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Sex Reversal in C57BL/6J XY Mice Caused by Increased Expression of Ovarian Genes and Insufficient Activation of the Testis Determining Pathway

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Abstract

Sex reversal can occur in XY humans with only a single functional WT1 or SF1 allele or a duplication of the chromosome region containing WNT4. In contrast, XY mice with only a single functional Wt1, Sf1, or Wnt4 allele, or mice that over-express Wnt4 from a transgene, reportedly are not sex-reversed. Because genetic background plays a critical role in testis differentiation, particularly in C57BL/6J (B6) mice, we tested the hypothesis that Wt1, Sf1, and Wnt4 are dosage sensitive in B6 XY mice. We found that reduced Wt1 or Sf1 dosage in B6 XY mice impaired testis differentiation, but no ovarian tissue developed. If, however, a YB6 chromosome replaced the YB6 chromosome, these otherwise genetically identical B6 XY mice developed ovarian tissue. In contrast, reduced Wnt4 dosage increased the amount of testicular tissue present in SF1+/−/−, B6 XYWnt4+/+, and B6 XYWnt4+/− fetuses. We propose that Wt1B6 and Sf1B6 are hypomorphic alleles of testis-determining pathway genes and that Wnt4B6 is a hypermorphic allele of an ovary-determining pathway gene. The latter hypothesis is supported by the finding that expression of Wnt4 and four other genes in the ovary-determining pathway are elevated in normal B6 XX E12.5 ovaries. We propose that B6 mice are sensitive to XY sex reversal, at least in part, because they carry Wt1B6 and/or Sf1B6 alleles that compromise testis differentiation and a Wnt4B6 allele that promotes ovary differentiation and thereby antagonizes testis differentiation. Addition of a “weak” Sry allele, such as the one on the YP0S chromosome, to the sensitized B6 background results in inappropriate development of ovarian tissue. We conclude that Wt1, Sf1, and Wnt4 are dosage-sensitive in mice, this dosage-sensitivity is genetic background-dependant, and the mouse strains described here are good models for the investigation of human dosage-sensitive XY sex reversal.

Introduction

Testis differentiation in humans is gene dosage-sensitive. For example, heterozygosity for WT1 or SF1 (NR5A1) mutations and duplications containing WNT4 has been associated with XY sex reversal [1–10]. Testis differentiation in humans also appears to be genetic background-sensitive because fathers who are heterozygous for SF1 mutations or hemizygous for SRY mutations can pass these mutations to their heterozygous/hemizygous XY daughters [4,11,12]. In contrast, initial studies in mice suggested testis differentiation is dosage-insensitive because heterozygosity for Wt1 or Sf1 null alleles or transgenic Wnt4 over-expression did not cause XY SR [13–17]. This apparent human/mouse species difference led to the hypothesis that some genetic events leading to testis development differed between these species [18]. However, given the evidence that in mice genetic background plays a critical role in testis differentiation [19,20], we examined the possibility that Wt1, Sf1 and/or Wnt4 would be haploinsufficient for normal testis determination on specific genetic backgrounds.

Work by our group and others has shown that testis determination in C57BL/6J (B6) inbred mice is particularly sensitive to genetic perturbation and a number of inherited gonadal sex reversal conditions have been identified in which B6 XY mice develop ovarian tissue [19–23]. The founding member of this group, B6-YP0S sex reversal, occurs when the Y Chromosome (Chr) from Mus domesticus poashinus (YP0S) is transferred to the B6 genetic background, replacing the Mus musculus-derived YB6 Chr [19]. B6 XY YP0S fetal gonads develop as either ovotestes (gonads that contain both ovarian and testicular tissue) or ovaries, but not normal testes. The YP0S Chr is not inherently defective, however, because it is testis-determining when transferred to most other inbred strain backgrounds, including DBA/2J (D2), BALB/cBy, C3H/HeJ and 129, or when present in F1 hybrids, such as in (D2×B6)/F1 mice [24–27]. Furthermore, not all M. domesticus-derived Y Chr (i.e., Sry alleles) behave like YP0S (SryYP0S) when
Author Summary

It has been proposed that mice do not adequately model human disorders of sex development because testis determination is gene dosage-sensitive in humans, but initial studies suggested it is gene dosage-insensitive in mice. For example, XY humans with reduced functional \(WT1\) or \(SF1\) gene-dosage or increased \(WNT4\) gene-dosage can be sex reversed, whereas the equivalent XY mice were not sex-reversed on the genetic backgrounds previously reported. However, because testis determination in C57BL/6J mice is very sensitive to disruption, we tested the hypothesis that these genes are dosage-sensitive in C57BL/6J XY mice. We found that C57BL/6J-YAKR mice with reduced \(WT1\) or \(SF1\) gene-dosage were sex-reversed, whereas decreased \(Wnt4\) gene-dosage partially rescued testis development in four genetic systems where C57BL/6J XY mice develop ovarian tissue. Our results demonstrate that \(WT1\), \(SF1\), and \(Wnt4\) are dosage-sensitive in mice, that genetic background affects this sensitivity (as we suspect is true in humans), and that the mouse models described here are good models for human dosage-sensitive sex reversal. The results from these and other experiments lead to the hypothesis that the \(WT1\) and/or \(SF1\) alleles found in C57BL/6J mice are relatively “weak” testis-determining genes and their \(Wnt4\) allele is a hyperactive ovary-determining gene.

transferred to B6. For example, testis differentiation in B6 XY mice containing the BUB/BnJ Y Chr appears to be normal whereas testis differentiation in B6 XY mice containing the AKR/J YChr is transiently delayed [28]. B6 XYAKR mice do not develop permanent ovaries or ovotestes like B6 XYB6POS mice. However, if B6 XYAKR mice are heterozygous for either the \(T^{sh}\) or \(T^{hp}\) deletion on Chr 17 they develop bilateral ovaries. In contrast, B6 XYB6 or B6 XYB6BUR mice heterozygous for the \(T^{dh}\) deletion develop testes. Thus, the B6-YAKR consomic strain is more sensitive to XY sex reversal than the B6 strain. We previously showed that the transient delay in testicular cord differentiation is caused by the presence of particular SRY protein isoforms found in \(M.\) domesticus mice and that the reduced expression of some of these variants, such as the \(SRY^{POS}\) allele, causes permanent sex reversal [29].

Further investigations revealed that B6-YPOS sex reversal occurs because the \(Sry\) testis-determining gene present on the YPOS Chr does not properly induce testis development if specific interacting genes, designated \(tda\) (testis determining autosomal) genes, are homozygous for B6 alleles. Two \(tda\) loci that differ between the B6 and D2 genomes and play a role in B6-YPOS sex reversal were mapped to Chr 2 (\(tda2\)) and 4 (\(tda1\)) [24]. Intriguingly, the \(Wt1\) (Wilms’ tumor 1) and \(SF1\) (steroidogenic factor 1, officially \(NR5A1\); nuclear receptor subfamily 5, group A, member 1, also known as \(Ad4BP\); adrenal 4-binding protein) map to the \(tda2\) chromosomal region and \(Wnt4\) (wingless-related MMTV integration site 4) to the \(tda1\) region. Thus, we investigated \(Wt1\) and \(SF1\) as candidates for \(tda2\), and \(Wnt4\) as a candidate for \(tda1\).

The \(Wt1\) and \(SF1\) genes share a number of characteristics in addition to those noted above, some of which result from the fact that \(Wt1\) is a direct activator of \(SF1\) expression [30] including: 1) both encode zinc finger transcription factors [31–33]; 2) both are expressed in XX and XY mouse urogenital ridges from about embryonic day (E) 9.5 until E12.5, when expression becomes sexually dimorphic and cell-type restricted; and 3) the gonad progenitor (i.e., genital ridge) is initially present in fetal mice that are homozygous for a null allele of either gene, but differentiation of the genital ridge does not progress and it regresses via apoptosis [14–17]. The \(Wt1\) gene encodes a member of the WNT family of secreted signaling molecules that regulate cell-cell interactions during development. \(Wt1\) is initially expressed in XX and XY genital ridges; thereafter expression is down-regulated in XY gonads but maintained in XX gonads [34]. Homozygosity for a \(Wt1^{POS}\) mutation causes partial sex reversal of XX gonads resulting in the development of a testis-like coelomic vessel and the presence of cells expressing steroidogenic enzymes [34,35]. In contrast, the gonadal phenotype in homozygous \(Wnt4^{null}\) null XY mice is relatively less severe; the gonads develop as testes, but the cords are initially fewer in number and disorganized [36]. To our knowledge, \(Wt1\) heterozygotes have no reported gonad differentiation defects. In XY mice, transgenic \(Wnt4^{POS}\) over-expression also causes testis differentiation defects but not sex reversal [13,35].

To examine the possibility that the \(Wt1\), \(SF1\), and \(Wnt4\) genes would be haploinsufficient on the B6 genetic background, we transferred null alleles to the B6 and D2 backgrounds and determined if reduced dosage influenced gonadal development in XYB6 and XYAKR mice. Gonad morphology and marker gene expression in mutant versus normal fetal mice was analyzed using immunohistochemistry and confocal microscopy, RNA in situ hybridization, and quantitative RT-PCR. The results of these experiments indicated that testis differentiation was compromised at a very early stage and that \(Sry\) expression was significantly reduced in \(Wt1^{POS}+/-\) or \(SF1^{POS}+/-\) B6 XYAKR but not in \(Wt1^{POS}+/-\) or \(SF1^{POS}+/-\) (D2×B6)F1 XYAKR fetal gonads. We also found that testis development was mostly rescued in \(Wt1^{POS}+/-\) and \(SF1^{POS}+/-\) B6 XYAKR fetuses that contained two \(Sry\) alleles. These results are consistent with the hypothesis that the \(Wt1^{B6}\) and \(SF1^{B6}\) alleles behave as hypomorphic alleles of testis-determining pathway genes in B6 XY mice if other genes in the genome are homozygous for the B6 allele. In contrast, reduced \(Wnt4\) dosage did not exacerbate the transient delay in testis differentiation in B6 XYAKR but rather appeared to ameliorate the phenotype, which suggests that the B6-derived \(Wnt4^{POS}\) allele (\(Wnt4^{POS}\)) is a hypermorphic allele of a gene in the ovary determination pathway. We also found that B6 \(Wt1^{POS}/-\) XYAKR, B6 \(SF1^{POS}/-\) XYAKR, and B6 XYB6POS fetuses with reduced \(Wnt4\) dosage developed more testicular tissue than \(Wnt4^{POS}/+\) fetuses. These data are consistent with the hypothesis that \(Wnt4^{POS}\) is an early functioning allele of an ovary-determining gene that antagonizes testis differentiation in XY mice. Expression of \(Wnt4\) and four other ovary-biased or ovary-specific genes was found to be elevated in E12.5 B6 vs. D2 XX ovaries. Together, our results suggest that B6 mice are sensitive to XY sex reversal compared to D2 mice because genes in the ovary determination pathway function earlier in the B6 genome than they do in the D2 genome.

