Loss of \textit{Wnt4} and \textit{Foxl2} leads to female-to-male sex reversal extending to germ cells

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The discovery that the \textit{SRY} gene induces male sex in humans and other mammals led to speculation about a possible equivalent for female sex. However, only partial effects have been reported for candidate genes experimentally tested so far. Here we demonstrate that inactivation of two ovarian somatic factors, \textit{Wnt4} and \textit{Foxl2}, produces testis differentiation in XX mice, resulting in the formation of testis tubules and spermatogonia. These genes are thus required to initiate or maintain all major aspects of female sex determination in mammals. The two genes are independently expressed and show complementary roles in ovary morphogenesis. In addition, forced expression of \textit{Foxl2} impairs testis tubule differentiation in XY transgenic mice, and germ cell-depleted XX mice lacking \textit{Foxl2} and harboring a \textit{Kit} mutation undergo partial female-to-male sex reversal. The results are all consistent with an anti-testis role for \textit{Foxl2}. The data suggest that the relative autonomy of the action of \textit{Foxl2}, \textit{Wnt4} and additional ovarian factor(s) in the mouse should facilitate the dissection of their respective contributions to female sex determination.

\section*{INTRODUCTION}

Sex determination provides a paradigm for the study of the mechanisms of cell fate choice and their role in the formation of alternative organs. The essential genetic determinants of sex determination in invertebrate model organisms were largely known by the early 1990s (1). In mammals, the mechanism of testis formation is now comparably well understood. Classical results in cytogenetics and experimental embryology have demonstrated that a ‘testis organizer’, induced by the testis determining factor (\textit{SRY}), drives male differentiation in mammalian embryos (2–7). In addition, testis determination was disrupted in over 10 distinct gene knockout models in mice, and was found to be associated with mutations affecting several additional genes in humans (7,8). This has created a strong framework for the study of mammalian male sex determination at a molecular level. By contrast, two decades have elapsed since genetic evidence for an ‘ovary organizer’ (the ovarian determinant, \textit{Od}) was first presented (9), and over a decade since an ‘anti-testis’ activity (termed \textit{Z}) was inferred to be necessary for ovary differentiation (10). But the molecular bases of mammalian female sex determination are still poorly understood.

Based on natural mutations in goats or human patients, \textit{Foxl2} and \textit{RSPO1} were proposed as candidate female sex determining genes (11–13). In part because of the difficulties in studying human tissues, characterization of \textit{RSPO1} action awaits the creation of an experimental model (13). As for \textit{Foxl2}, its role has been in doubt because of disparate effects of mutations in goat and human (8). Experimental ablation of \textit{Foxl2} in mice gave suggestive results, showing only partial secondary sex reversal on several genetic backgrounds (14). In addition to \textit{Foxl2}, a number of ovarian genes have been inactivated in knockout models, including \textit{Wnt4} (discussed subsequently), but no complete XX sex reversal has ever been observed in mice (9,15). This has given rise to intense speculation on the possible mechanism(s) of ovary differentiation in mammals (7,15–19). Our data suggest a route to test and extend current hypotheses.

We focussed on two genes that show the earliest developmental effects when individually inactivated, i.e. the secreted glycoprotein \textit{Wnt4} and the transcription factor \textit{Foxl2} (14,20). In mice, \textit{Wnt4} and \textit{Foxl2} are expressed in ovarian somatic cells that activate reporter constructs for steroidogenic factor 1 (\textit{Sf1/Nr5a1}) or \textit{Sry} (21,22), including the epithelial/supporting cell lineage that induces testis sex determination in males.

