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Report

FOXL2 Is a Female Sex-Determining Gene in the Goat

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Summary

The origin of sex reversal in XX goats homozygous for the polled intersex syndrome (PIS) mutation was unclear because of the complexity of the mutation that affects the transcription of both *FOXL2* and several long noncoding RNAs (lncRNAs) [1, 2]. Accumulating evidence suggested that *FOXL2* could be the sole gene of the PIS locus responsible for XX sex reversal, the lncRNAs being involved in transcriptional regulation of *FOXL2* [2]. In this study, using zinc-finger nuclease-directed mutagenesis, we generated several fetuses, of which one XX individual bears biallelic mutations of *FOXL2*. Our analysis demonstrates that *FOXL2* loss of function dissociated from loss of lncRNA expression is sufficient to cause an XX female-to-male sex reversal in the goat model and, as in the mouse model, an agenesis of eyelids. Both developmental defects were reproduced in two newborn animals cloned from the XX *FOXL2*$^{-/-}$ fibroblasts. These results therefore identify *FOXL2* as a bona fide female sex-determining gene in the goat. They also highlight a stage-dependent role of *FOXL2* in the ovary, different between goats and mice, being important for fetal development in the former but for postnatal maintenance in the latter.

Results

In goats, the polled intersex syndrome (PIS) mutation associates an absence of horns in heterozygous and homozygous animals of both sexes (dominant trait) and a female-to-male sex reversal in biallelic affected XX animals (recessive and sex-limited trait) [1]. This syndrome arises from a complex regulatory deletion of 11.7 kb located ~300 kb upstream from the *FOXL2* gene and inducing (1) loss of *FOXL2* expression and (2) transcription arrest of three long noncoding RNAs (lncRNAs) named *PISRT1*, the "long transcripts" (also called *PISRT2*), and *PFOXic*; these lncRNAs are located around either the PIS region or *FOXL2* [1–3]. *FOXL2* transcription normally initiates between 34 and 36 days postcoitum (dpc) in a subpopulation of somatic cells located in the deepest medulla of the XX gonad [4, 5]. This developmental stage corresponds to the gonadic switch, i.e., the time point at which male sex determination is instructed by increase of *SOX9* expression in the XY gonad [4]. The biallelic PIS mutation yields an absence of *FOXL2* transcription from 34–36 dpc onward in XX developing gonads, associated with an upregulation of *SOX9* expression and differentiation of the somatic cells of the gonad as testicular cells, with a 4–5 day delay when compared to normal testis development [4]. These findings suggested that either *FOXL2* or the lncRNAs (or both) could act as female sex-determining genes. In mice, however, although *Foxl2* expression is also detected from the gonadic switch time point (i.e., 12 dpc) onward, as in goats, its biallelic loss of function does not induce sex reversal of somatic cells until after birth [6, 7]. This finding established *Foxl2* as necessary to maintain female identity of the somatic compartment of the gonad during postnatal life, but casted doubts on *Foxl2* acting as a female sex-determining gene at the gonadic-switch time period.

To clarify the involvement of *FOXL2* during ovarian differentiation in goats and to elucidate its role in PIS, we have generated *FOXL2* loss-of-function mutations in wild-type goats using a *FOXL2*-selective zinc-finger nuclease (ZFN) and optimized the protocol as detailed in the Experimental Procedures.

