL2* activation was also observed in primary mouse granulosa cells (Fig. 4B). Moreover, AMH-induced upregulation of FOXL2 was also confirmed on the protein level by immunoblot analysis (Fig. 4C), indicating that AMH positively regulates FOXL2 expression.

FIGURE 3



FOXL2-mediated inhibition of accelerated follicle growth resulting from Amh-depletion in the mouse ovary. (A) Scheme of in vivo mouse intraovarian plasmid delivery is shown. (B) Mouse ovaries were electroporated to deliver shRNA specific for mouse *Amh*, and/or plasmid DNA encoding human FOXL2 was administered in vivo by intraovarian injection. The resultant changes in Amh and/or Foxl2 protein levels were examined by immunoblot analysis of ovarian extracts. Three or four ovaries, each from a different mouse, were used for each group. Expression levels were normalized to *β*-actin, and the quantified values of Amh (left graph) and Foxl2 (right graph) expression are also presented. (C) Representative hematoxylin and eosin-stained ovarian sections prepared from each group are shown: I, control; II, FOXL2; III, shAmh; IV, shAmh and FOXL2. *Arrowheads* indicate primary follicles, and *asterisks* denote preantral follicles. (D) Relative ratios of ovarian follicles at different developmental phases after modulation of Amh and/or FOXL2 expression levels are presented. CL = corpus luteum. Values (means ± SEM) represent the numbers of follicles at the indicated phase, expressed as percentages of the total number of follicles, from at least three ovaries in each group. Each ovary was isolated from a different mouse. Significantly different values between groups are indicated with different letters ($P<.05$). (E) Immunohistochemical analysis of PCNA expression, performed using ovarian tissue from each group, is shown. Representative tissue from each group is presented.

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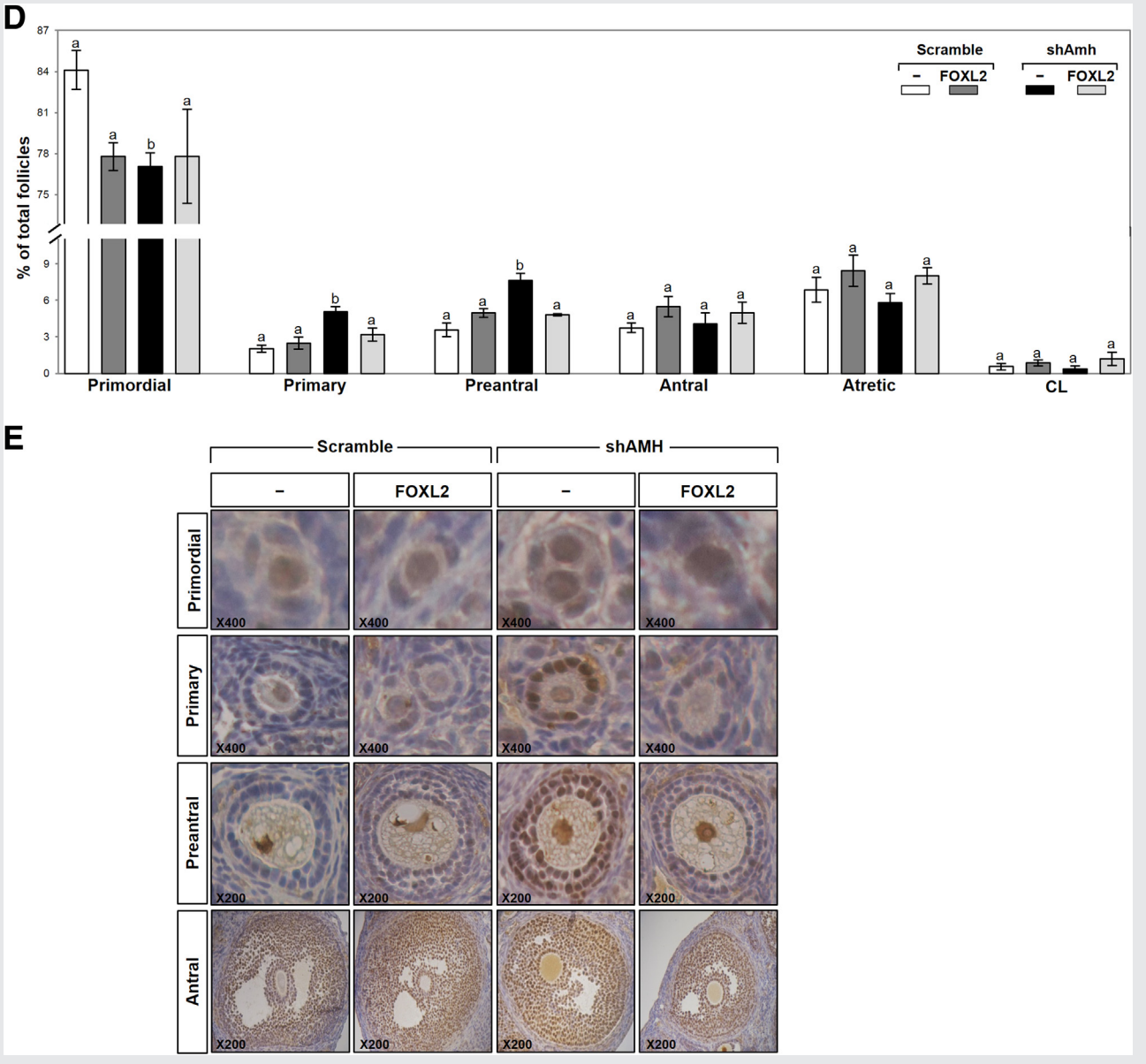
FIGURE 3 Continued



