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SPECIAL ISSUE REVIEWS–A PEER REVIEWED FORUM

# Balancing the Bipotential Gonad Between Alternative Organ Fates: A New Perspective on an Old Problem

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**The embryonic gonads give rise to one of two morphologically and functionally different organs, a testis or an ovary. Sex determination is the embryonic process that determines the developmental fate of the gonad. In mammals, sex determination is regulated by a DNA binding protein encoded on the Y chromosome, *Sry*, and it's downstream mediator, *Sox9*, which trigger testis determination in the bipotential gonad. However, evidence suggests that the extracellular signals. *Fgf9* and *Wnt4*, are also required to establish divergent organogenesis of the gonad. In this review, we discuss how these extracellular signals interface with cell-autonomous factors to determine the fate of the mammalian gonad, and we derive a model that could provide a molecular explanation for testis determination in vertebrates where *Sry* is absent. *Developmental Dynamics 235:2292–2300, 2006.* © 2006 Wiley-Liss, Inc.**

**Key words:** bipotential gonad; cell fate decisions; *Fgf*; *Sox9*; *Wnt*; signaling anagonism

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## INTRODUCTION

Unlike most developing organs in the embryo that follow a single developmental track, the gonad forms with the potential to develop as one of two alternative organs, an ovary or a testis. For this reason, the gonad primordium is called "the bipotential gonad." Consistent with this idea, each cell in the gonad primodia can follow one of two potential fates, differentiating into either an ovarian or testicular cell type. Remarkably, the fate decisions in individual cells are highly coordinated such that tissues with cells of discordant fate are rarely seen. These unusual features of gonad development present an ideal opportunity to study how cell fate decisions are made in a tissue and how individual fate decisions are coordinated in a community of cells to recruit the entire field of cells behind one of two organogenesis programs.

It may be useful to view the gonad as a patterning field where cells initiate differentiation in response to the interplay of a number of signals. The *Drosophila* genital disc is an example of a similar system because it is also a bipotential primodium where extracellular patterning signals and cell-autonomous factors that confer sex-specific identity are tightly coordinated to give rise to one of two alternative organogenesis programs (Keisman and Baker, 2001; Vincent et al., 2001; Christiansen et al., 2002). The sex of cells in the A/P organizer is controlled by one of two sex-specific splicoforms of the transcription factor Doublesex ($DSX^F$ or $DSX^M$). The sexually dimorphic growth and morphogenesis in the disc is conferred by rendering cells responsive or unresponsive to short- and long-range signals such as Hedgehog (Hh), Wingless (Wg), and Decapentaplegic (Dpp). In a second step, cells expressing $Dsx^M$ activate Fgf signals that recruit Fgfr-expressing cells into the male disc, leading to male-specific morphogenetic processes (Ahmad and Baker, 2002). In summary, the process of sex-specific devel-

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opment of the *Drosophila* genital disc initially depends on the cell-autonomous sex of the cells in the A/P organizer, but is integrated by the production and interpretation of non-cell-autonomous signals. Recent findings in the mouse suggest that the choice between alternative fates of the mammalian gonad may occur in a similar manner (Kim et al., 2006).

In this review, we will consider the sex-specific organogenesis of the mammalian gonad from this perspective, with particular emphasis on the relationship of three genes that have been shown to play essential sex-specific roles in gonad development, fibroblast growth factor 9 (*Fgf9*), wingless-related MMTV integration site 4 (*Wnt4*), and an *Sry*-related High Mobility Group (HMG)-box DNA binding protein, *Sox9*. Readers seeking a more general review of mouse gonadogenesis are referred to recent comprehensive reviews (Koopman et al., 2001; Lovell-Badge et al., 2002; Brennan and Capel, 2004; Park and Jameson, 2005).

