Involvement of *FOXL2* and *RSPO1* in Ovarian Determination, Development, and Maintenance in Mammals

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Abstract

In mammals, sex determination is a process through which the gonad is committed to differentiate into a testis or an ovary. This process relies on a delicate balance between genetic pathways that promote one fate and inhibit the other. Once the gonad is committed to the female pathway, ovarian differentiation begins and, depending on the species, is completed during gestation or shortly after birth. During this step, granulosa cell precursors, steroidogenic cells, and primordial germ cells start to express female-specific markers in a sex-dimorphic manner. The germ cells then arrest at prophase I of meiosis and, together with somatic cells, assemble into functional structures. This organization gives the ovary its definitive morphology and functionality during folliculogenesis. Until now, 2 main genetic cascades have been shown to be involved in female sex differentiation. The first is driven by *FOXL2*, a transcription factor that also plays a crucial role in folliculogenesis and ovarian fate maintenance in adults. The other operates through the WNT/CTNNB1 canonical pathway and is regulated primarily by R-spondin1.

Here, we discuss the roles of *FOXL2* and *RSPO1*/WNT/CTNNB1 during ovarian development and homeostasis in different models, such as humans, goats, and rodents.

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How sex is determined is a longstanding biological question in the fields of evolution, development, and reproduction. Long before the advent of modern science, the origins of the sexes in the animal kingdom already garnered much debate. Presently, the debate has intensified due to the controversy surrounding gender theories. In mammals, the genetic sex of an individual is conferred at conception through transmission of the X or the Y chromosome from the father's sperm. After this primary step, the first signs of gonadal dimorphism become evident. The bipotential gonad develops as either an ovary (XX embryos) or a testis (XY embryos). The secretion of hormones triggers the development of secondary sex characteristics, including male or female internal and external genitalia.

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Disruption of factors involved in these sexual developmental pathways may lead to disorders of sexual development (DSD), which are rare diseases with an estimated incidence of 1 out of 4,500 births in humans [Hughes, 2008]. Studies of DSD cases highlight numerous key genes involved in sexual development and gonadal differentiation. Such studies continue to represent a promising way of increasing our knowledge of sexual development [Bashamboo et al., 2010].

The morphogenesis of the gonad is initially similar in both XX and XY embryos. The gonad, along with the adrenal gland, is derived from the adreno-gonadal primordium, which is initially only a few layers of cells located on the ventromedian part of the mesonephros [Bandiera et al., 2015]. In mice, gonadal development begins at 9.5 days post coitum (dpc), which corresponds to 4 weeks post fertilization (wpf) in humans and 25 dpc in goats. At this time, cells from the coelomic epithelium proliferate, while cells from the underlying mesonephros are recruited to the gonad [Schmahl et al., 2004; Lin and Capel, 2015]. Around 6 wpf in humans, corresponding to 34–36 dpc in goats and 12.5 dpc in mice, the somatic precursors are specified into supporting cells (male: Sertoli, female: granulosa) as well as steroidogenic cells (male: Leydig, female: theca) [Karl and Capel, 1998; Albrecht and Eicher, 2001]. In contrast, primordial germ cells are not specified within the gonad but instead are derived from the allantois core domain [Mikedis and Downs, 2014]. During their migration they proliferate, and after colonizing the gonad, they differentiate into spermatogonia (male) or oogonia (female) in a process that is closely linked to their somatic environment. Primary sex determination and gonadal differentiation are controlled by genetic networks that act to promote one developmental pathway (i.e., testis) over the other (i.e., ovary).

Male sex determination is initiated in the somatic cells of the gonad by expression of the *SRY/Sry* gene (sex-determining region of the Y), a *Sox* family gene located on the paternally inherited Y chromosome [for review, see Carre and Greenfield, 2014]. In mice, via the distal TESCO (testis-specific enhancer of *Sox9* core) element [Sekido and Lovell-Badge, 2008], *SRY* directly induces expression of the transcription factor *Sox9*, which is required to induce testis differentiation [Houston et al., 1983; Chaboissier et al., 2004; Barrionuevo et al., 2006; Sekido and Lovell-Badge, 2008]. *SOX9* is necessary for the differentiation of Sertoli cells [Lavery et al., 2011], which support and nourish spermatogenesis. While *SOX9* is an essential factor for testis differentiation in many vertebrates, *SOX9/Sox9* upregulation seems to be

different among species harboring different testis-determining genes, even among eutherian mammals that are all under *SRY*-determination control [Elzaia et al., 2014]. In mice, after *Sox9* upregulation is achieved, its expression is maintained by 2 feedback loops involving FGF9 [Colvin et al., 2001; Kim et al., 2007] and Prostaglandin D2 (PGD2) [Malki et al., 2005; Moniot et al., 2009] (fig. 1). Activation of these genetic cascades in the XY genital ridges induces morphological and cellular changes that are characterized by strong proliferation [Schmahl et al., 2000] and endothelial cell migration from the adjacent mesonephros [Coveney et al., 2008; Combes et al., 2009]. This contributes to the organization of polarized Sertoli cells into sex cords and future seminiferous tubules that enclose primordial germ cells [Nel-Themaat et al., 2011]. As soon as they differentiate, Sertoli cells secrete a variety of signals that stimulate the differentiation of steroidogenic Leydig cells. The Leydig cells then produce androgens that, on the one hand, trigger the virilization of the embryo [Yao et al., 2002], and on the other, inhibit the development of the female internal duct via AMH (anti-Müllerian hormone) secretion.

Ovarian Differentiation

Interactions between germ cells and somatic cells are central to ovarian development and function. These interactions regulate germ cell proliferation, meiotic entry and arrest, as well as the formation of the primordial follicle pool [Ford et al., 1975; Palmer and Burgoyne, 1991]. The latter is required to ensure several years/decades of fertile life as well as the endocrine function necessary for puberty, ovulation, fertilization, and establishment of gestation.

The differences between mice and most other species is the timing between sex determination and meiosis entry, as well as the morphogenetic events that take place during this period. In mice, sex determination ends at 12.5 dpc and meiosis starts one day later at 13.5 dpc. During this time, no histological changes are observed in the female gonad. In contrast, the time between the end of sex determination (humans 6 wpf, goats 36 dpc) and the start of meiosis in germ cells (humans 11 wpf, goats 55 dpc) is longer in humans (5 weeks) and goats (19 days) and is characterized by morphological changes [Pailhoux et al., 2002; Le Bouffant et al., 2010]. In humans and goats the developing ovary is organized into 2 compartments before the beginning of meiosis: (i) the cortex, which is a hyper-cellular layer containing germ cells and (ii) the me-

Fig. 1. Highlights of *FOXL2* and *RSPO1* in ovarian differentiation.

dulla, which is centrally located and consists mainly of somatic cells rich in fibrovascular tissue [Konishi et al., 1986; Pannetier et al., 2006, 2012]. The cortex contains packed ovigerous cords formed by somatic and germ cells (oogonia), with the somatic cells appearing as though they are embracing the oogonia with thin overlapping cytoplasmic projections [Francavilla et al., 1990]. Differentiated steroid producing cells arise from the highly vascularized medulla. In particular, steroidogenic cells producing estrogens are observed in the goat fetal ovary. In humans, steroidogenic enzymes are expressed and fetal ovaries are able to produce estrogens at this stage of development. This was determined by the capacity of fetal ovaries to convert radiolabeled androgen into estrone and estradiol [George and Wilson, 1978]. Unlike in domestic ruminants, where 17β -estradiol secretion was

measured [Mauleon et al., 1977], formal proof of estrogen production by the early fetal ovary has not yet been reported in humans.

