Trends in Genetics

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Review

Genetic Control of Gonadal Sex Determination and Development

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Sex determination is the process by which the bipotential gonads develop as either testes or ovaries. With two distinct potential outcomes, the gonadal primordium offers a unique model for the study of cell fate specification and how distinct cell populations diverge from multipotent progenitors. This review focuses on recent advances in our understanding of the genetic programs and epigenetic mechanisms that regulate gonadal sex determination and the regulation of cell fate commitment in the bipotential gonads. We rely primarily on mouse data to illuminate the complex and dynamic genetic programs controlling cell fate decision and sex-specific cell differentiation during gonadal formation and gonadal sex determination.

Introduction

A fundamental goal of developmental biology is to shed light on the mechanisms behind cell fate determination and the differentiation of progenitor cells into distinct cell types. In mammals, the testes and ovaries initially develop from bipotential primordia comprising progenitor cells that can differentiate into either testicular or ovarian cells. This process is called **gonadal sex determination** (see Glossary). This unique ability of the gonadal progenitor cells provides a valuable model to explore how gene regulation and the chromatin landscape regulate cell fate determination and sex-specific differentiation.

The decision to adopt an ovarian or testicular fate relies on robust genetic networks, as well as a delicate balance of expression levels for several protestis and pro-ovary factors. In particular, initiation of the male pathway in XY gonads is driven by activation of the testis pathway and simultaneous repression of the ovarian pathway, while the commitment of XX gonads toward the female fate mostly depends on the continuous activation of female-promoting genes [1]. Accumulating evidence suggests that **epigenetic** regulation also contributes to the bipotential state of the developing gonad and is a key element fine-tuning the expression levels of sex-determining genes.

In this review, we lay out our current understanding of the complex genetic programs mediating gonadal sex determination in mice, as well as their epigenetic control. We first focus on how the bipotential gonad and its progenitors are established prior to sex determination. We then review the dynamics of gene expression at play during sex fate choice and the establishment of sexual dimorphisms. Finally, we highlight how the process of gonadal sex determination is controlled by epigenetic mechanisms regulating gene expression during gonadal development.

Establishment of the Bipotential Gonad Prior to Sex Determination

In mammals, the **genital ridges**, or bipotential gonads, constitute the primordia from which ovaries and testes differentiate. In the mouse, they develop at around embryonic day E9.5 in a precise region of the ventral surface of the mesonephros (Figure 1A,B) and form paired narrow

Highlights

New technologies in molecular biology, such as single-cell RNA sequencing and epigenetic techniques, are emerging and being refined at a fast pace. They provide an opportunity to gain new insights into mechanisms driving the fate of progenitor cells and the transcriptional epigenetic dynamics underlying the process of sex determination.

A single multipotent progenitor cell population undergoes fate restriction and differentiates into either the supporting or the interstitial/stromal progenitors of the gonad. The supporting cells differentiate from these early progenitors in two sequential steps with first a commitment toward the supporting-cell lineage followed by sex-specific differentiation toward pregranulosa and Sertoli cells.

Accumulating evidence suggests that epigenetic mechanisms contribute to establish the male fate. The expression of the male determining gene *Sry* is regulated by histone modification and DNA methylation.

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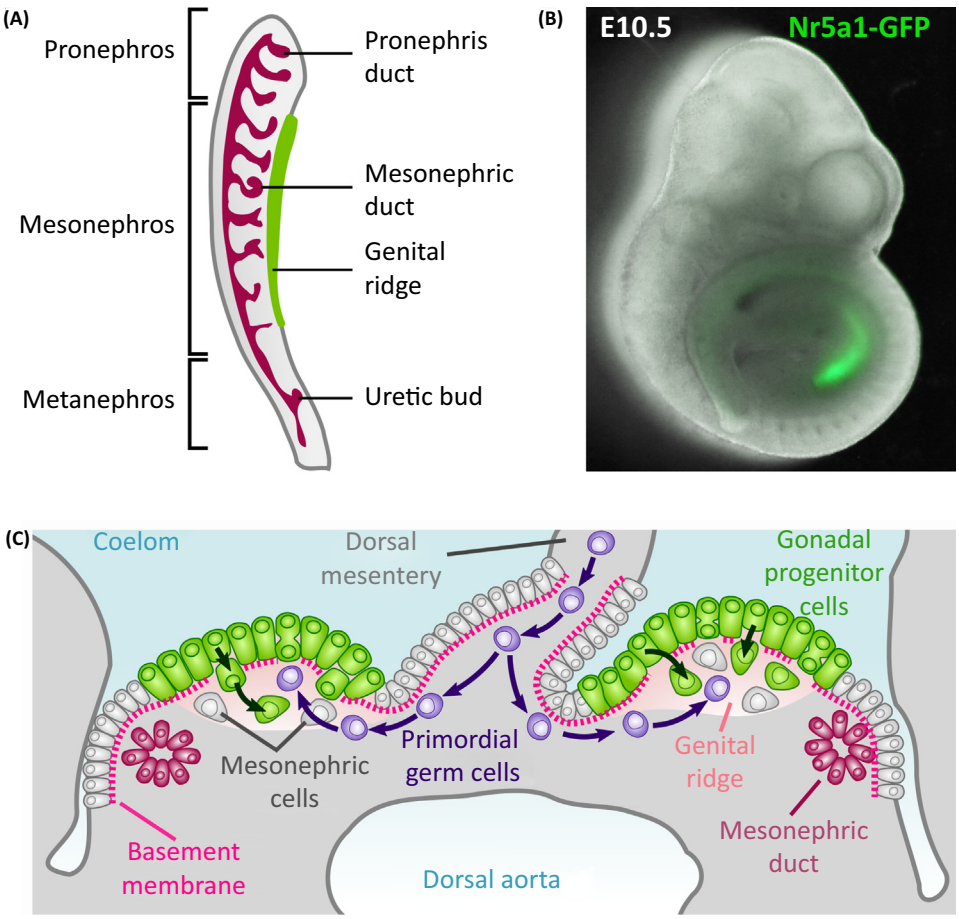

