FOXL2 activates *P450 aromatase* gene transcription: towards a better characterization of the early steps of mammalian ovarian development

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Abstract

Previous studies have equated *FOXL2* as a crucial actor in the ovarian differentiation process in different vertebrate species. Its transcriptional extinction in the polled intersex syndrome (PIS) leads primarily to a drastic decrease of aromatase (*CYP19*) expression in the first steps of goat ovarian development. In this study, we provide a better characterization of early ovarian development in goat, and we provide experimental evidence demonstrating that *FOXL2* represents a direct transcriptional activator of the *CYP19* gene through its ovarian-specific promoter 2. Moreover, the ovarian location of *FOXL2* and *CYP19* proteins, together with their expression profiles in the female gonads, stress the involvement of *FOXL2* co-factor(s) for regulating *CYP19* transcription. Expressional analyses show that activin- β A can be considered as a strong candidate for being one of these *FOXL2* co-factors. Finally, we discuss evidence for a role of activin and estrogens in somatic and germinal cell proliferation occurring before germ cell meiosis. This period, of 20 days in goat, seems to have no equivalent in mouse. This species-specific difference could explain the phenotype discrepancy observed between XX goat PIS^{-/-} and XX mouse *Foxl2*^{-/-}.

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Introduction

Recent accumulating evidence indicates that the transcription factor *FOXL2* is a crucial actor in early ovarian differentiation and maintenance. Natural mutations affecting *FOXL2* have been shown to be involved in two syndromes leading to ovarian dysfunction in mammals. First, heterozygous mutations of *FOXL2* induce premature ovarian failure in XX BPES (blepharophimosis-ptosis-epicanthus inversus syndrome) type I patients (Crisponi *et al.* 2001). Secondly, complete loss of ovarian expression of this gene was found to be associated with XX female-to-male sex-reversal in goats homozygous for the polled intersex syndrome (PIS) mutation (Pailhoux *et al.* 2001). As in humans, *Foxl2* invalidation in mouse leads to premature ovarian failure, but only in the homozygous state (Schmidt *et al.* 2004, Uda *et al.* 2004). In *Foxl2* null ovaries, granulosa cells fail to undergo squamous to cuboidal transition leading to arrest of folliculogenesis and oocyte atresia. During gonad development, *FOXL2* displays an ovarian-specific expression profile. The female-specific expression profile

has been demonstrated in different mammalian species such as mouse (Pannetier *et al.* 2003), goat (Pannetier *et al.* 2005), and humans (Cocquet *et al.* 2002), but also in other vertebrates such as birds (Govoroun *et al.* 2004), turtles (Löffler *et al.* 2003) and fishes (Baron *et al.* 2004, Wang *et al.* 2004).

FOXL2 is a member of the forkhead/HNF-3-related gene family of transcription factors that are highly conserved and involved in many developmental processes and in cellular differentiation (for review see Carlsson & Mahlapuu 2002). As the other member of the family, *FOXL2* possesses a well-conserved winged helix domain that binds DNA to a 7-bp core recognition motif (5'-G/A-T/C-C/A-A-A-C/T-A-3') (Kaufmann & Knochel 1996). Another feature of *FOXL2* is the presence of a polyalanine domain of 14 alanines, located to the C-terminus with respect to the DNA binding domain. Although the physiological function of this polyalanine tract remains unknown, its expansion from 14 to 24 residues accounts for 30% of the reported mutations in BPES patients (De Baere *et al.* 2003). The 14 to 24 expansion induces extensive nuclear and

cytoplasmic FOXL2 protein aggregation (Caburet *et al.* 2004). Until now, two FOXL2 target genes have been described in two different tissues, the gonadotropin releasing hormone receptor (*GnRHR*) gene in the pituitary (Ellsworth *et al.* 2003) and the steroidogenic acute regulatory (*StAR*) gene in the adult ovary (Pisarska *et al.* 2004). Interestingly, the regulatory role of FOXL2 on these two targets appears to be different. While, FOXL2 contributes to a transcriptional activation of *GnRHR*, via the GRAS element (GnRH-receptor activating sequence), it leads to a transcriptional repression of *StAR*. Interestingly, in the developing ovary of chicken (Govoroun *et al.* 2004) and rainbow trout (Baron *et al.* 2004), the spatio-temporal expression profile of *FOXL2* is strongly correlated with those of the *CYP19* gene encoding aromatase, an enzyme responsible for the conversion of androgens into estrogens (for review see Conley & Hinshelwood 2001). Then, one could hypothesize that the *CYP19* gene may represent a direct target of FOXL2.

In non-mammalian species such as fishes, reptiles, and birds, estrogens have been shown to be crucial for proper ovarian differentiation. Consequently, male-to-female sex reversal with the development of ovaries or ovotestes occurs after treatment with estradiol in male rainbow trout (Krisfalusi & Cloud 1999), reed frog (Hayes & Menendez 1999), reptiles (Pieau *et al.* 1999), and chicken (Scheib 1983). Conversely inhibition of estrogens, by the use of anti-aromatase, in embryos that would otherwise develop as females results in testis development in fishes (Guiguen *et al.* 1999), reptiles (Pieau *et al.* 1999) and birds (Elbrecht & Smith 1992, Wade & Arnold 1996). This crucial impact of estrogens in female gonad differentiation has also been demonstrated for different marsupial species, two opossums (Burns 1955, Fadem & Tesoriero 1986) and the tammar wallaby (Coveney *et al.* 2001). In these metatherian mammals, estrogen treatment at the time of testis differentiation induces a male-to-female sex reversal.