FOXL2 Ablation in Goats Leads to Female-To-Male Sex Reversal and Ablephary

This work reports the first gene targeting in goats using ZFN mRNAs microinjected into fertilized oocytes. All of the mutations obtained arise at the expected ZFN-cutting site, located 72 bp downstream of the ATG start codon and upstream of the region coding the forkhead DNA-binding domain. Four goat fetuses carrying *FOXL2* mutations were produced in the same experiment (Table S1 available online). They were recovered at 56 dpc, a stage corresponding to the onset of female germ cell meiosis, i.e., 20 days after the gonadic switch in goats [4]. Among them, one XY (#922) and two XX (#923 and #925) fetuses carrying a monoallelic deletion of *FOXL2* displayed no obvious, pathological phenotype. They were therefore considered as controls. Fetus #921 carried two *FOXL2*-mutated alleles, one with a 4 bp deletion and the other with a 54 bp deletion/+2 bp insertion (Figure S1). This fetus appeared with an absence of eyelids (Figure 1A) and with male external genitalia (Figure 1B), although PCR sexing revealed an XX genotype (Figure 1C). Importantly, no FOXL2 protein was detected in the gonad of fetus #921 (Figure 2A), confirming an efficient *FOXL2* loss of function. In agreement with a characteristic phenotype of female-to-male sex reversal, male-specific genes such as *SOX9* and *DMRT1* were detected in the gonad of fetus #921, in Sertoli-like cells that were organized in seminiferous cords highly similar to those observed in the control testis #922 (Figures 2B and 2C). In addition, Leydig cell differentiation was evident in the sex-reversed gonad #921, as attested by expression of CYP17 enzyme in the interstitial cells (Figure 2C), while CYP17 expression had stopped in the control ovary #925 (Figure 2C). It is worth noting that steroidogenesis has accordingly stopped in the normal ovary at this stage [5]. In contrast, testosterone

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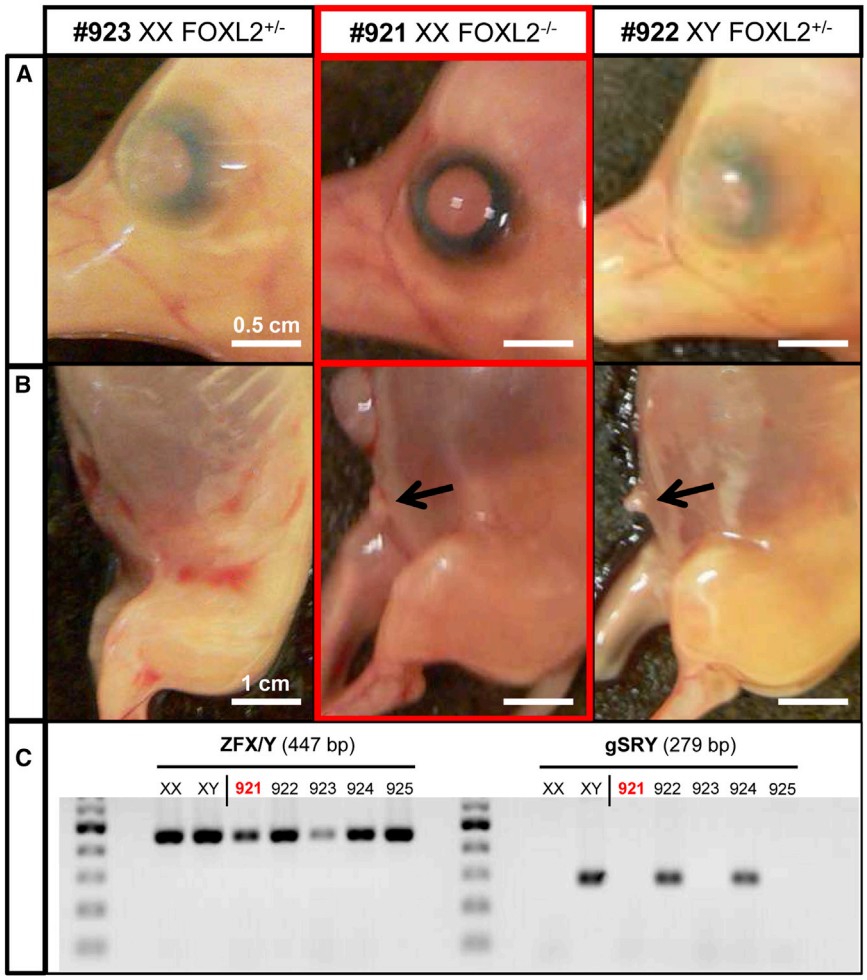

Figure 1. External Appearance of Goat Fetuses Recovered from Gene Targeting Experiments

(A) Absence of eyelids in an XX *FOXL2*$^{-/-}$ fetus (#921) but not in heterozygous male (#922) and female (#923) fetuses of the same litter.