The process of meiosis seems to follow a central to cortical gradient and is asynchronous in humans and goats. Less mature germ cells are located at the periphery of the ovary, with progressively more mature germ cells found deeper in the tissue [Anderson et al., 2007]. In mice, the germ cells enter meiosis in an anterior-posterior wave beginning at 12.5 dpc [Menke et al., 2003; Yao et al., 2003]. Around 16.5 dpc, the meiotic germ cells are visible in the cortical region of the ovary where they start to organize into ovigerous cords [Yao et al., 2004; pers. data].

Folliculogenesis, characterized by the assembly of oocytes and somatic cells into follicular functional structures, is the last step of ovarian differentiation. This pro-

cess requires the function of granulosa cells that are essential to the development and growth of ovarian follicles and oocytes. The origin of these cells remains a mystery.

Origin of the Different Somatic Cell Types

Ultrastructural studies in various rodent models suggest the existence of several sources of granulosa cells [for review, see Liu et al., 2010]. Somatic progenitors are present in the coelomic epithelium of the bipotential gonad, and cells from the rete ovarii are located at the gonad-mesonephros border. The experiments conducted by Albrecht and Eicher [2001] showed that Sertoli and granulosa cells have a common origin and their progenitors come from the coelomic epithelium [Karl and Capel, 1998; Mork et al., 2012]. In addition, histological studies and electron microscopy have traced the origin of granulosa cells to the ovarian surface epithelium in other mammalian species, such as ewes and cows [Sawyer et al., 2002; Hummitzsch et al., 2013].

For a long time, the absence of molecular markers specific for XX somatic cells has delayed the analysis of ovarian development. Transcriptomic analyses complemented by immunostainings have shown that there are 5 major cell populations in the mouse ovary at 14.5 dpc [Nef et al., 2005; Jameson et al., 2012a; Maatouk et al., 2012; Rastetter et al., 2014]. In addition to germ cells (DDX4-positive), interstitial cells (NR2F2-positive) and vascular cells are found together with 2 fetal pregranulosa subpopulations expressing either FOXL2 or LGR5 at 14.5–16.5 dpc [Rastetter et al., 2014].

FOXL2 is a forkhead-type transcription factor detected as early as 12.5 dpc in pregranulosa and granulosa cells of the XX gonad [Löffler et al., 2003]. A recent study used a MitoTracker dye to label the coelomic epithelium at 11.5 dpc and showed that granulosa cell precursors are derived from epithelial cells of this structure [Mork et al., 2012]. The ingress of these cells within the gonad is continuous until the second week postpartum. Additional experiments using a *GFP-Cre^{ERT2}* transgene under the control of the endogenous *Foxl2* promoter demonstrated that these epithelial cells give rise to 2 types of follicles [Zheng et al., 2014]. At 16.5 dpc, *Foxl2* expressing cells contribute to the formation of ovarian follicles that are localized in the medullar part of the postnatal ovary. These follicles are then either used such as in the case of an early pregnancy or undergo atresia [Mork et al., 2012; Zheng et al., 2014]. In addition, a second population of precursors that do not express *Foxl2* contributes to the granulosa cells of the cortical follicles. These follicles act as a reserve and are activated during the entire reproduc-

tive life of females [Zheng et al., 2014]. At 14.5–16.5 dpc, Rastetter et al. [2014] determined that this second subpopulation of granulosa cell precursors express Leucin-Rich Repeat-containing G-protein coupled Receptor 5 (LGR5), which is an R-spondin receptor. Importantly, although only a subset of follicular cell precursors express *Foxl2* during fetal life, all granulosa cells express *Foxl2* once follicles are formed.

Moreover, it has been shown that steroidogenic theca cells originate from both WT1-positive progenitor cells, which are localized both in the coelomic epithelium and within the gonad, and GLI1-positive mesenchymal cells that migrate from the mesonephros [Liu C et al., 2015].

Taken together, these data have advanced the cellular and molecular characterization of ovarian differentiation.

Due to the fact that morphological differentiation of the testis was considered more ‘spectacular’ than that of its female counterpart, and also because it was clear since the 1960s that the main sex-determining factor was testicular [Ferguson-Smith, 1965, 1966], the study of the molecular pathways governing ovarian development has been delayed for almost 20 years in comparison to that of testicular differentiation. However, the recent discovery of genetic loss-of-function mutations leading to the masculinization of an XX individual despite the absence of the *SRY* gene demonstrated the existence of genes capable of triggering ovarian differentiation and female determinism. Indeed, the identification of the molecular network responsible for regulating female fate has advanced dramatically during the past 20 years. In 1999, a loss-of-function mouse model of *Wnt4* was shown to exhibit sex reversal from female to male [Vainio et al., 1999]. This was followed by the identification of *FOXL2*, a gene deregulated in an intersex syndrome in goats [Pailhoux et al., 2002]. The genetic role of this gene as a major sex-differentiating factor was deciphered in 2014 [Boulanger et al., 2014]. In 2005, 46,XX *SRY*-negative men were reported [Radi et al., 2005]. One year later, the gene *RSPO1* was identified as being a key factor in human ovarian differentiation [Parma et al., 2006].

FOXL2: A Central Player in the Mammalian Ovary

In this review, we will focus on the functions of the gene *FOXL2* primarily in the ovary (fig. 1). However, *FOXL2* should be viewed as a crucial factor for female reproduction acting on 3 levels of the female reproductive system: in the pituitary where it controls the production

of gonadotropins [Fortin et al., 2014], in the ovary [for review, see Georges et al., 2014a], and in the uterus [Bellessort et al., 2015]. Indeed, *Foxl2* loss-of-function in any of these 3 tissues, using conditional deletion of *Foxl2* alone (ovary, uterus) or combined with *Smad4* (pituitary), leads to female infertility in mice. Apart from its reproductive role, *FOXL2* is also involved in eyelid formation [Crisponi et al., 2001] and, more generally, in the development of various cranio-facial structures [Heude et al., 2015]. Finally, the null *Foxl2/FOXL2* mutation triggers neonatal lethality in most newborn mice [Schmidt et al., 2004; Uda et al., 2004] and goats [Boulanger et al., 2014]. Lethality of *Foxl2* mutant mice may be associated with cartilage and skeletal defects [Marongiu et al., 2015]; however, we cannot exclude that *FOXL2* does not have additional roles in development as a recent paper has demonstrated its involvement in angiogenesis in the uterus [Bellessort et al., 2015].