In eutherian mammals, the role of estrogens in ovarian development is poorly documented. Indeed, hormonal treatments are not easy to perform because gonad differentiation takes place during fetal life in the maternal uterus. Moreover, the most studied model is the mouse, a species that has no ovarian steroidogenesis during fetal life. Accordingly, the major steroidogenic activator, steroidogenic factor (*Sf-1*), is not expressed in mouse ovaries from 13.5 days *post-coitum* (dpc) to 18.5 dpc (Ikeda *et al.* 1994). The absence of estrogen action on early ovarian development in the mouse is also confirmed by the absence of severe phenotypes in ovaries of newborn mice knocked-out for *CYP19* (Fisher *et al.* 1998), *StAR* (Hasegawa *et al.* 2000), or estrogen receptors (*ERα*, *ERβ*) (Couse *et al.* 1999, Dupont *et al.* 2000).

By contrast with mouse, ovaries from domestic ruminants produce estrogens from the onset of their

development. In sheep, ovaries are steroidogenically active at the time of morphological sexual differentiation as attested by direct hormonal dosages or *CYP19* expression studies (Mauléon *et al.* 1977, Payen *et al.* 1996, Quirke *et al.* 2001). Interestingly, in XX sex-reversed *PIS*^{-/-} goat fetuses, the first affected gene following *FOXL2* extinction is *CYP19* (Pailhoux *et al.* 2002, 2005). Indeed, *CYP19* transcription is found drastically decreased in XX *PIS*^{-/-} fetuses from 36 dpc, the earliest stage of its normal expression in goats. This decrease suggests that FOXL2 could play a direct regulatory role on *CYP19* transcription.

In order to investigate further a putative enhancing effect of FOXL2 on *CYP19* transcription, (i) immunolocalization of both proteins in goat fetal ovaries has been performed, (ii) the *CYP19* promoter used in ovarian development has been determined, and (iii) a direct transcriptional effect of FOXL2 on *CYP19* promoter 2 has been demonstrated by transfection assays in COS7 and granulosa cells. Furthermore, a direct interaction between FOXL2 and the promoter of *CYP19* was also confirmed in a human granulosa cell line (KGN), by chromatin immunoprecipitation assay.

Materials and methods

Animals and tissue samples

All goat fetuses were obtained from horned pregnant females, following hormonal treatments as previously described (Pailhoux *et al.* 2002). Day 0 *post-coitum* corresponded to the day of mating. The genetic sex of all fetuses was determined by PCR amplification of *SRY* and *ZFY/ZFX* genes on liver genomic DNA (Pailhoux *et al.* 2002). For each fetus, one gonad was frozen in liquid nitrogen for molecular analysis; the other one was fixed for immuno-histological studies. For granulosa cell culture, ovine ovaries were collected from adult cyclic Romanov ewes from a local slaughterhouse for immediate use.

All procedures were approved by the agricultural and scientific research Government committees in accordance with the guidelines for the Care and Use of Agricultural Animals in Agricultural Research and Teaching in France (approval A37801).

RT-PCR

RNA extraction, DNase treatment, and cDNA synthesis were conducted as previously described (Pailhoux *et al.* 2001). Briefly, 2 µl (corresponding to 0.5 µg reverse transcribed total RNA) of each RT mix was amplified in 50 µl PCR by using 0.5 U Taq polymerase (TaKaRa), 200 µM of each dNTP, and 150 nM of each primer. The sequences of the primers used and the PCR conditions are given in Table 1.

Table 1 Primers and PCR conditions

Primer names	Primer sequences	Temp./MgCl ₂
Aromatase 1 (E9)	5'-CTGTCGTGGACTTGGTCATG-3'	58 °C/1.5 mM
Aromatase 2 (E10)	5'-GGGGCCCAAAGCCAAATGGC-3'	
5'RACEAro1	5'-TGCAGGCACTGCCGATCCCC-3'	58 °C/1.5 mM
5'RACEAro2	5'-CAGTGGGAAATGAGGGGCCCC-3'	
5'RACEAro3	5'-GAGGATGTGTCCTCATAATTCC-3'	
5'RACEAro4 (5A4)	5'-GAAATCCAGTGAGCAGCAGG-3'	
5'RACEAro7 (5A7)	5'-CAAAGCGCCAATCTCTACGG-3'	
5'RACEAro8 (5A8)	5'-GTGGGAGACCTTGGTGCCTG-3'	
5'RACEAro9 (5A9)	5'-GCCCTTCAAAGGACTTATCAC-3'	
pAro1	5'-GTCCATGGGGTTGCAACGAG-3'	58 °C/1.5 mM
pAro2	5'-CCAGCACATGGTGCAAGAG-3'	
pAro3	5'-GAGAACTCAGTCACTCTACC-3'	
pAro4	5'-CCTGAGACTCTACCAAGGTCA-3'	
pAro5	5'-GACTCGTGACTATAAATTTG-3'	
pAro-R	5'-TTACTTGATCTCAGGGGAAG-3'	
Inhibin- α -1	5'-TGCCCCCTACCCCTTTCCAG-3'	55 °C/1.5 mM
Inhibin- α -2	5'-CTTAGATGCAAGCACAGTGC-3'	
Activin- β A-1	5'-GGCAAGGTCAACATTTGCTG-3'	55 °C/1.5 mM
Activin- β A-2	5'-ACATGGACATGGGTCTCAGC-3'	
Activin- β B-1	5'-GAGTCGCACCGGCCCTTCGT-3'	58 °C/1.5 mM
Activin- β B-2	5'-CCCATAGTAGCCGGTGGGCG-3'	
ActRII-1	5'-AACGAGGCACCAAGTGTGAT-3'	60 °C/1.5 mM
ActRII-2	5'-AGTACAGCGAGAAGCCAGTT-3'	
3 β HSD-1	5'-CTCGTGACCGGAGGAGGAGG-3'	58 °C/1.5 mM
3 β HSD-2	5'-GCTGGAATAGTGAGCTTCC-3'	
ER α -1	5'-CTTCCCGCCGCTCAACAGCG-3'	67 °C/1.5 mM
ER α -2	5'-CTCTCTGGCGCTTGTGCTTC-3'	
ER β -1	5'-GACATGCTCCTGGCAACGAC-3'	55 °C/1.5 mM
ER β -2	5'-GCAGGTGTTCCATGCCCTTG-3'	
FOX L2-9	5'-GGCCCCCTGAGCCAGCGCCC-3'	58 °C/1.5 mM
FOX L2-10	5'-CCCCGACGCTGAGGTGCCCG-3'	
SPO11-Cup	5'-TGCAACATTTTCAAGCGCTCC-3'	56 °C/2.0 mM
SPO11-Ddown	5'-TCAGAAGGGAGRAGACCAAG-3'	
β -actin-1	5'-CATCCGTAAAGACCTCTATGCC-3'	55 °C/2.5 mM
β -actin-2	5'-TAGAGCCACCAATCCACACAG-3'	
ChIP-CYP19up	5'-AACCTGCTGATGAAGTCACAA-3'	54 °C/2.5 mM
ChIP-CYP19down	5'-TCAGACATTTAGGCAAGACT-3'	
ChIP-IMP3up	5'-AGTTGTTGTCCCTCGTGACC-3'	54 °C/2.5 mM
ChIP-IMP3down	5'-GTCCACTTTGCAGAGCCTTC-3'	
ChIP-G3PDHup	5'-ACCACAGTCCATGCCATCAC-3'	54 °C/2.5 mM
ChIP-G3PDHdown	5'-TCCACCACCCTGTTGCTGTA-3'	

