

# Forkhead transcription factors in ovarian function

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## Abstract

Since the discovery of the conserved *forkhead* (*Fkh*) DNA binding domain more than 20 years ago, members of the *Fkh* or forkhead box (FOX) family of transcription factors have been shown to act as important regulators of numerous developmental and homeostatic processes. The human genome contains 44 *Fkh* genes, several of which have recently been reported to be essential for female fertility. In this review, we highlight the roles of specific FOX proteins in ovarian folliculogenesis and present our current understanding of their molecular function. In particular, we describe what we have learned from loss-of-function studies using mouse models as well as human genetics and illustrate how different stages of folliculogenesis, both in oocytes and in somatic granulosa and theca cells, are regulated by FOXC1, FOXL2, and FOXO subfamily members.

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## Introduction

Female fertility depends on a delicate balance of hormonal stimuli and cellular interactions, which ultimately enable conception and a successful pregnancy. Current estimates state that 10–15% of couples worldwide remain childless due to infertility, with genetic etiology making up a significant proportion (Matzuk & Lamb 2002). Research using genetic linkage analysis or genome-wide association studies as well as genetically modified mouse models is uncovering a plethora of novel candidate genes for fertility disorders and is leading to a better understanding of female reproductive function (Matzuk & Lamb 2008). In addition to hormones and their receptors as well as signaling molecules, transcription factors have emerged as powerful regulators of ovarian function. It is becoming evident that loss of forkhead (*Fkh*) factors is detrimental to female fertility, which is the subject of this review (Choi & Rajkovic 2006, Richards & Pangas 2010).

## Mammalian folliculogenesis

Mature follicles of the mammalian ovary consist of a single oocyte encased by several layers of somatic granulosa cells and a large, fluid-filled cavity (the antrum). Around birth, the primordial follicle pool is established when oocytes are surrounded by a single squamous layer of granulosa cells. This finite nongrowing population, containing oocytes arrested in meiosis, represents the reproductive potential of the female. On follicular activation, primary follicles are recruited from the primordial pool and thus committed to subsequent

stages of folliculogenesis. This transition is marked by oocyte growth and proliferation of the adjacent granulosa cells that become cuboidal. Antral follicles are formed when fluid-filled spaces develop between the multi-layered granulosa cells. During preantral to antral transition, the oocyte resumes meiosis, and after stimulation by pituitary gonadotropins, FSH, and LH, the mature oocyte is expelled from the follicle and moves into the oviduct as an unfertilized egg. Concurrently, the remaining granulosa cells undergo luteinization to form an endocrine gland, the corpus luteum, which secretes progesterone to prepare the uterus for implantation (Matzuk & Lamb 2002, Matzuk *et al.* 2002).

It is important to note that the size of the primordial follicle pool formed perinatally and the rate of follicular depletion after birth determine the duration of female fertility (Erickson 2001). Research performed during recent years is starting to provide insight into the nature of the molecular communication that leads to the selective periodic activation and maturation of a subset of primordial follicles (see below).

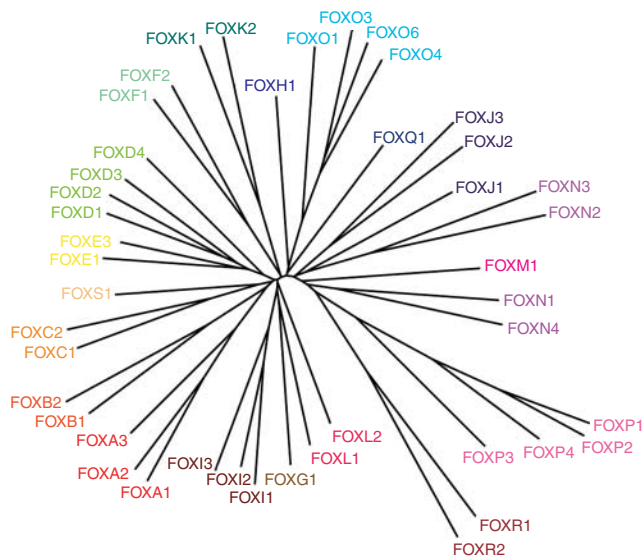
## The *Fkh* family of transcription factors

The forkhead box (FOX) family of transcription factors is named after the ectopic head structures observed in the mutants of the *Drosophila* gene *Fkh*, which became the family's founding member (Weigel *et al.* 1989, Weigel & Jäckle 1990). In humans (and in mice), the *Fkh* family has been extended to include 44 members that share a common DNA binding domain of up to 110 amino acids. This helix-turn-helix structure consists of three

$\alpha$ -helices, three  $\beta$ -sheets, and two 'wing' regions flanking the third  $\beta$ -sheet and resembling a butterfly, hence the alternative name 'winged helix'. Most FOX proteins bind to DNA as monomers (Lehmann *et al.* 2003).

