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To Be or Not To Be a Testis

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Abstract

Work that established the testis as the driver of male development, and the Y-chromosome as the bearer of the male-determining gene, established a working model, and set the stage for the molecular age of mammalian sex determination. The discovery and characterization of *Sry*/*SRY* at the top of the hierarchy in mammals launched the field in two major directions. The first was to identify the downstream transcription factors and other molecular players that drive the bifurcation of Sertoli and granulosa cell differentiation. The second major direction was to understand organogenesis of the early bipotential gonad, and how divergence of its two distinct morphogenetic pathways (testis and ovary) is regulated at the cellular level. This review will summarize the early discoveries soon after Sry was identified, and focus on my study of the gonad as a model of organogenesis.

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Setting the stage for the molecular investigation of sex determination

Sex determination is a very old field that has fascinated scientists and non-scientists alike for thousands of years. Early Greek philosophers suggested that the sex of the child depended on its position in the womb or on whether the semen came from the right of left testis. Aristotle, argued against these theories, and instead proposed that the sex of the child depended on the heat of copulation (for review, see Lesky, 1951).

It was Alfred Jost, working in France at the end of World War II, who set the stage for the molecular era when he established the critical importance of gonad sex specification and differentiation into either a testis or ovary (Ford et al., 1959; Jost, 1947). Jost surgically removed the gonads from rabbit embryos *in utero* and showed that this resulted in all female offspring. This pivotal experiment established the idea that the ovary was not essential to develop as a female, but the presence of a testis was essential to develop as a male. By 1959, advances in cytogenetics made it possible to identity the Y-chomosome as the mediator of male fate. At the time, this result was surprising as the number of X chromosomes had been found to mediate sex determination in *Drosophila* (Bridges, 1921). However, examination of human aneuploids with XXY and XO karyotypes showed that it was the presence or absence of the Y that controlled male sex (Ford et al., 1959; Jacobs and Strong, 1959; Welshons and Russell, 1959). Putting these results together, Anne McLaren and others suggested a working model in which the testis-determining signal (called *Tdy*) acted to control the development of the gonad as a testis or ovary (McLaren, 1991)(Fig. 1).

Identification of *Sry*

The race was on to identify the critical determinant, *Tdy*. Candidates came and went, with very little evidence to support them. For a while, it was believed that a Y-linked antigen (H-Y) discovered through male to female skin grafting experiments, must be the male determinant (Bennett et al., 1977). This idea was followed by the discovery of an evolutionarily conserved Bkm repeat sequence on the Y – also proposed to act as the male determinant for a time (Singh et al., 1984). However, in 1989, David Page at MIT put forward the first evidence-based molecular candidate, *ZFY*, relying on the investigation of patients whose sex chromosome genotype was discordant with their physiological development as male or female (Page et al., 1987). The Page lab found that the zinc finger protein, *ZFY*, was deleted in an XY female patient, and present in an XX male. The fact that ZFY was likely to act as a transcription factor also fit with predictions.

However, very soon after these findings were published, investigators on the other side of the Atlantic began to raise doubts that *ZFY* was the right gene. First, in the absence of germ cells in the mouse, *Zfy* was not expressed, but testis development occurred normally, strongly implying that *Zfy* could not be the right gene (Koopman et al., 1989). Second, in a group of 4 XX patients with Y-chromosome translocations and male characteristics, the 35 kb region of the Y chromosome that was translocated did not include *ZFY*, again, inconsistent with the candidacy of *ZFY* as the male-determining gene (Palmer et al., 1989).

Based on the idea that the mammalian sex-determining gene would be conserved among mammals, the Goodfellow and Lovell-Badge labs in London used tiling probes across the 35 kb region of the Y-chromosome present in the 4 XX patients, to identify a Y-specific fragment present on the Y chromosome in a large group of mammals, including human, chimp, rabbit, pig, horse, cattle, and tiger. This led to the identification of a gene encoding an HMG-box domain that was named *SRY* (sex determining region of the Y) (Sinclair et al., 1990).

Validation of SRY as the male sex-determining gene came from several directions. The orthologous gene was cloned from the mouse genome and used with a 14 kb surrounding region to produce an XX transgenic mouse (Randy) that developed as a male (Koopman et al., 1991). Randy was sterile, but otherwise had all male physiological attributes as well as male mating behavior. In addition, mutations within *SRY* were identified in several XY female patients (Berta et al., 1990). Furthermore, an XY female mouse generated years earlier and predicted to have lost *Tdy*, was shown to be deleted for the entire *Sry* locus (Gubbay et al., 1992).