Figure 1. Gonad Formation. (A) Schematic representation of a urogenital ridge showing the pronephros, mesonephros, and metanephros. The gonads (in green) develop from the thickening of the coelomic epithelium (CE) on the ventromedial surface of the mesonephros. Adapted from [78]. (B) Whole Tg(Nr5a1-GFP) mouse embryo at E10.5 (merge of bright field and UV light). GFP fluorescence is localized in the somatic cells of the developing gonads. (C) Schematic representation of a transverse section of the genital ridge during gonadal formation. The growth of the genital ridges occurs by the proliferation of the CE (shown in green) and subsequently by the fragmentation of the underlying basement membrane (pink broken line), allowing the delamination of the proliferating CE into the inner mesenchymal region of the mesonephros. Concurrently with the thickening, the genital ridges are colonized by the migrating primordial germ cells (shown in purple). Adapted from [79].

bands of proliferating cells on either side of the dorsal mesentery (Figure 1C). The growth of the genital ridges occurs via proliferation of the **coelomic epithelium (CE)** and subsequently via fragmentation of the underlying basement membrane, allowing the delamination of the proliferating CE into the inner mesenchymal region of the mesonephros (Figure 1C) [2]. It is unclear whether the fragmentation is an active or a passive mechanism that is initiated by an increase of the number of cells. Thickening of the genital ridges starts at E10.3, following an anteroposterior axis (Figure 1B). Concurrently with the thickening, the genital ridges are colonized by the migrating primordial germ cells (Figure 1C) [3–7].

The precise molecular mechanisms that define the location of the gonads on the surface of the mesonephroi remain to be elucidated. One interesting lead might reside in a recent

Glossary

Assay for transposase-accessible chromatin using sequencing (ATAC-seq): a rapid and sensitive technique to assess genome-wide chromatin accessibility. It can help to identify accessible DNA regions equivalent to DNase I hypersensitive sites. This method probes DNA accessibility with hyperactive Tn5 transposase, which inserts sequencing adapter sequences into accessible regions.

ChIP-seq: identifies the genome-wide binding sites of DNA-associated proteins by combining chromatin immunoprecipitation (ChIP) with next-generation sequencing. In particular, a profile of the chromatin landscape in a particular cell type can be obtained from ChIP-seq for histone modifications indicative of active (H3K4me2/3, H3K27ac, H3K9ac, H4K16ac) or silenced (H3K27me3, H3K9me2/3) gene promoters.

Coelomic epithelium (CE): refers to the epithelium that lines the surface of the body wall and abdominal organs, including the outermost layer of the male and female gonads. Proliferation and delamination of these epithelial cells into the inner mesenchymal region of the mesonephros give rise to the somatic lineage precursors of the urogenital ridges.

Disorders of sex development (DSDs): congenital conditions associated with atypical development of chromosomal, gonadal, or anatomical sex. A small portion of these disorders affects gonad development and differentiation, including 46,XX testicular DSD and 46,XY DSD with partial or complete gonadal dysgenesis. Currently, the majority of DSD patients with gonadal defects do not receive a genetic diagnosis.

DNaseI-seq: refers to a technique for genome-wide sequencing of regions exhibiting DNase I hypersensitivity. DNase I hypersensitive sites are regions of chromatin where the genetic material is sensitive to cleavage by the endonuclease. They include promoters, enhancers, insulators, silencers, and other features related to transcriptional activity.

Epigenetics: the study of heritable changes in gene expression that do

study in chicken embryos, which suggests that Sonic Hedgehog signaling mediated by the cytokine bone morphogenetic protein 4 (BMP4) initiates gonadogenesis by establishing the dorsoventral patterning of the mesoderm and by inducing ingression of the coelomic cells [8]. Activation of Hedgehog signaling in the mesonephric capsule progenitor cells (epithelial cells in the flanked regions of the genital ridges) induces the ectopic formation of gonads. However, significant differences between mouse and chick gonadal morphogenesis [9] call for a similar study in mammals to establish whether this process is conserved among vertebrates.

Full development of the genital ridges requires the correct ingression and the proliferation of coelomic epithelial cells to establish the pool of gonadal somatic progenitor cells prior to gonadal sex determination. Many important factors involved in both ingression and cell proliferation have been identified by studying mutant mice (Table 1). To our knowledge, GATA4 is the earliest transcription factor expressed specifically in the genital ridges. GATA4 is present in the CE cells of the rostral part of the genital ridges as early as E10.0 and its expression extends along the rostrocaudal axis until E10.4 [2]. It is involved in the initiation of the formation of the genital ridges by controlling the basement membrane fragmentation and the CE cell proliferation. It also controls the expression of *Nr5a1* (also called *Sf1*) and *Lhx9*. Whether it does so in a direct or indirect manner remains unclear. These two critical genes are also expressed specifically in the gonadal progenitor cells [10–12] (Table 1) and their expression pattern follows *Gata4* expression with a slight delay. It is worth noting that no expression of these two genes is observed when the *Gata4* gene is deleted [2]. The origin and the establishment of the gonadal somatic cell lineages are not yet fully resolved. Experiments tracing cell lineage *in vitro* prior to gonadal sex determination have revealed that, in XX and XY gonads, the ingressing CE cells constitute the most important source of gonadal somatic cells in both sexes and contribute to the supporting cell and the steroidogenic cell lineages [13,14]. These results are corroborated by transcriptomic reconstruction of the gonadal CE cell differentiation trajectories that shows that both supporting and steroidogenic precursor cells derive from a common progenitor cell population [15,16]. This suggests that CE cells are multipotent and that their fate is controlled either by asymmetrical cell division driven by molecular determinants or by external signals from other cells in the gonads [13,17–19]. Recently, NUMB – an inhibitor of the Notch signaling – has been found to be involved in the polarity of the CE cells. NUMB is distributed asymmetrically in the daughter cells during cell division. Its disruption in XY bipotential gonads leads to the accumulation of undifferentiated cells as well as a reduction of Sertoli and Leydig cells [20]. While it is clear that the supporting cells derive from the CE, the steroidogenic cell lineage derives from multiple sources. Studies demonstrated that while the vast majority of the steroidogenic cell precursors reside in the gonads and might derive from the CE, a significant portion (~30%) originate from cells migrating from the mesonephros after gonadal sex determination [13,21–27]. This phenomenon has been observed in both sexes, suggesting that they probably derive from a common mesonephric steroidogenic precursor niche.

