Signaling Pathways Involved in Mammalian Sex Determination and Gonad Development

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Abstract

The development of any organ system requires a complex interplay of cellular signals to initiate the differentiation and development of the heterogeneous cell and tissue types required to carry out the organs' functions. In this way, an extracellular stimulus is transmitted to an intracellular target through an array of interacting protein intermediaries, ultimately enabling the target cell to elicit a response. Surprisingly, only a small number of signaling pathways are implicated throughout embryogenesis and are used over and over again. Gonadogenesis is a unique process in that 2 morphologically distinct organs, the testes and ovaries, arise from a common precursor, the bipotential genital ridge. Accordingly, most of the signaling pathways observed throughout embryogenesis also have been shown to be important for mammalian sex determination and gonad development. Here, we review the mechanisms of signal transduction within these pathways and the importance of these pathways throughout mammalian gonad development, mainly concentrating on data obtained in mouse but including other species where appropriate. © 2016 S. Karger AG, Basel

Cellular signaling is crucial to life. Throughout development, cells must communicate in order to coordinate the differentiation of a vast array of heterogeneous cell types within any given organ system. Additionally, cellular interactions within the mature organism are necessary in order to maintain homeostasis and carry out day-to-day functions. Typically, these interactions occur as a result of induction, wherein a cell releases a signal to induce a change in another cell. A cell's competence, that is its ability to elicit a cellular change in response to a signal, is largely dependent on the presence or absence of a signaling receptor. Binding of this receptor by its ligand leads to receptor activation, resulting in the recruitment of protein intermediaries responsible for transducing the signal within the intracellular signaling cascades. In many cases this ultimately results in the activation of transcription factors, causing a change in gene expression, which dictates cell fate decision, differentiation, proliferation, migration, and apoptosis.

Despite the importance of signaling in all cellular processes, the number of signaling pathways identified to date that have been implicated throughout embryogenesis is limited; however, there is a substantial amount of cross-talk between these pathways. Integration and divergence of these pathways is vital throughout development to ensure that target cells interpret and respond to con-

text-dependent cues. Consequently, the cumulative effect of combined pathways may result in a response far different than the sum of its parts. Furthermore, many of these pathways elicit their effects in a dose-dependent manner and as such the time, location, and duration of signaling largely affects the nature of the response.

Gonadogenesis is a unique and complex process wherein morphologically distinct organs, testes and ovaries, arise from a common precursor. Since the discovery of the male-determining gene *SRY* in 1990, much research has gone into elucidating the mechanisms of sex determination and gonad differentiation. Given the importance of cell signaling throughout embryogenesis, it is not surprising that most of the signaling cascades characterized to date have been implicated in this process. However, there is a lack of knowledge regarding the importance of their cross-talk and cumulative effects during gonad development. In this review we will first summarize the cellular and morphological mechanisms underlying mammalian sex determination and gonad development before describing in detail the mechanisms of signal transduction pathways involved throughout these processes.

Cellular and Morphological Events during Mammalian Gonad Development

The development of both testes and ovaries starts with the the same precursor structures, the genital ridges. First described in 1870 [Waldeyer, 1870], the genital ridges develop through thickening and proliferation of the surface epithelium on the ventromedial side of the mesonephros at around 9.5 days post coitum (dpc) in mice and during the fourth week of gestation in humans [Byskov, 1986]. These somatic cells are joined by primordial germ cells (PGCs) that have made their journey from the base of the allantois [Anderson et al., 2000] where they have been specified as a result of inductive signaling at approximately 7.0 dpc in mice [Lawson et al., 1999] and during the third week of gestation in humans [Ginsburg et al., 1990]. Mouse PGCs reach the developing genital ridges at around 10.0 dpc [Tam and Snow, 1981; McLaren, 1984]. They proliferate rapidly with incomplete cytokinesis, thus forming germ cell cysts in both XX and XY developing gonads [Pepling and Spradling, 1998; Lei and Spradling, 2013]. In the mouse these germ cell cysts are not maintained but instead they fragment into smaller cysts and single germ cells during development [Lei and Spradling, 2013].

The genital ridges remain indistinguishable between males and females until approximately 12.0 dpc. After this, the developing testis shows massive morphological changes which are induced by the key regulator of sex determination in mammals, the sex-determining region on the Y chromosome gene *Sry*, the founding member of the family of *Sox* genes encoding HMG (high mobility group)-domain transcription factors [Bowles et al., 2000]. Gain-of-function experiments in which *Sry* was introduced into XX mice [Koopman et al., 1991] and experiments utilizing sex-reversed XY mice with mutations in the sex-determining region *Tdy* [Gubbay et al., 1990] demonstrated that *SRY* is both sufficient and necessary for testis development.

Testis Development

Sry is expressed in the supporting cell precursors and drives their differentiation into Sertoli cells [Sekido et al., 2004]. First described in 1865, Sertoli cells are nurse cells with the primary function of supporting germ cells [Sertoli, 1865]. It is believed that the origin of Sertoli cell precursors lies within the coelomic epithelium [Karl and Capel, 1998] and that these pre-Sertoli cells generate signals to induce proliferation within the XY genital ridge, thus producing more Sertoli cells [Schmahl et al., 2000]. This may dictate the fertility of the individual as Sertoli cells only proliferate during fetal and early neonatal development [Orth, 1982], and the final number of Sertoli cells in the adult dictates the number of germ cells that can be supported [Sharpe et al., 2003].

As Sertoli cells differentiate they assume a polarized morphology and extend cytoplasmic protrusions around germ cells to form testis cords [Combes et al., 2009]. Testis cords are the precursor of the seminiferous tubules in adult testes, which ultimately play a role in sperm maturation and transport. Testis cords form over a 24-hour period from 11.5 to 12.5 dpc in mice [Coveney et al., 2008] and between weeks 7 and 8 of human gestation [Hanley et al., 2000]. In this time, Sertoli cells surround germ cells to form aggregates of cells. Following this, endothelial cells from the mesonephros migrate into the developing testis and segregate these Sertoli/germ cell clusters into evenly distributed cords throughout the testis [Combes et al., 2009]. Later during development, the testes enlarge and testis cords elongate, eventually forming the seminiferous tubules. PGCs within the testis cords will enter mitotic arrest from around 13.5 dpc [Western et al., 2008, 2011] and only re-enter the cell cycle after birth to start the process of spermatogenesis.

Sertoli cells instruct interstitial mesenchymal cells to differentiate into peritubular myoid (PM) cells. These cells surround the testis cords, forming a single layer in mice [Gardner and Holyoke, 1964] and 3–4 layers in humans [Ross and Long, 1966]. Extracellular matrix proteins, secreted by both PM and Sertoli cells, form a basal lamina between these 2 cell types [Skinner et al., 1985] that is important for the integrity of testis cords. PM cells become contractile later in development, which is necessary for the transport of sperm from the testis into the epididymis [Ellis et al., 1981; Maekawa et al., 1996]. At around the same time (from 12.5 dpc), fetal Leydig cells differentiate from interstitial mesenchymal precursor cells. First described in 1850 [Leydig, 1850], Leydig cells are largely responsible for the production of androgens that are important for virilisation of the reproductive tract and development of secondary sexual characteristics [Geissler et al., 1994].