(B) Appearance of the external genitalia in the three fetuses.

(C) PCR sexing demonstrating that *FOXL2*$^{-/-}$ fetus #921 has an XX genotype.

See also Figures S1 and S2 and Table S1.

production in fetus #921 was functionally attested by the complete masculinization of its external genitalia (i.e., fully differentiated scrotum and urogenital orifice under the umbilicus; Figure 1B), which occurs between 40 and 50 dpc in normal XY goat fetuses [4].

To rule out ZFN-induced mosaicism in fetus #921 and to analyze later stages of gonadic development in the absence of *FOXL2*, we expanded skin fibroblasts from this founder in vitro and used them for goat-cloning experiments [8]. Upon nuclear transfers into enucleated oocytes (n = 44), followed by embryo transfers (n = 16) into synchronized recipient mothers (n = 9), two XX goats (#03036 and #03037) were recovered by cesarean section at 150 dpc, the developmental stage corresponding to birth. Both of them displayed the same absence of eyelids and female-to-male sex reversal as did fetus #921 (Figure S2), demonstrating that it had a nonmosaic composition. Interestingly enough, both #03036 and #03037 exhibited a male reproductive system, as in *PIS*$^{-/-}$ XX goats [9], with testis-like gonads descended into the scrotum and fully differentiated epididymes and vas deferens, but no residual Müllerian duct derivatives. However, the location of their urogenital orifices slightly differs from that of each other and from that of fetus #921. It was near the scrotum in #03037, between the scrotum and the umbilicus in #03036, and just under the umbilicus (i.e., in a "normal" male position) in fetus #921 (Figures 1B and S2). Assuming that FOXL2 deletion was the sole genetic alteration in all three fetuses, this suggests that androgen levels were likely at the limit of the masculinizing threshold in *FOXL2*$^{-/-}$ mutants during the time period when the urogenital bud is sensitive to hormone signaling (i.e., from 40 to 50 dpc), possibly because of the 4- to 5-day-long delay for testis differentiation in the mutant situation [4].

The *FOXL2*$^{-/-}$ XX Gonads Display a Testis-like Gene Expression Profile

As expected in a situation of sex reversal, expression of female-specific somatic genes (i.e., *RSPO1*, *RSPO2*, *CYP19*, and *FST*) was downregulated in the XX *FOXL2*$^{-/-}$ gonad at 56 dpc (Figure 3A), and, conversely, expression of male-specific somatic genes (i.e., *SOX9*, *AMH*, and *CYP17*) was upregulated (Figure 3B), as in the *PIS*$^{-/-}$ gonad. Moreover, germ cell fate was also sex reversed in XX *FOXL2*$^{-/-}$ #921 gonads, as previously described in XX *PIS*$^{-/-}$ testes at 56 dpc [4]. Indeed, meiosis initiation (attested by expression of *STRA8* [10]) was restricted to control female ovaries (i.e., *FOXL2*$^{+/-}$ or *FOXL2*$^{+/+}$) (Figure 3C). Interestingly enough, the strong expression of *VASA* in control female gonads compared to the others suggested a higher number of germ cells in the ovaries than in the testes and the XX sex-reversed gonads (*FOXL2*$^{-/-}$ and *PIS*$^{-/-}$) at this stage (Figure 3C). In keeping with this possibility, VASA-positive cells were much more abundant on tissue sections from *FOXL2*$^{+/-}$ ovaries than from *FOXL2*$^{+/-}$ testes and *FOXL2*$^{-/-}$ sex-reversed gonads (Figure S3). This indicates that the female-to-male sex reversal in XX *FOXL2*$^{-/-}$ gonads occurred prior to the period of germ cell proliferation in a normal ovary, which takes place in goats between 36 and 56 dpc [4, 5].