The identification of *Sry* as the Y-linked gene that controls sex determination in mammals launched the molecular age of mammalian sex determination. SRY was predicted to act at the top of a pathway to drive testis development. It was therefore an immediate priority to clone the transcript for *Sry*, understand its regulation, and begin to identify the downstream genes activated by SRY. This turned out to take some time.

By RT-PCR analysis, *Sry* was found to be expressed in the early mouse gonad, during the bipotential period (as predicted), but at very low levels and for approximately 48 hours (Hacker et al., 1995). Since it was also expressed in the adult testis where material was not limiting, efforts to clone the cDNA were directed at adult testis libraries. The problem was that screens of testis cDNA libraries turned up transcripts with circular permutations (Fig. 2A). This seemed likely to be an artifact, so it was several years before an RNA protection assay revealed that the *Sry* transcript in the adult testis is circular—the first circular transcript documented (Capel et al., 1993b). In contrast, the transcript in the early gonad is linear, as a result of an alternative start site that eliminates most of the 5' UTR, including the 5' end of the inverted repeats surrounding the *Sry* coding region (Fig. 2B,C)(Capel et al., 1993a; Hacker et al., 1995). The regulatory region upstream of *Sry* is complex. Deletion of Y-chromosome sequences spanning Sx1 repeats located >14kb upstream of the *Sry* promoter led to low expression of *Sry* and development of XY females (Capel et al., 1993a). These findings have not been explained, but may stem from chromatin position effects, or alterations in the epigenetic landscape (Kuroki et al., 2017).

Genetic studies identified other key genes in the sex determination pathway

The identification of other genes responsible for Disorders of Sexual Development in humans by many others helped to build the molecular pathway, upstream and downstream of *SRY*. A key step was the discovery of *SOX9*, identified at the chromosomal breakpoint in XY campomelic dysplasia patients who were sex-reversed to female (Foster et al., 1994). Experiments showing that gain or loss of *Sox9* had very similar effects to gain or loss of *Sry* led to the idea that *Sox9*

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might be the primary (and perhaps only) target of SRY (Chaboissier et al., 2004; Vidal et al., 2001). Much attention in the field turned to addressing the question of how *Sox9* is regulated. Twenty-five years later, this line of work culminated in the discovery of an enhancer element more than 500 kb upstream of *Sox9* based on analysis of the chromatin landscape (Garcia-Moreno et al., 2019; Maatouk et al., 2017a). Deletion/ mutation of this enhancer leads to male to female sex reversal in mice and humans (Gonen et al., 2018).

Several other seminal discoveries in human patients, goats, and mice framed the field of ovary development. *FOXL2* was identified as the gene responsible for XX Blepharophimosis/ Ptosis/Epicanthus inversus Syndrome (BPES), which is associated with premature ovarian failure in humans (Crisponi et al., 2001; Schmidt et al., 2004). Loss of *Foxl2* in goats led to female-to-male sex reversal (Pailhoux et al., 2001). However, in mice, loss of *Foxl2* led to arrested ovarian somatic cell differentiation, atresia, and infertility, but did not lead to sex-reversal of females to male (Schmidt et al., 2004). However, Camerino and co-workers discovered another key gene regulating ovary development in a consanguineous family with XX males, *RSPO1* (Parma et al., 2006). Loss of *Rspo1* in XX mice led to variable sex-reversal with evidence of some well-formed testis structures, strong SOX9 expression after birth, and ambiguous genitalia (Chassot et al., 2008; Tomizuka et al., 2008). RSPO1 is a secreted activator of Wnt signaling, another gene involved in ovary development in mice and humans (Biason-Lauber, 2012; Mandel et al., 2008; Vainio et al., 1999).

The Gonad as a Model of Organogenesis

Apart from being an interesting model for identification of the transcription factors that act in a cascade to control sex-determination, the gonad is an outstanding model of organogenesis. It is unique in that (unlike a kidney or a lung) the gonad arises as a bipotential organ with the ability to develop as either a testis or an ovary. Development of the gonad hinges on whether or not *Sry* or *Sox9* is expressed to trigger the testis pathway. In the absence of either of those genes, and barring other mutations, the gonad develops as an ovary. These two pathways diverge rapidly after *Sry* is expressed, and show remarkably different morphogenesis strategies. It was therefore very interesting to understand the cell biology of this branchpoint in gonad development. Developments in confocal imaging made this a particularly good time to tackle this problem.

