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Abstract

Primary sex determination is the decision by which the bipotential embryonic gonad commits to either the testicular or ovarian fate. The developing gonad constitutes a unique paradigm for the study of lineage specification, cell fate commitment and the exploration of how distinct cell populations diverge from multipotent progenitors. After the separation of the adreno-gonadal primordium into two distinct primordia, somatic progenitor cells of the gonadal primordium undergo several cell fate decisions and sex-specific cell differentiation. The specification of the supporting and steroidogenic cell lineages into either Sertoli and Leydig cells in the testis, or granu-

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losa and theca cells in the ovary is essential for germ cell development and endocrine function of the gonads. In this review, we focus on the early events leading to gonad formation, including the identity of gonadal progenitors, the genetic networks involved in cell lineage specification, the mutual antagonism of the pro-testis and pro-ovary networks and the importance of timing of developmental events orchestrating testis and ovary development. We discuss, and put into perspective, a number of experiments performed in mice or humans that have shed light on sex-determining mechanisms and, where possible, the clinical significance and limitations of such model organism data.

1. INTRODUCTION

Sexual reproduction is an evolved strategy to generate biological diversity and novelty at each generation and is of fundamental importance to all vertebrates and many invertebrates. At the core of this strategy is the generation of distinct sexes, with distinct reproductive organs, producing distinct gametes. Mammals use a chromosomal sex-determining mechanism: XY individuals develop testes; XX individuals form ovaries. While the gonads are germ cell factories they are also endocrine glands, shaping the development of the reproductive organs and ensuring sexual dimorphism and fertility. Thus, the development of gonads is crucial to sex identity. The process by which the embryo develops either a testis or an ovary is known as gonadal sex determination or primary sex determination. Once differentiated, the gonads produce sex hormones that promote development of sexually dimorphic structures that characterize distinctive male and female anatomy, a process also known as secondary sex determination.

The study of gonadal sex determination is a paradigm of developmental biology, based as it is on the sexually dimorphic development of a gonadal primordium, the genital ridge. This primordium is commonly described as "bipotential": whether it is found in an XY or XX embryo it has the capacity to develop into either a testis or an ovary. It is something of a dogma in the field of mammalian sex determination that most of the male and female gonadal cell-types arise from precursor cell lineages that are homologous, i.e., the Sertoli cells of the testis and the granulosa cells of the ovary arise from a single precursor lineage in this bipotential primordium. Recent studies in the mouse suggest that the story is more complicated than this (see Sections 4 and 5). Nevertheless, this framework allows us to characterize sex determination as a process that funda-

mentally concerns cell lineage commitment or cell fate determination. Questions that still fascinate developmental biologists include how an embryonic cell lineage can become increasingly specialized throughout development, giving rise to multiple daughter lineages, and how the final differentiated cell-types achieve a stability, i.e., how they maintain their differentiated state. Both these questions will be discussed in this review. The first question is increasingly being answered by careful study of transcription factors and signaling molecules required for sexually dimorphic gonad development and an analysis of the transcriptomes and epigenomes that such molecules regulate. Techniques for performing such analyses are becoming increasingly sophisticated and we will discuss these.

Sex determination raises some distinct questions: How is sexually dimorphic development of a bipotential embryonic primordium achieved? What is the relationship between the testis- and ovary-determining gene regulatory networks? What is the consequence of disrupting these networks by genetic mutation? The answers to these questions, mostly discovered over the last 25 years, are fascinating and sometimes unexpected. In this review, we will discuss the mutual antagonism that exists between the pro-testis and pro-ovary developmental pathways, the importance of timing of developmental events and why gonadal sex reversal is a relatively common phenotype when key genes are disrupted.

The significance of sex determination, however, goes beyond the insights its study offers into the role of genes in organogenesis and development more broadly. In humans, mutations in key sex-determining genes can give rise to a group of developmental abnormalities known as disorders (or differences) of sex development (DSD). Diagnosing and managing such conditions requires an understanding of human sex determination and the molecular mechanisms underpinning it. While human fetal material and human cells are increasingly used as tools for experimentation, the mouse has been the primary model for understanding mammalian sex determination. Here, focusing on events prior to overt differentiation of the gonads, we will discuss a number of experiments performed in mice which have shed light on sex-determining mechanisms and, where possible, the clinical significance of such model organism data.