## THE PATTERNING FIELD OF BIPOTENTIAL CELLS

The mammalian gonad forms on the surface of the mesonephros within the urogenital ridge. This occurs through proliferation of the mesothelium (coelomic epithelium) in a restricted region on the medio-lateral side of the mesonephros, and through the allocation of cells from the mesonephros. At the same time, germ cells are completing their migration from the base of the allantois along the gut tube, and through the mesonephros to the site where the gonad is forming. Until this stage (~10.5 days post coitum (dpc) in the mouse), there are no differences in the mesonephros or gonad between XX and XY embryos (Swain and Lovell-Badge, 1999; McLaren, 2000).

Among somatic cells in the early gonad, the so-called "supporting cells" are the first to adopt a sex-specific fate. These cells initiate differentiation either as follicle (granulosa) cells in XX gonads or as Sertoli cells in XY gonads (Jost et al., 1981; McLaren, 1991). Prior to the identification of the mammalian sex-determining gene on the Y chromosome, *Sry*, analysis of XX XY chimeric testes indicated that Sertoli cells show a strong bias for the presence of a Y chromosome, whereas all other lineages in the testis could be XX or XY with equal probability. This finding suggested that a cell-autonomous testis-determining factor (TDF) from the Y chromosome was required only in Sertoli progenitors (Palmer and Burgoyne, 1991). In accord with this prediction, it was later shown that somatic supporting cell precursors in the early XY gonad express *Sry* (Albrecht and Eicher, 2001; Sekido et al., 2004; Taketo et al., 2005; Wilhelm et al., 2005). *Sry* expression initiates differentiation of Sertoli precursors and is considered the first step of "primary sex determination."

It is clear that the fate of other cell types in the gonad depends on the fate of supporting cell progenitors. Steroidogenic cells, producing either female or male hormones, differentiate after the specification of the supporting cell lineage. It is not clear whether precursor cells specific to the steroidogenic lineage exist in the bipotential gonad, or the lineage differentiates from multipotent somatic precursors in response to the confluence of signals from supporting cell and connective/endothelial cell lineages (Racine et al., 1998; Habert et al., 2001; Yao et al., 2002) (Fig. 1).

The fate of germline cells also depends on whether they find themselves in a testis or ovary environment, and not on their sex chromosome constitution (Fig. 1). Germ cells reflect a commitment to the ovarian pathway at 12.5–13.5 dpc when they enter meiosis in the XX gonad, or undergo mitotic arrest in the XY gonad. Entry into meiosis in XX gonads seems to depend on the activity of retinoic acid. Testis somatic cells prevent germ cells from entering meiosis, likely through the male-specific expression of Cyp26B1, an enzyme that degrades retinoic acid (Koubova et al., 2006; Bowles et al., 2006). It is not yet clear whether mitotic arrest requires an independent set of signals in XY gonads. However, preventing the XY germ cells from entering meiosis seems to be important for testis organogenesis as there is evidence that meiotic germ cells promote the ovarian pathway and interfere with testis development (Yao et al., 2003).

Differentiation of Sertoli cells and the development of the testis do not require germ cells (Merchant, 1975; De Franca et al., 1994). Morphological evidence suggests that the initial fate specification of the somatic cells of the XX gonad also does not depend on the presence of germ cells (Merchant-Larios and Centeno, 1981). However, germ cells are required for the formation and maintenance of ovarian follicles in postnatal life, and loss of germ cells from the ovary results in degeneration of ovarian structure and, frequently, to expression of Sertoli cell markers (Hashimoto et al., 1990; Taketo et al., 1993)