Origin of gonadal supporting cells

The gonad forms on the coelomic surface of the intermediate mesoderm, just above the mesonephric ducts that are forming in the interior of the tissue (Karl and Capel, 1995). There are three major lineages in the early gonad, all of which are bipotential: supporting cells (which can become Sertoli or granulosa cells), steroidogenic cells (which can become Leydig or theca cells), and germ cells (which migrate from the posterior of the embryo and arrive coincident with gonad formation). Labeling of single cells in the coelomic epithelium (CE) with a vital dye (Karl and Capel, 1998), or lineage tracing of dividing cells (Schmahl et al., 2000), showed that Sertoli cells arise from this surface epithelium prior to embryonic day (E)11.5. After this

timepoint, other gonadal cell types arise from the CE, but it is no longer competent to give rise to Sertoli cells. Cells in the CE report active Notch signaling, but as cells leave the CE, NUMB accumulates asymmetrically at their basal surface, and governs the competence to differentiate. In gonads lacking *Numb* and *Numbl* (Numb-like), large groups of cells in both XX and XY gonads fail to differentiate, reducing populations of supporting and steroidogenic cells in both sexes (Lin et al., 2017).

Patterns of proliferation differ between the developing testis and ovary. Whereas supporting cells in the ovary remain quiescent (until after birth) (Mork et al., 2012a), cells in the testis reenter the cell cycle and expand the population of Sertoli progenitors (Schmahl and Capel, 2003; Schmahl et al., 2000). Changes in proliferation of Sertoli cell progenitors in the CE are contingent on the expression of *Fgf9* and *Fgfr2* which are upregulated for a brief period after *Sry* is expressed (Colvin et al., 2001; Schmahl et al., 2004). Loss of *Fgf9* leads to disruption of testis development and sex reversal, even though expression of *Sry* and *Sox9* initially occur normally (Kim et al., 2006). This may be because the reduced Sertoli population cannot stabilize testis development, but other explanations are possible, including a failure to propagate the activation of SOX9 across the gonad field (Hiramatsu et al., 2009).

At least some of the somatic cells in the ovary also arise from the CE (Karl and Capel, 1998; Mork et al., 2012a). However, the window of competence to give rise to granulosa cells extends over a longer period of development. DiI-labeling of the CE in the XX gonad showed that granulosa cells (which express FOXL2) continue to arise until E14.5. FOXL2+ cells in the interior of the gonad surround clusters of germ cells (germ cell cysts) and enter cell cycle arrest. They remain in arrest until birth, when germ cell cysts break apart and granulosa cells surround some individual oogonia, re-enter active cell cycle, and proliferate to form the first wave of growing follicles. When the fetal population of granulosa cells was lineage traced, they were shown to contribute to growing follicles in the medulla, but not to the cortical population. Instead, between birth and P7, a new group of FOXL2+ follicle cells move in from the CE and surround individual oogonia in the cortex of the ovary. This population constitutes the primordial follicle pool, the so called “ovarian reserve” (Mork et al., 2012a). Progress has been made on the break-down of germ cell cysts, but the hand-off to the new population of granulosa cells arising from the CE is still not clear (Lei and Spradling, 2013, 2016). In many ways, this system is similar to Drosophila, where the follicle cells that surround germ cells as they first arise are called “escort cells”, whose job is to chaperone the germ cells and transfer them to the definitive follicle cells (Sahai-Hernandez et al., 2012).

These findings required a reassessment of the standard model of sex determination in which the Sertoli and granulosa cells of the adult testis and ovary directly stem from the supporting cell precursors of the bipotential gonad. Although the CE of the gonad, which expresses LGR5 in fetal, neonatal, and adult life, appears to be a major, if not the only, source of both Sertoli cells and granulosa cells, fetal and adult granulosa cells are born at different stages of development. Whereas the original *Sry*-expressing cells and their progeny are believed to account for all of the Sertoli cells present in the adult (Sekido et al., 2004), the number of FOXL2-positive cells increases in the absence of intrinsic proliferation by recruitment from the CE after the

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bipotential period has concluded. The first cells to emerge from the CE (prior to E11.5) include stromal cells and the bipotential supporting cell precursor population (Karl and Capel, 1998), competent to differentiation as granulosa cells, or to activate the *Sry* promoter and differentiate as Sertoli cells (Mork et al., 2012a). In some sense, the fetal granulosa cells act as "place keepers" for the ovarian pathway until the definitive adult population arises.