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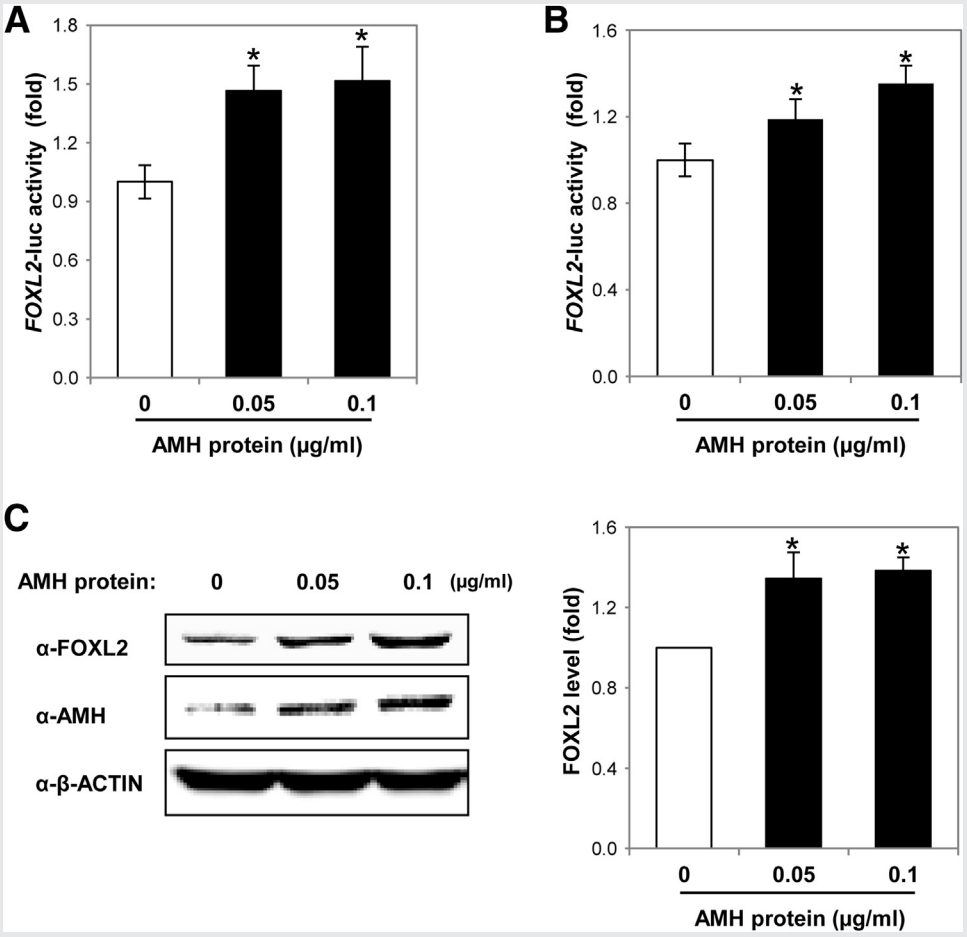
DISCUSSION

Folliculogenesis is a highly coordinated developmental process, which generates competent oocytes in addition to nurturing granulosa cells and theca cells. Several millions of primordial follicles are endowed in the fetal ovary; the majority of these follicles remain degenerate, whereas some are recruited for growth (28, 29). Although the signaling network governing follicular development has remained elusive, proteins in the TGF-β family are considered to be prime intraovarian candidates for regulating these events (30). AMH, a dimeric glycoprotein member of the TGF-β superfamily, is produced by granulosa cells in the primordial, primary, preantral, and small antral follicles. Furthermore, the serum level of AMH has been shown to be proportional to the number of developing follicles (31–33). Accumulating evidence indicates that AMH plays a crucial role in regulating the number of follicles for initial follicle recruitment from the primordial pool and preantal follicle development (20). Factors including steroidogenic factor-1, SOX-9, DAX, WT1, and GATA-4 are known to be involved in AMH regulation (34–36). However, the mechanisms by which AMH regulates ovarian follicular development are unknown. Here we presented data indicating that FOXL2 is the critical transcriptional activator of *AMH*, thus restricting early follicular growth. In vivo mouse gene delivery experiments revealed that Amh-knockdown

