Cell fate commitment during mammalian sex determination

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Abstract

The gonads form bilaterally as bipotential organs that can develop as testes or ovaries. All secondary sex characteristics that we associate with 'maleness' or 'femaleness' depend on whether testes or ovaries form. The fate of the gonads depends on a cell fate decision that occurs in a somatic cell referred to as the 'supporting cell lineage'. Once supporting cell progenitors commit to Sertoli (male) or granulosa (female) fate, they propagate this decision to the other cells within the organ. In this review, we will describe what is known about the bipotential state of somatic and germ cell lineages in the gonad and the transcriptional and antagonistic signaling networks that lead to commitment, propagation, and maintenance of testis or ovary fate.

Formation of the gonad

Gonads form as paired, bilateral organs that are composed of several lineages of somatic cells as well as the population of germ cells. Precursors of many of the somatic cells in the gonad arise from proliferation of the SF1 (steroidogenic factor 1, aka NR5A1)-positive cells in the coelomic epithelium (CE) overlying the region of the intermediate mesoderm called the mesonephros. The CE begins to thicken in this region at approximately embryonic day (E) 10.0 and contributes to at least two distinct somatic precursor lineages that are bipotential: first, supporting cell precursors, which give rise to Sertoli cells in the testis or fetal granulosa cells in the ovary, and second, steroidogenic progenitors, which give rise to Leydig cells in the testis or theca cells in the ovary [1,2]. Genes including *Wt1* (Wilms tumor 1 homolog) [3], *Lhx9* (LIM homeobox protein 9) [4], *Emx2* (empty spiracles homeobox 2) [5], *Sf1* [6], *M33* (Cbx2, chromobox 2) [7,8], *Gata4* [9] and *Six1/4* (sine oculis-related homeobox 1/4) [10•] are essential to establish the bipotential population of somatic cells in the gonad.

The bipotential stage

The early somatic progenitors are capable of adopting either male or female fate. In accord with classic theory in the field, the transcriptomes of whole XX and XY gonads are nearly indistinguishable at E10.0 through E11.2 [11••,12]. At this bipotential stage, genes that are later associated with testis fate (i.e. *Sox9* (Sry (sex determining region of the Y)-box 9) and *Fgf9* (fibroblast growth factor 9)) and ovary fate (i.e. *Wnt4* (wingless-type MMTV integration site family, member 4) and *Rspo1* (R-spondin homolog 1)) are expressed at

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similar levels in XX and XY gonads [11••]. This is also true if different cell types in the XX and XY gonad are isolated by flow cytometry and analyzed separately at E11.5 [13]. These results suggest that the bipotential plasticity of the mammalian gonad results from a transient balanced transcriptional state in which many genes later associated with male or female fate are expressed at similar levels in supporting cell precursors of both XX and XY gonads. Although the gonad is poised to follow either pathway at this bipotential stage, the supporting cell lineage expresses more genes later associated with the female than the male pathway, suggesting a female bias in the underlying program [13].

The first steps of male or female fate commitment

Sex determination initiates by tilting the balance in the transcription network toward the male or female fate. The switch to initiate the male pathway in the poised supporting cell progenitors is the Y-linked gene, *Sry*. An *Sry* transgene, driven in the XX gonad from its own promoter, caused differentiation of a testis [14]. This experiment showed that first, *Sry* is the only gene from the Y chromosome that is required for male sex determination, and second, the molecular environment of the XX gonad is fully competent to activate *Sry* and initiate testis development (for a recent excellent review focused on the regulation of *Sry* itself, see [15]).

