Published in final edited form as:

Fertil Steril. 2010 June ; 94(1): 353–356. doi:10.1016/j.fertnstert.2009.09.050.

Human Forkhead L2 Represses Key Genes in Granulosa Cell Differentiation Including Aromatase, P450scc, and Cyclin D2

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Abstract

FOXL2 is expressed in granulosa cells of small and medium ovarian follicles, functions as a repressor of the human StAR gene, a marker of a granulosa cell differentiation, and its mutation is associated with premature ovarian failure in women with BPES type I. We now report that FOXL2 also represses the transcription of aromatase, P450scc, and cyclin D2, three other key genes involved in granulosa cell proliferation, differentiation, and steroidogenesis, and that a FOXL2 mutation found in BPES type I patients also fails to repress aromatase transcription, further supporting a role for FOXL2 in follicle maturation.

Keywords

FOXL2; Premature ovarian failure; Granulosa cells; Aromatase; P450scc; Cyclin D2 (CCND2)

The duration of a woman's reproductive life is determined before birth by a limited numbers of primordial follicles. Primordial follicles develop to primary, secondary (medium) and then to antral (large, mature) follicles. Most antral follicles go through atresia, however, under proper stimulation with gonadotropins during the reproductive period, a few survive and develop further to the preovulatory stage. Every ovulation cycle uses many primordial follicles and eventually the pool of resting primordial follicles is exhausted, resulting in menopause (1). Although the average age of menopause is 51 years of age, 1% of women are reported to undergo menopause before the age of 40 (2), known as premature ovarian failure (POF). Extensive research has identified genes involved in POF (3,4), which include the gene encoding FOXL2 (5,6).

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Capsule:

Human FOXL2 is a repressor of aromatase, P450scc, and cyclin D2 promoters. It may inhibit the expression of these genes in granulosa cells of small follicles.

FOXL2 is a member of the winged helix/forkhead transcription family. FOX (forkhead box) family members are expressed in all eukaryotes and play important roles in the establishment of the body axis and in the development of embryonic tissue. Forkhead transcription factors bind to a common consensus DNA sequence, 5' [(G/A)(T/C)(C/A) A A (C/T) A] 3' in the promoters of target genes (7). FOXL2 is mainly expressed in the ovary and in the developing eyelid. In the mouse, FOXL2 expression starts at 12.5 dpc in female gonads, but not in developing male gonads, and is found in ovarian somatic cells in newborns (8). In the adult female, granulosa cells of early follicles express high levels of FOXL2 (8). Female FOXL2^{-/-} mice exhibit hypoplastic uterine tubes and ovaries and are infertile (8,9). In humans, mutations in the FOXL2 gene are known to cause Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome (BPES) (5,10), which is an autosomal dominant disorder. There are two types of BPES: type I which presents with a complex eyelid malformation in association with POF, and type II, in which only the eyelid defects are seen.

Previously, we have shown that FOXL2 is localized to undifferentiated granulosa cells in small and medium follicles, and binds to the human steroidogenic acute regulatory (StAR) gene promoter, suppressing its activity (11). StAR is a key molecule in steroidogenesis and is a marker of granulosa cell differentiation. StAR immunoreactivity is detected in granulosa cells of large preovulatory follicles, but not in small and medium immature follicles (12). We hypothesize that FOXL2 binds to the StAR promoter and suppresses StAR expression in granulosa cells until follicles reach later stages of development and differentiation. Mutations in FOXL2, with loss of repressor activity, could lead to accelerated differentiation of granulosa cells and follicle recruitment, leading to early depletion of ovarian follicles and POF. We also identified FOXL2 consensus sites in the promoter regions of other key genes that are involved in granulosa cell proliferation, differentiation, and steroidogenesis, including aromatase, P450scc, and cyclin D2. Aromatase is the enzyme that converts C-19 androgens to C-18 estrogens, which is a key product of differentiated granulosa cells (13). P450scc cleaves the side chain of cholesterol to produce pregnenolone, the first committed step and rate limiting process in steroid hormone synthesis (14). Cyclin D2 regulates cyclin-dependent kinases 4 or 6 (Cdk4 or Cdk6) to control G1 phase progression of the cell cycle and is involved in granulosa cell proliferation (15). In this study, we investigated whether FOXL2 also functions as a repressor of these three genes.