Ovarian Development

In contrast to the developing testis, morphological changes in the developing ovary have a later onset, with the developing ovary being in an apparently inert state until approximately 13.5 dpc. At this stage, germ cells at the anterior pole start entering meiosis, followed by other germ cells in an anterior-to-posterior order [Menke et al., 2003; Yao et al., 2003; Bullejos and Koopman, 2004]. Germ cells within a cyst, and even within different fragments of the same cyst, enter meiosis synchronously [Lei and Spradling, 2013]. By 15.5 dpc, most germ cells have entered meiosis and arrest in prophase I [Borum, 1961; Miles et al., 2010]. In addition, many germ cells are lost through apoptosis. The remaining cells will form primordial follicles, single oocytes surrounded by a single layer of flattened granulosa cells, shortly after birth in mouse [Epifano and Dean, 2002] and from week 21 in human gestation, being completed at birth [Gougeon, 1996]. In mouse there are 2 types of follicles: medullary follicles that are activated shortly after birth and contribute to puberty and early fertility, and cortical follicles that are activated gradually throughout life [Mork et al., 2012; Zheng et al., 2014]. It is unclear if these 2 populations of follicles also exist in human. However, it has been shown that growing follicles exist in the medullary region throughout infancy and childhood [Lintern-Moore et al., 1974; Peters et al., 1976], suggesting that the mouse ovary reflects the situation in the human ovary. In accordance with the presence of the 2 types of follicles, 2 classes of pre-granulosa cells can be found in the fetal ovary, medullary and cortical pre-granulosa cells, which might arise from a sin-

gle precursor cell lineage [Mork et al., 2012; Hummitzsch et al., 2013] but are distinguishable by marker gene expression from 13.5 dpc [Rastetter et al., 2014]. The assembly of the follicles is completed by the recruitment of steroidogenic precursor cells to differentiate into theca cells [Hirshfield, 1991]. Recently, it has been shown that there are 2 sources of theca cell precursors: mesenchymal cells that reside within the ovary and a second population that migrates into the ovary from the mesonephros [Liu et al., 2015].

Importance of Signaling Pathways in Embryogenesis

The formation of any organ in the body depends on cell-cell communication to accomplish the coordinated differentiation and development of different cell types and tissues. This cell-cell communication is achieved by signaling pathways by which one type of cell generates a signal that is received by other cells that are competent to respond [Spemann and Mangold, 1924; Waddington, 1940]. Surprisingly, only a small number of signaling pathways are involved in organogenesis, including WNT (wingless-type MMTV integration site family), NOTCH, TGF β (transforming growth factor beta), which includes BMPs (bone morphogenetic proteins), HH (hedgehog), Hippo, nuclear receptors, for example the retinoic acid receptor (RAR), and RTKs (receptor tyrosine kinases) such as FGFR (fibroblast growth factor receptor), EGFR (epidermal growth factor receptor), and IR (insulin receptor). Activation initiates a signaling cascade within the receiver cell that usually results in a change of gene expression. Accordingly, the effectors of these pathways are mainly transcription factors at promoters and enhancers of genes. However, signaling pathways also regulate the function of other proteins involved in transcriptional regulation, such as RNA polymerase II, histones, and histone modifying enzymes, as well as chromatin remodeling complexes [for reviews see Klein et al., 2013; Yang et al., 2013].

Almost all of the above-mentioned signaling pathways have been shown to play a role during sex determination and gonad development. Only the Hippo pathway, which was originally identified in *Drosophila* for its role in controlling organ size [Pan, 2010], has not been implicated to date. In the following sections we will discuss the molecular mechanisms of how these signaling pathways function and what role they play during mammalian gonad development (see also table 1).

Table 1. Signaling components involved in different stages of gonad development. Loss-of-function phenotypes in the fetal gonads, observed through mouse knockout models and/or the use of inhibitors in ex vivo gonad cultures of different signal pathway components

Component	Loss-of-function phenotype	Reference
Bipotential genital ridge/sex determination		
GADD45γ	XY gonadal sex reversal caused by reduced phosphorylation of p38α MAPK and GATA4 and therefore a lack of <i>Sry</i> expression	Gierl et al. [2012]; Warr et al. [2012]
MAP3K4	XY gonadal sex reversal caused by a dramatic reduction in <i>Sry</i> and <i>Sox9</i> expression	Bogani et al. [2009]
INSR/IGF1R/IRR	XY gonadal sex reversal due to a delay/lack of <i>Sry</i> upregulation	Nef et al. [2003]; Pitetti et al. [2013]
FGFR2	background-dependent partial to complete male-to-female sex reversal due to failure to maintain <i>Sox9</i> expression	Kim et al. [2007]; Bagheri-Fam et al. [2008, 2015]; Siggers et al. [2014]
FGF9	background-dependent partial to complete male-to-female sex reversal due to failure to maintain <i>Sox9</i> expression	Colvin et al. [2001]; Schmahl et al. [2004]; DiNapoli et al. [2006]
BMP7	significant reduction in PGC numbers of both XX and XY at 11.5 dpc as a result of reduced germ cell proliferation	Ross et al. [2007]
Testis differentiation		
WNT4	compromised Sertoli cell differentiation at early stages and therefore delayed sex cord formation, suggesting a testicular role for WNT4 downstream of <i>Sry</i> but upstream of <i>Sox9</i>	Jeays-Ward et al. [2004]
WNT5a	hypoplastic testes at early stages caused by a decrease in somatic cell proliferation	Chawengsaksophak et al. [2012]
DHH	defects in fetal and adult Leydig and peritubular myoid cell differentiation, anastomotic testis cords	Bitgood et al. [1996]; Clark et al. [2000]; Pierucci-Alves et al. [2001]; Yao and Capel [2002]; Yao et al. [2002]
HHAT	reduction in testis size from 12.5 dpc with a decrease in testis cord number and alteration in size and shape of testis cords, as well as a lack of Leydig cell differentiation	Callier et al. [2014]
HES1	increase in Leydig cell numbers, abnormal testis cord formation as well as germ cell loss. In contrast, constitutively active Notch signaling results in Leydig cell loss	Tang et al. [2008]
ALK4/5/7 and ALK5 alone	reduction in Sertoli cell proliferation and therefore disrupted testis cord formation; PGCs enter meiosis instead of mitotic arrest	Miles et al. [2013]; Wu et al. [2013]
ActivinβA	reduction in Sertoli cell proliferation at 13.5 dpc and therefore a failure in fetal testis cord elongation	Archambeault and Yao [2010]; Mendis et al. [2011]
SMAD4	knockout specifically in Sertoli cells results in a similar phenotype as activinβA with reduced Sertoli cell proliferation and failed testis cord elongation; knockout in PGCs causes entry into meiosis instead of mitotic arrest in some PGCs	Archambeault and Yao [2010]; Wu et al. [2015]
SMAD2	PGC-specific knockout results in downregulation of <i>Nodal</i> and <i>Nanos2</i> and therefore a defect in male germ cell differentiation.	Wu et al. [2015]
TGFβ2	transient reduction in testis cord number	Memon et al. [2008]
TGFBR3	defect in testis cord formation at early stages and reduced fetal Leydig cell function	Sarraj et al. [2010]
PDGFRA	defects in cell proliferation and endothelial cell migration from the mesonephros and therefore defective testis cord formation. In addition, fetal Leydig cell differentiation is impaired	Brennan et al. [2003]
PTGDS	transient abnormal SOX9 protein localization in cytoplasm and reduced <i>Sox9</i> mRNA levels, causing a delay in testis cord formation, which recovered at later stages	Moniot et al. [2009]
HPGDS	defect in initial SOX9 translocation into the nucleus at 11.5 dpc	Moniot et al. [2011]
PTGDS/HPGDS	phenotype in somatic cells is similar to PTGDS single knockout; however, in addition, the double knockout displays a significant increase in germ cell proliferation due to the regulation of cell cycle genes by PGD ₂	Moniot et al. [2014]
DP2	same phenotype as PTGDS/HPGDS double knockout, showing that PGD ₂ acts through DP2 receptor	Moniot et al. [2014]
FGF9	male-specific reduction in germ cell number due to elevated apoptosis	DiNapoli et al. [2006]
p38 MAPKs	PGCs in a testis enter meiosis instead of mitotic arrest, suggesting p38 inhibits RA signaling	Ewen et al. [2010]; Wu et al. [2015]
Ovary differentiation		
INSR/IGF1R	delay in ovarian differentiation in XX and XY mutant gonads	Pitetti et al. [2013]
β-Catenin	partial female-to-male sex reversal with the formation of a coelomic vessel, androgen-producing, adrenal-like cells and loss of germ cells; in addition, <i>Wnt4</i> expression was reduced, but <i>Rspo1</i> unaffected	Liu et al. [2009]
RSPO1	partial female-to-male sex reversal with the formation of a coelomic vessel, androgen-producing, adrenal-like cells and loss of germ cells due to a decrease in proliferation; in addition, granulosa cells differentiate prematurely and trans-differentiate into Sertoli-like cells postnatally	Chassot et al. [2008, 2012]; Tomizuka et al. [2008]; Maatouk et al. [2013]
WNT4	partial female-to-male sex reversal with the formation of a coelomic vessel, androgen-producing, adrenal-like cells and loss of germ cells due to an increase in apoptosis; in addition, granulosa cells differentiate prematurely and trans-differentiate into Sertoli-like cells postnatally	Vainio et al. [1999]; Jeays-Ward et al. [2003]; Maatouk et al. [2013]
FST	formation of the coelomic vessel as well as loss of germ cells through apoptosis; FST acts downstream of WNT4 to inhibit endothelial cell migration and formation of the coelomic vessel	Yao et al. [2004]