2. BEGINNINGS: FORMATION OF THE ADRENO-GENITAL PRIMORDIUM (AGP)

Both the gonads and the adrenal cortex share a common developmental origin, the adreno-gonadal primordium (AGP) (see Fig. 1). In the mouse, the AGP is visible at around 9.5 days *post coitum* (dpc) as a thickening of the coelomic epithelium on the ventral surface of the mesonephros (Hatano, Takakusu, Nomura, & Morohashi, 1996; Ikeda, Shen, Ingraham, & Parker, 1994). It is composed of a population of uncharacterized progenitor cells expressing the orphan nuclear receptor steroidogenic factor 1 (SF1, also named NR5A1 or AD4BP) (Hatano et al., 1996; Morohashi, 1997) and primordial germ cells (PGCs). Germ cells arise at approximately 6.25 dpc in a cluster of 45 epiblast cells in the proximal region of the pre-gastrulation mouse embryo (Seki et al., 2005). These PGCs proliferate, relocate to the endoderm and are incorporated into the hindgut invagination. PGCs migrate anteriorly along the hindgut, through the dorsal mesentery, and ultimately colonize the AGP at around 10.0 dpc (Ginsburg, Snow, & McLaren, 1990; Molyneaux, Stallock, Schaible, & Wylie, 2001). By 10.5 dpc, the AGP separates to give rise to the adrenal and gonadal primordia (Morohashi, 1997). Together with PGCs, ingressing SF1-positive (SF1$^+$) cells along the rostro-caudal axis contribute to the formation of the gonadal primordium. In contrast, the more medial SF1$^+$ cells, located in the rostral region of the AGP, migrate and condense to form the adrenal primordium at the cranial end of the mesonephros. AGP specification and its separation into two distinct primordia, the gonadal and adrenal, remain poorly understood. What is known is that the transcription factors Wilms' tumor 1 protein (WT1) and SF1 play central roles in AGP precursor specification (Kreidberg et al., 1993; Luo, Ikeda, & Parker, 1994). While both WT1 and SF1 are expressed in the AGP, WT1 is switched off within the adrenal primordium soon after it separates, but remains expressed in the gonadal primordium (Vidal & Schedl, 2000).
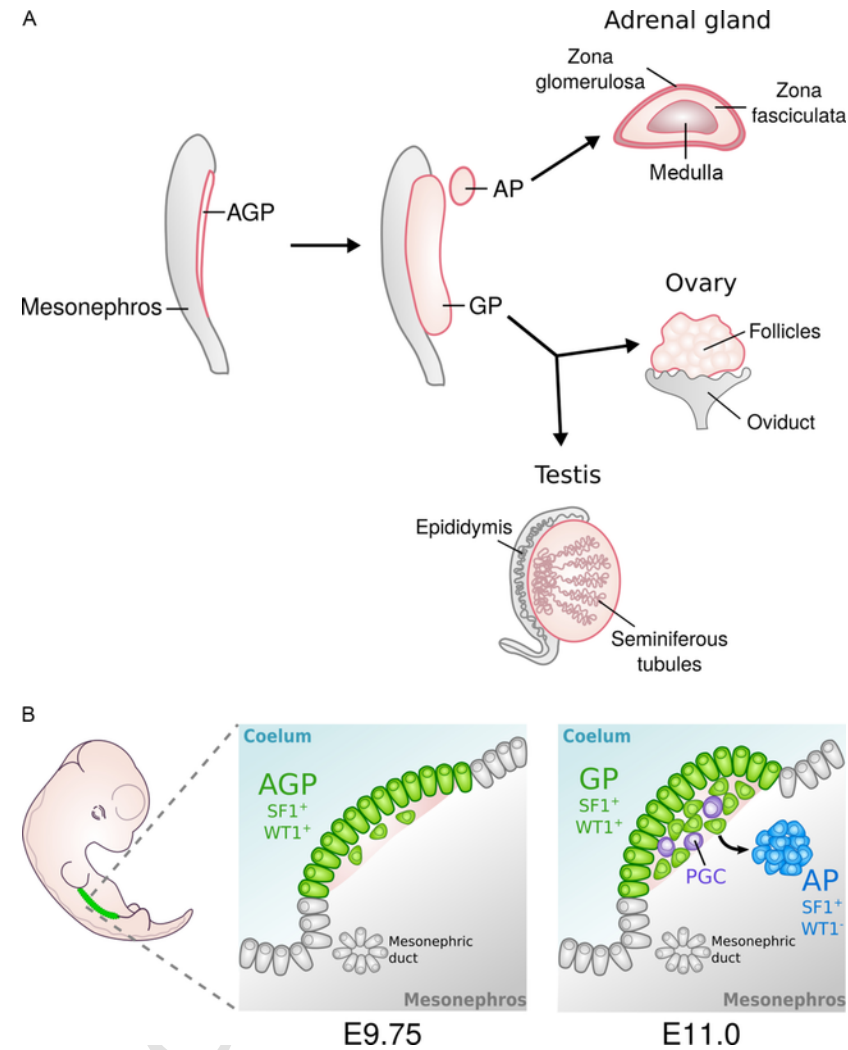
Fig. 1. Adrenogonadal primordium (AGP) development in the mouse. (A and B) Schematic representations of AGP differentiation into gonadal primordium (GP) and adrenal primordium (AP).

3. GENITAL RIDGE FORMATION

The gonadal primordium (also known as the genital ridge) first appears on the coelomic surface of the mesonephros at around 10.0 dpc in

the mouse or at gestational week 4 in the human embryo. The cells of the coelomic epithelium possess a unique molecular signature which includes the GATA zinc finger transcription factor GATA4 and WT1, as well as SF1. Formation of the genital ridge results from the proliferation of these coelomic epithelial cells, which then undergo epithelial-to-mesenchymal transition (EMT). By 11.5 dpc, the basement membrane beneath the coelomic epithelium is discontinuous, thereby permitting ingression or cell-oriented division of these progenitor cells into the gonad. Cell lineage tracing experiments using organ culture indicate that the coelomic epithelium is itself a source of supporting cell precursors for the male and female gonads (Karl & Capel, 1998). This developmental potential is, however, temporally regulated. Labeling of coelomic epithelial cells prior to 11.5 dpc in XX and XY gonads revealed subsequent ingression into the gonad; in XY gonads these cells contributed to the Sertoli cell population and interstitial lineages. Labeling after 11.5 dpc resulted in the incorporation of cells only into the interstitial compartment of the testis. This migratory potential ceased by 12.5 dpc. In the XX gonad, ingressing cells had no obvious fate restriction in the same developmental period. From these data, we can infer that at least some Sertoli cell progenitors arise in the coelomic epithelium. It is unclear whether there are other sources. Interestingly, deleting the Notch signaling inhibitors *Numb* and *Numbl* in the developing gonad from early stages disrupted the polarity of coelomic epithelial cells and eliminated or reduced cell numbers of multiple gonadal cell lineages, including supporting cells and Leydig cells, and led to clusters of undifferentiated cells within the gonad (Lin, Barske, DeFalco, & Capel, 2017). The paired-like homeobox protein EMX2 and the homeodomain proteins SIX1 and SIX4 also contribute to the regulation of EMT and subsequent ingression of gonadal progenitor cells. In the forming genital ridges of *Emx2*$^{-/-}$ mouse mutants, coelomic epithelial cells again lose their cell polarity and the number of migrating cells is reduced, resulting in impaired gonad formation (Kusaka et al., 2010). In the case of *Six1* and *Six4* double knockout mutants, genital ridges exhibit delayed/reduced EMT and subsequent ingression of progenitor cells, resulting in smaller gonads and adrenal glands (Fujimoto et al., 2013). Overall, these data suggest that asymmetrically-dividing coelomic epithelial cells are multipotent progenitors of gonadal somatic cell lineages.

SF1 is a critical factor for genital ridge formation and development. It acts in a dose-dependent manner by modulating the expression of target genes implicated in proliferation, survival and differentiation of gonadal progenitor cells. As a consequence, mouse embryos lacking one functional copy of *Sf1* exhibit underdeveloped adrenal glands and reduced cellular proliferation, while complete inactivation of *Sf1* leads to regression of the gonads by 12.5 dpc due to apoptosis of somatic cells and complete agenesis of the adrenal gland. In addition, impaired gonadal and adrenal formation is associated with downregulation or ectopic upregulation of *Sf1*, as is also the case for mice with targeted inactivation of *Gata4* (Hu, Okumura, & Page, 2013), *Wt1* (Kreidberg et al., 1993), the polycomb factor M33 (*Cbx2*) (Katoh-Fukui et al., 2005), the transcription co-factor *Cited2* (Val, Martinez-Barbera, & Swain, 2007), the homeodomain protein *Pbx1* (Schnabel, Selleri, & Cleary, 2003), the transcription factor Odd-skipped related 1 (*Odd1*) (Wang, Lan, Cho, Maltby, & Jiang, 2005), the LIM homeobox protein *Lhx9* (Birk et al., 2000) and the Insulin/IGF1R signaling pathway (Pitetti et al., 2013).

