

Somatic Sex Reprogramming of Adult Ovaries to Testes by FOXL2 Ablation

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SUMMARY

In mammals, the transcription factor SRY, encoded by the Y chromosome, is normally responsible for triggering the indifferent gonads to develop as testes rather than ovaries. However, testis differentiation can occur in its absence. Here we demonstrate in the mouse that a single factor, the forkhead transcriptional regulator FOXL2, is required to prevent transdifferentiation of an adult ovary to a testis. Inducible deletion of *Foxl2* in adult ovarian follicles leads to immediate upregulation of testis-specific genes including the critical SRY target gene *Sox9*. Concordantly, reprogramming of granulosa and theca cell lineages into Sertoli-like and Leydig-like cell lineages occurs with testosterone levels comparable to those of normal XY male littermates. Our results show that maintenance of the ovarian phenotype is an active process throughout life. They might also have important medical implications for the understanding and treatment of some disorders of sexual development in children and premature menopause in women.

For a video summary of this article, see the PaperFlick file with the Supplemental Data available online.

INTRODUCTION

Sex determination in vertebrate species exhibits a broad variety of mechanisms based either on genotype or environmental factors (Barske and Capel, 2008; Guiguen et al., 2009). In almost all mammals the heterogametic sex is male, propagated by the inheritance of a Y chromosome. The discovery of a single gene, *Sry*, on the Y chromosome and its subsequent functional analysis has demonstrated that SRY is necessary and sufficient to initiate testicular development (Koopman et al., 1991; Sinclair

et al., 1990). SRY stands at the top of a genetic cascade that directs the differentiation of the bipotential gonad toward a testis fate through activation of its direct target gene *Sox9* (Dinapoli and Capel, 2008; Sekido and Lovell-Badge, 2009). When misexpressed in XX mice or humans, SOX9, which belongs to the same family of HMG-box transcription factors as SRY but is encoded by an autosomal gene, is also able to induce testis formation (Bishop et al., 2000; Vidal et al., 2001). In the absence of SRY or SOX9 function the bipotential gonad develops as an ovary (Barrionuevo et al., 2006; Chaboissier et al., 2004). Subsequent to gonadal differentiation, the different types and levels of hormones produced by the testes and ovaries dictate the differentiation of most secondary sexual characteristics (Wilhelm and Koopman, 2006), others being dependent on the direct action of Y- and X-linked genes (Arnold, 2009).

XX male sex reversal could result from gain of function mutations (GOF) in genes that promote testis development or loss of function mutations (LOF) in genes that oppose them or actively promote ovary development. In humans, most XX males have a functional SRY gene due to abnormal X-Y interchange during male meiosis, however some rare cases lack SRY (Pannetier et al., 2004). Duplications affecting SOX9 can be responsible (Huang et al., 1999), a situation that reflects experimental manipulation of mice where complete XX sex reversal can be achieved by ectopic expression in the developing XX gonad of SRY or SOX9, or of other SOX proteins that mimic these (Bishop et al., 2000; Koopman et al., 1991; Vidal et al., 2001). XX gonads can also show testicular development in culture when treated with FGF9 or prostaglandin D2, both of which are involved in positive autoregulatory loops required to maintain high levels of SOX9 expression or activity (Kim et al., 2006; Moniot et al., 2009; Wilhelm et al., 2007). The common denominator in all these cases of primary XX male sex reversal is the activation of SOX9 in the indifferent gonad. In the mouse, SRY has to function within a narrow time window to upregulate *Sox9* otherwise the gene is repressed and ovaries develop (Hiramatsu et al., 2009). Candidates for genes that oppose the male pathway include *Nr0b1* (also called *Dax1*), *Wnt4*, *Rspo1*, and *Foxl2*.

DAX1 is thought to recruit corepressors when it is complexed with steroidogenic factor 1 (SF1 or Nr5a1). The latter is a member of the nuclear receptor superfamily required for gonadal development in both sexes, but with an especially important role in testis development as it contributes to the activation of male pathway genes, including *Sry* and *Sox9* (Sekido and Lovell-Badge, 2008). Over-expression of DAX1 can lead to XY female development, however, LOF mutations have not revealed a role in ovaries (Bouma et al., 2005; Swain et al., 1998). WNT4 and RSPO1 both serve to stabilize β -catenin, and ectopic expression of a stable form of β -catenin in XY gonads can lead to ovary development (Maatouk et al., 2008). However, LOF mutations of *Wnt4* and *Rspo1* in the mouse lead to only partial XX male sex reversal, suggesting that additional factors still ensure that SOX9 activity is repressed (Chassot et al., 2008a; Jeays-Ward et al., 2003; Vainio et al., 1999). Cases of human XX male development have been attributed to homozygous null mutations in *RSPO1* (Parma et al., 2006), or with LOF mutations in *WNT4* associated with SERKAL syndrome (Mandel et al., 2008), which might suggest that human sex determination is more sensitive than mouse to levels of β -catenin. However, care has to be taken in interpreting gonadal phenotypes after birth by which time ovotestes may have resolved into testes, or other secondary changes may have occurred. In *Wnt4* mutant XX gonads, Sertoli-like cells begin to appear at late fetal stages and Leydig-like cells increase in number (Vainio et al., 1999). This was associated with dysgenesis and loss of oocytes, and was thought to reflect other circumstances where Sertoli and Leydig-like cells appear in an XX gonad; for example in Freemartins, or when fetal ovaries are grafted to the kidney capsule, in adult XX mice carrying mutations in both estrogen receptors or in the aromatase gene (*Cyp19a1*), or in Sertoli/Leydig tumors in postmenopausal women (Couse et al., 1999; Guiguen et al., 2009; Sekido and Lovell-Badge, 2009). However, in none of these cases has it been shown whether the appearance of Sertoli- and Leydig-like cells in an ovary could be due to transdifferentiation from granulosa and theca cells as suggested by Burgoyne et al. (1988), or differentiation from populations of immature precursor cells.

FOXL2 mutations in the human germline lead to the autosomal dominant blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) associated with premature ovarian failure in females (Crisponi et al., 2001). Furthermore, deregulated *FoxL2* expression has been described in the goat polled intersex syndrome (PIS) where XX male sex reversal occurs (Pailhoux et al., 2001). Similarly, global deletion of *Foxl2* (*Foxl2*^{-/-}) during development in mice leads to ovary dysgenesis and infertility (Schmidt et al., 2004; Uda et al., 2004). Although the *Foxl2*^{-/-} mutant phenotype becomes apparent only late in fetal development with derepression of some testis specific genes, it could reflect the much earlier loss of *Foxl2* expression, which is ovary-specific from E12.5 (Ottolenghi et al., 2005; Schmidt et al., 2004).

Understanding the plasticity of an adult ovary at the cellular level and the extent to which “terminally” differentiated gonadal cell types can switch to a new fate would provide molecular insight into not just the maintenance of the differentiated state in the mammalian gonad, but perhaps also well known postnatal sex conversion phenomena in lower vertebrates.

Here, we show that upon conditional loss of *Foxl2* in the adult ovary the two major female-specific somatic cell lineages switch their cell fate revealing a rare example of true adult lineage reprogramming in vivo. While granulosa cells, which support oocytes, are reprogrammed into testis-specific Sertoli-like cells, the steroidogenic theca cell lineage upregulates *17-beta hydroxysteroid dehydrogenase type 3* (*Hsd17b3*), the rate-limiting enzyme in testosterone biosynthesis resulting in male-like levels of testosterone in the blood. Using a combination of genetically based oocyte-ablation experiments we demonstrate that this reprogramming is cell autonomous and oocyte-independent. We show that FOXL2 represses the testis differentiation program mainly through repression of *Sox9*-regulatory sequences that are required for its testis-specific expression. We extend our analysis by demonstrating that FOXL2 and estrogen receptor (ESR1) cooperate in *Sox9* repression in vivo, consequently providing a mechanism by which loss of estrogen signaling can lead to gonadal sex reversal.