Expression of PIS-Regulated Genes Persists in *FOXL2*$^{-/-}$ XX Gonads

In addition to shutting down *FOXL2* gene expression in the female gonad, the PIS mutation leads to a transcriptional silencing of three lncRNAs [1–3]. Among these three lncRNAs, *PFOXic* arises from the bidirectional *FOXL2* promoter (Figure 3D) and has been proposed to regulate the level of FOXL2 protein by a mechanism of "transcriptional overflow" [3]. *PFOXic* is a polyadenylated lncRNA showing an expression level comparable to that of *FOXL2* [3]. The

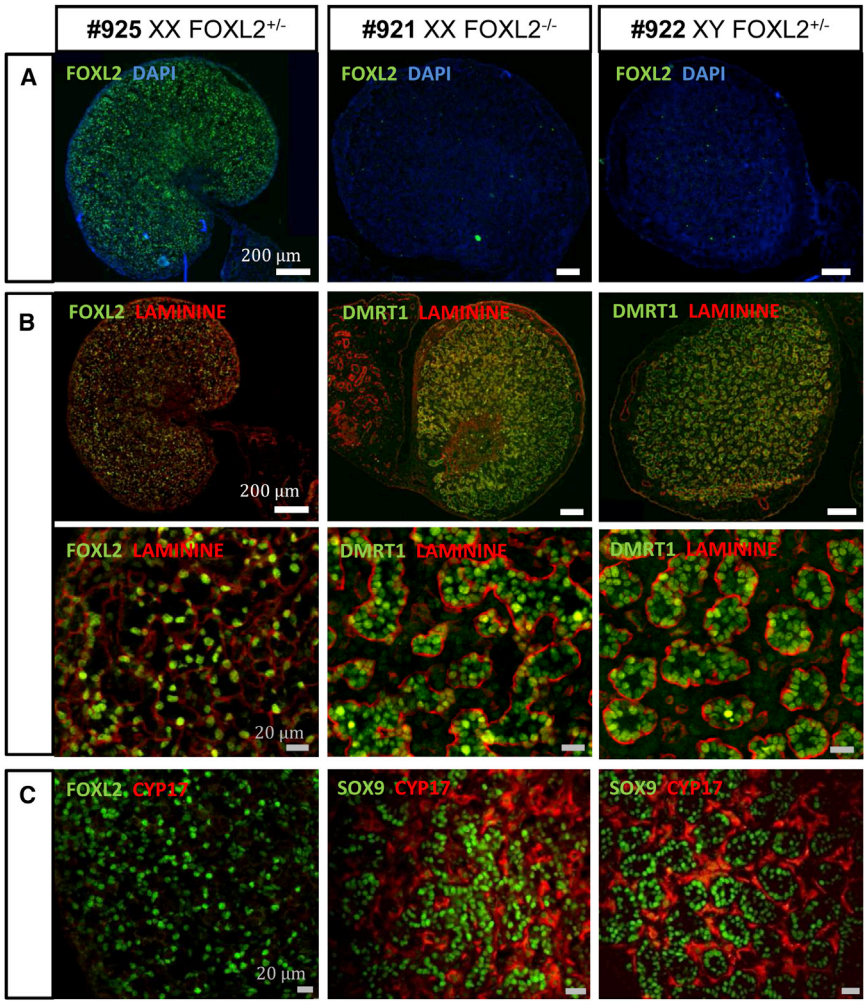

Figure 2. *FOXL2* Knockout Yields a Gonad with Testicular Characteristic Features

(A) FOXL2 is detected in the control female gonad (#925, green signal), but neither in the male control gonad (#922) nor in the XX *FOXL2*$^{-/-}$ gonad (#921), as expected. Nuclei are counterstained with DAPI (blue signal).