Sry gene expression initiates just after E10.5 (10 tail somites (ts)) based on an RNase protection study [16]. Using *in situ* hybridization, expression is detectable in the middle of the gonad at ts14 (~E11.0) and expands toward the anterior, then posterior poles [17]. The timing and level of expression of *Sry* are critical. XY mice carrying a weak allele of *Sry* that shows a decrease/delay in expression, are susceptible to male-to-female sex reversal [18–20]. Experiments that drive *Sry* expression in XX gonads using a heat shock promoter, revealed a requirement for *Sry* in the 6-h time window between E11.0 and E11.25 [21]. If expression is delayed, the testis pathway is aborted and ovarian development ensues. Exactly why the window of opportunity to initiate the male pathway closes at E11.25 remains unclear. Downstream of *Sry* expression, *Sox9* is the earliest gene to be upregulated in the male pathway at E11.2, closely followed by *Cited4* (*Cbp*/p300-interacting transactivator-4, with Glu/Asp-rich carboxy-terminal domain, and *Sox13* (SRY-box 13) at E11.4, and a larger group at E11.6 [11••]. Many of these genes are critical to establish male fate [22–24].

Genes associated with the female pathway become dimorphic slightly later, between E11.4 and E11.6, including *Wnt4*, *Rspo1*, *Irx3* (Iroquois related homeobox 3), *Lhx9*, *Fst* (follistatin), and *Lef1* (lymphoid enhancer binding factor 1) [11••,13]. The downstream effect of WNT4/RSPO1 signaling is the stabilization of β-catenin [25,26]. β-Catenin accumulates in the nucleus [27,28] where it may interact with the transcription factor LEF1 leading to the activation of downstream genes, as in other systems [29]. Stabilization of β-catenin in the XY gonad leads to down-regulation of SOX9 and male to female sex-reversal [30]. Antagonism between SOX9 and β-catenin may underlie the molecular decision in individual cells. However, loss of *Wnt4* and/or *Rspo1* and/or *β-catenin* does not lead to complete female to male sex reversal until perinatal stages [27,31–33].

Female fate is also regulated by the transcription factor *Foxl2* (forkhead box L2). FOXL2 co-operates with BMP2 (bone morphogenetic protein 2) to up-regulate the expression of *Fst* [34]. In goats, loss of function of *Foxl2* leads to female to male sex reversal in fetal life [35**]. However, in mice and humans, disruption of the female pathway does not occur until neonatal stages. Although loss of *Foxl2* in combination with *Rspo1* or *Wnt4* slightly accelerates the sex reversal phenotype in mice [36,37], no gene has been discovered whose loss leads to female to male sex reversal at early fetal stages. Since the bipotential gonad is initially biased toward the ovarian fate, a gene with a comparable role to *Sry* may not be required to initiate the female pathway. It may be sufficient *not* to initiate the male pathway.

Supporting cell precursors enter a quiescent state by E12.5 in XX gonads [38], consistent with the upregulation of negative regulators of the cell cycle observed in transcriptome studies [13,39]. The quiescent state of progenitor cells in the ovary may protect them from switching fate until proliferation resumes around the time of birth [38,40•]. In contrast, supporting cell progenitors in the XY gonad upregulate proliferation immediately downstream of *Sry*, and blocking proliferation disrupts the male pathway [2,41]. Whether proliferation is important for intracellular fate commitment or is required to establish a threshold population of Sertoli progenitors is still unclear.

Sexually dimorphic expression can result from activation in one sex, repression in the other sex, or a combination of both mechanisms. All of these patterns were evident in a study in which the gene expression profile for each gene was compared in XX and XY gonads at fine time points between E11.0 and E12.0 [11**]. Enrichment of male pathway genes occurred primarily through activation in the XY gonad with a minor contribution from repression of male genes in the XX gonad. In contrast, about half of the genes that became enriched in the female gonad did so due to repression in the XY gonad (Figure 1). This is a critical feature of the counterbalanced system that controls sex determination and gonadal fate: to establish a fate decision in the gonad, it is not sufficient to activate one of the alternative pathways — it is also necessary to repress the other.

Propagation of fate commitment across the gonad field

Subsequent to the primary fate decision in both differentiation pathways, feedback mechanisms are activated that canalize the chosen sexual fate. This occurs within individual cells and across the gonad field.