RESULTS

Somatic Ovary-to-Testis Transdifferentiation upon Conditional Deletion of *Foxl2*

To determine FOXL2 function during folliculogenesis in sexually mature female mice we created a conditional allele, flanking the single *Foxl2* coding exon with loxP sites. Animals homozygous for this are referred to as *Foxl2*^{ff} (Figure S1). We also took advantage of a *R26CreERT2* line that allows ubiquitous induction of Cre recombinase activity upon tamoxifen (TM) administration. This gave recombination efficiencies greater than 95% in adult ovaries when crossed to an *R26lacZ* reporter strain (data not shown). For conditional deletion of *Foxl2*, 8-week old *Foxl2*^{ff} or *R26CreERT2;Foxl2*^{ff} female mice were treated for 5 days with TM. Histological examination 3 weeks later unexpectedly revealed gonadal sex reversal in XX *R26CreERT2;Foxl2*^{ff} (“conditional mutant”) mice (Figure 1A). Structural changes affected nearly the whole gonad, where the typical follicular structure of the ovary now resembled in appearance the seminiferous tubules of the testis. Some degenerating oocytes were still present whereas in most tubule-like structures germ cells appeared to be lost. Instead of granulosa cells, cells with all the morphological characteristics of testicular Sertoli cells were present. These displayed a prominent basal lamina, tripartite nucleoli and numerous veil-like cytoplasmic extensions pointing toward the lumen (Figure 1A). To obtain a global view of the extent of lineage reprogramming, we performed genome-wide gene expression analysis of wild-type ovaries, reprogrammed sex-reversed ovaries and testes. This revealed a large gene set upregulated and expressed in common between reprogrammed sex-reversed gonads and testes, including well-characterized Sertoli and Leydig cell markers. This substantiated the morphologically observed cell type switch at the molecular level (Figures 1B–1D and Table S1 available online). *Foxl2* mRNA was no longer detectable in conditional mutant XX gonads (Figure S2). Consistent with the mRNA profiling, in situ hybridization studies demonstrated robust expression of Sertoli cell markers and genes involved in testis determination

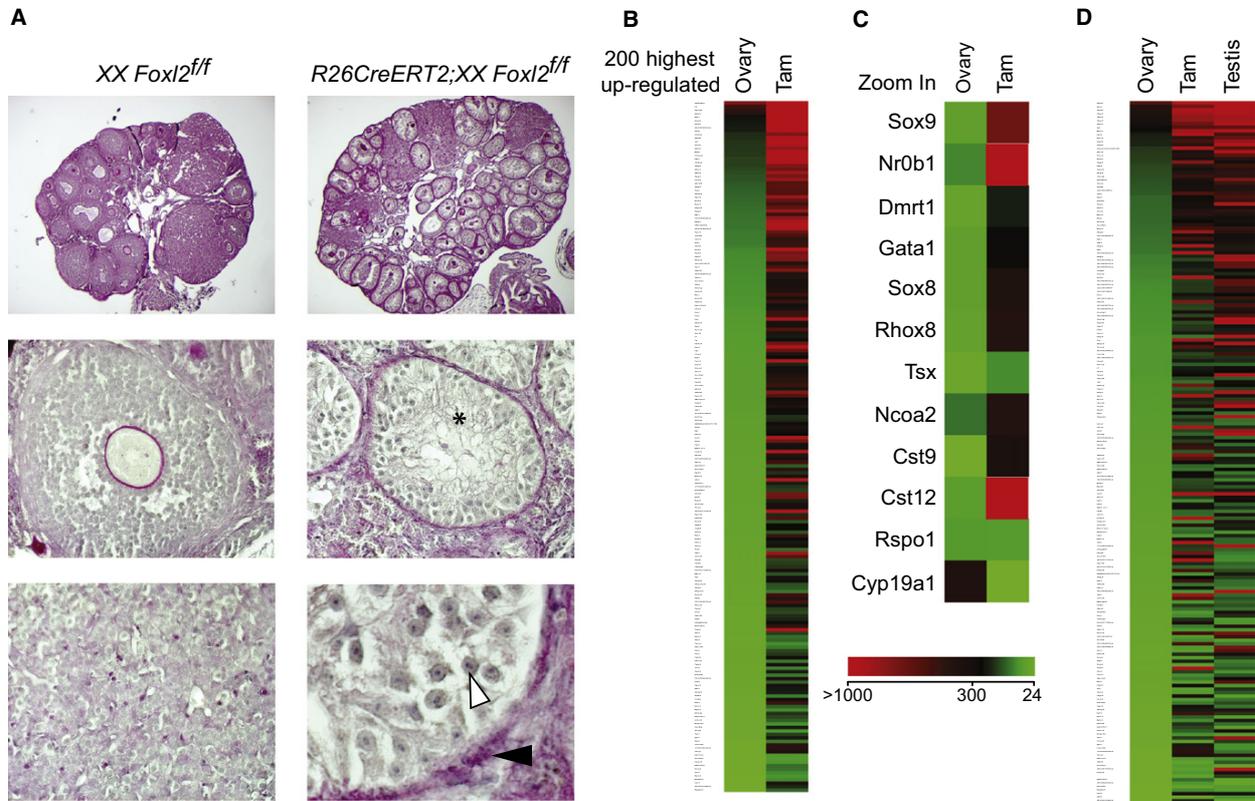


Figure 1. Postnatal Gonadal Sex Reversal in XX *Foxl2* Conditional Mutant Mice

(A) PAS staining showing morphology of XX *Foxl2^{ff}* (control) and XX *R26CreERT2;Foxl2^{ff}* mutant gonads three weeks after TM administration. Left panel: wild-type, right panel: mutant at different magnifications. Note cytoplasmic extensions (asterisk), tripartite nucleoli (white arrow) and prominent basal laminae (black arrow) in mutant structures, which are features of testicular Sertoli cells.

(B–D) Genome wide expression profiling of XX *Foxl2^{ff}* (control) and XX *R26CreERT2;Foxl2^{ff}* mutant gonads three weeks after TM administration. (B) Heat map of the 200 genes that have the highest fold change out of the 915 genes that are upregulated at least 1.8-fold in XX *R26CreERT2;Foxl2^{ff}* mutant gonads after TM administration (Colors red and green representing absolute expression levels). (C) Zoom in on a set of particular important genes selected out of the 915 up-regulated ones. (D) Heat map showing the 200 genes that are at least 1.8-fold upregulated in both XX *R26CreERT2;Foxl2^{ff}* mutant gonads and testis compared to ovaries (for gene names, see Table S1).

including *Gata1*, *Tif2*, *Dax1*, *Dhh*, *Dmrt1*, and notably *Sox9* (Figure S2).

Although *Foxl2^{ff}* control ovaries were normal after TM administration, the phenotype seen in the conditional *Foxl2* deletion experiment could result from a cooperative effect between *Foxl2* loss and a context-dependent antagonistic estrogen activity of TM. To rule out this possibility we used a noninducible Cre-line, in which regulatory sequences from the α -glycoprotein subunit (*aGSU*) gene drive the expression of *Cre* in the ovary only after sexual maturation. *aGSU;Foxl2^{ff}* mice have the same gonadal phenotype (Figure S3), directly demonstrating that deletion of *Foxl2* alone is sufficient to cause postnatal sex reversal in the XX gonad.

Cell Autonomous Reprogramming of Granulosa into Sertoli-Like Cells

The short time frame of 3 weeks between TM administration and appearance of the somatic sex-reversed phenotype suggested transdifferentiation of granulosa into Sertoli-like cells, but to

rule out any developmental and/or hormonal phenomena we examined when the molecular changes first occurred. Whereas 2 days after the start of TM administration FOXL2 was still detected by immunohistochemistry, a day later FOXL2 was absent from XX *R26CreERT2;Foxl2^{ff}* but not control gonads (Figure 2A and data not shown). In contrast SOX9 immunostaining first appeared 4 days after the start of TM administration in the former. Thus for a period of about 1 day neither FOXL2 nor SOX9 was detectable by immunohistochemistry suggesting that high expression levels of both factors are mutually exclusive. SOX9 expression started randomly within follicles where it was often first detected in mural but not in cumulus granulosa cells and thus spatially divided from the oocyte (Figure 2A). One week after the start of TM administration SOX9 was expressed in all cells in a typical granulosa cell location, but many had already started to transdifferentiate into Sertoli-like cells despite the presence of an oocyte (Figure 2B). These results strongly suggest that granulosa cells undergo cell autonomous reprogramming into Sertoli-like cells upon loss of *Foxl2*. In contrast