(B and C) the XX *FOXL2*$^{-/-}$ mutant gonad (#921) is clearly organized as the control testis (#922). It is surrounded by the tunica albuginae (B, top panels). Well-organized seminiferous cords contain SOX9 and DMRT1-positive Sertoli cells (green signals) and are surrounded by a basal lamina that is positive for laminin (red signal; B, bottom panel, and C). In addition, CYP17-positive cells (red signal) were detected in the interstitial tissue (C).

Scale bars represent the indicated measurements. See also Figure S3.

other *PISRT1* and *PISRT2* lncRNAs are transcribed from a highly conserved promoter region located ~300 kb upstream of *FOXL2* (Figure 3D). These lncRNAs are transcribed at a very low level when compared to *FOXL2/PFOXic*, but their expression is always positively correlated to that of *FOXL2/PFOXic* [1, 2, 11]. They are thus considered to be enhancer lncRNAs, acting in *cis* on *FOXL2/PFOXic* transcription [2, 8, 12, 13].

To test whether sex reversal in fetus #921 could be due to deregulation of one of these lncRNAs, we measured the expression of all four PIS-regulated genes at 56 dpc in *FOXL2* mutant and controls gonads (Figure 3D). Their expression was also assessed in XX *PIS*$^{-/-}$ gonads for comparison purpose. Independently of *FOXL2* genotype (i.e., *FOXL2*$^{+/+}$, *FOXL2*$^{+/-}$, or *FOXL2*$^{-/-}$), *PISRT1* RNA levels were identical in the XX gonads, while *PISRT2* and *PFOXic* RNA levels were ~50% lower in *FOXL2*$^{-/-}$ than in *FOXL2*$^{+/+}$ and *FOXL2*$^{+/-}$ XX gonads (Figure 3D). These results show that PIS-regulated gene transcription is not shut down in XX *FOXL2*$^{-/-}$ gonads as it is in the context of the PIS mutation [1, 2]. In other words, the PIS locus remained transcriptionally active in *FOXL2*$^{-/-}$ gonads, and sex reversal occurred independently of the loss of lncRNAs. Thus, it is unlikely that altered levels of *PISRT1*, *PISRT2*, and *PFOXic* are instrumental to sex reversal in XX *FOXL2*$^{-/-}$ mutants.

Discussion

FOXL2 Is a Critical Gene for Eyelid Development in Mammals

PIS$^{-/-}$ goats harbor normal eyelids. Indeed, the PIS mutation doesn't affect *FOXL2* expression in developing eyelids [1]. By contrast, we show here that *FOXL2* loss of function in goats leads to absence of eyelids (ablephary), a pathological phenotype never described until now in this species, but already observed in mice knocked out for *Foxl2* [6, 7]. In addition, *FOXL2* haploinsufficiency in humans is responsible for blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES; MIM 110100) [14]. Thus, either total or partial loss-of-function mutations of *FOXL2* are responsible for agenesis or malformation of eyelids in three distinct orders of mammals (namely, primates, rodents, and artiodactyls). As a result, *FOXL2* can be considered as a crucial actor of eyelid development in mammals.

FOXL2 Is a Female Sex-Determining Gene in the Goat

We show that *FOXL2* loss of function dissociated from loss of lncRNA expression in goat ovaries leads to an early sex-reversal phenotype, identical to the one previously described in goats carrying the PIS mutation [1, 2]. This observation demonstrates that the loss of *FOXL2* gene product is responsible for the female-to-male sex reversal in *PIS*$^{-/-}$ goats. We therefore identify *FOXL2* as a genuine female sex-determining gene in the goat.