The results reported here show that the \(Wt1\), \(SF1\), and \(Wnt4\) genes are dosage-sensitive during mouse primary sex determination, as they are in humans, and that this effect is modulated by genetic background in the mouse, as is probably the case in humans. We propose that the dosage-sensitive C57BL/6J XY mouse strains described here are powerful models for further investigation of human dosage-sensitive XY sex reversals. In addition, our results support the candidacy of \(Wt1\), \(SF1\), and \(Wnt4\) as \(tda\) genes, and suggest the testable hypothesis that B6 XYB6POS sex reversal is caused by the additive effects of: 1) hypomorphic \(Wt1^{B6}\) and \(SF1^{B6}\) alleles, and reduced activation of the \(Wnt4\) differentiation pathway, 2) a hypermorphic \(Wnt4^{POS}\) allele and hyperactivation of the ovary differentiation pathway, and 3) reduced function and expression of the \(SRY^{POS}\) allele.
Results

Wt1+/− and Sf1+/− B6 XYAKR mice are sex-reversed

To examine the possibility that Wt1 and/or Sf1 would be haploinsufficient on the B6 genetic background and to investigate their candidacy as ida genes, we transferred null alleles to the B6 and D2 backgrounds and determined if reduced dosage influenced gonadal development in XBy6 and XYAKR mice. We reasoned that if the B6 alleles of Wt1 and/or Sf1 function as hypomorphs in the testis determination pathway, then further reducing their dosage in B6 XY mice should cause sex reversal, whereas reducing their dosage in D2 XY mice should not. Sex reversal was not evident in Wt1+/− or Sf1+/− B6 or D2 XY weanling or adult mice, and the mice were fertile. To increase the sensitivity of Wt1 dosage in B6 XY mice should cause sex reversal, whereas reducing (D2 × B6)F1 XYAKR gonads analyzed were tested, whereas the 33 Sf1+/− B6 XYAKR gonads analyzed were ovotestes (3) or ovaries (31). These results are consistent with the weaning analysis and show that sex reversal is the result of primary (gonadal) sex reversal.

Morphological and marker gene analysis of E13.5

Wt1+/− and Sf1+/− B6 XYAKR gonads

To gain further insight into the mechanism of sex reversal in Wt1+/− and Sf1+/− B6 XYAKR gonads, whole-mount immunohistochemistry (WHIC, Figure 1) and whole-mount RNA in situ hybridization (WISH, Figure 2) were used to analyze morphology and marker gene expression in E13.5 gonads. Overall, the morphological analyses were consistent with the E14.5–16 analyses: Wt1+/− B6 XYAKR gonads were classified as ovaries, ovotestes and testes and Sf1+/− B6 XYAKR gonads were classified as ovaries and ovotestes. The Wt1+/− and Sf1+/− B6 XYAKR gonads that developed as ovaries or ovotestes were phenotypically indistinguishable regardless of which gene was mutant. However, some Wt1+/− B6 XYAKR gonads appeared to be very similar in morphology and marker gene expression pattern to those observed in control +/+ B6 XYAKR gonads, whereas none of the Sf1+/− B6 XYAKR gonads were phenotypically similar to +/+ B6 XYAKR gonads.

Generally, Wt1+/− and Sf1+/− B6 XYAKR gonads that developed as ovaries were morphologically indistinguishable from normal XX ovaries and strongly expressed ovary-specific markers, such as FOXL2 (Figure 1F), Wnt4 (Figure 2F), Foxl2 (data not shown) and Bmp2 (data not shown) in a pattern similar to XX ovaries. Additionally, XY ovaries generally did not express testis-specific Sertoli cell markers, such as AMH (Figure 1F) or SOX9 (data not shown), or Leydig cell markers, such as P450scc (Figure 2L), or markers such as GATA4, WT1 (data not shown) or FGRF2 (Figure 1L) in a testis-like pattern. However, about 20% of XY mutant gonads that had ovarian morphology contained cells that expressed Sertoli cell-specific markers, such as AMH or SOX9 (data not shown), or expressed markers, such as PDGFRα and FGRF2 in a testis-like pattern (Figure 1L). However, these cells were not organized in a pattern that resembled testicular cords. We conclude that Sertoli cell differentiation was initiated in some XY gonads that were morphologically ovarian, but was insufficient to initiate or sustain cord formation.

In Wt1+/− and Sf1+/− B6 XYAKR ovotestes, testis markers generally were expressed in central regions containing cords whereas ovary markers were expressed at the anterior and posterior poles within regions lacking cords. For example, Figure 1C shows part of an ovotestis in which the central region

Table 1. External sexual phenotype of Wt1+/− and Sf1+/− weaning age mice.

| Maternal genotype | Paternal genotype | #/+− Females | #/+− Hermaphrodites | #/+− Males | Total #/+− XY pups | Total XY pups |
|-------------------|------------------|--------------|---------------------|----------|-------------------|-------------|
| B6 Wt1+/−         | B6 +/+ XYAKR     | 8 (21%)      | 11 (28%)            | 20 (51%) | 39                | 81          |
| B6 Wt1+/−         | AKR +/+ XYAKR    | 0 (0%)       | 0 (0%)              | 20 (100%)| 20                | 47          |
| B6 Sf1+/−         | B6 +/+ XYAKR     | 16 (80%)     | 4 (20%)             | 0 (0%)   | 20                | 95*         |
| D2 Sf1+/−         | B6 +/+ XYAKR     | 0 (0%)       | 0 (0%)              | 13 (100%)| 13                | 18          |

*Approximately 30% of B6 Sf1+/− pups die prior to weaning.
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Table 2. Gonad phenotype in E14.5–16 Wt1+/– and Sf1+/– XY fetuses.

| Maternal genotype | Paternal genotype | f/– Ovaries | f/– Ovotestes | f/– Tests | f/– Abnormal testesa | Total f/– XY gonads |
|-------------------|-------------------|------------|-------------|----------|----------------------|-------------------|
| B6 +/–            | B6 Wt1+/– XYAKR   | 0 (0%)     | 0 (0%)      | 28 (100%)| 0 (0%)               | 28                |
| B6 Wt1+/–         | B6 +/+ XYAKR      | 5b (10%)   | 23 (48%)    | 8 (17%)  | 12 (25%)             | 48                |
| B6 Wt1+/–         | D2 +/+ XYAKR      | 0 (0%)     | 0 (0%)      | 24 (100%)| 0 (0%)               | 24                |
| D2 Wt1+/–         | B6 +/+ XYAKR      | 0 (0%)     | 0 (0%)      | 12 (100%)| 0 (0%)               | 12                |
| B6 Sf1+/–         | B6 +/+ XYAKR      | 0 (0%)     | 0 (0%)      | 20 (100%)| 0 (0%)               | 20                |
| D2 Sf1+/–         | B6 +/+ XYAKR      | 0 (0%)     | 0 (0%)      | 14 (100%)| 0 (0%)               | 14                |

aCord development is somewhat delayed at the anterior and/or posterior ends but the gonad is not an obvious ovotestis.

The presence of cords and strongly expressed AMH, while the adjacent posterior region lacked cords and expressed FOXL2. Figure 2J shows a ovotestis that expressed Pthl1 in the central region that contained testicular cords, but not in the ovarian regions at the anterior and posterior poles. [Pthl1 expression in ovotestes was not as strong as that observed in B6 XYAKR control gonads (compare Figure 2H and 2J)]. Conversely, Figure 2D shows an ovotestis expressing Wnt4 in the anterior and posterior regions, but not in the central testicular region. Occasionally, testis- and ovary-specific markers were expressed in the same region (Figure 1E), where FOXL2 expression was detected in specific cells within a region containing well-formed testicular cords and strongly expressing the Sertoli cell marker AMH, which we noted a few cells that appeared to express both AMH and FOXL2 (Figure S2A). However, because only whole-mount tissue was examined and because AMH is localized to the cell surface and FOXL2 to the nucleus, we were not able to exclude the possibility that the expression was in adjacent cells. Nevertheless, it is possible that some supporting cell progenitors can express both granulosa and Sertoli cell markers under certain conditions.

Because Wt1 is functionally upstream of Sf1 and is necessary for the initiation of Sf1 expression [30], we examined Sf1 expression in Wt1+/– B6 XY AKR gonads using WHIC and found that expression levels appeared normal (Figure 1N–1O). Additionally, quantitative real-time RT-PCR (qRT-PCR) analysis did not detect a significant difference in Sf1 expression in E12 gonads from Wt1+/– B6 XY AKR vs. +/+ B6 XY AKR fetuses (data not shown). On the other hand, Wnt4 expression appeared to be reduced in Wt1+/– and Sf1+/– B6 XX ovaries compared to +/+ B6 XX ovaries, and Pthl1 expression was strongly reduced in Wt1+/– B6 XX and B6 XY mesonephros (Figure 2). Overall, the morphological and marker gene expression analyses showed that the testis determination pathway is aberrant at an early stage, that the ovarian pathway is activated in Wt1+/– and Sf1+/– B6 XY AKR gonads, and that gene expression is affected in both XX and XY heterozygous gonad/mesonephros complexes.