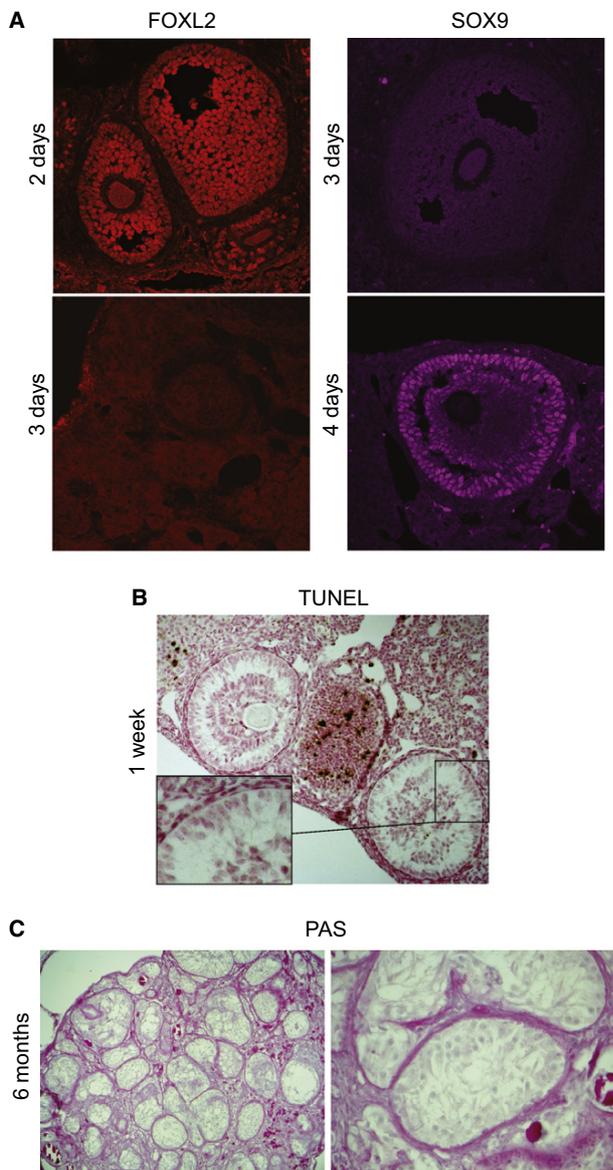


Figure 2. Time-Course Analysis of XX *R26CreERT2;Foxl2^{fl/fl}* Gonads after Tamoxifen Administration

(A) FOXL2 and SOX9 immunostaining on ovarian sections of XX *R26CreERT2;Foxl2^{fl/fl}* gonads 2, 3, and 4 days after start of TM administration. In half of the follicles SOX9 expression is first seen in mural but not cumulus granulosa cells and thus spatially divided from the oocyte. (B) TUNEL staining on conditional mutant XX gonads one week after TM treatment, showing apoptotic nuclei (brown) in follicles which did not show signs of transdifferentiation, but not follicles that have started to transdifferentiate into Sertoli-like cells as evident by the appearance of the characteristic veil-like cytoplasmic extensions of Sertoli cells (see inset). (C) PAS staining of XX *R26CreERT2;Foxl2^{fl/fl}* gonads 6 months after a one-week TM administration showing gross morphology. Note that Sertoli-like cells are still present in the mutant XX gonads.

to granulosa cells which undergo programmed cell death (apoptosis) at the end of a follicle life cycle, major apoptosis is normally not seen in Sertoli cells. Consistent with this, the Sertoli-like cells in XX *conditional mutant* gonads do not undergo apoptosis and were still present after 6 months (Figures 2B and 2C). Together these findings further support the notion that true lineage reprogramming has occurred, resulting in a stable cell type conversion.

Transdifferentiation of Granulosa Cells Is Independent of Oocyte Function

It has been proposed on the basis of indirect evidence that oocytes are required to maintain granulosa cell fate, and that in their absence the latter can transdifferentiate into Sertoli-like cells (Burgoyne et al., 1988; Guigon and Magre, 2006; McLaren, 1991). We addressed this possibility by taking advantage of a mouse strain that expresses *Cre* under the control of *Gdf9* regulatory sequences exclusively in oocytes of postnatal ovaries. First, we generated *Gdf9Cre;Foxl2^{fl/fl}* mice which were fully fertile demonstrating that FOXL2 does not play a role in oogenesis (data not shown). Next we crossed *Gdf9Cre* mice with a *R26DTA* mouse line in which Diphtheria toxin expression is activated upon *Cre* expression resulting in cell ablation. *Gdf9Cre;R26DTA* mice were viable and analysis of ovaries from 8-week old females demonstrated a complete absence of oocytes (Figure 3A), yet FOXL2 was still expressed and not SOX9 (Figure 3B). To substantiate this finding further, we compared a set of informative genes by quantitative RT-PCR in 8-week-old wild-type, *Gdf9Cre;R26DTA* (oocyte-depleted) and *conditional mutant* ovaries. Unlike the latter, the oocyte-depleted ovaries showed no significant deregulation of *Sox9*, *Foxl2*, *Dmrt1*, *HSD17b3*, *testatin (Cst9)* or *17-beta hydroxysteroid dehydrogenase type 1 (Hsd17b1)*, which converts estrone into estradiol (Figure 3C and data not shown). Finally, we ablated oocytes in 8-week old sexually mature female mice in case this had different consequences from deleting oocytes during sexual maturation. In mice, only cells that express the human Diphtheria toxin receptor (DTR) are susceptible to cell ablation upon Diphtheria toxin (DT) administration. We therefore generated *Gdf9Cre;R26iDTR* animals and compared their ovaries with those of control *R26iDTR* mice 9 days after DT administration. This is when follicles activated at the time of DT administration should have reached an antral stage (Hoage and Cameron, 1976) and by when 'granulosa' cells in XX *R26CreERT2;Foxl2^{fl/fl}* gonads express SOX9 after TM administration. As expected, in control *R26iDTR* ovaries, TUNEL assays revealed apoptosis in atretic but not preantral follicles. In contrast, both oocytes and surrounding granulosa cells underwent apoptosis in preantral follicles of *Gdf9Cre;R26iDTR* ovaries with no SOX9 expression detectable (Figure 3D and data not shown). Taken together these experiments demonstrate that granulosa cells become reprogrammed into Sertoli-like cells in XX *R26CreERT2;Foxl2^{fl/fl}* gonads upon TM administration in a cell-autonomous way. Moreover, although consistent with oocyte health being essential for follicle growth (Eppig, 2001), our findings challenge a long-standing hypothesis in the field that oocytes are directly required to maintain somatic cell identity during ovarian development (Guigon and Magre, 2006; McLaren, 1991).

