

Sex and the circuitry: progress toward a systems-level understanding of vertebrate sex determination

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In vertebrates, the gonad arises as a bipotential primordium that can differentiate as a testis or ovary. Cells are initially primed to adopt either fate by balanced antagonistic signaling pathways and transcription networks. Sexual fate is determined by activating the testis or ovarian pathway and repressing the alternative pathway. A complex, dynamic transcription network underlies this process, as approximately half the genome is being transcribed during this period, and many genes are expressed in a sexually dimorphic manner. This network is highly plastic; however, multiple lines of evidence suggest that many elements of the pathway converge on the stabilization or disruption of *Sox9* expression. The single gene mutational approach has led to the identification of ~30 additional genes involved in vertebrate sex determination. However, >50% of human disorders of sexual development (DSDs) are not explained by any of these genes, suggesting many critical elements of the system await discovery. Emerging technologies and genetic resources enable the investigation of the sex determination network on a global scale in the context of a variable genetic background or environmental influences. Using these new tools we can investigate how cells establish a bipotential state that is poised to adopt either sexual fate, and how they integrate multiple signaling and transcriptional inputs to drive a cell fate decision. Elucidating the genetic architecture underlying sex determination in model systems can lead to the identification of conserved modules correlated with phenotypic outcomes, and critical pressure points in the network that predict genes involved in DSDs in humans. © 2012 Wiley Periodicals, Inc.

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INTRODUCTION

Genetic sex is established at conception when an X or Y-bearing sperm fertilizes an X-bearing ovum. However, male or female sexual development is not initiated until midgestation, as the result of the decision within the bipotential gonad to differentiate as a testis or ovary. Sexually dimorphic differentiation of the gonad is the first step in male and female development, occurring prior to the production

of hormones, which control subsequent steps of phenotypic sexual development.

Two decades have passed since the discovery that *Sry* (sex-determining region of Chr Y) acts as a dominant genetic determinant of sex in mammals. We have since learned that this 'dominant determinant' acts more like a nudge.¹ It is expressed at low levels in the testis, and may influence the underlying transcriptional network by directly upregulating only one gene, *Sox9* (SRY-box containing gene 9). The idea that the female is a default pathway has been discarded, and a more complex and nuanced view of sex determination is emerging. Cells of the gonad primordium are initially poised between sexual

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fates by balanced antagonistic intercellular signaling pathways and intracellular transcription networks. Sexual fate is established during a brief temporal window, and requires both the activation of the testis or ovarian pathway and repression of the alternative pathway. A large, dynamic transcription network underlies this process, as evidenced by microarray profiles showing that fully half of all genes in the genome are expressed in XX and XY gonads during the critical window of sex determination.

Approximately 10% of expressed genes exhibit a sexually dimorphic pattern within 24 hours following the onset of *Sry* expression. However, mutational analyses in mice have confirmed roles in early gonadogenesis and/or primary sex determination for only approximately 30 genes. More than 50% of human sex reversal cases remain unexplained by variation in *SRY* or any of these known genes. Moreover, numerous genomic regions that modify sex reversal phenotypes in inbred mouse strains have been mapped, and many of these do not harbor genes with known roles in sex determination. These deficiencies in our understanding call for a more global approach.

It seems likely that an evolutionarily conserved framework underlies sex determination, but if so, what is its structure? How is sexual plasticity conferred at the level of the underlying transcription network, maintained for several days, and ultimately disrupted to drive the testis or ovarian pathways? How is a high level of expression variability tolerated in the network? If many genes must act in a combinatorial manner to buffer this variability, what are the limits of the system? What are the critical nodes in the network, and how are they regulated? Is there an underlying modularity in the network associated with various aspects of the sexual fate decision?

Answers to these questions will require approaches that provide a systems-level perspective to integrate multiple sources of global molecular data (including genetic variability within and among species, as well as differences at the level of the transcriptome and proteome) and link them to phenotypic outcomes. In this focus article, we take a systems perspective to review the critical first 36 hours of gonadogenesis in mice, culminating in gonadal sex determination. We place special emphasis on the establishment and maintenance of sexual fate within a single cell, as well as the spatiotemporal establishment of sexual fate across the entire field of the gonad. Throughout, we highlight holes in our current understanding and recent studies that seek to address these gaps. We conclude with a discussion of emerging systems-level approaches and genetic resources and how they will provide a broader perspective of the

gonad as a differentiating organ system. Readers who seek a more detailed molecular understanding of testis and ovary development are directed to recent comprehensive reviews by Wainwright et al.² and Liu et al.³