## GENETIC CONTROL OF DIVERGENT PATHWAYS

A number of genes have been identified that are required in both sexes for formation of the founding population in the bipotential gonad. These include *Wilms' Tumor 1* (*Wt1*), *LIM homeobox protein 9* (*Lhx9*), and *Steroidogenic factor 1* (*SF1*) (Shimamura et al., 1997; Hanley et al., 1999; Birk et al., 2000; de Santa Barbara et al., 2000; Mazaud et al., 2002). In fact, many of the genes that later show dimorphic patterns of expression are initially expressed in both XX and XY gonads. There is a growing list of genes that show this basic expression pattern. Here we will focus on three genes shown to be required for sex-specific development of the gonad, two genes encoding signaling molecules, *Fgf9* and *Wnt4*, and *Sox9*. *Fgf9* is required for testis development, but seems to be dispensable in the ovary (Colvin et al., 2001; Schmahl et al., 2004). *Wnt4* is required for ovary development, but, interestingly, loss of *Wnt4* also causes a delay in testis development consistent with a functional role in patterning the bipotential gonad (Vainio et al., 1999; Colvin et al., 2001; Jeays-Ward et al., 2003, 2004; Schmahl et al., 2004). *Sox9* is dispensable for ovary development, but critical for establishing the testis pathway (Kent et al., 1996; Morais da Silva et al., 1996; Koopman, 1999; Chaboissier et al., 2004).

### Ovary Development

In the absence of *Sry*, the network of transcription factors and extracellular signals in the bipotential gonad leads

to development of an ovary. This is not to say that the ovarian pathway is passive, but simply that the gonad appears to be entrained by existing genetic signals to develop along the ovarian pathway if *Sry* does not intervene.

Morphological landmarks of ovarian development are not obvious until just before birth when medulla-cortical regions show distinct patterns of organization, and ovarian follicles begin to form around oocytes. This has been a disadvantage in studying the ovary because few tools were available to recognize alterations in the pattern of ovary development until near birth. As predicted by Eicher and Washburn in 1986 (Eicher and Washburn, 1986), transcriptome analysis has highlighted the transcriptional changes in somatic cells of XX and XY gonads and revealed an active ovarian molecular program well before morphogenesis is obvious (Bouma et al., 2004; Nef et al., 2005; Beverdam and Koopman, 2006).

While *Sox9* can be used as an early marker of commitment to the Sertoli lineage at 11.5 dpc, no gene essential for primary commmitment to the follicle cell lineage has been identified, although experimental evidence suggests that the ovarian pathway is established by 12.5 dpc (Tilmann and Capel, 1999). *DAX1* was initially identified as a testis antagonist in humans (Zanaria et al., 1995) and was shown to oppose the male pathway in mice (Swain et al., 1998). However, it is not required for ovary development (Yu et al., 1998), but instead is required for testis development (Meeks et al., 2003; Bouma et al., 2005). *Foxl2*, a basic helix-loop-helix transcription factor, is the best candidate at present for a determinant that stabilizes follicle cell differentiation in the somatic cells of the XX gonad. This gene was initially identified from studies in sex-reversed goats (Pailhoux et al., 2001, 2002; Baron et al., 2005). *Foxl2* expression begins specifically in somatic cells of the XX gonad at 12.5 dpc and continues into adult life; however, the gene is not required for the initial differentiation of follicle cells or for the assembly of ovarian follicles (Loffler et al., 2003; Ottolenghi et al., 2005). In *Foxl2* mutants, follicle cells begin to express male markers after birth as though they have transdifferentiated to their male counterparts, Sertoli cells (Ottolenghi et al., 2005). Mutants in estrogen receptors, or aromatase, the enzyme that produces estrogen, also show transdifferentiation of follicle cells after birth (Couse et al., 1999; Britt and Findlay, 2003). However, in both these cases, germ cells were lost first, thus it was impossible to determine whether the loss of follicle cell fate was a primary effect of the mutation or a secondary consequence of germ cell loss. In the case of *Foxl2* mutants, loss of follicle cell fate occurs in the absence of oocyte loss, suggesting a primary effect on follicle fate (Ottolenghi et al., 2005).

Although *Wnt4* is initially expressed in both sexes, expression becomes XX-specific at 12.5 dpc as a result of down-regulation of *Wnt4* in XY gonads (see Fig. 3). Unlike other candidates that have been tested, loss of *Wnt4* shows a primary effect on differentiation of the ovary. Expression of the downstream genes *follistatin* and *Bmp2* is eliminated, the morphological organization of the tissue into cortical and medullary domains is lost prior to birth, germ cells undergo apoptosis, and the gonad shows partial differentiation as a testis (Vainio et al., 1999; Jeays-Ward et al., 2003; Yao et al., 2004). Thus *Wnt4* plays a primary role in establishing ovary development.