Despite significant efforts, details about the formation of the genital ridges and the characteristics of the somatic cells constituting the bipotential gonads remain poorly understood. This is mostly due to the lack of specific marker genes and reporters, the small size of the tissue, and the difficulty of accessing it. New, arising technologies, such as **single-cell RNA-seq (scRNA-seq)** and cyclic single-molecule fluorescence *in situ* hybridization [28] hold great potential for the study of cells from the whole mesonephric region during genital ridge formation, including the mesonephros, the mesonephric duct, and the genital ridges.

not involve changes in the DNA sequence of the genome. Epigenetic marks include histone protein modifications, such as methylation, acetylation, and phosphorylation, and DNA cytosine modifications, such as methylation.

Genital ridges: the precursors of the gonads; also known as bipotential gonads. They develop as paired thickening layers on the ventral surface of the mesonephroi at around embryonic day E9.5 in mouse embryos. The genital ridges form through the proliferation of the CE and the thickening of the underlying mesonephroi.

Gonadal sex determination: refers to the developmental decision that directs the bipotential gonad or genital ridge to develop into a testis or an ovary. Once differentiated, the gonads produce sex hormones that promote the development of sexually dimorphic structures that characterize male and female.

Sex-determining region of the Y chromosome (SRY): also known as testis-determining factor (TDS); a member of the SOX protein family of transcription factors. *Sry* acts as the master gene that initiates male sex determination in almost all mammals by upregulating *Sox9* expression and initiating the differentiation of supporting progenitors into Sertoli cells.

Single-cell RNA-seq (scRNA-seq): provides the transcriptional profile of individual cells. By profiling the transcriptome of thousands of individual cells, scRNA-seq provides an atlas of every cell type present in a particular tissue, including rare occurrences. In addition, when performed in time series it allows the reconstruction of cell developmental trajectories and provides transcriptional dynamics occurring during cell lineage commitment and differentiation.

Supporting progenitors: play a crucial role during the process of sex determination. In the presence of a Y chromosome carrying the male-determining gene *Sry*, progenitors from the supporting lineage will differentiate as pre-Sertoli cells and testis will form. In the presence of two X chromosomes, they will commit toward the granulosa fate and an ovary will develop. The term

Transcriptional Events Underlying Gonadal Sex Determination and Sex-Specific Cell Differentiation

Because gonadal sex determination is mostly a cell-autonomous process driven by antagonistic genetic programs, intensive efforts have been deployed to uncover the dynamics of gene expression at play during sex fate choice and the establishment of sexual dimorphisms. Time-series transcriptomic studies of XX and XY mouse gonads during sex determination were first conducted using microarrays technologies [1,29–34] and more recently using high-throughput techniques such as RNA-seq [35] and scRNA-seq [15,16] (Figure 2). Although evaluation of the dynamics of gene expression during gonadal sex determination is pitted with technical difficulties (Box 1 and Figure 3), these complementary studies contributed greatly to the understanding of the genetic programs driving gonadal somatic cell sex determination.

supporting cells refers to the role of the Sertoli and the granulosa cells in nursing and sustaining the development of the gametes.

Table 1. Critical Factors Involved in the Genital Ridges and Early Gonadal Development

Gene	Full name	Phenotype	Refs
Gata4	GATA-binding protein 4	Conditional knockout of *Gata4* induced at E8.75 impairs epithelial proliferation and basement membrane fragmentation; Gata4 controls the expression of *Nr5a1* and *Lhx9*	[2]
Nr5a1 (*Ad4BP*,*Sf1*)	Nuclear receptor subfamily 5, group A, member 1	Constitutive knockout of *Nr5a1* causes the degeneration of the gonadal ridge by apoptosis and complete absence of both the adrenal glands and the gonads	[10,11]
Wt1	Wilms' tumor 1	Constitutive induced mutation of *Wt1* leads to a disruption of urogenital development, in particular to the absence of gonads, caused by increased cell death	[48,80]
Lhx9	LIM homeobox 9	Constitutive *Lhx9* knockout mice show absence of gonads due to coelomic epithelial cell proliferation failure at E10.5	[12]
Emx2	Empty spiracles homeobox 2	Constitutive *Emx2* knockout mice display absence of gonads caused by impaired epithelial cell migration through the basement membrane, but adrenal gland development is not affected	[81]
Six1 and *Six4*	SIX homeobox 1 and 4	*Six1*/*Six4* double knockout results in smaller gonads with male-to-female sex reversal due to impaired *Sry* expression; *Six1*/*Six4* regulates *Nr5a1* and *Zfpm2*, a direct regulator of *Sry*	[82]
Insr and *Igf1r*	Insulin receptor and IGF receptor 1	*Insr* and *Igf1r* double knockout affects the expression of *Nr5a1* and reduces the proliferation rates of the somatic progenitor cells in both XX and XY prior to gonadal sex determination; mice exhibit male-to-female sex reversal and complete absence of adrenal glands	[83,84]
Numb	NUMB endocytic adaptor protein	Conditional knockout of *Numb* and *Numbl* at E8.25 using tamoxifen-inducible ROSA-CreER leads to disrupted cell polarity in the CE, reduce numbers of supporting and steroidogenic cells, and accumulation of undifferentiated cells in the developing gonad	[20]
Nrg1	Neuregulin 1	Conditional *Nrg1* knockout using WT1-Cre$^{Tg/+}$ results in a decrease of coelomic epithelial cells and a reduced number and delayed differentiation of Sertoli cells	[85]
Sry	Sex-determining region of the Y chromosome	Sry initiates a dramatic increase in somatic cell proliferation at the CE of XY gonads starting at E11.25 in two distinct stages; proliferation was observed initially largely in NR5A1-positive cells and contributed to the Sertoli cell population, and later in NR5A1-negative cells below the CE that did not give rise to Sertoli cells	[13,14,86]
Rspo1 and *Wnt4*	R-spondin 1 and Wingless-type MMTV integration site family, member 4	Constitutive knockout of *Wnt4* and *Rspo1* results in impaired proliferation of the cells of the CE in XY gonads leading to a reduced number of Sertoli cells and the formation of a hypoplastic testis	[87]

