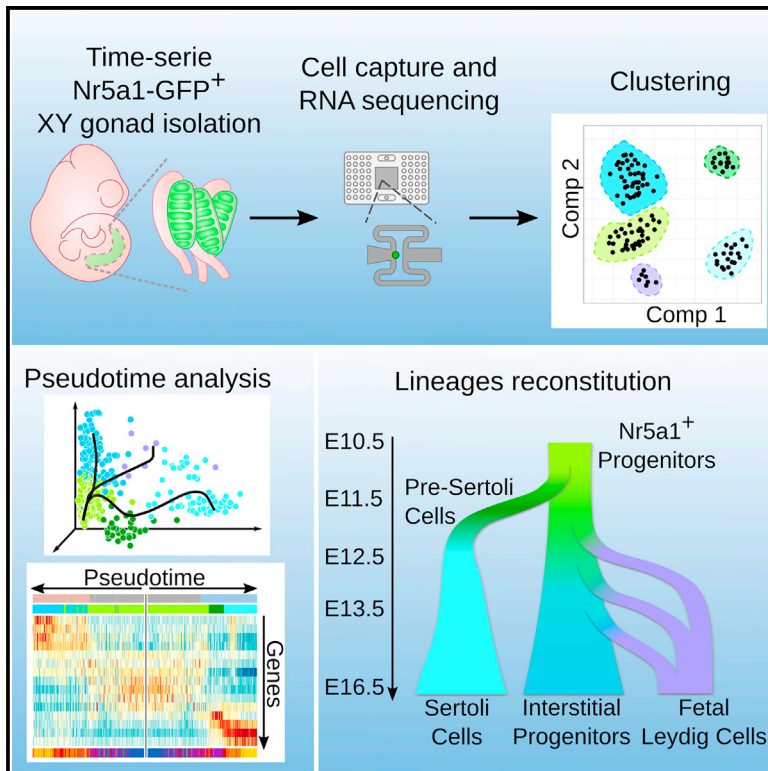


Cell Reports

Deciphering Cell Lineage Specification during Male Sex Determination with Single-Cell RNA Sequencing

Graphical Abstract



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In Brief

Using single-cell RNA sequencing of gonadal somatic cells during male sex determination, Stévant et al. identify a single Nr5a1-expressing progenitor cell population before sex determination that undergoes temporal fate specification with competence windows to differentiate first toward Sertoli cells or later to fetal Leydig cells.

Highlights

- A single Nr5a1⁺ progenitor cell population was detected prior sex determination
- Progenitors are able to give rise to first Sertoli and later to fetal Leydig cells
- Sertoli cell differentiation is characterized by a highly dynamic genetic program
- The remaining interstitial progenitors gradually acquire a steroidogenic fate

Data and Software Availability

GSE97519



Deciphering Cell Lineage Specification during Male Sex Determination with Single-Cell RNA Sequencing

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SUMMARY

The gonad is a unique biological system for studying cell-fate decisions. However, major questions remain regarding the identity of somatic progenitor cells and the transcriptional events driving cell differentiation. Using time-series single-cell RNA sequencing on XY mouse gonads during sex determination, we identified a single population of somatic progenitor cells prior to sex determination. A subset of these progenitors differentiates into Sertoli cells, a process characterized by a highly dynamic genetic program consisting of sequential waves of gene expression. Another subset of multipotent cells maintains their progenitor state but undergoes significant transcriptional changes restricting their competence toward a steroidogenic fate required for the differentiation of fetal Leydig cells. Our findings confirm the presence of a unique multipotent progenitor population in the gonadal primordium that gives rise to both supporting and interstitial lineages. These also provide the most granular analysis of the transcriptional events occurring during testicular cell-fate commitment.

INTRODUCTION

Testis development is a powerful model for the study of cell-lineage specification and sex-specific cell differentiation. Prior to sex determination, the gonadal ridge is composed of primordial germ cells and uncharacterized somatic precursor cells. These precursors primarily originate from the overlying coelomic epithelium (Svingen and Koopman, 2013) and communally express the transcription factors NR5A1 (nuclear receptor subfamily 5 group A member 1, also known as SF1 and Ad4BP) (Hatano et al., 1996; Luo et al., 1994), WT1 (Wilms' tumor suppressor 1), GATA4 (GATA transcription factor 4), LHX9 (Lim homeobox gene 9) (Birk et al., 2000; Mazaud et al., 2002), and NUMB (NUMB, en-

docytic adaptor protein) (Lin et al., 2017). During gonadal ridge formation, proliferating NR5A1⁺ cells delaminating from the coelomic epithelium are able to give rise to most of the somatic cells of the gonad, including both the supporting and the steroidogenic cells that differentiate as Sertoli and fetal Leydig cells, respectively, following the initiation of male sex determination (DeFalco et al., 2011; Karl and Capel, 1998; Schmahl et al., 2000). Sertoli cells are the first somatic cell type to differentiate around embryonic day (E)11.0 in the mouse (Albrecht and Eicher, 2001; Koopman et al., 1990) driven by the expression of the testis determining factor SRY (Sex-determining region of chromosome [Chr] Y) in the supporting cell lineage (Gubbay et al., 1990; Sinclair et al., 1990). Sertoli cells are essential to the coordination of testis development, forming the testis cords that encapsulate and direct germ cell differentiation, and orchestrating the differentiation of the other somatic cells, notably, the steroidogenic cell lineage at the origin of fetal Leydig cells, which develop from E12.5 (Habert et al., 2001). The increase in fetal Leydig cell number between E12.5 and E15.5 occurs through the recruitment and differentiation of interstitial Leydig progenitor cells rather than by mitotic division of differentiated fetal Leydig cells (Barsoum and Yao, 2010; Migrenne et al., 2001; Miyabayashi et al., 2013; Wen et al., 2016).

Whether Sertoli and fetal Leydig cells derive from a single or from two predefined precursor populations has been a matter of debate (Karl and Capel, 1998; Schmahl et al., 2000; Svingen and Koopman, 2013). Recently, it has been shown that disruption of coelomic epithelium cell polarity affects both steroidogenic and supporting lineage commitment (Lin et al., 2017). Moreover, lineage tracing and knockout studies unraveled Leydig-to-Sertoli trans-differentiation capacity (Zhang et al., 2015) and showed that both lineages derive from WT1⁺ cells present at E10.5 in the genital ridge (Liu et al., 2016).

A more complete understanding of lineage specification during testis development is critical for identifying the main regulators of normal testicular development and tissue homeostasis. However, a detailed characterization of cell heterogeneity of bipotential gonads prior to sex determination has been hampered by the absence of specific markers for precursor populations.