Signaling Pathways Regulating Genital Ridge Formation and Sex Determination

During the early stages, XX and XY genital ridges increase in size through proliferation of somatic cells within or near the coelomic epithelium [Schmahl et al., 2000]. This proliferation is dependent on WNT signaling (fig. 1A) [Chassot et al., 2012], with 2 ligands of the WNT signaling pathway being expressed: WNT4 (wingless-type MMTV integration site family, member 4) [Nusse et al., 1991] and RSPO1 (R-spondin homolog 1) [Kamata et al., 2004]. Null mutations of *Wnt4* or *Rspo1* in mouse result in a reduction of proliferation in the developing genital ridges of both sexes, especially within the coelomic epithelium [Chassot et al., 2012].

The canonical WNT signaling pathway is crucial throughout embryonic development as a mediator of cellular differentiation and morphogenesis. Activation of this pathway ultimately leads to the accumulation and nuclear translocation of β -catenin, which acts as a co-activator of the transcription factors TCF/LEF (T cell factor/lymphoid enhancer factor). All TCF/LEFs bind to the DNA through a HMG domain that also induces bending of the DNA [Giese et al., 1991; Love et al., 1995]. These factors repress the transcription of target genes in the absence of active WNT signaling by interacting with co-repressors such as members of the AES (amino terminal enhancer of split, also called Groucho-related gene, GRG)/TLE (transducing-like enhancer of split) family. Interaction of TCF/LEF with β -catenin removes these co-repressors and enables transcription of WNT target genes [for reviews, see Miller and Moon, 1996; Clevers and Nusse, 2012]. The signal transduction components that link the WNT receptors complex FZD (frizzled)/LRP6 or LRP5 to β -catenin is the β -catenin destruction complex, which consists of the scaffold protein AXIN1, the tumor suppressor APC (adenomatous polyposis coli), CSNK1 (casein kinase 1), and GSK3 (glycogen synthase kinase 3). In the absence of ligand binding, CSNK1 and GSK3 phosphorylate β -catenin, which results in the recruitment of an E3 ubiquitin ligase and subsequent targeting of β -catenin for proteasomal degradation by ubiquitination [Aberle et al., 1997]. Upon WNT ligand binding to its receptors, the scaffolding protein DVL1 (dishevelled) is recruited, leading to the phosphorylation of LRP6 and the translocation of the destruction complex away from cytoplasmic β -catenin to the membrane-bound receptors. This disruption of the association of β -catenin with the destruction complex ultimately leads to the accumulation of β -catenin in the cytoplasm and translocation to the nu-

cleus to act as a transcriptional co-activator [for review see Bienz and Clevers, 2003; Mosimann et al., 2009].

In addition to WNT4 and RSPO1, a knockout mouse model of another signaling pathway, namely the double deletion of the receptor tyrosine kinases INSR (insulin receptor) and IGF1R (insulin-like growth factor 1 receptor), also displays a reduction in proliferation in XX and XY genital ridges [Pitetti et al., 2013]. Interestingly, there appears to be cross-talk between these 2 pathways (fig. 1A), as *Wnt4* expression is significantly reduced in the *Insr/Igf1r*-null genital ridges [Pitetti et al., 2013], and *Igf1r* expression is greatly reduced in the *Rspo1*- and *Wnt4*-null gonads [Chassot et al., 2012].

Things change quickly in the developing testis through the upregulation of the male-determining gene *Sry* from the Y chromosome (fig. 1B). Recently, the combination of several studies nicely demonstrated that this upregulation is under the control of a signaling pathway involving MAP (mitogen-activated protein) kinases. The MAPK cascades control proliferation, differentiation, and apoptosis of cells. Each MAPK pathway is a conserved cascade of 3 protein kinases. A breadth of differing extracellular stimuli, including mitogens, cytokines, growth factors, and cellular stressors, such as heat shock and UV irradiation, induce these pathways through the activation of a MAP kinase kinase kinase (MAP3K). MAP3Ks, in turn, phosphorylate and activate specific MAP kinase kinases (MAP2Ks), which then activate MAP kinases (MAPKs). The 3 conventional MAPK pathways described to date are the extracellular signal-regulated protein kinases (ERK1 and ERK2), cJUN N-terminal kinases (JNK1, JNK2 and JNK3), and the p38 MAP kinases (p38 α , p38 β , p38 γ , p38 δ). These kinases activate and regulate the activities of a number of downstream target regulatory proteins and transcription factors. In addition, the 3 MAPK signaling pathways are not isolated, but there is considerable cross-talk between these pathways, and as such these cascades share some of their components.

Through the use of a forward genetic screen, Bogani et al. [2009] identified a recessive mouse mutant, named *boy-girl* (*byg*), which exhibited XY gonadal sex reversal as a result of a mutation leading to a premature stop codon in the *Map3k4* gene. The role of MAP3K4 in murine sex determination was further supported by the analysis of *Gadd45g* (growth arrest and DNA damage response) [Gierl et al., 2012; Warr et al., 2012]. GADD45 γ disrupts the auto-inhibitory domain of MAP3K4, thereby inducing auto-phosphorylation and activation of MAP3K4 [Miyake et al., 2007], which in turn activates the downstream p38 MAP kinases [Takekawa et al., 1997]. p38 MAPKs then phos-

Fig. 1. Signaling pathways involved in genital ridge proliferation and the regulation of *Sry* expression. **A** At 9.5 dpc, WNT signaling, activated by WNT4 and RSPO1, as well as insulin signaling through the receptors INSR and IGF1R, results in rapid proliferation of somatic cells in the early genital ridges, with cross-talk existing between these 2 pathways. **B** At 10.5 dpc, MAPK signaling plays a crucial role in upregulating *Sry* expression. In this way, GADD45γ, possibly activated by insulin signaling, disrupts the auto-inhibitory domain of MAP3K4, inducing its auto-phosphorylation and activation, ultimately resulting in the activation of the downstream p38 MAP kinases via a MAP2K. However, it is not yet known which MAP2K is important in this regulation. p38 MAPKs then activate GATA4 which, in conjunction with its co-factor FOG2, binds to the *Sry* promoter activating *Sry* transcription and initiation of testis differentiation. **C** SRY, in conjunction with SF1, directly upregulates *Sox9* transcription. In order to maintain *Sox9* expression, 2 positive feedback loops are established. First, SOX9 upregulates *Fgf9* enabling the FGF9 protein to signal through its receptor FGFR2 to maintain *Sox9* expression. Secondly, SOX9 upregulates *Ptgds* expression, which results in the production of PGD₂. This ultimately leads to nuclear translocation of the SOX9 protein where it can maintain its own expression by binding its own promoter in conjunction with SF1.