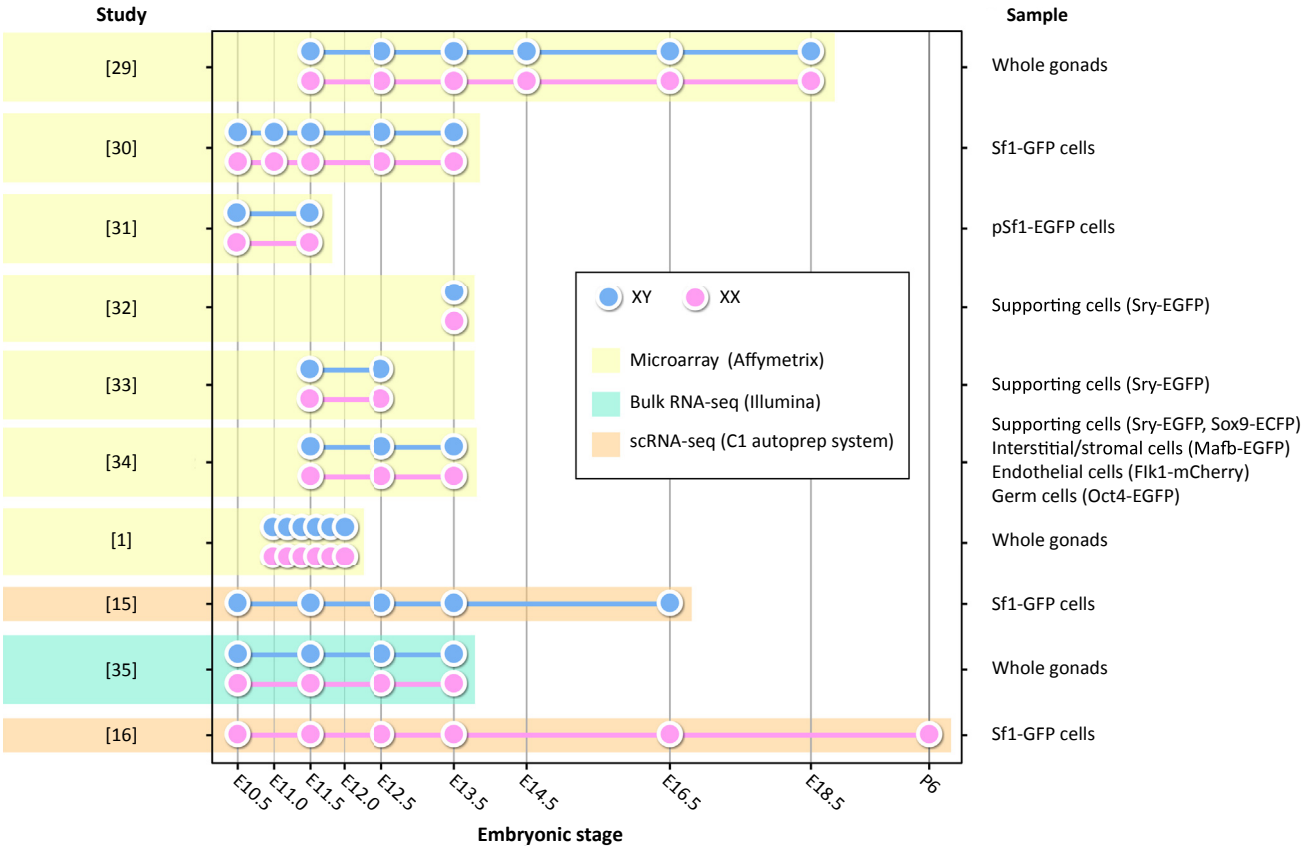

Figure 2. Description of the Existing Large-Scale Transcriptomic Studies of Mouse Gonadal Sex Determination. The blue and pink dots indicate the embryonic time points covered by the studies (in XY and XX gonads, respectively), while the pale-colored rectangles inform about the method employed [microarrays, RNA-seq or single-cell (sc) RNA-seq]. The input cells for each study are indicated on the right of the graph. See also [1,15,16,29–35].

Supporting-Cell Commitment and Differentiation

As the bipotential gonads form at E10.5, the gonadal cells do not display any sexual dimorphism at the transcriptomic level, with the exception of few genes located on the sex chromosomes [30,35]. The CE-derived somatic progenitor cells (expressing *Gata4* and *Sf1*) express epithelial-like stem cell related genes as well as proliferation genes and do not yet show any transcriptomic signature of a commitment toward the supporting or the steroidogenic fate [15,16]. Supporting-cell commitment and sex-specific differentiation occur sequentially, with an initial decision whereby XY and XX multipotent progenitors adopt a supporting-cell fate and share a similar transcriptomic identity followed by sex-specific differentiation into Sertoli and pregranulosa cells [15,16]. More precisely, around E11.0–E11.5 the commitment of the supporting-cell lineage from the multipotent somatic progenitor cells is mediated by a common genetic program comprising the rapid upregulation of hundreds of genes in both XX and XY [15,16]. This program poises the expression of pro-Sertoli (*Fgf9*, *Dmrt1*) and pregranulosa (*Wnt4*, *Runx1*, and *Dax1*) genes at the onset of supporting-cell commitment [1,16,34]. Moreover, this upregulation of genes also coincides with the activation of the **sex-determining region of the Y chromosome (*Sry*)** gene and its direct target SRY-related high mobility group (HMG) box 9 (*Sox9*) in XY supporting cells [15,16]. It is likely that the genetic program engaging