Existing time course transcriptomic assays using sample pools of purified testicular cells have provided valuable evidence of the dramatic changes occurring during cell differentiation (Jameson et al., 2012; Munger et al., 2013; Nef et al., 2005), but higher resolution is needed to evaluate cell-type heterogeneity and the precise dynamics of gene expression during cell-lineage specification.

In this study, we utilized single-cell RNA sequencing on NR5A1⁺ cells to perform an unsupervised reconstruction of somatic cell-lineage progression in the developing testis prior to, during, and after sex determination. At E10.5, in the bipotential gonads, we observed a single uncommitted somatic cell population. This multipotent progenitor cell population first gives rise to Sertoli cells around E11.5, which is driven by a rapid and highly dynamic differentiation program. Then, the remaining progenitors evolve transcriptionally to express steroidogenic precursor cell markers, defining the origin of Leydig cells.

RESULTS

Somatic Cell Purification and Single-Cell RNA Sequencing

To isolate and RNA-sequence individual somatic cells of the gonads prior, during, and after sex determination, we collected gonads from *Tg(Nr5a1-GFP)* male mice at five different developmental stages of testicular differentiation (E10.5, E11.5, E12.5, E13.5, and E16.5) (Nef et al., 2005; Pitetti et al., 2013; Stallings et al., 2002). *Nr5a1* is specifically expressed in the somatic cells of the developing gonads as early as E10.5 and later in Sertoli cells, fetal Leydig cells, and interstitial cells (Figures 1A and 1B; Figure S1).

The NR5A1-GFP⁺ cells from the genital ridges were sorted by fluorescence-activated cell sorting (FACS) and individually captured and processed with the Fluidigm C1 Autoprep System (Figure 1C; Figure S1). We prepared and sequenced 435 single-cell libraries, of which 400 passed the quality filters (Figure S2; Table S1; Supplemental Experimental Procedures). Cells expressed a total of 16,459 protein-coding genes (reads per kilobase per million mapped reads [RPKM] > 0), with a median of 5,628 genes per cell (Figure 1D). A strong correlation ($r = 0.82$, Spearman correlation) between the levels of *GFP* and endogenous *Nr5a1* transcripts confirmed the reliability of the *Tg(Nr5a1-GFP)* reporter (Figure 1E; Figure S3).

Single-Cell Transcriptomics Identifies Six Gonadal Somatic Cell Populations

To identify the different cell populations captured in our experiments, we selected the highly variable genes and performed hierarchical clustering on the significant principal components, and we visualized the cell clusters with t-SNE (t-distributed stochastic neighbor embedding; van der Maaten et al., 2008) (Supplemental Experimental Procedures). We obtained six cell clusters, each mixing different embryonic stages (Figures 2A and 2B). The dendrogram (Figure 2C) reveals the relative distances between the cell clusters. Cluster 1 (C1) is the most distant, and clusters 2 (C2) and 3 (C3) are the most similar. We found 2,802 differentially expressed genes (q value < 0.05) between the six cell clusters (Data S1). GO terms of the cluster

upregulated genes (Figure 2D; Data S2) associate C1 with angiogenesis, C2 and C3 with Wnt signaling and urogenital system development, cluster 4 (C4) with sex differentiation, cluster 5 (C5) with steroid metabolic process, and cluster 6 (C6) with cell cycle.

To identify cell clusters, we looked at the expression enrichment of known marker genes in the six cell clusters (Figure 2E; Figure S4; Data S3). C1 is composed of three cells from E11.5, E13.5, and E16.5 expressing endothelial cell-marker genes *Pecam1* (Platelet endothelial cell adhesion molecule), *Esam* (Endothelial cell adhesion molecule), and *Cdh5* (Cadherin 5) (Giannotta et al., 2013; Lampugnani et al., 1993; Nasdala et al., 2002) consistently with the associated GO terms. C2 contains 183 cells arising from E10.5 to E13.5. It is the only cell cluster containing the E10.5 cells and is highly similar to C3, which contains 106 cells from E12.5 to E16.5. C2 and C3 are enriched in genes specific to the interstitial progenitors (Figure S4). They both express progenitor-related genes such as *Nr2f2* (nuclear receptor subfamily 2, group F, member 2, also known as *Coup-TFII*), *Pdgfra* (Platelet-derived growth factor receptor alpha), and *Lhx9* (Brennan et al., 2003; Mazaud et al., 2002; Qin et al., 2008); therefore, we called C2 “early progenitors” and C3 “interstitial progenitors,” in accordance with both their embryonic stages and the marker gene expression. C4 is composed of 31 cells from E11.5 to E13.5 expressing pre-Sertoli cell markers *Sry*, *Nr0b1* (nuclear receptor subfamily 0, group B, member 1, also known as *Dax1*), and *Gadd45g* (growth arrest and DNA damage-inducible gamma) (Hanley et al., 2000; Kashimada and Koopman, 2010; Warr et al., 2012). C5 contains seven cells arising from E12.5, E13.5, and E16.5 expressing fetal Leydig cell markers *InsI3* (Insulin-like 3), *Hsd3b1* (Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1), and *Star* (Steroidogenic acute regulatory protein) (Ge et al., 2006; Nef and Parada, 2000). Finally, C6 is composed of 70 cells arising from E12.5 to E16.5 and is enriched in Sertoli cell marker genes *Sox9* (SRY-box 9), *Amh* (Anti-Müllerian hormone), and *Dhh* (Desert hedgehog) (Clark et al., 2000; Josso et al., 1977; Wilhelm et al., 2005).

Overall, with our single-cell RNA sequencing (RNA-seq) experiments on *Nr5a1-GFP*⁺ cells, we captured and identified six somatic cell populations in the developing testis. We detected a single progenitor cell population in the developing testis that remains identifiable from E10.5 until E13.5, the committed pre-Sertoli cells from E11.5, the differentiating Sertoli cells from E12.5, the interstitial progenitor cells from E12.5, and the fetal Leydig cells from E12.5. We also detected three endothelial cells, although they are not known to be *Nr5a1*⁺ cells.