Recruitment of other cell types in the gonad

Cell types other than supporting cells are present in the early gonad. Some of the cells that make up the interstitial or stromal populations can also be lineage traced to the CE, but others are recruited from sources extrinsic to the gonad. Previous work from Anne McLaren's lab suggested that cells from the adjacent tissue, the mesonephros, migrated into the mouse gonad (Buehr et al., 1993), but methods at the time did not lend themselves to a definitive analysis. The development of the ROSA-βgal (Soriano, 1999), and subsequently, the ROSA-GFP (Giel-Moloney et al., 2007) mouse lines facilitated tissue recombination experiments to measure cell migration between tissues. These experiments showed that vascular endothelial cells migrate into the XY but not the XX gonad. Migration was contingent on expression of *Sry* (Capel et al., 1999; Coveney et al., 2008; Martineau et al., 1997; Tilmann and Capel, 1999), and cells could be induced to migrate into the XX gonad when it was sandwiched between the mesonephros and an XY gonad. Experiments that block migration disrupted testis morphogenesis, suggesting that the vasculature plays a critical role in the structural reorganization of gonadal cells into testis tubules. Further experiments showed that endothelial cells act through Vegf and Pdgf signaling to trigger the expansion of interstitial tissue around vessels, which serves to sub-divide the gonad and reorganize domains into approximately 12 cord-forming units (Cool et al., 2011). The vascular niche is also important for the regulation of the steroidogenic progenitors that give rise to Leydig cells (Defalco et al., 2013; Tang et al., 2008). These progenitors arise both from the coelomic epithelium and from specialized cells along the gonad-mesonephros border (Defalco et al., 2011; Kumar and DeFalco, 2018).

Other signals downstream of *Sry* are important for inducing testis-specific cell types in the XY gonad. For example, both *Dhh* (Yao and Capel, 2002; Yao et al., 2002) and *Pdgfra* (Brennan et al., 2003) are required to induce Leydig cell development. In part, the requirement for *Pdgfra* may reflect the fact that fetal Leydig cell progenitors are regulated by Notch signaling within a vascular niche (Defalco et al., 2013; Tang et al., 2008). Yolk sac-derived macrophages also play a role in the structural organization of the testis, by engulfing Sertoli cells that are not enclosed in cords, eliminating wayward germ cells, and cleaning up other debris during morphogenesis of the testis (DeFalco et al., 2014). Whether they also produce important cytokines is not yet clear.

Recently, neuronal development was found to differ between testis and ovary development. While neurons derived from the neural crest invade the ovary during the last quarter of fetal development, they are restricted to the tunica albuginea of the testis, likely by repulsive cues downstream of *Sry* (McKey et al., 2019). Neither the function of neurons in the ovary, nor the reason they are absent from the testis is yet understood. Whether recruitment of theca cell

progenitors from the adjacent mesonephros (Liu et al., 2016) is related to recruitment of neurons in the ovary has not yet been determined.

Cell Fate Determination

The gonad is also an outstanding model of cell fate determination. Gonadal sex determination can be reduced to a question of whether the bipotential supporting cells that enter the gonad from the CE initiate differentiation as Sertoli or granulosa cells. Transcriptome analysis of early gonadal populations (Jameson et al., 2012b; Munger et al., 2009) and more recently from single cell analysis (Stevant et al., 2019) indicates that the cells in XX and XY bipotential gonads are initially nearly identical. The only differences at early stages arise from genes that are specific to the sex chromosomes such as *Xist*, *Utx* and *Eif2s3x* (only present in XX) and *Ddx3y*, *Eif2s3y* and *Jarid1d* (only present in XY) (Munger et al., 2009).

Downstream of *Sry*, the fate of XY gonadal cells depends on expression of *Sox9* and the ability to repress the Wnt/β-catenin pathway that drives the ovary fate (Fig. 3)(Kim and Capel, 2006; Kim et al., 2006; Lavery et al., 2012; Nicol and Yao, 2015) *Wnt4* and *Rspo1* may be involved in the establishment of cells in the gonad field. Together these genes regulate proliferation of CE precursors that give rise to Sertoli cells (Chassot et al., 2012), and loss of *Wnt4* was previously shown to affect the SOX9 population (Jeays-Ward et al., 2004). However, once this population is established, repression of *Wnt4* signaling is required to stabilize testis fate (Maatouk et al., 2008). Loss of *Fgf9* leads to the reversion to ovary fate after SOX9 expression is established, but if *Wnt4* is also lost, SOX9 expression and the testis pathway are rescued (Jameson et al., 2012a). These results strongly suggest that there is a second stabilization step controlling testis fate governed by repression of *Wnt4* signaling. This occurs at multiple levels including through the Wnt antagonist, ZNRF3 (Harris et al., 2018).