Rapid amplification of cDNA ends (RACE)

To determine the *CYP19* promoters used in the goat ovary, 5' RACE experiments were carried out on 5 μ g DNase-treated total RNA purified from goat ovaries at two developmental stages (1 month old and adult). Reverse transcription of *CYP19* specific RNA was performed by using the primer 5'RACEAro1 (Table 1). Reverse transcription assays and PCR amplifications were performed using the 5' RACE system from GibcoBRL, according to the manufacturer's instructions. Briefly, two rounds of PCR using nested primers were carried out. The first round of PCR was performed with 5'RACEAro2, the second round with either 5'RACEAro3 or 5'RACEAro4 (Table 1). All these 4

primers were located in exons 2 and 3 of *CYP19* that are present in all mRNA isoforms. They were designed from the goat *CYP19* mRNA sequence (Genbank accession number AY148883). The PCR products obtained after the second round of PCR and showing a 34 bp difference in size, which is the length present between the two primers used, 5'RACEAro3 and 5'RACEAro4, were cloned. Then, at least three independent clones per PCR product were sequenced.

Plasmid constructs

Different lengths of CYP19 promoter 2 fragments were obtained by PCR using pAro1, pAro2, pAro3, pAro4 or

pAro5 as forward primers, and pAro-R as reverse primer (Table 1). These different promoters were cloned upstream of the *luciferase* gene in the pGL3-basic vector (Promega). Goat *FOXL2* ORF was obtained by PCR and inserted in the pSG5 vector (Stratagene) as previously described (Govoroun *et al.* 2004). Constructions 3 xGRAS-Luc and Foxl2-VP16 were gratefully obtained from Dr Clay's laboratory (Ellsworth *et al.* 2003). All plasmids were amplified in DH5 α bacteria, and large amounts of plasmids were obtained by using the anion exchange columns nucleobond AX (Macherey-Nagel, Düren, Germany).

Cell culture conditions and transfection assays in COS7 cells

COS7 cells were cultured in Dulbecco's modified Eagle's medium and Ham's F-12 medium (Eurobio, Courtaboeuf, France) containing 10% (v/v) heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μ g/ml) and L-glutamine (300 μ g/ml), in a 5% CO₂ atmosphere at 37 °C. For transfection, 1.3×10^5 cells were seeded in 12-well Petri dishes and transfected the following day using FuGENE 6 reagent (Roche), according to the manufacturer's instructions. The amount of transfected DNA was 1 μ g of the reporter vectors with 0.25 μ g Foxl2-VP16 or pVP16 alone (negative control). Each combination of plasmids was tested in triplicate in each culture and in three independent experiments. Seventeen hours after transfection, cells were harvested by scraping in 1 ml PBS, pelleted by centrifugation and resuspended in 50 μ l lysis buffer (25 mM glycylglycine, 1 mM EDTA, 8 mM MgSO₄, 1% Triton, 15% glycerol, 1 mM dithiothreitol). A 10 μ l-extract was used for the measurement of luciferase activity. Each tube of extract was placed in a luminometer equipped with one reagent injector (Berthold, Bad Wildbad, Germany), and the reaction was initiated by the injection of 100 μ l substrate solution containing 23 μ l luciferine (1 nM) and 77 μ l of its buffer (25 mM glycylglycine, 15 mM MgSO₄, 5 mM ATP).

Ovine granulosa cells (oGC) primary culture and transfection assays

In each independent culture experiment, ovarian follicles from three Romanov ewes were quickly dissected, pooled and classified according to size. Ovine GC were recovered from small antral follicles (1–3 mm in diameter) as previously described (Le Bellego *et al.* 2002). They were seeded at 200 000 cells/well in 24-well plates and cultured in McCoy's 5a medium (Sigma, L'Isle d'Abeau Chesnes, France) supplemented with 3% fetal ovine serum. After 48 h, the medium was changed and oGC were transiently transfected as previously described (Pierre *et al.* 2004) using DAC30 transfection

reagent with a DNA/DAC30 ratio of 1/2 (w/w) as specified by the manufacturer (Eurogentec, Seraing, Belgium). Ovine GC were transfected with pARO or GRAS luciferase reporter genes (250 ng/well) both alone or in combination with plasmids expressing goat *FOXL2* or mouse Foxl2-VP16 (250 ng/well). Empty pSG5 or pVP16 plasmids were used in control conditions. After 48 h, cells were lysed before the luciferase assay (Promega, Charbonnières, France). Each combination of plasmids was tested in triplicate in each culture and in three independent experiments.