BUILDING A BIPOTENTIAL DEVELOPMENTAL NETWORK

In mice, the gonads arise around embryonic day 10 (E10.0) as paired thickenings of the coelomic epithelium covering the ventral medial surface of the mesonephros. Both the timing and spatial aspects of the initial cell proliferation that gives rise to the gonad appear quite precise, yet surprisingly little is understood about what underlies the specification and boundaries of this gonad field. Mutational analyses have identified a handful of genes that are required for this first phase of proliferation in both sexes, including *SF1/Nr5a1* (nuclear receptor subfamily 5, group A, member 1), *Wt1* (Wilms tumor 1 homolog), *M33/Cbx2* (chromobox homolog 2), *Lhx9* (LIM homeobox protein 9), *Emx2* (empty spiracles homolog 2), and *Igf1r* (insulin-like growth factor I receptor)/ *Insr* (insulin receptor)/ *Insr* (insulin receptor-related receptor). Disruption of any of these genes results in varying degrees of gonad dysgenesis. Microarray analyses of gonad somatic cells at this early stage revealed a vast, active transcription network that is similar in both sexes before E11.5.^{4,5} Thousands of genes are expressed early in the bipotential gonad, including a large number of homeobox, zinc finger, and SOX (*Sry*-box) transcription factors. Many of these genes may play a role in specifying the spatial boundaries for the initial waves of cell proliferation, or in regulating the plasticity of this cell population.

For the first 36 hours of their development, the gonad primordia remain bipotential, competent to develop as testes or ovaries irrespective of genetic sex. The early gonad primordium consists of two to three somatic precursor cell types, including supporting cell precursors (so called because of their role in supporting the development of germ cells throughout reproductive life) and steroidogenic cell precursors. In addition, primordial germ cells, which are specified extra-embryonically at the base of the allantois, finish their migration through the hindgut and begin to colonize the gonad as early as E10.5. Surprisingly, all of these precursor cell types are themselves initially bipotential and proceed to adopt sex-specific fates in the testis and ovary.

The plasticity of the gonad at the bipotential stage may stem in part from a balanced network state established by the antagonistic intercellular

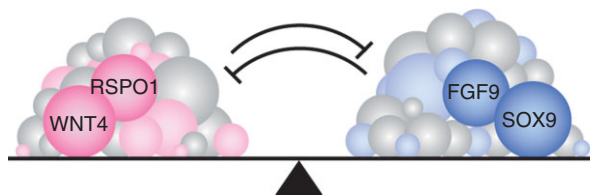


FIGURE 1 | Many genes may be involved in establishing a bipotential state in the early gonad. Balanced antagonistic signaling pathways, including fibroblast growth factor 9 (FGF9) and wingless-related MMTV integration site 4 (WNT4), hold somatic precursor cells in an undifferentiated state. The transcriptome is highly active and complex during this window, which suggests that many more genes and pathways are involved in conferring this plasticity.

signals WNT4 (wingless-related MMTV integration site 4) and FGF9 (fibroblast growth factor 9).⁶ Before E11.5, *Fgf9* and *Wnt4* are expressed in both XY and XX gonads—*Fgf9* is expressed along the anteroposterior (AP) axis in the coelomic domain at the surface of the gonad, while *Wnt4* is expressed at the gonad-mesonephros border. These opposing signaling pathways may hold gonadal cells in an undifferentiated state. It is not clear how the boundaries of these two expression domains are established and maintained, nor is it known by what mechanism cells process this balanced signaling state to remain undifferentiated. However, based on the large number of genes expressed at this stage, it seems likely that many other factors contribute to this balanced network (Figure 1).

What is the transcription signature of the bipotential network that exists in early XY and XX gonads? One clue from a recent study⁷ suggests an

intracellular battle of the sexes. We quantified gene expression in E11.5 XY gonads from a large genetically heterogeneous population from an intercross of C57BL/6J (B6) and 129S1/SvImJ (129S1) inbred strains, and found an unexpectedly high level of expression variability across individuals, despite the fact that samples were stage-matched and all were at or past the close of the bipotential window. Using this expression variability, which was driven by genetic variation segregating in the population, we identified two distinct clusters of coexpressed genes (Figure 2). Surprisingly, when these genes were annotated based on their later association with the testis or ovarian differentiation pathway (based on known functional data or sexually dimorphic expression in XY/XX microarray experiments), cluster membership was largely restricted to testis or ovary-associated genes. Thus, at the end of the bipotential window as the testis pathway is first being activated, a clear transcriptional signature of the ovarian pathway is still evident in XY gonads.