Outside of the conserved Fkh domain, there is little protein sequence similarity between different clades of the family, which include transcriptional activators as well as repressors (see Fig. 1). The FOX family is highly conserved throughout evolution, and their nomenclature is based on a phylogenetic classification of Fkh domain protein sequences from more than ten species (FOXA through FOXS; Hannenhalli & Kaestner 2009).

Fkh factors have been shown to influence a diverse range of biological processes. For instance, FOX genes are necessary for the establishment of the body axis, for the development of cell types from all three germ layers, for metabolic and immunoregulatory processes, as well as cell cycle control and cellular survival. Oncogenic fusion proteins with members of the FOXO subfamily have been implicated in rhabdomyosarcoma and leukemia. Furthermore, the expression levels of several Fkh genes have been correlated with various types of cancer. Currently, 11 different human developmental disorders have been associated with mutations in FOX genes, with an interesting prevalence of eye abnormalities. Namely, mutations in FOXC1, FOXC2, FOXE3, and FOXL2 lead to ocular phenotypes in mice and humans (Lehmann *et al.* 2003, Hannenhalli & Kaestner 2009).



**Figure 1** The forkhead transcription factor family. Phylogenetic tree of all known human forkhead family members, based on similarity of protein sequences. The nomenclature of subfamilies is derived from a phylogenetic classification of forkhead domain protein sequences from more than ten species (including invertebrates). Alignment and dendrogram of human FOX protein sequences was performed using CLUSTALW.

In the following paragraphs, we will focus on those mammalian Fkh family members that have been associated with development of the female gonads and ovarian folliculogenesis to date.

### FOXC1

In addition to its role in eye development, one study has implicated FOXC1 in early ovarian function. *Foxc1* homozygous mutant mice display impaired migration of primordial germ cells (PGCs) to the gonadal anlage. In those animals, PGCs are initially specified correctly in the absence of FOXC1, but do not migrate efficiently to the genital ridges, with many PGCs remaining trapped in the hindgut, and the developing gonad being smaller and disorganized. To circumvent the neonatal lethality of the knockout mice, the authors performed transplantation experiments and found that in wild-type adult hosts, *Foxc1* mutant follicles do not develop beyond preantral stages (Mattiske *et al.* 2006). This report indicates that FOXC1 may be required for maturation of follicles beyond the early antral stage, but its potential target genes and regulatory mechanism remain to be elucidated. Despite a substantial amount of data on FOXC1 mutations causing Axenfeld–Rieger malformations and glaucoma observed in human patients, there are no data available at present on human allelic variants of FOXC1 with regard to female fertility (Tumer & Bach-Holm 2009).

### FOXL2 and blepharophimosis/ptosis/epicanthus inversus syndrome

Mutations in the human FOXL2 gene are the cause of the autosomal dominant blepharophimosis/ptosis/epicanthus inversus syndrome (BPES, MIM \*605597; Crisponi *et al.* 2001). The characteristic dysplasia of the eyelids observed in BPES patients can be associated with premature ovarian failure (POF; BPES type I) or not (BPES type II). POF is defined as the onset of menopause before the age of 40 years. There is a certain variance in the clinical spectrum associated with FOXL2 mutations, with infertility ranging from primary amenorrhea to irregular menses followed by POF and ovarian appearance varying from ostensibly normal to streak gonads (De Baere *et al.* 2001). Depending on the type and position of the mutation within the FOXL2 coding sequence, a putative genotype–phenotype correlation could be derived: mutations closer toward the N-terminus, leading to the production of a truncated FOXL2 protein, most likely lead to loss of function and cause both ovarian failure and eyelid abnormalities (BPES type I), whereas extensions such as frameshifts or duplications represent hypomorphic alleles and result in BPES type II (De Baere *et al.* 2003, 2009, Beysen *et al.* 2009).