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**RESULTS**

We investigated the effects of loss of Wnt4 and Foxl2 on the genital and gonadal phenotype of single and double knockout mice. As reported (28,29), Foxl2-null ovaries and genitalia were very similar to wild-type females at birth (Fig. 1A and B). XX mice lacking Wnt4 harbored male internal genitalia and gonads resembling small testes (cf. Fig. 1C–E) (20). Testis-shaped newborn ovaries lacking Wnt4 alone contained cell aggregates that often resembled follicles and were positive for the antimullerian hormone (AMH, which is expressed in both Sertoli cells and postnatal ovarian granulosa cells). However, they showed no testis-like tubules and were negative for the antimullerian hormone (AMH, which is expressed in both Sertoli cells and postnatal ovarian granulosa cells). They showed no testis-like tubules and were negative for the antimullerian hormone (AMH, which is expressed in both Sertoli cells and postnatal ovarian granulosa cells).

The strikingly complementary phenotypes associated with loss of Wnt4 or Foxl2 suggested that their combined ablation might lead to complete XX sex reversal. We generated mice lacking both these genes and confirmed this possibility, providing an experimental model for a sex-determining action of ovarian genes in mammals.
Figure 2. Differentiation of germ cells in Foxl2−/− Wnt4−/− newborn ovaries relative to ovary and testis, assessed by immunohistochemistry (A–F) and electron microscopy (G–I). Wild-type ovaries contain oocytes (A, D, G) and testes contain spermatogonia (C, F, I); but the double knockout ovaries contain some oocytes (B) and some spermatogonia (E and H). (A–C): meiotic (oocyte) markers, SCP3 (green) and γH2AX (red); (D–F): spermatogonial marker, PLZF (green), and supporting cell marker, WT1 (pink). (A–F) DAPI marks all nuclei in blue. (G–I) show an oocyte (G), and type A spermatogonia, the endoplasmic reticulum was poorly developed (H and I: arrowheads). Abundant chromatin clumps in the oocyte nucleus (G, arrowhead) contrast with homogenous electron density in the spermatogonial nuclei (H and I: arrowheads). Bars: (A–F): 40 μm; (G–I): 2 μm.

In accordance with the phenotype of Wnt4−/− and Foxl2−/− single knockout ovaries (discussed earlier) germ cells located in the cortical regions of Wnt4−/− Foxl2−/− double knockout ovaries resembled late prophase oocytes and expressed two meiotic markers, the synaptonemal complex protein SYCP3 and the phosphorylated histone γH2AX (Fig. 2A–C). Instead, in the ovarian medulla of the double knockout mice, the tubules harbored well differentiated spermatogonia. These showed positive immunostaining for the promyelocytic leukemia zinc finger protein, PLZF/ZBTB16, a marker for adult-type spermatogonia (33) in light microscopy (Fig. 2D–F), and showed characteristic morphology at higher resolution, by electron microscopy (Fig. 2G–I). In electron micrographs, spermatogonial could be easily distinguished from somatic cells by their much larger size, round or ovoid shape, and round nucleus with a light outline devoid of indentations. In addition, as expected for spermatogonia, their nuclei were homogenously granulated, except for a more electron-dense, highly reticulated nucleolus located near the nuclear membrane (Fig. 2H and I, arrows); also characteristic of spermatogonia, the endoplasmic reticulum was poorly developed and the cytoplasm contained few organelles (34). Compared to spermatogonia, primordial germ cells and oogonia have centrally located nucleoli and contain abundant organelles; in addition, oogonia are characterized by a considerably smaller size, and highly reduced nucleo-cytoplasmic ratio (35); and meiotic prophase oocytes (cf. control ovary, Fig. 2G) are larger than oogonia but have heterogeneous nuclei that contain abundant irregular clumps of chromatin (35).