Chromatin immunoprecipitation

To perform the chromatin immunoprecipitation (ChIP) assay, KGN cells (Nishi *et al.* 2001) were grown to confluence in DMEM-F12 medium supplemented with 10% FBS. Formaldehyde cross-linked cells were scraped into a conical tube in 2 ml PBS supplemented with protease inhibitors (1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml pepstatin), centrifuged for 4 min at 500 *g*, and the cell pellet was resuspended in 10⁶ cells per 200 μ l SDS lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA, ChIP assay kit). Resuspended cells were sonicated five times for 20 s, with pauses of 30 s between each sonication round. The sonicated supernatant was diluted to 1 ml in RIPA buffer without SDS (300 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA and 1% NP40). To reduce non-specific background, samples were pre-cleared for 2 h at 4 °C using pre-immune serum and 7.5% protein A-sepharose gel in RIPA buffer with protease inhibitors. Pre-cleared extracts were then incubated overnight in the presence of either 1% pre-immune serum (negative control) or with the anti-C terminal *FOXL2* antibody previously described (Cocquet *et al.* 2002). Washes and the rest of the experiment were carried out according to the protocol from the Upstate Biotechnology kit. In order to exclude a potential contamination with non-specifically adsorbed DNA, we attempted to amplify insulin-like growth factor II mRNA-binding protein 3 (IMP3) and glyceraldehyde-3-phosphate dehydrogenase (G3 PDH) genes, which are not supposed to be targets of *FOXL2*. This was performed on both the material immunoprecipitated with the pre-immune serum (negative control), and with the anti-*FOXL2* antibody. The sequences of the primers used in the PCRs are indicated in Table 1. *CYP19* primers have been described previously (Ghosh *et al.* 2005). PCR conditions were as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 40 s, and then 72 °C for 5 min. *Taq* polymerase was purchased from Invitrogen.

In situ hybridization

The cDNA used for riboprobe synthesis was obtained by RT-PCR on goat adult ovary with 3 β hydroxysteroid

dehydrogenase (3 β HSD)-1 and 3 β HSD-2 primers (Table 1), and then subcloned into pGEM-T Easy vector. Riboprobes were generated by transcription in the presence of digoxigenin-labeled deoxy-UTP and the appropriate SP6 or T7 RNA polymerase. Freshly dissected goat ovaries were fixed for 1 h in 4% paraformaldehyde in phosphate saline buffer (PBS) at 4 °C. After washing in PBS with increasing concentrations of sucrose (0, 12%, 15%, and 18%), tissue specimens were embedded in Jung tissue freezing medium (Leica Instruments, Wetzlar, Germany) and frozen at -80 °C. Sections (7 μ m thick) were produced and stored at -80 °C. After thawing, frozen sections were de-lipidized in chloroform for 1 min, and re-hydrated in PBS. Then, sections were post-fixed for 20 min with 2% paraformaldehyde in PBS, pH 9, and treated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8. After 2 h prehybridization in 50% formamide, 2 $\times$ sodium saline citrate, 5 $\times$ Denhardt's solution, 50 μ g/ml yeast tRNA, 250 μ g/ml salmon sperm DNA, 4 mM EDTA and 2.5% dextran sulfate at 55 °C, hybridization was carried out overnight at 55 °C in a moist chamber with riboprobes diluted in the same buffer without salmon sperm and EDTA. The slides were washed twice in 2 $\times$ SSC and were then treated for 30 min at 37 °C with ribonuclease A (20 mg/ml) in NTE (500 mM NaCl, 10 mM Tris-HCl pH 7.5, 5 mM EDTA). After 4 washings in 2 $\times$ SSC, slides were incubated for 1 h in 0.1 $\times$ SSC, 30% formamide at 60 °C. Then sections were washed for 1 h in Tris pH 7.5 100 mM, NaCl 150 mM, 0.5% blocking reagent (Roche), and incubated with an alkaline phosphatase anti-DIG antibody (Roche) (diluted 1/500) in the same buffer. After 3 washes with 100 mM Tris pH 7.5, 150 mM NaCl, slides were rinsed for 20 min with alkaline phosphatase buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl₂) and incubated overnight with the staining solution NBT/BCIP Ready-to-use tablets (Roche) containing 0.24 mg/ml of levamisole (Sigma). The reaction was stopped with water and slides were mounted in glycerol-gelatin medium (Sigma).

Immunostaining

Sections were air dried and re-hydrated in PBS. FOXL2 anti-C terminal antibody (1/500 or 1/1000), P450-aromatase (1/50, Serotec), α -inhibin (1/100, Serotec), β A-activin (1/200, Serotec, Oxford, UK), c-Kit (1/50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ER α (1/50, Santa Cruz Biotechnology) or ER β (1/200, Upstate biotechnology) were then applied onto the tissue sections for 1 h at room temperature. After three washings, sections were incubated with biotinylated anti-rabbit secondary antibody (1/200, VECTOR, Burlingame, CA, USA) (FOXL2, c-Kit, ER α and ER β) or biotinylated anti-mouse secondary antibody (1/200, VECTOR) (P450-aromatase, inhibin- α and activin- β A)

for 30 min at room temperature and rinsed in PBS. An ABC system (VECTOR) was then applied to sections for 30 min. After three washes with PBS, peroxidase activity was revealed with 3,3'-diaminobenzidine (VECTOR). Slides were rinsed and permanently mounted in EUKKIT (Sigma) mounting medium.

For double immunostaining, FOXL2 staining was followed by P450-aromatase staining. Peroxidase activity was revealed with VECTOR-SG substrate (VECTOR) for FOXL2 and with DAB for P450-aromatase.

Histological processing

Freshly dissected gonads were fixed in Bouin's fluid for 24 h, dehydrated, embedded in paraffin and sectioned at 7 μ m. Sections were mounted and stained according to the trichrome technique of Tuchmann du Plessis.