Identification of FOXL2 as an Ovarian Factor

The first hint suggesting that *FOXL2* is involved in ovarian development came from the analysis of patients exhibiting eyelid malformations and suffering from premature ovarian failure (POF), 2 symptoms of type I BPES (blepharophimosis, ptosis, and epicanthus inversus syndrome, MIM#110100) [Crisponi et al., 2001] (fig. 1). POF is defined as amenorrhea for at least 4 months before the age of 40 years, combined with decreased serum concentration of estradiol and increased serum concentration of follicle stimulating hormone (FSH) [De Vos et al., 2010]. It is a heterogeneous disorder affecting approximately 1% of women under the age of 40, 1:10,000 women under the age of 20, and 1:1,000 women under the age of 30. In the same year 2001, *FOXL2* was shown to be involved in polled intersex syndrome (PIS) in goats where its loss of expression in the ovary, together with that of 3 long non-coding RNAs (*PISRT1*, *PISRT2*, and *PFOXic*), leads to early testis differentiation and, as a result, XX female-to-male sex-reversal [Pailhoux et al., 2001; Pannetier et al., 2012]. Later, *FOXL2/Foxl2* was shown to be a highly conserved transcription factor with a gonadal sex-dimorphic pattern of expression specific to the ovary from the beginning of its differentiation until adulthood [Pailhoux et al., 2002; Loffler et al., 2003]. However, in mice *Foxl2* expression was also observed transiently and at very low levels in the XY gonad, although the protein was not detected [Auguste et al., 2011]. Since these first reports, the role of *FOXL2* as a master regulator of ovarian differentiation has been abundantly described in various vertebrate species, such as fish [Baron et al., 2004; Nakamoto et al., 2006;

Liu et al., 2007; Yamaguchi et al., 2007; Amberg et al., 2010; Hu et al., 2014; Liu H et al., 2015; Wang et al., 2015], amphibians [Oshima et al., 2008; Okada et al., 2009], reptiles [Rhen et al., 2007; Alam et al., 2008; Moore et al., 2010; Shoemaker-Daly et al., 2010], and birds [Govoroun et al., 2004]. More recently, *FOXL2* ovarian expression was also reported in invertebrate species, such as crustaceans [Ma et al., 2012; Li et al., 2015; Meng et al., 2015] and molluscs [Dheilly et al., 2012; Liu et al., 2012; Zhang et al., 2014; Shi et al., 2015]. In total, ovary-specific *FOXL2* expression has been described in more than 40 species. In the future it will be very interesting to study the involvement of *FOXL2* in sex determination and ovarian differentiation in some of these species by using novel gene editing techniques. Such functional data will help decipher the role of *FOXL2* as a putative master regulator of ovarian development and female fertility across a wide range of species.

Structure and Regulation of FOXL2

On the first glance, *FOXL2* appears to be a rather simple gene. Its human form contains only 1 exon which is ~3 kb long and encompasses an open reading frame of 1,131 bp. The human *FOXL2* protein is composed of 376 amino acids and has 2 important domains: a highly conserved DNA-binding domain known as the 'forkhead box' or winged-helix domain and a poly-Alanine domain of 14 residues highly prone to mutations. Indeed the expansion of this domain to 24 residues is responsible for 30% of BPES cases [Caburet et al., 2004]. In direct contrast to its relatively simple structure, the regulation of *FOXL2* appears to be highly complex. Whereas intragenic mutations are found in about 70% of BPES patients, several translocation breakpoints and microdeletions upstream of *FOXL2* have been characterized in humans and goats [Beysen et al., 2005; Pailhoux et al., 2001], highlighting the existence of long-range regulation of this gene. Interestingly, the deletions upstream of the gene described in humans include the PIS/*PISRT1* region that is located 300 kb upstream of *FOXL2* [Beysen et al., 2005; D'Haene et al., 2009; Verdin et al., 2013] (fig. 2). To date, the shortest deletion downregulating *FOXL2* expression in humans is a 7.4-kb deletion that encompasses the *PISRT1* noncoding gene as well as a region 3 kb upstream of its promoter. This region is highly conserved and may be more important than the *PISRT1* transcript itself for *FOXL2* long-range regulation. This deletion leads to *FOXL2* haploinsufficiency that results in BPES. In addition, this region around *PISRT1* is able to interact with the proximal region of the *FOXL2* promoter [D'Haene et

Fig. 2. Schematic representation of the *FOXL2* locus in goat, human, and mouse. The *FOXL2* gene (pink) is represented together with long noncoding RNAs (green) that were found to be associated with its expression. Both DNA strands are shown: the forward strand being represented as a black line and the reverse strand as a grey one. The distance between poly-adenylation signals of *PISRT1*

and *FOXL2* is indicated for each species. Note that 2 distinct poly-adenylation signals have been described for *Foxl2* in mice (dark versus light pink). The PIS mutation is positioned on the goat chromosome 1, while the Smallest Region of deletion Overlap (SRO) described in human BPES patients is localized on human chromosome 3 (both in red). Scale bar = 1 kb.

al., 2009]. It is worth mentioning that in all analyzed vertebrate genomes, *FOXL2* was found in synteny with the *PISRT1* surrounding region, which contains highly conserved elements traceable in species extremely distant from mammals, such as *Xenopus* or Medaka [pers. data]. In the future, it will be important to understand how these distal regulatory elements direct *FOXL2* tissue- and developmental stage-specific expression. One could imagine that some human XX sex reversal cases result from an ovarian-specific loss-of-function of *FOXL2*, as reported in the XX PIS sex-reversed goats [Pailhoux et al., 2001].

In addition to this critical 300-kb distal regulation, complex proximal *FOXL2*/*Foxl2* regulation has also been highlighted by 2 transcriptional features. These include the existence of a bidirectional promoter in goats [Pannetier et al., 2005] and the presence of antisense transcripts overlapping the whole *Foxl2* transcriptional unit in mice [Cocquet et al., 2005].