Pseudotime Reconstruction Identifies Cell-Lineage Specification from the E10.5 Cells

To understand the lineage specification from the E10.5 progenitor cells and identify the mechanisms controlling cell-fate decision, we performed an *in silico* reconstruction of the cell lineages using diffusion map and ordered them along a pseudotime (Figures 3A and 3B; Supplemental Experimental Procedures). We obtained three cell lineages—the interstitial progenitor cell lineage, the Sertoli cell lineage, and the fetal Leydig cell lineage—in accordance to the previous cell clustering. These three

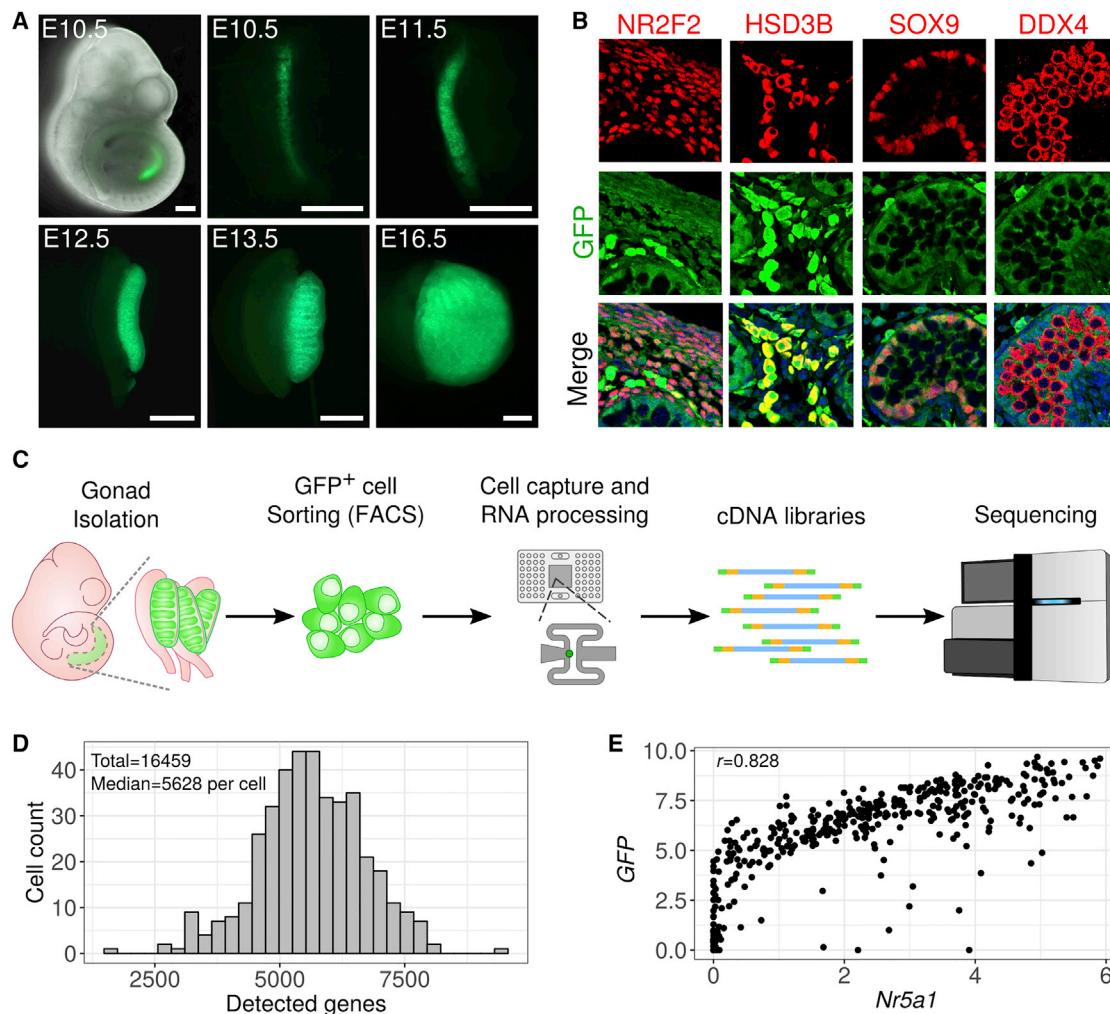


Figure 1. Experimental Protocol

(A) Images of E10.5 whole *Tg(Nr5a1-GFP)* mouse embryo (merge of bright-field and UV light) and XY gonads at five stages of development under UV light (scale bars, 500 μ m).

(B) Co-immunofluorescence of GFP and marker genes for interstitial progenitors (NR2F2), fetal Leydig cells (HSD3B), Sertoli cells (SOX9), and germ cells (DDX4) at E16.5. GFP co-localizes with the somatic cell markers but not with the germ cell marker.

(C) Experimental design. XY gonads at each stage were collected, *Nr5a1*-GFP⁺ cells were sorted by FACS, single-cell captured, and harvested cDNA was processed Nextera for libraries, and sequenced.

(D) Distribution of the number of detected genes per cell.

(E) Correlation between the expression of the *Nr5a1-GFP* transgene and the endogenous *Nr5a1* gene ($\log(\text{RPKM}+1)$).

lineages share a common origin starting from the E10.5 cells. No specific cell lineage was found for the endothelial cells which were assigned to the interstitial progenitor cell lineage.

To compare how the lineages acquire their identity, we selected cell-cluster-enriched genes (differentially expressed genes with q values $< 1e-5$) and looked at their expression dynamics along the predicted pseudotime from their common origin at E10.5 until E16.5 (Figure 3C). The small number of fetal Leydig cells prevents a high-resolution understanding of their differentiation process (data not shown). Figure 3C plots the divergence of early progenitors (from 0 pseudotime [vertical line at E10.5]) toward Sertoli (right) or interstitial (right) cell fate at E16.5.

The comparison of the interstitial progenitor and the Sertoli cell lineage (Figure 3C; Data S4) reveals that 45% (162 out of 357 genes; gene profiles G5, G6, and G7) of the interstitial-progenitor-enriched genes are also expressed in the common progenitor cells. GO terms associated with these genes are relative to urogenital system development and epithelium development (Data S5). Among these genes, we found regulators of the Wnt signaling pathway, such as *Gpc3* (Glypican 3) and *Sfrp1* (Secreted frizzled-related protein 1) (Figure 3D). *Gpc3* modulates insulin growth factor 2 (IGF2) interaction with its receptor and has been described as responsible for the X-linked recessive Simpson-Golabi-Behmel syndrome in human. Patients display genital abnormalities, including testicular dysplasia and cryptorchidism