## Testis Development

Divergence of testis development is easily recognized because the organization of testis cords at 12.5 dpc is an obvious morphological landmark of the early stage of testis organogenesis. Beginning at 10.5 dpc, *Sry* is transiently expressed in gonadal somatic cells of XY embryos (Hacker et al., 1995; Bullejos and Koopman, 2001, 2005; Sekido et al., 2004; Taketo et al., 2005; Wilhelm et al., 2005). This diverts the fate of supporting cells to the Sertoli lineage, marks the initiation of testis-specific development of the tissue, and provides a discrete starting point for analysis of cell fate decisions in the gonad. If this single gene is deleted from the Y chromosome in an XY mouse, the gonad differentiates as an ovary (Lovell-Badge and Robertson, 1990; Gubbay et al., 1992). Conversely, if this single gene is expressed in an XX embryonic gonad, Sertoli cells differentiate, and together with germ cells, undergo testis cord morphogenesis (Koopman et al., 1991).

Although expression of *Sry* is transient, it normally results in up-regulated expression of *Sox9*, which is continually expressed thereafter (Sekido et al., 2004; Bullejos and Koopman, 2005). *Sox9* is an HMG-type DNA binding factor closely related to *Sry* and required for Sertoli cell differentiation. Although *Sry* is specific to mammals, *Sox9* is conserved among all vertebrates so far tested (Morrish and Sinclair, 2002). Deletion of *Sox9* in mice leads to ovary development in XY gonads (Chaboissier et al., 2004; Barrionuevo et al., 2006). Furthermore, ectopic expression of *Sox9* leads to Sertoli cell development and testis morphogenesis in XX gonads (Bishop et al., 2000; Vidal et al., 2001). These results are similar to the effect of deleting or exogenously expressing *Sry* itself, suggesting that establishment of *Sox9* expression is the critical step downstream of *Sry* required to establish differentiation of Sertoli cells and morphological development of the testis.

It is clear that expression of *Sry* in supporting cell progenitors is a cell-autonomous first step in Sertoli fate commitment. However, a threshold number of Sertoli cells is required for testis development (Palmer and Burgoyne, 1991; Schmahl and Capel, 2003). Evidence suggests that paracrine signals are involved in the testis pathway. For example, Sertoli cells can be recruited from the XX population in an XX ↔ XY chimera so long as >30% of the chimeric gonad is XY (Palmer and Burgoyne, 1991). In XX-Sxr[a] mice, where the *Sry* gene has been transposed to an X chromosome, cells that have randomly inactivated the X chromosome carrying *Sry* can nonetheless be recruited to the Sertoli pathway (Jamieson et al., 1998). In addition, XX cells are recruited to express Sertoli cell markers in an ex vivo cell mixture between XX and XY cells (Wilhelm et al., 2005). The extracellular signals responsible for this "community effect" are not well understood; however, several signaling pathways have been identified that are involved in testis organogenesis (Colvin et al., 2001; Adams and

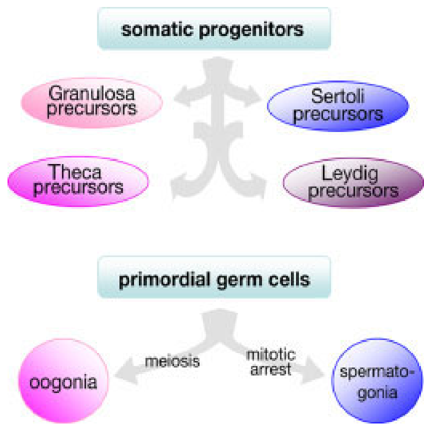


**Fig. 1.** Bipotential cells in the early gonad. Somatic progenitors give rise to follicle and theca cells in the ovary or Sertoli and Leydig cells in the testis. Primordial germ cells differentiate as oogonia in an ovarian environment (characterized by entry into meiosis) or spermatogonia in a testis environment (characterized by mitotic arrest).