The presence of a bidirectional *FOXL2* promoter was first described in goats, directing *FOXL2* transcription in the sense direction and *PFOXic* (promoter FOX inverse-complementary) in the antisense direction [Pannetier et al., 2005]. This bidirectional promoter is conserved in humans and drives the expression of the *FOXL2NB* gene (*FOXL2* neighbour, ENSG00000206262), although *FOXL2NB* and *PFOXic* genes and transcripts are different (i.e., exons span different genomic regions). They are predicted to produce different proteins of 173 aa and 163 aa that do not share an identity with any known protein. In addition, neither of these putative proteins have been described in other species, including mice, suggesting that either the resulting transcript is a noncoding one that should be considered as a transcriptional relic, and/or that the conserved function remains on the bidirectional promoter. Divergent, bidirectional transcription of protein-coding genes was reported many years ago [Beck and

Warren, 1988], and the recent sequencing and annotation of various whole genomes has revealed that bidirectional organization of coding genes is a common phenomenon [Trinklein et al., 2004]. In this context, lncRNA transcription from bidirectional promoters is described as a means of creating a local RNA pol II reservoir, or as a way of imposing a physical barrier against repressive chromatin spreading [Wei et al., 2011]. We believe that *FOXL2NB/PFOXic* transcriptional activity keeps the promoter region open to decrease stochastic variation of *FOXL2* expression. In mice, Ensembl annotation reports an lncRNA transcribed from the opposite strand of the *Foxl2* promoter. However, this transcript, labelled as *Foxl2os* (*Foxl2* opposite strand), actually originates from a region located between the 2 *Foxl2* poly-adenylation signals encompassing the *Foxl2* gene and is transcribed in an antisense direction [Cocquet et al., 2005]. High-throughput sequencing data available in the ENCODE database also support the existence of this antisense transcription in mice. The transcription of lncRNAs from the opposite strand of protein-coding sense genes might be part of self-regulatory circuits that allow genes to regulate their own expression [reviewed in Pelechano and Steinmetz, 2013]. Thus, even if different strategies have been selected across different species (bidirectional promoter or antisense transcription), the same goal of tightly regulating *FOXL2/Foxl2* expression has been conserved.

FOXL2 Regulators

In the ovary, the *FOXL2* protein is first detected at 12.0/12.5 dpc in mice and 34/36 dpc in goats [Pailhoux et al., 2002; Auguste et al., 2011]. In both species these stages correspond to the end of the sex determination period (i.e., many Sertoli cells are differentiated and cord formation is underway in the XY testis counterpart), demonstrating that *FOXL2/Foxl2* is not expressed in the undifferentiated gonad. This contrasts with the expression of some other crucial gonad-differentiating genes such as *Sry*, *Sox9*, *Rspo1*, and *Wnt4* [Gasca et al., 2002; Chassot et al., 2012]. According to this peculiar expression pattern (i.e., expression in early differentiating ovaries), *FOXL2* is widely used as a primary marker of granulosa cell differentiation. But the question remains: which factors are responsible for primary *FOXL2/Foxl2* upregulation? These factors are expressed in the genital ridges, but is *FOXL2/Foxl2* specifically activated by a female-specific factor or is *FOXL2/Foxl2* expression linked to an advanced developmental stage of the female supporting cells? Or are both scenarios occurring? *SOX9* and *FOXL2* are usually mutually exclusive at the cellular level [Hers-

mus et al., 2008; Wilhelm et al., 2009]; however, recent studies of mouse gonads demonstrate the presence of both *FOXL2* and *SOX9* proteins in the same cell type [Maatouk et al., 2013; Matson et al., 2011]. It seems clear that enhancing the male pathway with *SRY* and *SOX9* obliterate *FOXL2/Foxl2* expression but the precise molecular mechanisms behind this observation remain unclear. Some factors have been already pointed out as being important for *FOXL2/Foxl2* upregulation. Among them, GATA factors appear to be good candidates since it has been shown that *Foxl2* expression is drastically decreased in *Gata4*, *Gata6*, or *Fog2* mutant mice [Manuylov et al., 2008; Padua et al., 2014]. However, this could also be the result of an indirect effect due to defective differentiation of XX supporting cells. To date, the best candidate for promoting the upregulation of *Foxl2* expression in mouse ovaries is the WNT signaling pathway, and there are many reasons for this. First, *Wnt4* is upregulated before *Foxl2* at 11.5 dpc [Nef et al., 2005]. Second, overexpression of CTNNB1 in mice is sufficient to allow ovarian differentiation in XY gonads with *Foxl2* expression [Maatouk et al., 2008]. Third, ablation of *Ctnnb1* is associated with a drastic reduction of *Foxl2* expression [Manuylov et al., 2008]. Whether or not CTNNB1 directly activates *Foxl2* expression is still an open question. The IGF system is also considered a potential *FOXL2/Foxl2* activator since it has been reported that IGF3 positively regulates *FOXL2* in the gonads of tilapia [Li et al., 2012]. Apart from this limited data on *FOXL2/Foxl2* transcriptional regulators, it has also been reported that *FOXL2* mRNA stability could be affected by the microRNA133b, which has been shown to stimulate ovarian estrogen synthesis by targeting *FOXL2* in the human granulosa cell line KGN [Dai et al., 2013]. However, the physiological role of miR133b in vivo has not been studied.

FOXL2 Ovarian Functions

The functions of *FOXL2* in the developing ovary are related to folliculogenesis. In mice, loss-of-function mutations lead to failure of granulosa cell progression from squamous to cuboidal shape, preventing the formation of primary follicles and resulting in female infertility [Schmidt et al., 2004; Uda et al., 2004]. In addition, *Foxl2*^{-/-} mice have no eyelids. In humans, the development of eyelids is not impaired, but malformations have been reported. This may be due to differences in the level of expression of *FOXL2/Foxl2* between both species. Indeed, *Foxl2* mutations in mice are null mutations and lead to no expression of the gene, while mutations in humans are heterozygous and most likely lead to only reduced lev-

els of expression. In a previous study in humans, ovarian defects were documented for 2 patients who then underwent microbiopsies [Meduri et al., 2010]. One ovary showed some differences in histology, while the other did not show any noticeable abnormalities. However, an acceleration of follicular recruitment was noted in both ovaries, possibly explaining the occurrence of POF.

Surprisingly, *Foxl2* is expressed shortly after the beginning of ovarian differentiation in mice but does not seem to play a particular role before folliculogenesis. In humans, only heterozygous mutations of *FOXL2* have been reported, and the effects of a complete loss-of-function of this gene are unknown. In human fetuses, *FOXL2* expression is detected in the female gonad as soon as sex determination proceeds, preceding the onset of meiosis. This is consistent with different roles for *FOXL2* in ovarian function, which may include follicle growth and sex determination [Duffin et al., 2009]. This is supported by recent results obtained from a third mammalian species. In XX goats bearing a PIS homozygous mutation (XX *PIS*^{-/-}), *FOXL2* transcription is specifically extinguished in gonads, leading to early testis differentiation and female-to-male sex reversal [Pailhoux et al., 2001; Pannetier et al., 2012]. As the goat phenotype did not fit with the one observed in *Foxl2*^{-/-} female mice, long noncoding RNAs disrupted by the PIS mutation together with *FOXL2* were first incriminated [Boulanger et al., 2008]. However, it was recently demonstrated in goats that the *FOXL2* loss-of-function alone (independent of lncRNAs) was sufficient to explain the early sex reversal process of the ovary [Boulanger et al., 2014]. Indeed, XX *FOXL2*^{-/-} mutants have fully differentiated testes in goats and have no eyelids in mice, and the majority of full knockouts die shortly after birth [Boulanger et al., 2014]. This ultimately leads to the question of whether or not this neonatal lethality is a common trait in other vertebrate and invertebrate species.