4. RECONSTRUCTING DEVELOPMENTAL LINEAGE TRAJECTORIES AND CELL FATE IN THE DEVELOPING GONAD BY SINGLE-CELL RNA SEQUENCING (SCRNA-SEQ)

A major goal in the field of sex determination, and more broadly in developmental biology, is to understand cell fate potential and the specification of progenitor cells. Lineage tracing is an important tool for following a group of cells and observing their descendants. There are a variety of complementary approaches available for in vivo lineage tracing (Spanjaard & Junker, 2017). Currently, the most popular approach for lineage tracing relies on genetic labeling of cells using a combination of inducible recombinases and fluorescent reporters, which provides exquisite control by enabling cell-type and stage-specific labeling of cells. While powerful, there are several limitations and technical challenges associated with the use of genetic labeling by means of the Cre/*lox* system. In particular, it requires prior knowledge of the progenitor cells (i.e., identification of cell-specific marker genes) and the availability of specific, inducible Cre lines that recapitulate the endogenous expression of these key markers. In addition, while this method provides data on the

cell fate potential of labeled cells, it gives no information about the intermediate cellular states, their molecular identity and the potential branch points along the route (Fletcher, Das, & Ngai, 2018).

Single-cell transcriptomics based on RNA sequencing (scRNA-seq) has emerged as a powerful technique for measuring gene expression in individual cells. Within a tissue or organ of interest, it allows the identification and classification of cell populations in a comprehensive and unbiased manner, a perspective that would be obscured in bulk level analysis. When performed in times-series, scRNA-seq data can be analyzed with lineage prediction tools to reconstruct developmental trajectories of cell lineages and characterize the transcriptional dynamics controlling their differentiation. This approach has been applied to the sex determination process and has provided new insights into the differentiation of the somatic progenitor cells during testis and ovarian differentiation (see Section 5).

5. SPECIFICATION OF THE SOMATIC LINEAGES IN THE DEVELOPING GONADS

A population of coelomic epithelial cells that expresses *Gata4* and *Sf1* is thought to give rise to the somatic lineages of the genital ridge. However, little is known about these early somatic progenitors. In particular, it is unclear whether there are distinct subclasses of progenitors, with discrete characteristics, sex differences and cell fate. The classical model of gonadal sex determination states that the bipotential gonad contains at least two pre-established somatic cell lineages: the supporting and the steroidogenic cell lineages. The supporting and steroidogenic progenitors will differentiate, respectively, into Sertoli cells and Leydig cells in the testis and granulosa cells and theca cells in the ovary (Svingen & Koopman, 2013). This model has been recently challenged with the report of Leydig-to-Sertoli cell trans-differentiation (Zhang et al., 2015) and the observation that both lineages derive from WT1$^+$ cells present at E10.5 in the genital ridge (Liu, Rodriguez, & Yao, 2016).

Recent studies taking advantage of scRNA-seq have provided new insights into these somatic progenitor cells and the fate decisions that lead to their differentiation into the supporting and steroidogenic cell lineages (for a review, see Stevant & Nef, 2018). scRNA-seq has shown great effectiveness in identifying the different cell types composing the develop-

ing gonads. It also helps to reconstruct the cell lineages and the expression dynamics controlling cell differentiation during the process of sex determination. scRNA-seq performed on XY and XY mouse gonads during sex determination identified a single multipotent *Sf1*^{+} progenitor cell population prior to the onset of sex determination (Stevant, Kuhne, et al., 2018; Stevant, Neirijnck, et al., 2018). These early somatic progenitors, present at 10.5 dpc, express genes related to epithelial stem cell identity and also negative regulators of cell differentiation, consistent with their coelomic epithelial origin. XX and XY early progenitors have a similar transcriptomic identity and do not exhibit obvious sexual dimorphism. Although the presence of additional rare populations of progenitor cells cannot be excluded, these findings support the "common progenitor identity" hypothesis and demonstrate the absence of a cell-lineage-specific fate prior to sex determination. Reconstruction of the fates and sex-specific differentiation of the gonadal *Sf1*^{+} cell lineage, based on these scRNA-seq studies, is depicted in Fig. 2.

It has been shown that supporting cell commitment and sex-specific differentiation into either Sertoli or granulosa cells occurs sequentially. Initially, both XY and XX multipotent progenitors that adopt a supporting cell fate share a similar transcriptomic identity, before initiating the sex-dependent genetic program leading to their differentiation into Sertoli and granulosa cells, respectively. Both Sertoli and granulosa cell differentiation are mediated by dynamic and highly complex transcriptional programs, including thousands of genes differentially expressed in granulosa and Sertoli cells between 10.5 and 16.5 dpc. Both genetic programs are complex, with sequential waves of activation or inactivation of genes, reflecting the process of Sertoli or granulosa cell commitment and differentiation. The narrow, transient expression profile of numerous genes suggests that they are required during a precise stage of cellular differentiation (e.g., *Nr0b1*, *Sry*), while prolonged expression profiles might reveal a role in maintenance of cell identity (e.g., *Sox9*, *Dmrt1*). However, XX and XY supporting cells initially share similar transcriptomic profiles and then bifurcate toward either the Sertoli or granulosa cell branch (Stevant, Kuhne, et al., 2018). This indicates that supporting cell commitment is initially sex-independent and disconnected from sex-specific differentiation into either Sertoli and granulosa cells.