At first glance, this suggests that the testis pathway is being superimposed on an underlying ovarian pathway. However, there is also evidence for the expression of genes later associated with the male pathway in E11.5 XX gonads, even though *Sry* is not present.⁸ This finding suggests that the early gonad progenitors in both XY and XX gonads are primed to adopt either fate, and sex determination proceeds as a consequence of a tug-of-war between testis and ovarian gene subnetworks. Perhaps this dual lineage priming is a characteristic of ‘bipotential cells’ in general. This idea is consistent with the identification of XX patients that develop a testis in the absence of

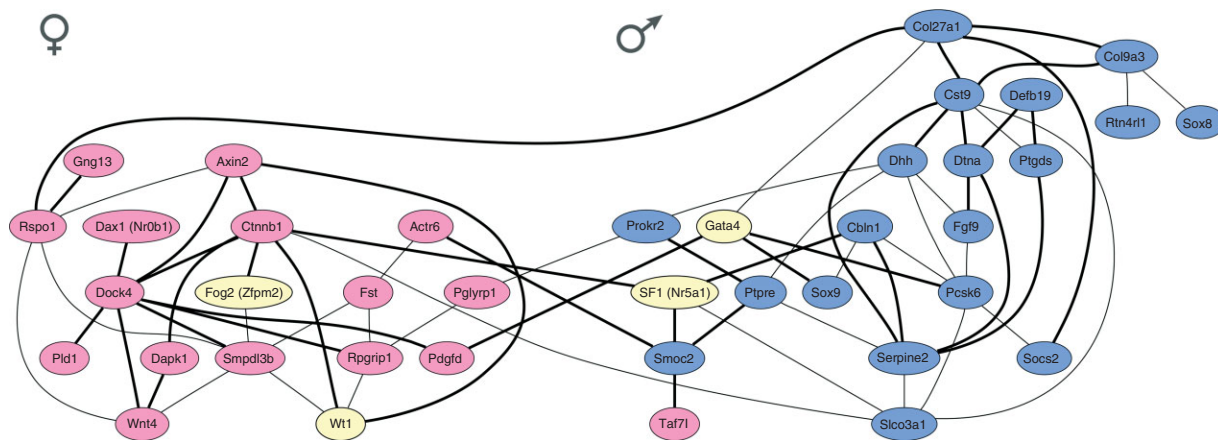


FIGURE 2 | Evidence for a transcriptional tug-of-war underlying sex determination. A coexpression network was estimated for a subset of 40 genes quantified in 68 XY gonad samples from a mixed F2 intercross of B6 and 129S1. Testis-associated genes that are enriched in XY gonads at E11.5 are highlighted in blue, whereas ovarian genes are highlighted in pink, and genes with known roles in sex determination that are not expressed in a sexually dimorphic pattern at E11.5 are highlighted in yellow. Thick black edges represent more robust coexpression relationships (partial correlation coefficient ≥ 0.33), while thinner edges are less robust but still significant (partial correlation coefficient ≥ 0.25). (Reprinted with permission from Ref 7. Copyright 2009 Cold Spring Harbor Laboratory Press)

SRY. The cause of female-to-male sex reversal in some of these cases is disruption of the WNT4/RSP01 (R-spondin homolog)/ β -catenin signaling pathways that oppose testis development in XX gonads^{9,10}; however, it is likely that many other genes are involved.

Interestingly, the gonad retains the ability to form a testis or ovary irrespective of genetic sex from its origin at E10.0 until \sim E11.5. However, if the testis pathway is not engaged by this time, the ovarian pathway is stabilized and resistant to perturbation.¹¹ This defines a critical window, and suggests that elucidating the expression dynamics of the network as it evolves will be important.

DISRUPTING THE BIPOTENTIAL STATE

Primary sex determination refers to the decision within the bipotential gonad to become a testis or an ovary, a process in mammals that normally hinges on the presence or absence of the Y chromosome. In a series of experiments analyzing XY–XX chimeric testes,¹² Palmer and Burgoyne showed that only the somatic supporting cell lineage (i.e., pre-Sertoli cells) exhibited a significant bias for the presence of the Y chromosome, suggesting that the testis-determining gene on the Y acts autonomously in supporting cell precursors, and sexual differentiation of all other cell types proceeds from intercellular interactions with this population. Shortly thereafter, *Sry* was identified as the Y-linked genetic switch underlying male sex determination in mammals and shown to be expressed in pre-Sertoli cells as predicted.^{13–15}

Sry encodes a high mobility group (HMG)-box transcription factor that is transiently expressed in pre-Sertoli cells from \sim E10.5 to E12.0.¹⁶ Little is known about the regulatory mechanisms underlying *Sry* expression in a brief and tightly constrained spatiotemporal pattern. *Sry* is first expressed in cells at the center of the gonad, and then spreads to the anterior and then posterior poles.^{17,18} Expression is extinguished following the same pattern, and individual cells are believed to express *Sry* for <7 hours.¹⁵ Loss-of-function experiments implicated a few genes as potential regulators of *Sry* expression, including *Wt1*,^{19,20} *Nr5a1/SF1*,²¹ *Lhx9*,²² *Emx2*,²³ *Cbx2/M33*,²⁴ *Igf1r/Insr/Insrr*,²⁵ and the combination of *Gata4* (GATA binding protein 4)/ *Fog2/ Zfp2* (zinc finger protein, multitype 2).²⁶ However, as many of these genes are also involved in the formation of the gonad primordium, it is difficult to determine whether they directly regulate *Sry* or affect the initial proliferation of the pre-Sertoli lineage. In a recent expression QTL (eQTL) study, we mapped a novel autosomal

modifier of *Sry* expression on Chromosome 1.⁷ ChIP assays will be necessary to identify direct regulators of *Sry*.

