HHS Public Access

Author manuscript

Fertil Steril. Author manuscript; available in PMC 2023 July 09.

Published in final edited form as:

Fertil Steril. 2009 April ; 91(4 Suppl): 1392–1394. doi:10.1016/j.fertnstert.2008.04.054.

Expression of forkhead transcription factors in human granulosa cells

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Abstract

Members of the forkhead box O1 (FOXO) family of transcription factors are expressed in granulosa cells during various stages of follicle development, and evidence from rodent and other model systems suggests that they may be involved in regulating follicular activation and oocyte maturation. In this report, we show that FOXO1, FOXO3, and FOXO4 are expressed in human luteinized mural granulosa cells, which may suggest that these transcription factors are also involved in human folliculogenesis and luteinization.

Female fertility is determined by the size of the primordial follicle pool formed during fetal life and by the rate of depletion of the pool after birth (1). In addition to reduced ovarian complement, early depletion of the follicular pool due to excess follicular activation and/or atresia can occur and results in infertility (2, 3). The events surrounding follicle activation, development of the preovulatory follicle, and follicular atresia are controlled both by ovarian factors, such as IGF-1 and estrogen, and by the gonadotropins LH and FSH (4).

The forkhead family of transcription factors is highly conserved in evolution, and its members are known to play critical roles in regulating cellular differentiation and proliferation in many systems (5). In the rodent ovary, members of the *Foxo* (forkhead box O1) subfamily, which includes *Foxo1* (FKHR), *Foxo3* (FKHRL1), and *Foxo4* (AFX), have been shown to be expressed in granulosa cells during various stages of follicle development and are thought to play important roles in oocyte maturation, ovulation, and possibly luteinization (6–11). Specifically, *Foxo1* has been shown to be negatively regulated by FSH and IGF-1 via the PI3-kinase pathway (12) and appears to mediate proliferation and differentiation of murine granulosa cells in response to FSH (11). Furthermore, studies using

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M.P. is Medical Editor of the babycenter.com website sponsored by Johnson and Johnson and is Editor of ASRM News. D.T. is currently employed by Celera. F.-T.K. has nothing to disclose. P.Z. has nothing to disclose. S.K. has nothing to disclose. A.K. has nothing to disclose.

porcine granulosa cells have shown that *Foxo1* is a regulator of cell cycle progression (7). *Foxo3*-null female mice have been shown to have abnormal ovarian follicular development with early degeneration of oocytes, resulting in age-dependent infertility (6). This appears to be due to early activation of primordial follicles in these mice, which leads to the early depletion of functional oocytes and the rapid onset of infertility (9, 10), a phenotype which resembles that of women with premature ovarian failure (POF). More recently, a transgenic mouse model with constitutively active expression of *Foxo3* in oocytes was also shown to be infertile, resulting from retarded oocyte growth and follicular development (13). Furthermore, mutations that result in loss of activity of some of the forkhead transcription factors have been shown to be related to human infertility. Watkins et al. (14) recently identified potentially causal mutations in the FOXO3A and FOXO1A genes in women with POF, and mutations in the related FOXL2 forkhead transcription factor (15, 16) have also been linked to POF (17).

To date, studies of the forkhead transcription factors have largely involved the rodent ovary, and there have been no studies to determine whether these transcription factors are expressed in the human ovary. Therefore, the present study was undertaken to determine whether the FOXO family of transcription factors are expressed in human granulosa cells. Future studies will be necessary to determine their role in the human ovary.

To determine whether members of the FOXO family of forkhead transcription factors are expressed in the human ovary, we first tested for the presence of human *FOXO1*, *FOXO3*, and *FOXO4* transcripts in commercially available human ovarian cDNA (Human Ovary PCR-Ready cDNA; Ambion, Austin, TX). Polymerase chain reaction (PCR) was used to identify transcripts of human *FOXO1*, *FOXO3*, and *FOXO4*, with β-actin as a control. Ovarian cDNA (2.5 ng) was used in a 50-μL PCR using the HotStar Taq DNA Polymerase Kit (Qiagen, Valencia, CA). The primers used to amplify *Foxo* cDNA fragments were as follows: *FOXO1* (5′-cagccctggatcacagtttt-3′ and 5′-catccccttctccaagatca-3’); *FOXO3* (5′-gcaagcacagagttggatga-3′ and 5′-caggtcgtccatgaggtttt-3′); and *FOXO4* (5′-ttgaggccagagtctgaggt-3′ and 5′- aggatgctgcaaagagaagc-3′). The PCR cycling profile consisted of an initial denaturation step at 95°C for 15 min, followed by 35 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s. After PCR, the amplified products were subjected to electrophoresis on 1.5% agarose gels, which were then stained with ethidium bromide to visualize the anticipated fragments. As a control, β-actin was amplified in a similar fashion. Bands of the anticipated sizes were obtained, and a band for each gene was eluted, purified using a QIAquick gel extraction kit (Qiagen) and confirmed by sequencing.