In addition, scRNA-seq data reveal that XY interstitial cells and XX stromal cells are transcriptionally similar and evolve to express a set of
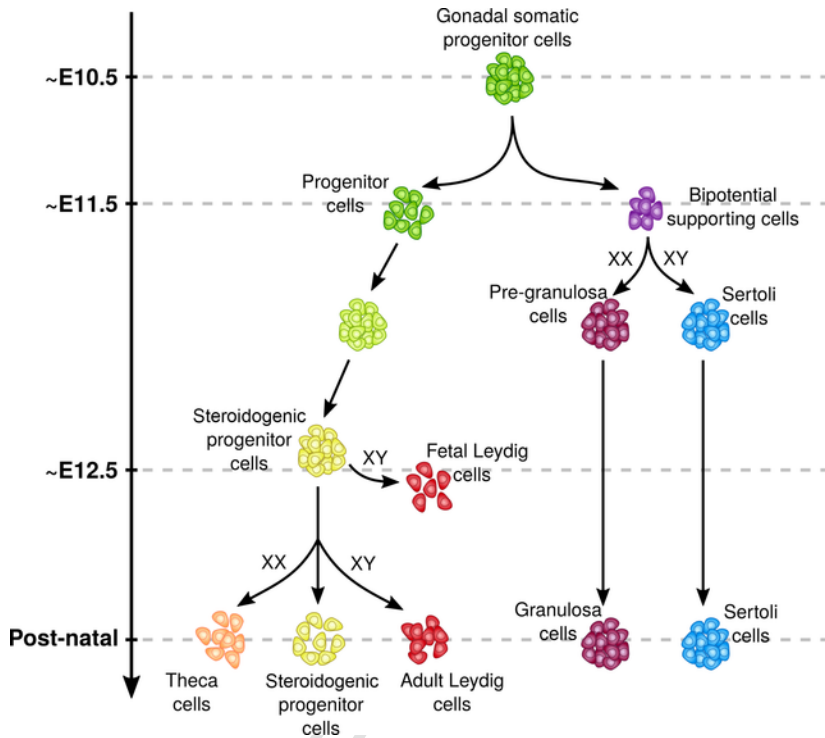
Fig. 2. Model of cell lineage specification during sex determination. (A) Single-cell RNA sequencing data identified a unique *Sf1*+ progenitor cell population at 10.5 dpc, at the origin of the supporting and steroidogenic lineages in XX and XY gonads. Around 11.5 dpc, a subset of these multipotent cells commit toward the supporting lineage. Rapidly, pre-Sertoli cells initiate the sex-specific genetic program leading toward Sertoli cell differentiation. In contrast, pre-granulosa cell differentiation into granulosa cells is delayed and progenitors remain in an early stage of differentiation. XX and XY supporting progenitors share similar transcriptomes prior to the sex-specific differentiation into either Sertoli or granulosa cells. Another subset of multipotent cells maintain their progenitor state and, by 12.5 dpc, undergo gradual transcriptional changes, acquiring a steroidogenic fate by progressively expressing *Pdgfra*, *Arx* or *Ptch1*. The transcriptomes of XX and XY steroidogenic precursors are similar and do not display strong sexual dimorphism.

genes that specify their fate as steroidogenic cell precursors, at the origin of both fetal Leydig cells and theca cells (Stevant, Kuhne, et al., 2018). In contrast to the highly dynamic program mediating granulosa and Sertoli cell differentiation, the remaining XX and XY progenitor cells that do not commit toward the supporting lineage display much less variation in gene expression and undergo progressive transcriptomic changes through time, switching from a multipotent identity to a steroidogenic

precursor identity. Initially, the XX and XY progenitor cells express multipotent markers such as *Wt1* and *Cbx2*. Then, starting at 11.5 dpc, the progenitor cells lose expression of these genes and gradually express markers of steroidogenic cell precursors such as *Wnt5a*, *Arx*, *Pdgfra*, *Gli2*, and *Tcf21*.

6. MOLECULAR CONTROL OF GONADAL CELL FATE CHOICE: GENE NETWORKS AND SIGNALING EVENTS

6.1. SRY and SOX9: Initiating testis determination

The Y-linked testis-determining gene, *SRY*, encodes an HMG box transcriptional regulator that initiates testis determination in the developing XY gonad by driving differentiation of supporting cell precursors into Sertoli cells. The control of expression of *Sry* has been studied in great detail in the mouse and it reveals a complex story of genetic and epigenetic regulation aimed at ensuring timely expression of *Sry*. The consequences of a delay to (or reduction in) *Sry* expression are discussed below.

6.1.1. Early events: Cell proliferation

It has been clear for many years that the number of somatic cells that have a Y chromosome, and so express *Sry*, in the developing XY gonad is an important determinant of gonadal fate. The injection of XY embryonic stem cells into an XX blastocyst can generate chimeric male mice, but only if sufficient numbers of XY cells are incorporated into the gonadal cell lineages (Burgoyne, Buehr, & McLaren, 1988; Patek et al., 1991). It is likely, therefore, that supporting cell precursors must be present in sufficient numbers to permit testis determination. What is known about the origin of the supporting cell lineage and the role played by cell proliferation in testis determination?

Transcriptome characterization by scRNA-Seq of *Sf1*⁺ cells at 10.5 dpc confirms the presence of proliferating, multipotent progenitors possessing an epithelial identity as might be expected for coelomic epithelial cells (Stevant, Neirijnck, et al., 2018). In particular, these cells significantly overexpress genes related to cell division, as well as *Fox* genes (*Foxc1*, *Foxc2* and *Foxd2*) essential for patterning of the intermediate

mesoderm (Kume, Deng, & Hogan, 2000) from which the testis develops, stem cell maintenance- and renewal-related genes (*Sall1*, *Sall4*) (Basta, Robbins, Kiefer, Dorsett, & Rauchman, 2014; Yang, Gao, Chai, & Ma, 2010), and *Cbx1* and *Cbx2*, necessary for *Sry* expression (Katoh-Fukui et al., 2012). Two genes known to repress miRNA activity and to be involved in stem cell proliferation are also detected: *Trim71* and *Lin28* (Rybak et al., 2009; Yang et al., 2015).

Given the role that the proliferative coelomic epithelium plays in the provision of supporting cell precursors (see part 3 above), is there any direct evidence for a role for cell proliferation in sex determination? The use of cell proliferation inhibitors, again in organ culture models, revealed an 8-h window, between approximately 10.8 and 11.2 dpc, that was critical for establishing expression of pro-testis genes and testis cord formation (Schmahl & Capel, 2003). Inhibition of cell proliferation either side of this window did not block testis development. This window overlaps with the developmental potential of coelomic epithelial cells to contribute to Sertoli cells (see above) and also with the onset of *Sry* expression. However, unlike male-specific cell proliferation in the coelomic region after 11.5 dpc, initiated by SRY (Schmahl, Eicher, Washburn, & Capel, 2000), there is no indication that these early events are sex-specific. Indeed, this absence of sex-specificity is predicted by the fact that XX embryos transgenic for *Sry* are (often) capable of initiating and completing testis determination (Koopman, Gubbay, Vivian, Goodfellow, & Lovell-Badge, 1991); this suggests that events required for *Sry* expression and subsequent testis determination can occur in an XX embryo. Thus, molecular and cellular events "upstream" of the initiation of Sry expression occur in both sexes—whether they result in testis determination depends on whether *Sry* is present or not.