It was initially assumed that *Sry* would be found to directly regulate numerous genes and pathways, however 20 years later the consensus view is that the sole function for *Sry* during sex determination may be to upregulate the expression of one critical downstream target, *Sox9*.²⁷ In fact, *Sry* is not conserved across vertebrates, and appears to act as the male trigger only in eutherian mammals. Other genes can act as the genetic switch, such as *DMRT1* (DM-related transcription factor 1) in the chicken²⁸ and its related homolog *DMY* (DM-related transcription factor on Chr Y) in some species of medaka fish.²⁹ In other vertebrate species, environmental mechanisms (such as temperature or even population density) can act as the trigger of testis development.³⁰ Perhaps most surprisingly, coincident genetic and environmental sex determining mechanisms have been found within a single species, illustrating the flexibility at the top of the testis pathway.^{31,32} This suggests that many genes could potentially assume the role of the genetic sex determination (GSD) switch. However, *Sox9* seems to be a conserved element of the testis pathway in all systems that have been investigated. Thus, the constraint limiting the evolution of new potential switch genes may be their ability to activate/stabilize *Sox9* expression during a critical temporal window of development. Importantly, this emerging model does not preclude the possibility that the genetic or environmental switch in some species activates the ovarian rather than the testis pathway, although it does predict that an ovarian switch would directly or indirectly repress *Sox9* expression.³³

Like *Sry*, *Sox9* encodes a HMG-box transcription factor that is thought to bind and bend DNA. *Sox9* is expressed at basal levels in both XY and XX gonads before E11.0, but is thereafter upregulated by SRY in XY gonads and extinguished in XX gonads. *Sox9* expression is tightly regulated and buffered against perturbation by a complex network of intra and intercellular feedforward loops. Indeed, this regulatory complexity provides strong support for the importance of *Sox9* to the testis differentiation pathway. Following the initial ‘nudge’ by SRY,¹ *Sox9* expression is amplified and then maintained by multiple feedforward loops (Figure 3). SOX9 is able to bind its own promoter with higher affinity than SRY,²⁷ and consequently displaces SRY and activates its own expression in an autoregulatory loop. In addition, multiple intercellular signaling pathways, including FGF9⁶ and PGD2 (Prostaglandin D2),³⁴ act as independent feedforward loops to amplify *Sox9*