Our findings therefore imply that the function of *FOXL2* in sex and ovarian differentiation occurs at distinct stages in goats and mice, respectively. Even though *Foxl2* is expressed as early as 12.0 dpc in the mouse (i.e., at the time of the so-called gonadic switch), its loss of function does not yield female-to-male sex reversal [6, 7, 15]. However, *Foxl2* ablation after birth (i.e., at the time of follicle formation) yields the transdifferentiation of two ovarian somatic cell lineages, namely granulosa and theca cells into Sertoli and Leydig cells, respectively [16]. This somatic cell sex reversal is not linked to the germ cell fate, but occurs because FOXL2 is no longer present

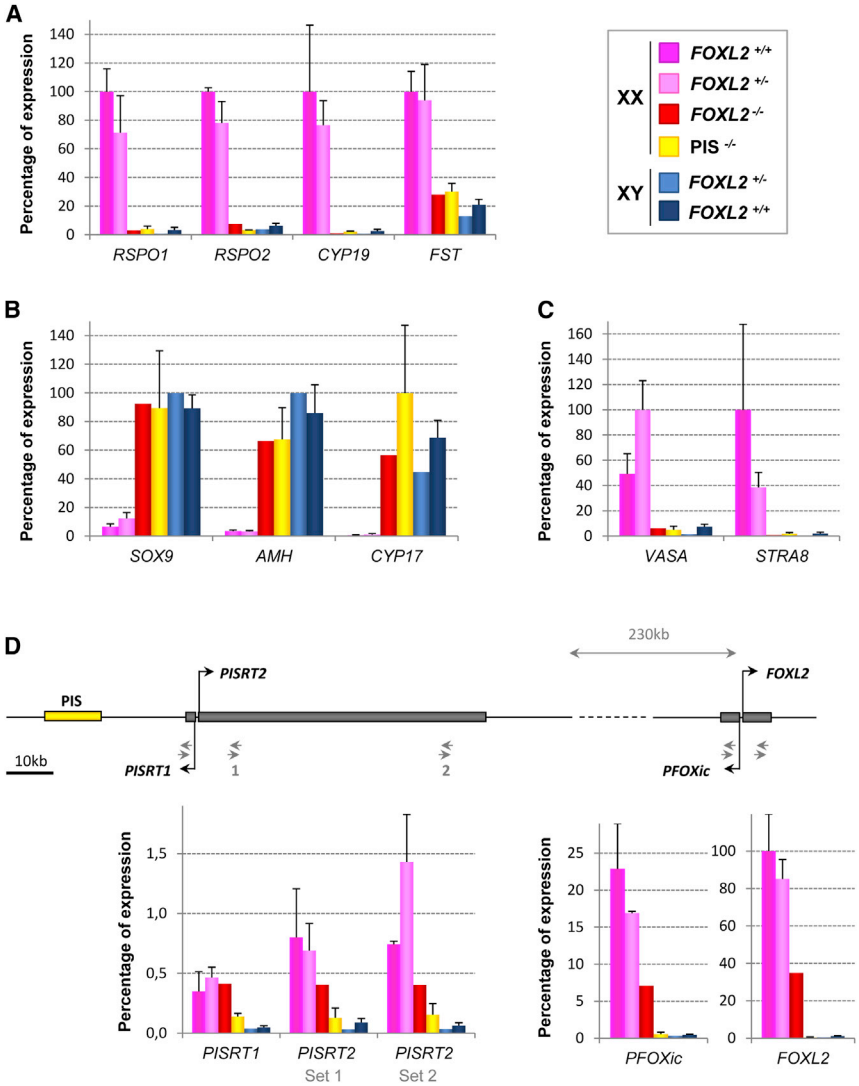

Figure 3. *FOXL2* Knockout Yields Gonadic Down-regulation and Upregulation of Female-Specific and Male Specific Genes, Respectively, without Altering PIS-Regulated lncRNA Expression

(A) Expression of somatic female-specific genes that are all decreased in the XX *FOXL2*$^{-/-}$ gonad (red bars) to levels similar to those observed in the control *PIS*$^{-/-}$ (yellow bars) and male (blue bars) gonads. Note that *RSPO2*, *CYP19*, and *FST* are FOXL2 target genes.

(B) Expression of somatic male-specific genes that are all increased in the XX *FOXL2*$^{-/-}$ gonad (red bars) to levels similar to those observed in the control *PIS*$^{-/-}$ (yellow bars) and male (blue bars) gonads. Note that *SOX9* and *AMH* are Sertoli cell-specific markers, while *CYP17* is a Leydig cell-specific marker.