Results

FOXL2 and CYP19 co-localize in the same cellular type of goat 45 dpc ovaries

In order to test if FOXL2 could act as a modulator of *CYP19* gene expression, we first examined the cellular localization of both proteins in the developing ovary by immunohistochemistry. In addition, 3 β -HSD mRNAs, encoding a crucial enzyme of steroid biosynthesis, were detected by *in situ* hybridization (Fig. 1). The experiments were performed at the 45 dpc stage, when the higher levels of *CYP19* transcripts were detected by RT-PCR (Fig. 2B). As previously shown, at 36 dpc (Cocquet *et al.* 2002) FOXL2 was detected in the majority of somatic cells in the 45 dpc ovaries (Fig. 1C,E). However, no signal was detected either in the region located just below the surface epithelium or in the germ cells, which were concentrated in this region. On the other hand, CYP19 protein and 3 β -HSD mRNA were found expressed by somatic cells located in the deeper medulla part of the ovary (Fig. 1A,B). By a double immunostaining approach, it appeared that all CYP19-expressing cells were also positive for FOXL2, but there were many cells positive for FOXL2 that did not express aromatase (Fig. 1E,F). These results indicated that FOXL2 could represent a direct transcriptional activator of *CYP19*. However, FOXL2 is not sufficient alone to trigger fetal *CYP19* expression, as some FOXL2-positive cells do not express CYP19.

CYP19 is transcribed via its promoter 2 during goat fetal ovarian development

In human and bovine, the *CYP19* gene uses different tissue-specific promoter regions to transcribe different non-coding first exons (named 1.1, 1.2, 1.3,...etc; and 2)

Figure 1 3β -HSD, FOXL2 and CYP19 expression in a 45 dpc goat ovary. (A) 3β -HSD mRNA detected by *in situ* hybridization. (B) CYP19 protein and (C) FOXL2 protein detected by immunohistochemistry. (E and F) Double immunolocalization of FOXL2 (blue nuclear staining) and CYP19 (brown cytosolic staining). Note that all CYP19 positive cells also expressed FOXL2. (D) Negative control, sense probe for 3β -HSD. Higher magnification (x4) of a positive region (inside the rectangle) is presented for A, B, C and F in the upper right corner. A line represents the thinness of the cortical region (co) where the majority of germ cells are located. Scale bar in A (for A-D), 200 μ m, scale bars in E and F, 50 μ m.

(Furbass *et al.* 1997, Bulun *et al.* 2004). In bovine, a species closely related to goat, *CYP19* promoter 2 (located in front of exon 2, the first coding exon) has been shown to be used mainly in adult ovaries (Furbass *et al.* 1997). In order to determine the promoter used in fetal goat ovaries, and to test its putative responsiveness to FOXL2, we carried out 5' RACE experiments on *CYP19* transcripts from ovarian goat samples at 30 days *post-partum* (dpp) and at adulthood. In both cases, 5' RACE-products, ranging from 0.15 to 0.35 kb in length, were detectable after the second round of PCR. Each band was cloned independently and three clones of each were sequenced. Two and three different alternative first exons were detected at 30 dpp and adulthood respectively (Fig. 2A). At 30 dpp, transcripts corresponding to a transcriptional initiation at promoter 2a (promoter 2, distal TATA box) and a newly characterized promoter 1.6, located at $\sim$ 850 bp upstream from the ATG initiator codon on the goat sequence, were isolated. At the adult stage, transcripts generated from promoters 2a, 2b (promoter 2, proximal TATA box), and 1.1 were isolated. Promoter 1.1 has previously been shown to be used mainly in bovine placenta (Furbass *et al.* 1997). The presence of two TATA boxes, separated by 28 bp in the promoter 2, can also be seen for the bovine and the ovine species (Furbass *et al.* 1997, Vanselow *et al.* 1999).

The use of the different ovarian promoters isolated by RACE, together with promoter 1.5 previously described in sheep (Vanselow *et al.* 1999), were then tested on developing ovaries mRNAs by promoter-specific RT-PCR (Fig. 2B). The promoter-specific expression profiles obtained were compared with the one obtained with primers located in exons 9 and 10, which are able to detect all *CYP19* transcripts (Fig. 2B). In this way, no transcripts initiated from promoters 1.6 and 1.5 can be detected after 35 PCR cycles (data not shown). Transcripts initiated from promoter 1.1 were detected at 40 dpp and in the adult, in agreement with results recently published in a bovine species (Lenz *et al.* 2004). More interestingly for our purpose, only the promoter 2 seems to be used in the developing goat ovaries before the onset of germ cell meiosis (55 dpc), as attested by the expression profile of the meiosis-specific gene *SPO11* (Fig. 2B). Indeed, *CYP19* expression profiles were found to be highly similar when detected with primers located in exons 9 and 10 (present in all transcripts), or when detected in the 5' non-coding region of exon 2, specifically present in transcripts generated from promoter 2. Furthermore, the fact that *FOXL2* expression profile did not peak as did those of *CYP19* before germ cell meiosis, reinforces the idea that FOXL2 is not sufficient alone to trigger fetal *CYP19* expression (Fig. 2B).

Figure 2 *CYP19* promoters used during goat ovarian development. (A) Schematic representation of the three different promoter regions found by 5' RACE analyses; two distal promoters transcribed exon 1.1 and exon 1.6, and one proximal promoter, with two TATA boxes, transcribed directly exon 2 (exon 2a=distal TATA box, exon 2b=proximal TATA box). Promoter specific primers (5A9, 5A8, 5A7) located in the different 5' non-coding exons are indicated, with the reverse one (5A4) located in the common coding part of the different *CYP19* transcripts. (B) RT-PCR expression studies of aromatase tested for all transcripts, with primers located in exons 9 and 10 (E9/E10) or for transcripts specifically initiated at promoters 1.1 or 2, compared with expression profiles of *FOXL2* and *SPO11*, a meiosis-specific factor. E, exon; Days pp, days post partum.