Although XX ⇔ XY chimeras typically have a similar number of XX and XY cells in the fetal gonad (as in other organs), more than half develop as males. This result suggests that a threshold number of XY cells can establish the testis pathway throughout the organ, but in cases where this cell threshold is not met, the gonad develops as an ovary. In the adult testes of chimeras, most of the Sertoli cells are XY, however, a small number of XX Sertoli cells are reproducibly found, indicating that XY supporting cells in the gonad can recruit XX cells (that do not have a Y chromosome or *Sry* gene) to Sertoli fate, presumably through paracrine signals [42,43]. These results indicate that in addition to the fate determination step that occurs in each supporting cell (likely based on antagonism between SOX9 and β-catenin), there is a community decision that takes place across the field of the gonad.

Two paracrine signaling molecules downstream of SOX9, FGF9 and PGD2 (prostaglandin D2 synthase), are known to contribute to propagation and maintenance of the male fate decision [44–50]. *Fgf9* is required to signal from the central part of the gonad and establish *Sox9* expression in the two ends [51]. Fgf signaling functions to antagonize Wnt signaling [45]. Elimination of the expression of *Fgf9* or its receptor *Fgfr2* (fibroblast growth factor receptor 2), leads to upregulation of the Wnt pathway and male-to-female sex reversal [44,52–54]. On the other hand, elimination of *Wnt4/Rspo1/β-catenin* leads to female-to-male sex reversal near birth [27,31–33], suggesting that the *Fgf9* and *Wnt4* signaling pathways have antagonistic roles in sex determination. Deletion of both *Fgf9* and *Wnt4* or *Fgfr2* and *Wnt4* rescues the male-to-female sex reversal phenotype in the XY gonad. In double mutants both somatic cells and germ cells express markers associated with male development, strongly suggesting that the primary role of Fgf signaling in XY gonad development is to repress the Wnt pathway [55]. Similar results were obtained by paired deletion of several other sets of antagonistic factors. For example, deletion of *Sox9* leads to male to female sex reversal, but rescue of the male fate occurs when *Sox9* and *Rspo1* are simultaneously deleted [56]. Similarly, when *Wnt4* and *ActivinβB* were simultaneously deleted, the female fate was rescued [57]. These studies and others indicate a multi-layered input into sex determination and suggest that many coding genes and perhaps microRNAs [58] affect the balance between male and female fate.

Relatively little is known about how other somatic lineages across the gonad field commit to a testis or ovary fate. However, it is clear that this occurs downstream of the fate commitment step in the supporting cell precursors. The steroidogenic precursor lineage in XX and XY gonads shares an indistinguishable transcriptional profile at E11.5 [13]. At E12.5, XX and XY steroidogenic progenitors begin to show divergent expression patterns downstream of morphological changes that occur in the XY gonad including the formation of the male-specific vasculature. Disruption of *Dhh* [23,24] or *Pdgfra* [59,60] leads to defects in Leydig cell development in the testis, but it is somewhat unclear whether these effects are direct, or are indirectly mediated by effects on the vasculature. Disruption of the male-specific vasculature also disrupts Leydig cell development, likely through *Notch/Jag1* signaling [61•]. In the fetal mouse ovary, NR2f2 and MafB label the progenitors of a cell type distinct from the supporting cell lineage [62•,63]. Steroidogenic cells in the ovary begin to produce hormones around birth. Like the steroidogenic lineage in the testis, they likely have a mixed origin, and differentiate in response to induction signals in their testis or ovary environment [64,65].

Germ cell fate commitment

Germ cells arise at the base of the allantois in the E6.25 embryo, migrate within the gut epithelium, and arrive in the gonad during the early stages of gonadogenesis. During these stages, the germ cell genome is stripped of most of its methylation [66]. Like ES cells, germ cells carry bivalent histone marks on many developmentally regulated genes [67•], are highly pluripotent, and can readily give rise to embryonic germ cells (EG cells) [68]. Fate determination in the germ cell lineage involves a repression of pluripotency and a commitment to differentiate as one of two specialized gamete precursors: spermatogonia or oogonia.