In order to confirm further the presence and activity of spermatogonial in Wnt4−/− Foxl2−/− newborn ovaries, we measured the level of RNA markers specific to spermatogonia. Many genes have been reported to be male germ cell specific in studies limited to adult tissues; but whenever fetal ovaries were subsequently tested, oocytes were also found positive. To look for more specific male germ cell markers, we combined available microarray data on whole gonads and isolated cell types from adult mice and embryos (21,30,36,37) (see Materials and Methods). The analysis yielded 10 candidate markers (Supplementary Material, Table S1), including a well-characterized multiplicity gene, termed ‘Xlr-related, meiois regulated’ (Xmr). Detailed RNA and protein analyses have demonstrated that Xmr expression is limited to spermatocytes within the testis and is absent in the ovary throughout development (38). Using microarray hybridization and quantitative real-time PCR, we verified that Xmr was expressed in the Wnt4−/− Foxl2−/− newborn ovary as it is in testis, and was undetectable or at baseline levels in control and single knockout ovaries (Fig. 3A and Supplementary Material, Fig. S2). Profiles similar to Xmr were shown by additional genes (see Materials and Methods and Supplementary Material, Fig. S1). However, validation of these genes as specific markers for spermatogonia requires further cyto/histo logical expression studies, including tests for expression in fetal oocytes.

The Wnt4−/− Foxl2−/− XX mice thus harbored both male and female germ cells and can be regarded as XX true hermaphrodites, a condition that falls in the range of SRY-negative forms of XX sex reversal in humans (39). Neonatal lethality associated with Wnt4 loss (20) prevented studies of germ cells at later stages, but it seems likely that the presence of two X chromosomes would block progression through spermatogenesis (4).

We used RNA profiling of whole newborn gonads to look for clues to the mechanism of extensive sex reversal in the double knockout mice. The expression pattern of genes required for testis determination or early testis differentiation is sometimes only transiently or not at all male-specific (see Materials and Methods and Supplementary Material, Fig. S3). We therefore focussed on functionally characterized genes that are male-specific throughout development and postnatal life. Some of the reliable testis markers were partly upregulated in the Wnt4−/− single knockout ovaries, but reached testis-like levels only in the Wnt4−/− Foxl2−/− knockout animals. These genes included a cytochrome P450 enzyme, Cyp26b1 (Fig. 3A), which antagonizes meiosis and thus oogenesis in embryonic testes (40); and 17β-hydroxysteroid dehydrogenase 3, Hsd17b3, which is required for testosterone synthesis (41 and data not shown).

Other testis-specific genes were upregulated only in newborn ovaries lacking both Wnt4 and Foxl2. They included several critical testis factors primarily or selectively expressed in the supporting cell lineage, i.e. Sox9, Dmrt1, Dmrt3, Ptdgs and Gdnf (Fig. 3A) (reviewed in 7 and 8). The doublesex/mab3-related transcription factors Dmrt1 and Dmrt3 are strong candidate for a role in testis determination in

**Figure 2.** Differentiation of germ cells in Foxl2−/− Wnt4−/− newborn ovaries relative to ovary and testis, assessed by immunohistochemistry (A–F) and electron microscopy (G–I). Wild-type ovaries contain oocytes (A, D, G) and testes contain spermatogonia (C, F, I); but the double knockout ovaries contain some oocytes (B) and some spermatogonia (E and H). (A–C): meiotic (oocyte) markers, SCP3 (green) and γH2AX (red); (D–F): spermatogonial marker, PLZF (green), and supporting cell marker, WT1 (pink). (A–F) DAPI marks all nuclei in blue. (G–I) show an oocyte (G), and type A spermatogonia, the endoplasmic reticulum was poorly developed (H and I: arrowheads). Abundant chromatin clumps in the oocyte nucleus (G, arrowhead) contrast with homogenous electron density in the spermatogonial nuclei (H and I: arrowheads). Bars: (A–F): 40 μm; (G–I): 2 μm.
mammals and other vertebrates (42–45); prostaglandin D2 synthase, *Ptgds*, has been shown to provide positive feedback for *Sox9* during mouse testis determination (46,47); and the glial cell-derived neurotrophic factor, *Gdnf*, is necessary and sufficient to maintain spermatogonia (48). Limited upregulation of *Dmrt1* and *Dmrt3* in the *Wnt4*/*Foxl2* double knockout ovary compared to *Sox9* may reflect either incomplete male differentiation in the somatic compartment, or reduced numbers of male germ cells [as the latter express *Dmrt1*, and likely, *Dmrt3*, but not *Sox9* (37,49–51)]. As reported, *Foxl2*/*Wnt4* ovaries activate *Sox9*, *Dmrt1* and other testis-determining genes only later, starting about 1 week postnatum (14). Thus, some features of sex reversal occur with loss of either gene, but loss of both *Wnt4* and *Foxl2* results in maximal early upregulation of testis genes and the induction of male gametogenesis.