A multigene qRT-PCR strategy was used to quantify gene expression changes in E12 +/– vs. +/+ B6 XY AKR gonads, and E14 +/– ovaries vs. +/+ B6 XY AKR testes (Table 3). These quantitative results were consistent with the morphological and marker gene analyses. For example, Adamb19, an ovary-specific gene, was significantly up-regulated in E14 Wt1+/– and Sf1+/– B6 XY AKR ovaries vs. +/+ B6 XY AKR control testes, and Cyp11a1 and Cyp17a1, both Leydig cell specific genes, were expressed at significantly lower levels in Wt1+/– and Sf1+/– B6 XY AKR ovaries.

Ovary-specific markers are expressed at the anterior and posterior of B6 XY AKR gonads

During analysis of marker gene expression in E13.5 Wt1+/– and Sf1+/– B6 XY AKR gonads using WHIC and WISH we observed the expression of ovary-specific somatic cell markers in gonads from +/+ B6 XY AKR fetuses. As expected, testis-specific markers, such as SOX9, AMH and Pthl1, were expressed in the central regions that contained testicular cords (Figure 1, Figure 2, and Figure S2). However, FOXL2, Wnt4, and Fst often were expressed in the anterior and posterior regions that lacked cords (Figure 1, Figure 2, Figure S2, and data not shown), indicating that the ovary differentiation pathway was activated in these regions. WISH analysis of the meiotic germ cell markers Stra8 and Rec8 [37] and the ovary-specific somatic cell marker Brc3 [38] was used to determine if activation of the ovary differentiation pathway extended to the germ cells and to quantify the occurrence of ovary-specific marker gene expression (Figure S2B). We found that 11 of 13 B6 XY AKR gonads clearly expressed either Stra8 or Rec8 at one or both poles and that 7 of 7 gonads expressed Brc3 at both poles. In the 9 gonads where Stra8 or Rec8 were expressed at both poles, expression was higher at the anterior pole than the posterior pole, suggesting that the anterior-to-posterior wave of meiotic germ cell gene expression is maintained despite the presence of intervening testicular tissue [37]. It is important to note that testicular cords are present throughout B6 XY AKR gonads after about E14.5. Therefore, these results are consistent with the idea that B6 XY AKR gonads develop as transient ovotestes [39,40].

Testis and adrenal gland development is abnormal in B6 XY AKR fetuses that are double-heterozygous for Wt1 and Sf1

Because the Wt1+/– and Sf1+/– B6 XY AKR sex reversal phenotypes appear to be very similar and because Wt1 is necessary for activation of Sf1 expression in fetal gonads [30], we examined if B6 XY AKR mice were sex reversed when doubly heterozygous for Wt1 and Sf1 (Wt1+/–; Sf1+/–). We first attempted to ascertain the external sexual phenotype of the double heterozygotes at weaning. However, no Wt1+/–; Sf1+/– mice were recovered among the B4 progeny from B6 Wt1+/–×B6 Sf1+/– matings (Table 4). Because our previous results suggested that lethality
could be sensitive to genetic background, progeny from B6 Wt1+/− × D2 Sf1+/− matings were analyzed. Among the 25 offspring were 4 Wt1+/− × Sf1+/− mice, indicating that lethality was rescued in (B6×D2)F1 hybrids. To determine at which developmental stage the lethality occurred, E14.5–15.5 fetuses from B6 Wt1+/− × B6 Sf1+/− matings were analyzed. Among the 12 offspring were 3 Wt1+/− × Sf1+/− mice (2 XY and 1 XX), indicating that lethality occurred between E15.5 and weaning. Histological examination of the three Wt1+/− × Sf1+/− fetuses showed that each had bilateral adrenal agenesis, which likely would cause neonatal lethality (Figure 3A and 3B). Both of the Wt1+/− × Sf1+/− XY fetuses had bilateral testes that appeared morphologically normal, but were smaller than those in wild-type XY littersmates.

To extend these observations, gonad development was examined in E13.5 fetuses from B6 Wt1+/− × B6 Sf1+/− matings using WHIC (Figure 3C–3J). Of the 15 fetuses examined, 7 were Wt1+/− × Sf1+/−, which is consistent with the E14.5–15.5 survival data. Of the 9 XY B6 fetuses, 4 were Wt1+/− × Sf1+/−, 4 were Wt1+/+, Sf1−/−, and 1 was Wt1+/−; Sf1+/+. All 8 Wt1+/− × Sf1+/− XY gonads were classified as ovotestes, with large central testicular regions (Figure 3I and 3J). (Ovotestes in E13.5 double heterozygous fetuses appear to ovotestes, with large central testicular regions (Figure 3I and 3J).) The 8 gonads from Wt1+/− × Sf1+/− XY fetuses were classified as testes but their cords appeared to be less well developed and more disorganized than +/+ B6 XY gonads (Figure 3). None of the XY offspring from these intercrosses were Wt1+/+ × Sf1−/−, so we examined gonad development in E13.5 B6 Wt1+/− × B6 XY B6 fetuses generated from B6 Wt1+/− × B6 Sf1+/− × B6 XY B6 crosses. All 6 fetuses from Wt1+/− × B6 XY B6 were classified as testes. However, their cords appeared to be less well-developed and more disorganized than normal B6 XY gonads: 4 displayed disorganized anterior and posterior poles and 2 had disorganized posterior poles (Figure 3E and 3F). In comparison, of the 6 fetuses from +/+ × B6 XY B6 testes, 2 had testicular cords throughout and 4 had disorganized posterior poles. In summary, the degree of testicular cord development in the E13.5 B6 XY B6 fetuses could be ranked from most complete to least complete as: Sf1+/+; Wt1+/− × Sf1−/−; Wt1+/− × Sf1+/−; Wt1+/− × Sf1+/−; Wt1+/− × Sf1+/−. These results indicate that testis development is compromised in Wt1+/− × Sf1+/− B6 XY fetuses, even in the absence of the Y AKR Chr, and that testis development is further compromised in mice that are double heterozygous for Wt1 and Sf1 null alleles.

Sry expression is reduced in Wt1+/− and Sf1+/− B6 XY AKR gonads and sex reversal is partially rescued by increased Sry dosage

Because the morphological and marker gene expression analyses in E13.5 gonads suggested an early defect in the testis determination pathway, Sry expression in E11.5 Wt1+/− and Sf1+/− B6 XY AKR vs. +/+ B6 XY AKR gonads/mesonephros complexes was quantified. (Sry is not expressed in the mesonephros.) During peak Sry expression (E11.75), transcript levels in both mutants were less than 50% of the controls. We found that Sry transcript levels were reduced in Wt1+/− vs. +/+ samples at E11.25 (15–17 ts, t29 = 2.125, p < 0.0179), E11.5 (18–20 ts, t25 = 3.653, p < 0.0012), and E11.75 (21–23 ts, t5 = 3.511, p < 0.0014) but not significantly different at E12.0 (24–26 ts, Figure 4A). Sry transcript levels also were reduced in Sf1+/− vs. +/+ samples at E11.5 (18–20 ts, t29 = 2.224, p < 0.0422) and E11.75 (21–23 ts, t5 = 1.47, p < 0.0016), but were not significantly different at E12.25 (15–17 ts) or E12.0 (24–26 ts, Figure 4A). These results suggest that sex reversal in Wt1+/− × Sf1+/− B6 XY AKR gonads is at least partially due to a failure to sufficiently up-regulate Sry expression and properly initiate the testis determination pathway. However, because pairwise comparisons between Wt1+/− vs. Sf1+/− samples did not reveal significant differences in Sry expression at any of the embryonic stages analyzed, differences in the level of Sry expression do not explain the differences in the severity of the Wt1+/− and Sf1+/− phenotypes.

To test the hypothesis that sex reversal in Wt1+/− and Sf1+/− B6 XY AKR gonads is due, at least in part, to reduced Sry expression, gonad morphology was examined by WHIC in gonads from E13.5 fetuses that had increased Sry dosage due to the presence of the Y AKR Chr. [Classification was done independently by two investigators (SMC and KHA). This rearranged Y Chr carries two Sry alleles, one from the AKR/J inbred strain (M. domestica) and one from the RII inbred strain (M. musculus).] Gonads in Wt1+/− × Sf1+/− × B6 XY AKR,Sry fetuses consistently developed more testicular tissue than gonads in Wt1+/− × Sf1+/− × B6 XY AKR,Sry fetuses; however, it appeared that cord differentiation in the Wt1+/− × Sf1+/− × B6 XY AKR,Sry gonads was not completely normal (Figure 4B, Table 5). Of the 8 Wt1+/− × B6 XY AKR,Sry fetuses analyzed, 7 were classified as abnormal testes because the testicular cords appeared to be more disorganized and less well developed than those in +/+ B6 XY AKR,Sry gonads. In fact, these cords appeared to be similar to the abnormal cords in Wt1+/− × B6 XY B6 testes. One of the Wt1+/− × B6 XY
XYAKR,Sxr gonads was classified as an ovotestis because it lacked cords in a small region at the posterior pole and had disorganized testicular cords in the remainder of the gonad. Of the 8 $Sf1^{+/-}B6$ XYAKR,Sxr gonads analyzed, 3 were classified as abnormal testes, with testicular cords throughout the gonad, and 5 were classified as ovotestes due to the absence of testicular cords at the posterior pole. The cords in these 8 gonads also were somewhat disorganized and less differentiated. In contrast, all 17 of the $+/+$ B6 XYAKR,Sxr gonads examined developed robust, well-organized testicular cords throughout the anterior, center and posterior regions. These data indicate that increasing $Sry$ dosage in $Wt1^{+/-}$ and $Sf1^{+/-}B6$ XYAKR gonads by replacing the YAKR Chr with the YAKR,Sxr Chr dramatically increased the amount of testicular tissue that develops, but did not fully rescue cord differentiation.