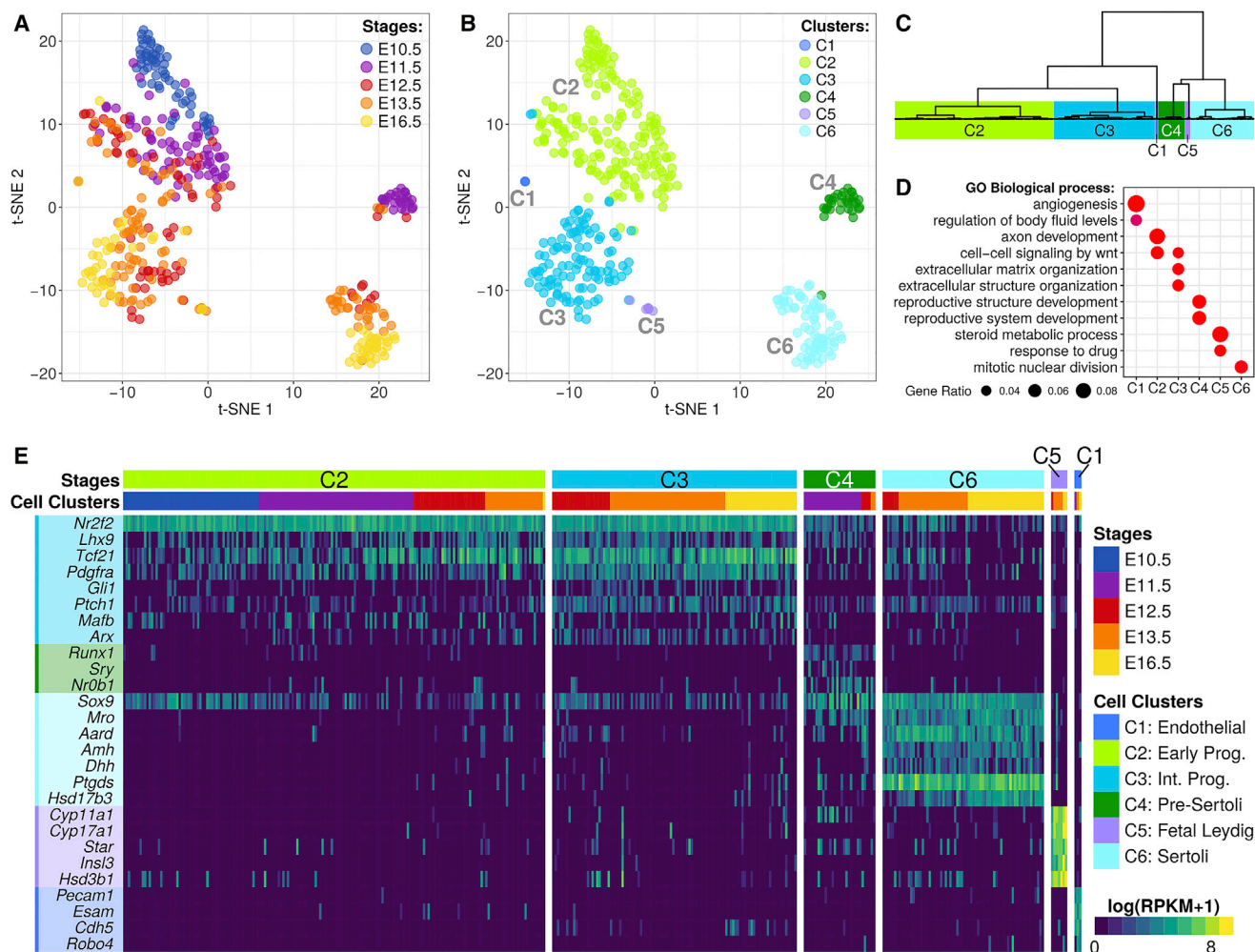


Figure 2. Clustering and Identification of the Cell Populations

(A and B) Two-dimensional t-SNE (t-distributed stochastic neighbor embedding) representation of 400 GFP⁺ single cells. Cells are colored by embryonic stages (A) and by cell clusters (B). The t-SNE representation well recapitulates the cell clusters defined by hierarchical clustering.

(C) Dendrogram resulting from the hierarchical clustering on the principal components shows the relative distances between the cell clusters.

(D) Top results from a Gene Ontology (GO) enrichment test showing the terms associated with the upregulated genes from each of the six cell clusters.

(E) Heatmap showing the expression of well-known marker genes of testicular somatic cell types in the previously computed cell clusters.

(Griffith et al., 2009). *Sfrp1* is a secreted negative regulator of the Wnt signaling pathway that has been identified as important for testis descent and normal testicular development (Warr et al., 2009). We also found *Pbx1* (Pre B cell leukemia homeobox 1), which has been previously described as being expressed in the interstitial compartment of the testis and is required for a normal urogenital differentiation (Schnabel et al., 2003). The other interstitial-progenitor-specific genes display a gradual increase in expression in time from E12.5 to E16.5 (P1 to P4).

In addition, 22% (Figure 3C; 75 out of 340 genes; gene profiles G11 and G12) of the Sertoli-cell-enriched genes are also expressed in the common progenitor cells. Among them, we found *Wt1* (Figure 3E), which is necessary for genital ridge formation and Sertoli cell differentiation and maintenance; *Bex4* (Brain expressed X-linked 4), which is expressed in Sertoli cells, but the function of which is poorly understood (Yu et al., 2017); and

Lsr (Lipolysis-stimulated lipoprotein receptor), of which the function is unknown in testis development. The other Sertoli cell-specific genes display a sharp and strong increase in expression at the exact moment of cell differentiation (G13 to G15).

The lineage reconstruction and the predicted pseudotime allow us to accurately describe the genetic program driving cell differentiation. With this analysis, we showed that the lineage specification of the interstitial progenitor cells display linear and smooth processes by slowly increasing the expression of their specific genes. In contrast, the Sertoli cell lineage differentiates from the common progenitor cells by downregulating the progenitor genes and rapidly upregulating Sertoli cell-specific genes.

Progenitor Cells Gradually Acquire a Steroidogenic Fate

To provide a deeper evaluation of the transcriptional changes of these progenitor cells from E10.5 to E16.5, we performed a