phorylate and activate the transcription factor GATA4 [Gierl et al., 2012; Warr et al., 2012], which, together with its co-factor FOG2, binds to the *Sry* promoter [Gierl et al., 2012], resulting in the upregulation of *Sry* transcription and therefore the induction of the testis differentiation program (fig. 1B) [Gierl et al., 2012; Warr et al., 2012]. Conse-

quently, if any one of these factors is mutated in mouse, male-to-female sex reversal is observed due to reduced *Sry* expression [Tevosian et al., 2002; Miyamoto et al., 2008; Bogani et al., 2009; Gierl et al., 2012; Warr et al., 2012].

Nonetheless, the question still remains as to what causes the testis-specific upregulation of *Gadd45g*. GADD45

proteins have been implicated in signaling in response to physiological and environmental stress including methylating agents such as methylmethanesulfonate, γ and UV irradiation, as well as inflammatory cytokines such as IL2 and IL6 [Beadling et al., 1993; Liebermann and Hoffman, 1998; Zhang et al., 1999]. Possible inducers of *Gadd45g* during testis determination are the insulin receptors (fig. 1B). It has been shown that the combined null mutation of *Insr* and *Igf1r* [Pitetti et al., 2013] or the triple mutation also including *Irr* (insulin receptor-related receptor) [Nef et al., 2003] leads to reduced *Sry* expression and consequently male-to-female sex reversal. Interestingly, the *Ir/Igf1r*-double knockout also displayed reduced expression of *Gadd45g* [Pitetti et al., 2013], suggesting that this could be the cause of decreased *Sry* levels. It would be interesting to test if overexpression of *Gadd45g* can rescue the sex reversal phenotype caused by the loss of the insulin receptors.

In contrast in humans, mutations in a gene encoding another MAP3K, namely *MAP3K1*, result in 46,XY DSD with partial or complete gonadal dysgenesis [Pearlman et al., 2010]. Interestingly, the identified mutations seem to result in a gain- rather than loss-of-function of the *MAP3K1* protein shown by an increase in phosphorylation of the downstream kinases p38 and ERKs as well as an increase in binding to interacting factors such as RHOA and AXIN1 [Pearlman et al., 2010; Loke et al., 2014]. Over-expression of the mutated *MAP3K1* proteins in the human pluripotent testicular embryonic carcinoma cell line NT2/D1 resulted in a decrease in expression of genes associated with testicular differentiation such as *SRY*, *SOX9*, and *FGF9*, as well as an increase in gene expression that is usually associated with ovarian development including *WNT4*, β -catenin, and *FOXL2* [Loke et al., 2014]. However, it remains to be tested if the mutated *MAP3K1* proteins are sufficient to also tilt this balance of the sex differentiation pathways in vivo by, for example, generating knock-in mouse models. Certainly the opposite, i.e. loss-of-function mutations in *Map3k1*, does not result in any significant defects in testis differentiation [Warr et al., 2011], demonstrating that *Map3k1* is not necessary for murine testis determination.

Control of Testis Development

The expression of *Sry* in the developing testis causes a rapid increase in proliferating somatic cells, while the rate remains unchanged in the developing ovary [Schmahl et al., 2000]. This increase in proliferation is one of the ear-

liest events after the expression of *Sry*. *SRY* has been shown to directly upregulate the related factor *SOX9* [Sekido and Lovell-Badge, 2008], which in turn leads to the upregulation of the proliferation-promoting factor *FGF9* (fig. 1C) [Colvin et al., 2001; Schmahl et al., 2004; Kim et al., 2006, 2007; Bagheri-Fam et al., 2008; Bradford et al., 2009].

The FGF family of signaling molecules consists of 18 ligands (FGF1–10 and FGF16–23) in mice and humans, which signal through 4 tyrosine kinase receptors FGFR1–4 [for review see Thisse and Thisse, 2005; Turner and Grose, 2010]. To signal, FGF ligands are released, they bind to heparin sulphate proteoglycans on the cell surface, and form a dimer with another FGF ligand [Yayon et al., 1991]. These ligands then bind the extracellular immunoglobulin domains of an FGFR enabling dimerization of the receptor, which brings the intracellular domains of each FGFR into close proximity, causing the trans-phosphorylation of critical tyrosine residues, thereby initiating signaling [Plotnikov et al., 1999; Schlessinger et al., 2000]. The predominant signaling cascades used by FGFRs are the RAS/MAPK pathways, but they also activate the PI3K (phosphatidylinositol 3-kinase)/AKT (thymoma viral proto-oncogene) and the PLC γ (phospholipase C γ) pathways.

FGF9, directly or indirectly upregulated by *SOX9* in the developing testis [Kim et al., 2006], signals through FGFR2 to maintain *Sox9* expression [Bagheri-Fam et al., 2008; Kim et al., 2007], thereby forming a positive feedback loop (fig. 1C). Mice homozygous for a null mutation in *Fgf9* or *Fgfr2* exhibit male-to-female sex reversal, with all testis-specific cellular events being disrupted, including cell proliferation, mesonephric cell migration, Sertoli cell differentiation, and testis cord formation [Colvin et al., 2001; Kim et al., 2007; Bagheri-Fam et al., 2008; Siggers et al., 2014]. Accordingly, mutations in *FGFR2* in human have been identified that result in 46,XY DSD [Bagheri-Fam et al., 2015]. Interestingly, there are 2 alternative splice forms of FGFR2, FGFR2b and FGFR2c, affecting the extracellular domain and therefore ligand binding [Zhang et al., 2006]. These splice forms are expressed in a cell-specific manner, with the b isoform predominantly expressed in epithelial cells and the c isoform in mesenchymal cells [Gilbert et al., 1993; Orr-Urtreger et al., 1993; Yan et al., 1993; Alarid et al., 1994]; however, to date it is only known that FGFR2c is important for *FGF9* signaling in the developing testis [Bagheri-Fam et al., 2015] but not if FGFR2b also plays a role.

Fig. 2. Signaling pathways involved in Leydig cell differentiation. The pathway shown to be both necessary and sufficient to drive Leydig cell (yellow cell) differentiation is the DHH pathway. To activate signaling, DHH first undergoes a series of post-translational modifications including palmitoylation by the acyltransferase HHAT. Once secreted from Sertoli cells (blue cell), the active ligand binds PTCH1, which then releases its inhibition of SMO, ultimately resulting in full-length GLI proteins being translocated into the nucleus where they act as transcriptional activators of

hedgehog target genes. The second pathway implicated in Leydig cell differentiation is NOTCH signaling with activation of the pathway inhibiting recruitment of Leydig cells from undifferentiated progenitor cells. Binding of the ligands DLL1, 3, and 4 as well as JAG1 and 2 to the NOTCH receptor on neighboring cells initiates proteolytic cleavage, releasing the intracellular domain, NOTCH-ICD, which translocates to the nucleus, binds RBPJ, and regulates target genes such as *Hes1* that ultimately inhibits differentiation of Leydig cells.