Box 1. Technical Challenges in Gonadal Sex Determination

The developing testis and ovary are complex organs comprising numerous cell lineages that evolve rapidly during the process of gonadal sex determination. The biggest challenges to the study of gonadal sex determination resides in the cell heterogeneity in the developing gonads, as well as in the asynchrony of cell differentiation at any given time point. Performing a classical transcriptomic analysis on the whole gonad can lead to averaging artifacts. Specific reporter genes or markers can be used to purify cell types prior to transcriptomic analysis; however, unknown cell populations are excluded from the analysis, and the specificity of the reporter has to be verified to avoid contamination. Moreover, the transcriptomic information obtained for each cell type still represents a mixture of asynchronous differentiating cells, masking the precise chronology of gene expression. Finally, the exogenous sources of gonadal cells should not be neglected as it has been recently shown that a significant proportion of fetal Leydig cells, peritubular myoid cells, endothelial cells, and pericytes are derived from migrating cells of the adjacent mesonephros [46]. In this particular context, scRNA-seq represents a technology of choice to explore the cellular landscape of the differentiating gonads and precisely reconstruct the transcriptomic events driving sex determination (Figure 3). The big advantage of performing RNA-seq in a tissue with nonsynchronous cells is that it allows the collection of snapshots of cells at different differentiation stages. Because cell differentiation is a continuous process, it is possible to order cells by looking at expression transitions of hundreds of genes from one state to another. Following this concept, time-series scRNA-seq in a nonsynchronous tissue represents *in silico* cell lineage tracing.

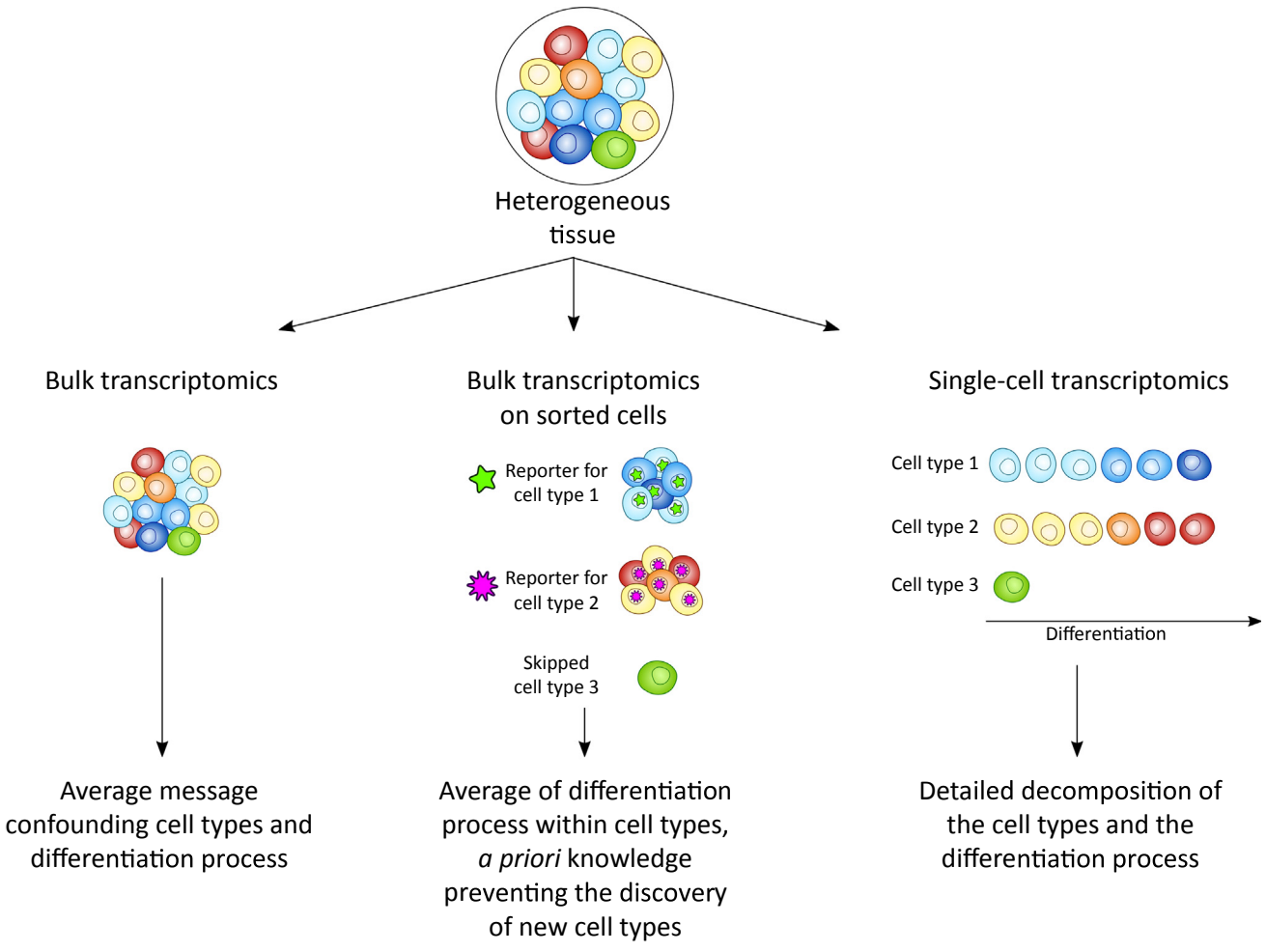

Figure 3. Comparison of the Information Obtained from Transcriptomic Studies Using a Bulk of Whole Gonadal Cells (Left), a Bulk of Sorted Cell Populations (Center), or Isolated Single Cells (Right).