We then tested whether *FOXO1*, *FOXO3*, and *FOXO4* transcripts were present in human granulosa cells. We obtained luteinized mural granulosa cells from ten consecutive patients undergoing IVF for various reasons under an Institutional Review Board–approved protocol. The cumulusoocyte complexes were removed, and granulosa cells were obtained from the follicular aspirates. The granulosa cells were separated from red blood cells by a previously described protocol using Percoll gradient (18). Briefly, the follicle aspirates were centrifuged at 1800 rpm for 10 min, the supernatent was removed, and the cells were resuspended in 10 mL 1× phosphate-buffered saline (PBS) and spun at 1500 rpm for 10 min. The supernatant was aspirated, and the cells were resuspended in 4 mL of 1× PBS and layered on Percoll.

After centrifugation at 2000 rpm for 30 min, the luteinized mural granulosa cells were washed in 1× PBS and spun at 1500 rpm for 10 min.

The cell pellet was then lysed in RLT buffer (Qiagen), and total RNA was extracted using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. Reverse transcription of total RNA was then performed using the Omniscript Reverse Transcription Kit (Qiagen) as described in the manufacturer's protocol. The PCR was used to identify the transcripts of human *FOXO1*, *FOXO3*, and *FOXO4* as described above, again using β-actin as a control. Five microliters of the reverse transcription reaction was used in a 50-μL PCR using the HotStar Taq DNA Polymerase Kit (Qiagen). Positive control samples (human ovary cDNA [Ambion]) and negative control samples (sterile distilled water) were performed for each primer. The PCR primers and the PCR cycling profile were as described above. After PCR, the amplified products were subjected to electrophoresis on 1.5% agarose gels, which were then stained with ethidium bromide to visualize the anticipated fragments. As a control, β-actin was amplified in a similar fashion. A band for each gene was gel eluted, purified, and confirmed by sequencing as described above.

FOXO1 was found to be expressed in luteinized mural granulosa cells from all ten patients, as demonstrated by the 170-bp band in Figure 1a. The presence of a 186-bp band demonstrates the presence of *FOXO3* in the luteinized mural granulosa cells of all patients (Fig. 1b). *FOXO4*, as demonstrated by the 173-bp band, was also present in luteinized mural granulosa cells of all patients, regardless of the cause of infertility (Fig. 1c).

This is the first time that members of the FOXO subclass of forkhead transcription factors, specifically *FOXO1*, *FOXO3* and *FOXO4*, have been shown to be expressed in human luteinized mural granulosa cells. Previously, these transcription factors have been shown to be expressed in rodent and porcine granulosa cells, where they are thought to play important roles in normal follicular development, maturation, ovulation, and possibly luteinization (6–11). *Foxo1* is selectively expressed in granulosa cells of growing follicles, where it is differentially regulated by hormones, including FSH and LH. Activation of Foxo family members has been shown to block cellular proliferation by inducing expression of genes such as $p27^{kip}$ and Fas ligand, which cause cell cycle arrest and apoptosis (19–21). Studies by Park et al. (11) demonstrate that proliferation and differentiation of granulosa cells in response to FSH plus activin requires removal of Foxo1-dependent repression of the cyclin D2 promoter. In addition to transcriptional regulation of *Foxo1*, FSH and IGF-1 also stimulate rapid PI3K-dependent phosphorylation of Foxo1 (12). Phosphorylation of Foxo1 results in its exclusion from the nucleus, providing additional control of its transcriptional activity (8). Thus, Foxo1 appears to be a tightly-controlled key regulator of granulosa cell proliferation and differentiation.

Loss of Foxo3 transcriptional activity in $Foxo3a^{-/-}$ female mice leads to global follicular activation, resulting in oocyte death, premature depletion of the follicular pool, and secondary infertility, which suggests that Foxo3 normally functions as a repressor of follicular activation (6, 9). *Foxo3* and *Foxo4* are expressed in luteal cells (12), which may suggest additional roles for these transcription factors in follicular differentiation.