A second, more unusual, signaling pathway has been identified that helps to maintain *Sox9* expression in the developing testis [Adams and McLaren, 2002]. This regulatory loop involves SOX9 initiating a positive feedback loop via the direct transcriptional activation of *Ptgds* (prostaglandin D synthase) [Wilhelm et al., 2005], which produces PGD₂ (prostaglandin D₂). PGD₂, via binding to its DP receptor, initiates an intracellular cascade including the activation of PKA (cAMP-dependent protein kinase A), which phosphorylates the SOX9 protein, leading to its nuclear translocation [Malki et al., 2005; Moniot et al., 2009]. In the nucleus, SOX9 then binds to its promoter to maintain its own expression (fig. 1C) [Sekido and Lovell-Badge, 2008]. Accordingly, *Ptgds* transcription is abolished in *Sox9*-null Sertoli cells, and *Ptgds* knockout testes have reduced *Sox9* mRNA levels, abnormal SOX9 protein localization, and therefore delayed testis cord formation at early stages [Moniot et al., 2009]. However, at later stages testis cord structure appeared normal, demonstrating that the *Sox9/Ptgds* regulatory loop represents only a secondary mechanism to maintain *Sox9* expression.

Sertoli Cells Are the Control Centers of Testis Differentiation

While *Sry* expression is transient, *Sox9* expression in Sertoli cells continues throughout life. Sertoli cells are the first to differentiate and are necessary to instruct other precursor cells to differentiate into testis-specific cell types, including the differentiation of PM and fetal Leydig cells, the prevention of entry into meiosis of germ cells in the testis, and the degradation of the Müllerian duct [Svingen and Koopman, 2013].

Leydig cells differentiate at around 12.5 dpc in mouse testes and 8–9 weeks of gestation in humans [Habert et al., 2001] and increase in number at least 2-fold during fetal development. However, this increase in Leydig cell number is not due to proliferation [Byskov, 1986; Kerr and Knell, 1988; Migrenne et al., 2001] but is most likely the result of new recruitment from progenitor cells [Orth, 1982] driven by 2 signaling pathways; DHH as well as NOTCH signaling (fig. 2) in vasculature-associated cells in the interstitium [Tang et al., 2008]. A third pathway has been implicated with the ligand PDGFA (platelet-derived growth factor alpha) expressed in Sertoli cells and its receptor PDGFRα present on interstitial cells [Brennan et

al., 2003]. Knockout of *Pdgfra* in the testis results in impaired fetal Leydig cell differentiation [Brennan et al., 2003]. These mice, however, also exhibit a reduction in Sertoli cell proliferation and mesonephric cell migration [Brennan et al., 2003], suggesting that the Leydig cell phenotype might not be a direct but a secondary effect.

The pathway that has been shown to be both necessary and sufficient to drive Leydig cell differentiation is the DHH pathway [Clark et al., 2000; Pierucci-Alves et al., 2001; Yao and Capel, 2002; Yao et al., 2002; Barsoum et al., 2009]. Detailed description of the HH signaling pathway can be found elsewhere [for example Varjosalo and Taipale, 2008; Hui and Angers, 2011; Robbins et al., 2012; Briscoe and Therond, 2013]. We will focus on the steps and molecules shown to be important for testis differentiation. To activate signaling through this pathway, the hedgehog ligand itself has to be processed to be fully active. After translation and removal of the signal sequence, the HH protein is cleaved into a C-terminal domain without signaling activity and an N-terminal domain that is linked to cholesterol at its C-terminus [Porter et al., 1996]. This cholesterol-bound protein part is further processed by palmitoylation at its N-terminus by the acyltransferase HHAT (hedgehog acyltransferase) for full activity (fig. 2) [Chamoun et al., 2001; Lee and Treisman, 2001]. This step has been shown to be crucial for Leydig cell differentiation, as mutations in the *Hhat* gene in mouse and human result in testicular dysgenesis with impaired testis cord formation and a loss of Leydig cell differentiation [Callier et al., 2014]. Subsequent binding of HH to its 12-pass transmembrane protein receptor PTCH1 (patched 1) releases PTCH1 inhibition of the 7-pass transmembrane protein SMO (smoothened), resulting in pathway activation through the effector transcription factors of the GLI-Kruppel family, enabling transcription of hedgehog target genes (fig. 2).

While different hedgehog ligands are important throughout development, the main hedgehog ligand involved in the developing gonads is DHH. DHH is expressed in Sertoli cells, while expression of PTCH1 is found on the surface of interstitial cells of the developing testis, including PM cells, Leydig cells, and endothelial cells [Bitgood et al., 1996; Clark et al., 2000]. While the phenotype of *Dhh*-null mice is variable depending on the genetic background, these mice typically show defective testis organization including abnormal development of PM cells, the absence of a basal lamina surrounding the testis cords, severely defective differentiation of fetal and adult Leydig cells and, subsequently, impaired spermatogenesis [Bitgood et al., 1996; Clark et al., 2000; Pierucci-

Alves et al., 2001; Yao and Capel, 2002; Yao et al., 2002]. This phenotype is largely consistent with 46,XY partial or complete gonadal dysgenesis observed in human patients with mutations in *DHH* [Umehara et al., 2000; Canto et al., 2004, 2005]. In contrast, mutation of the DHH receptor PTCH1 results in constitutive activation of the pathway [Ingham et al., 1991]. Mice homozygous for *Ptch1* null mutations die between 9 and 10.5 dpc during embryogenesis [Goodrich et al., 1997], too early to investigate a gonadal phenotype. However, similar to the null mutation of *Ptch1*, knockout of *Smo* in mice also results in the activation of the pathway. Conditional ablation of this gene in SF1-positive, somatic cells of embryonic mouse gonads resulted in the differentiation of functional fetal Leydig cells in the developing ovary [Barsoum et al., 2009], demonstrating that the HH pathway is not only necessary but also sufficient for Leydig cell differentiation.

Interestingly, in recent years it became obvious that in vertebrates a number of signaling pathways, and specifically hedgehog signaling, take place within the primary cilia of cells [Christensen et al., 2012; Clement et al., 2013; Oh and Katsanis, 2013; Mukhopadhyay and Rohatgi, 2014]. These microtubule-based organelles can be found on most vertebrate cell types [Satir and Christensen, 2007]. Primary cilia have sensory roles but, more importantly, also function as signaling centers on the cell surface. The formation, maintenance, and function of the primary cilium depends on intraflagellar transport (IFT) associated with 2 protein complexes, IFTA, which is required for retrograde (from tip to base) transport, and IFTB that is essential for anterograde (from base to tip) transport [Pedersen et al., 2008]. Defects in cilia formation due to mutations in IFT proteins lead to pleiotropic disorders in human collectively referred to as ciliopathies that display some aspects of mis-regulated HH signaling [Tobin and Beales, 2009; Toriello and Parisi, 2009]. Some of these syndromes such as the Bardet-Biedl syndrome are also often associated with hypoplastic testes [Green et al., 1989; Forsythe and Beales, 2013], which could be due to the role of HH signaling in Leydig cell differentiation. Accordingly, primary cilia are present on interstitial cells including Leydig and PM cells in mouse and human testis [Wainwright et al., 2014; Nygaard et al., 2015]. However, a hypomorphic mutation of the IFTA gene *Wdr19* (also called *Ift144*) did not display a Leydig cell phenotype but instead resulted in hyperplastic gonads in both XX and XY embryos due to an extension of the embryo trunk [Wainwright et al., 2014]. It would be important to investigate the gonadal phenotype of other mouse models of

ciliopathies [Norris and Grimes, 2012] to establish the function of these organelles and the role they play in HH signaling during gonad differentiation.