the cells into the supporting-cell lineage also confers on them their bipotential properties. This is supported by the fact that transgenic expression of *Sry* in XX supporting cells in the same time window as the XY supporting cells is sufficient to drive Sertoli cell development [36,37]. By E12.5, the supporting-cell progenitors have adopted their sex-respective identity by engaging their differentiation as Sertoli cells in XY and pregranulosa cells in XX. The differentiation of the supporting cells into Sertoli or pregranulosa cells follows different courses. In XY, a massive wave of activation of male-specific genes, such as *Amh* and *Dhh*, is observed between E11.5 and E12.5, soon after *Sry* expression and upregulation of *Sox9*. In parallel, a large proportion of the genes that are upregulated at the time of the supporting-cell commitment is downregulated at this stage [1,15,16,34,35]. In XX supporting-cell progenitors, female-specific genes, such as *Foxl2*, are upregulated from E12.5 onward and less gene repression is observed compared with XY [16,34,35]. Further analysis determined that XX and XY bipotential **supporting progenitors** at E11.5 exhibit female-biased priming, suggesting that the natural progression of these progenitors is to adopt the female identity unless SRY activation induces the repression of the female program and promotes Sertoli cell differentiation [34]. Transcriptomic analysis of granulosa cells at a later embryonic stage (E13.5 and E16.5) and at an early postnatal day (P6) revealed that the differentiation of granulosa cells occurs over several days. This is in contrast to Sertoli cells, which differentiate within a 24-h period. Between E12.5 and E16.5, pregranulosa cells gradually upregulate female-specific genes but also progressively downregulate the same set of genes that is rapidly downregulated in pre-Sertoli cells between E11.5 and E12.5 [16]. After birth, the granulosa cells complete their differentiation as the process of folliculogenesis begins. In summary, the temporal differences in gene expression observed between XX and XY supporting-cell progenitors is likely to be due to a delay of cell differentiation in the pregranulosa cells compared with the Sertoli cells.

Interstitial/Stromal Cell Specification as Steroidogenic Progenitors

In parallel with the supporting-cell differentiation, the remaining CE-derived progenitor cells also undergo transcriptomic changes during gonadal development [15,16,34]. In early testis development, these progenitor cells are confined within the interstitial compartment, while the Sertoli cells enclose germ cells to form the testis cords. In the early developing ovary, no particular structures are yet observed, and the progenitor cells appear to be intermingled with the pregranulosa cells and the germ cells but are later found in the stromal compartment of the ovaries during folliculogenesis. The interstitial and the stromal compartments both comprise heterogeneous cell populations. They are thought to contain the major source of steroidogenic progenitor cells that differentiate as fetal Leydig cells from E12.5 in testes and theca cells during the first week postpartum in ovaries. They are also the source of the vasculature development from migrating mesonephric endothelial cells [18,38–40].

The study of the interstitial and stromal cells is challenging due to their heterogeneity and the lack of specific markers (Box 1). A first transcriptomic study attempted to isolate these cells with a Mafb-eGFP transgene [34]. They found that the interstitial and the stromal cells display gradual sexual dimorphism from E11.5 to E13.5, partly driven by the differentiation of fetal Leydig cells among the Mafb-eGFP positive cells [34]. More refined studies using scRNA-seq on Nr5a1-expressing cells [15,16] showed that the CE-derived interstitial/stromal progenitor cells gradually upregulate genes known as markers of steroidogenic precursor cells such as *Pdgfra*, *Arx*, and *Ptch1* [41–44]. XX stromal cell progression appears to be slightly delayed compared with that of the XY interstitial cells. Gradual transcriptomic sexual dimorphism is observed from E12.5, concomitantly with the differentiation of the supporting cells. However, the observed sexual dimorphism is driven by differences in the level of expression of genes rather than the expression of genes specific to one or the other sex. Unfortunately, the low

number of fetal Leydig cells, together with the absence of theca cells in the dataset, prevented study of the steroidogenic cell differentiation program. Whether the steroidogenic precursor cells migrating from the mesonephroi display a similar transcriptome progression remains unknown.

Alternative Splicing (AS) and Sex Determination

AS is a ubiquitous regulatory mechanism involving the selection of specific exons/introns to produce different transcripts from a single gene, thus expanding the complexity of the proteome [45,46]. While it is well established that AS plays a major role in the development of various organs and contributes to cell differentiation and lineage determination [47], the extent of AS occurring during the process of gonadal sex determination and its functional relevance remain unclear. Recently, an RNA-seq study performed in mouse fetal gonads during the process of sex determination detected widespread stage- and sex-specific regulation of transcript isoform usage during gonadal development [35]. Although it remains difficult to predict the exact molecular consequences of these differential splicing events, AS has been reported in genes known to have important functions in gonadal development. It includes the Wilms' tumor suppressor gene *Wt1* [48] and the FGF9 receptor fibroblast growth factor receptor 2 (*Fgfr2*) [49] as well as *Lef1*, a key mediator of the canonical WNT signaling pathway [50–55]. Overall, these findings suggest an important regulatory role of AS in sex determination and early gonadal development.

Evaluation of the dynamics of gene expression during gonadal sex determination allows us to better understand how sexual dimorphisms are established upstream from the gonadal morphological changes and revealed the complexity of the transcriptomic program at play during testicular and ovarian development. However, efforts still need to be deployed to elucidate the gene networks responsible for sex fate decision and in particular to understand the mechanisms of activation and repression of gene expression.

Mechanisms of Gene Expression Regulation during Gonadal Development

The transcriptome is a direct readout of the genetic program responsible for cell fate decision and differentiation, but it is also the result of many upstream events occurring at the DNA level to promote or prevent transcription. Transcription factors play key roles in the control of gene expression by binding on enhancer and promoter regions. The accessibility of the enhancer regions is an important mechanism of gene expression regulation. It involves 3D DNA conformation, chromatin modifications, and DNA demethylation. There is increasing evidence for the role of epigenetics and DNA regulatory elements in gonadal sex determination, starting with the activation of the testis-determining factor *Sry* itself [56].

Regulation of *Sry* Expression

The mechanisms controlling the precise spatiotemporal expression of *Sry* are not yet fully resolved [57]. Bisulfite sequencing highlighted two loci upstream of *Sry* that exhibit dynamic methylation status in a time period that coincides with *Sry* expression [58–60] (Figure 4A). The first region overlaps with the transcription start site (TSS) of the untranslated circular *Sry* RNA (region I), while the second overlap with the TSS of the translated *Sry* transcript that is expressed during gonadal sex determination (region II). These two regions exhibit CpG dinucleotide hypermethylation at E8.5, when *Sry* is not expressed. Around E11.5, both regions are hypomethylated in the gonad, while the hypermethylated status is maintained in other tissues. By E15.5, region I remains hypomethylated while region II is hypermethylated again [58,60]. These results suggest that DNA methylation might be responsible for the tissue specificity and temporal regulation of *Sry* by protecting *cis*-regulatory regions.