C-terminal of the DNA binding domain, the FOXL2 sequence, encodes a polyalanine stretch that appears to be a mutational hotspot. Polyalanine tracts are frequently found in association with transcriptional repression, but currently there is no evidence that alanine tracts affect transcriptional control *per se* (Brown & Brown 2004). About 30% of human BPES patients carry mutations that lead to polyalanine expansions, mainly resulting in BPES type II (De Baere *et al.* 2003). Despite the intra- and inter-familial phenotypic variability observed for the various types of FOXL2 mutations, truncations before the polyalanine tract are almost certainly predicted to result in POF (Beysen *et al.* 2009, De Baere *et al.* 2009).

Interestingly, not all BPES patients carry mutations within the FOXL2 coding sequence but some individuals have been identified that exhibit genomic rearrangements several hundred kilobases upstream of the gene itself (Crisponi *et al.* 2004, Beysen *et al.* 2005). Apparently, long-range *cis*-regulatory elements are required for the correct spatiotemporal expression pattern of FOXL2, and translocations or microdeletions separating the FOXL2 transcriptional start site from these enhancers lead to loss of FOXL2 expression and thus BPES.

### FOXL2 in mouse ovary development

Consistent with human BPES in which ovary and eyelid development are affected, *Foxl2* homozygous mutant mice are born with open eyes and display eyelid hypoplasia as well as other craniofacial malformations and most importantly female infertility (Schmidt *et al.* 2004, Uda *et al.* 2004). In mice, *Foxl2* expression is sexually dimorphic, starting at e12.5 in female gonads but is not detectable in developing male gonads. In fact, *Foxl2* expression is one of the earliest markers of ovarian differentiation, and it continues throughout adulthood in somatic granulosa and theca cells of all follicular stages (Schmidt *et al.* 2004).

Phenotypic analysis of homozygous knockout mice has revealed that FOXL2 is required for ovarian granulosa cell differentiation and proliferation. In *Foxl2* mutants, granulosa cells within primordial follicles do not undergo the squamous-to-cuboidal transition, preventing development of follicles beyond this point. Therefore, two inhibitors of primordial follicle activation, activin- $\beta$ A and anti-Mullerian hormone, which are normally secreted from late stage follicles, are almost undetectable in *Foxl2*<sup>-/-</sup> ovaries. Consequently, GDF9 expression, which can be considered a marker of primordial follicle activation, is detected in nearly all *Foxl2* mutant follicles. Lacking support of functional granulosa cells, oocytes of activated mutant follicles subsequently undergo atresia, which results in progressive follicular depletion and infertility (Schmidt *et al.* 2004, Uda *et al.* 2004, Uhlentaut & Treier 2006). Taken together, the defects in granulosa cell differentiation in the absence of FOXL2 lead to global

activation and premature depletion of the primordial follicle pool, followed by oocyte atresia, and subsequently cause infertility.

### FOXL2 in female sex maintenance

In mammals, the bipotential gonad can develop either into an ovary or into a testis, depending on the absence or presence of the Y chromosome. The male or female gonad then governs sexual differentiation and establishes the phenotypic sex of the organism by secretion of gender-specific hormones and signaling molecules. As a consequence of their common origin, the fully developed testes and ovaries consist of three main cell types, which are similar in function and analogous to one another: the germ cells, oocytes and spermatocytes, the supporting somatic cells, granulosa and Sertoli cells, and the steroidogenic theca and Leydig cells in the ovary and in the testis (Kim & Capel 2006).

The first molecular event during sex determination is expression of *Sry*, a transcription factor encoded by the Y chromosome, in male gonadal somatic cells. *Sry* initiates expression of the related transcription factor SOX9 that promotes differentiation and proliferation of Sertoli cells. The cascade of events triggered by *Sry* and its downstream target SOX9 leads to the formation of testis cords, Leydig cell differentiation, regression of the Mullerian duct, and subsequent masculinization of the embryo (Brennan & Capel 2004).

In the absence of a Y chromosome, gonads develop as ovaries, which has led to the prevailing view that the female pathway is a passive default program. However, several lines of evidence have recently been put forward demonstrating that the ovarian pathway also requires an active course of events. For example, in mice, lack of *Wnt4* leads to masculinization of the XX gonad (Jeays-Ward *et al.* 2003). Furthermore, a recessive mutation in the human autosomal gene *RSPO1* that results in full XX female-to-male sex reversal was reported (Parma *et al.* 2006). Interestingly, RSPO1 is a member of the R-spondin family of secreted ligands that have been shown to synergize with WNT proteins in the activation of  $\beta$ -catenin (Capel 2006).