We have demonstrated expression of three FOXO family members, *FOXO1*, *FOXO3*, and *FOXO4* in human granulosa cells. Evidence from studies in rodents and other model systems, together with the identification of mutations in the human *FOXO1* and *FOXO3* genes in women with POF (14), suggest that the FOXO class of transcription factors may also play important roles in human folliculogenesis and luteinization. Future directions will include the quantification of the expression of these transcription factors and correlation with diagnosis of infertility, specifically relating to ovarian function, such as advancing age, diminished ovarian reserve, and anovulation. In addition, correlation of *FOXO* gene expression levels with clinical parameters, including ovarian response, E_2 levels, and eggs retrieved, may provide further insights into the roles of these genes in human folliculogenesis.

Acknowledgments

Supported by a grant from the NIH (R01HD047603) (to M.D.P.) and a grant from the Helping Hands of Los Angeles Inc. (to M.D.P.).

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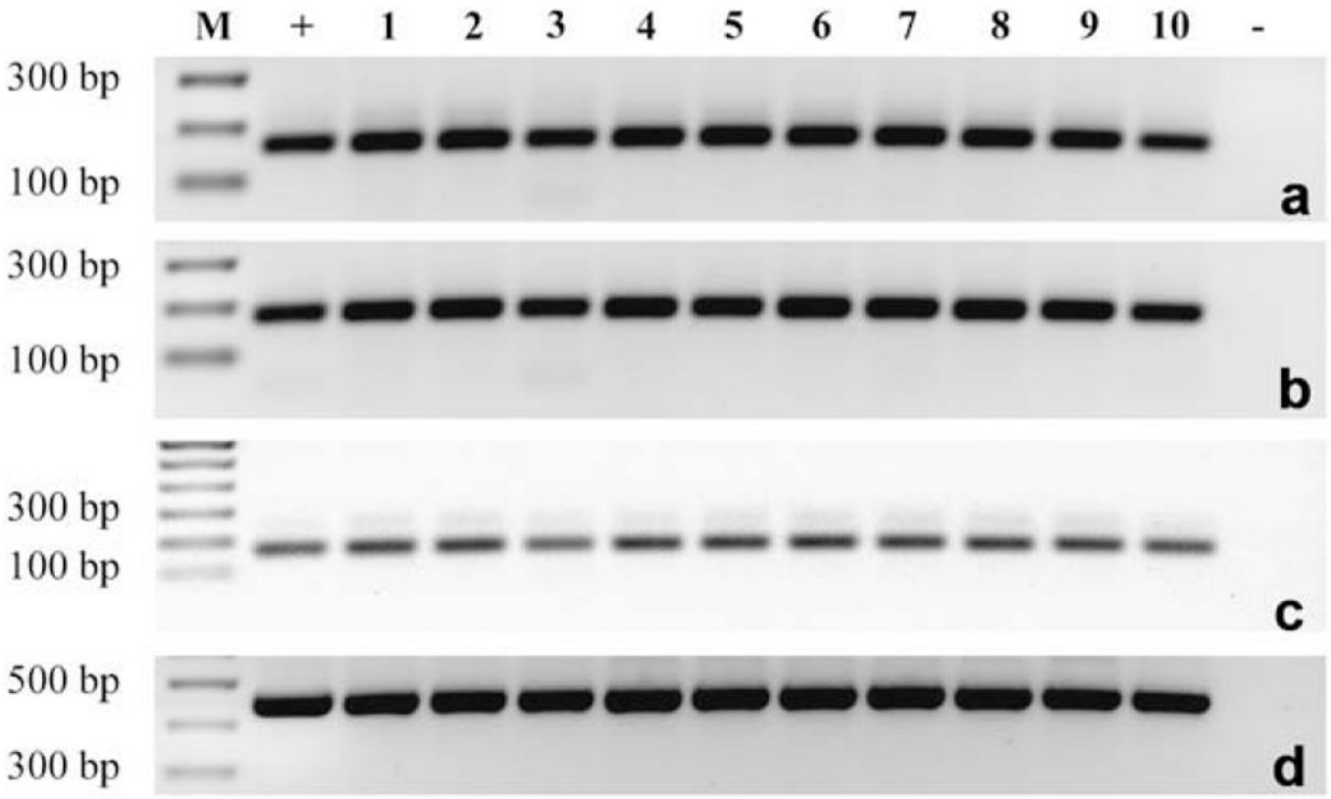

Figure 1.

Expression of *FOXO1*, *FOXO3*, and *FOXO4* in human luteinized mural granulosa cells. Total RNA isolated from human granulosa cells taken from ten different patients were reverse-transcribed and polymerase chain reaction (PCR) amplified with gene-specific primer pairs. Human luteinized mural granulosa cells from all ten patients were positive for the 170-bp product of *FOXO1* (**a**), the 186-bp product of *FOXO3* (**b**), and the 173-bp product of *FOXO4* (**c**), as well as for *β*-actin (**d**). M = molecular weight marker; + = positive control; − = negative control.