While HH signaling is required for Leydig cell differentiation, activation of the Notch pathway inhibits the recruitment of Leydig cells from undifferentiated progenitor cells (fig. 2) [Tang et al., 2008]. Mammals have 4 NOTCH receptors, NOTCH1–4, and 5 canonical NOTCH ligands, DLL1 (Delta like 1), 3 and 4 and JAG1 (Jagged 1) and 2, that are all structurally related, single-pass transmembrane proteins [Artavanis-Tsakonas et al., 1999; D'Souza et al., 2008]. Binding of the receptor to the ligand on a neighboring cell initiates proteolytic cleavage to release the intracellular domain, NOTCH-ICD, which translocates into the nucleus, binds to RBPJ (recombination signal binding protein for immunoglobulin kappa J region), and regulates downstream target genes such as members of the *Hes* (hairy and enhancer of split) family [De Strooper et al., 1999; Tani et al., 2001; Gordon et al., 2008; Tien et al., 2009]. In developing mouse testes, *Notch1* and *Notch4* are expressed in endothelial cells of the coelomic vessel [Brennan et al., 2002; Tang et al., 2008], whereas *Notch2* is expressed in Sertoli cells at early stages before the expression shifts to interstitial cells where it is co-expressed with *Notch3* from 13.5 dpc [Tang et al., 2008]. Inhibition of NOTCH signaling by blocking the proteolytic cleavage led to an increase in Leydig cell numbers, whereas constitutive activation reduced Leydig cell numbers [Tang et al., 2008], showing that NOTCH signaling restricts the number of cells that are recruited from progenitor cells to differentiate into Leydig cells (fig. 2).

In contrast to Leydig cells, which do not actively proliferate and increase in number through new recruitment from progenitor cells, Sertoli cells undergo 2 phases of proliferation; first, at the early stages to generate sufficient pre-Sertoli cells for testis determination and secondly to elongate the testis cords into highly coiled structures, which will eventually form the seminiferous tubules [Orth, 1982; Sharpe et al., 2003]. The first phase of proliferation is, as mentioned above, mainly driven by FGF9 [Willerton et al., 2004]. In contrast, proper Sertoli cell proliferation and testis cord expansion from 15.5 dpc depends on *Inhba* (inhibin beta-A), expressed and secreted from Leydig cells [Archambeault and Yao, 2010]. *Inhba* encodes activin β A, which is a member of the TGF (transforming growth factor) superfamily.

The TGF superfamily of growth factors comprises more than 30 ligands in mammals, including TGF β 1–3, BMPs (bone morphogenetic proteins), activin A and B, NODAL, GDFs (growth differentiation factors), and AMH (anti-

Müllerian hormone). The receptors in these signaling pathways are single-pass transmembrane proteins with an intracellular serine-threonine kinase domain. The ligands bind as homo- or heterodimers to their respective type II receptor, initiating complex formation with the type I receptor and activating the intracellular signal transduction by SMADs [Shi and Massague, 2003; Itman et al., 2006]. Receptor binding of TGF β isoforms, activin, and NODAL results in the phosphorylation SMAD2 and 3, while receptor binding of BMPs, GDFs, and AMH leads to phosphorylation of SMAD1, 5, and 8. These receptor-regulated SMADs then form a heterotrimeric complex with the common mediator SMAD4 and translocate into the nucleus to regulate gene expression by direct DNA binding or through the interaction with other transcription factors [Wakefield and Hill, 2013; Weiss and Attisano, 2013]. Irrespective of the binding ligand or receptor combinations used for signaling, SMAD phosphorylation, SMAD-complex formation, nuclear accumulation, and transcriptional regulation are central to effective signaling for all members within the TGF β superfamily of growth factors. SMAD nuclear accumulation and transcriptional activity is further regulated by post-translational modifications such as phosphorylation by ERK/MAPK, GSK3 β and CDKs, thus SMAD proteins serve as a possible mediator in the integration of several signaling pathways.

In addition to *Inhba*, also the founding members of the TGF superfamily, TGF β 1–3, are expressed in fetal testes with TGF β 1 expression in Sertoli cells, TGF β 2 in both Sertoli and Leydig cells, and TGF β 3 expressed in PM and germ cells [Memon et al., 2008]. While compensatory mechanisms between the TGF β ligands obscure the elucidation of a role for single members of this family in testis development, loss of the TGF β receptor TGFBR3 leads to abnormal testis cord structure and compromised fetal Leydig cell function, implicating this signaling pathway in testis differentiation [Sarraj et al., 2010]. This was further confirmed by blocking the 3 receptors ACVR1B (activin A receptor type 1B, also called ALK4), TGFBR1 (transforming growth factor beta receptor 1, also called ALK5), and ACVR1C (activin A receptor type 1C, also called ALK7) in ex vivo gonad culture using a potent inhibitor, thereby preventing testis cord formation [Miles et al., 2013].

Signaling Pathways Driving Ovarian Differentiation

In the absence of a functional SRY protein, *Sox9* expression is not upregulated and the ovarian program of gene expression will prevail. In the fetal ovary this is

mainly driven by one signaling pathway, the WNT/ β -catenin pathway activated by RSPO1 and WNT4, as well as the transcriptional regulator FOXL2.

Wnt4 and *Rspo1* are initially expressed in somatic cells of the genital ridges of both XX and XY individuals before being downregulated in the developing testis and maintained in the ovary from around 12.5 dpc in the mouse [Vainio et al., 1999; Parma et al., 2006]. Both WNT4 and RSPO1 signal through the canonical WNT pathway, causing the stabilization and nuclear translocation of β -catenin. Accordingly, ectopic expression of constitutively active β -catenin in the developing testis leads to male-to-female sex reversal [Maatouk et al., 2008]. In contrast, the deletion of either *Wnt4* or *Rspo1* only results in partial female-to-male sex reversal associated with the appearance of testis-specific vasculature and ectopic androgen-producing cells, as well as a reduction in germ cell number [Vainio et al., 1999; Jeays-Ward et al., 2003; Chassot et al., 2008; Tomizuka et al., 2008]. In the absence of WNT4, ovarian markers such as *Bmp2* and *Fst* (follicle-stimulating hormone receptor-like receptor), which encode TGF β superfamily ligands, are not expressed [Yao et al., 2004]. Indeed, FST acts downstream of *Wnt4* to inhibit the formation of testis-specific vasculature in the ovary while simultaneously maintaining the survival of germ cells [Yao et al., 2004]. Similar to the mouse phenotype, heterozygous WNT4 mutations in humans lead to reproductive phenotypes in females [Bisason-Laubert et al., 2004, 2007; Philibert et al., 2008, 2011], and XX patients with heterozygous mutation in *RSPO1* display female-to-male sex reversal associated with the skin disorder palmoplantar hyperkeratosis with squamous cell carcinoma [Parma et al., 2006].

Interestingly, there is a difference in the cause for the reduced germ cell numbers between *Wnt4* and *Rspo1*-null ovaries. The loss of *Rspo1* results in a decrease in germ cell proliferation, which is already evident at 12.5 dpc [Chassot et al., 2012]. In contrast in *Wnt4*-null ovaries, germ cell proliferation is unaffected but apoptosis is increased [Yao et al., 2004]. This indicates that these 2 signaling molecules, although functioning through the same mediator β -catenin, do not have completely overlapping functions during ovarian development.