We have recently shown that the ovarian pathway has to be actively maintained throughout adulthood and that it requires continuous suppression of the key testis differentiation gene *Sox9* by *Foxl2*. Apparently, *Sox9* must be repressed in females both during embryonic development by R-spondin1 and WNT4 acting via  $\beta$ -catenin and later in adulthood by FOXL2 (Sinclair & Smith 2009). Mutation of *Foxl2* in adult mice using the tamoxifen-inducible Cre/loxP system leads to de-repression of *Sox9*, causing transdifferentiation of ovarian granulosa and theca cells into testicular Sertoli and Leydig cells respectively. Moreover, loss of FOXL2 triggers upregulation of an entire suite of testis genes with concomitant downregulation of ovarian genes and



loss of oocytes. Furthermore, gonads of sex-reversed XX *Foxl2* mutant mice are able to produce testosterone at levels comparable to those of normal male XY testes, demonstrating true adult lineage reprogramming in a cell autonomous manner *in vivo*. Finally, we have shown that FOXL2 synergistically interacts with estrogen receptors  $\alpha$  and  $\beta$  to directly repress the gonad-specific *cis* regulatory element of *Sox9* known as testis-specific enhancer of *Sox9*, core sequence (TESCO). In mice, FOXL2 is therefore not only necessary for sustaining folliculogenesis beyond primary stages but also necessary for repressing reprogramming of the somatic cell compartment within the postnatal ovary and for maintenance of the female phenotype throughout life (Ottolenghi *et al.* 2005, Uhlenhaut *et al.* 2009).

Our studies have also challenged the long-standing dogma in the field stating that oocytes are required to maintain granulosa cell fate and that, without them, granulosa cells would transdifferentiate into Sertoli-like cells. In particular, we have used mouse genetic cell ablation experiments to deplete oocytes from postnatal ovaries, which does not result in loss of granulosa cell identity, demonstrating that the ovarian cell fate switch observed in FOXL2 mutants is cell autonomous and is not indirectly caused by loss of germ cells (Uhlenhaut *et al.* 2009).

### **FOXL2 in the goat polled intersex syndrome**

Interestingly, lack of FOXL2 function may be the underlying cause of the female-to-male sex reversal observed in goats with polled intersex syndrome (PIS), which have a chromosomal deletion that encompasses noncoding elements ~200 kb upstream of *Foxl2* and that are influencing its transcription. In goats, the PIS mutation seems to affect *Foxl2* expression, which leads to de-repression of the male sex determination pathway. Consequently, the 11.7 kb deleted in the goat PIS map to the conserved nongenic sequences upstream of the human FOXL2 gene mentioned previously that cause BPES through a position effect. The existence of a conserved distant regulatory element required for proper FOXL2 expression, lying far upstream of the gene and containing the region homologous to PIS is thus very likely (Pailhoux *et al.* 2001).

### **Transcriptional targets of FOXL2**

In addition to repressing *Sox9*, and consistent with its role in female sex maintenance, *Foxl2* has been implicated in the regulation of steroidogenic genes such as *Star* and aromatase and of pivotal ovarian genes such as follistatin (Moumne *et al.* 2008, Kuo *et al.* 2009, Kashimada *et al.* 2011). Other potential target genes in somatic cells including regulators of the cell cycle and apoptosis, which together with the observation

that some granulosa cell tumors carry a p.Cys134Trp mutation in the FOXL2 protein sequence, may point to a potential role as a tumor suppressor (Shah *et al.* 2009, Benayoun *et al.* 2010).

### **FOXO family members in the ovary**

The FOXO proteins arguably constitute the most well-known Fkh subfamily, since the orthologs of the *Caenorhabditis elegans* longevity gene *Daf16* were discovered to be central mediators of insulin signaling and the determination of life span (at least in invertebrates). As mentioned above, FOXOs not only play a role in aging and energy metabolism but are also involved in tumor suppression. The most well-defined FOXO target genes include the cell cycle inhibitors *p27* and *p21*, stress response, and proapoptotic genes such as *MnSOD*, *TRAIL*, and *CD95L* as well as the glycogenolytic gene *G6pc* (Paik *et al.* 2007, Salih & Brunet 2008, Hedrick 2009, Maiese *et al.* 2009).