Figure 2. WISH analysis of the ovary somatic cell marker $Wnt4$ and the testis Leydig cell marker $Ptch1$ in E13.5 gonad/mesonephros complexes. $Wnt4$ was expressed throughout $+/+$ XX ovaries, as expected (A), and at the anterior and/or posterior poles of $+/+$ B6 XYAKR gonads in the regions without cords, but not in the central region containing cords (B). Expression was detected at the anterior and/or posterior poles of $Wt1^{+/-}$ (D) and $Sf1^{+/-}$ (data not shown) B6 XYAKR ovotestes and throughout $Wt1^{+/-}$ (data not shown) and $Sf1^{+/-}$ B6 XYAKR ovaries (F). $Wnt4$ expression was reduced in B6 $Wt1^{+/-}$ XX (C) and $Sf1^{+/-}$ XX (E) ovaries compared to B6 $Wt1^{+/-}$ XX (A) ovaries. Expression also was reduced in B6 $Wt1^{+/-}$ mesonephroi (C and D), but not in $Sf1^{+/-}$ mesonephroi (E and F). $Ptch1$ expression was restricted to central regions containing testicular cords in $+/+$ B6 XYAKR gonads (H) and was not detected in $+/+$ B6 XX ovaries (G), as expected. Expression was not detected in $Wt1^{+/-}$ and $Sf1^{+/-}$ B6 XX ovaries (I and K). $Ptch1$ was expressed in the central regions of $Wt1^{+/-}$ (J) and $Sf1^{+/-}$ (data not shown) B6 XYAKR ovotestes but at notably lower levels than in $+/+$ B6 XYAKR gonads, and it was not expressed in $Wt1^{+/-}$ (data not shown) and $Sf1^{+/-}$ (L) XYAKR ovaries. $Ptch1$ expression was reduced in the anterior region of B6 $Wt1^{+/-}$ mesonephroi (I and J), but not in $Sf1^{+/-}$ mesonephroi (K and L). The gonad/mesonephros complex in each panel is oriented with the gonad above the mesonephros and separated by a dotted black line with the anterior (cranial) pole to the left. All images are at 10× magnification.

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Table 3. Quantitative RT–PCR analyses of E12 +/- versus +/+ B6 XYAKR gonads and E14 +/- B6 XYAKR ovaries (ovotestes excluded) versus +/- B6 XYAKR testes.

| Stage | Gene | Fold change in Wt1+/-- | Significant (p<0.05) |
|-------|------|------------------------|----------------------|
| E12   | Bmp2 | 2.4                    | T                    |
|       | Cbln4| -5.4                   | G, T                 |
|       | Dhh  | -6.0                   | G, T                 |
|       | Mro  | -6.0                   | G, T                 |
|       | Ptgds| -11.82                 | G, T                 |
|       | Defb19| -3.4                  | T                    |
| E14   | Bmp2 | 10.7                   | G, T                 |
|       | Fgr2 | 2.4                    | G                    |
|       | Fst  | 15.5                   | G, T                 |
|       | Wnt4 | 12.1                   | G, T                 |
|       | Aard | -4.1                   | G, T                 |
|       | Amh  | -20.8                  | G, T                 |
|       | Cbln4| -3.3                   | G, T                 |
|       | Cbln4| -3.9                   | G, T                 |
|       | C10a1| -2.1                   | G                    |
|       | C10a3| -6.9                   | G, T                 |
|       | Cst9 | -5.1                   | G, T                 |
|       | Cyp11a1| -4.7                | G, T                 |
|       | Cyp17a1| -8.2                  | G, T                 |
|       | Cyp26b1| -4.0                 | G, T                 |
|       | Dhh  | -7.1                   | G, T                 |
|       | Etd  | -18.3                  | T                    |
|       | Hhip | -4.1                   | G, T                 |
|       | Mro  | -2.1                   | G, T                 |
|       | Ptgds| -8.0                   | G, T                 |
|       | Ren1 | -4.8                   | G, T                 |
|       | Sostdc1| -4.7                 | G, T                 |
|       | Sox9 | -2.0                   | G, T                 |
|       | Defb19| -8.6                 | G, T                 |
| E14   | Bmp2 | 7.2                    | G, T                 |

Table 3. Cont.

| Stage | Gene | Fold change in Sf1+/- | Significant (p<0.05) |
|-------|------|------------------------|----------------------|
| Fgr2  | 2.9  | G                      |                      |
| Fst   | 25.9 | G, T                   |                      |
| Sry   | 56.9 | G, T                   |                      |
| Wnt4  | 3.0  | G                      |                      |
| Aard  | -3.4 | T                      |                      |
| Amh   | -29.5| T                      |                      |
| Cbln1| -9.6  | G, T                   |                      |
| Cbln4| -4.3  | G, T                   |                      |
| Col9a3| -4.6  | T                      |                      |
| Cst9  | -7.3  | T                      |                      |
| Cyp11a1| -66.9 | T                      |                      |
| Cyp17a1| -89.9 | T                      |                      |
| Cyp26b1| -8.3  | T                      |                      |
| Dhh   | -9.1  | P = 0.06               |                      |
| Etd   | -22.3 | G, T                   |                      |
| Hhip  | -6.1  | G                      |                      |
| Hsdb1| -14.7 | G, T                   |                      |
| Mro   | -2.4  | G, T                   |                      |
| Ptgds | -15.4 | G, T                   |                      |
| Ren1  | -28.9 | T                      |                      |
| Scarb1| -2.8  | G, T                   |                      |
| Sostdc1| -8.2  | G, T                   |                      |
| Sox9  | -3.2  | G, T                   |                      |
| Defb19| -18.5 | G, T                   |                      |
| Vnn1  | -2.6  | G, T                   |                      |

G = GPR score equal or greater than 0.4.
T = Student’s t-test.

The Wt1 and Sf1 B6 alleles require additional interacting genes to be homozygous for the B6 allele to function as hypomorphs

The results presented above suggest that the Wt1B6 and Sf1B6 alleles are hypomorphic alleles. One possibility is that the Wt1B6 and Sf1B6 alleles behave as intrinsic hypomorphs. An alternative possibility is that the Wt1B6 and Sf1B6 alleles function as hypomorphs only when one or more additional interacting gene(s) are homozygous for B6 alleles. To distinguish between these two alternatives, genetic crosses were designed that would generate hybrid (D2 x B6)F1 XY AKR mice that are heterozygous for the null (knockout) allele of Wt1 or Sf1 and the Wt1B6 or Sf1B6 allele, respectively (e.g. Wt1KO/Wt1B6 or Sf1KO/Sf1B6) (Table 2). These F1 hybrid mice are genetically identical to Wt1B6/Sf1B6 and Wt1+/-- B6 XYAKR mice at the Wt1 and Sf1 loci; however, the remainder of the genome is different because it is (D2 x B6)F1 hybrid (except for the regions immediately around the Sf1KO and Wt1KO alleles that are derived from the 129 strain). Because Wt1+/-- and Sf1+/-- (D2 x B6)F1 XY AKR mice are not sex reversed, we conclude that the Wt1B6 and Sf1B6 alleles function as hypomorphs only when other genes (modifier genes) are homozygous for B6 alleles.

Molecular analysis of the difference between the B6 and D2 alleles of the Wt1 and Sf1 genes

The B6 and D2 alleles of the Wt1 and Sf1 genes could encode different protein variants and/or have different expression...
patterns. A PCR primer-walking strategy using cDNA and genomic DNA as template was employed to determine the DNA sequence of the Wt1 and Sf1 genes present in the D2 genome and these sequences were compared to the mouse B6 reference sequence (data not shown). For Wt1, the ORF, 5’ and 3’ UTRs, and about 1 kb of the upstream flanking region were sequenced. One synonymous difference was identified in the ORF: a T (B6) vs. C (D2) substitution at the third position of codon 339, which was confirmed by its presence in dbsNP Build 128 (NCBI, rs27444896). There were no differences in the 5’ UTR and upstream flanking region. Four differences were identified in the 3’ UTR, two of which were confirmed by their presence in dbsNP (rs33129987 and rs13467742). Two single base pair differences in homopolymer length were not confirmed in dbsNP. The functional significance, if any, of the 3’ UTR polymorphisms is not readily apparent.

For Sf1, the ORF, 5’ and 3’ UTRs, and ~1.5 kb of the upstream and 500 bp of the downstream flanking regions were sequenced. One synonymous and one nonsynonymous difference were identified in the ORF. The synonymous polymorphism was a T (B6) vs. C (D2) substitution at the third position of the third codon. This polymorphism is represented in dbsNP (rs3142930) for certain inbred strains, but not for DBA/2J. The non-synonymous polymorphism was a G (B6) vs. T (D2) substitution in the first position of codon 172 resulting in an alanine (B6) vs. serine (D2) amino acid substitution and was confirmed by its presence in dbsNP (rs3142929). This polymorphism was independently discovered by Frigeri and colleagues who first identified it in ACTH resistant derivatives of the mouse Y1 adenocortical tumor cell line [41]. There were no differences in the 5’ and 3’ UTRs. The upstream and downstream regions each had one difference, both in CA repeat units: The upstream CAAA repeat had 17 units in B6 vs. 22 units in D2. Neither polymorphism is present in dbSNP and their functional significance is not readily apparent.

To gain insight into whether the presence of the Sf1<sup>AT72</sup> (B6) vs. Sf1<sup>AT72</sup> (D2) allele was correlated with B6 XY sex reversal, and thus of potential functional significance, we took advantage of the fact that when females from different inbred strains are mated to B6 XYPOS sex reversal in B6 XYPOS, B 6X YAKR, and Sf1<sup>AT72</sup> alone is not strictly correlated with B6 XY sex reversal.

The Wt1 gene encodes multiple splice isoforms. Of particular interest here, alternative splicing of exon nine gives rise to two protein isoforms that differ by three amino acids and are referred to as the +KTS and -KTS isoforms. Both isoforms and their correct relative expression are necessary for normal gonad development [42]. For example, Fraser syndrome in humans is associated with XY sex reversal and caused by reduced expression of the Wt1 +KTS isoform [43]. Thus, it was possible that B6 XY<sup>AKR</sup> Wt1<sup>+</sup> - sex reversal could be caused by altered +KTS/ -KTS isoform ratios and qRT-PCR using primers that span the exon nine splice junction was employed to examine if the ratio of +KTS/ -KTS splice isoforms is different in E10.5–14.5 B6 vs. D2 XX and XY gonad/mesonephros complexes. We found that the relative expression of the Wt1 +KTS and -KTS isoforms did not differ between B6 and D2 gonad/mesonephros complexes. We thus conclude that the presence of an Sf1<sup>AT72</sup> allele. We further corroborated that Sf1<sup>AT72</sup> does not affect expression of the Wt1<sup>B6</sup> and Sf1<sup>B6</sup> alleles function as hypomorphs because they are expressed at lower levels or with different temporal profiles than the Wt1<sup>D2</sup> and Sf1<sup>D2</sup> alleles, respectively.