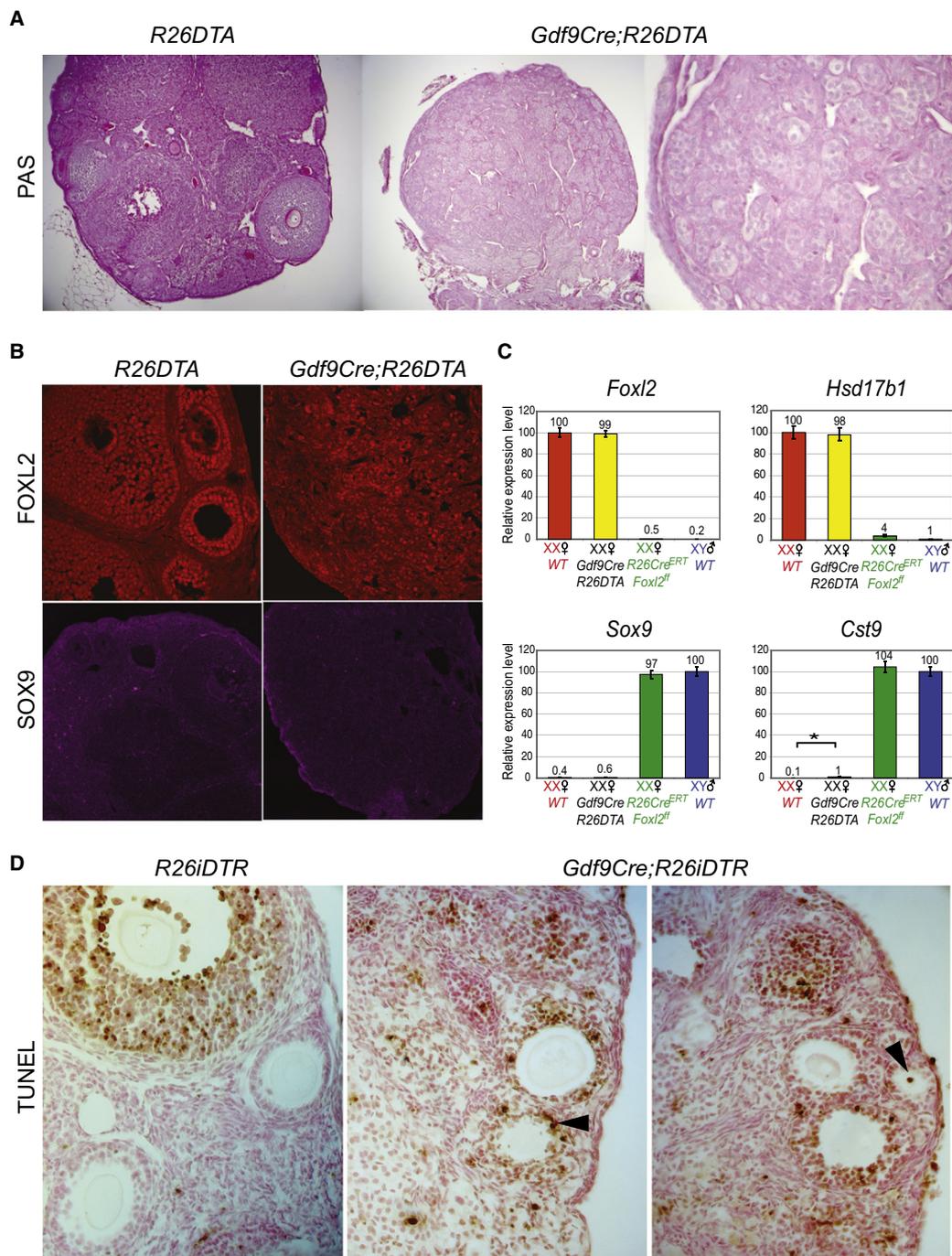


Figure 3. Diphtheria Toxin-Mediated Oocyte Ablation in Adult Ovaries

(A and B) PAS, FOXL2 and SOX9 immunostaining on ovarian sections of 8-week old *Gdf9Cre;R26DTA* and control XX gonads. Despite the absence of oocytes in *Gdf9Cre;R26DTA* ovaries the remaining somatic cells still express FOXL2 but not SOX9. Consistent with the lack of SOX9, no tripartite nucleoli are observed in these cells. (C) No significant change in the expression levels of representative genes by qRT-PCR is detected in *Gdf9Cre;R26DTA* in contrast to XX *R26Cre^{ERT2};Foxl2^{fl/fl}* mutant ovaries. The data are represented as mean \pm SD ($^*p < 0.001$).

(D) Detection of apoptosis by TUNEL staining. Positive TUNEL staining in atretic but not preantral follicles is seen in control ovaries. In contrast positive TUNEL staining is observed in oocytes and surrounding granulosa cells (black arrows) in different preantral stage follicles of *Gdf9Cre;R26iDTR* ovaries. Representative pictures from two different ovaries 9 days after the start of DT administration are shown.

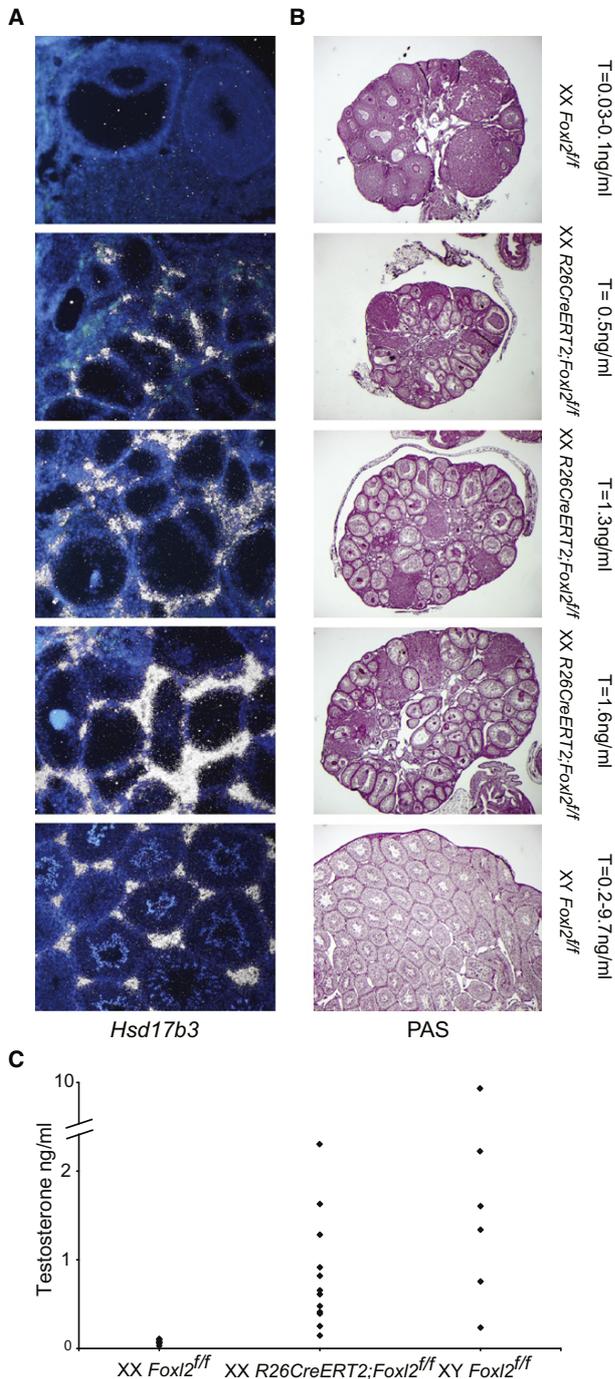


Figure 4. XX *R26CreERT2;Foxl2*^{ff} Gonadal Sex-Reversed Mutant Mice Have Testosterone Levels Similar to Wild-Type XY Males

(A) In situ hybridization on XX control and XX sex-reversed gonads and control testis showing ectopic expression of the testosterone-producing enzyme HSD17B3 in cells surrounding Sertoli-like cells of *R26CreERT2;Foxl2*^{ff} sex reversed gonads, in a pattern similar to that seen in normal testicular Leydig cells. White signal, antisense probe; blue staining, nuclei.