Epigenetic Regulation

Recent analysis of histone methylation patterns confirm that genes associated with both testis and ovary pathways in supporting cells are bivalent in the E10.5 gonad, marked with both H3K27me3 (repressing) and H3K4me3 (activating) histones marks (Garcia-Moreno et al., submitted). These genes are initially expressed at similarly low levels in XX and XY gonads. At E13.5, after sex determination has occurred, genes associated with the testis pathway in XY gonads are stripped of their H3K27me3 repressive marks, but genes associated with the ovary pathway retain their bivalent status. Symmetrical changes occur in the XX gonad, where genes associated with the ovary pathway are stripped of their H3K27me3 repressive marks, but genes associated with the testis pathway retain their bivalent status. These findings may explain the ability of Sertoli and granulosa cells to reverse fate under some circumstances in adult life (Matson et al., 2011; Uhlenhaut et al., 2009).

Several lines of evidence suggest that the polycomb repressive complex 1 (PRC1) is required for testis development. Loss of *Cbx2*, the component of the PRC1 complex that recognizes the

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H3K27me3 mark, led to ovary development in XY mice and humans (Biason-Lauber et al., 2009; Katoh-Fukui et al., 2012; Katoh-Fukui et al., 1998). Although this was originally believed to be due to a failure of *Sry* expression, it is more likely due to a failure to repress ovary fate and stabilize SOX9 expression. Interestingly, *Lef1*, which encodes a protein downstream of Wnt signaling, is a direct target of CBX2, as is another key gene in the ovary pathway, *Foxl2* (Garcia-Moreno et al., PLoS Gen, in press). The identification of regulatory elements across the genome in XX and XY supporting cells using DHS and ATAC-seq methods (Garcia-Moreno et al., 2019; Maatouk et al., 2017b), combined with histone data (Garcia-Moreno et al., submitted) and ChIP-seq approaches for important transcription factors, may reveal how male or female fate is stabilized by repression of the alternative fate.

Questions remain about how the male or female pathway is activated in mammals. Interestingly, experiments in the red-eared slider turtle, *T. scripta*, may provide some insight. *T. scripta* has a temperature-dependent sex-determining system, in which the temperature of incubation of the egg controls whether the gonad differentiates as a testis or ovary. Evidence from a transcriptome time course of gonadal expression at male- and female-producing temperatures revealed that *Kdm6b* is male-specific at the earliest stages of turtle gonad formation (Czerwinski et al., 2016). KDM6B is a histone demethylase that specifically removes H3K27me3 repressive marks from target genes. Depletion of *Kdm6b* in the turtle, using a virally transduced shRNA, led to female development at the male-producing temperature (Ge et al., 2018). Furthermore, KDM6B was shown to bind the promoter of *Dmrt1*, a gene that was previously shown to drive male sex-determination in *T. scripta* (Ge et al., 2017)(and many other species of fish, reptiles, amphibians, and birds). Loss of *Kdm6b* was associated with a failure to remove H3K27me3 marks from the *Dmrt1* locus, and a failure to activate the gene at the male-producing temperature (Ge et al., 2018). These findings suggest that removal of repressive histone marks may be the key to activation of the male pathway and point toward the exploration of the orthologous H3K27me3 enzymes in mammals.

These experiments have taught us a lot about how cell fate is established in the gonad (which controls the sexual development of the organism), and the findings are likely to be widely applicable to other less accessible and less dramatic cell fate decisions. Many important questions remain. It is known from a group of genetic and gain of function experiments that there is a narrow window in development when Sertoli fate can be initiated in gonadal supporting cells (Hiramatsu et al., 2009). If *Sry* is expressed too late, or at a reduced level, Sertoli cell commitment fails. The molecular explanation for this narrow window of competence to initiate the testis pathway has not been discovered. However, one possibility is that the ovary pathway is on a steady upward trajectory that must be intersected before a female factor accumulates to an insurmountable level (Fig. 4). Antagonism could play out as a competition between the relative levels of a testis and an ovary protein. Even if this is the case, it is unclear which factors are involved in this competition. Another remaining puzzle is the explanation for why in many mutants that impair the male pathway, the central region of the XY gonad shows a stronger tendency to stabilize as testicular while the peripheral regions develop ovarian tissue (Eicher et al., 1982). While this was originally believed to be the result of earlier expression of

Sry in the center of the gonad (Bullejos, 2001), this pattern does not hold up to close scrutiny, thus is unlikely to be the explanation (Bunce et al., unpublished).