To test the hypothesis that the Wt1<sup>B6</sup> and Sf1<sup>B6</sup> alleles function as hypomorphs because they are expressed at lower levels or with different temporal profiles than the Wt1<sup>D2</sup> and Sf1<sup>D2</sup> alleles, respectively, each sample was genotyped by qRT-PCR using primers that span the Sf1<sup>B6</sup> allele. To analyze a subset of the samples (data not shown). In summary, these molecular analyses suggest that Wt1<sup>B6</sup> is a hypomorph due to reduced expression and that Sf1<sup>B6</sup> is a hypomorph possibly due to altered protein structure and/or the presence of other modifier B6 loci.

Reducing Wnt4 dosage increases the amount of testis tissue that develops in multiple cases of B6 XY sex reversal.

To examine the possibility that Wnt4 would be haploinsufficient on the B6 genetic background and to investigate its candidacy as a tda gene, we transferred a Wnt4 null allele onto the B6 genetic background and assessed if Wnt4 heterozygosity exacerbated XY sex reversal in B6 XY<sup>AKR</sup>, B6 XY<sup>AKR</sup> Wt1<sup>+</sup> - Wt1<sup>+</sup>, and Wt1<sup>+</sup> Wt1<sup>+</sup> - B6 XY<sup>AKR</sup> fetuses. WHIC was used to examine gonad morphology and marker gene expression at E13.5. Each gonad/mesonephros complex was assessed at dissection for the presence of cords and male-specific vasculature, and classified as an ovary, ovotestis, or testis (Table 7). Classification was done prior to

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**Table 4. Survival of Wt1<sup>+/−</sup>; Sf1<sup>+/−</sup> offspring.**

| Offspring genotypes | Genotype | Wt1<sup>+/−</sup> | Sf1<sup>+/−</sup> | Sf1<sup>−/−</sup> | Wt1<sup>−/−</sup> | Wt1<sup>+/−</sup> | Sf1<sup>+/−</sup> | Sf1<sup>−/−</sup> | Wt1<sup>−/−</sup> | Total |
|---------------------|----------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|-------|
| Weaning             | B6 Wt1<sup>+/−</sup> | 43               | 34               | 7                | 0                | 84               |
|                     | B6 Sf1<sup>+/−</sup> | 3                | 4                | 2                | 3                | 12               |
| E14.5–15.5          | B6 Wt1<sup>+/−</sup> | 6                | 3                | 10               | 4                | 23               |
Figure 3. Histological and WHIC analysis of Wt1+/−; Sf1+/− B6 XYB6 fetuses. (A and B, 10× magnification) Hematoxylin and eosin staining of E14.5–15.5 fetal sagittal sections showed the absence of adrenal glands in Wt1+/−; Sf1+/− B6 XYB6 and XX (data not shown) fetuses. The inset panels in A and B are a magnified view showing that normal testicular cords were present in both Wt1+/++; Sf1+/+ and Wt1+/−; Sf1+/− B6 XYB6 fetuses (white dotted line outlines a section of a single cord). (C–J) WHIC analysis of E13.5 B6 XYB6 gonad/mesonephros complexes stained with SOX9 (Sertoli cells, green) and PECAM (germ cells and vascular endothelial cells, red). Panels C, E, G and I show 10 × images taken with 0.7 × zoom. Panels D, F, H, and J show 20 × images. The gonads in all wild-type and single heterozygous E13.5 B6 XYB6 gonads were classified as testes, but testis cords in Wt1+/−; Sf1+/− (E and F) and Wt1+/++; Sf1+/+ (G and H), B6 XYB6 gonads were less developed and more disorganized than cords in Wt1+/++; Sf1+/+ (C and D) B6 XYB6 gonads. B6 Wt1+/−; Sf1+/− gonads, however, were more similar to wild-type gonads than were Wt1+/++; Sf1+/− gonads. Testis differentiation was most affected in Wt1+/−; Sf1+/−/ B6 XYB6 gonads and these were classified as ovotestes: Sertoli cells in the anterior and posterior poles often expressed SOX9 but were not organized into cords (I and J).

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ascertainment of the fetal genotype and was done by a single investigator (SMC) for consistency. A subset of complexes of each genotype was processed for WHIC and examined by two investigators (SMC and KHA) to confirm the initial classifications (Figure 6). In all four experimental groups, reducing Wt1 expression increased the area containing testicular cords and reduced the area without obvious structure in XY gonads. In B6 XYPOS fetuses, 5 of the 12 gonads analyzed from Wt1+/−/+ fetuses were classified as ovaries and none were classified as testes, whereas 7 of the 28 gonads analyzed from Wt1+/−/− fetuses were classified as ovaries and 4 were classified as testes. These data indicate that reducing Wt1 dosage in B6 XYPOS fetuses decreased the percentage of ovaries from 42% to 25% and increased the percentage of testes from 0% to 14%. In B6 XYAKR fetuses, 12 of the 16 gonads analyzed from Wt1+/−/+ fetuses were classified as ovotestes and 4 were classified as testes, whereas 6 of the 18 gonads analyzed from Wt1+/−/− fetuses were classified as ovotestes and 12 were classified as testes. Thus, reducing Wt1 dosage in B6 XYAKR mice decreased the percentage of ovotestes from 75% to 33% and increased the percentage of testes from 25% to 67%. In B6 Wt1+/− XYAKR fetuses, 3 of the 12 gonads analyzed from Wt1+/−/+ fetuses were classified as ovaries, 5 as ovotestes, and 4 as testes, whereas none of the 6 gonads analyzed in Wt1+/−/− fetuses were classified as ovaries, 1 was an ovotestis and 5 were testes. Thus, reducing Wt1 dosage in Wt1+/− B6 XYAKR mice decreased the percentage of ovaries from 25% to 0%, decreased the percentage of ovotestes from 42% to 17%, and increased the percentage of testes from 33% to 83%. In Sf1+/− B6 XYAKR fetuses, all 8 gonads analyzed from Wt1+/−/+ fetuses were classified as ovaries, whereas 2 of the 12 gonads analyzed from Wt1+/−/− fetuses were classified as ovaries, 3 as ovotestes, and 7 as testes. Thus reducing Wt1 dosage in B6 Sf1+/− XYAKR mice decreased the percentage of ovaries from 100% to 17%, increased the percentage of ovotestes from 0% to 25%, and increased the percentage of testes from 0% to 58%. The WHIC analyses confirmed these results (Figure 6).

These data indicate that Wt1B6 acts like an anti-testis gene. Using qRT-PCR we found that Sry expression in Wt1B6/+ vs. Wt1B6/+ B6 XYAKR was not significantly different during peak Sry expression in E11.75 (21–23 ts) gonad/mesonephros complexes (data not shown), suggesting that Wt1B6 compromises the testis pathway downstream of Sry [36]. Together, these data suggest that Wt1B6 behaves as a hypermorph allele of a gene in the ovary determination pathway, rather than a hypomorphic allele of a gene in the testis determination pathway.

Elevated expression of ovary-biased genes in B6 versus D2 XX ovaries

The Wt1 results presented above suggested the possibility that the ovary differentiation pathway is prematurely- or hyper-activated in B6 gonads, thereby suppressing the testis determination pathway. As with Wt1 and Sf1, the B6 and D2 Wt1 alleles could encode different protein variants or have different expression patterns. We determined the DNA sequence of the Wt1 ORF present in the D2 genome and found that it did not differ from that in B6, which was confirmed by data present in dbSNP Build 128 (data not shown). We used qRT-PCR to compare the expression of Wt1 and seven other genes in the ovary differentiation pathway in E12.5 B6 vs. D2 XX ovaries to test the hypothesis that Wt1B6 is an expression hypermorph and to investigate the possibility that the ovary differentiation pathway is prematurely- or hyper-activated in B6 gonads (Figure 7). The seven genes were chosen because their expression normally is ovary-biased at E12.5 (i.e., their expression is either ovary-specific or higher in ovaries vs. testes) [34,38,44–46]. We found that the expression of Wt12 (p<0.0004), Adams19 (p<0.026), Fst (p<0.0001), Igfph2 (p<0.0001), and 1700106J16Rik (p<0.0001) was significantly higher in B6 vs. D2 ovaries; that expression of Inr3 (p<0.177) and Bmp2 (p<0.323) was not significantly different; and that expression of Rps11 (p<0.0002) was significantly lower. These results demonstrate that Wt1 and other genes known, or thought to be, in the ovary differentiation pathway are hyperactivated in E12.5 B6 vs. D2 XX gonads.

Discussion

It has been suggested that some genetic events leading to testis determination differ between humans and mice because several genes that are haplosufficient in humans appeared to be haplosufficient in mice [18]. However, based on our studies of B6-XYPOS sex reversal we hypothesized that genetic background would have a significant influence and that some of these genes would be haplosufficient on the B6 genetic background. We chose to analyze the Wt1, Sf1, and Wt1 genes for two main reasons: 1) they are dosage-sensitive in humans but initial studies suggested they were dosage-insensitive in mice, and 2) they are good candidates for idio genes based on genetic location and because gonad differentiation is abnormal in homozygous knockout mice. Our analyses show that these three genes can be dosage-sensitive in mice, depending on the genetic background, and provide insights into the mechanisms of B6 XY sex reversal.