Appearance of Testosterone Producing Leydig-Like Cells upon Loss of *Foxl2*

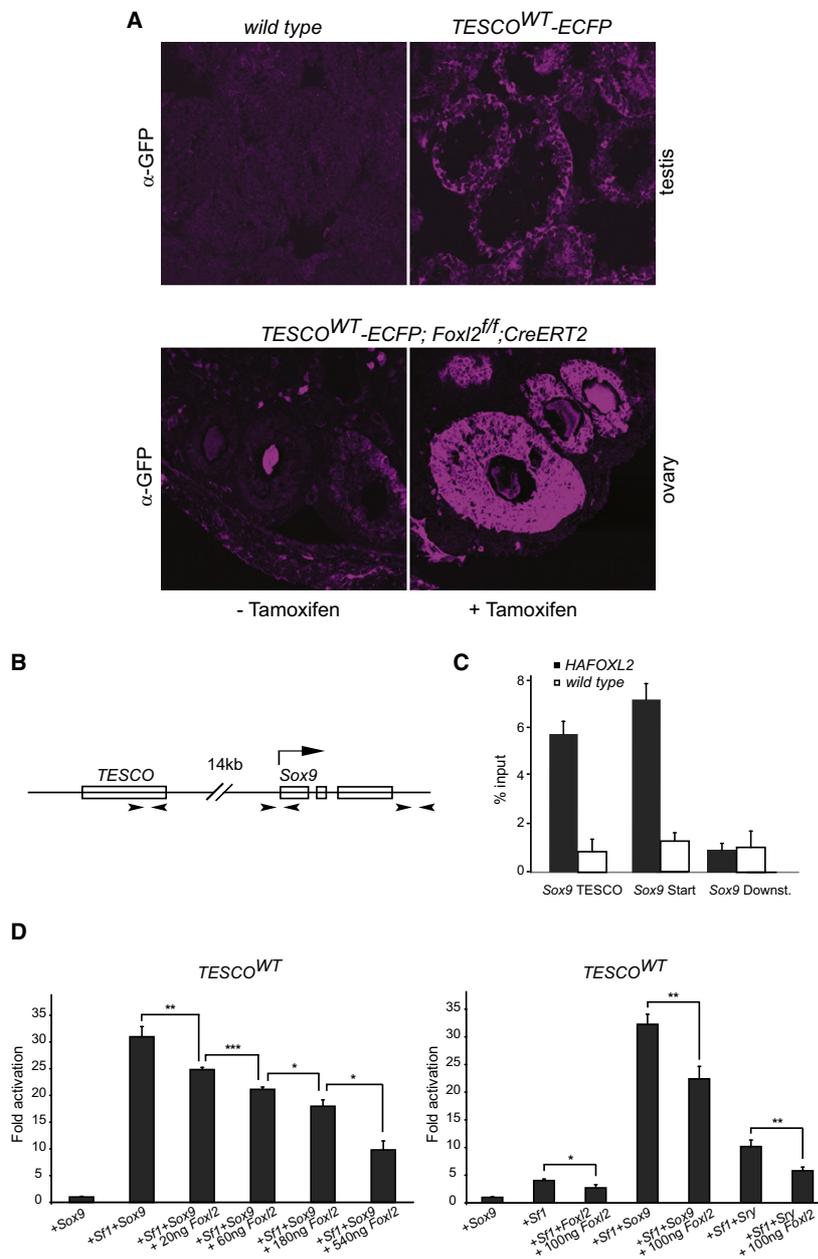
It has been suggested that the supporting cell lineages of the testis and ovary arise from a common precursor during development (Albrecht and Eicher, 2001). In contrast, the origin of the steroidogenic lineages is less clear. Adult Leydig and theca cells are both thought to be recruited from surrounding progenitor populations after birth (Haider, 2004; Skinner, 2005). To ask whether FOXL2-positive gonadal precursor cells give rise to theca cells we generated a *Cre* replacement allele of the *Foxl2* locus termed *Foxl2*^{Cre} (Figure S1). Using this allele in combination with the *R26lacZ* reporter we could demonstrate that *Foxl2* expressing cells indeed give rise to the theca cell lineage (Figure S4). To further facilitate the analysis of FOXL2 function, we generated a mouse line (*Foxl2*^{HAFoxl2}) in which the endogenous *Foxl2* locus is modified so that FOXL2 is N-terminally tagged with a HA-epitope. These retain full FOXL2 activity as homozygous *Foxl2*^{HAFoxl2/HAFoxl2} female mice are fertile (Figure S1 and data not shown). Immunohistochemical detection of HA-tagged FOXL2 protein demonstrated maintenance of FOXL2 expression in theca cells of the adult ovary (Figure S4). Desert hedgehog (DHH), which has been shown to be required for specification of Leydig cells in the fetal testis (Yao et al., 2002), is upregulated in XX *Foxl2*-deleted sex-reversed gonads as were many Leydig cell markers including *Hsd17b3* (Figure S2). *Hsd17b3* was found to be expressed in sex-reversed gonads in a similar pattern to adult testis (Figure 4A). We therefore asked if the putative Leydig-like cells produced circulating testosterone. Indeed similar levels were found in XX gonadal sex-reversed mice and wild-type XY littermates, with a direct correlation with expression levels of *Hsd17b3* (Figures 4A and 4B). Whether loss of *Foxl2* within theca cells is sufficient on its own to switch them into Leydig-like cells or rather makes them permissive for paracrine inducing molecules, such as DHH or PDGF from the Sertoli-like cells remains to be investigated (Brennan et al., 2003; Schmahl et al., 2008). However, the differentiation of Leydig cells in α GSUCre:*Foxl2*^{ff} XX mice, where *Cre* should not be active in theca cells, implies that such paracrine factors are sufficient.

Repression of *TESCO* by FOXL2 in the Adult Ovary

The rapid upregulation of *Sox9* expression suggested direct transcriptional repression of *Sox9* by FOXL2 in the ovary. Recently, the cis-regulatory element for testis-specific *Sox9* expression, referred to as *TESCO*, has been identified (Sekido and Lovell-Badge, 2008). Consistent with the activity of a *TESCO*-ECFP transgene during embryonic development (Sekido and Lovell-Badge, 2008), ECFP was detected in Sertoli cells of the adult testis but not in granulosa cells of the adult ovary (Figure 5A). To ask whether *TESCO* is activated upon loss of *Foxl2* in adult

(B) PAS staining of control ovaries and testes, and XX sex-reversed gonads, showing the morphological similarity between mutant “follicles” and seminiferous tubules. Note that the levels of serum testosterone, as well as the levels of *Hsd17b3* expression correlate with the number of tubule-like structures in XX *R26CreERT2;Foxl2*^{ff} sex-reversed gonads.

(C) Serum testosterone levels (ng/ml) as measured by RIA of control *Foxl2*^{ff} XX females (n = 6), XY males (n = 6) and XX *R26CreERT2;Foxl2*^{ff} gonadal sex-reversed mutant mice (n = 12).



ovaries, the *TESCO-ECFP* reporter was crossed into the *R26CreERT2;Foxl2^{fl/fl}* genetic background. We observed strong *TESCO* activation after tamoxifen-induced *Foxl2* deletion suggesting that FOXL2 may regulate *TESCO* in vivo (Figure 5B). Examination of the *TESCO* sequence indeed revealed predicted forkhead (FOX) binding sites (Figure S5). We therefore asked if FOXL2 binds to this *Sox9* cis-regulatory element in vivo. To perform FOXL2 chromatin immunoprecipitation (ChIP) assays from adult ovaries we took advantage of our *Foxl2^{HAFOXl2}* mouse line. We used an HA-epitope specific antibody on DNA-protein cross-linked material isolated from 8-week old *Foxl2^{HAFOXl2}* and

TESCO-regulatory element by SF1, SOX9/SF1 or SRY/SF1 in vitro.

FOXL2 and ESR1 Cooperatively Suppress Granulosa Cell Reprogramming

A similar phenotype with appearance of Sertoli-like cells expressing *Sox9* in postnatal ovaries has been described in *Esr1/Esr2* double mutant XX mice (Couse et al., 1999). Furthermore, forkhead transcriptional regulators have been shown to be essential for estrogen receptor DNA binding in other systems such as breast cancer cell lines (Carroll et al., 2005).

Figure 5. Testis-Specific Enhancer of *Sox9*, *TESCO*, Is Directly Repressed by FOXL2

(A) *TESCO* drives expression of an *ECFP* reporter in the adult testis but not ovary. ECFP expression is detected by immunohistochemistry with an α -GFP antibody (note unspecific staining in oocytes and some interstitial cells of the ovary). However, upon *Foxl2* deletion, *TESCO* activity is strongly induced in granulosa cells within one week after the start of TM administration in ovaries of XX *R26CreERT2;Foxl2^{fl/fl}* mice.

(B) Schematic drawing of the *TESCO-Sox9* genomic region indicating the primer pairs used for ChIP analysis (see Figure S5 for exact positions of binding sites).

(C) FOXL2 binds to *TESCO* sequences in vivo as demonstrated by ChIP comparing the relative enrichment obtained through a HA-epitope specific antibody from wild-type FOXL2 and HAFOXL2 ovaries.

(D) FOXL2 can repress *TESCO* driven luciferase reporters in transient transfection assays. Dose dependent repression of *TESCO* is shown for FOXL2 with SF1 and SOX9.

Error bars show standard deviations of three biological replicates performed in triplicate, asterisks denote (*p < 0.05, **p < 0.01, ***p < 0.001).

wild-type ovaries and directly compared the relative enrichment obtained for specific DNA loci between the two samples. With *Foxl2^{HAFOXl2}* ovaries we obtained a 6-fold enrichment using primers that spanned *TESCO* and a 7-fold enrichment around the *Sox9* transcription start site compared to wild-type ovaries (Figure 5C), consistent with the looping model of distant regulatory elements in transcriptional initiation. In contrast, no relative enrichment was seen for other *Sox9* genomic regions that served as controls. To further demonstrate that FOXL2 can negatively modulate *TESCO*, we performed transient transfection reporter assays employing the same DNA sequences. As shown in Figure 5D, FOXL2 attenuated activation of the