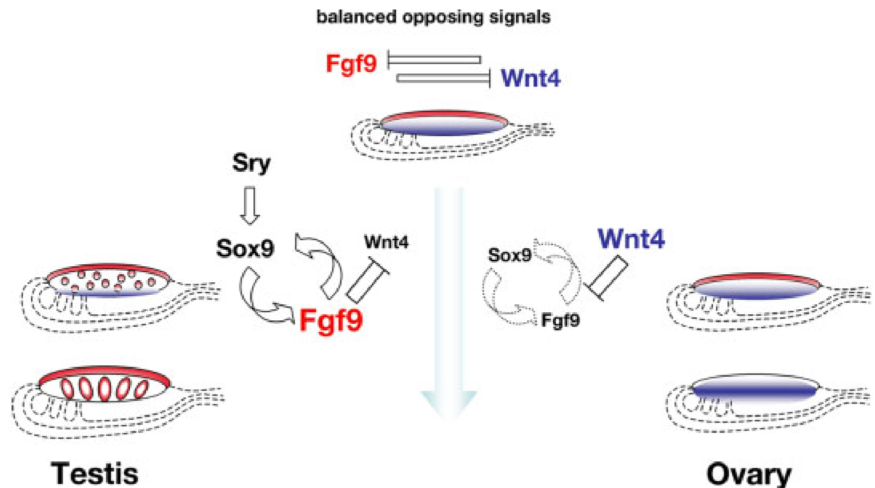


**Fig. 3.** Model of balanced opposing signals between *Fgf9*and *Wnt4*. In XY gonads, *Sry* upregulates *Sox9* to establish a feed-forward loop that upregulates *Fgf9* and silences *Wnt4*. In XX gonads, *Wnt4* dominates and silences *Fgf9* and *Sox9*.

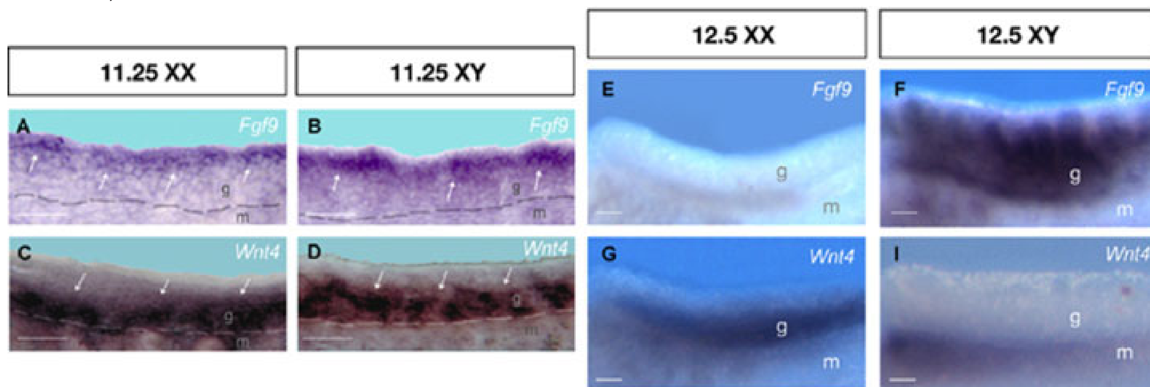


**Fig. 2.** *Fgf9* and *Wnt4* mRNA expression in 11.25- and 12.5-dpc gonads. *Fgf9* and *Wnt4* are expressed in reciprocal domains in both XX and XY gonads during the bipotential stage (11.25 dpc). Expression of *Fgf9* resolves to a male-specific pattern, whereas *Wnt4* resolves to a female-specific pattern, at 12.5 dpc. Modified from Kim et al. (2006).