(C) Expression of germ cell-specific (*VASA*) and meiosis-specific (*STRA8*) genes, which are all robustly expressed in control ovaries (pink bars) and barely detectable in the XX *FOXL2*$^{-/-}$ gonad (red bars), the control *PIS*$^{-/-}$ gonad (yellow bars), and the testis (blue bars). This attests a higher number of germ cells in ovaries than in testes and illustrates the meiotic initiation restricted to the ovaries.

(D) Top: schematic representation of the PIS locus. Broken arrows indicate transcription start sites; gray boxes stand for the locations of *PISRT1*, *PISRT2*, and *PFOXic* lncRNAs and for the *FOXL2* gene, as indicated; the yellow box indicates the sequence altered in the PIS mutation; and small arrow pairs indicate the locations of primer sets used for PCR analysis. Bottom: expression of all four PIS-regulated transcripts, which are all maintained at a substantial level in the XX *FOXL2*$^{-/-}$ gonad (red bars) as in control female gonads (pink bars), while they were almost undetectable in the testes (blue bars).

Errors bars represent the mean ± SEM of triplicates.

to synergize with the estrogen receptor alpha (ESR1) to repress *Sox9* expression after birth [16, 17]. Thus, FOXL2, in conjunction with ESR1, is essential to maintain female identity of the somatic compartment in the postnatal gonad [16].

To our point of view, the difference in estrogen production, for which FOXL2 seems to play a critical function, represents the most probable explanation for the distinct outcomes of *FOXL2*/*Foxl2* loss of functions in goats and mice at the time of gonadic switch. Indeed, FOXL2 enhances aromatase expression in goats, as in humans and in many nonmammalian species through its direct action on *CYP19A1* ovarian-specific promoter [5, 18–22], thereby favoring synthesis of estrogens by the differentiating ovary, long before germ cell meiotic entry [23, 24]. Accordingly, sheep [25, 26], rabbit [27], and human [28] XX gonads are able to synthesize estrogens at the time of the gonadic switch. In contrast, estrogens are not produced at the early stages of ovarian differentiation in the mouse [29], and, in keeping with this, neither *Esr1;Esr2*- nor *Cyp19a1*-null mutants display sex reversal at birth [30, 31]. Therefore, the fact that *Foxl2* has lost its feminizing action during fetal development in the mouse is most likely related to the fact that estrogen signaling is dispensable to female sex determination during the fetal period in this species.

The regulatory mechanism involving estrogens for sex determination exists also in nonmammalian vertebrates like fishes, amphibians, reptiles, and birds [32], making the mouse rather an exception. It is therefore tempting to speculate that *FOXL2* is actually, in humans as in goats, a female sex-determining gene whose functioning in the mouse has been restricted to the maintenance of the female identity of somatic cells in the postnatal ovary [16]. However, any female-to-male sex reversal in humans due to *FOXL2* mutations should be associated with an agenesis of the eyelids, a situation that has never been described to our knowledge. It is, however, possible that some cases result from complex regulatory mutations, leading to an ovarian-specific *FOXL2* loss of function as the PIS mutation does in the goat.

Experimental Procedures

Research involving goat experimentations conformed to the principles for the use and care of animals in agricultural research and teaching, in compliance with French and European regulations on animal welfare. Goat eggs have been produced in vivo. They have been injected with two mRNAs encoding each monomer of a specific ZFN engineered by Sigma or with a tricistronic mRNA encoding EGFP-ZFN1-ZFN2 and engineered by ourselves. In the tenth experiment, 23 EGFP-positive embryos were transferred

into eight recipient goats, from which five fetuses were recovered at 56 days of gestation (fetus #921, #922, and #923 on recipient number 09072; fetus #924 and #925 on recipient number 09025). Day 0 corresponded to the day of natural mounting by male goats. PCR sexing, quantitative RT-PCR, and IHC analyses were performed as previously described [4, 8]. Detailed experimental procedures are given in the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.12.039.

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