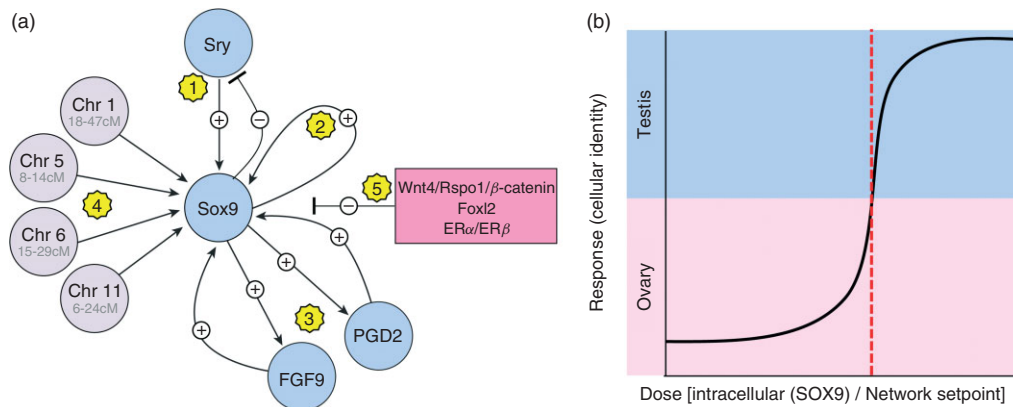


FIGURE 3 | Sex determination within individual XY supporting cells depends on the level of *Sox9* (SRY-box containing gene 9) expression. (a) The complexity of *Sox9* regulation points to its importance as the conserved regulatory hub of the testis differentiation pathway. *Sox9* is expressed at basal levels early in both XY and XX gonads. 1. A pulse of *Sry* (sex-determining region of Chr Y) expression activates the initial upregulation of *Sox9* in XY somatic supporting cell precursors. *Sox9* may or may not be involved in extinguishing *Sry* expression. 2. Following the initial activation by SRY, SOX9 is able to bind its own promoter with higher affinity than SRY and act in an autoregulatory feedforward loop. 3. Intercellular prostaglandin D2 (PGD2) and fibroblast growth factor 9 (FGF9) signaling pathways act as independent feedforward loops to amplify *Sox9* expression downstream of *Sry*-mediated activation. 4. Many other genes are likely to directly or indirectly regulate *Sox9* expression or function, including unidentified genes underlying expression QTLs (eQTLs) on mouse Chromosomes 1, 5, 6, and 11. 5. Multiple genes associated with the ovarian differentiation pathway, including *Wnt4* (wingless-related MMTV integration site 4)/*Rspo1* (R-spondin homolog)/ β -catenin, *Foxl2* (forkhead box L2), and the estrogen receptors $ER\alpha$ and $ER\beta$, are likely to repress *Sox9* expression to canalize the ovarian pathway. It is important to note that one or more of the genes underlying the *Sox9* eQTLs could influence *Sox9* expression indirectly by activating or repressing female pathway genes. (b) Within individual gonad supporting cells, sexual fate is likely to be a binary decision. The intracellular concentration of SOX9 most likely must reach a critical threshold (denoted by a red dotted line) relative to the underlying setpoint of the network before E11.5 to impose a testis-specific state on the transcription network. Cells that fail to express SOX9 above this threshold adopt the alternative ovarian-specific network state.

expression in pre-Sertoli cells. These signaling pathways may act as a failsafe mechanism to recruit cells to the Sertoli fate in extreme cases where too few *Sox9*-expressing cells are specified at the outset.¹² We have mapped at least four additional modifiers of *Sox9* expression (eQTLs)⁷ that do not harbor genes with known functions during sex determination. Two of these regions, on Chromosomes 1 and 11, also mapped as strain-specific modifiers of sex reversal (phenotypic QTLs) in the hemizygous *Dax1*-/Y (*Nr0b1*, Nuclear receptor subfamily 0, group b, member 1) and Y^{DOM} mouse models, and both QTLs enhanced susceptibility to or protection from sex reversal by affecting *Sox9* expression.^{35,36} Finally, *Sox9* expression is regulated directly or indirectly by ovarian pathway genes (see below).^{6,37} The complexity in the regulation of SOX9 that has been uncovered by recent experiments reveals many negative and positive feedback loops that may be a more general characteristic of this mutually antagonistic and highly canalized system.

STABILIZING THE SEX DETERMINATION DECISION

Within gonad supporting cells, we hypothesize that the sexual fate decision operates as a bistable switch

followed by stabilization mechanisms. Despite the high level of expression variability observed during sex determination, individual cells seem to adopt one of only two network states.³⁸ Concurrent testis and ovary-associated network motifs are present in uncommitted supporting cells early during the bipotential period (see Figure 2). However, as this critical window closes around E11.5, the intracellular transcription network begins to resolve to a testis or ovary-specific state. These sexually differentiated states appear to be quite stable and robust to a wide range of fluctuations in gene expression levels. State switching (i.e., transdifferentiation from a *Sox9*-expressing Sertoli cell to a *Foxl2* (forkhead box L2)-expressing granulosa cell³⁷) is rare. This observation, along with strict temporal constraints,³⁹ suggests that the cellular establishment of a sex-specific transcriptional state in response to a lineage-specific differentiation factor is bistable, nonlinear, and ultrasensitive.

All current evidence in vertebrates points to SOX9 as the critical differentiation factor in males⁴⁰ (Figure 3(b)). It seems likely that the nuclear concentration of SOX9 must reach a critical threshold (denoted in Figure 3(b) by a red dotted line) before E11.5 to impose a testis-specific state on the underlying transcription network.^{41,42} Below this

threshold, *Sox9* expression may be insufficient to drive the testis pathway, thus supporting cells adopt an ovarian network state. This is the case in all XX cells where *Sox9* is initially expressed at a low level but is never upregulated above the threshold, as well as in XY cells with defects in one or more of the positive feedback pathways outlined in Figure 3(a). Indeed, one requirement for bistable switch-like behavior is the presence of a feedforward loop. PGD2³⁴ and FGF9⁹⁶ signaling pathways, as well as SOX9 autoregulation of its own enhancer,²⁷ fulfill this requirement.

The ability to repress the alternative pathway may be of equal importance for establishing a bistable differentiation switch in gonad supporting cells. In XX or XY gonads, the supporting cell fate is defined as much by the genes that are silenced as by the genes that are expressed.⁸ XX cells silence testis pathway genes and XY cells silence ovarian pathway genes. Surprisingly, evidence suggests that the sexual fate decision must be actively maintained and the alternative sex must be repressed well after sex is determined. In a case where repression of *Sox9* is not maintained in adult females, XX cells transdifferentiate to the alternative cell fate (i.e., granulosa cells transdifferentiate to Sertoli-like cells).³⁷ Similarly, loss of *Dmrt1* leads to loss of *Sox9* in the adult testis and reprogramming of XY Sertoli cells to a granulosa-like fate.⁴³