Another remaining question is why *FOXL2/Foxl2* knockout leads to early XX sex reversal in goats and not in mice. Interestingly, in mice loss-of-function of *Foxl2* in a differentiated and functional ovary during adulthood induces sex reversal, but this does not occur before birth with a null mutation of this gene. Following *Foxl2* disruption in mice, *Sox9* upregulation was observed shortly afterwards, leading to the modification of the supporting cell fate from granulosa to Sertoli-like cells [Uhlenhaut et al., 2009]. To regulate female identity in mice, *FOXL2* was shown to work alongside estrogen receptors (ESR) as both of them are able to fix the TESCO element and the *Sox9* proximal promoter, thereby repressing *Sox9* expression [Uhlenhaut et al., 2009]. Surprisingly, neither ChIP-

on-Chip (chromatin immunoprecipitation on chip) data obtained from mouse ovary (focused on promoter region) nor ChIP-sequencing experiments performed from mouse granulosa cells (pan genomic study) confirmed this direct interaction [Benayoun et al., 2011; Georges et al., 2014b]. Despite these results, it is clear that the absence of *FOXL2* in highly differentiated granulosa cells producing estrogens leads to prompt *Sox9* upregulation and the activation of the male signaling pathway in mice.

These highly differentiated somatic cells can be observed very early during ovarian differentiation in goats. One major ovarian developmental difference between mice and goats is the period from the end of gonadal sex determination to the onset of germ cells meiosis, a process spanning 20 days in goats (36–55 dpc) and only 1 day in mice (12.5–13.5 dpc). During this period, *FOXL2*-expressing cells are steroidogenically active and produce estrogens in goats [Pannetier et al., 2006]. This directly contrasts with observations made in mouse ovaries where steroid production has never been detected before meiosis [Greco and Payne, 1994]. Thus, highly differentiated cells (i.e., highly specialized cells producing steroids) are present as soon as sex determination proceeds in the fetal goat ovary (*CYP19A1* first detection at 36 dpc). The downregulation of *FOXL2* expression in these highly differentiated cells rapidly leads to *SOX9* de-repression and upregulation, which can be detected about 4 days later in XX *PIS*^{-/-} sex-reversed goat gonads [Pailhoux et al., 2002; Elzaïat et al., 2014]. As this event takes place at the very early stages of sex determination and gonadal differentiation, a complete sex reversal of the genetic program is possible in goats, leading to the differentiation of testes in XX fetuses. According to this, one criterion to observe early sex reversal in XX mammals would be the differentiation of highly specialized somatic cells and in particular the production of estrogens in the early fetal gonad before the onset of meiosis. Besides bovidae, several mammalian species were also shown to produce estrogens before meiosis onset in the ovary (e.g., rabbits) [George et al., 1978] or at least to present the enzymatic capacity to produce estrogen (e.g., humans) [George and Wilson, 1978]. Although the role of this estrogen production (or putative synthesis) is unknown and deserves further attention especially in bovidae and rabbits, it attests to a high degree of differentiation of somatic cells within the early fetal ovary.

Recent data obtained from studies in goats even more complicate this system. Indeed, following *FOXL2* disruption in XX *PIS*^{-/-} gonads and before the upregulation of *SOX9*, another gene known to belong to the male genetic

cascade was found to be overexpressed [Elzaïat et al., 2014]. This gene, *DMRT1*, has been shown to be involved in sex reversal phenotypes in humans [Ledig et al., 2012; Murphy et al., 2015]. In particular, a single nucleotide mutation in the coding sequence of *DMRT1* was shown to induce a 46,XY complete gonadal dysgenesis, clearly demonstrating the essential function of this gene in testicular differentiation [Murphy et al., 2015]. In addition, 2 *DMRT1* consensus binding sites have been described within the 'RevSex' region, both of which play important roles in controlling *SOX9* expression in humans [Hyon et al., 2015]. These observations in humans and in goats could contribute to the re-evaluation of the *DMRT1* position in the testis determination pathway as it can contribute to *SOX9* gene expression activation. These hypotheses are speculative and need more experiments to be demonstrated. In rodents, *DMRT1* does not appear to be involved in early testis determination as attested by the analysis of *Dmrt1*^{-/-} XY mice that have normal testis until postnatal day 20 [Raymond et al., 2000]. Interestingly, in adult female gonads, when *Foxl2* deletion is induced, *Dmrt1* upregulation is observed after *SOX9* overexpression [Uhlenhaut et al., 2009]. Therefore, in mice, *Dmrt1* appears to act downstream of *Sox9* in the masculinization genetic cascade, which is the exact opposite of the observations made in goats. Although *Sox9* is a *FOXL2* target gene in adult mice female gonad, the situation may be slightly different in goats. Indeed, *DMRT1* is more likely to be targeted by *FOXL2* in the early fetal goat ovary.

FOXL2 Partners and Target Genes

As soon as ovarian differentiation occurs in fetal life and until obtaining a fully functional ovary in adulthood, *FOXL2* is expressed in the female gonad. Despite this constant expression, *FOXL2* functions most likely vary during different stages of ovarian differentiation and/or maintenance. Encompassing the developmental process, one could expect that different protein partners will be available, leading to the activation or repression of different target genes.

The identification of *FOXL2* partners in the adult ovary was partially elucidated through yeast 2-hybrid screenings (Y2H) whereby 12 interactive proteins were discerned [Lee et al., 2005; Park et al., 2010; L'Hôte et al., 2012]. Among them, *CXXC4* and *CXXC5* are known as negative regulators of the WNT/CTNNB1 pathway, disrupting WNT signal transmission from receptors to downstream effectors [Andersson et al., 2009; Kojima et al., 2009]. But these proteins also act in the nucleus, where their interaction with *FOXL2* inhibits its activity inde-