To do this, we generated reporter vectors for the aromatase, P450scc and cyclin D2 promoters. The human p27 promoter was used as a control. The ovary-specific human aromatase promoter (-1067-bp, the translation start site as +1: GenBank accession no. S85356), the human P450scc promoter (-1430-bp: GenBank accession no. M60421), the human cyclin D2 (-1624-bp) promoter (a generous gift from Dr Masataka Nakamura (16)), and the human p27 promoter (-1899-bp: sequence obtained from the UCSC Genome Browser) were amplified from genomic DNA using Hotstar Taq polymerase (Qiagen, Valencia, CA) according to the manufacturer's protocol. The PCR products were electrophoresed, extracted, and subcloned into pGL2 luciferase reporter vector (Promega, Madison, WI). The human wild-type FOXL2 and mutated FOXL2 expression vectors were prepared as previously described (11). The mutated human FOXL2 construct contains a premature stop codon (p.Q219X), corresponding to one found in families with BPES type I (10,17). We previously showed that this mutant lacks the proline and alanine rich repressor region and loses its activity as a repressor of the StAR promoter.

For the reporter assays, CHO cells (1×10^5 /well in a 24-well plate) were cultured in DMEM/F12 supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. The following day, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with the aromatase, P450scc, cyclin D2, or p27 promoter luciferase reporter vectors (500 ng per well) plus wild-type or/and mutant FOXL2 (0–200 ng per well). The total

DNA concentration in each well was maintained at 1 µg by adding the empty pcDNA3 expression vector, including 50 ng of indicator plasmid, pCMV-beta-galactosidase. Transfections were performed in an Opti-MEM® Media (Invitrogen, Carlsbad, CA) for 4 h, cells were then cultured in fresh medium with 10% FBS for 24 h. Twenty-four hours after transfection, the cells were washed with PBS once and lysed in 100 µl of 1× Reporter Lysis Buffer (Promega, Madison, WI). Cells were completely lysed by freeze/thawing. Twenty microliters of each sample was used for measurement of luciferase activity using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenberg, Germany). Beta-galactosidase activity was measured by the same plate reader, after the addition of 50 µl of beta-galactosidase substrate [200 mM Sodium Phosphate Buffer, 2 mM Magnesium Chloride, 100 mM beta-mercaptoethanol, and 1.33 mg/ml ortho-Nitrophenyl-β-galactoside (ONPG)] to 5 µl of cell lysate from each sample in a 96-well plate. Results were normalized using the beta-galactosidase activity. Normalized luciferase activity of each sample was compared to normalized luciferase activity when the empty expression vector backbone (pcDNA3) without FOXL2, was transfected (control). Values are shown as % of control. Each condition was tested in quadruplicate. Each experiment was also repeated at least three times. A paired t-test using 2 groups was used to compare the effects of wild-type and mutant FOXL2 at each concentration for each promoter. Significance was at $p < 0.05$ and $p < 0.07$.

We found that in the presence of wild-type FOXL2, aromatase, P450scc, and cyclin D2 promoter activities were repressed in a dose-dependent manner. However, wild-type FOXL2 had no effect on p27 (Fig. 1A–D: Wild-type FOXL2). In contrast, the luciferase activity of the aromatase promoter was upregulated in the presence of mutant FOXL2 (Fig. 1A: Mutant FOXL2), indicating that not only is mutant FOXL2's repressor activity lost but mutant FOXL2 may function as an enhancer of aromatase promoter activity. Mutant FOXL2 had no effect on P450scc promoter activity, demonstrating loss of repressor activity (Fig. 1B: Mutant FOXL2), but retained minimal repressor activity on the cyclin D2 promoter (Fig. 1C: Mutant FOXL2). However, this effect was only 20% from baseline, and was not dose-dependent. When wild-type and mutant FOXL2 was transfected with the p27 promoter, there was no difference in transcriptional activity between wild-type and mutant FOXL2 (Fig. 1D). The results indicate that wild-type FOXL2 is a repressor of aromatase, P450scc, and cyclin D2 promoters (but not p27) and the transcriptional repressor activity of FOXL2 is impaired or modulated when it is mutated.

These results confirm our hypothesis that, in addition to controlling the expression of the StAR gene, FOXL2 also represses the activities of other key genes involved in granulosa cell proliferation, differentiation, and steroidogenesis: aromatase, P450scc, and cyclin D2. Moreover, mutant FOXL2 (Q219X mutation), upregulates aromatase activity but has no effect on the P450scc and cyclin D2 promoters. This may suggest that mutant FOXL2 affects the first and final step in steroidogenesis which may lead to the POF phenotype in patients with FOXL2 Q219X heterozygous mutations.