McLaren, 2002; Yao et al., 2002; Brennan et al., 2003; Nef et al., 2003; Schmahl et al., 2004; Malki et al., 2005; Wilhelm et al., 2005).

*Fgf9* is the best studied signaling molecule in this group, and the only case where deletion of a single signaling gene causes a failure of Sertoli cell differentiation and complete sex reversal (Colvin et al., 2001; Schmahl et al., 2004; Kim et al., 2006). To distinguish between a role of *Fgf9* in the primary commitment to Sertoli cell fate from a role during subsequent Sertoli differentiation and morphogenesis in the gonadal field, the genetic interactions between *Fgf9*, *Sry*, and *Sox9* were examined. This epistatic analysis revealed that *Fgf9* forms a regulatory network in conjunction with *Sry* and *Sox9* during the early steps of Sertoli cell specification. In the absence of *Fgf9*, *Sox9* expression is not maintained, despite the normal expression of *Sry* and initial upregulation of *Sox9*, and the male pathway is aborted prior to Sertoli cell differentiation and testis cord formation. These data have the important implication that the cell-autonomous activity of *Sry* is not sufficient to establish the male pathway without *Fgf9*. Remarkably, in this failing environment, somatic cells do not die, but instead undergo cell fate transitions. *Wnt4* expression is upregulated in $Fgf9^{-/-}$ XY gonads and somatic cells in the gonad switch to the female developmental pathway (Kim et al., 2006). Moreover, ovarian markers normally expressed downstream of *Wnt4* in XX gonads are upregulated in the $Fgf9^{-/-}$ XY gonads (DiNapoli et al., 2006). This data indicate that *Fgf9* is a critical component that drives divergence of the testis pathway but it is not essential for ovary development.

## ANTAGONISM BETWEEN WNT4 AND FGF9

*Fgf9* is expressed in Sertoli precursor cells, and may act as an autocrine factor to maintain SOX9 expression. However, it seems likely that it also acts as a paracrine factor through Fgf receptors in neighboring cells. In in vitro culture systems, ectopic FGF9 induced SOX9 expression in XX cells. Also consistent with a paracrine role, addition of FGF9 to XX gonads in culture blocked *Wnt4* expression. Both of these experiments suggest that FGF9

acts non-cell-autonomously to block female signals and recruit the entire gonad cell community to the male pathway (Kim et al., 2006).

In a reciprocal manner, *Wnt4* is involved in suppressing the male pathway. *Fgf9* is de-repressed in *Wnt4* mutant XX gonads. Surprisingly, *Sox9* expression is also upregulated in $Wnt4^{-/-}$ XX gonads, despite the fact that *Sry* is not present in XX embryos. This finding indicates that *Sox9* upregulation in the gonad is not absolutely dependent on *Sry*, as *Sox9* can be upregulated in XX gonads simply by downregulating *Wnt4* (Kim et al., 2006). This finding has important implications for sex determination in XX humans that develop a testis (Berta et al., 1990), and in species where *Sry* is absent and the male pathway is established by other genetic/environmental switches (see below).

*Sox9* expression is not maintained in $Wnt4^{-/-}$ XX gonads, despite the coincident upregulation of *Fgf9*. The explanation for this result is not yet clear, but there are several possibilities. It has recently come to our attention that *Wnt4* transcripts are still detectable in the mutant mesonephric mesenchyme (Stark et al., 1994). This raises the possibility that the *Wnt4* mutation acts as a hypomorphic allele of *Wnt4* in gonadal tissue. However, it seems more likely that other Wnts expressed in the gonad (Nef et al., 2005) compensate for the loss of *Wnt4*. Alternatively, maintenance of *Sox9* expression may normally be stabilized by another male-specific factor such as Fgf receptors, Fgf-binding proteins, or other downstream targets of *Sry*.