The Wt1 and Sf1 genes are dosage-sensitive on the B6 genetic background

Our results show that the Wt1 and Sf1 B6 alleles are haplosufficient for normal testis differentiation when present on the B6 genetic background. We found that Wt1+/− and Sf1+/− B6 XYB6 mice developed as males; however, testis differentiation was transiently delayed and was further compromised in double heterozygous Wt1+/−; Sf1+/− B6 XYB6 fetuses. These data are consistent with evidence that Wt1 activates Sf1 transcription [30]. When the genetic background was further sensitized by substituting the YB6 Chr with the Y AKR Chr, testis differentiation was transiently delayed and was further compromised in double heterozygous Wt1+/−; Sf1+/− B6 XYB6 mice. In contrast, all Wt1+/− and Sf1+/− B6 XYAKR mice developed testes. Taken together, these results show that the Wt1 and Sf1 B6 alleles are hypomorphic
Modeling Dosage-Sensitive Sex Reversal in Mice

A

Sry expression relative to Gapdh

Stage

15-17 ts E11.25
18-20 ts E11.5
21-23 ts E11.75
24-26 ts E12.0

+/+
Sf1+/-
Wt1+/-

B

AMH/PECAM

+/+ XY<sup>AKR</sup>
Wt1+/- XY<sup>AKR</sup>
Sf1+/- XY<sup>AKR</sup>

+/+ XY<sup>AKR,Sxr</sup>
Wt1+/- XY<sup>AKR,Sxr</sup>
Sf1+/- XY<sup>AKR,Sxr</sup>

+/+ XY<sup>AKR</sup>
Wt1+/- XY<sup>AKR</sup>
Sf1+/- XY<sup>AKR</sup>

+/+ XY<sup>AKR</sup>
Wt1+/- XY<sup>AKR</sup>
Sf1+/- XY<sup>AKR</sup>
relative to the D2 alleles. Analysis of morphology and marker gene expression in E13.5 Wt1+/− and Sf1+/− B6 XYAKR gonads showed that some developed as ovaries and were indistinguishable from control XX ovaries in that they expressed ovary-specific markers, and did not express Sertoli cell or Leydig cell-specific markers. These data indicate that the testis-determining pathway is interrupted at a very early stage in these XY gonads.

In addition to defects in testis differentiation, we found that double heterozygous Wt1+/−; Sf1+/− fetuses lacked adrenal glands and that neonates die shortly after birth, presumably from adrenal insufficiency. Because the gonads and adrenal cortex share a common origin [47], because the morphological defects we observed occur early in the development of these organs, and because the lethality and sex reversal phenotypes were sensitive to genetic background in a similar way, we hypothesize that the initial defect occurs during the adrenogonadal progenitor stage and that one or more (de)gene(s) plays a role in the differentiation of this progenitor into the adrenal cortex and gonads. While it has been known that Wt1, Sf1 and Wt4 are necessary for adrenal cortex differentiation [14–17,34,35,48], our results show that Wt1 and Sf1 interact genetically during adrenal development. These results are consistent with results reported for fetuses that are double heterozygous for Sf1 and Cited2, a transcription cofactor that interacts with Wt1 [49]. Given that Wt1 is thought to directly activate Sf1 transcription in the gonadal progenitor, our results suggest that this genetic pathway is important for the differentiation of the adrenogonadal progenitor. It will be interesting to determine how Wt4 fits into this genetic pathway in the adrenogonadal progenitor.

Wt1 and Sf1 regulate Sry expression, but reduced Sry transcript levels are not solely responsible for sex reversal in Wt1+/− or Sf1+/− B6 XY mice

Consistent with these indications of an early defect in testis differentiation, we found that Sry expression was markedly reduced in heterozygous mutant gonads. Our unpublished data indicated that reduced expression results from both a direct effect on Sry expression and an indirect effect on the differentiation of pre-Sertoli cells (manuscript in preparation). Interestingly, increasing Sry dosage by replacing the YAKR Chr in Wt1+/− or Sf1+/− B6 XYAKR mice with the YchrCl dramatically increased the amount of testis tissue that developed but cord differentiation remained somewhat abnormal. Our previous experiments showed that the SryYAKR and SryYchrCl allelic expression is reduced in ovaries (Figure 2). In B6 XX ovaries indicating that Sry dosage levels alone do not predict the expression of critical genes other than Sry is likely to be affected. We consistently found that the Sf1 heterozygous phenotype was more severe than the Wt1 heterozygous phenotype. However, the reduction in Sry expression was not statistically different between the two heterozygotes, which supports our hypothesis that genes and cellular processes other than those controlled by SRY contribute to the phenotypic differences. Our result agree with previous reports suggesting that Sry expression levels alone do not predict whether a given Y Chr causes testis differentiation defects on the B6 genetic background [50] and that sex reversal is caused by both the presence of particular SRY protein isoforms found in M. domesticus mice and reduced Sry expression [29].

Table 5. Increasing Sry dosage partially rescues the sex reversal phenotypes of E13.5 B6 XYAKR, Wt1+/−, Sf1+/− and Sf1+/− B6XYAKR gonads.

| Genotype examined | Ovaries | Ovotestes | Abnormal testes | Testes | Total gonads |
|-------------------|---------|-----------|----------------|--------|-------------|
| B6 Wt1+/− XYAKR   | 3 (25%) | 7 (58%)   | 2 (17%)        | 0 (0%) | 12          |
| B6 Wt1+/− XYAKR,St | 0 (0%)  | 1 (13%)   | 7 (88%)        | 0 (0%) | 8           |
| B6 Sf1+/− XYAKR   | 21 (95%)| 1 (5%)    | 0 (0%)         | 0 (0%) | 22          |
| B6 Sf1+/− XYAKR,St| 0 (0%)  | 5 (63%)   | 3 (38%)        | 0 (0%) | 8           |
| B6 Sfx+/− XYAKR,St| 0 (0%)  | 0 (0%)    | 0 (0%)         | 17 (100%)| 17          |

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contrast, Wnt4 expression was elevated in Wt1+/+ and Sf1+/+ B6 XYAKR gonads compared to +/+ gonads, but we suspect that this increase is due to the presence of more ovarian tissue in these gonads. Expression of Wnt4 also was reduced in Wt1+/+ XX and XY mesonephroi. Because Sf1 is not expressed in mesonephroi, reduced Wnt4 expression indicates in this tissue WT1 regulates Wnt4 expression independently of SF1. Pch1 expression also was reduced in Wt1+/− XX and XY mesonephroi. Interestingly, Pch1 is strongly expressed in the region where the mesonephric tubules form and the number of tubules is reduced in Wt1−/− mesonephroi [14]. This result suggests that Wt1 regulates Pch1 expression and that Pch1 could have a role in the formation of mesonephric tubules. However, these WISH results, though quite intriguing, require confirmation with a more quantitative method such as qRT-PCR.

Co-expression of ovarian and testicular markers suggests reduced antagonism of the ovary and testis differentiation pathways in B6 XY gonads

Analysis of E13.5 B6 XYAKR gonads showed that the anterior and posterior regions that lacked testicular cords expressed multiple ovarian somatic cell markers and that the germ cells within these domains had initiated meiosis. Therefore, these regions were not simply undifferentiated, but rather had initiated the ovary differentiation program in both somatic and germ cells. By E15.5, testicular cords are present throughout B6 XYAKR gonads and it is unclear what happens to the ovarian regions between these two stages. Two possibilities are that these ovarian cells might transdifferentiate into the analogous testicular cell types, or regress via apoptosis. A recent analysis showed that the ovarian regions in Sox9

Table 6. Strain distribution pattern of the Sf1A172 versus Sf1S172 alleles.

| Inbred strain | F1 phenotype | Ncol genotype | rs3142929 confirmed |
|---------------|--------------|----------------|---------------------|
| C57BL/6J      | B6-like      | +              | +                   |
| C57BL/10J     | B6-like      | +              | −                   |
| NZB/BINJ      | B6-like      | −              | +                   |
| DBA/2J        | D2-like      | −              | +                   |
| 129S1/SvImJ   | D2-like      | −              | +                   |
| C3H/HeSnJ     | D2-like      | −              | not available       |
| SM/J          | D2-like      | −              | +                   |
| C58/J         | D2-like      | −              | +                   |
| BALB/cBy      | D2-like      | −              | +                   |

*Indicates an imputed value.
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Figure 5. Wt1 but not Sf1 transcript levels were significantly lower in B6 versus D2 gonad/mesonephros complexes. Real-time qRT-PCR analysis of Wt1 and Sf1 expression normalized to Gapdh (2−ΔCt) showing mean transcript levels +/- standard error. Wt1 and Sf1 expression were determined in the same B6 and D2 samples, therefore the samples sizes are only shown above the corresponding error bars for the Wt1B6 and Sf1B6 comparisons. An asterisk denotes statistically significant differences in pairwise comparisons (α = 0.05). Wt1 expression was significantly lower in B6 vs. D2 samples at E10.5 (10–13 ts, t15 = 2.063, p = 0.0284), E11.0 (14–17 ts, t40 = 5.355, p < 0.00002), E11.5 (18–21 ts, t29 = 1.699, p < 0.0500), E12.5 (27–30 ts, t13 = 3.441, p < 0.0003), and E13.5 (t12 = 5.985, p < 0.00003). Wt1 expression also was lower in B6 than in D2 samples at E12.0 (27–30 ts) but the difference did not reach statistical significance (t13 = 1.741, p < 0.0526). Sf1 expression was lower in B6 vs. D2 gonad/mesonephros complexes at E11.0 (t40 = 1.466, p < 0.0753), E11.5 (t29 = 1.182, p < 0.1235), and E12.0 (t13 = 1.208, p < 0.1243), but these differences were not statistically significant.
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transgenic XX ovotestes regress via apoptosis [51] and our preliminary analyses suggest that there is an increased number of apoptotic cells within the ovarian regions in B6 XYAKR ovotestes (data not shown). In these regions it was clear that most cells expressed either ovarian (FOXL2) or testicular (AMH) markers and that these cells could be interspersed. We propose that co-expression of ovarian and testicular markers in E13.5 B6 XY ovotestes suggests that antagonism between these opposing differentiation pathways is reduced in these XY gonads. Contrary to results reported by Bradford and colleagues [52], we did observe a few cells at the boundaries between testicular and ovarian domains that appeared to express both granulosa and Sertoli cell markers. We could not unequivocally demonstrate co-expression using the markers and techniques presented here, however, the possibility that cells expressing both female and male supporting cell progenitor markers might be present during fetal gonad differentiation warrants further investigation.