The findings also strongly suggest that additional gene(s) independently contribute to female sex determination. In particular, some cortical oocytes persist in the absence of *Wnt4* and *Foxl2*, possibly resulting from the action of additional female sex determining gene(s). A candidate (13) is *Rspos1*, whose RNA levels correlated with oocyte markers in all conditions tested (Fig. 3B). *Dax1/Nr0b1* might also be involved (52,53). Our data are consistent with a requirement of *Wnt4* for *Dax1* expression (54), but they also suggest a possible repressive action by *Foxl2* (Fig. 3B).

Consistent with at least partial autonomy, *Foxl2* and *Wnt4* were each still expressed when the other was ablated (Fig. 3B). Some ovarian genes were partly downregulated in the absence of either *Foxl2* or *Wnt4*, suggesting additive effects (e.g. follistatin, Fig. 3B). Many follicle-specific genes were downregulated in *Foxl2*/*Wnt4* ovaries that expressed *Wnt4* (14 and 29, and data not shown) and some of them were maintained at higher levels or even upregulated in *Wnt4*/*Foxl2* ovaries that expressed *Foxl2*. They included aromatase/Cyp19a1, the enzyme that catalyzes the formation of estrogens from androgens (Fig. 3C). This suggests a critical selective role for *Foxl2* promoting ovary morphogenesis.

Independent evidence supports an early anti-testis function of *Foxl2*. As a first approach, we generated and compared mice that either lack or retain *Foxl2* and harbor a hypomorphic mutation for another gene required for ovarian development, Kit. Several mutations that disrupt the *Kit* tyrosine kinase receptor have been found to lead to migratory defects in primordial germ cells. The resulting germ cell depletion impaired ovary differentiation without inducing sex reversal (23). In newborn ovaries from mice that are wild-type for *Foxl2* but homozygous for the *Kit<sup>Wv</sup>* mutation, *FOXL2* was expressed (55 and Fig. 4A and B), and consistent with absent sex reversal, testis markers were not detected (Fig. 4E and F, and data not shown). In the *Foxl2*/*Kit<sup>Wv</sup>*/<sup>Wv</sup> double mutant (Fig. 4D and H), ovaries derepressed SOX9 more rapidly than in *Foxl2*/*Wnt4*/*Kit<sup>Wv</sup>*/<sup>Wv</sup> single knockout mice (Fig. 4C and G). SOX9 expression was widespread by the time of birth (cf. Fig. 4E–H).

Molecular sex reversal in *Foxl2*/*Kit<sup>Wv</sup>*/<sup>Wv</sup>** XX mice extended to other somatic testis markers (e.g. *Dmr1*, data not shown), but the phenotype was still limited to somatic cells and was incomplete. The few surviving germ cells expressed meiotic markers and were likely female; steroidogenic genes were not active, accounting for normal female genitalia; and no morphologically recognizable testis tubules were detectable until at least 8 days postnatum, after which high mortality of *Foxl2*/*Kit<sup>Wv</sup>*/<sup>Wv</sup> mice prevented further studies (data not shown).

Incomplete testis differentiation in *Foxl2*/*Kit<sup>Wv</sup>*/<sup>Wv</sup>** XX gonads possibly reflects the complex role of *Kit* in both testis and ovary differentiation (56). But in this case as in the other models tested, *Foxl2* ablation was accompanied by *Sox9* derepression, though with distinct timing and outcomes on ovary differentiation. *Foxl2* thus seems to act as an effective repressor of *Sox9* in the supporting cell lineage.