6.1.2. Regulation of Sry expression

A number of studies have demonstrated that mouse *Sry* is expressed in a narrow temporal window between 10.5 and 12.5 dpc. RNAse protection (Hacker, Capel, Goodfellow, & Lovell-Badge, 1995), wholemount in situ hybridization (Bullejos & Koopman, 2001; Warr et al., 2012), qRT-PCR (Jeske, Bowles, Greenfield, & Koopman, 1995; Koopman, Münsterberg, Capel, Vivian, & Lovell-Badge, 1990) and immunodetection of expression from an epitope-tagged *Sry* transgene (Sekido, Bar, Narvaez, Penny, & Lovell-Badge, 2004) all indicate an onset at around 10.5 dpc, with

cells in the center of the gonad initiating expression. The basis of this initial spatial restriction is unclear, but it may relate to the source of supporting cell precursors and where they first enter the gonad. This early urogenital ridge *Sry* expression is germ cell-independent and is extinguished by 12.5 dpc.

Loss of *Sry* causes complete XY gonadal sex reversal of the fetal and adult gonad, i.e., XY female development (Lovell-Badge & Robertson, 1990). Thus, ensuring that *Sry* is expressed in the appropriate place and time is vital for the development of maleness. Loss of function genetic studies have revealed a role for a relatively large number of genes in the regulation of *Sry* expression (see Fig. 3). Not surprisingly, several transcription factors are implicated, along with signaling molecules and, more recently, chromatin modifiers. What have studies of these factors revealed about the regulation of *Sry*?

The insulin family of growth factors plays an essential role in mediating different aspects of adreno-gonadal development, such as adrenal specification, testicular differentiation, and ovarian development (Griffeth, Bianda, & Nef, 2014; Pitetti et al., 2013). The action of insulin, and the related growth factors insulin-like growth factor 1 (IGF1) and IGF2 is mediated by the insulin receptor (INSR) and the IGF type I receptor (IGF1R), two membrane-associated tyrosine kinase receptors. Mouse embryos lacking both *Insr* and *Igf1r* exhibit: (i) complete agenesis of the adrenal cortex; (ii) ovary development and a completely female phenotype in XY mutants and (iii) a delay in ovarian differentiation so that *Insr;Igf1r* mutant gonads, irrespective of genetic sex, remain in an extended undifferentiated state, before the ovarian differentiation program ultimately is initiated at around 16.5 dpc. The primary gonadal defect is due in part to the early failure in genital ridge expansion following reduced proliferation rates of somatic progenitor cells in both XX and XY gonads prior to sex determination. The absence of insulin/IGF signaling affects also the expression of transcription factors essential for adreno-gonadal development and function, such as *Wt1*, *Lhx9*, *Bmp2*, *Dax1* and *Sf1*, but also 50 additional genes defined as lineage-primed by Jameson, Natarajan, et al. (2012) prior to and/or at the time of sex determination. Together, reduced proliferation and premature alteration of the testicular and ovarian genetic programs in these cells could explain both adrenal agenesis and the incapacity of mutant gonads to develop into either ovaries or testes at the time of sex determination. The reduction of
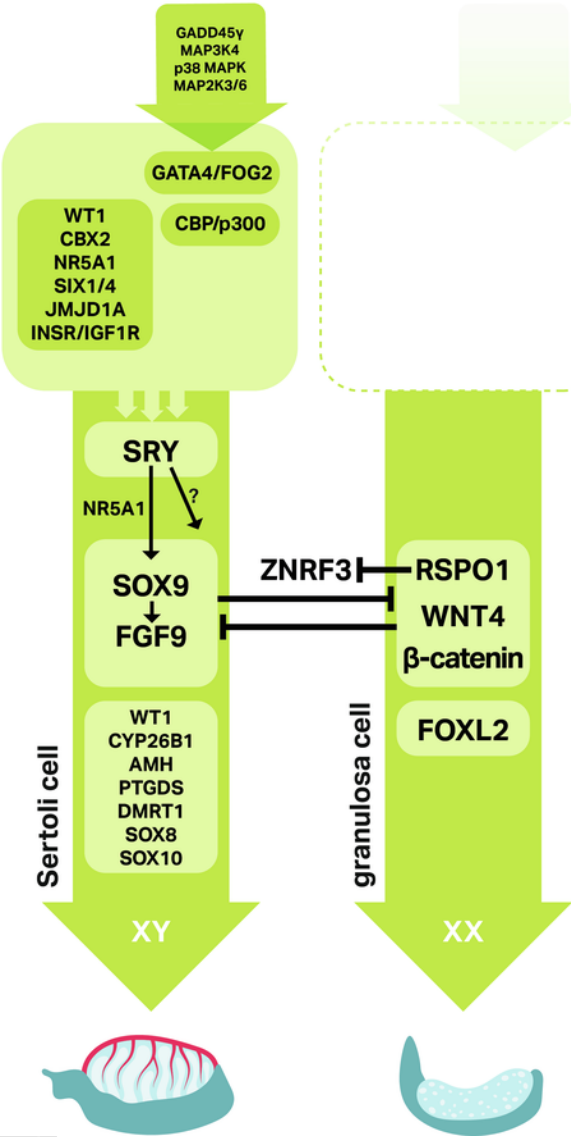
Fig. 3. Mutually antagonistic genetic networks regulating sex determination in mammals. A schema is shown that includes key (but not all) molecules that function in XY testis and XX ovary development. *Sry* expression is controlled by a number of transcription factors, signaling molecules and chromatin modifiers. These pro-*Sry* factors are also present in the XX gonad (dotted empty box), explaining the possibility of *Sry* transgene expression in XX gonads. SRY promotes *Sox9* expression (arrow), but it may have other targets. SOX9 drives Sertoli cell differentiation with the assistance of FGF9/FGFR2. The

SRY-SOX9-FGF9 pro-testis axis inhibits (hammered lines) the pro-ovarian network of RSPO1-WNT4/CTNNB1-FOXL2, which itself inhibits the male pathway and drives granulosa cell differentiation. RSPO1 directly inhibits the anti-WNT factor, ZNRF3, which sequesters Frizzled receptor. Other potential regulatory relationships are shown, with question marks where these are unclear or uncertain. The specification of supporting cell fate results in the commitment of other lineages to testicular or ovarian fates.

Sf1 expression (and *Gadd45g*—see below) and the failure of *Sry* upregulation support the idea that IGFs act upstream of SRY action and are important for regulating AGP development.