| Maternal genotype | Paternal genotype | Fetal genotype | Ovaries | Ovotestes | Testes | Total gonads |
|-------------------|-------------------|----------------|---------|-----------|--------|--------------|
| B6 Wnt4+/-- | B6 XYPOS | B6 Wnt4+/+ XYPOS | 5 (42%) | 7 (58%) | 0 (0%) | 12 |
| B6 Wnt4+/-- | B6 XYAKR | B6 Wnt4+/+ XYAKR | 7 (25%) | 17 (61%) | 4 (14%) | 28 |
| B6 Wnt1+/-- | B6 Wnt4+/+ XYAKR | B6 Wnt4+/+ XYAKR | 0 (0%) | 12 (75%) | 4 (25%) | 16 |
| B6 Wnt4+/+ | B6 WT1+/+ | B6 Wnt4+/+ XYAKR | 6 (33%) | 12 (67%) | 12 (67%) | 18 |
| B6 Wnt4+/+ | B6 Sf1+/+ | B6 Wnt4+/+ XYAKR | 1 (17%) | 5 (83%) | 5 (83%) | 6 |
| B6 Sf1+/+ | B6 Wnt4+/+ XYAKR | B6 Sf1+/+ | 8 (100%) | 0 (0%) | 0 (0%) | 8 |
| B6 Sf1+/+ | B6 Wnt4+/+ XYAKR | B6 Sf1+/+ | 2 (17%) | 3 (25%) | 7 (58%) | 12 |

Figure 6. Reduced Wnt4 dosage partially rescued cord differentiation in B6 XY genotypes that typically develop ovarian tissue. Representative images are shown for WT1+/+ (A and B) and Sf1+/+ (C and D) B6 XYAKR E13.5 gonads that were either Wnt4+/+ (A and C) or Wnt4+/-- (B and D). The amount of tissue expressing the Sertoli cell marker AMH (green) was consistently greater in Wnt4+/-- vs. Wnt4+/+ gonads for all four sex reversals examined. Germ and vascular endothelial cells are labeled by PECAM staining (red).

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Why are the Wt1 and SF1 B6 alleles haploinsufficient?

To determine why the Wt1B6 and SF1B6 alleles are haploinsufficient and function as hypomorphs, we compared the coding sequences and expression levels of the B6 and D2 alleles. The results indicated that the Wt1B6 and SF1B6 allele encode the same protein and that the ± KTS Wt1 isoform expression ratio was the same in B6 and D2 gonad/mesonephros complexes. However, we found that Wt1 expression throughout the initial stage of testis differentiation was lower in complexes from B6 fetuses, suggesting that in the B6 genetic background the Wt1B6 allele is an expression hypomorph. In contrast, we were unable to detect significantly lower SF1 expression in complexes from B6 fetuses but we did identify one coding polymorphism in SF1, suggesting that the SF1B6 allele is haploinsufficient not because its expression is reduced but possibly because the SF1B6 protein variant has reduced function. It appears that two SF1 variants are present among the common inbred mouse strains: SF1A172 (present in B6) and SF1S172 (present in D2). However, we found that homozygosity for the SF1A172 allele in F1 hybrids was not sufficient to cause sex reversal in the presence of the YPOS Chr [as in (NZB/BIN)×B6 XYPOS F1s] and that trans-heterozygosity for the SF1A172 and SF1S172 alleles was not sufficient to prevent sex reversal in the presence of the YPOS Chr [as in (BALB/cBy×B6 XYPOS) F1s]. These data show that the SF1A172 polymorphism alone does not account for sex reversal in the presence of the YPOS Chr. Therefore, if the SF1A172 protein variant does indeed have reduced function during testis determiniation, then homozygosity for B6 alleles at other loci likely is necessary to cause SF1B6 to function as a hypomorph.

The Wnt4 B6 allele is dosage-sensitive in the B6 genetic background and may contribute to hyperactivation of the ovary differentiation pathway

Our genetic results suggest that the Wnt4B6 allele functions as a hypermorp in the ovary determination pathway. To ascertain if the ovary determination pathway was hyperactive in B6 vs. D2 gonads we quantified the expression of eight ovary-biased or ovary-specific genes in E12.5 B6 vs. D2 XX ovaries. We chose this developmental stage because existing data suggested that the ovary determination pathway was hyperactive in B6 vs. D2 XX ovaries, indicating that the ovary determination pathway is activated earlier and/or to a greater degree in B6 vs. D2 ovaries. These results are consistent with those reported in an eQTL study of B6 vs. 129 gonads [53]. The B6 vs. D2 expression differences do not appear to be caused by gross differences in development between these two strains because we did not detect differences in...
limb morphology or in the size of the gonads or mesonephroi at the 27–28 ts stage used for these analyses (data not shown). However, not all eight genes were up-regulated in B6 ovaries: two were not significantly different, and one was down-regulated. We find it intriguing that Rspo1, which maps close to Wnt4 within the tda1 region, had reduced expression in B6 ovaries. However, because the Rspo1 and Wnt4 knockout phenotypes are very similar in that ovary and not testis differentiation is primarily affected, and because Wnt4 expression is greatly reduced in Rspo1−/− XX gonads [54,55], we think it is unlikely that Rspo1 is tda1 because we would expect that a strain with reduced Rspo1 expression like B6 would be more sensitive to XY sex reversal, whereas B6 is, in fact, more sensitive. We note that for Rspo1 no SNPs that differ between the B6 and D2 strains are present in dbSNP (Build 128), indicating that the Rspo1 protein does not differ between these strains. Furthermore, we suggest that the interaction between Rspo1 and Wnt4 may be indirect because Rspo1 expression is decreased in B6 mice while Wnt4 expression is increased. Nevertheless, these data do not formally exclude Rspo1 as a tda1 candidate.

Are Wt1, Sf1, and Wnt4 tda genes?

More than twenty-five years ago, two of the authors hypothesized that the B6-derived tda genes could function later than normal in the testis determination pathway and/or, because the testis and ovary determination pathways antagonize each other, could function earlier than normal in the ovary determination pathway [25,56]. Thus, if a tda gene functions in the testis-determining pathway, then reducing its dosage in B6 XY mice should cause sex reversal, whereas reducing its dosage in D2 XY mice should not. Similarly, if a tda gene functions in the ovary-determining pathway and antagonizes testis differentiation, then reducing its dosage should ameliorate B6 XY sex reversal. The Wt1 and Sf1 genes fulfill the criteria as late functioning testisdetermining genes and Wnt4 fulfills the criteria as an early function ovary-determining gene that antagonizes testis differentiation. Furthermore, our results show that B6 XYPOS, Wt1+/− B6 XYAKR, and Sf1+/− B6 XYAKR sex reversal share similar gonadal phenotypes and that these three sex reversals are sensitive to genetic background in a similar way. Additionally, we have recently determined that a major modifier of Sf1+/− B6 XYAKR sex reversal that differs between the B6 and D2 genomes, maps to the distal region of Chr 4, which suggests that the tda1 modifier gene plays a prominent role in both Sf1+/− B6 XYAKR and B6 XYPOS sex reversal (manuscript in preparation). However, while our data are consistent with the hypothesis that tda2 is comprised of Wt1 and Sf1 and that tda1 is Wnt4, the genetic intervals defined by the linkage analyses are fairly broad, and further experiments are needed to directly test this hypothesis and to exclude the possibility of other candidate genes in the tda1 and tda2 intervals.

Undoubtedly, tda genes in addition to tda1 and tda2 contribute to B6 XYPOS sex reversal because, as shown in the original linkage analysis, some XYPOS fetuses that were homozygous for B6 alleles at the tda1 and tda2 loci contained normal appearing testes [24]. Nevertheless, it appears that tda1 and tda2 play an important role in many cases of B6 XY sex reversal because we have mapped a major modifier of Dax1−/− sex reversal that differs between the B6 and D2 genomes to the same chromosome region as tda1 [22], and have recently determined that major modifiers of Gata4+/− B6 XYAKR sex reversal [23] map to the same chromosome regions as tda1 and tda2 (manuscript in preparation).

An additive model to explain B6 XY sex reversal

Overall, our data suggest that the B6 inbred strain is sensitive to XY sex reversal, at least in part, due to hypomorphic alleles of Wt1 and/or Sf1 that compromise the testis determination pathway, as well as a hypermorphic allele of Wnt4 that antagonizes testis development. We suggest that in XY gonads of most inbred strains, such as B6a[2], the testis determination pathway reaches a critical threshold that is sufficient to inactivate the ovary determination pathway prior to the developmental stage when the ovary determination pathway reaches a threshold that is sufficient to inactivate the testis determination pathway. In contrast, in a few inbred strains such as B6, the testis determination pathway is compromised and the ovary determination pathway is hyperactivated, thus the interval between the developmental stages at which these two thresholds are reached is greatly reduced. We propose that this interval is even further reduced or eliminated in B6 mice carrying certain M. domesticus-derived Sry alleles that do not efficiently activate the testis determination pathway, thus allowing ovotestes to develop transiently in B6 XYAKR mice and allowing stable ovotestes and ovaries to develop in B6 XYPOS mice, for example. In summary, we propose that sex reversal in B6 XYPOS gonads is the result of the additive effects of 1) hypomorphic Wt1B6 and/or Sf1B6 alleles, 2) a hypermorphic Wnt4B6 allele and hyperactivation of the ovary pathway, 3) reduced function of M. domesticus-derived Sry proteins, 4) reduced expression of the SypPOS allele, and 5) the presence of other unidentified tda genes.

Mouse models of human dosage-sensitive sex reversal

These studies demonstrate that Wt1, Sf1, and Wnt4 are dosage-sensitive in mice, as they are in humans, and show that this effect is modulated by genetic background, as we suspect it is in humans. They also demonstrate that the Wt1+/− and Sf1+/− B6-YAKR mice are good models for the corresponding human XY sex reversals involving heterozygous mutations in these genes. We suggest that these heterozygous mice will be very useful for the elucidation of Wt1 and Sf1 functions during gonad differentiation because the heterozygous phenotype is far less severe than the homozygous phenotype, which is a complete abrogation of gonad differentiation and subsequent apoptosis at the genital ridge stage. Thus, we think it is likely that these strains will be particularly useful in providing further insights into the roles of Wt1 and Sf1 in organogenesis and human disease.