Germ cells differentiate as spermatogonia or oogonia irrespective of their genetic sex, but based on cues from their somatic environment. Unlike the supporting cell lineage, germ cells at the bipotential stage express more genes associated with a male fate, than with a female fate [13], suggesting that female germ cells take the more divergent path at this stage of development. At E11.5, XX and XY germ cells have very similar transcriptomes, The only differences are detected in levels of a few Y-linked and X-linked genes [11••]. Regardless of these differences, both XX and XY germ cells are capable of responding to either male or female gonadal cues up until E12.5, after which their fate is fixed [69].

XX and XY germ cells enter meiosis in an ovarian environment, or in cases where they are lost in the mesonephros or adrenal. In contrast, in a testis environment germ cells arrest in G0 of the mitotic cell cycle [70]. Recently a molecular explanation for this behavior has emerged that provides both a repressor of meiosis in the XY gonad, as predicted by McLaren and Southee [69], and an activator of meiosis in the XX gonad, as proposed by Byskov *et al.* [71].

In XX gonads, retinoic acid (RA) signaling initiates meiotic entry via activation of *Stra8* (stimulated by RA gene 8). RA is synthesized in the mesonephros, and may be transported by the mesonephric tubules that are physically connected with the anterior end of the gonad. This could explain the anterior-to-posterior wave of *Stra8* expression that triggers meiotic entry between E13.5 and E15.5 [72–74]. The timing of meiotic entry is controlled by polycomb complex 1 (PRC1), which represses expression of *Stra8* prior to E13.5 in XX germ cells [75•]. Germ cells in the female gonad progress through leptotene, zygotene and pachytene, and arrest in diplotene near the time of birth.

RA is also produced from the mesonephros in XY embryos (as in XX), however, the P450 enzyme CYP26B1 (cytochrome P450, family 26, subfamily b, polypeptide 1) is synthesized early in the male pathway by somatic cells in the testis, and functions to degrade RA and block activation of *Stra8* and meiosis [76,77]. In mouse genetic models where *Cyp26b1* is disrupted, germ cells enter meiosis in the testis [77,78].

Additional factors regulate the divergence of male and female germ cell fates. In addition to their role in somatic sex determination, the antagonistic signaling pathways, Wnt/Rspo and Fgf, also affect germ cell fate. Wnt/Rspo signaling acts through β-catenin to regulate proliferation of XX germ cells and promote their entry into meiosis [79]; whereas, loss of *Wnt4* leads to an anterior to posterior loss of female germ cells [40•]. In contrast, loss of *Fgf9*, which is upregulated early in the Sertoli cell differentiation pathway leads to sex-specific apoptosis of germ cells in the testis and up-regulation of meiotic markers [80,81].

FGF9 is required for the up-regulation of *Nanos2* [81,82], a key RNA-binding protein (RBP) that is critical for initiating the male pathway in germ cells. RBPs, including NANOS2/3 [83,84•], TDRD1, a tudor domain containing RBP [85,86], DND1 (dead end homolog 1) [87,88], and PUMILIO [89] play a prominent role in male germ cells. In the absence of *Nanos2*, male germ cells enter meiosis and/or undergo apoptosis [90]. NANOS2 and NANOS3 associate in the CNOT complex to control adenylation of multiple mRNA targets and promote degradation of mRNAs encoding meiotic genes [83,84•]. *Dnd1* also promotes

translation of several negative regulators of the cell cycle, including *P21* (Cdkn1a, cyclin-dependent kinase inhibitor 1A) and *P27* (Cdkn1b, cyclin-dependent kinase inhibitor 1B) [87,91]. Accumulation of negative cell cycle regulators is believed to bring male germ cells into mitotic arrest in G0, where they remain until perinatal stages when mitosis resumes prior to the establishment of the spermatogonial stem cell population [92,93]. Some evidence suggests that cell cycle arrest in male germ cells is critical to repress pluripotency and establish spermatogonial fate. Germ cells that fail to enter G0 by E15.5 in the male gonad are susceptible to teratoma formation, a strong indication that germ cells have not repressed their pluripotent state [87,94,95].