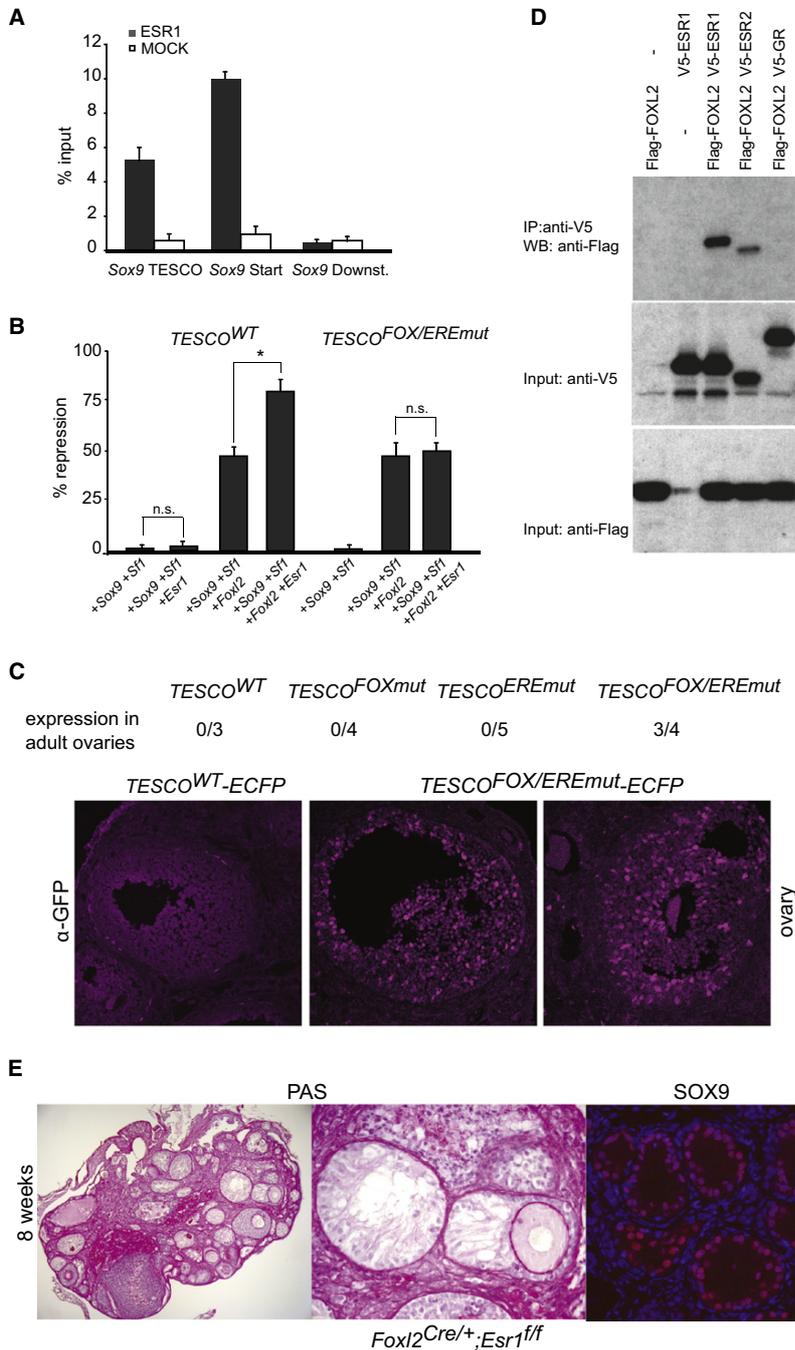


Figure 6. FOXL2 and ESR1 Together Regulate TESCO Activity

(A) ChIP analysis demonstrates ESR1 binding to *TESCO* sequences in adult ovaries. Error bars show standard error of the mean, from three biological replicates.

(B) FOXL2 and ESR1 synergize in repressing a *TESCO* driving luciferase reporter, which is activated by SOX9 and SF1 in transient transfection assays in a DNA binding dependent manner, while the synergistic repression is abolished when both FOX and ERE binding sites are mutated. The data are represented as mean \pm SD (* $p < 0.001$).

(C) Transgenic founder analysis of mutated *TESCO-ECFP* reporters. The number of transgenic founders analyzed for each construct is indicated. Expression of the *TESCO-ECFP* transgenic reporter is only seen in follicles upon mutation of FOX and ERE sites simultaneously (see Figure S6). Immunostaining for ECFP in ovarian follicles from two different independent *TESCO^{FOX/EREmut}-ECFP* lines are shown next to a *TESCO-ECFP* line. (D) Coimmunoprecipitation of Flag-tagged FOXL2 and V5-tagged nuclear hormone receptors from HEK293 cells demonstrating a strong physical interaction between FOXL2 and ESR1 or ESR2. Under the same conditions glucocorticoid receptor (GR) does not interact demonstrating the specificity of the FOXL2/ESR1/2 interaction.

(E) PAS staining of gonads from 8-week old *Foxl2^{Cre/+};Esr1^{fl/fl}* XX mice demonstrating the presence of Sertoli-like cells. This phenotype is not seen in ovaries of *Foxl2^{Cre/+};Esr1^{wild}* mice (data not shown) and has also not been reported in *Esr1* null mutant ovaries (Couse et al., 1999). SOX9 immunostaining on sections from another *Foxl2^{Cre/+};Esr1^{fl/fl}* XX gonad demonstrating the expression of SOX9 in cells that have a morphological Sertoli-like appearance.

Interestingly, nuclear hormone receptor DNA binding motifs are present in the *TESCO* sequence, raising the possibility that estrogen receptors are also binding to this *Sox9* regulatory element in the adult ovary (Figure S5). As with FOXL2, we observed a 5- to 10-fold enrichment of ESR1-occupied sequences in ChIP assays over mock control (Figure 6A). Although ESR1 was not able to repress the *TESCO* regulatory

element on its own, together with FOXL2 synergistic repression was observed in transient transfection assays (Figure 6B). To show that the repression of the *TESCO* regulatory element by FOXL2 and ESR1 is mediated through the putative forkhead and estrogen receptor (ERE) binding sites within *TESCO* we mutated the corresponding sequences (Figure S6). Mutation of all putative forkhead binding sites in *TESCO* did not lead to a loss of repression in transient transfection assays. This could be

estrogen receptor binding sites (which may in addition be bound by FOXL2) in *TESCO* abolished the observed synergistic repressive effect of ESR1 together with FOXL2 in our transient transfection assay (Figure 6B).

Although *in vitro* assays are informative, DNA regulatory elements may be under additional constraints when integrated into the genome. We speculated that relief of repression of *TESCO* may lead to the expression of the mutated *TESCO* elements in the adult ovary. This would still require the presence of factors able to activate the element, and it is notable that both SF1 and SOX8, a close homolog of SOX9 are present in the adult ovary albeit at low concentrations (Salmon et al., 2005). We therefore analyzed *TESCO* elements harboring different sequence mutations in transgenic animals to find that neither mutation of the forkhead sites alone nor mutation of the putative estrogen receptor binding sites alone, leads to derepression in adult ovaries. However when both types of site were simultaneously mutated, derepression of the *TESCO* element was observed during folliculogenesis (Figure 6C). Although concordant with our *in vitro* cotransfection results, the lack of derepression of the *TESCO* element upon mutation of all putative forkhead binding sites *in vivo* could be due to the presence of alternative sites, as discussed above, to functional redundancy between FOXL2 and ESR1 (although *Foxl2* mutations are far more severe than even *Esr1/2* double null mutations), or to the ability of FOXL2 and ESR1 to recruit each other via protein-protein interaction in the absence of the other's DNA binding site. We therefore tested if FOXL2 and ESR1/ESR2 can interact at the protein level. Indeed, coimmunoprecipitation experiments from HEK293 cells expressing FLAG-tagged FOXL2 and V5-tagged ESR1 or ESR2 demonstrated a strong protein-protein interaction between these proteins in contrast to glucocorticoid receptor, which does not show any significant interaction with FOXL2 under the same conditions (Figure 6D). Finally, we tested the observed interaction of FOXL2 and estrogen receptors *in vivo* by generating female mice with the genotype *Foxl2^{Cre/+};Esr1^{fl/fl}* in which all granulosa cells, but not oocytes have lost ESR1 and one *Foxl2* allele utilizing a floxed *Esr1* allele. The appearance of Sertoli-like cells expressing SOX9 in 8-week old ovaries of *Foxl2^{Cre/+};Esr1^{fl/fl}* mice, which is neither observed in *Foxl2^{Cre/+}* nor in *Esr1^{-/-}* mice, further supports the suggested cooperation of FOXL2 with estrogen receptors in *Sox9* repression *in vivo* (Figure 6E and data not shown).

DISCUSSION

Although sex determination is essential for successful reproduction of most species within the animal kingdom the underlying molecular mechanisms are marginally conserved from *Drosophila* to man. At least vertebrates have a common set of transcriptional regulators known to be involved, including DAX1, DMRT1, SF1, SOX9, FOXL2 and WT1, as well as some signaling molecules such as FGF9 and WNT4 (Barske and Capel, 2008; Guiguen et al., 2009; Shoemaker and Crews, 2009). However, their molecular interplay and epistatic relationships seem highly variable and are still far from being understood.