In bipotential XY gonads, the levels of ovarian pathway genes likely provide an initial set point for the network and establish a state-switching threshold for SOX9 expression. Evidence from studies of genetic background effects on XY sex reversal suggests that *Sox9* must be expressed above some threshold relative to this underlying baseline to adopt and maintain the Sertoli cell fate (Figure 3(b)). The ovarian gene-derived set point may vary among individual supporting cells in the gonad, and consequently each cell may have a unique threshold for establishment and maintenance of the male pathway. New gene expression platforms are sensitive enough to accurately quantify gene expression in single cells,^{44,45} and used in combination with laser capture microdissection (LCM) to isolate individual fluorescently labeled early gonad somatic cells (e.g., SF1-eGFP⁴), may make it now possible to map dynamic gene expression changes in individual cells across this gonad field at the earliest stages of differentiation. Importantly, community-effect mechanisms exist that may override the microheterogeneity in the population and recruit the entire field to the testis or ovarian pathway under normal conditions (see below).

PROPAGATING THE DECISION ACROSS THE GONAD FIELD

All data to date suggest that sexual fate of the gonad depends on the number of specified pre-Sertoli cells (i.e., cells that are expressing SOX9 above a critical threshold) present in the gonad when the bipotential window closes around E11.5 (Figure 4). This critical timepoint appears to be set by the expression of ovarian pathway genes in the bipotential XY gonad. Once the ovarian pathway has progressed to a 'point of no return' (termed the ovarian canalization threshold, OCT), the number of specified pre-Sertoli cells in the XY gonad must surpass a threshold (termed the testis canalization threshold, TCT) to direct testis development. It is unclear how ovarian pathway genes stably repress the testis differentiation pathway after this critical timepoint. Expression of female pathway genes above the OCT may render individual XY cells unable to express SOX9 at high enough levels to

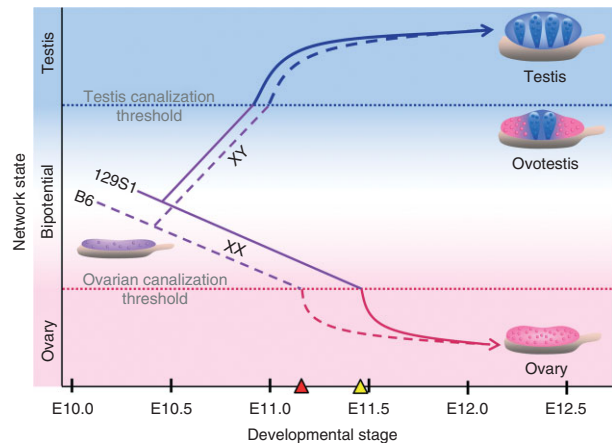


FIGURE 4 | Sexual fate of the XY gonad depends on the number of specified pre-Sertoli cells (i.e., cells that are expressing SOX9 (SRY-box containing gene 9) above a differentiation threshold) present in the gonad when the bipotential window closes. This critical timepoint appears to be set by the expression of ovarian pathway genes in the bipotential XY gonad. Once the ovarian pathway has progressed to a 'point of no return' (the ovarian canalization threshold, OCT; denoted as a red dotted line), the number of specified pre-Sertoli cells in the XY gonad must surpass a threshold (the testis canalization threshold, TCT; blue dotted line) to direct testis development. If the number of pre-Sertoli cells does not meet this threshold by this critical timepoint, the XY gonad full or partially sex reverses to an ovary or ovotestis. Variation in the expression of ovarian pathway genes in XY gonads among inbred strains may affect the duration of the bipotential window. For example, the higher expression or earlier onset of ovarian pathway genes in B6 XY gonads (dashed purple line) relative to 129S1 (solid purple line) may cause the bipotential window to close earlier in B6 (denoted by red triangle; compare to yellow triangle = critical timepoint in 129S1). In XX gonads, the ovarian pathway proceeds past the OCT and is canalized toward ovarian development.

activate the testis pathway. Alternatively, the ovarian pathway may repress the testis pathway by altering chromatin structure, effectively limiting the exposure of downstream testis pathway effectors to SOX9, and blocking establishment of the Sertoli fate.