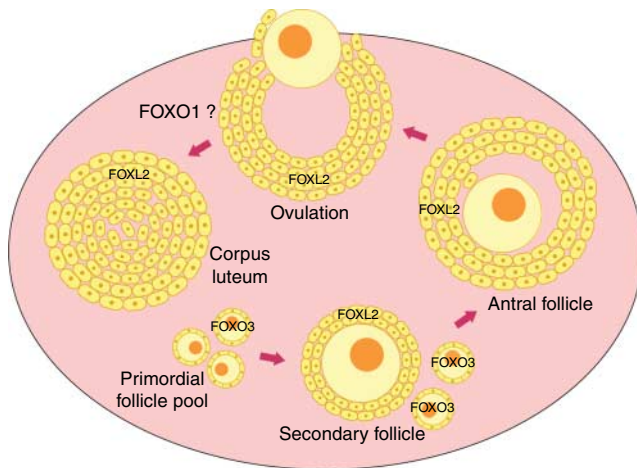
Interestingly, the activity of FOXOs is regulated by a mechanism that so far appears to be unique to this subfamily: FOXOs are phosphorylated in response to activation of PI(3)kinase (PI3K)/AKT signaling downstream of the insulin receptor, which was shown to result in nuclear exclusion, thereby turning off FOXOs' transcriptional functions (Hannenhalli & Kaestner 2009).

There are four Foxo family members, FOXO1, FOXO3, and FOXO4, expressed almost ubiquitously and share partially overlapping functions, whereas expression of FOXO6 is mainly restricted to the brain, where its cellular localization is differently regulated (Hedrick 2009).

### **FOXO3 in primordial follicle activation**

A number of elegant mouse genetic experiments during recent years have provided insight into the role of FOXO3 in oocyte quiescence. *Foxo3* homozygous knockout mice show POF and age-dependent female infertility, but the underlying mechanism is very distinct from the *Foxl2* gene. In *Foxo3* null mice, primordial follicles are assembled normally but then immediately undergo global activation, resulting in early depletion of the entire primordial follicle pool (Castrillon *et al.* 2003, Hosaka *et al.* 2004). This means that young females can give birth to normal size litters but reach menopause much more quickly, suggesting that FOXO3 functions in suppressing the initiation of follicular growth and controlling the rate of utilization of the reproductive potential.

As described above, the PI3K pathway that regulates nucleocytoplasmic shuttling of FOXO3 is also important within oocytes. In mice, FOXO3 was reported to localize to the nucleus during primordial follicle assembly and to be exported to the cytoplasm on follicular activation.



**Figure 2** Schematic showing the role of FOXL2 and FOXO family members during ovarian folliculogenesis. A follicle consists of an oocyte surrounded by one or more layers of somatic granulosa cells. The primordial follicle pool represents the female reproductive potential. On follicular activation, granulosa cells undergo a transition from squamous to cuboidal morphology, and secondary follicles are formed when granulosa cells proliferate to form several layers. The appearance of a fluid-filled cavity marks the preantral to antral transition. After surge of LH, the oocyte is expelled from the ovary, ready to undergo fertilization. The remaining granulosa cells form the corpus luteum, which secretes progesterone, adapted from Matzuk & Lamb (2002). FOXL2 is required throughout female lifetime to maintain somatic cell identity and to prevent transdifferentiation of ovarian granulosa and theca cells into their testicular counterparts, Sertoli and Leydig cells. FOXL3 functions as a suppressor of primordial follicle activation in oocytes and preserves oocyte quiescence and thus the duration of female fertility.

Oocyte-specific ablation of *Pten* (a potent PI3K antagonist) using the Cre/loxP system was shown to result in PI3K-induced AKT activation, phosphorylation, and nuclear export of FOXO3, thereby triggering primordial follicle activation and consequently leading to POF (John *et al.* 2008). Essentially mimicking the *Foxo3* loss-of-function phenotype, these findings demonstrate the importance of the PI3K signaling pathway in preserving follicular quiescence.