In mammals, SRY is the dominant switch initiating testes development, and it does so by directly upregulating the expression of *Sox9* in cells of the supporting cell lineage (Sekido and Lovell-Badge, 2008). Once above a critical threshold, SOX9 initiates several positive autoregulatory loops that maintain the high levels of SOX9 expression and activity that are necessary for Sertoli cell differentiation and function. These “feedforward” mechanisms, which make continued expression of SRY unnecessary, include SOX9 binding together with SF1 to its own regulatory region, as well as the upregulation of *Fgf9* and *prostaglandin D synthase (Ptgds)*, each of which make extracellular signaling molecules that act back on early Sertoli cells (Kim et al., 2006; Wilhelm et al., 2007). The maintenance of high levels of SOX9 and subsequent Sertoli cell differentiation and testis development can be overridden by manipulating WNT pathway activation through overexpression of a stable form of β -catenin in XY gonads (Maatouk et al., 2008). This finding has its conceptual counterpart in the XX gonad where loss of either the WNT pathway activator *R-Spondin* or *Wnt4* can lead to *Sox9* upregulation and XX male sex reversal, albeit with weak or partial phenotypes (Chassot et al., 2008b; Tomizuka et al., 2008; Vainio et al., 1999). Misexpression of *Sox9* itself can give robust XX male sex reversal (Bishop et al., 2000; Vidal et al., 2001). However, these examples of sex reversal, together with numerous others in different mammalian species, occur as a result of an altered cell fate decision of the common supporting cell lineage precursor during development. They do not address the question whether ‘terminally’ differentiated Sertoli or granulosa cells can switch or *flip-flop* from one fate to that of the other as suggested by Burgoyne et al. (1988). The demonstration here that upon loss of *Foxl2* mature granulosa/theca cells undergo transdifferentiation into Sertoli/Leydig-like cells with high levels of testosterone production is one of the few documented examples of *in vivo* lineage reprogramming in an adult organism (Zhou and Melton, 2008).

FOXL2 and SOX9: Yin and Yang of Sex Maintenance

Like Yin and Yang, FOXL2 and SOX9 oppose each other's action to ensure together the establishment and maintenance of the different female and male supporting cell types respectively (Figure 7). The observed upregulation of *Sox9* as well as its testis-specific regulatory element *TESCO* upon loss of FOXL2 function in mature granulosa cells not only demonstrates that FOXL2 represses *Sox9* expression but also begs the question as to the identity of the positive factors required for *Sox9* upregulation in the absence of SRY? These could include extrinsic factors known to be involved in positive feedback loops in the early testis, such as FGF9, PGD2 or related family members, which by themselves are each capable of stimulating high levels of SOX9 in the early embryonic gonad *in culture*, or it could involve intrinsic factors able to mimic SOX9 (Sekido and Lovell-Badge, 2009). As *Sox9* and *Foxl2* are also expressed in a mutually exclusive way in the gonads of many lower vertebrate species, the suggested trans-repression between the two factors seems to be an evolutionary conserved mechanism (Barrionuevo et al., 2006; Chaboissier et al., 2004; Uhlenhaut and Treier, 2006). It is therefore tempting to speculate that this mechanism is central to the maintenance of the gonadal sexual

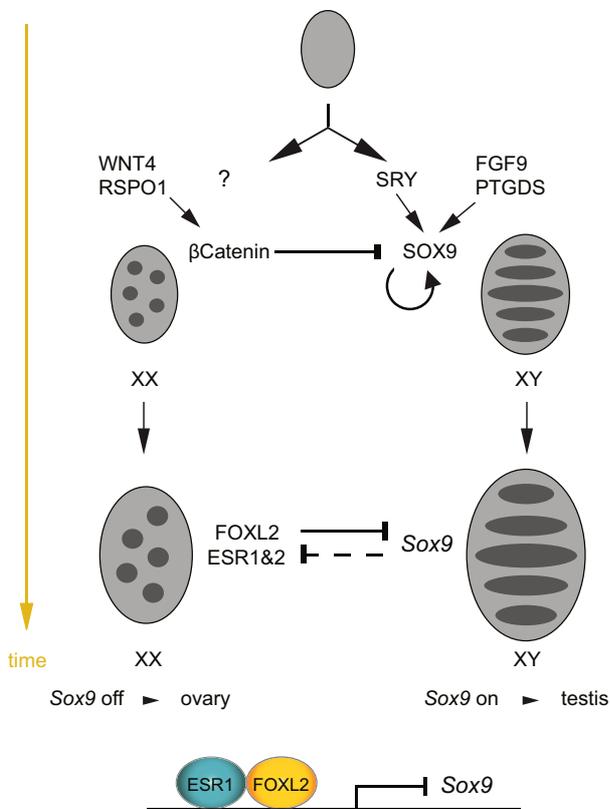


Figure 7. Model of Sox9 Regulation Required for Maintenance of Gonadal Phenotype in Mammals

During initial phases of sex determination, SRY upregulates Sox9 expression, and subsequent positive autoregulatory loops involving SOX9 itself, together with FGF9 and prostaglandin D2 signaling, activate and maintain Sox9 expression in male gonads, whereas β -catenin stabilized by WNT4 and RSPO1 signaling suppresses Sox9 expression in female gonads. After birth β -catenin activity declines and thus in adult female gonads, FOXL2 and estrogen receptors (ESR1/2) are required to actively repress Sox9 expression to ensure ovarian somatic cell fate. The transcriptional repression of Sox9 by FOXL2 and estrogen receptors is necessary throughout the lifetime of the female to prevent transdifferentiation of the somatic compartment of the ovary into a testis (PTGDS, prostaglandin D synthase).

phenotype in vertebrates and supports the model in which the decisive issue that determines the outcome of mammalian sex development and maintenance is whether SOX9 can establish an auto-regulatory self-maintaining loop or not (Kim et al., 2006; Sekido and Lovell-Badge, 2008). Although *Foxl2* is upregulated in Sox9-conditional null mutant XY gonads (Barrionuevo et al., 2006), direct repression of *Foxl2* expression by SOX9 remains to be demonstrated.

As shown here, the maintenance of female gonadal sex requires continuous expression of FOXL2 to suppress male development. Due to the infertility of human females heterozygous for FOXL2 mutations, the homozygous loss-of-function phenotype has not yet been reported. In the mouse global *Foxl2* deletion, such as a null deletion (*Foxl2*^{-/-}) or replacement with lacZ (*Foxl2*^{lacZ/lacZ}) leads to ovary dysgenesis but not to sex

reversal (gonadal or otherwise) during development in contrast to the deletion at adult stages reported here (Schmidt et al., 2004; Uda et al., 2004). Due to the observed upregulation of SOX9 during perinatal stages in *Foxl2*^{-/-} ovaries, it was suggested that granulosa cells have transdifferentiated into Sertoli-like cells (Ottolenghi et al., 2005). However, our comparison of genome wide gene expression profiles revealed that postnatal *Foxl2* null mutant XX gonads do not show upregulation of most of the 200 testis-specific genes observed in the conditional mutant, such as *Rhox8*, *Sox8*, *Ncoa2/TIF2*, *Tsx* and *Ins13*, suggesting that despite expression of SOX9 they have not transdifferentiated into bonafide Sertoli-like cells (Figures S7 and S8). Importantly, we find that *R-Spondin1* (*Rspo1*), which is still robustly expressed in P3 wild-type ovaries, is more than 2-fold upregulated in *Foxl2* null mutant ovaries (Chassot et al., 2008a). In contrast, *Rspo1* is no longer significantly expressed in adult ovaries nor is it upregulated in the conditional mutant (Figure S8).