A loss of germ cells was once thought to cause trans-differentiation of granulosa cells into Sertoli-like cells [Burgoyne et al., 1988; McLaren, 1991; Guigon and Magre, 2006]. Accordingly, gonads in the *Wnt4*- and *Rspo1*-null mice not only show a reduction in germ cells but also an upregulation of the Sertoli cell marker AMH at later stages during fetal development [Vainio et al., 1999; Chassot et al., 2008]. However, 2 lines of evidence

speak against this hypothesis. First, it has been demonstrated recently that a loss of germ cells is not sufficient to cause trans-differentiation of granulosa cells into Sertoli cells [Uhlenhaut et al., 2009]. And secondly, it appears that most of the AMH expression in the *Wnt4*- and *Rspo1*-mutant ovaries [Vainio et al., 1999; Chassot et al., 2008] is not a sign of granulosa cell trans-differentiation but rather precocious maturation, as demonstrated by the lack of other Sertoli cell markers such as DMRT1 and continuous expression of granulosa cell markers such as activated BMP signaling [Maatouk et al., 2013]. This premature differentiation of granulosa cells appears to be independent of the loss of germ cells; however, the presence of germ cells at earlier stages is required [Maatouk et al., 2013]. Only after birth do granulosa cells in these mutant ovaries start to express SOX9 and display signs of trans-differentiation [Maatouk et al., 2013].

Cross-Talk between the Testicular and Ovarian Pathway of Gene Expression

Gonadal identity is not, as believed for a long time, fixed to one sex once differentiation has taken place, but rather the sexual phenotype of cells must be actively maintained, while concurrently repressing that of the other sex. This ultimately results in a paradigm wherein genes and factors expressed in a testis antagonize those expressed in an ovary and vice versa in order to maintain gonadal identity.

Antagonistic relationships in the fetal gonad involve FGF9 signaling repressing the RSPO1/WNT/ β -catenin signaling pathway and vice versa [Kim et al., 2006]. Knockout of *Fgf9* or its receptor *Fgfr2* resulted in the upregulation of *Wnt4* expression and testis-to-ovary sex reversal [Colvin et al., 2001; Kim et al., 2006, 2007; Bagheri-Fam et al., 2008; Siggers et al., 2014]. Similarly, partial ovary-to-testis sex reversal was observed when *Wnt4* [Vainio et al., 1999; Jeays-Ward et al., 2003], *Rspo1* [Chassot et al., 2008; Tomizuka et al., 2008], or β -catenin [Manuylov et al., 2008; Liu et al., 2009] was ablated.

Interestingly, double knockout studies involving the combination of male- and female-determining pathways such as *Wnt4* and *Fgf9* or *Wnt4* and *Fgfr2* [Jameson et al., 2012], *Rspo1* and *Sox9* [Lavery et al., 2012], or *Ctnnb1* (β -catenin) and *Sox9* [Nicol and Yao, 2015] rescued the male-to-female sex reversal phenotype in XY gonads of the single knockout of *Sox9*, *Fgf9*, or *Fgfr2*. In contrast in XX gonads, the partial female-to-male sex reversal of the single knockout of *Wnt4*, *Rspo1*, or *Ctnnb1* was not res-

cued in the double-knockout gonads. Hence, both XY and XX gonads display testicular features, although XY were more masculinized than XX double knockout gonads. This suggests that the expression of testis-specific genes other than *Sox9* and *Fgf9* requires the repression of the β -catenin pathway rather than the direct activation by SOX9. A possible explanation lies in the redundancy that exists among SOX transcription factors. For example, SRY and SOX9 [Li et al., 2014], as well as SOX9, SOX8, and SOX10 [Schepers et al., 2003; Polanco et al., 2010] have been shown to activate similar target genes. Accordingly, the masculinization of *Sox9*/ β -catenin double knockout XY gonads was associated with the expression of *Sry* and *Sox8* [Nicol and Yao, 2015]. In contrast, for the expression of ovarian-specific genes the activation by the β -catenin pathway is absolutely essential.

Surprisingly, sexual identity of gonadal cells not only has to be sustained during fetal development but also postnatally. This maintenance of adult sexual identity is mediated by 2 transcription factors, testis-expressed DMRT1 (doublesex and mab-3 related transcription factor 1) and ovary-expressed FOXL2 in cooperation with ESR1 and 2 (oestrogen receptors 1 and 2) [Uhlenhaut et al., 2009; Matson et al., 2011; Minkina et al., 2014]. Inducible deletion of *Foxl2* in adult XX mouse ovaries leads to immediate upregulation of *Sox9* and, subsequently, reprogramming of granulosa and theca cells into Sertoli and Leydig-like cells [Uhlenhaut et al., 2009]. FOXL2 and ESR1 and 2 have been shown to directly bind to the gonad enhancer of *Sox9*, called TESCO, to repress *Sox9* transcription [Uhlenhaut et al., 2009]. Conversely, DMRT1 binds to a number of regulatory regions to repress ovarian-promoting genes and to activate testis-promoting genes [Minkina et al., 2014]. Hence, deletion of *Dmrt1* in adult testes results in the activation of *Foxl2* and the downregulation of *Sox9* expression, leading to trans-differentiation of Sertoli cells into granulosa-like cells [Matson et al., 2011].

Signaling Pathways Driving Germ Cell Development

Like the gonads themselves, the gametes of both males and females arise from a common precursor. PGCs arise as a result of inductive signaling from both the extraembryonic ectoderm and the visceral endoderm during gastrulation [Lawson et al., 1999]. As a result, PGCs are specified independently of the 3 germ layers at approximately 7.0 dpc in mice and during the third week of gestation in humans [Chiquoine, 1954; Ginsburg et al., 1990]. This

specification is induced by BMP4 and BMP8B, secreted by the extraembryonic ectoderm, and BMP2 produced by the visceral endoderm [Lawson et al., 1999; Ying et al., 2000; Ying and Zhao, 2001]. BMP4 is sufficient to induce PGC specification and as BMP2, although less efficient [Ohinata et al., 2009]. In contrast, BMP8B does not have a direct effect on PGCs but is necessary for PGC specification if the visceral endoderm is present [Ohinata et al., 2009], suggesting that it counteracts negative signals. BMPs function through the ALK2 receptor to induce phosphorylation and activation of SMAD1 and SMAD5 [De Sousa Lopes et al., 2004]. Accordingly, heterozygous mutations in any of the genes encoding these signaling molecules, i.e. *Bmp4*, *Bmp2*, *Bmp8b*, *Alk2*, *Smad1*, or *Smad5*, result in a reduced PGC number, while null mutations result in a complete loss of PGCs [Chang and Matzuk, 2001; Tremblay et al., 2001; Hayashi et al., 2002; Aubin et al., 2004; Chu et al., 2004; De Sousa Lopes et al., 2004; Yamaji et al., 2008; Ohinata et al., 2009]. Activation of the BMP signaling pathway results in the expression of 2 key genes, *Prdm1* (PR domain containing 1, also called *Blimp1* for B-lymphocyte-induced maturation protein 1) and *Prdm14* (PR domain-containing protein 14). PRDM1 is a potent transcriptional repressor of the somatic cell fate within PGCs, while *Prdm14* acts as a transcriptional regulator, critical for germ cell pluripotency and epigenetic reprogramming [Ohinata et al., 2005; Vincent et al., 2005; Yabuta et al., 2006; Kurimoto et al., 2008; Yamaji et al., 2008]. In addition to BMP, WNT signaling is also essential for germ cell fate at this time, with *Wnt3* mutants exhibiting a loss of both *Prdm1* and *14* expression [Ohinata et al., 2009].