pendently of the promoter [L'Hôte et al., 2012]. Thus, *CXXC4* and *CXXC5* may be able to interfere with the 2 major female pathways, modulating their activity at least in the adult ovary. In addition, Steroidogenic Factor 1 (SF1, also known as NR5A1) was also found by Y2H to directly interact with *FOXL2* [Park et al., 2010]. This interaction was shown to prevent SF1 from binding to its target DNA on the *CYP17A1* promoter in human granulosa cells [Park et al., 2010]. The trapping of SF1 by *FOXL2* may impact other SF1-activated genes and may explain some *FOXL2* downregulating effects on different targets such as *Cyp26b1* [Kashimada et al., 2011a] or on genes encoding steroidogenic enzymes (see below). Another important protein for female fertility, *NOBOX*, has also been shown to be trapped by *FOXL2*. In vitro studies showed that *NOBOX* and *FOXL2* were interacting together via their DNA-binding domain (homeodomain/forkhead interaction), preventing each other from acting on their specific DNA-binding sites [Bouilly et al., 2014]. Like *FOXL2*, *NOBOX* plays a crucial role in the progression of primordial follicles to primary follicles [Rajkovic et al., 2004], and mutations in its DNA sequence are responsible for POF [Qin et al., 2007]. In addition to controlling *NOBOX* activity, *FOXL2* was also shown to directly activate *AMH* gene expression, which is another essential component for the ovarian reserve [Park et al., 2014]. One could propose that the mutual antagonism between *FOXL2* and *NOBOX* may regulate the balance between these 2 factors involved in the same steps of folliculogenesis in order to promote female fertility.

Other partners have been described in the pituitary where *FOXL2* plays a crucial role particularly on gonadotropin gene expression. In this tissue, interplay with the Activin signaling system as well as with the Smad3 protein was observed [Ellsworth et al., 2003; Blount et al., 2009]. In the ovary, *FOXL2* cooperation with BMP2 was described, suggesting that *FOXL2*'s partnership with Smad3 is conserved [Kashimada et al., 2011b]. Additionally, in both the pituitary and ovary, *FST* (follistatin) is targeted by *FOXL2* and Smad3 [Blount et al., 2009; Kashimada et al., 2011b; Nonis et al., 2013]. This phenomenon seems to occur very early during ovarian differentiation in goats [Elzaïat et al., 2014]. In mice, *Fst* expression becomes *FOXL2* dependent only after birth when folliculogenesis proceeds. In comparison the WNT4/CTNNB1 pathway first controls its activation during fetal life [Schmidt et al., 2004; Yao et al., 2004; Ottolenghi et al., 2005; Kashimada et al., 2011b].

Lastly, *FOXL2* has been shown to interact with steroid hormone nuclear receptors such as PR [Ghochani et al.,

2012], ESR1 (ER α), and ESR2 (ER β) [Uhlenhaut et al., 2009]. Gene regulation via or along with ESR2 may represent an important part of the transcriptional effects of FOXL2 [Georges et al., 2014b]. Indeed, genes related to steroid production or signaling have been shown to be FOXL2 targets. *StAR*, *CYP11A*, *CYP17A1*, and *HSD17B3* were described to be downregulated by FOXL2, limiting androgen production in the ovary [Pisarska et al., 2004; Park et al., 2010; Rosario et al., 2012; Elzaïat et al., 2014]. In contrast, a direct activating effect has been demonstrated on *CYP19A1*, which favors female hormone synthesis through the conversion of androgens into estrogens [Pannetier et al., 2006; Fleming et al., 2010]. FOXL2 was also shown to repress *Sf1* gene expression during ovarian development in mice [Takasawa et al., 2014] and to control *Dlx5/6* expression which is involved in steroid hormone production [Bouhali et al., 2011]. A reciprocal regulation exists between DLX5/6 and FOXL2, which may contribute to the homeostatic control of steroidogenesis within the ovary [Bouhali et al., 2011]. In addition to controlling estrogen synthesis, FOXL2 directly activates *ESR2* expression and shares common targets with this estrogen receptor [Georges et al., 2014b]. Hence, it appears as though FOXL2 plays an important part in estrogen signaling as the aforementioned data points toward the existence of a feed-forward loop in which FOXL2 stimulates both estrogen production and receptivity [Georges et al., 2014b].

In addition to these observations, large-scale identification of FOXL2 putative targets has been performed by numerous transcriptomic studies using microarray or RNA sequencing technologies. Different approaches were used to modify FOXL2 expression and observe its effects. These include natural loss-of-function mutations, knockouts, knockdowns, or overexpression. Consequences on downstream pathways were observed in vivo at different stages of ovarian development (from early fetal differentiation until adulthood). They were also observed in vitro on immortalized or primary cultured granulosa cells in different mammalian species such as mice, goats, and humans [Batista et al., 2007; Garcia-Ortiz et al., 2009; Uhlenhaut et al., 2009; Escudero et al., 2010; Rosario et al., 2012; Elzaïat et al., 2014; Georges et al., 2014b]. Comparisons of these different studies have been attempted [Escudero et al., 2010; Caburet et al., 2012]. Nevertheless, even if some FOXL2 regulated pathways are common to different developmental stages or are shared within species, each study is relevant in particular if it is designed from in vivo conditions. Criticizers of transcriptomic strategies to determine FOXL2 target

genes argue that the resulting data are not providing direct evidence of real FOXL2 regulatory function. ChIP on in vivo material would be definitive evidence but may prove difficult to execute.

To date, only one study has provided a wide description of FOXL2 direct binding sites using a ChIP sequencing strategy [Georges et al., 2014b]. This analysis was performed on primary cultures of mouse granulosa cells, which may be representative of in vivo circumstances, but only during one specific window of female gonadal development (prepubertal stage). Correlations of FOXL2 peaks with the transcriptional effects induced by FOXL2 knockdown indicated that FOXL2 was mainly interacting with its targets through intronic regulatory elements. In addition, FOXL2 repressed genes were not enriched in FOXL2 peaks, suggesting their repression may be mostly indirect, at least in these experiments [Georges et al., 2014b]. One could argue that FOXL2's interactions with its partners (e.g., SF1-trapping) may be responsible for these negative regulations.

Another Genetic Pathway Involved in Ovarian Development: The WNT Signaling Pathway

In addition to FOXL2, studies carried out in both human patients and mouse models have established a crucial role for the WNT/CTNNB1 signaling pathway in ovarian development. In 1999, a study of mice that were deficient for *Wnt4* [Vainio et al., 1999] and later the description of 46,XX SRY-negative patients carrying mutations in *WNT4* leading to a syndrome associated with virilization (MIM#158330) [Biaison-Laubert et al., 2004] revealed the importance of Wnt4 in ovarian differentiation. In 2006, a human syndrome characterized by palmoplantar hyperkeratosis and a predisposition to squamous cell carcinomas also lead to sex reversal in 46,XX SRY-negative patients (MIM#610644). Importantly, no activating mutations in *SOX9* or other 'male' genes were found in these patients. Instead, mutations in the *R-spondin1* (*RSPO1*) gene were identified as the causative event triggering this syndrome [Parma et al., 2006] (fig. 1). *RSPO1* is an activator of the WNT/CTNNB1 canonical signaling pathway [Kazanskaya et al., 2004] which has been very well-documented in various reviews [de Lau et al., 2014]. Briefly, canonical WNT signaling is activated by the binding of a WNT ligand to a complex constituted by a Frizzled receptor and LRP5/6 co-receptors. This binding triggers CTNNB1 cytoplasmic stabilization and, as a result, translocation into the nucleus where it interacts with