Recent investigations have revealed a signaling pathway that is required for normal expression of *Sry*: GADD45γ-MAP3K4-MAP2K3/ 6-p38 MAPK (Bogani et al., 2009; Gierl, Gruhn, von Seggern, Maltry, & Niehrs, 2012; Johnen et al., 2013; Warr et al., 2012, 2014; Warr, Siggers, Carre, Wells, & Greenfield, 2016). GADD45γ, a member of the GADD45 family that is implicated in active DNA demethylation, can activate MAP3K4 (Miyake, Takekawa, Ge, & Saito, 2007), a kinase at the top of a mitogen-activated protein kinase (MAPK) phospho-relay signal transduction system that can phosphorylate the transcription factor GATA4. Since GATA4 (and its cofactor FOG2) is known to be required for *Sry* expression, this phosphorylation is thought to be an important component in activating the onset of *Sry* transcription. One interesting observation about mutants lacking *Map3k4* or *Gadd45g* is that XY gonadal sex reversal is associated with a significant reduction in levels of *Sry* transcript at 11.5 dpc, but levels recover by around 12.0 dpc, indicating a delay in reaching peak expression. This delay is thought to result in sex reversal due to a failure to repress expression of pro-ovarian genes in the mutant XY gonadal somatic cells. Careful experimentation with an inducible *Sry* transgene indicates a critical window (between 11.0 and 11.25 dpc) for SRY to establish the male pathway during XY gonad development: any delay to *Sry* activation beyond 11.3 dpc caused a sex reversal phenotype (Hiramatsu et al., 2008). Below, we discuss the inhibitory interactions between the testis- and ovary-determining pathways in more detail.

The regulation of *Sry* transcription must ultimately be explained at the level of chromatin, by describing epigenomic events required at the locus. Such events create a chromatin context conducive to recruitment of transcription factors and co-factors. Interestingly, demethylation of several CpG dinucleotides in the vicinity of the proximal *Sry* promoter in XY gonadal somatic cells is one of the earliest events in testis determination, preceding *Sry* expression itself (Gierl et al., 2012). Again, mouse

knockout studies have also shed light on chromatin regulators required for normal *Sry* expression. The balanced activity of the histone demethylase KDM3A (JMJD1A), and the histone methyltransferase GLP/G9A, regulates levels of the inhibitory heterochromatin mark H3K9me2 at the *Sry* promoter, permitting accumulation of the positive mark, H3K4me3, and activation of *Sry* expression (Kuroki et al., 2013, 2017). Control of histone acetylation has also been shown to be required for normal *Sry* expression: loss of three out of four functional copies of the genes encoding the histone acetyltransferases CBP and p300 in gonadal somatic cells causes XY gonadal sex reversal, associated with loss of *Sry* expression and reduced levels of acetylated H3K27 at the *Sry* promoter (Carre et al., 2018). It is unclear why such commonly employed chromatin regulators as KDM3A and CBP/p300 are required for testis determination, but are apparently dispensable for fetal ovary development.

The upshot of *Sry*/SRY accumulation in supporting cell precursors of the XY gonad is the rapid up-regulation of another key testis-determining gene, *Sox9*, which also encodes an HMG box transcription factor. This up-regulation is mediated by the cooperation between SF1 and SRY and their occupancy of gonadal enhancers 5′ to the *Sox9* locus (Gonen et al., 2018; Gonen, Quinn, O'Neill, Koopman, & Lovell-Badge, 2017; Sekido & Lovell-Badge, 2008). SOX9 then drives Sertoli cell differentiation by its regulation of numerous target genes (Rahmoun et al., 2017). Loss of *Sox9* also results in complete gonadal sex reversal (Barrionuevo et al., 2006). We now turn to the phenomenon of gonadal sex reversal and the relationship between the pro-testis and pro-ovary genetic networks.

6.2. Testis determination: Opposing the ovarian genetic program

When genes functioning in testis determination, such as *Sry* and *Sox9*, are disrupted, gonadal sex reversal can occur. This might be complete, resulting in XY ovary development, or partial, causing ovotestis development (Wilhelm et al., 2009). But why? The explanation consists in the mutually antagonistic relationship between the testis- and ovary-determining gene networks: when one is disrupted, it fails to appropriately inhibit the other and that alternate pathway becomes dominant. In numerous cases of testis-determining gene knockouts, XY gonadal sex reversal occurs due to the ectopic activation of pro-ovary pathways of gene expression. One example of this phenomenon is offered by the analysis of

mouse knockouts for the fibroblast growth factor gene, *Fgf9*, and the canonical WNT signaling molecule, *Wnt4*. XY fetuses lacking FGF9 undergo complete gonadal sex reversal, when on the sensitized C57BL/6J background, and this is associated with activation of canonical WNT signals (Colvin, Green, Schmahl, Capel, & Ornitz, 2001; Kim et al., 2006). The corollary of this is that loss of *Wnt4* in the XX gonad results in partial masculinization and up-regulation of *Fgf9* (Kim et al., 2006). (In the case of knockouts for other pro-ovary genes, such as *Foxl2*, *Ctnnb1* (which encodes β-catenin) or *Rspo1*, XX gonadal sex reversal is also only partial and occurs postnatally: possible explanations for this will be addressed below.) Thus, FGF9 and WNT4 act in a mutually antagonistic fashion. Indeed, analysis of XY fetuses lacking both *Fgf9* and *Wnt4* reveals that testis development can occur in the absence of both, suggesting that the primary role of FGF9 is to oppose the anti-testis activity of WNT4 (Jameson, Lin, & Capel, 2012). Other pairwise deletions of testis- and ovary-determining genes, such as *Sox9/Rspo1* (Lavery et al., 2012) and *Sox9/Ctnnb1* (Nicol & Yao, 2015), also result in XY testicular differentiation, suggesting that testis determination can occur when anti-testis genes such as *Rspo1* and *Ctnnb1* are absent, even if key testis-determining genes are also missing. These data reveal the importance of the anti-ovary roles of testis-determining genes like *Fgf9* and *Sox9*. They also suggest that other pro-testis factors, such as other SOX proteins, can positively drive testis development in the absence of SRY and SOX9.

Mechanistic understanding of these mutually antagonistic interactions is sketchy. For example, it is not immediately obvious how two secreted signaling molecules, FGF9 and WNT4, might interact at the molecular level. One recent study reveals a key antagonistic molecular interaction in the sex-determining mechanism (Harris et al., 2018). As mentioned above, canonical WNT signals are an important determinant of ovarian fate. WNT4 activity results in the accumulation of nuclear β-catenin and the activation/inhibition of target genes (Bernard et al., 2012). RSPO1, another key ovarian determinant, is a secreted molecule known to potentiate canonical WNT signaling, and it does so through its negative impact on two widespread transmembrane E3 ubiquitin ligases, ZNRF3 and RNF43 (Hao et al., 2012; Koo et al., 2012). ZNRF3/RNF43, in turn, inhibit WNT signals by increasing membrane turnover of the WNT receptor, Frizzled (FZ). A study of XY fetuses lacking ZNRF3 reveals variable testis determination defects, ranging from complete gonadal sex reversal

to morphologically abnormal testis development, often within a single fetus (Harris et al., 2018). These defects are associated with ectopic canonical WNT signals at 11.5–12.5 dpc in the mutant XY gonad and disruption to *Sox9* expression. These data suggest a model in which high levels of RSPO1 in the XX gonad result in inhibition of ZNRF3, itself a negative regulator of ovary development. In the XY gonad, *Rspo1* expression is negligible (in contrast to the XX gonad) and, as a consequence, ZNRF3 can actively inhibit WNT4/FZ-mediated signals.