Although FGF9 also has been assigned a role in up-regulation of $p15^{INK4B}$ (*Cdkn2b* cyclin-dependent kinase inhibitor 2B) [81], if both *Fgf9* and *Wnt4* are deleted, the XY gonad embarks on the testis pathway, and XY germ cells fail to enter meiosis and express normal markers of the male pathway [55]. This has been interpreted to mean that other Fgfs can compensate for this function of *Fgf9*, or that there are layers of antagonistic signals that control germ cell fate similar to those that control somatic cell fate.

Maintaining Sertoli or granulosa cell fate in adult life

Transdifferentiation from one committed fate to another is an unusual phenomenon that occurs naturally when germ cells are depleted from the adult ovary: in this situation granulosa cells transdifferentiate to Sertoli cell fate [96,97]. While transdifferentiation was regarded as an indirect effect of germ cell loss, in the past decade, several genes have been identified that are directly involved in active maintenance of supporting cell fate in adult life.

In the female gonad, *FoxL2* plays a key role in maintaining ovarian fate postnatally. In cooperation with the estrogen receptors 1 and 2 (ESR1/2), FOXL2 antagonizes the male pathway by direct binding to the TESCO regulatory region of SOX9 to repress its expression. When FOXL2 is lost in adult life, granulosa cells transdifferentiate prior to germ cell loss, and theca cells also begin to produce testosterone [98]. Null mutations in *Esr1/Esr2*, or in P450 aromatase (*Cyp19a1*) also result in transdifferentiation in the postnatal ovary accompanied by germ cell loss [99,100]. While it is difficult in these instances of transdifferentiation to disentangle the effects of germ cell loss, it is likely that they are due at least in part to direct effects of estrogen on maintenance of granulosa cell fate.

In the adult testis, DMRT1 (doublesex and mab-3 related transcription factor 1) binds to regulatory regions of testis-promoting and ovary-promoting genes. DMRT1 activates testis-promoting genes such as *Sox8*, *Sox9* and *Ptgdr* (prostaglandin D receptor), and represses ovary-promoting genes such as *FoxL2*, *Wnt4*, and *Rspo1*. DMRT1 may be important to antagonize the influence of RA, which is produced in the adult testis to drive entry of spermatogonia into meiosis, and may have a feminizing influence on Sertoli cells [101••]. These results suggest that maintenance of testis or ovary fate is an active process in adult life.

Summary

Commitment of the bipotential gonad to testis or ovary fate is the result of antagonistic male and female pathways that compete to control the differentiation of supporting cell precursors, likely through regulation of SOX9 and β-catenin (Figure 2). Sertoli or granulosa cell fate commitment involves the activation of one program and the repression of the alternative pathway of development. The decision is then propagated across the gonad field, and controls the fate of other somatic lineages as well as the germ cell lineage. In the adult testis and ovary, transdifferentiation can occur between Sertoli and granulosa cell fates. Many differentiated cells in other organs share a common bipotential progenitor, however, it is unclear whether the phenomenon of transdifferentiation between differentiated fates occurs naturally in other systems, or whether it is specific to gonadal cells. It could be an evolutionary remnant of the ability of some fish to switch sex in adult life [102] or of some vertebrates' ability to function as 'natural hermaphrodites' by maintaining a gonad with seasonally expanding ovarian and testicular regions such as the mole and alligator [103,104]. It will be interesting to see how this plasticity of cell fate is related to the epigenetic landscape at the bipotential stage, and in Sertoli and granulosa cells during fetal and adult life.