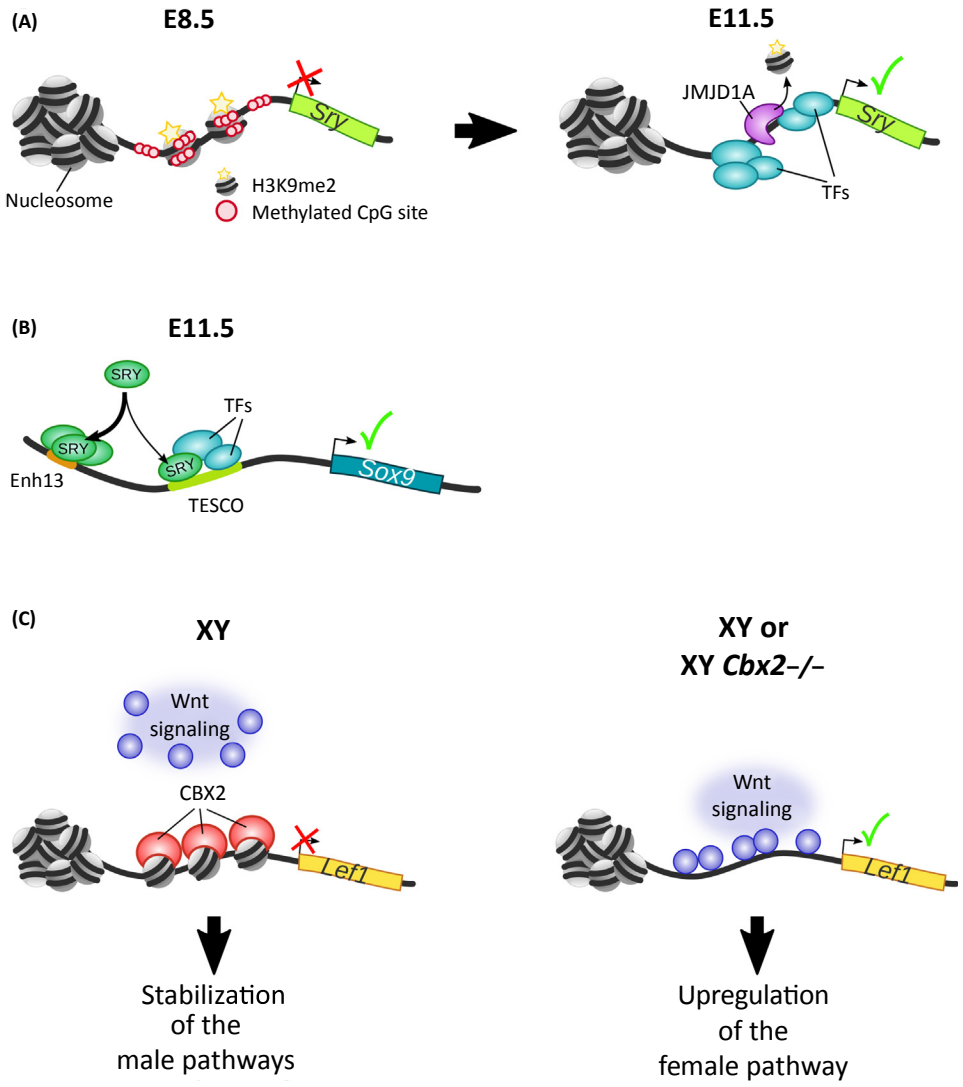

Figure 4. Epigenetic Regulation of Sex Determination. (A) Dynamic of the accessibility of the *cis*-regulatory regions controlling *Sry* expression. Prior to sex determination (E8.5), the *cis*-regulatory regions upstream to the *Sry* gene exhibit high DNA CpG methylation and dimethylated state of lysine residue 9 on histone H3, a histone modification associated with transcriptional repression. These marks prevent access to the region by transcription factors and thus inhibit *Sry* expression. Around E11.5, the same regulatory regions appear demethylated and histone H3 is demethylated by the action of JMJD1A. Thereby, transcription factors have access to the *cis*-regulatory regions and trigger the expression of *Sry* [61]. (B) Over-expression of *Sox9* in XY is controlled by the TESCO enhancer regions but also by the recently identified Enh13 region, which is preferentially bound by SRY. Deletion of Enh13 is sufficient to cause male-to-female sex reversal in XY mice [65]. (C) In XY mice, CBX2 prevents the activation of the *Lef1* gene via the Wnt pathway by acting as a protector of DNA compaction. This allows stabilization of the male pathway and assures normal testicular development. In XX and in XY mice lacking *Cbx2*, Wnt signalling activates *Lef1* gene expression, causing upregulation of the female pathway and the development of ovaries [74].

In addition to DNA demethylation, *Sry* expression requires the demethylation of dimethylated Lys9 on histone 3 (H3K9me2), a histone mark associated with transcriptional repression, by the histone demethylase JMJD1A (Figure 4A). Disruption of *Jmjd1a* results in frequent sex reversal due to accumulation of H3K9me2 around the *Sry* promoter, leading to lower expression [61].

Conjointly with the H3K9 demethylation, the *Sry* promoter displays enrichment of permissive histone marks, including H3 Lys4 trimethylation (H3K4me3) and H3 acetylation (H3ac) [60].

Together, DNA methylation and histone modifications are actively involved in the spatiotemporal expression of *Sry* by making the enhancers and the promoter accessible for the binding of multiple transcription factors (reviewed in [62]).