FOXL2 is predominately expressed in the granulosa cells of the small and medium follicles however, FOXL2 expression is absent in the granulosa cells of large antral follicles and the corpus luteum (11). Unlike FOXL2 expression, but similar to StAR, aromatase and P450scc are present in granulosa cells of large follicles and the corpus luteum but not in small and medium follicles (18). In addition, cyclin D2 expression in granulosa cells increases when the follicles reach the preantral/antral stages (type 4–7), during rapid granulosa cell proliferation (19). Since FOXL2 is a transcriptional repressor of StAR, aromatase, P450scc, and cyclin D2, all genes involved in granulosa cell differentiation and proliferation, FOXL2 may function as a determinant of tissue differentiation, similar to other forkhead transcription factors (20–24), by inhibiting premature differentiation and proliferation of granulosa cells. This may in turn

control the number of primordial follicles that remain dormant and prevent premature depletion of ovarian follicles, however, further studies are necessary to elucidate these mechanisms.

The forkhead family of transcription factors are known to bind to the common consensus sequence, 5' [(G/A)(T/C)(C/A) A A (C/T) A] 3', which is found in the aromatase, P450scc, and cyclin D2 promoters, as well as the StAR promoter. We identified 11 candidate FOXL2 binding sites in the –1067-bp aromatase promoter, 20 in the –1430-bp P450scc promoter, and 14 in the –1624-bp cyclin D2 promoter. Recently, Benayoun et al. reported that FOXL2 has very high affinity to 5' [G T C A A G G (T/C)] 3' which differs from the known common consensus sequence of other forkhead members (25). The authors suggested that this unique FOXL2 consensus site would facilitate tighter regulation of gene expression by FOXL2 but not other FOX members. We identified this newly described binding site in the aromatase promoter only, but not the P450scc and cyclin D2 promoters, which may explain the differential effects of FOXL2 on the transcriptional regulation of these genes. Further, we (26) and others (27) have demonstrated that FOXL2 is sumoylated, a postranslational modification that enhances protein stability and plays a role in subcellular protein localization (28). Thus, postranslational modifications may also help determine differential effects on transcriptional regulation of these genes.

FOXL2 has been shown to colocalize with aromatase in ovaries of many species, suggesting that FOXL2 and aromatase may work in concert during ovarian development. In the frog (*Rana rugosa*), medaka, Nile tilapia, and Japanese flounder, FOXL2 upregulates aromatase promoter activity (29–32), in contrast to our findings which demonstrate that FOXL2 suppresses human aromatase promoter activity. Human FOXL2 contains a polyproline and polyalanine carboxyl terminus which we have previously shown to be a transcriptional repressor domain, and which is absent in the frog (*Rana rugosa*), medaka, Nile tilapia and Japanese flounder (29–32). Thus, FOXL2 may lose its activity as a transcriptional repressor in these species, and may even function as transcriptional activator of aromatase, as we found in our studies using truncated human FOXL2, which also lacks the polyproline and polyalanine tract. Taken together, these results suggest that human FOXL2 may play a complex role in the transcriptional regulation of ovarian genes, particularly StAR and aromatase. In humans, repression of key ovarian genes by FOXL2 may inhibit premature differentiation and proliferation of granulosa cells, and loss of this repressor activity may contribute to the premature depletion of ovarian follicles and POF seen in women with BPES type I.

Acknowledgments

The authors thank Dr Masataka Nakamura (Human Gene Sciences Center, Tokyo Medical and Dental University, Tokyo, Japan) for his generous gift of the Human cyclin D2 (–1624-bp) promoter construct.

Financial support: This work was supported by R01HD047603 from the National Institute of Child Health and Human Development (NICHD) and the Office of Research on Women's Health (ORWH), Bethesda, Maryland (MP) and by a grant from the Helping Hands of Los Angeles, Inc., Los Angeles, California (MP).

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Fig. 1. Effects of wild-type FOXL2 and mutant FOXL2 on (A) aromatase, (B) P450_{scc}, (C) cyclin D2, and (D) p27 promoters

Each promoter reporter vector was co-transfected into CHO cells with 0, 10, 30, 50, or 100 ng of wild-type FOXL2 or mutant FOXL2. Twenty-four hours after transfection, promoter activity was determined by measuring luciferase activity in cell lysates. Results are reported as % of control, where the control was transfected with the empty pCDNA3 vector (control is set at 100%). In order to statistically compare the effects of wild-type FOXL2 and mutant FOXL2 on each promoter, paired t-tests were performed comparing the effects of wild-type FOXL2

and mutant FOXL2 at different concentrations for each promoter. Significance was at $p < 0.05$ for aromatase and P450_{scc}, and $p < 0.07$ for cyclin D2.