As a second independent approach, we performed gene overexpression studies. Studies with transgenic mice have
previously shown that \textit{Wnt4} can induce vascular anomalies in embryonic testes (57). To test for a comparably early action of \textit{Foxl2}, we generated transgenic mice expressing mouse \textit{Foxl2} under a ubiquitous heat-inducible promoter. This promoter is preferentially active in mouse pre-Sertoli cells and leads to XX maleness when joined to the \textit{Sry} gene (58). XY \textit{Foxl2} transgenic embryos showed disorganized tubules and in some cases produced ovotestis-like gonads at 13.5 dpc [as indicated by staining for the AMH, which is a testis tubule marker at this stage (Fig. 4I–K, green)]. The assay cannot distinguish between endogenous and transgenic FOXL2 expression in XY \textit{Foxl2} transgenic embryos (Fig. 4J, red), but the downregulation of AMH at the poles of the gonad (Fig. 4J, upper right of the white arrowheads) and the disruption of the tubular organization (Fig. 4J, yellow arrowhead) is consistent with a spatially organized anti-testis action. This phenotype is comparable to that observed in several mouse models of partial XY sex reversal (59), including mice transgenic for another candidate anti-testis gene, \textit{Dax1} (52). All the data are thus consistent with \textit{Foxl2} antagonism of embryonic testis differentiation to a significant though incompletely characterized extent.

\section*{DISCUSSION}

In mammals, sex determination in the embryonic gonad governs most features of phenotypic sex, and the \textit{SRY} gene located on the Y chromosome determines male sex (reviewed in 7). XX individuals can also be male, when translocations or transgenic constructs activate \textit{SRY} or the downstream transcription factor \textit{Sox9} (6,31,32,60). It has been suggested that XX maleness might also ensue if a putative master female regulatory gene (\textit{Od/Z}) (9,10) were inactivated. In fact, complete XX sex reversal involving the disruption of ovarian factors has been inferred in humans and other mammals (10,39), and natural mutations were found to affect \textit{Foxl2} in goats (11,12) and \textit{RSPO1} in humans (13). However, establishing the role of the genes involved requires the creation of experimental models, and in mice, the most favorable current mammalian experimental system, only partial forms of sex reversal affecting specific gonadal cell lineages have thus far been reported (9,15).

The \textit{Wnt4} \textit{Foxl2} double knockout ovaries produce testis-like tubules (Figs 1–3) and spermatogonia (Fig. 2 and Supplementary Material, Fig. S2), thus providing a first experimental model of XX sex reversal by loss of ovarian gene activity in mammals. This demonstrates that female sex determining genes are required to suppress an alternative male fate in the ovary, acting as a female equivalent of \textit{Sry} (9,10). Thus, \textit{Wnt4} and \textit{Foxl2} are part of the putative primary \textquoteleft ovary organizer\textquoteright{} or act immediately downstream of it. Indeed, the data formally reflect either primary or secondary gonadal sex reversal, and result from corresponding defects of induction or maintenance of female sex determination (4).
In either case, the outcome of the loss of Wnt4 and Foxl2 has primary sex reversal-like features, indicating that these genes are bona fide Z factors (10). Genes acting upstream or downstream of Wnt4 and Foxl2 in the ovary may thus be involved in cases of human 46,XX maleness and 46,XX true hermaphroditism that are not accompanied by extragonadal anomalies (10,39).

Can Wnt4 and Foxl2 repress weak alleles of Sry, thus accounting for a second postulated action of the ovary organizer, Od (9)? The partial anti-testis action observed in XY transgenic embryos is consistent with this possibility (Fig. 4I–K, discussed earlier). However, more detailed studies are required to test if the sex determining action of Wnt4 and Foxl2 starts early enough to interfere with Sry under physiological conditions. Studies of embryonic ovaries lacking Wnt4 and Foxl2 could help to discriminate between these possibilities. However, logistical barriers to such studies in ongoing work include the wide range of interindividual variation among the double knock-out mice (discussed earlier) and the complex mouse breeding schemes required to circumvent high mortality and infertility in the single knock-out mice (14,20).