We suggest that antagonistic activity between WNT4 and FGF9 balances the bipotential gonad between two alternative fates. At the bipotential stage, *Fgf9* is expressed in the cortical domain of XX and XY gonads including the coelomic epithelial layer, while *Wnt4* is expressed in the medullary region overlapping the mesonephric border. In XX gonads, the *Fgf9* expression domain is lost while the *Wnt4* domain expands, whereas, in XY gonads, the reverse is true (Fig. 2).

In XY gonads, *Sry* influences the balance between WNT4 and FGF9 by the initial up-regulation of *Sox9*. *Sox9* upregulates *Fgf9*, which is required to maintain *Sox9*. This creates a feed-forward loop that triggers proliferation of Sertoli progenitors (Schmahl et al., 2004), thus expanding the number of cells expressing *Fgf9*. This leads to the predominance of FGF9 signals in the gonadal field, stabilization of SOX9, repression of *Wnt4*, and commitment to the testis pathway (Fig. 3). There is evidence that FGF9 is involved in initiating cell migration into the XY gonad (Colvin et al., 2001), a process also known to be mediated by Fgfs during development of the *Drosophila* genital disk (Ahmad and Baker, 2002). Proliferation and cell migration rapidly change the morphology of the XY gonad and initiate testis cord formation, which also is believed to stabilize Sertoli cell differentiation. Through these mechanisms, Fgf signaling links commitment to Sertoli cell fate and morphological development of the testis.

## INTRACELLULAR RESPONSE TO EXTRACELLULAR SIGNALS: COMMITMENT TO THE SERTOLI OR FOLLICLE CELL FATE

How extracellular signaling from FGF9 or WNT4 implements intracellular molecular machinery to produce sex-specific cellular responses in gonadal cells has not been investigated. Immunostaining using an antibody raised against FGFR2 detects the protein on the membrane of coelomic epithelial cells in XX and XY gonads and in the nuclei of cells specifically within the XY gonad, suggesting that it may be an important element of FGF signaling (Schmahl et al., 2004). It will be useful to test whether FGF9 signaling utilizes the mitogen-activated protein kinase (MAPK) pathway or other intracellular pathways, and how FGFR2 functions in the nuclei of XY cells. Receptors for the WNT4 have not been characterized in the gonad. In the kidney, where *Wnt4* is essential for the mesenchymal to epithelial transition, WNT4 binds Frizzled receptors and activates both canonical and noncanonical pathways to active target genes required for kidney tubulogenesis (Stark et al., 1994; Lyons et al., 2004). However, it is not known whether canonical (β-catenin/LEF/TCF) or noncanonical Wnt signals are active in gonads to direct the transcriptional regulation of the ovary-specific genes required for commitment to the female pathway.

An important question for future experiments is how the antagonism between *Fgf9* and *Wnt4* generates opposing outcomes, which appears to be stabilization or destabilization of SOX9 expression. The intracellular pathways downstream of Fgf and Wnt signaling have been studied in other systems. For example, during chondrogenesis, *Sox9* is required for chondrocyte cell fate determination and differentiation from mesenchymal progenitors. Fgfs increase the expression of *Sox9* in chondrocytes through the MAPK pathway (de Crombrugghe et al., 2001). Many different Wnts including *Wnt4*, *-5a*, *-5b*, and *-9a*, are expressed during chondrogenesis and have different effects on chondrocyte differentiation (Hartmann and Tabin, 2000; Yang et al., 2003). In particular, *Wnt4* regulates the rate of chondrocyte differentiation through the canonical β-catenin pathway (Hartmann and Tabin, 2000; Church et al., 2002; Enomoto-Iwamoto et al., 2002). Inactivation of β-catenin or overexpression of *Sox9* in chondrocytes produces a similar phenotype, delayed chondrocyte differentiation and dwarfism (Akiyama et al., 2004), indicating that the two genes are acting on a common pathway but in opposite directions. Wnt/β-catenin signaling has also been shown to act as a molecular switch between osteoblast and chondrocyte cell fates in common mesenchymal progenitors (Day et al., 2005; Hill et al., 2005). Studies in an amphibian system demonstrated an essential interaction between β-catenin and XSox during embryonic axis formation and mesoderm development (Zorn et al., 1999). A frequent theme in all of these systems is that the fate of responding cells depends on the cooperative relation between Fgf signals and Sox gene expression on the one hand, and the opposing activity of Wnt signals on the other.