Figure 3 Transfection assays. (A) Schematic representation of the 1249 bp fragment encompassing *CYP19* promoters 2 (2a, 2b), 1·5 and 1·6. Circles represent the putative *FOXL2* binding sites with no mismatches (black with a 0 inside), one mismatch (dark gray with a 1 inside) or two mismatches (light gray with a 2 inside) in the first three bases of the consensus. The putative Smad-binding site (gray square) near a *FOXL2* degenerated site (empty circle) and the putative HNF3/*FOXQ1* (black triangle) are also indicated. The location of the 5' bases of the five different restricted *CYP19* promoters cloned in front of the luciferase reporter gene are indicated by dotted lines (-1104, -507, -180, -110, and -40). (B) Comparative induction test of native goat *FOXL2* (gFOXL2) and chimerical Foxl2-VP16 on the reporter construct containing three copies of the GRAS element cloned in front of the prolactin promoter driving the *luciferase* gene. (C) Multiple analyses of *luciferase* reporter gene driven by different fragments of *CYP19* promoter. The different plasmid constructs (pCYP19-Luc) are indicated in the left margin. In COS7 cells, plasmid constructs were transiently co-transfected with a Foxl2-VP16 expression vector or VP16 vector alone. In sheep granulosa cells (oGC) plasmid constructs were transiently co-transfected with a gFOXL2 expression vector or pSG5 vector alone. In both cases, cells were harvested and assayed for luciferase activity. Results are presented as fold induction obtained with Foxl2-VP16 or gFOXL2 compared with VP16 alone or pSG5 alone, in COS7 or oGC respectively.

FOXL2 is an inducer of the *CYP19* promoter 2

To determine if the *CYP19* promoter 2, used in 45 dpc goat ovaries, could represent a direct target of *FOXL2*, a 1249 bp fragment encompassing promoter 2 has been isolated by PCR from goat genomic DNA (Genbank

accession number DQ122857). This fragment contained respectively 1104 bp upstream and 145 bp downstream of the transcriptional start site of promoter 2a (+1) and encompassed three putative *CYP19* promoters, 1·6, 1·5 and 2 (Fig. 3A). Based on the FOX recognition 7-bp motif (5'-G/A-T/C-C/A-A-A-C/T-A-3') (Kaufmann &

Knochel 1996), the core of this motif (A-A-Y-A) was searched on this goat fragment. Among twelve detected sites, 2 presented no mismatch, 5 had one mismatch and 5 two mismatches compared with the consensus sequence (Fig. 3A). Moreover, one HNF-3/FOXQ1 (position -50/-44), and one putative binding site for the Smad factors linked to a degenerated site for the FOX family position (-128/-114) have been pointed out by the Genomatix MatInspector software (<http://www.genomatix.de>).

In order to delineate the putative FOXL2 responsive elements in the 1249 bp of the goat *CYP19* promoter, a successive deletion approach was used. Different 5'-ends of the promoter 2 were cloned upstream of the luciferase reporter gene and transiently transfected alone or in combination with FOXL2-expressing vectors in COS7 cells as well as in primary oGC. First of all, using the GRAS-containing reporter construct previously shown to be a Foxl2 target in the mouse (Ellsworth *et al.* 2003), we have tested the functionality of the goat FOXL2 protein (gFOXL2) compared with the already described (Ellsworth *et al.* 2003) mouse Foxl2-VP16 fusion protein. As indicated in Fig. 3B, Foxl2-VP16 was able strongly to activate the GRAS construct in COS7 cells as well as in oGC. However, gFOXL2 appeared to be functional preferentially in oGC, which suggests that a natural FOXL2 co-factor is missing in the COS7 cells. Based on these results, goat *CYP19* promoter constructs were tested for Foxl2-VP16 action in COS7 cells and for gFOXL2 action in oGC.

In COS7 cells, a threefold induction of luciferase activity was seen with Foxl2-VP16 on the -507/+145 *CYP19* promoter construct (Fig. 3B). This induction was lost after a further deletion (-180/+145), indicating a major role of the 327-bp region between -180 and -507, containing four putative FOXL2 binding sites. Interestingly, the same result was observed in oGC for gFOXL2 activity. However, in this closer physiological system, the sensitivity of the goat *CYP19* promoter to gFOXL2 was much higher as shown by the 7.5-fold induction on the -507/+145 construct. By contrast, Foxl2-VP16 was unable to activate this construct (0.61 ± 0.05 -fold induction). Moreover, the luciferase activity noticed for the smallest constructs was also significantly induced by gFOXL2 in oGC, indicating a possible role on the proximal part of promoter 2 (-40/+145) where two putative FOXL2 binding sites were located.

In order to verify that FOXL2 directly interacts with the *CYP19* promoter we have carried out a chromatin immunoprecipitation assay in a human granulosa cell line (KGN), by using the FOXL2 anti-C terminal antibody. A parallel immunoprecipitation was carried out with the pre-immune serum (negative control). Figure 4 shows that, on the one hand, markers like IMP3 and G3 PDH, which are not supposed to be targets of FOXL2, cannot be amplified from the DNA of the chromatin

Figure 4 Chromatin immunoprecipitation assay. Amplification of the *CYP19* promoter was positive only in the chromatin immunoprecipitated with the anti-FOXL2 antibody. No signal was detected with the pre-immune serum. We failed to amplify DNA fragments from *IMP3* and *G3 PDH* genes, not supposed to be targets of FOXL2, which excludes a potential non-specific chromatin adsorption. Human DNA stands for the positive control of PCR.

immunoprecipitated by the anti-FOXL2 antibody. This is also true for the precipitation in the presence of the pre-immune serum. This shows that non-specific chromatin adsorption was negligible in our conditions of immunoprecipitation and washing. On the other hand, strong PCR amplification was obtained with primers specific for the ovarian promoter of *CYP19*. These primers amplified a 260 bp fragment located just upstream of the distal TATA box. As expected, no amplification was obtained when the pre-immune serum was used in the precipitation. We also tested a pair of primers specific for the *CYP19* ORF and no amplification was obtained in any case, showing that the precipitated chromatin contained specifically the granulosa-specific promoter region of the gene. We repeated this assay three independent times and the results were always the same.