6.3. Ovary development: Granulosa cell heterogeneity, germ cells and XX gonadal sex reversal

In addition to WNT signals, the transcription factor FOXL2 plays an important role in the specification of ovarian granulosa cell fate (Auguste et al., 2011; Boulanger et al., 2014; Pannetier, Chassot, Chaboissier, & Pailhoux, 2016). However, recent studies suggest a cellular heterogeneity in precursors of the granulosa cell lineage (Mork et al., 2012; Rastetter et al., 2014; Zheng et al., 2014). Lineage tracing experiments indicate that FOXL2-positive somatic cells in early fetal gonads give rise to granulosa cells of ovarian medullary follicles, which are activated before puberty and contribute to early fertility. These cells are thought to be homologous to SRY-positive cells of the XY gonad and originate in the coelomic epithelium. LGR5, which is a receptor of RSPO1, marks another population of fetal ovarian somatic cells; these lack FOXL2 and give rise to cortical granulosa cells, which form the primordial follicles of the definitive ovarian reserve. Although distinct granulosa cell populations have been observed based on the mutually exclusive expression of FOXL2 and LGR5 (Rastetter et al., 2014), recent studies suggest that these two populations represent granulosa cells at different stages of differentiation. Both ovarian granulosa cells of medullary and cortical follicles are derived from a common precursor cell that expresses GATA4 (Gustin et al., 2016). These GATA4-positive precursors may undergo one of two differentiation processes: proliferation or entry into mitotic arrest. The first set of precursor cells directly contributes to medullary granulosa cells by differentiating at the onset of ovary development via maintained expression of the cell cycle inhibitor p27 and activation of FOXL2 expression. Around 15.5 dpc and postnatal day 4 (PND4), a second population of precursors contributes indirectly by initially establishing a proliferative pregranulosa cell pool regulated by WNT4-LGR5 and β-catenin

(Mork et al., 2012). Subsequently, these cells will differentiate in response to unknown signals that initiate the expression of p27 and FOXL2, thus contributing to the life-long cortical follicular reserve. It is unclear what are the factors and signals regulating the balance between WNT/β-catenin signaling, which promotes proliferation of supporting cells in the developing ovary and initiation of p27, and FOXL2 expression required for differentiation of granulosa cell precursors.

Nevertheless, it is remarkable to compare the temporal discordances between, on the one hand, early differentiation of Sertoli cells and the rapid formation of cords in the testis, with, on the other, the delay in formation of ovarian follicles and the late differentiation of granulosa cells of cortical follicles. Such temporal delays in specifying ovarian cell fate have been also observed when comparing the transcriptional changes occurring during supporting cell lineage differentiation in XX and XY gonads. Differentiation of both granulosa and Sertoli cells is mediated by a complex, highly dynamic program. Initially, XX and XY supporting cells share a common transcriptomic profile, which then bifurcates toward either the Sertoli or the granulosa branch. Differentially expressed genes between XX and XY supporting cells at each embryonic stage reveal that sexual dimorphism in XX supporting cells appears delayed compared to XY supporting cells (Stevant, Kuhne, et al., 2018).

For many years, it had been a dogma in the field of sex determination that, while germ cells are not required for testis determination, they do play a role in development of the ovary during folliculogenesis. Germ cells are critical for morphological development of the ovary at birth: clusters of germ cells break down and primordial follicles form. In addition, postnatal XX gonadal sex reversal in mutants such as *Wnt4* and *Rspo1*, involving somatic cell transdifferentiation to a SOX9-positive Sertoli cell fate, occurs after germ cell loss, suggesting a link between germ cells and inhibition of testicular somatic cell fate (Guigon & Magre, 2006). Determining whether germ cells play a role in the specification of somatic cell fate in the fetal ovary requires separating the effects of germ cell depletion from any independent effects of somatic cell disruption. Two recent studies have reported such an approach. Targeted ablation of germ cells in the developing XX gonad, using Cre-regulated diphtheria toxin expression, revealed that specification of granulosa cell fate, indicated by FOXL2 expression, was not affected by their absence (Uhlenhaut et al., 2009). In addition, early germ cell depletion using the chemotherapy drug busulfan, or genetic disruption using the *Kit*^{Wv} allele

that disrupts germ cell migration, did not disrupt the establishment or maintenance of granulosa cells based on a variety of molecular markers (Maatouk et al., 2012).

However, other data suggest that meiotic germ cells may still play a role in XX gonadal sex reversal. In XX gonads lacking *Rspo1* and *Wnt4*, in which perinatal partial sex reversal occurs, somatic cell transdifferentiation occurs when germ cells survive the pre-meiotic stage of development and die only after meiotic entry. This may indicate that granulosa cells acquire the competence to transdifferentiate only following contact with meiotic germ cells (Maatouk et al., 2012). Analysis of *Wnt4*- and *Rspo1*-deficient gonads indicates that prior to transdifferentiation, mutant pregranulosa cells exit mitotic arrest and undergo precocious differentiation (Maatouk, Mork, Chassot, Chaboissier, & Capel, 2013). Since depletion of germ cells in these mutants prior to meiosis prevents this exit from quiescence, it appears that germ cell-somatic cell interactions around the time of meiosis are normally important for maintenance of quiescence and these are disrupted in the absence of WNT4/RSPO1, leading to precocious differentiation and transdifferentiation. It has been suggested that *Sox9* up-regulation cannot occur in quiescent pregranulosa cells, even in the absence of WNT4 or RSPO1, and that this may account for the absence of fetal XX gonadal sex reversal in such cases. This could be tested by attempting an early disruption to mitotic arrest of pregranulosa cells in conjunction with deletion of *Wnt4/Rspo1/Foxl2* and assaying for fetal female-to-male gonadal sex reversal.