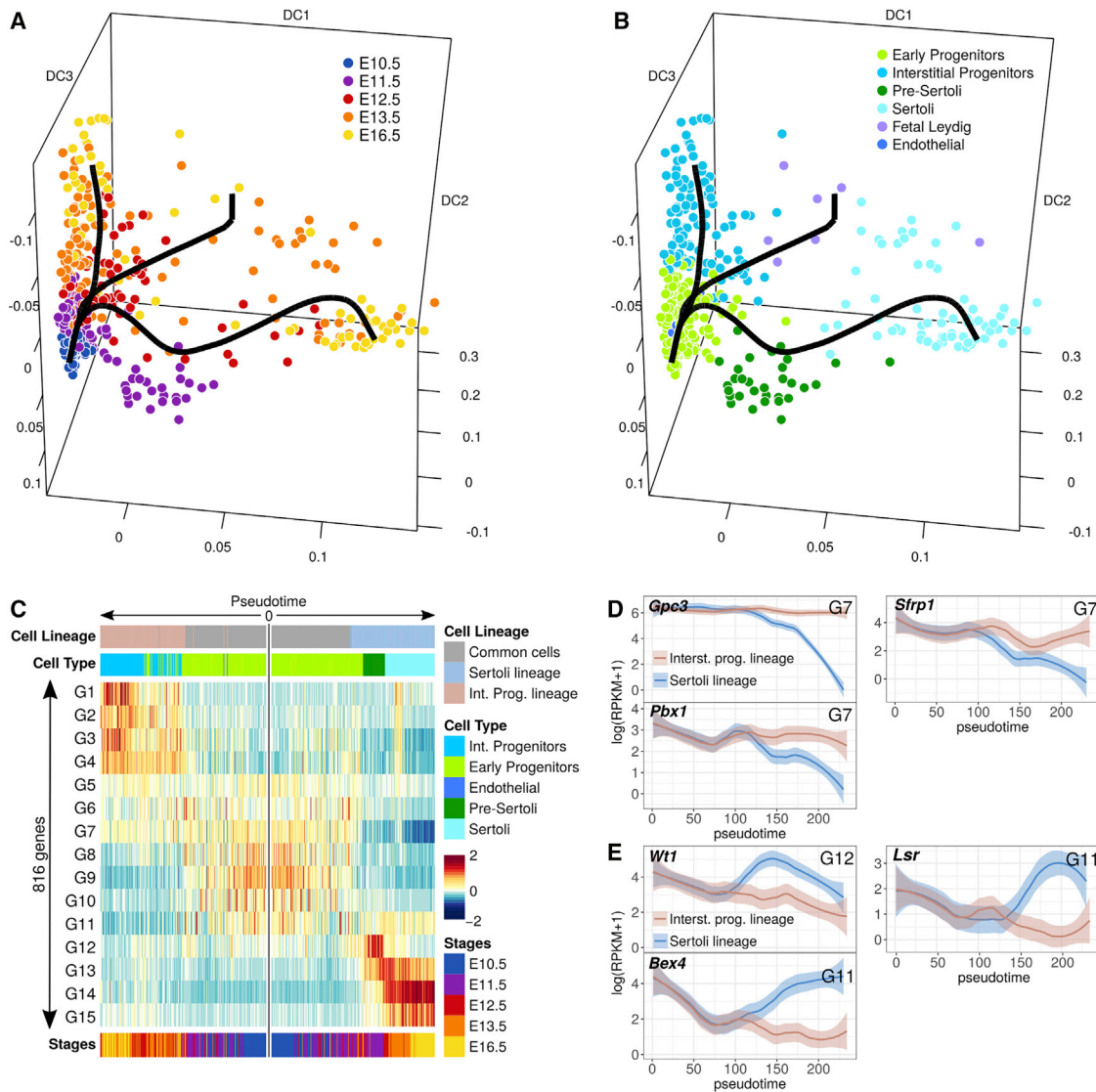


Figure 3. Cell Lineage and Pseudotime Prediction Shows Divergence of Sertoli, Interstitial, and Leydig Lineages from a Common Progenitor Population

(A and B) Diffusion map on the most variable genes and reconstruction of the cell lineages. Dots represent cells, and the black lines represent the estimated cell lineages. Embryonic stages are shown in (A) and cell clusters are shown in (B). The lineage reconstruction predicted one common origin for the interstitial progenitors, the Sertoli cells, and the fetal Leydig cells.

(C) Heatmap representing the transcriptomic progress of the progenitor and Sertoli cell-lineage-specific genes through the pseudotime. Genes were grouped by expression profiles with k-means. The heatmap shows the normalized average expression of each gene group. Early progenitor cells common to all lineages are located in the center (0) of the map, with divergence of Sertoli cells at the right and evolution of progenitor cells at the left. Sertoli cells display a dramatic cell identity acquisition, in contrast to interstitial progenitors that display a more gradual cell-fate specification.

(D and E) Expression profiles of genes that becomes restricted to interstitial progenitor (D) or Sertoli (E) lineage. The solid line represents the loess regression, and the fade band indicates the 95% confidence interval of the model.

differential expression analysis as a function of the pseudotime on the whole transcriptome ([Supplemental Experimental Procedures](#)). We identified 1,734 genes presenting a dynamic expression over the pseudotime (q value < 0.05) and classified them according to their expression pattern (P1 to P9; [Figure 4A](#); [Data S6](#)).

The expression profile P1 shows genes that are expressed twice during the cell development: once at E10.5 and once

around E12.5–E13.5. These genes are related to mitotic cell division.

The expression profiles P2 to P4 present genes expressed between E10.5 and E11.5 and are related to developmental processes, regulation of gene expression, and epithelium development, consistent with the cellular identity of the NR5A1⁺ cells of the genital ridge before sex determination. These three expression profiles also contain genes related to