Sox9 Cis-Regulatory Regions

During male gonadal sex determination, SRY upregulates *Sox9* by targeting testis-specific enhancer of *Sox9* expression (TES), which includes the core element TESCO [63]. Deletion of TES and TESCO in XY mice reduces *Sox9* expression by ~50% without provoking sex reversal [64]. These results confirmed the important role of TES/TESCO to control *Sox9* expression levels in the testis and suggested the presence of additional enhancers that remained to be identified. A more recent study combined **assay for transposase-accessible chromatin using sequencing (ATAC-seq)** on XY and XX gonads at E10.5 and E13.5 with **ChIP-seq** for H3K27ac to screen for new putative *Sox9* enhancers. Sixteen candidate enhancer regions were screened using transgenic mice carrying the enhancer regions upstream to a *LacZ* reporter. Two of the 16 candidates showed testis-specific activity (Enh13 and Enh14). Deletion of Enh14 did not result in alteration of *Sox9* expression. However, the homozygous deletion of Enh13, a 557-bp element located 565 kb upstream from the *Sox9* TSS, resulted in complete XY male-to-female sex reversal (Figure 4B). The study also demonstrated that SRY preferentially binds to Enh13 rather than on TESCO, indicating that Enh13 is critical for the upregulation of *Sox9* whereas TESCO is responsible for the stabilization of *Sox9* upregulation [65].

Stabilization of Male and Female Fate

Sertoli cells and granulosa cells come from a common progenitor cell population and maintain the ability to transdifferentiate into their opposite-sex counterpart even after birth [66–70]. Although many transcription factors controlling the differentiation of the Sertoli cells have been identified, the way they act to control cell fate decision remain poorly understood.

The epigenetic regulator chromobox protein homolog 2 (CBX2) is part of the polycomb repressive complex 1 (PRC1), which binds to trimethylated Lys27 on histone 3 (H3K27me3) to maintain chromatin compaction and repress gene expression [71]. Disruption of *Cbx2* in XY mice embryos results in ovary development [72,73]. It was originally proposed that *Cbx2* acts as an activator of the male fate through indirect positive regulation of *Sry* [56,73]. However, recent findings suggest instead that *Cbx2* is required to stabilize the male fate by blocking the upregulation of bivalent female-determining genes [74]. By directly binding to *Lef1*, a Wnt downstream target in XY gonads, CBX2 inhibits Wnt signaling and promotes the stabilization of the male fate and the differentiation of the Sertoli cells. In XX E13.5 gonads, or in XY gonads that lack *Cbx2*, *Lef1* promotes the upregulation of the female pathway that antagonizes the male fate, allowing the differentiation of the pregranulosa cell (Figure 4C).

Two complementary studies recently monitored open chromatin regions (using **DNAseI-seq** and ATAC-seq) and histone modification indicative of active enhancers and promoters (ChIP-seq for H3K27ac) in purified XX and XY gonadal supporting cells before (E10.5) and after (E13.5 and E15.5) gonadal sex determination in mice [75,76]. At E10.5, the XX and XY progenitor cells have similar chromatin accessibility landscapes, consistent with their transcriptional state [1,30]. Differentiation of the supporting lineage into Sertoli or granulosa cells is accompanied by an increase in open chromatin regions that neighbor Sertoli- or granulosa-promoting genes, respectively. These open chromatin regions are enriched for transcription factor binding motifs

that are known to promote supporting-cell development. Interestingly, in Sertoli cells, granulosa-promoting genes display accessible but inactive regulatory regions (i.e., depleted in H3K27ac histone modification) that are enriched in binding motifs for DMRT1 and SOX9. This suggest that the progranulosa genes are repressed by Sertoli cell transcription factors immediately after gonadal sex determination to restrict the cell fate to the Sertoli type. These findings are consistent with expression data indicating that commitment to the male fate requires both upregulation of Sertoli-promoting genes and simultaneous repression of granulosa-promoting genes [1].

Until recently, most of our knowledge about the antagonistic genetic programs underlying gonadal sex determination was based on the observation of sex reversals induced by targeted knockout genes. The integration of genome-wide screening of chromatin state and transcription factor binding sites starts to uncover the regulatory networks that control the fine-tuned timing of gene expression observed during gonadal sex determination.

Concluding Remarks

Although we have limited the scope of this review to embryonic sex determination, it is now evident that the initial decision for gonads to differentiate into either testes or ovaries is not permanent and has to be actively maintained throughout life [68,70]. Disruption of the delicate balance of the genetic programs required to maintain cell identity in adult gonads results in a change of fate of somatic gonadal cell types, with Sertoli cells transdifferentiating into granulosa cells and vice versa. Unfortunately, there is only scarce information about the epigenetic and transcriptional changes driving adult transdifferentiation and how similar/divergent they are compared with the process of embryonic sex determination. With the emergence of scRNA-seq as well as other genome-wide techniques describing the chromatin landscape at single-cell resolution (e.g., single-cell ATAC-seq), it is now possible to reconstruct the transcriptomic programs and the dynamics of chromatin regulatory landscapes driving transdifferentiation of adult testis and ovarian cells.

It is likely that future progress in our understanding of the transcriptomic and epigenetic mechanisms regulating embryonic sex determination and adult testis and ovarian cell transdifferentiation will improve our capacity to identify causative variants in patients with **disorders of sex development (DSDs)**. Currently, the majority of DSD cases related to defects in gonadal development and differentiation do not receive a genetic diagnosis [77]. It is hypothesized that a significant proportion of pathogenic variants or in/dels may be localized in critical regulatory intergenic regions that are not covered by classical exome sequencing. We expect that a better understanding of the regulatory regions controlling the fine balance of expression of key sex-determining genes, in the mouse as well as in humans, will improve the success rate for the identification of causal variants in patients with DSDs.

Outstanding Questions

Ovaries and testes are two complex – and very different – organs that arise from a common primordium. A comprehensive atlas of all of the cell lineages and cell types originating or migrating in these two organs during both embryo and child development, and later in adult life, will be required to obtain a basic framework to understand the full gonadal development and function.

The exact epigenetic modifications and transcriptional events mediating cell lineage specification and sex-specific differentiation during the process of primary sex determination remain unidentified or unclear. What are the molecular mechanisms underlying the early stages of gonadal primordium establishment? How are the supporting and interstitial/stromal cell lineages defined at the onset of sex determination? Is sex fate decision maintained by constant and active repression by sexual antagonistic factors or by epigenetic modifications?

To what extent can genetic variation in intergenic regions such as promoters, enhancers, and silencers impact the fine-tuning of key sex-determining genes and explain undiagnosed DSD cases?

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