Furthermore, studies using a transgenic mouse model with constitutively active FOXO3 expressed in oocytes confirmed that FOXO3 suppresses follicular maturation. The authors used the *Zp3* promoter to express a mutated *Foxo3* cDNA without AKT phosphorylation sites, which resulted in female infertility. Ovulation in these mice was almost completely prevented, due to oocyte growth and folliculogenesis being greatly impaired (Liu *et al.* 2007).

Taken together, these analyses lead to the conclusion that the rate of primordial follicle pool depletion and the duration of fertility are controlled by the phosphorylation and localization of FOXO3, which prevents follicular activation when nuclear and allows maturation when inactivated and exported to the cytoplasm (see Fig. 2).

Which specific target genes are regulated by FOXO3 in order control follicular activation is still an open question, but the cell cycle inhibitor p27 and the enzyme galactose-1-phosphate uridylyltransferase (Galt) have been implicated as potential mediators (Halperin *et al.* 2008, Brosens *et al.* 2009).

### FOXO1 and FOXO4

Despite the fact that two other FOXO proteins, FOXO1 and FOXO4, are also expressed in the mammalian ovary (Richards *et al.* 2002), we are still awaiting a detailed characterization of their cellular localization and function during folliculogenesis. *Foxo1* knockout mice have been generated, but homozygous mutants are early embryonic lethal and do not develop post day e10.5 due to vascular defects, which makes it impossible to analyze their reproductive phenotypes (Hosaka *et al.* 2004). FOXO1 was reported to be regulated both at its expression level and via phosphorylation in response to FSH and LH in granulosa cells. In addition, FOXO1 has been implicated in putatively regulating genes involved in lipid and sterol biosynthesis, suggesting that it may play a role in follicular steroidogenesis (Liu *et al.* 2009).

*Foxo4* null mice are viable and do not show any overt developmental defects or histological abnormalities and thrive as well as their wild-type littermates (Hosaka *et al.* 2004). To date, there is no evidence for reproductive deficiencies or impaired fertility of female *Foxo4* mutant mice, but one could speculate on a functional redundancy between members of the same subfamily (Paik *et al.* 2007; see Table 1).

### FOXOs and human reproduction

In spite of the well-documented ability of FOXO proteins to block cell cycle progression via regulation of cyclins or CDK inhibitors, evidence for a role in ovarian cancer

**Table 1** Forkhead family members currently known to play a role in ovarian folliculogenesis, as reported from mouse or human genetic and phenotypic characterizations.

Gene	Ovarian expression	Mouse mutant reproductive phenotype	Human fertility disorder
<i>FOXC1</i>	Somatic cells (granulosa, theca)	PGC migration and late folliculogenesis affected	
<i>FOXL2</i>	Granulosa and theca cells (all stages)	Defects in granulosa cell development; XX sex reversal	BPES, POF
<i>FOXO1</i>	Granulosa cells (preovulatory follicles)	?	POF?
<i>FOXO3</i>	Oocytes of primordial follicles	Suppression of primordial follicle activation impaired	POF?
<i>FOXO4</i>	Not characterized	None	
<i>FOXO6</i>	Probably none	?	

or POF is still lacking (Brosens *et al.* 2009, Hedrick 2009). Two studies have reported the identification of rare SNPs or mutations in *FOXO3* and *FOXO1* loci in small cohorts of POF patients, but causal relationships and the effect of these mutations for FOXO protein function remain to be explored (Watkins *et al.* 2006, Gallardo *et al.* 2008).

## Conclusion and perspectives

The primordial follicle pool inside the ovary presents a limited resource that must be carefully protected and efficiently exploited to sustain female fertility. Once this pool of primordial follicles is depleted, the fertile period ends and menopause ensues. Two prominent members of the Fkh family of transcription factors, FOXL2 and FOXO3, both serve an essential maintenance function that is necessary throughout the entire female reproductive lifespan. FOXL2 functions throughout life to stabilize somatic cell fate in both granulosa and theca cells to maintain 'femaleness', whereas FOXL3 acts within oocytes to keep primordial follicles in check in order to preserve the resting follicle pool and thus the female reproductive potential. As we learn more about the molecular mechanisms that are used by Fkh factors to regulate gene expression and consequently mammalian physiology, we gain a deeper understanding of transcriptional processes that are ongoing throughout the reproductive lifespan and that are necessary for female fertility. Undoubtedly, future experiments such as genomic approaches in combination with mouse and human genetics will lead to the identification of additional direct target genes of Fkh transcription factors and might implicate additional family members in ovarian function.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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