Materials and Methods

Animals and genotyping

Null (knockout) alleles of Wt1 (Wt1tm1Jae) [14], Sf1 (N5sf1tm1Bb) [16], and Wnt4 (Wnt4tm1Amc) [34] were transferred to the B6 and D2 inbred strain backgrounds using standard backcrossing. No sex ratio disturbances were noted in heterozygous progeny during construction of these congenic strains. Experimental results reported here were conducted after backcross generation N10 or greater was achieved. The B6-YPOS, B6-YAKR, B6-YAKRtm1 and D2-YAKR consomic strains were previously described [19,20,23,29,57]. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Jackson Laboratory and Boston University Laboratory Animal Science Center are AALAS accredited and all animal procedures were approved by The Jackson Laboratory Animal Care and Use Committee (protocols LAH:93-26, LAH:95-01, LAH:95-02) or the Boston University Animal Care and Use Committee (protocol AN-13935).

The presence of a Wt1, Sf1, or Wnt4 knockout allele was determined by multiplex PCR using primers to detect the neomycin cassette and the myogenin gene as a control [23]. In crosses involving multiple knockout alleles, gene-specific PCR assays were used to amplify both the normal and the targeted
alleles [46,58]. The genetic sex of fetuses was determined by PCR using Y Chr specific primers and myogenin control primers [59]. The B6-Y<sup>A</sup>KKR<sup>A</sup>Y strain carries a rearranged Y Chr containing two Sry alleles: the <i>M. domesticus</i>-derived Sry<sup>AKKR</sup> allele and the <i>M. musculus</i>-derived Sry<sup>Y</sup> allele. The presence of the <i>M. domesticus</i> and/or <i>M. musculus</i> Sry alleles was determined by PCR followed by digestion with NalI restriction enzyme using primers that flank a polymorphism within the Sry gene [29].

**Embryo isolation and staging, and determination of sexual phenotype**

Timed matings were used for all embryonic experiments. Noon on the day of vaginal plug detection was designated as E0.5. Embryos between E10.5 and 12.5 were precisely staged by counting the number of tail somites (E10.5~8 ts, E11.5~18 ts, E12.5~30 ts) [60] and those between E12.5 and 16 were staged by limb morphology and the extent of cranial vessel formation [61]. To classify the gonads of individual E14.5~16 fetuses, the gonads were removed from the body cavity and immediately examined with the aid of an inverted microscope using transmitted light, and then photographed [62]. Tests were identified as having distinctive cords and vasculature, and an incipient tunica albuginea. Ovaries were identified as having a reticular appearance and lacking the structures noted above in the testes. Ovotestes contained a region with a reticular appearance at one or both ends of the gonad with the remainder containing cords. The sexual phenotype of weaning age (~3 weeks postpartum) mice was determined by examining the external genitalia, noting the anal-genital distance, and observing whether mammary gland-associated yellow-pigmented hairs were present [63]. Gonads from E13.5 fetuses were classified at the time of dissection, when the observer was blind to the fetal genotype. Gonads without any testicular cords were classified as ovaries, those with testicular cords only in the central region of the gonad were classified as ovotestes, and those with testicular cords throughout were classified as testes. Those with disorganized testicular cords throughout the gonad were classified as abnormal testes.

**Whole-mount immunohistochemistry and RNA in situ hybridization**

Gonad/mesonephros complexes were fixed overnight in 4% paraformaldehyde in PBS at 4°C. Whole-mount indirect fluorescent immunohistochemistry (WHIC) was performed essentially as previously described [64], except that a blocking buffer containing 3% BSA, 10% donkey serum, 0.1% Triton-X, 0.02% sodium azide in PBS was used when the anti-PDGFRα or anti-MIS (AMH) antibodies were employed. Primary antibodies and dilutions are listed in Table S1. Secondary antibodies were donkey anti-IgG conjugated with Cy3 or Cy5 (Jackson Immunoresearch, 1:500) or AF488 (Molecular Probes, 1:750). Samples were imaged using a Zeiss LSM510 confocal microscope.

Standard protocols for whole-mount in situ hybridization (WISH) were used with minor changes [46,65]. Gene-specific probes are listed in Table S1. The Red9 probe was synthesized from a full-length cDNA clone [IMAGE ID: 6339595, GenBank: BC052155.1]. Antisense and sense probes were tested in parallel, and only signal specific to the antisense probes was reported as gene-specific expression. Each result reported was replicated in a minimum of four samples.

**RNA extraction, reverse transcription, and quantitative RT–PCR**

Total RNA was isolated using the RNeasy mini kit (Qiagen). Samples were treated with Turbo DNase (Ambion) and subsequently treated with DNase inactivation reagent using the DNA-free protocol (Ambion). RNA Samples were determined to be free of DNA by PCR using primers specific for the M<sub>myo</sub> or L<sub>hx1</sub> genes [29]. cDNA was synthesized using oligo-dT primers and reverse transcriptase (RT). The RT reaction was tested for the presence of intact cDNA by PCR amplification with primers specific for the Hprt transcript [46].

Relative Sry expression in mutant vs. normal gonad/mesonephros complexes was determined using TaqMan Universal PCR Master Mix, and Sry (Mm00441712_s1) and Gapdh (Mm99999915_g1) Assays-on-Demand following the manufacturer’s standard protocol (Applied Biosystems). Relative Wt and Sf<sup>1</sup> expression in B6 vs. D2 gonad/mesonephros complexes was determined using Wt (Mm00460570_m1), Sf<sup>1</sup>(Mm00446826_m1) and Gapdh TaqMan Assays-on-Demand. Relative expression of ovary pathway genes in B6 vs. D2 ovaries was determined using Power SYBR Green PCR Master Mix (Applied Biosystems) and the following primers: <i>Adams19</i> (5′-CCGATGCCTCGTGTTATTA and 3′-GTTGCGGTTGACCTATGAT: 165 bp product), <i>Wt1</i> (5′-AGGAGTGCTCTGCAAGACATCAG and 3′-CCGATGTGTGTGACAGAGTGCG: 149 bp product), Igf1r2 and Irg6 (as in [46], Fst, Bmp2, and Hprt as in [44], and Irg6 as in [39]).

Quantitative RT-PCR (qRT-PCR) assays were performed on an ABI PRISM 7900HT Sequence Detection System using SDS 2.3 software to determine the average cycle threshold (Ct) value from three technical replicates for each sample. Average Ct values for Hprt or Gapdh were subtracted from the average Ct values for each gene for each sample, to determine the normalized average Ct value (ΔCt). When expression was profiled across developmental stages, the results were represented as expression relative to the endogenous control gene (2<sup>−ΔΔCt</sup>), when expression was compared within a single developmental stage, the results were determined by subtracting the average ΔCt for D2 ovaries according to the 2<sup>−ΔCt</sup> method [66]. In pairwise comparisons of gene expression levels with <i>a priori</i> predictions about the direction of the effect, such as detecting lower expression of Wt1 and Sf<sup>1</sup> or higher expression of Wt1 and other ovarian genes in B6 samples, statistically significant differences were identified by one-tailed unpaired Student’s t-tests (<i>α</i> = 0.05) using Microsoft Excel. For statistical analysis of Sry expression, one-way ANOVA was used to test for a significant effect of genotype in multiple comparisons and two-tailed unpaired Student’s t-tests (<i>α</i> = 0.05) in pairwise comparisons within a developmental stage using JMP version 8 software (SAS Institute Inc.).

Multi-gene quantitative real time RT-PCR was conducted using RNA extracted from E12 gonad-mesonephros complexes and E14 gonads as described in [44]. Only E14 XY<sup>A</sup>KKR mutant gonads lacking any testicular cords (classified as ovaries) were used for this analysis.

**Genotyping assay for the presence of Sf<sup>1</sup><sup>A172</sup> versus Sf<sup>1</sup><sup>6172</sup> alleles**

The presence of the Sf<sup>1</sup><sup>A172</sup> and Sf<sup>1</sup><sup>6172</sup> alleles was determined using a previously published genotyping assay [41]. Briefly, a region of Sf<sup>1</sup> exon 3 including codon 172 was amplified from genomic DNA using 5′-TGCGGTGTCGATCAGTCGGAAGGG and 3′-CCAGGTGAAGAATGGGAGGATGAAAGG as primers and the PCR product digested using NcoI. Genomic DNA containing the Sf<sup>1</sup><sup>A172</sup> allele contains a NcoI recognition site resulting in two fragments of 331 and 262 bp, while the Sf<sup>1</sup><sup>6172</sup> allele lacks the NcoI recognition site resulting in an undigested 593 bp fragment. Genomic DNA was obtained from mice in the Eicher Laboratory mouse colony or purchased from the JAX Mice DNA Resource (C58/J) (000669),
found in regions containing AMH-expressing Sertoli cells and incipient testicular cords (right panel, 40× magnification). B) WISH analysis. The ovary-specific somatic cell marker Brachyury (Brd) and meiotic germ cell markers Stra8 and Rec8 were expressed in the cranial (anterior) and/or caudal (posterior) poles of B6 XY<sup>AKR</sup> gonads. When Stra8 and Rec8 were expressed at both poles in B6 XY<sup>AKR</sup> gonads at this developmental stage, expression was higher at the cranial (left) vs. the caudal pole (right). The gonad/ mesonephros complex in each panel is oriented with the gonad above the mesonephros and the anterior pole to the left. (TIF)

**Table S1** WHC and WISH markers. Pertinent information on the antibodies and riboprobes used to analyze morphology and marker gene expression during fetal gonad differentiation. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: KHA EME. Performed the experiments: SMC LLW MCM GJB EME KHA. Analyzed the data: SMC LLW MCM GJB EME KHA. Wrote the paper: SMC EME KHA.

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