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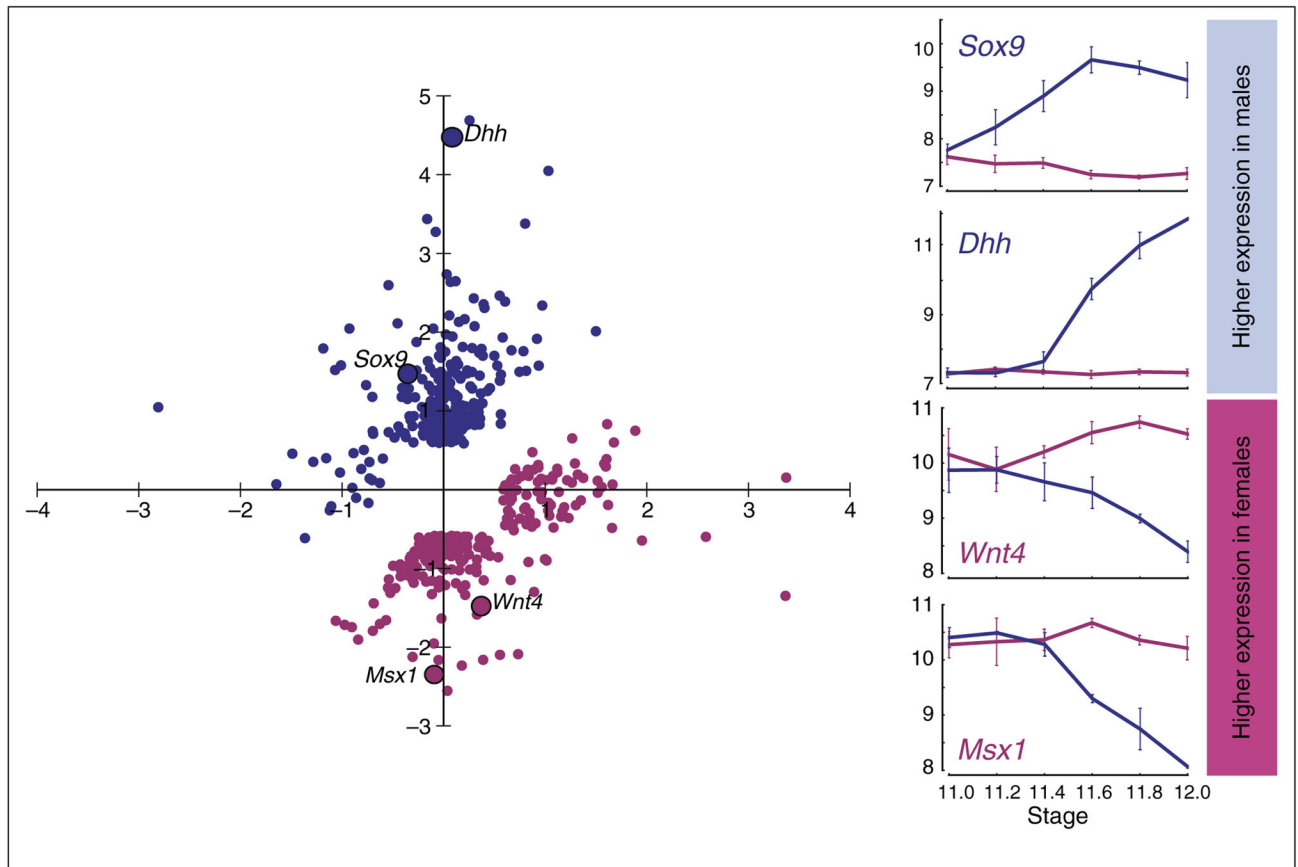
Figure 1.