The WNT Pathway and FOXL2 Act Independently during Gonadal Development

Mutations in *R-Spondin1* lead to female-to-male sex reversal in at least some humans and partially in mice, as does the global disruption of murine *Wnt4*. This has led to the realization that the WNT/ β -catenin pathway is important for development of the female gonad (Chassot et al., 2008b; Maatouk et al., 2008; Parma et al., 2006; Tomizuka et al., 2008; Vainio et al., 1999). In all these cases *Foxl2* is still expressed, suggesting that *Foxl2* is not regulated by WNT signaling and acts independently to regulate SOX9 activity. We speculate that defined temporal developmental windows of *Rspo1* expression are essential to allow the mutually exclusive expression of Sox9 and *Foxl2* and its deregulation may explain in part the variable outcome of *Foxl2* deletion seen in different species (Figure 7). This also argues the independence of the two “anti-testis” pathways represented by FOXL2 and R-Spondin1/ β -catenin and that (as a minimum) both need to be lost to allow robust differentiation of testicular cell types. In contrast to a previous report we do not find a synergistic interaction between *Wnt4* and *Foxl2* (Ottolenghi et al., 2007)(Figure S9), and in accordance with the observed upregulation of *Rspo1* in *Foxl2* null mutants these data further suggest that negative regulation of SOX9 activity through the WNT pathway is epistatic and dominant over FOXL2 activity during XX gonadal development at least until birth.

The Steroid Connection: Interaction of FOXL2 and ESR1

In contrast to eutherian mammals, other vertebrate species, even those employing genetic based sex determination mechanisms, are sensitive to the application of exogenous hormones (Barske and Capel, 2008; Guiguen et al., 2009; Shoemaker and Crews, 2009). In particular estrogens have long been known to be important for ovarian differentiation and they are able to trigger male to female sex reversal in fish, reptiles and birds, whereas decreasing estrogen levels in these species has the opposing effect resulting in phenotypic males from females (Guiguen et al., 2009; Nakamura, 2009). Exogenous estrogens can even trigger XY ovary development in marsupials (Coveney et al., 2001). The molecular mechanisms underlying these

phenomena are far from being understood. The demonstration that FOXL2 and estrogen receptor interact in controlling granulosa cell identity at least in part through repression of *Sox9* expression suggest that similar mechanisms operate in lower vertebrate species, as *Foxl2* is one of the few conserved ovarian differentiation genes (Uhlenhaut and Treier, 2006). Furthermore it validates in vivo the proposed importance of forkhead factors for estrogen receptor function (Carroll et al., 2005).

While this early dependence on estrogens for ovary differentiation is not evident in most eutherian mammals, this may not be true of all. In contrast to the mouse, where there is no ovarian steroidogenesis during fetal development, ruminants such as the goat show precocious production of estrogens from the earliest stages of ovary development. This is evident from the onset of expression of CYP19a1. Pannetier et al. (2006) have shown that *Cyp19a1* is regulated by FOXL2 in the fetal goat ovary, as we show here for adult mice (Figure 1B). Therefore, in animals with PIS, estrogens will be absent in addition to FOXL2 from early stages. As the PIS phenotype is evident much earlier than in *Foxl2* null mutant XX mice, this suggests that estrogen activity may have replaced the RSPO1/WNT/ β -catenin pathway as the predominant anti-testis or ovary differentiation mechanism during fetal development in goats. Our results therefore strongly suggest that FOXL2 deficiency is the sole underlying cause of female-to-male sex reversal seen in the goat polled intersex syndrome (Pailhoux et al., 2001).

ESR1/2 null mutant mice show a phenotype similar to that of the *Foxl2* conditional mutation, with post-pubertal appearance of Sertoli-like cells and testis-tubule like structures in ovaries (Couse et al., 1999). The origin of the Sertoli-like cells was not resolved, indeed it was attributed to the loss of oocytes, which we show here is more likely to be a consequence of Sertoli cell differentiation than a cause. Thus our demonstration that 'terminally' differentiated somatic ovarian cell types can cell autonomously switch their fate not only extends our understanding of how mammalian sex is maintained but also reveals the presence in mammals of an evolutionary conserved mechanism of vertebrate sex determination.

FOXL2, SOX9, DMRT1, DAX1, and CYP19a1: Who Depends on Whom?

Although we have demonstrated that FOXL2 regulates *Sox9* expression, many of the other suggested interactions within the proposed transcriptional network underlying sex determination/maintenance are mainly based on temporal gene expression patterns and have not been rigorously tested. DMRT1 is required for postnatal testis differentiation in mice and its homologs have been shown or suggested to be master sex-determining genes in the chicken and in some medaka species (Guiguen et al., 2009; Raymond et al., 2000; Smith et al., 2009). The observation that one of the strongest upregulated genes in the ovary upon *Foxl2* deletion is *Dmrt1*, and that FOXL2 and DMRT1 appear to exhibit opposing effects on the regulation of estrogen production in fish, suggests that FOXL2 (together with estrogen receptor) may directly repress *Dmrt1* transcription. Similarly, upregulation of *Dax1*, an established negative regulator of SF1, upon conditional *Foxl2* deletion in

the ovary may be due to derepression mechanisms. Alternatively, activation of these two genes may result from upregulation of SOX9. The temporal appearance of SOX9 and DMRT1 in granulosa cells upon *Foxl2* deletion supports the latter suggestion (Figure S10). A similar unresolved matter is the transcriptional regulation of *Cyp19a1*, the strongest downregulated gene upon conditional *Foxl2* deletion, in which FOXL2 and DMRT1 have been implicated (Figure 1B) (Guiguen et al., 2009). The establishment of the inducible system of somatic ovarian reprogramming presented here will now allow the definite clarification of the transcriptional interaction network underlying the maintenance of mammalian gonadal sex.

In conclusion, we show that the mammalian ovarian phenotype has to be maintained throughout adulthood, mainly by active repression of the Sertoli cell-promoting gene *Sox9*, and that FOXL2 (acting together with estrogen receptors) is the critical factor responsible for this. The realization that granulosa/theca cells in the adult ovary retain the plasticity to transdifferentiate into long-lived Sertoli/Leydig cells with subsequent testosterone production also has important implications for reproductive biology, in particular the treatment of sex differentiation disorders in children, as well as premature ovarian failure and female menopause, both of which are associated with declining estrogen levels and occasional signs of masculinization.

EXPERIMENTAL PROCEDURES

Mouse Strains

All mouse strains used are described in Supplemental Experimental Procedures.

Histochemistry

In situ hybridisation was performed on 20 μ m cryosections as described in Supplemental Experimental Procedures. For immunohistochemistry, 20 μ m cryosections of 2% paraformaldehyde fixed ovaries were used (Leica 3050S). Sections were incubated in blocking solution (PBS containing 0.4% Triton-X, 5%) for 1 hr at room temperature followed by overnight incubation with the primary antibodies at 4°C. The sections were washed (0.4% Triton-X in PBS) and incubated for 1 hr at room temperature with the secondary antibodies. FOXL2 was detected with a rabbit antibody described in Schmidt et al. (2004) diluted 1:100. SOX9 was detected using an antibody described in Stolt et al. (2003) diluted 1:200. ECFP was detected using an α -GFP antibody (Torrey Pines Biolabs) diluted 1:500. DMRT1 was detected using an antibody described in Raymond et al. (2000) diluted 1:2000.

Serum Testosterone Measurements

Serum blood was collected from the orbital sinus of mice. Serum was frozen at -20°C until assayed. Serum testosterone levels were measured by RIA in collaboration with Dr. A. F. Parlow at the National Hormone and Peptide Program, HUMC.

Protein Coimmunoprecipitation

HEK293 cells were transfected with expression vectors for murine Flag-tagged FOXL2 and V5-tagged ESR1, ESR2, or Glucocorticoid Receptor (GR) cDNAs using Fugene (Roche). After 48 hr, Co-IPs were performed by incubating cleared cell lysates in the presence of Flag M2 beads (Sigma) overnight at 4°C, followed by washing and standard Western blotting using either anti-V5-HRP or anti-Flag-HRP antibodies. The nuclear receptor cDNAs were a generous gift from J. Jonker (Salk Institute).

Diphtheria Toxin Oocyte Ablation Experiment

Diphtheria toxin (DT) (List Biological Laboratories, Campbell, CA) was dissolved in PBS and 8-week old female mice were injected three times i.m with a 48 hr gap between each injection (50 μ g/kg). Mice were killed and ovaries dissected for analysis 9 days after the first injection.

Statistical Analysis

Values are given as the means \pm SEM. Data were analyzed by Student's *t* test. Statistically significant threshold was accepted as $p < 0.05$.

ACCESSION NUMBERS

The genome-wide profiling data discussed in this publication have been deposited in the NCBI GEO repository, accession number GSE16853.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, two tables, ten figures, and a video summary and can be found with this article online at [http://www.cell.com/supplemental/S0092-8674\(09\)01433-0](http://www.cell.com/supplemental/S0092-8674(09)01433-0).

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