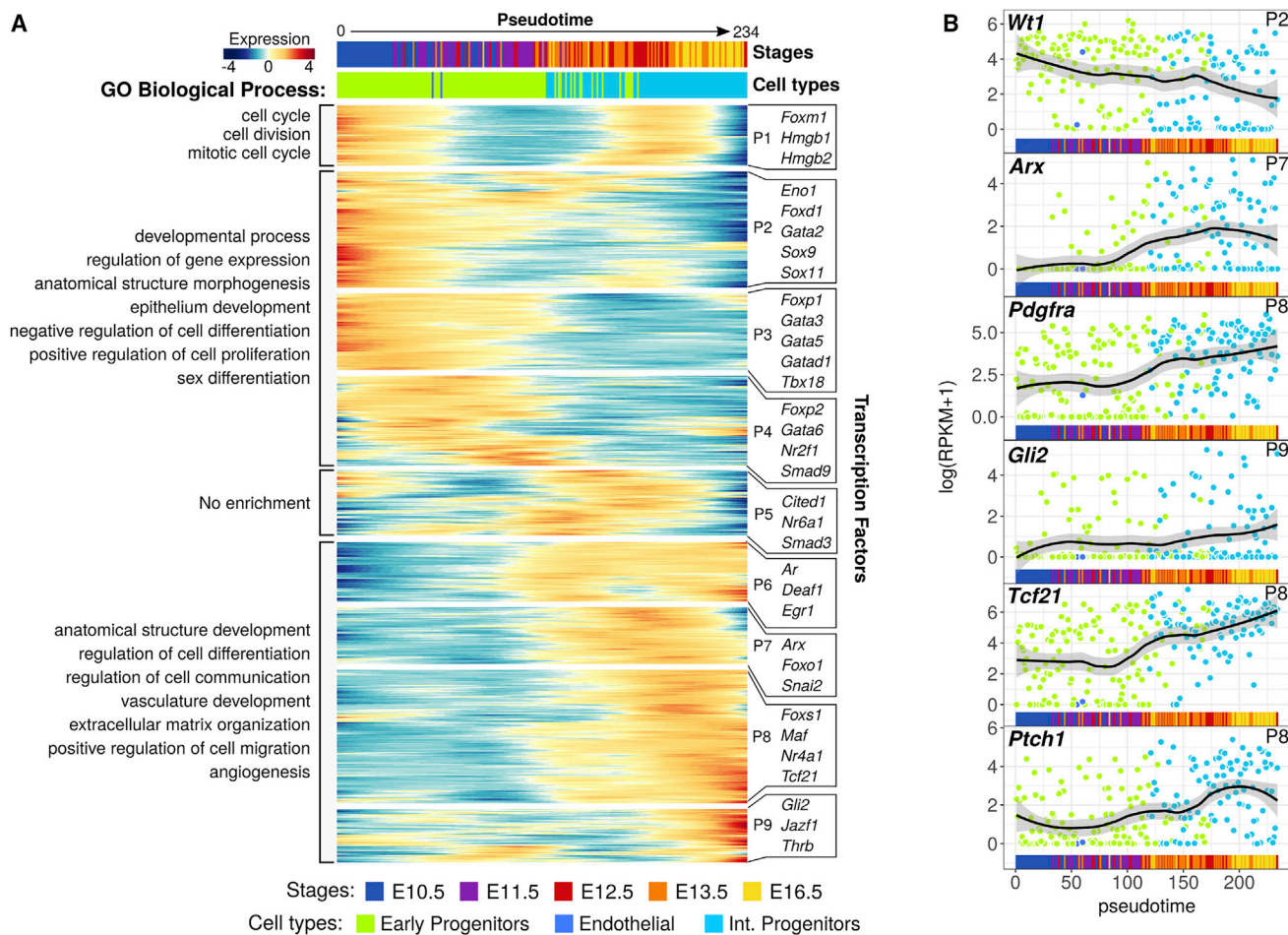


Figure 4. Dynamics of the Progenitor Transcriptome from E10.5 Onward

(A) Heatmap showing the gene expression dynamics of the progenitor cells from E10.5 onward. Genes were grouped by similar expression profiles with hierarchical clustering. GO terms from enrichment analysis reveal the evolution of the function of the progenitor cells. On the right are listed some of the transcription factors expressed in each expression profile.

(B) Expression profiles of relevant genes reflecting the acquisition of a steroidogenic fate. Each dot represents a cell, the solid line represents the loess regression, and the fade band indicates the 95% confidence interval of the model.

stem cell maintenance and renewal (*Sall1* [Spalt-like transcription factor 1], *Lin28a* [Lin-28 homolog A], and *Trim71* [Tripartite motif-containing 71]) (Basta et al., 2014; Cuevas et al., 2015; Yang et al., 2015), the negative cell differentiation regulator *Foxp1* (Forkhead box P1) (van Keimpema et al., 2015; Li et al., 2012; Takayama et al., 2008), and *Wt1* and *Cbx2* (Chromobox 2), necessary for *Sry* expression and, thus, Sertoli cell differentiation (Kato-Fukui et al., 2012; Wilhelm and Englert, 2002) (Data S7).

Expression profiles P6 to P9 contain genes expressed from E12.5 onward. Associated GO terms are related to extracellular matrix organization, angiogenesis, and also to the regulation of cell migration. Moreover, we found that these four expression profiles contain genes known as markers of fetal Leydig cell precursors such as *Arx* (Aristaless related homeobox), *Pdgfra*, *Gli2* (GLI-Kruppel family member GLI2), *Tcf21* (Transcription factor 21), and *Ptch1* (Patched 1) (Figure 4B) (Barsoum and Yao, 2011; Bhandari et al., 2012; Brennan et al., 2003; Cui et al.,

2004; Inoue et al., 2016; Liu et al., 2016; Miyabayashi et al., 2013).

The transcriptome dynamics of the interstitial progenitor cell lineage reflect a change in cell identity, from multipotent epithelial cells expressing stem cell markers at E10.5 to steroidogenic precursor cells at the origin of fetal Leydig cells.

The Sertoli Cell Differentiation Program Is Regulated by Waves of Transcription Factors

Expression dynamics of key genes such as *Sry*, *Sox9*, *Fgf9* (Fibroblast growth factor 9) or *Dhh* is consistent with published reports and emphasize the complex gene expression kinetics at play during Sertoli cell-lineage specification (Figure 5A). By applying the same procedure as we did for the progenitor cells, we identified the gene expression profiles driving Sertoli cell differentiation in the fetal testis from the E10.5 progenitor state prior to sex determination until E16.5. We identified 2,319 genes differentially expressed as a function of the

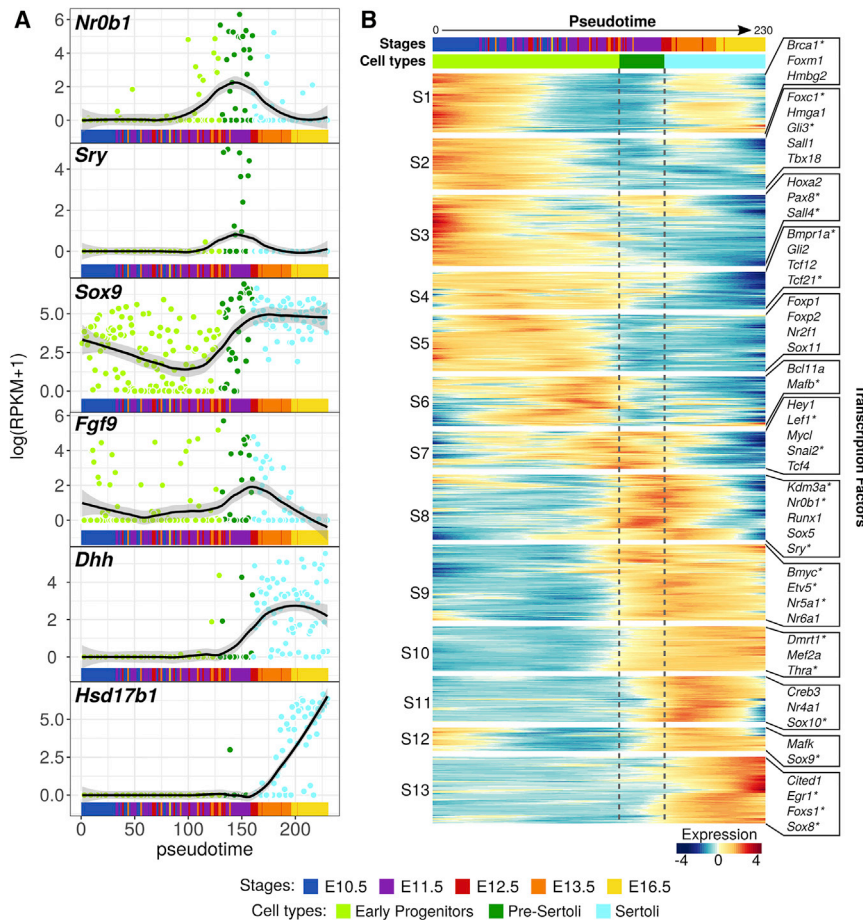


Figure 5. Sertoli Cell Transcriptome Dynamics

(A) Expression profiles of known marker genes important for Sertoli cell differentiation. Each dot represents a cell, the solid line represents the loess regression, and the fade band indicates the 95% confidence interval of the model.