Germ cell development in the fetal gonad

Germ cells are another major constituent of the fetal gonad. They arise at the base of the allantois at ~E6.5 and migrate through the gut to the site where the gonad is forming, arriving ~E10.5. Initially, gonadal germ cells are found in clusters formed by aggregation (based on E-cadherin) as well as clonal divisions (Mork et al., 2012b). Although germ cells are not required for the structural development of the testis, their absence may delay testis cord formation (McLaren, 1985; Merchant, 1975). After birth, germ cells are required in the ovary for follicle formation and maintenance. In the absence of germ cells, or when they are lost in adult life, the ovary undergoes degeneration (Guigon et al., 2005; Guigon and Magre, 2006). However, fetal ovary development in the absence of germ cells proceeds normally until birth (Maatouk et al., 2012).

Upon arrival in the gonad, germ cells proliferate rapidly in both XX and XY gonads, but by E12.5, when the somatic cells of the gonad initiate sex-specific behavior, the fate of germ cells diverges between the testis and ovary (Schmahl et al., 2000). Experiments indicate that the chromosome constitution of germ cells (XX or XY) can be dominated by their gonadal environment: XX germ cells that arrive in a testis environment enter a male differentiation pathway, whereas XY germ cells that arrive in an ovary environment enter a female pathway (Adams and McLaren, 2002). In the ovary, germ cells up-regulate *Stra8* (stimulated by retinoic acid (RA)) in response to RA produced in the mesonephros (Bowles et al., 2006; Koubova et al., 2006). They initiate meiosis in a wave that proceeds from anterior to posterior (Yao et al., 2003). Although retinoic acid is produced in the mesonephroi of both the ovary and the testis, its meiosis-inducing effect is blocked in the testis by expression of the RA catabolic enzyme, CYP26B1 (Bowles et al., 2006; Koubova et al., 2006), and perhaps other factors produced by Sertoli cells. Instead of entering meiosis, male germ cells undergo a period of mitotic arrest extending from ~E15.5 until the end of fetal life (McLaren, 1984). Experiments suggest that meiotic germ cells antagonize testis cord formation (Yao et al., 2003).

Fgf9 is required for germ cell survival and development in the testis but not the ovary environment (DiNapoli et al., 2006). In *Fgf9* mutants, germ cell numbers were significantly reduced in the testis and could not be rescued unless exogenous FGF9 was added by E11.5, suggesting that transition to dependence on FGF9 occurs between E10.5 and E11.5. It is still unclear what entrains germ cells to the presence of FGF9 in the testis. Both suppression of meiosis, and expression of Nanos2, an RNA-binding protein required for male germ cell development, are downstream of FGF9 (Bowles et al., 2010).

A large number of RNA-binding proteins are required for male germ cell development. One of these is DND1. In 2003, the classic *Ter* mutation was mapped to *Dnd1*, which was shown to be expressed in germ cells (Youngren et al., 2005). The *Ter* mutation in *Dnd1* (*Dnd1*$^{Ter/Ter}$) leads to severe germ cell loss in both sexes, owing to BAX-mediated cell death pathways (Cook et al.,

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2009). However, on some genetic backgrounds, germ cells in male *Dnd1*$^{Ter/Ter}$ mutants fail to undergo mitotic arrest, and give rise to a very high incidence of testicular teratomas that arise between E16.5 and birth (Cook et al., 2011). The higher incidence of teratomas in the left testis compared to the right is correlated with differences in vascular architecture, oxygen availability, and metabolic profile (Bustamante-Marin et al., 2015). Transcriptional profiling comparing wild type and *Dnd1*$^{Ter/Ter}$ germ cells prior to the formation of teratomas, as well as DND1-RNA-immunoprecipitation experiments, showed that DND1 regulates genes associated with pluripotency, the cell cycle, and chromatin regulators (Ruthig et al., 2018).