In conclusion, altogether these results indicate that FOXL2, by its co-localization with aromatase, its positive action on *CYP19* promoter and its direct interaction with the latter can act as a direct transcriptional regulator of the *CYP19* gene expression. However, (i) the existence of FOXL2 expressing cells, negative for *CYP19* expression, (ii) the observed decrease of *CYP19* expression at 60 dpc while *FOXL2* expression remains stable and, (iii) the fact that gFOXL2 functions preferentially in its normal cellular context, i.e. granulosa cells, are key indicators of the existence of FOXL2 co-factors necessary to fully control *CYP19* transcription during ovarian development.

Activin- β A is a good candidate for being a FOXL2 co-factor

Due to the increasing impact of numerous members of the *TGF β* (transforming growth factor) family on ovarian

Figure 5 RT-PCR expression studies of the activin/inhibin system, 3β HSD and both estrogen receptors during goat ovarian development. Note the peak of expression centred on 45 dpc for *inhibin- α* , *activin- β A* and *3 β HSD*, similar to those observed for *CYP19*. *ActRII*, activin receptor II.

differentiation and maintenance, and the fact that Smad factors have previously been described as FOXL2 collaborators on the GRAS element (Ellsworth *et al.* 2003), the expression profiles of different members of the activin/inhibin system have been determined during

goat ovarian development. By RT-PCR analyses, two subunits of the system, *inhibin- α* and *activin- β A* showed an expression profile very similar to that found for *CYP19*. They were expressed between 36 and 55 dpc with a peak at 45 dpc (Fig. 5). The third subunit, *activin- β B* and the *activin receptor II* are expressed at a high level from the first steps of ovarian differentiation (40 and 36 dpc respectively) until adulthood (Fig. 5). Follistatin has also been tested and was expressed only after birth (data not shown), in agreement with previous studies performed in humans (Martins da Silva *et al.* 2004).

On the other hand, to confirm a physiological role of *CYP19* in early ovarian development, expression of *3 β -HSD* and estrogen receptors has also been analyzed (Fig. 5). The presence of *3 β -HSD* transcripts between 36 and 55 dpc is consistent with active steroidogenesis. Finally, these female steroids can induce some effects via their receptors ER α and ER β , which are both expressed from the first steps of ovarian differentiation (40 and 36 dpc respectively) until adulthood (Fig. 5).

In order to gain further insights into the physiological role of these genes, immuno-histological studies have been performed on 45 dpc ovarian samples with different antibodies directed against *inhibin- α* , *activin- β A*, ER α , ER β and c-Kit, used as a germ cell marker. No signal was obtained for *inhibin- α* despite the fact that this antibody cross-reacts with the goat protein in fetal testes, confirming similar results observed in human and sheep (Braw-Tal *et al.* 1994, Martins da Silva *et al.* 2004). This absence of detectable *inhibin- α* protein in ovaries of different species, despite the presence of mRNA, could be a sign of the existence of a post-transcriptional regulatory process of this gene. No specific signal was observed for ER α . These negative results can be due to the absence of a cross-reaction of the antibodies with the goat protein or to a too small quantity of protein, attested by the low level of transcripts detected by RT-PCR (Fig. 5). In contrast, ER β can be detected and was expressed by all ovarian cellular types, somatic as well as germ cells (Fig. 6C). Interestingly, immuno-detection

Figure 6 Activin A (A), c-Kit (B) and ER β (C) immunolocalization in a 45 dpc goat ovary. Higher magnification (x4) of a positive cortical region (inside the rectangle) is presented in the upper right corner for each experiment. A line represents the thinness of the cortical region (co) where the majority of germ cells (gc) are located. Scale bar 200 μ m.

A **B**

Figure 7 Ovarian development in goat during the 20-day period preceding germ cell meiosis. (A) Upper pictures: transverse sections of goat ovaries at 36, 43, 50 and 56 dpc respectively from left to right (m stands for mesonephros). Lower pictures present a fivefold magnification of the region encompassed in the upper rectangles. Note the crucial increase in the gonad during this period, especially in the cortical area (co) where germ cells (gc) are localized. Scale bars in upper pictures, 100 μ m; in lower pictures, 20 μ m. (B) Schematic representation of the 'inverse follicle' organization of the goat fetal ovary.

of activin- β A revealed a germ cell-specific expression (Fig. 6A), which is attested by the same cortical staining as those obtained for c-Kit (Fig. 6B). Once again, this result fits well with those reported in humans, showing that activin A promotes germ cell survival and proliferation before follicle formation (Martins da Silva *et al.* 2004).

Discussion

In the present study, experimental evidence showed that the *CYP19* promoter 2 could represent a physiological target of FOXL2 during the early steps of goat ovary development. First, both proteins were found in the same cellular type located in the medulla of the 45 dpc

female gonads, around the *rete ovarii*. Secondly, a direct transcriptional enhancing effect of FOXL2 on the *CYP19* promoter 2 was reproducibly noticed *in vitro*, following co-transfection experiments in sheep primary granulosa cell cultures. Thirdly, a direct physical link between FOXL2 and the promoter of *CYP19* could be established by chromatin immunoprecipitation. Altogether, these results reinforced the hypothesis that one of the roles of *FOXL2* can be to enhance estrogen production during the early steps of ovarian differentiation in goats. This role has previously been envisaged by studying the ontogenesis of XX sex-reversal in *PIS*^{-/-} goat fetuses (Pailhoux *et al.* 2002, 2005). In this pathology, *FOXL2* expression remained undetectable from the early stages of ovarian differentiation (34 dpc) and *CYP19* expression has been found to be drastically decreased as the first consequence of downstream *FOXL2* suppression, before *SOX9* up-regulation and the occurrence of XX gonad sex-reversal.