6.4. Comparisons between human and mouse sex determination

At the outset of any comparison of mouse and human sex determination it should be made clear that several key sex-determining genes have conserved roles in these two species. A number of *SRY* and *SOX9* mutations have been reported to disrupt human testis determination, resulting in 46,XY gonadal dysgenesis and female presentation (Berta et al., 1990; Wagner et al., 1994). *RSPO1* mutations have been described in very rare cases of 46,XX testicular disorder of sex development (DSD) (Parma et al., 2006). Even this modest description of gene conservation already suggests that human sex determination in the fetal gonad, at around 6 weeks, consists of SOX protein-driven testis determination and canonical WNT signal-driven ovary development, each opposing the other. These

observations suggest that the mouse is an excellent model for studying potential mechanisms of human sex determination.

There is genetic evidence supporting a role for a number of human genes in sex determination. However, DSDs that disrupt human sex determination are rare and there are often limited numbers of mutations available for functional studies. Moreover, such functional studies rely on heterologous cell line assays or mouse mutant phenotypes. Nevertheless, mutations in the following human genes have been reported in DSDs and also support the case for the similarity between mouse and human testis determination: *FOG2* (*ZFPM2*) (Bashamboo et al., 2014), *GATA4* (Lourenco et al., 2011), *CBX2* (Biason-Lauber, Konrad, Meyer, DeBeaufort, & Schoenle, 2009), *SF1* (*NR5A1*) (Allali et al., 2011; Bashamboo et al., 2016), *DMRT1* (Murphy et al., 2015), *FGFR2* (Bagheri-Fam et al., 2015), *WT1* (Barbaux et al., 1997), *DHH* (Canto, Soderlund, Reyes, & Mendez, 2004; Clark, Garland, & Russell, 2000), *HHAT* (Callier et al., 2014) and *ZNRF3* (Harris et al., 2018). A homozygous *WNT4* mutation is associated with 46,XX testicular DSD (Mandel et al., 2008), as are structural variants associated with the mis-regulation of *SOX9*, *SOX10* and *SOX3* (Benko et al., 2011; Polanco, Wilhelm, Davidson, Knight, & Koopman, 2010; Sutton et al., 2011).

Differences between mouse and human are also sometimes apparent, as one might expect. One interesting example involves the role of MAPK signals in testis determination. As discussed earlier, MAP3K4 is required for mouse testis determination (Bogani et al., 2009). However, mutations in *MAP3K1* are associated with 46,XY complete gonadal dysgenesis in humans (Granados et al., 2017; Pearlman et al., 2010), but loss of *Map3k1* does not disrupt mouse testis determination (Warr et al., 2011). The simplest explanation for this apparent discrepancy is that reported human *MAP3K1* mutations have gain-of-function effects, perhaps disrupting protein interactions required for testis determination (Loke et al., 2014). But inferring causality in an individual case of mutation is not without risk.

One other significant difference consists in the frequency of human sex reversal reported in individuals heterozygous for a mutation. In contrast, homozygosity for a mutant allele tends to be required for disruption to sex determination in mice. However, how these observations are interpreted requires some care. There are cases where mouse XY sex reversal occurs in the absence of one copy of a gene, such as in the case of *Fog2*

and *Gata4* (Bouma, Washburn, Albrecht, & Eicher, 2007), *Map3k4* (Warr et al., 2014) and *Znrf3* (Harris et al., 2018). In all these cases, sex reversal requires the highly sensitized B6.Y^{AKR} genetic background, suggesting that genetic background may be similarly important in cases of human disorders of sex development. Indeed, a growing body of evidence suggests an oligogenic basis for some DSDs (Camats, Fernandez-Cancio, Audi, Schaller, & Fluck, 2018; Mazen et al., 2016; Robevska et al., 2018).

7. FUTURE RESEARCH PERSPECTIVES

Sex determination is a complex developmental process by which the bipotential gonads develop as either testes or ovaries. Despite several decades of research revealing the delicate balance of pro-male and pro-female genes mediating sex determination, the majority of DSD cases related to gonadal development and differentiation remain undiagnosed at the genetic level (Bashamboo, Eozenou, Rojo, & McElreavey, 2017). The success rate of identifying causal variants through exome sequencing in patients with DSD is rather low compared to other genetic disorders, suggesting that a significant proportion of pathogenic variants and in/dels may be localized in intergenic regions, perhaps regulating the balance of expression of key sex-determining genes. In particular, regions such as enhancers, silencers and insulators are potentially attractive candidates, but the lack of knowledge regarding the locations of these crucial gene-regulatory elements limits our ability to study the regulatory mechanisms that control gene expression. We might reasonably expect that in the near future, with the advent of new, more sensitive genome-wide approaches, a precise characterization of the dynamics of gene expression during the process of sex determination will be available for each of the major cell-lineages of the gonad, both in the mouse model and human (Stevant & Nef, 2018; Stevant, Neirijnck, et al., 2018). In parallel, we are also starting to witness the emergence of genome-wide data describing the chromatin landscape in the major cell types of the gonad (Garcia-Moreno, Plebanek, & Capel, 2018; Maatouk et al., 2017). These genome-wide assays include DNaseI hypersensitive site sequencing (DNaseI-seq), ATAC-Seq, chromatin immunoprecipitation followed by sequencing (ChIP-seq) of histone modifications that mark promoters of active or silenced genes (H3K4me2/3, H3K27ac, H3K9ac, H4K16ac,

H3K27me3, H3K9me2/3) and chromosome conformation capture methods such as Hi-C, all of which will be instrumental in identifying active enhancer regions regulating the complex genetic network that underlies sex determination. Indeed, the observation that open regions of chromatin in developing Sertoli cells are detectable at gene loci expressed specifically in granulosa cells (Maatouk et al., 2017) suggests that such regions may have a dual use, being occupied by repressive or activating protein complexes depending on the sex of the gonad. Such sites may therefore mediate the mutual antagonism of the testis- and ovary-determining networks on a genome-wide scale.

Another major limitation in the field is the lack of an in vitro system to test whether a putative candidate gene or potential pathogenic variant is important for the process of sex determination. So far, the classic method of validation relies on in vivo loss- or gain-of-function experiments using mouse functional genomics. The generation of knock-out mice using traditional methods, or CRISPR/Cas9-mediated genome editing, is still expensive and time consuming. Unfortunately, there are no reliable somatic gonadal cell lines, such as gonadal somatic precursor cell lines, Sertoli or granulosa cell lines, although attempts have been made (Buganim et al., 2012). Here also, significant resources are currently being invested to develop culture protocols aimed at differentiating human or mouse iPS/ES cells toward the Sertoli or granulosa cell lineage, in order to derive stably reprogrammed cell lines. The development of such in vitro systems may be extremely important in testing and validating the potential pathogenic impact of DSD variants and also in establishing genome-wide or proteome-wide screens for novel genes and pathways that regulate gonadal lineage specification.

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