These findings have led to the idea that reprogramming of pluripotency in germ cells and up-regulation of genes essential for spermatogonial stem cell differentiation require cell cycle arrest. Experiments are in progress to investigate the mechanisms involved in this transition from fetal gonocyte to spermatogonial stem cell, responsible for the lifetime fertility of the male.

A new venture: Can the adult testis and ovary be rescued?

Infertility is often the outcome of chemotherapy, which is frequently used for treatment of cancers and immune disorders. Can a deeper understanding of the origin of cell types and mechanisms of organogenesis during fetal life help us to devise a scheme to rescue the adult testis and ovary after damage? This question led us to establish models of ovarian and testicular damage in the hope of devising a means of rescue for one or both organs.

In female FVB mice, three consecutive IP injections with a cytotoxic cocktail of busulfan and cyclophosphamide led to complete infertility (Batchvarov et al., 2016). However, when an isogenic ovary fragment from a healthy female homozygous for a GFP transgene was grafted to the left ovary of CTx-treated hosts, follicle development in the left host ovary was rescued. In contrast, the ungrafted right ovary underwent complete degeneration. Some host (non-GFP) pups were born as late as the 6th litter after grafting, suggesting long-term rescue of host fertility. Investigation of the ovary during and soon after the CTx treatments indicates that granulosa cells in growing follicles are the primary target of the cytotoxic drugs. Experiments are ongoing to determine the mechanism through which follicles are rescued by the graft.

While (unsuccessfully) trying to block neuronal development in the ovary, we discovered a drug that can severely deplete the Sertoli cell population in the testis. Four days after treatment, Sertoli cells were depleted, but the basal lamina of testis cords and other cell types in the testis were intact. This created a scaffold for engraftment of a new population of Sertoli cells that rescued spermatogenesis from remaining host spermatogonial stem cells. This approach might be used to rescue infertility by the replacement of a defective Sertoli cell population in a host. Alternatively, a delay of seven days between treatment with the drug and injection of donor cells from a neonatal mouse, led to the engraftment by many cell types in the testis, including spermatogonial stem cells, peritubular myoid cells, and Leydig cells (Yokonishi et al., submitted). Using this method, it might be possible to establish xenogeneic spermatogenesis in the mouse testis by matching the species origin of somatic and germ cells. Whether a testis

423 depleted for Sertoli cells has the capacity for repair when donor cells are not provided is
424 currently under investigation.
425
426 It would be especially rewarding if the many years of basic science research in the mouse and
427 turtle one day paid off in the clinic.
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705

Figure Legends

Fig. 1. Working model of mammalian sex determination (circa 1991). The gonad is initially bipotential. A gene from the Y-chromosome (*Tdy*) is expressed in the early XY gonad and initiates testis development. Ovary development was known to require oocytes, but no key genes in the ovary pathway were known at that time (after McLaren, 1991).

Fig. 2.A) In the adult testis, *Sry* is transcribed from a promoter upstream in the 5' inverted repeat (TSS). Multiple circular permutations of the *Sry* transcript were recovered from adult testis libraries, leading to the hypothesis that the transcript was circular. **B)** The *Sry* transcript in the adult testis was predicted to undergo circularization based on pairing between the long terminal repeats that bring a splice donor near a splice acceptor. **C)** In the fetal testis, transcription initiates from a promoter internal to the 5' repeat (GSS), creating a linear transcript. The conserved DNA binding domain (HMG box) is shown in diagonal stripes.

Fig. 3. Opposing signals control the fate of the gonad. When SRY triggers SOX9 upregulation, FGF9 is expressed and represses *Wnt4* and the female pathway. In the absence of SOX9 upregulation, ovarian development ensues, based on Wnt and downstream signaling.

Fig. 4. Model to explain the narrow developmental window when Sertoli fate can be initiated. The bipotential gonad is initially advancing on an ovarian trajectory (solid red line) based on the accumulation of a female regulator. If the male regulator is expressed at the right stage and level (solid blue line), it can intersect the trajectory of the female regulator and divert the gonad to testis fate. However, if the ovarian factor is advanced (broken red line), or the male regulator is delayed (broken blue line), the initiation of Sertoli fate fails.

Disclosures

I have nothing to disclose.