Following specification, PGCs migrate, driven by morphogenic gradients, towards the developing gonadal ridges [Fujimoto et al., 1977]. These migrating germ cells express 2 FGF receptors, FGFR1 and FGFR2. Signaling through these receptors by FGF2 and FGF7 via the MAP kinase pathways affects germ cell motility and number [Takeuchi et al., 2005]. In addition, the WNT and BMP pathways have also been implicated in the migration of PGCs. *Wnt5a*-null embryos of both sexes exhibit a significant reduction in the number of primordial germ cells colonizing the gonads due to both abnormal germ cell migration and an increase in apoptosis [Laird et al., 2011; Chawengsaksophak et al., 2012]. Similarly, a reduction in BMP signaling via the ablation of the receptor BMPRI1A results in a reduction in migratory germ cells [Dudley et al., 2010].

Upon colonization of the gonads between 10.0 and 11.0 dpc, PGCs undergo rapid proliferation, which is

Fig. 3. Signaling pathways driving germ cell development. **A** During ovary differentiation, retinoic acid (RA, purple) is produced by ALDH1A2 in the mesonephros, diffuses into the ovary where it binds to its receptor RAR and induces expression of *Stra8* in ovarian germ cells, which is necessary for the entry into meiosis. **B** In contrast, during testis differentiation, while also the XY mesonephros produces RA, Sertoli cells express the RA-degrading enzyme CYP26B1, preventing germ cells to enter meiosis at this stage. Instead, germ cells exit the cell cycle and arrest in a quiescent state. Testicular germ cells are characterized by the expression of *Nanos2*, which is induced by 2 signaling pathways. FGF9 signaling within somatic (blue) and germ (green) cells leads to the upregulation of 2 TGF β superfamily members, *InhbA* and *Nodal*, respectively. ActivinA and NODAL then signal by binding the type II receptors ALK4 and ALK7 in conjunction with the co-receptor TDGF1, which initiates complex formation with ALK5. This ultimately results in phosphorylation of SMAD2, which binds to SMAD4 and translocates into the nucleus to activate *Nanos2* expression. Additionally, NODAL signaling leads to the upregulation of *Nodal* itself as well as *Lefty1* and 2, which act as dose-dependent feed-back inhibitors of the pathway.

transiently reliant on BMP7 signaling [Ross et al., 2007]. Further, upon entering the gonads, PGCs begin to express FGF4 and FGF8 which then act in an autocrine manner to maintain proliferation of the germ cells [Kawase et al., 2004]. This proliferation of PGCs continues until 13.5 dpc in mice and 11–12 weeks of gestation in humans, when a stark difference in the morphology of germ cells within the testis and ovary is observed. Germ cells fated to the male lineage enter mitotic arrest, while those destined to become oogonia enter the first meiotic prophase (fig. 3) [McLaren and Southee, 1997].

While the timing of germ cell entry into meiosis differs between the sexes, *Stra8* (stimulated by retinoic acid gene 8) is important in the transition from mitosis to meiosis in both sexes, first expressed in the embryonic ovary at approximately 12.5 dpc [Menke et al., 2003] and postnatally in the developing testis. Expression of *Stra8* is stimulated by the presence of an active derivative of vitamin A, retinoic acid (RA), catalyzed by the enzyme ALDH1A2 (aldehyde dehydrogenase family 1, subfamily A2) (fig. 3) [Bowles et al., 2006; Koubova et al., 2006]. Entry into meiosis differs slightly between species, with mouse germ cells

entering meiosis in an anterior-to-posterior wave due to RA production by the mesonephros [Menke et al., 2003; Bullejos and Koopman, 2004; Bowles et al., 2006; Koubova et al., 2006], while human germ cells enter meiosis radially, beginning in the medulla, due to RA production by the ovary itself [Childs et al., 2011]. While RA is also produced by the mesonephros adjacent to a mouse testis, expression of the RA-degrading enzyme CYP26B1 (cytochrome P450, family 26, subfamily b, polypeptide 1) by Sertoli cells prevents meiotic entry of germ cells in the embryonic testis (fig. 3) [Bowles et al., 2006; Koubova et al., 2006].

Following retinoid signaling, germ cells in an ovary progress through the stages of the first meiotic prophase before arresting at the diplotene stage [Borum, 1961; Speed, 1982]. Meiosis resumes upon maturation into primordial follicles, a process dependent on NOTCH signaling [Trombly et al., 2009; Vanorny et al., 2014], before being completed at the time of fertilization.

In contrast to germ cells in the developing ovary, germ cells in a testis exit the mitotic cell cycle at the G1 phase to remain in a quiescent state (G0) until shortly after birth when mitosis recommences before eventually entering meiosis [McLaren and Buehr, 1990; Western et al., 2008; Spiller et al., 2009]. A distinctive feature of testicular germ cells at this time is the expression of *Pou5f1* (POU domain, class 5, transcription factor 1, also called OCT3/4), which is otherwise downregulated upon entry into meiosis. TGF β signaling may regulate germ cell quiescence at this time in addition to its role as an anti-proliferative and anti-apoptotic factor for primordial germ cells [Godin and Wylie, 1991; Richards et al., 1999; Moreno et al., 2010]. In addition, inhibition of p38 MAP kinases resulted in an upregulation of the meiotic markers *Stra8* and *Sycp3* [Ewen et al., 2010], suggesting that this signaling pathway also plays a role. In support of this, MAPK pathways are activated by FGF9, which has been shown to promote the differentiation of PGCs towards spermatogenesis (fig. 3), while promoting survival of germ cells within the fetal testis independent of Sertoli cells [Colvin et al., 2001; DiNapoli et al., 2006]. This occurs as a result of the upregulation of *Nanos2*, a hallmark of testicular primordial germ cells [Barrios et al., 2009]. Loss of *Nanos2* in testicular germ cells leads to a complete loss of spermatogonia [Tsuda et al., 2003], while ectopic expression of the gene in ovarian germ cells results in male-like germ cell differentiation through the suppression of *Stra8* expression [Suzuki and Saga, 2008].

Induction of *Nanos2* expression by FGF9 occurs through the upregulation of 2 TGF β superfamily members: NODAL, produced by germ cells, and *Inhba*, pro-

duced by testicular somatic cells (fig. 3). NODAL signaling has been shown to be active in XY but not in XX PGCs at the time of sex determination [Souquet et al., 2012; Spiller et al., 2012] and signals by binding to activin receptors in the presence of the co-receptor TDGF1 (teratocarcinoma-derived growth factor 1, also called Cripto). Ultimately, this leads to upregulation of *Nodal* itself as well as upregulation of 2 other TGF β molecules, LEFTY (left right determination factor) 1 and 2, both of which act as dose-dependent feedback inhibitors of the pathway.

NODAL and INHBA work redundantly and have dual functions in promoting the male germ cell fate through the upregulation of *Nanos2* and suppression of meiosis. Additionally, they act to prevent differentiation of female somatic cells within the developing testis [Souquet et al., 2012; Wu et al., 2013]. Signaling within these pathways occurs through the receptors ALK4 and ALK7 [Miles et al., 2013] and the SMAD2 pathway to promote male differentiation in a RA-independent manner [Wu et al., 2015].

Conclusions

While a lot of work has gone into elucidating the role of transcription factors in driving the differentiation of specific cell types within the fetal testis and ovary, their activity is ultimately regulated by signal transduction pathways that coordinate the development of the gonads as a whole. Roles for many of these pathways in gonadogenesis have been identified through mutation analysis in human and mouse as well as inhibitor studies using ex vivo gonad culture approaches; however, we are far from completely understanding their function. Moreover, cross-talk is known to exist between different signal transduction cascades and, as such, the cumulative effects of these pathways may greatly alter the response to signal-dependent cues. Thus, while no such examples have been elucidated to date, it would be interesting to further analyze the integration and divergence of these pathways within the developing gonad to better understand the complex molecular interplay dictating gonadogenesis and, consequently, fertility.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

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