TCF/LEF transcription factors to activate cell type specific target genes. Apart from the canonical pathway, many non-canonical pathways have been described. These include the Planar Cell Polarity (PCP), the WNT/ROR, and the WNT calcium Ca^{2+} -dependent pathways, which regulate processes such as cell polarity, directional migration, and orientation of cellular division during tissue morphogenesis [Niehrs, 2012]. R-spondins are secreted proteins that enhance WNT canonical signaling by interacting with LGR4/5/6 receptors. This interaction triggers the endocytosis of 2 E3 ligases, RNF43 and ZNRF3, which normally promote degradation of WNT receptors. As a result, by increasing the availability of WNT receptors, RSPO1 activates/maintains WNT signaling [de Lau et al., 2014]. R-spondins have also been shown to interact with other receptors to regulate WNT non-canonical pathways, such as WNT/PCP (through binding to Syndecan-4) or WNT/ Ca^{2+} (unidentified receptor) [Ohkawara et al., 2011; Scholz et al., 2016].

RSPO1 and WNT4 are both critical factors required for ovarian differentiation in mice and humans, and phenotypic analyses indicate that these genes have similar gonadal functions in both species [Vainio et al., 1999; Parma et al., 2006; Chassot et al., 2008; Mandel et al., 2008; Tomaselli et al., 2008; Tomizuka et al., 2008]. Moreover, gain-of-function experiments carried out in mice showed that CTNNB1 is sufficient to trigger ovarian differentiation when expressed in XY gonads [Maatouk et al., 2008], thus demonstrating the importance of WNT signaling in sex determination.

RSPO1 and WNT4 Expression and Function in the Ovary

While R-spondins can trigger both canonical and non-canonical WNT signaling, it is believed that in gonadal differentiation RSPO1 acts mostly through the canonical WNT signaling pathway, with WNT4 being the key ligand [Clevers and Nusse, 2012; Biason-Lauber and Chaboissier, 2015]. Prior to sex determination, RSPO1 and WNT4 are coexpressed in XX and XY gonads in mice, humans, and goats, contributing together to the gonadal development in both sexes [Pailhoux et al., 2002; Kocer et al., 2008; Tomaselli et al., 2011; Chassot et al., 2012]. In the human fetus, RSPO1 becomes strongly upregulated in the ovary around 6–7 wpf, while WNT4 and CTNNB1 expression remains similar in male and female gonads [Tomaselli et al., 2011]. In mice, Wnt4 is highly expressed in the XX gonads at the time of sex determination, while Rspo1 expression becomes dimorphic when sex determination occurs [Nef et al., 2005; Parma et al., 2006]. In

goats, RSPO1 and WNT4 become upregulated in the differentiating ovary after gonadal sex determination [Kocer et al., 2008; Pailhoux et al., 2002]. At this time, WNT4 is a key factor promoting ovarian differentiation together with RSPO1 by activating part of the ovarian-specific genetic cascade and antagonizing testicular differentiation in mice [Vainio et al., 1999; Kim et al., 2006; Chassot et al., 2008; Jameson et al., 2012b] (fig. 3).

Only a few things are known in humans and goats after the sex determination period. Nevertheless, in goats, it was shown that RSPO1 is mostly bound to the surface of the germ cells located in the cortical part of the fetal ovary, suggesting that RSPO1 has a regulatory function in oögonia [Kocer et al., 2008]. Interestingly, in mice, RSPO1 was demonstrated to promote the proliferation and differentiation of primordial germ cells [Chassot et al., 2011] whereas WNT4 was involved in their survival [Vainio et al., 1999; Naillat et al., 2010]. RSPO1, via WNT4 signaling, has also been shown to prevent male-specific vascularization and steroidogenic cell differentiation in the mouse fetal ovary. In addition, Rspo1 loss-of-function mutations, similarly to a Wnt4 knockout, triggered a partial sex reversal and the formation of an ovotestis in XX animals and in XX patients [Vainio et al., 1999; Parma et al., 2006; Chassot et al., 2008; Mandel et al., 2008; Tomaselli et al., 2008]. Ablations of the RSPO1 receptor genes, Lgr4 and Lgr5, have been performed in mice. Lgr5 deficient mice did not fully recapitulate the Rspo1 knockout phenotype. In XX Lgr5 knockout gonads, the germ cells exhibited a delay in meiosis entry without any sex reversal of the gonad [Rastetter et al., 2014]. Recently, genetic ablation of Lgr4 appeared to fully recapitulate the Rspo1 mutant phenotype, demonstrating that LGR4 might act as the main RSPO1 receptor during ovarian development [Koizumi et al., 2015] and may compensate for Lgr5 ablation.

It is worth mentioning that the expression of the Sertoli cell marker Sox9 is detected during late gestation in Rspo1 and Wnt4 knockout mice. This is preceded by the resumption of mitosis, as well as the precocious differentiation of the supporting cells before their transdifferentiation into Sertoli-like cells [Maatouk et al., 2013]. This observation must be interpreted in parallel with the observed phenotype in Foxl2 knockout ovaries, where transdifferentiation is observed only when granulosa cells have reached a particular stage of differentiation [Uhlenhaut et al., 2009]. However, the molecular bases regulating the observed transdifferentiation are still unknown. Germ cell ablation experiments in Wnt4 and Rspo1 loss-of-function gonads support a role of these cells in this trans-

Fig. 3. Mouse testis- and ovary-determining pathways. Gonadal differentiation is controlled by genetic networks which act to promote one developmental way (testis or ovary) over the other. Male- and female-determining pathways and their mutual antagonisms in mice between 10.5 dpc and birth are presented. Positive (arrows) and negative (hammered lines) interactions are shown and possible additional determining factors and targets are indicated by question marks. Dashed lines indicate possible direct or indirect interactions.

differentiation process [Maatouk et al., 2013]. Interestingly, it has been shown that meiotic germ cells antagonize testicular development from 12.5 dpc [Yao et al., 2003]. The majority of WNT-signaling mutant gonads are associated with delayed meiosis, thus allowing transdifferentiation to occur at a later stage [Maatouk et al., 2013]. However, the primary cause that triggers sex reversal is still unknown. As described above, these mutants commonly develop ovotestes. Knowing that there are 2 subpopulations of supporting cells, some being LGR5 positive and the other being FOXL2 positive at 16.5 dpc [Rastetter et al., 2014], the downregulation of WNT signaling regulators might affect one subpopulation only, providing a possible explanation for the development of ovotestes.

RSPO1/WNT/CNTNB1 Signaling and FOXL2

Interplay

An additional intriguing question is the nature of the interactions of FOXL2 and the WNT/CTNNB1 pathway during ovarian development.