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Figure 1

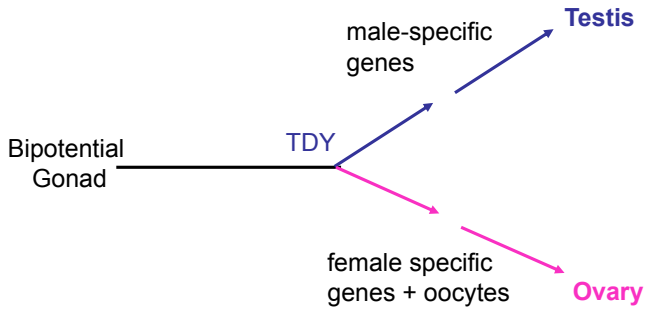

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Figure 2

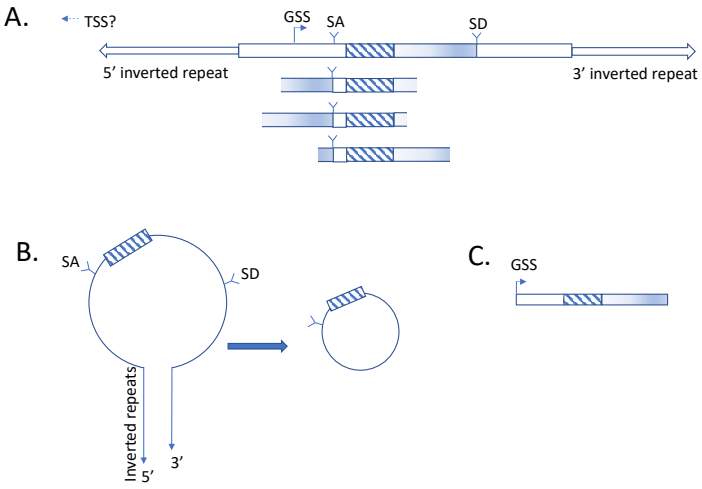

Figure 3

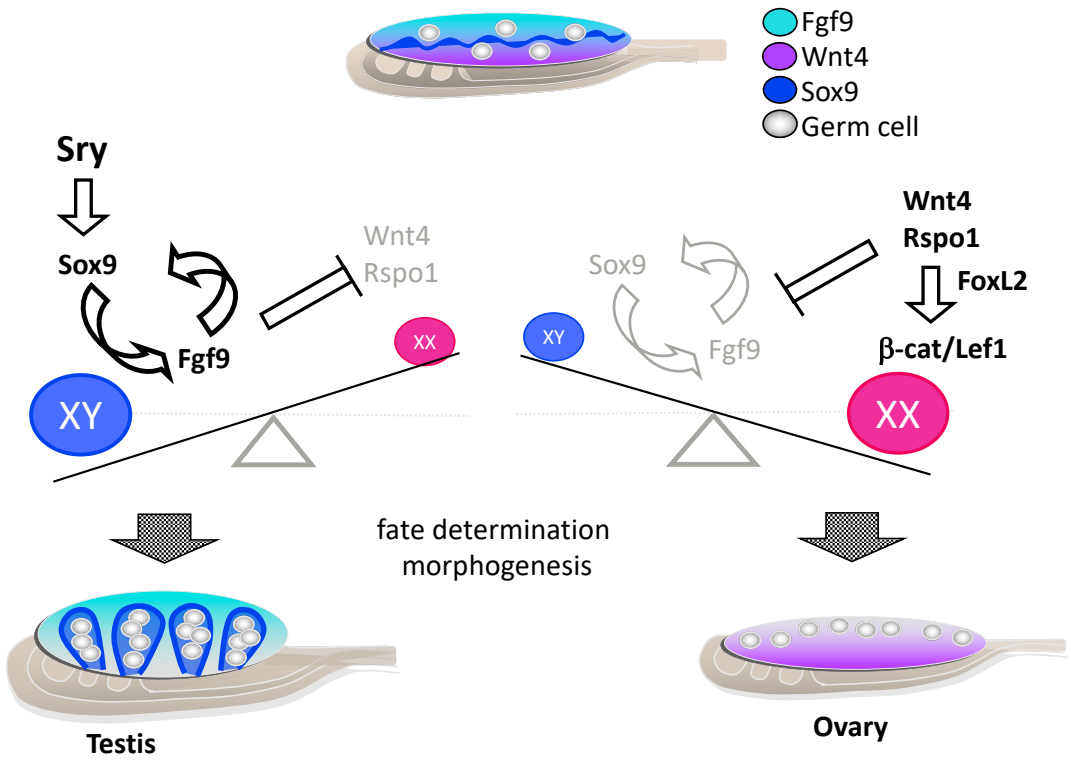

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Figure 4