(B) Heatmap showing the gene expression dynamics of the supporting lineage from the common progenitor cells at E10.5 to E16.5 Sertoli cells. The vertical dashed lines divides the progenitor cells and the pre-Sertoli and Sertoli cell clusters. On the right are listed some of the transcription factors expressed in each expression profile. Asterisks indicate the ones presenting a reproductive phenotype in MGI.

tion factor 1), which has a key role in maintaining Sertoli cell fate and repressing female promoting signals (Lavery et al., 2012; Matson et al., 2011; Minkina et al., 2014); and *Gata4*, which is required for testis differentiation and controls the expression of *Dmrt1* (Manuylov et al., 2011).

Our findings reveal that Sertoli cell differentiation is driven by a highly dynamic transcriptional program composed of several intermediate stages defined by peaks of co-expressed genes as well as the activation and repression of genes. The transcriptomic signature of Sertoli cells evolves with time as a reflection of the differentiation status and

pseudotime from E10.5 to E16.5 (q value < 0.05) and classified them into 13 gene profiles (Figure 5B; Data S7). 1,217 genes (profiles S7 to S13) characterize Sertoli cell differentiation from E11.5, including *Sry*, *Sox9*, *Amh*, and *Dhh*. We found transcription factors expressed in each of the expression profiles, with some associated with reproductive phenotypes in Mouse Genome Informatics (MGI: <http://www.informatics.jax.org>), indicated with an asterisk in Figure 5B (Blake et al., 2017).

Among the interesting expression profiles, we found 221 genes that follow the narrow expression pattern of *Sry* at the onset of Sertoli cell differentiation (S8). They include *Kdm3a*, a H3K9 histone demethylase that directly regulates the expression of *Sry* by modifying the chromatin conformation upstream of *Sry* (Kuroki et al., 2013). *Nr0b1*, also known as *Dax1*, encodes a member of the orphan nuclear hormone receptor family. *Nr0b1* is considered as a pro-testis (Meeks et al., 2003) and anti-testis gene (Swain et al., 1998).

In contrast to the narrow “Sry-like” expression pattern, expression profiles S9, S10, and S13 display a more persistent expression in differentiating Sertoli cells. Among them, we find *Nr5a1*, which co-regulates, with *Sox9*, the expression of *Amh* (expression profile 8; Data S7) (Lasala et al., 2011); *Dmrt1* (Doublesex and mab-3-related transcrip-

evolving functions of these supporting cells during early testis development.

DISCUSSION

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, whose progenitors will ultimately differentiate into Sertoli and fetal Leydig cells, respectively (Svingen and Koopman, 2013). This model has been challenged toward a common progenitor, with the recent findings of Leydig-to-Sertoli trans-differentiation capacity (Zhang et al., 2015) and that both lineages derive from *WT1*⁺ cells present at E10.5 in the genital ridge (Liu et al., 2016). However, the characterization of this multipotent somatic progenitor has, so far, remained elusive, and the possibility that different populations of *WT1*⁺ progenitors carrying a supporting or steroidogenic fate cannot be excluded.

Here, we aimed to evaluate the heterogeneity of *Nr5a1*⁺ cells by establishing the transcriptomic identity of each individual cell present in the gonadal somatic compartment. Strikingly, we identify a single homogeneous multipotent *Nr5a1*-GFP⁺ progenitor cell population in the bipotential gonad, whose transcriptomes progressively evolve and diverge toward both the

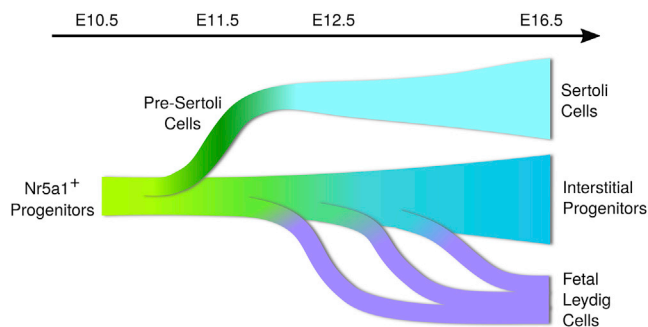


Figure 6. Model of Nr5a1-GFP⁺ Cell-Lineage Specification during Male Sex Determination

A unique *Nr5a1*⁺ progenitor cell population at E10.5 is able to later differentiate as Sertoli cells or as interstitial progenitors at the origin of fetal Leydig cells. Around E11.5, a subset of these multipotent cells will differentiate as Sertoli cells. Another subset of multipotent cells will maintain their progenitor state and, by E12.5, become restricted to the interstitial compartment of the developing testis. These interstitial progenitors undergo gradual transcriptional changes that restrict their competence toward a steroidogenic fate. Some of them will differentiate into fetal Leydig cells.

supporting and steroidogenic lineages from E11.5 onward (Figure 6). We cannot exclude the existence of additional rare *Nr5a1*-GFP⁺ cell types; however, our findings support the common progenitor identity hypothesis and demonstrate the absence of a cell-lineage-specific fate before sex determination, at least at the transcriptional level.

The presence of a unique progenitor for both supporting and steroidogenic cell lineages raises the question of the mechanisms underlying cell-fate decisions. We show that the progenitor cell lineage undergoes progressive transcriptomic changes through time, switching from a multipotent identity to a steroidogenic precursor identity. Before sex determination, cells express multipotent markers as well as *Wt1* and *Cbx2*, two co-factors necessary for *Sry* expression and Sertoli cell differentiation. From E11.5, when the Sertoli cells have differentiated, the progenitor cells lose expression of multiple genes, including these two factors, and, as a consequence, might lose the capacity to differentiate as Sertoli cells.