XY gonads in which the number of pre-Sertoli cells falls below the TCT at the critical timepoint will undergo full or partial sex reversal and develop as ovaries or intersex gonads (i.e., ovotestes), respectively. Ovotestes have a stereotypical arrangement of testicular and ovarian regions, with testicular tissue at the center and ovarian tissue at one or both poles. This order along the AP axis likely reflects the earlier onset of the testis pathway in the center relative to the poles.^{17,18} In many cases (e.g., Y^{DOM}-associated sex reversal), an intersex phenotype results from a failure at the top of the genetic cascade, in the level and/or timing of *Sry* expression.³⁹ However, failure to express *Sry* above a threshold by a specific time cannot fully account for all ovotestis phenotypes, and other cases provide insight into the antagonistic relationship among spatiotemporal constraints, intercellular signaling, and structure-mediated activation of testis development. For example, in *Dax1*-/Y and *Fgf9* mutants on the B6 strain background, supporting cells at the poles express *Sry* with normal timing and levels, but do not maintain *Sox9* expression above the threshold required for testicular development, illustrating the importance of positive feedback downstream of SRY-mediated activation.^{6,35} Undifferentiated supporting cell precursors are more likely to activate the testis pathway if surrounded by differentiated Sertoli cells. Diffusible extracellular ligands (e.g., FGF9⁶; PDGF, platelet-derived growth factor⁴⁶; DHH, desert hedgehog⁴⁷; and PGD2³⁴) likely play an important role in conferring this community effect. Surprisingly, an extracellular serine protease, *Serpine2* (serine peptidase inhibitor, clade E, member 2), mapped to a central position in the male coexpression network in Figure 2,⁷ suggesting that extracellular matrix (ECM) components may also influence the establishment of the testis pathway. Clearly, much work remains to elucidate the mechanisms and constraints controlling the propagation of sexual fate along the AP axis. Ovotestes provide a good model for dissecting these complex interactions.³⁸

Interestingly, strain variation in the expression of ovarian pathway genes in XY gonads may affect the exact timing of this critical 'point of no return' (Figure 4). For example, the higher expression, earlier onset, and/or delayed repression of many ovarian pathway genes in B6 XY gonads relative to 129S1 may cause the bipotential window to close earlier in B6 (denoted by red triangle; compare to yellow triangle =

critical timepoint in 129S1). The importance of timing to sex determination is not a new idea. Indeed, 25 years before global gene expression data for the gonad was available, Eva Eicher proposed that strain differences in the relative timing of the testis and ovarian pathways may underlie the sensitivity of B6 to sex reversal.⁴⁸

Recent experiments defined the boundaries of the bipotential window by artificially upregulating SRY at different stages in XX gonad explants from outbred (IRC/CD-1) embryos.¹¹ Extending this experimental approach to test gonad explants from specific inbred strains may expose genetic background differences in this temporal window. If this critical timepoint is earlier in B6, this may account for the observed susceptibility of the B6 genetic background to XY sex reversal in response to genetic perturbations that delay the activation of the testis pathway (e.g., Y^{DOM}). Despite this bias, sex reversal is not observed in wildtype B6 XY mice, and this observation may be accounted for by increased expression of *Sox9* in B6.⁷ Indeed, there may be strong balancing selection in the gonad for mutations that affect gene expression in compensatory pathways. In fact, in a genetically heterogeneous population of E11.5 XY gonads, expression levels for 50 sex-associated genes were highly variable across the population ($n = 82$), yet all XY gonads in this cross would likely have developed as testes. All evidence to date suggests that the buffering capacity of the network—the ability of the network to compensate for allelic variation—is extensive. Although the exact limits of this buffering capacity are not well-defined, this finding predicts that a surprising level of variation in gene expression can normally be tolerated in the system. Only certain combinations of discordant alleles will be selected against because they disrupt the ability of the pathway to resolve in both directions.

MODULARITY IN THE TRANSCRIPTIONAL NETWORK

Testis and ovary structure are well conserved across vertebrate species, yet the last decade of research has proven that there are many paths to these two destinations (for review, refer to Ref 49). Although critical regulatory network hubs (e.g., *Sox9*) and core cellular processes (e.g., cell proliferation and testis cord formation) are conserved and required for testis determination across vertebrate species, the relative timing of individual processes varies considerably.⁴⁰ For example, in *Trachemys scripta*, a temperature-dependent turtle species, SOX9 is expressed at high levels in all gonads at the bipotential stage regardless

of their incubation temperature.³³ Moreover, cord-like structures that develop specifically in the testis in mice are initially present in both sexes in turtles. Downregulation of SOX9 and loss of cord structures occurs at the female producing temperature in turtles, and results in differentiation of an ovary. Thus, although adult testes and ovaries are structurally very similar between turtles and mice, their differentiation occurs differently. The finding that many of the same genes are expressed in the turtle (e.g., *Sox9*, *Amb*/anti-Mullerian hormone, *SF1/Nr5a1*, *Dmrt1*, *Wt1*, and *Foxl2*), but in a different temporal sequence, makes sense in light of the reordering of the steps in differentiation. This interspecific modular heterochrony has been observed for other aspects of gonad development⁴⁹ and in other developing systems,⁵⁰ and may represent a more general characteristic of developmental networks. From an evolutionary perspective, the gonad is only constrained by its adult function (i.e., reproductive fitness), therefore much of the variability in gene expression levels and module timing is likely to be neutral.