Figure 5A and B schematizes two alternatives for the actions of Wnt4 and Foxl2. These two genes compensate for one another to repress Sox9 expression and male sex determination during ovary development. In one scenario (Fig. 5A), they act as early (primary) female sex determining genes, involved in the Od/Z ovary organizer in its simplest formulation (9,10). In particular, Wnt4 could start to be effective slightly earlier than Foxl2 (11.5 versus 12.5 dpc), consistent with the reported early transient derepression of SOX9 in Wnt4-null ovaries (61). In an alternative scenario (Fig. 5B), Wnt4 and Foxl2 would both act later. Such timing would be more consistent with a maintenance role in female sex determination, but nevertheless, their action would presumably be effective before germ cells enter meiosis [which is complete by 16.5 dpc in XX mice (62)], because oocytes are unlikely to transdifferentiate into spermatogonia. Unknown gene(s) may account for an early Od function independently of Wnt4 and Foxl2, but any such genes would dictate equivalent Z anti-testis actions in the developing ovary (Fig. 5B).

Mouse knockout models have previously shown that Wnt4 is required to activate follistatin (Fst), which together with Wnt4 antagonizes the expression and activity of inhibin beta B (Inhbb) (24,63). Derepression of Inhbb in the absence of Wnt4 and Fst accounts for male-like differentiation of vasculature and cortical stroma in Wnt4+/− and, to a lesser degree, Fst−/− ovaries (63). We found that Wnt4 and Foxl2 are additively required to maintain Fst expression at birth and possibly throughout fetal life (Fig. 3C and data not shown). This leads to an inferred partial model for molecular interactions underlying mouse ovary differentiation, as summarized in Figure 5C. Additional genes may act downstream of Wnt4 (in particular Dax1/Nr0b1 and, possibly FZD6), but their functions in the embryonic ovary are currently hypothetical (26 and 64, discussed earlier). As for Rspo1 (13), its expression is partly independent of Wnt4 and Foxl2, because it persists, though at reduced levels, in the Foxl2−/−Wnt4−/− double knockout ovaries at birth (Fig. 3B). In addition, we found normal RNA levels of Rspo1 in Wnt4−/− or Foxl2−/− single knockout ovaries during fetal life and in oocyte-depleted KitWv/Wv and Foxl2−/− KitWv/Wv ovaries at birth (data not shown). This is consistent with the proposal that Rspo1 may autonomously initiate female sex determination in mouse ovarian somatic cells (Fig. 5C) and with the possibility that RSPO1 and WNT4 might signal, in parallel to Wnt4 and Foxl2, and may signal through receptors in common with WNT4. Hammerhead lines indicate repressive interactions; the arrows indicate inductive actions. The time arrows indicate the inferred initiation of Wnt4 and Foxl2 actions during mouse development (dpc).

Figure 5. Schematics of two models of Wnt4 and Foxl2 action to suppress testis differentiation. (A) Outlines action based on the previously inferred ‘ovary organizer’ functions. Od and Z (see text). (B) Is based on an early maintenance mechanism, equivalent to Z but not Od (B). Sry (or Sox9 overexpression) represses Wnt4 and Foxl2 either directly (A) or via a putative upstream gene, Od (B), leading to testis differentiation, in the male embryo. In both models, Wnt4 and Foxl2 repress Sox9 in the ovary (Z action, indicated by a red double arrow in B, and they can repress Sry in model A but not B (Od action). (C) Summarizes data from this study and previous analyses on Wnt4−/−, Fst−/− and Inhbb−/− knockout mice (see text). The model further suggests that Nr0b1/Dax1 and, possibly, FZD6 may act downstream of Wnt4. Finally, early activity of Rspo1 may regulate an anti-testis pathway in parallel to Wnt4 and Foxl2, and may signal through receptors in common with WNT4. Hammerhead lines indicate repressive interactions; the arrows indicate inductive actions. The time arrows indicate the inferred initiation of Wnt4 and Foxl2 actions during mouse development (dpc).
traditionally assumed (4, 15). It is notable that Foxl2<sup>−/−</sup> Kit<sup>Wv/Wv</sup> double mutant ovaries show a sex reversal phenotype that is intermediate between the phenotypes resulting from loss of Foxl2 alone and loss of both Foxl2 and Wnt4 (Fig. 4A–H). This suggests that the combined ablation of other ovarian factors may produce a wide range of the degree or timing of sex reversal, and can aid in dissecting the regulatory circuit and mechanism of female sex determination (discussed subsequently). We suggest that in perinatal wild-type ovaries, after Wnt4 is downregulated, Foxl2 may be the dominant anti-testis factor as well as a major effector in ovary morphogenesis.