In goats, *RSPO1* expression was downregulated by around 50% in XX *PIS*^{-/-} sex-reversed gonads at 36 and 40 dpc [Elzaïat et al., 2014]. These observations are in favor of the coexistence of 2 separate pathways in the early differentiating ovary. The possible interplay between

RSPO1 and *FOXL2* is not obvious in goats, but another member of the R-spondin family appears to belong to *FOXL2* downstream pathways. Indeed, *RSPO2* exhibits a sex dimorphic pattern of expression in goats as soon as sex determination proceeds and it is strongly downregulated in *PIS*^{-/-} sex-reversed gonads [Kocer et al., 2008; Elzaïat et al., 2014]. This suggests that *RSPO2* could be a direct target of *FOXL2*.

In mice, as previously mentioned, 2 subpopulations of pregranulosa cells were characterized during fetal life; the first one expressing *FOXL2* and the second one *LGR5* [Rastetter et al., 2014]. This may indicate that *FOXL2* and *RSPO1* are 2 strictly separated pathways, giving rise to 2 subpopulations of pregranulosa cells. Nevertheless, *Foxl2* expression becomes restricted in *Rspo1* knockout gonads, while *Rspo1* expression is not affected in *Foxl2* loss-of-function mutants, suggesting that *Foxl2* expression could be partly dependent on *RSPO1* signaling [Auguste et al., 2011]. This effect could be indirect, since *LGR5* is not expressed in *Foxl2* expressing cells. It could also be a direct effect if an, as of yet, uncharacterized subpopulation of supporting cells co-expressing *FOXL2* and another *RSPO1* receptor, such as *LGR4*, exists. Given that ablation of *Lgr5* does not trigger sex reversal while *Lgr4* depletion does [Rastetter, 2014; Koizumi, 2015], WNT signaling could be enhanced via *LGR4* in these pregranulosa cells,

explaining the partial control of *Foxl2* expression by *RSPO1* in these pregranulosa cells. The precise localization of *LGR4* during ovarian development and its interaction with *FOXL2* remain to be investigated.

While *FOXL2* is a key factor for folliculogenesis in mice [Schmidt et al., 2004; Uda et al., 2004], genetic ablation of the *Foxl2* gene did not trigger an early embryonic phenotype in the mouse gonad. However, in *Rspo1/Foxl2* or *Wnt4/Foxl2* double mutants (XX *Foxl2*;*Rspo1* double-knockout or XX *Foxl2*;*Wnt4* double-knockout embryos), the sex reversal phenotype was amplified compared to the single *Rspo1* or *Wnt4* KO gonads [Ottolenghi et al., 2007; Auguste et al., 2011]. Thus, double mutants exhibit earlier transdifferentiation features, suggesting that there is an increased number of cells expressing Sertoli cell markers at a given time point. Nevertheless, in both XX *Foxl2*/*Wnt4* double-knockout and XX *Foxl2*/*Rspo1* double-knockout embryos, sex reversal was incomplete.

Conclusion and Future Directions

In conclusion, here we have summarized the key roles of the transcription factor *FOXL2* and the WNT signaling pathway during ovarian differentiation. These 2 genetic pathways act in a complimentary manner, and the phenotypic differences in different species resulting from their depletion may be related to the differentiation status of granulosa cell precursors. Concerning *FOXL2*, its loss-of-function mutation induces sex reversal either in early developmental stages in goats and in postnatal stages in mice. At these stages, the granulosa cells have acquired the ability to produce hormones, with *FOXL2* being involved in aromatase synthesis. Although *FOXL2* is expressed as early as 12.5 dpc in the mouse ovary, steroidogenesis is not detected before late gestation, suggesting that the differentiation of the granulosa cells is delayed or different in mice.

In mice, 2 types of granulosa cells have been described: those giving the medullar follicles and those promoting the formation of the cortical follicles after birth. The first ones are *FOXL2* positive and the second subpopulation of pregranulosa cells is positive for *LGR5*; however, it remains unclear whether these 2 types of granulosa cells arise from the same supporting cell precursors or from different ones [Mork et al., 2012; Gustin et al., 2016]. WNT signaling is involved in the proliferation of the granulosa cell precursors [Gustin et al., 2016], but it is not clear yet whether this pathway induces proliferation of all the granulosa cell precursors or just the *LGR*-positive

subpopulation. WNT signaling is also important for the first steps of differentiation, as highlighted by sex reversal triggered by *WNT4* depletion and *RSPO1* mutations in human and mice. However, it is not known whether this pathway also plays a key role in ovarian development in goats. Such knowledge would help to explain the evolution of early gonadal differentiation pathways in different species. Afterwards, during late gestation, all pregranulosa cells express *FOXL2*, and a part of all of them becomes steroidogenic. This suggests that *FOXL2* is an essential factor in granulosa cell differentiation. However, the fate of these early *FOXL2*-expressing cells disappears at puberty, suggesting that these follicles remain different from those of the second subpopulation.

In goats, *DMRT1* is the earliest known testis-differentiating factor to be expressed in female-to-male sex-reversed gonads associated with *FOXL2* downregulation. *DMRT1* is also a key factor for testis differentiation in other species such as Medaka [Masuyama et al., 2012] and chicken [Smith et al., 2009]. In humans, mutation of its genetic sequence is associated with gonadal dysgenesis [Ledig et al., 2012; Murphy et al., 2015], suggesting that some evolutionary features are conserved between these species. In mice, *SOX9* is expressed before *DMRT1*, suggesting that *SOX9* is the primary Sertoli cell-differentiating factor in *Foxl2* mutant gonads [Uhlenhaut and Treier, 2006]. This implies that the steroidogenic maturity of the granulosa cells is not the only status required for sex reversal in both species. The differentiation of granulosa cells is also partly specific for each species.

Whatever these differences may be, *FOXL2* is a key player in ovarian differentiation, and mutations of this gene impact the function of granulosa cells at various stages of their differentiation, ultimately leading to various ovarian pathologies [Verdin and De Baere 2012]. At the time of sex determination, *FOXL2* mutation induces sex reversal in XX goats [Boulanger et al., 2014], folliculogenesis defects in mice [Schmidt et al., 2004; Uda et al., 2004], and type I BPES with POF [Beysen et al., 2008] or non-syndromic POF without BPES in humans [Laissue et al., 2009].

Moreover, one mutation in *FOXL2* (C134W) is responsible for almost all granulosa cell tumors in adult women [Kobel et al., 2009; Shah et al., 2009]. In the context of this disease, it has been shown that this mutation alters the expression of different factors comprising aromatase [Kobel et al., 2009; Fleming et al., 2010; Kim et al., 2011; Rosario et al., 2012]; however, the mechanisms through which *FOXL2* mutation induces these cancers remain a mystery.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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