Variability is also observed among vertebrates in the duration of the bipotential window, and the cause and significance of this variation is not well understood. Species with GSD mechanisms (e.g., mouse and humans) differ greatly in the length of this period. In the mouse, the undifferentiated stage is short, lasting only ~36 hours from formation of the gonad at E10.0 until ~E11.5,¹¹ whereas in humans this period likely extends for 4–5 weeks from the initial specification around gestational week 3 to the upregulation of testis pathway genes during weeks 7–8. The length of the bipotential window is even more variable among species that utilize environmental mechanisms for sex determination, and can vary significantly between sexes in some species where the growth rate differs. For example, in the temperature-dependent turtle *T. scripta*, the bipotential (i.e., temperature-sensitive) period spans 10 days for eggs incubated at the female-producing temperature (31°C) but lasts twice as long for eggs incubated at the lower male-producing temperature (26°C).³³ Differences in growth rate, the duration of the bipotential stage, and the sequence in which morphological events occur may account for temporal differences in expression data. In future studies at the global level it may be possible to correlate specific expression modules with morphological differentiation events.

CONCLUSION

Over the past two decades, the single gene mutational approach (including fortuitous as well as intentional

mutations) has been the predominant experimental strategy for identifying and validating sex determination genes, and has successfully elucidated roles for nearly 30 genes, including *Sry* and *Sox9*. Loss and gain-of-function experiments will continue to play an important role in characterizing the function of single genes during development; however, a number of studies illustrate the limitations of this approach for sex determination. First, the gonad appears to be highly buffered against most perturbations, and good candidate sex determination genes (identified by sexually dimorphic expression or gene ontology/pathway membership) have been ablated with little or no phenotypic consequence.⁵¹ In one particularly heroic experiment, the function of all three members of the insulin receptor tyrosine kinase family was eliminated before a sex-reversed phenotype was observed.²⁵ This and other studies^{52,53} expose the high level of genetic redundancy and transcriptional buffering present in the gonad transcription network at the time of sex determination, and predict that roles for many individual genes during sex determination will be masked. Yet over 50% of human disorders of sexual development (DSDs) remain unexplained by variation in known sex determination genes. This suggests that numerous genes with critical roles in the process (i.e., having large effect sizes) await discovery, or that a majority of DSDs result from subtle changes in the dosage or expression of multiple genes. These genes with modest effects would likely escape detection in a single perturbation experiment, however in combination with other variant alleles or environmental insult may cause a sexual disorder. This is in line with evidence from recent genome-wide association studies (GWAS) showing that tens to hundreds of variants, most of which have small effects, associate with common diseases.⁵⁴

A wealth of new genetic resources (particularly in the mouse model), genome-wide analysis platforms, and computational methods have appeared on the landscape. These tools will enable the next leap forward in biomedical research by overcoming both the limitations of the single gene approach and the analytical hurdles associated with the identification of numerous small effect variants underlying complex disorders. For example, the emerging Collaborative Cross (CC) recombinant inbred panel and associated diversity outbred (DO) heterogeneous stock population model the complexity of the human population (the eight founder strains capture over 89% of the variation observed in mice⁵⁵) in a normally distributed⁵⁶ random manner. The 'pre-CC' (i.e., nearly inbred) strains and DO stock have already proven to be powerful resources for

mapping allelic variants associated with complex phenotypic as well as expression traits.^{56–59} Similarly, mapping studies in other mouse strains are being aided by the recently completed full sequences for 17 common inbred strains⁶⁰ and accurate imputed full sequences (from high density genotype datasets) for over 100 additional strains.^{61,62} As genome-wide analysis platforms continue to improve and lower in cost, it is feasible for a single investigator to quantify genetic variation (including alternative splicing and copy number variants^{63–65}) and molecular phenotypes (e.g., mRNA^{4,5,66}/protein⁶⁷ abundance) across the genome at single nucleotide resolution. These systems-level analyses are becoming increasingly common in the field of sex determination. Moreover, by utilizing advanced computational tools^{56,68,69} to integrate multiple types of molecular data, it is now possible to elucidate directed gene interactions at an unprecedented level.⁷ Loss and gain-of-function experiments

will continue to be the gold standard for candidate gene validation—in this regard, the application of gene delivery technologies^{70–72} to *in vitro* cell^{73,74} and *ex vivo* gonad explant culture^{70,75} models will make it feasible to artificially perturb the expression of many genes at low cost and high throughput. These assays will facilitate the identification of causative genes underlying QTLs and the elucidation of combinatorial interactions among small effect genes. Finally, much of the transcriptional architecture underlying sex determination is highly conserved between mouse and human. Therefore, a comprehensive understanding of the gonad transcription network in the mouse, coupled to an interspecies-derived template of where variation in the network is tolerated, should be highly informative for predicting causative genes from DNA-sequencing approaches in affected patients and elucidating the etiology of unexplained human DSD.

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