These findings impinge on long-standing discussions about the role of female somatic cells in mammalian germ cell sex determination. The determinative role of male somatic cells for spermatocyte formation from the bipotential gonad has been unequivocal, but it has been suggested that somatic cells may be dispensable for the induction of oocyte formation in the ovary (18, 65). This possibility was raised by the observation that XX and XY germ cells enter oogenesis when they migrate to abnormal locations outside the gonads (66, 67). However, indirect evidence against an autonomous mechanism of female germ cell sex determination in the ovary was provided by gene expression studies in wild-type and mutant mice (40, 68, 69). Several reports, including microarray analyses on isolated cells, indicate selective expression for Foxl2, and likely for Wnt4, in somatic cells (20, 21, 24, 27–30). Therefore, the presence of spermatogonia in XX gonads lacking Wnt4 and Foxl2 now demonstrates that female germ cell sex determination, or the early steps of its maintenance, indeed require somatic genes. However, we cannot exclude that this requirement may be confined to the spatially restricted domain of the ovarian medulla.

The sum of current studies thus suggests that multiple redundant anti-testis activities are deployed in the mouse fetal ovary, involving the independent action of Foxl2, Wnt4 and other factor(s) (possibly Rspo1; discussed earlier). This corroborates previous suggestions that several autonomous developmental modules may underlie female sex determination in mammals (16). However, the degree of interdependence among female sex determining genes may be greater in large mammals than rodents. Indeed, our data combined with previous studies (12, 15) strongly indicate that in goats, early complete XX sex reversal occurs after inactivation of Foxl2 — and most parsimoniously, of Foxl2 alone. Consequently, we suggest that positive feedback loops among female sex determining genes are stronger and/or are established earlier in goats than mice. Consistent with an evolutionary increase in the integration of pathways, most features of ovary differentiation overlap in time extensively in large mammals, whereas sequential phasing is the rule in rodents (70, 71). We note that a similar divergence in regulatory connectivity, on a larger evolutionary scale, was recently reported for another classical model of cell fate determination involving the myogenic pathway (72).

Independent of the degree of conservation of gene interactions in the regulatory hierarchy, the general role of the somatic factors Foxl2 and Wnt4 in mammalian female sex determination is now unequivocal. In addition, because of the greater autonomy of Foxl2, Wnt4 and other ovarian gene(s) in the mouse, this model system should facilitate the discrimination of their respective contributions to the establishment and/or maintenance of female sex.

**Materials and Methods**

**Mouse strains**

The generation of Wnt4 and Foxl2 knockout mice was previously reported (20, 29). We obtained Wnt4<sup>−/−</sup> and Kit<sup>Wv/Wv</sup> mice from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were euthanized ethically according to ACUC-approved NIA Animal Protocols. They were maintained on a mixed C57B6/J/129/SVJ genetic background. The construct used for the Foxl2 transgenics contained the 0.7 kb promoter from the mouse hsp68 gene (58) followed by the mouse Foxl2 coding sequence and a rabbit beta-globin 3'-UTR (the latter from the pTRE2 vector, Clontech). A 2.4 kb tandem insulator from chicken beta-globin was excised from pJC13-1 (the generous gift of Dr G. Fenselward) and placed upstream of the promoter sequence. Transgenic founders were produced by pronuclear injection (FVB/N strain) and three lines were studied (49, 50 and 191). Western blot detected the protein of the correct size in the adult testis of transgenic mice (data not shown). Mild heat treatment was performed on pregnant females between 10.5 and 12.5 dpc according to an established protocol (8–13 min at 41°C and 75% humidity, every 24 h) (73).