During canonical Wntsignaling,

β-catenin is transported to the nucleus where it converts TCF (T cell factor) into a transcriptional activator of target genes (Zorn et al., 1999; Weidinger and Moon, 2003). Sox genes may antagonize Wnt signaling by competition for binding to β-catenin, which could block β-catenin/TCF activation targets, alter target gene selectivity, or both. β-catenin has been shown to co-immunoprecipitate with SOX9 during chondrogenesis, and this physical interaction also has been shown to inhibit Wnt/β-catenin signaling (Akiyama et al., 2004). In another study of craniofacial development, it was shown that Fgf/FGFR2 signaling activates the expression of *Sox2* in osteoblasts. Association between SOX2 and β-catenin was shown to lead to down-regulation of numerous Wnt target genes (Mansukhani et al., 2005). Future experiments will determine whether similar intracellular molecular pathways mediate the interaction of FGF9, SOX9, and WNT4 in the XX and XY gonad to determine the fate of the supporting cell lineage.

## PRIMARY SEX DETERMINATION IN OTHER VERTEBRATES: TAKING THE MODEL ON THE ROAD

While most of the molecular work has been done in mice (with a critical input from genes identified in the human population), half a century of electron microscopy work on morphology of the developing gonad has been done in many vertebrates. It is clear that the basic bipotential plan of gonad development is a common theme across vertebrates. Curiously the switch that initiates the male pathway is highly variable across species. *Sry* is a consistent feature in eutherian and metatherian mammals; however, it is not found in monotremes, birds, reptiles, or fish (Zarkower, 2001; Graves, 2002). For example, in many reptiles, sex determination depends on environmental cues. This does not mean that there are no genetic differences in the population, but that any genetic differences that exist can be over-ridden by incubation at a specific temperature (Sarre et al., 2004). In species such as alligators and frogs, hormones or their mimics found in the environment may have a strong influence on early sexual development (Milnes et al., 2005; McLachlan et al., 2006).

The molecular regulation of sex determination in animals where *Sry* is absent is not understood. Establishment of *Sox9* expression, which reflects the outcome of antagonistic signaling between FGF9 and WNT4 in mammals, has proved to be a common feature of the testis pathway in other vertebrates (Koopman et al., 2001; Morrish and Sinclair, 2002). Antagonism between FGF9 and WNT4 could be a conserved mechanism that balances the gonad between two alternative fates. An array of genetic, environmental, hormonal, or behavioral switches could trigger an imbalance in these signals or their downstream pathways, leading to stabilization or destabilization of *Sox9* expression. This might explain the rapid evolution of diverse sex determining mechanisms that seem to have arisen frequently and independently in different species.

## FUTURE DIRECTIONS

Expression of both *Fgf9* and *Wnt4* has been reported in the bipotential gonad of *Rana rugosa* (Oshima et al., 2005; Yamamura et al., 2005), and also detected in *Trachemys scripta* (unpublished data) consistent with a balancing mechanism involving these signals in species of frogs and turtles. It will be interesting to investigate broader conservation of these expression patterns among other vertebrates. Functional studies will also be required to test the model in nonmammalian species.

If alterations in WNT4 or FGF9 downstream pathways can trigger testis development in the absence of *Sry*, this might explain how the testis pathway is initiated in cases of human XX sex-reversal to male (McElreavey et al., 1993). How intracellular pathways downstream of Fgf and Wnt signaling compete for control of supporting cell fate will be an exciting new direction of research. This will likely require molecular and biochemical experiments to investigate networks of protein interactions and transcriptional regulation. The links between molecular differentiation and the structure and relationship of cells in the developing organs should also prove to be an informative focus of future experiments.

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