Commitment to the male pathway involves the down-regulation of many genes associated with female development. Diagram depicting the change in the expression of each gene in the XX gonad (*X*-axis) and XY gonad (*Y*-axis) between E11.0 and E12.0. Both of these changes can contribute to sexually dimorphic expression. Line graphs show changes in expression of two representative male genes (*Dhh* and *Sox9*) and two representative female genes (*Msx1* and *Wnt4*) in the XY (blue line) and XX (red line) gonads. Higher expression of the male pathway genes, *Dhh* and *Sox9*, occur primarily through up-regulation in the XY gonad. Sexually dimorphic expression of many of the genes in the female pathway, *Msx1* and *Wnt4*, occurs through down-regulation in the XY gonad. Adapted from Munger SC *et al.* [11].
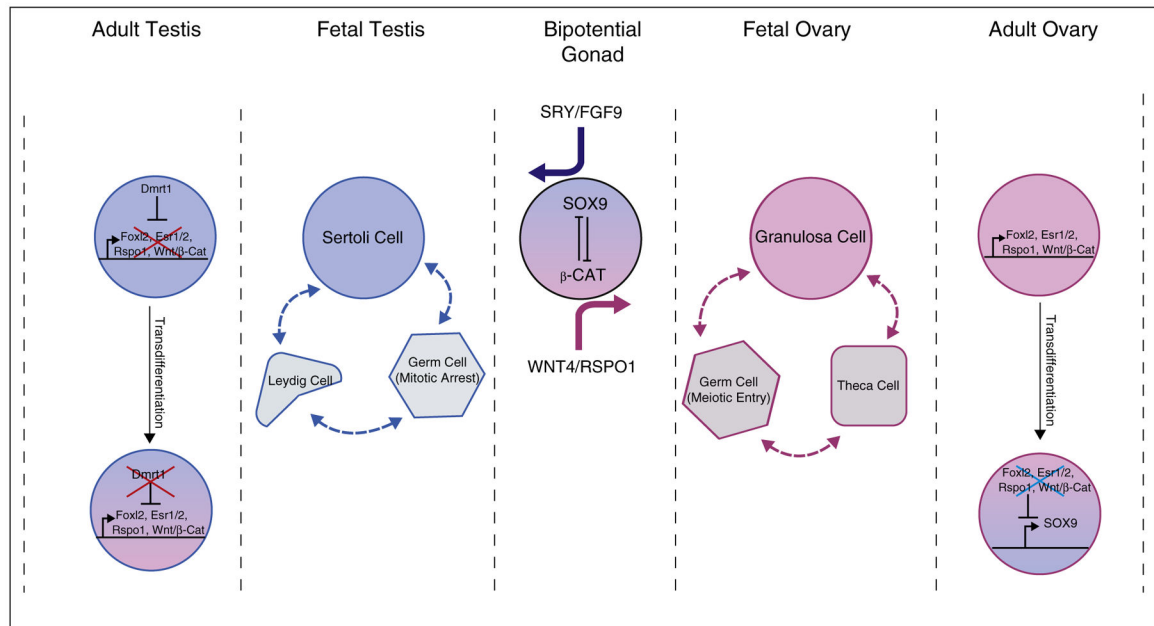
Figure 2.
Commitment and maintenance of gonadal cell fate. In XX and XY gonads at the bipotential stage, supporting cell precursors are exposed to male and female promoting signals that antagonize each other. In the XY gonad, expression of *Sry* triggers up-regulation of *Sox9* and *Fgf9*, which activate the male pathway and repress signals that promote the female pathway (WNT4/RSPO1 and β-catenin). Supporting cell precursors commit to Sertoli cell fate and orchestrate testis development by promoting Leydig cell differentiation from steroidogenic progenitors, and regulating mitotic arrest in germ cells. In the XX gonad, in the absence of *Sry* to initiate the male pathway, *Wnt4* and *Rspo1* maintain *β-catenin* signaling to promote the female pathway. Supporting cell precursors commit to granulosa cell fate and orchestrate ovary development by promoting Theca cell differentiation from steroidogenic progenitors, and regulating meiotic entry in germ cells. Reinforcing signals (dotted lines) exist between somatic and germ cells in the developing testis and ovary. In the adult testis, *Dmrt1* is required for maintaining Sertoli cell fate by repressing female promoting signals, while in the adult ovary, abolishing female promoting signals leads to loss of granulosa cell fate and up-regulation of *Sox*9 and other markers of Sertoli fate.