**RNA expression analysis**

For each genotype, gonads from three to five pups were separately processed and analyzed (thus representing biological replicates). Total RNA was obtained from dissected gonads by enzymatic extraction (Melt<sup>®</sup>, Ambion) followed by linear RNA amplification using Pico Ovation<sup>TM</sup> (NuGEN). Amplified RNA was affinity purified (Zymo Research) and labeled for MOE430 v.2.0 arrays (Affymetrix). As testis controls for the microarray studies, we used Wnt4<sup>−/−</sup> XY males in order to avoid possible confounding factors related to the requirement of Wnt4 for some aspects of testis differentiation (7); we verified that the levels of the genes tested were comparable to Wnt4<sup>+/−</sup> XY testes by real-time PCR (Supplementary Material, Table S2). Microarray data were processed with the Plier program on R (cran.r-project.org). In this study, we did not perform further statistical analyses of the data, but used the normalized expression values of specific markers of interest (Supplementary Material, Table S2), which were selected based on the literature or other analyses on publicly available microarray data (see Microarray analyses on public data sets). We validated these markers by quantitative real-time PCR (TaqMan) using an ABI 7900HT system (Applied Biosystems) (Supplementary Material, Table S2 and Fig. S2).

**Microarray analyses on public data sets**

For spermatogonial markers, we used the following criteria: (i) enrichment in the newborn testis relative to fetal ovary and epididymis (unpublished data set, GDS565 from the NIH public repository, www.ncbi.nlm.nih.gov/projects/geo/gds/);
(ii) enrichment in purified spermatogonia relative to later stages (37); (iii) undetectable expression in peri/postnatal oocytes (30); (iv) undetectable expression in somatic cells isolated from embryo-fetal testes (21) and (v) undetectable expression in somatic cells isolated from postnatal testes compared to whole gonads (36). The data processed by the authors of the referenced studies were analyzed by the Focus program, using contrast statistics to detect differential expression of genes and to identify correlated profiles (74). The final list of candidate spermatogonia markers was manually curated (Supplementary Material, Table S1). Of the five genes tested by real-time PCR, four of them were found to be testis-specific in our samples (Xmr, C530008M17, Clgn and Asb9), whereas a fifth gene (Ccdc13) showed a distinct profile, consistent with expression in oocytes (Supplementary Material, Table S1, Fig. S2 and data not shown).

For somatic testis markers, it is well-known that testis tubule formation and folliculogenesis in newborn ovaries upregulate a common set of genes that are silent in the fetal ovary; thus, many testis markers that are reliable when used in embryonic gonads, loose their sex specificity at birth (e.g. 14,75). Consistent with this pattern of expression, many genes upregulated in single knockout ovaries, notably in Wnt4−/− newborn ovaries and Foxl2−/− ovaries aged 7 dpn or older, were expressed in both embryonic testis (21) and adult ovary (based on the public microarray data set GDS565, at www.ncbi.nlm.nih.gov/projects/geo/gds/) (Supplementary Material, Fig. S3). We therefore restrict our discussion to genes that are known to be relevant for the phenotype and show sexually dimorphic expression throughout development (Fig. 3A–C).

Immunohistochemistry and electron microscopy

Protocols were as reported (29). Primary antibodies (tested for specificity by western blot) were from the following sources: FOXL2 (29), AMH/MIS, SOX9, PLZF (Santa Cruz Biotechnology), γH2AX (Upstate), SYCP3 (Abcam), WT1 (Dako).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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