The decision between a Sertoli fate and a progenitor fate during male sex determination remains elusive. However, we noticed that the progenitor cells express genes involved in negative regulation of cell differentiation during the same time frame as that of Sertoli cell differentiation. Notably, we found *Sox11* (SRY box 11), which has been recently identified as a potential co-repressor of the pro-Sertoli gene *Sox9* (Zhao et al., 2017), as well as *Sfrp1*, a negative regulator of the *Wnt* signaling pathway (Gauger et al., 2013). This suggests that the progenitor cells may have a repression program that allows cells to escape a Sertoli cell fate and conserve a progenitor identity, similar to a stem-cell-renewal process.

From E12.5, progenitor cells become restricted to the interstitial compartment of the testis and progressively lose *Wt1* gene expression, which is suspected to be the key regulator of Sertoli cell differentiation and maintenance (Zhang et al., 2015). Conversely, they gradually express markers of fetal Leydig cell precursors, such as *Arx*, *Pdgfra*, and *Tcf21* (Brennan et al.,

2003; Cui et al., 2004; Miyabayashi et al., 2013), and restrict their fate toward interstitial steroidogenic precursors that define the origin of Leydig cells. The maintenance of the steroidogenic cell precursors is known to be regulated by a differentiation repression program via *Notch1* and *Tcf21*. This mechanism maintains a pool from the fetal Leydig cell precursor cell population (Barsoum and Yao, 2010; Tang et al., 2008).

We also noticed that the interstitial progenitors express a significant amount of genes related to the regulation of cell migration, as well as angiogenesis. Our data suggest that the interstitial cells actively contribute to this cell migration and control the vascularization of the testis from E12.5, together with Sertoli cells. However, we found no clear evidence that these cells are themselves able to differentiate as endothelial cells.

Our results also reveal that Sertoli cell differentiation is mediated by a highly complex and dynamic transcriptional program composed of waves of transiently expressed genes. Our capacity to order the cells along a pseudotime reflecting their differentiation status allows the most accurate expression dynamics catalog compared to published data based on pools of non-synchronous cells (Jameson et al., 2012; Nef et al., 2005). In particular, the narrow transient expression profile of numerous genes suggests that they are required during a precise cellular differentiation stage, but not for maintenance of cell identity. We found that both *Nr0b1* and *Sry* are expressed in E11.5 pre-Sertoli cells during a very narrow window of time, supporting the hypothesis that both factors are critical for Sertoli cell differentiation and are required for *Sox9* upregulation (Bouma et al., 2005; Ludbrook and Harley, 2004). In contrast, a broad expression profile with a permanent activation in differentiating Sertoli cells might reveal a role in maintaining their identity. For example, the upregulation of *Sox9* and *Dmrt1* is consistent with their key role in maintaining Sertoli cell fate and repressing female promoting signals (Lavery et al., 2012; Matson et al., 2011; Minkina et al., 2014).

Testis formation is a complex developmental process requiring coordinated differentiation of multiple cell lineages. This study focused on the *Nr5a1*-GFP⁺ cells, and while we identified both the supporting and steroidogenic lineages using this approach, we cannot exclude the possibility that *Nr5a1*⁺ cells also contribute to both the Sertoli and the fetal Leydig cell lineages.

In the future, we expect single-cell expression studies to transform our understanding of gonadal development and sex determination. In particular, a similar single-cell ovarian development analysis would provide a high-resolution understanding of the mutually antagonist testis/ovarian genetic program at play during sex determination. Single-cell genomics will provide unparalleled insights into the stem cell origin of the different cell lineages, distinguish the different stages of differentiation, characterize cell-fate decisions and the regulatory mechanisms that govern the production of different cell types, and elucidate how different cell types work together to form a testis. We also expect that advances in single-cell genomics, such as chromatin immunoprecipitation sequencing (ChIP-seq), assay for transposase-accessible chromatin using sequencing (ATAC-seq), or, again, high chromosome contact map (Hi-C) (Pott and Lieb, 2015; Ramani et al., 2017; Rotem et al., 2015), will facilitate the

identification of epigenetic modifications and regions of open chromatin at the single-cell level. In combination, such single-cell approaches will serve as a basis for both understanding testis determination and its pathologies.

EXPERIMENTAL PROCEDURES

Mouse Strains and Isolation of Purified Nr5a1-GFP Positive Cells

Animals were housed and cared for according to the ethical guidelines of the Direction Générale de la Santé of the Canton de Genève (experimentation GE-122-15). The experiment has been performed using heterozygous *Tg(Nr5a1-GFP)* transgenic male mice (Stallings et al., 2002). We performed the experiments in independent duplicates for each embryonic stages, except for E10.5, where XX embryos were used as controls but not included in the study. At each relevant gestation day, *Tg(Nr5a1-GFP)* gonads were collected and dissociated. Several animals from different litters were pooled together to obtain enough material for the experiment (Table S1). GFP⁺ cells were sorted by FACS (Figure S1; Supplemental Experimental Procedures).

Tissue Processing and Immunological Analyses

Embryos at relevant stages of development were collected, fixed in 4% paraformaldehyde overnight at 4°C, serially dehydrated, and embedded in paraffin. Fluorescent images were acquired using a confocal laser scanning microscope and processed with ZEN software (Supplemental Experimental Procedures).

Single-Cell Capture and cDNA Libraries and Sequencing

Cells were captured and processed using the C1 Autoprep System (96-well, small-size IFC chip), following the official C1 protocol. Sequencing libraries were prepared using the Illumina Nextera XT DNA Sample Preparation Kit using the modified protocol described in the C1 documentation. Cells were sequenced at an average of 15 million (Supplemental Experimental Procedures).

Bioinformatics Analysis

The computations were performed at the Vital-IT Centre for high-performance computing of the SIB (Swiss Institute of Bioinformatics) (<http://www.vital-it.ch>). Data were analyzed with R v3.4.0. Cell clustering was performed on the highly variable genes with hierarchical clustering on principal components (HPCP), differential expression analysis with performed with Monocle2, diffusion maps were computed with Destiny, and lineage reconstruction was performed with Slingshot (for details, see the Supplemental Experimental Procedures).

Code Availability

The source code of the analysis is publicly available on GitHub at <https://github.com/IStevanc/scrRNAseq-XY>.

DATA AND SOFTWARE AVAILABILITY

The accession number for the FastQ files of the 435 single-cell RNA-seq libraries reported in this paper is GEO: GSE97519.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, one table, and seven data files and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.01.043>.

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AUTHOR CONTRIBUTIONS

Conceptualization, I.S. and S.N.; Investigation, I.S., Y.N., J.E., and C.B.; Formal Analysis and Data Curation, I.S.; Writing – Original Draft, I.S., Y.N., and S.N.; Writing – Review & Editing, I.S., Y.N., C.B., L.B.S., S.E.A., and S.N.; Funding Acquisition, S.N. and E.T.D.; Resources, S.N. and E.T.D.; Supervision, S.N. and E.T.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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