FOXL2 activates CYP19 promoter 2 *in vitro* and directly interacts with its promoter

For the first time, our study provides clear evidence for FOXL2 being a potential transcriptional activator protein. Until now, two targets have been described for this recently characterized transcription factor, the *GnRHR* promoter via the GRAS composite regulatory element (Ellsworth *et al.* 2003), and the *StAR* promoter (Pisarska *et al.* 2004). This last study reported a repressive role of the native human FOXL2 protein on the *StAR* promoter containing numerous putative FOX-binding elements, a similar situation as those described here for the *CYP19* promoter 2. The FOXL2 repressive activity was demonstrated *in vitro* by co-transfection experiments performed in Chinese hamster ovary (CHO) cells. In this system, the authors found a ~0.5-fold repression of FOXL2 on different lengths of the *StAR* promoter constructs. The *StAR* promoter was also tested in our primary granulosa cell system and a ~fivefold repression was noticeable with the goat FOXL2 native protein, confirming the reliability of our cellular system (S Fabre, unpublished observations). On the other hand, an enhancing effect of FOXL2 on the *GnRHR* gene in the pituitary was supposed (Ellsworth *et al.* 2003), but this effect was demonstrated with the use of a chimeric Foxl2-VP16 protein. This same chimeric construct was also needed to obtain an activation of *CYP19* promoter 2 in COS7 cells. These results argued for FOXL2 being a weak transcriptional activator alone, thus requiring specific co-factors to induce a strong enhancing effect. This also seemed to be the case for *CYP19* promoter 2 according to (i) the spatio-temporal profiles of both genes during ovarian development and (ii) the discrepancy of FOXL2 action depending on the cellular system used for

transfection experiments (no effect in COS7 cells versus an enhancing effect in sheep granulosa cells).

Estrogens are decisive in early ovarian development across vertebrates

Estrogens play a crucial role in ovary differentiation in lower vertebrates, such as fishes, reptiles and birds. In these species, sex-reversal can be achieved by estrogen or anti-aromatase treatments leading to a steroidogenesis inversion. This steroid-responsiveness of gonad differentiation also exists in metatherian mammals, but has been lost in eutherian mammals, for which gonad development takes place in the uterus bathed in maternal hormones. In rainbow trout and chicken, *FOXL2* expression profiles correlated with those of *CYP19* during ovarian development, both in normal or sex-reversed situations (Baron *et al.* 2004, Govoroun *et al.* 2004, Hudson *et al.* 2005), but a direct regulatory role of FOXL2 on *CYP19* transcription remains to be demonstrated in such species. Such a mechanism, pointed out in this study for the goat species, could represent a remnant of an ancestral mechanism. If this is true, it implies that FOXL2 could be one of the main factors acting in the early stages of ovary differentiation in lower vertebrates.

In goat, the first estrogen secretion occurred between the first step of gonad differentiation (36 dpc) and germ cell meiosis (55 dpc). Interestingly, this ~20-day period in goat is restricted to a ~1-day period in mouse, between 12 and 13 dpc. During this period in the female mouse gonad, there is no active steroidogenesis, no *CYP19* expression and, intriguingly, no Foxl2 protein. Indeed, Foxl2 was first detected around 13 dpc, when germ cell meiosis began in this species (Loffler *et al.* 2003, Pannetier *et al.* 2003). These observations reinforce the hypothesis that the primary switch controlling gonad differentiation could be different among mammals, especially between mouse and other mammals including human and goat. This species-specific difference should be taken into consideration for interpreting the phenotype discrepancy observed between XX goat *PIS*^{-/-} and XX mouse *Foxl2*^{-/-}.

In addition to this species-specific difference on steroidogenesis, the architectural organization of the female gonad also evolves differently between mouse and goat. During the ~20-day period preceding germ cell meiosis in goat, the cortical part of the ovaries considerably increases in size and a spatial organization into syncytial nests of germ cells intermingled with somatic cells takes place at 55 dpc (Fig. 7A) (Pailhoux *et al.* 2002, Sawyer *et al.* 2002). Such an organization is not visible in the mouse ovary at the equivalent developmental stage (before 13.5 dpc) but occurs later, after 16.5 dpc (Appert *et al.* 1998). According to these observations and to published data on the proliferative effect of estrogens and activin A at later stages of ovary

development, one could envisage a similar role of these molecules on both somatic and germ cells during these early stages of ovarian development in the goat. Indeed, it has been shown that activin A promotes germ cell survival and proliferation before primordial follicle formation in the human (Martins da Silva *et al.* 2004). Furthermore, an estradiol-induced mitogenic effect on prepubertal granulosa cells has also been reported in mouse (Robker & Richards 1998, Dupont *et al.* 2000). Moreover, it has also been shown that activin A exerts a differential effect on steroid synthesis, by enhancing estradiol secretion and inhibiting progesterone production, in cultured goat or sheep granulosa cells (Shidaifat 2001, Shidaifat *et al.* 2001). Altogether, these observations and our results lead us to propose a model of early ovarian organization in goat.

Early ovarian organization: towards a model of 'inverse follicle'

At the beginning of their differentiation, before germ cells enter meiosis, the organization of goat ovaries evolves from an un-organized blastema to a regionalized gonad with a cortex and a medulla (Fig. 7A). Throughout this period, and by the use of molecular markers, our study demonstrates a centripetal organization of the goat ovaries. Indeed, β HSD is expressed by a cellular population occupying a small circle in the deeper medulla, near the *rete ovarii*. Cells expressing CYP19 are located within and around this first circle, and germ cells are located in the cortical region, just below the coelomic epithelium (Fig. 7B). Interestingly, this structure resembles that of an ovarian follicle except that the cell types are inversely located (centripetal versus centrifugal). Moreover, the same molecules seem to be used at these early stages for a cross-talk between somatic and germ cells. Indeed, one can postulate that activin A secreted by germ cells acts, in the first instance, as a proliferative factor on themselves via an autocrine pathway, and then as an estrogen-enhancer factor on cells located in the center of the gonad, via a paracrine action. In consequence, estrogens could reinforce the proliferative action on both germinal and somatic cells, both expressing ER β . Experiments are currently in progress in order to test the veracity of this model.

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