FIGURE 4



Positive feedback regulation of FOXL2 by AMH. AMH-induced activation of the *FOXL2* promoter was examined in KGN (A) and isolated mouse granulosa (B) cells. Cells were transfected with the *FOXL2* promoter construct; at 6 hours post-transfection, cells were incubated with 0, 0.05, or 0.1 μg/mL recombinant human AMH protein for 24 hours. Promoter activation was then measured with a luciferase reporter assay. *Asterisks* indicate significantly different values compared with the control ($P<.05$). (C) KGN cells were treated with AMH protein and harvested 24 hours after incubation. The representative immunoblot result (left panel) and quantified immunoblot results from four independent experiments performed (right panel) are shown. Significantly different values between groups are indicated with different letters ($P<.05$).

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accelerated follicle growth; however, the acceleration was prevented by ectopic expression of FOXL2, indicating that Amh and FOXL2 collaboratively work to reserve ovarian follicles. In addition, we described an in vivo intraovarian plasmid transfection protocol and demonstrated that this protocol can be a useful methodology for investigating in vivo the regulatory relationships between ovarian factors of interest.

FOXL2 is a protein expressed in granulosa cells that is critical for proper folliculogenesis. The importance of FOXL2 is demonstrated by the findings that humans with mutations in FOXL2, or FOXL2 knockout mice, exhibit POF associated with early depletion of ovarian follicles (6, 37). FOXL2 regulates expression of genes involved in steroidogenesis, proliferation, apoptosis, differentiation, and the stress response. These genes include *StAR*, *aromatase*, *p21*, *FAS*, *TNF-R1*, *Caspase 8*, and *SIRT1* (22, 38–40). In this study, we identified *AMH* as a direct ovarian target gene that is regulated by FOXL2 (Fig. 1). Of particular note, mutant versions of FOXL2 found in patients with BPES type I and exhibiting POF failed to activate *AMH* transcription (Fig. 2A). In agreement with the findings presented here, the serum AMH level of a patient with POF carrying a mutation of FOXL2 was shown to be considerably lower than the normal level (41); moreover, infertile *Foxl2*^{lacZ} homozygous mutant ovaries have been shown to exhibit significantly decreased *Amh* expression (6). Cumulatively, these observations indicate that regulation of AMH by FOXL2 is a pathophysiologically relevant event and imply that defective AMH regulation by FOXL2 mutants may be involved in the pathogenesis of POF.

Moreover, we discovered positive cross talk between FOXL2 and AMH, in which FOXL2-regulated AMH positively stimulates *FOXL2* transcription (Fig. 4). This observation indicates that these two critical ovarian factors are regulated by a positive feedback loop. Therefore, the work described here

presents a novel regulatory circuit capable of controlling ovarian follicle reserve by FOXL2 and AMH. In addition, this study opens the possibility of using FOXL2 and AMH in preventive intervention against POF.

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SUPPLEMENTAL TABLE 1

Nucleotide sequences of primers.

Genes	Sequences (5′ → 3′)
Promoters	
hAMH	(F) ACGACGCGTGGGCAAGTTCTGGACACACTG
	(R) CTACTCGAGGTCCCTGGCAGCCTATCTCCC
hAMH FBE1 mutant	(F) ACGGCACCCCCCTACATAGGCCC
	(R) TGTTGGGGGGTGCCGTCCTTGAGG
hAMH FBE2 mutant	(F) TGCCCCCCCTCCCCACCTTCCA
	(R) AAGGTGGGGAGGGGGGGCAGGACAGA
hFOXL2	(F) ACGACGCGTGACTTGGAGATGAACTCGCCCGTGC
	(R) CTACTCGAGACAGAGAGGGGCTCCGGCCTCGCC
Protein coding constructs	
hFOXL2 wild-type	(F) CTAGAATTCAAATGATGGCCAGCTACCCC
	(R) CATTCGCGCCTCGATCTCTGACTCGAGTAG
hFOXL2 I80T mutant	(F) GGTGCCGGACAGCGTGAGCCT
	(R) CTGTCCGGCACCTACCAGT
hFOXL2 I84S mutant	(F) ATGCTGTACTGGTAGAT
	(R) CCAGTACAGCATCACGAA
hFOXL2 1-94 mutant	(F) CCGGTCTCGGGCCAAGCAG
	(R) GAGACCGGTCGCACA
hFOXL2 Q219X mutant	(F) AGCAGGAGGCATAGGGCA
	(R) CCTCCTGCTAGATGGCGGCAG
Recombinant protein constructs	
hAMH	(F) CTTCCATGGAAATGCGGGACCTGCCTCTC
	(R) CTTGCGGCCGCCCGGCAGCCACACTCGGT
hFOXL2	(F) CTAGGATCCATGATGGCCAGCTACCCCGAG
	(R) TAGCTCGAGTCAGAGATCGAGGCGCGAATG

Park. FOXL2 and AMH regulate folliculogenesis. Fertil Steril 2014.