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Single cell transcriptomics to define germ cell function in livestock

Jon M. Oatley, Colton Robbins, Michela Ciccarelli
School of Molecular Bioscience, College of Veterinary Medicine, Washington State University, Pullman, WA

Abstract

Spermatogenesis is a specialized developmental process that produces millions of sperm each day from puberty to old age. Crosstalk among testicular germ and somatic cells provides required direction for homeostasis of spermatogenesis and regeneration after an insult. Understanding spermatogenesis and the importance of sperm are critical. Sperm contribute genetic information that influences the traits of offspring, thus shaping performance, resiliency, and fitness of animal populations. Recent emergence of single cell RNA sequencing analysis (transcriptomics) opened a new avenue to explore reproductive biology by defining heterogeneity and crosstalk within complex cell lineages (e.g. male germline). New insights in the regulation of developmental processes at a molecular level provides invaluable information for researchers and clinicians to address underlying causes of infertility/subfertility and design novel advanced reproductive technologies.

Keywords: Single cell RNA sequencing, livestock, spermatogenesis, testis

Introduction

Spermatogenesis occurs in seminiferous tubules of testes that produce millions of sperm daily.[1] The foundation of this process is formed by activities of an undifferentiated spermatogonial population that contains a stem cell pool and resides at the basement membrane of seminiferous tubules.[2] A foundational pool of spermatogonial stem cells (SSCs) is established during neonatal development and sustained via self-renewal in adulthood.[3,4] Out of the SSC pool, transit amplifying progenitor spermatogonia arise that undergo a series of mitotic cell divisions to bolster the population prior to initiation of a differentiating transition in response to a pulse of retinoic acid.[5] Differentiating spermatogonia undergo further mitotic amplifying divisions while gaining competency for initiation of meiosis as preleptotene spermatocytes and progressively migrating through the seminiferous epithelium towards tubule lumen.[5] After 2 meiotic divisions (primary and secondary spermatocytes) haploid spermatids are formed that undergo morphogenesis to form elongate spermatids and are subsequently released into seminiferous tubules. Sertoli cells, the only somatic cell type in the seminiferous epithelium, nurture the entire spermatogenic process.

Interstitial tissue of testicular parenchyma that is present among seminiferous tubules includes Leydig cells, macrophages, and blood vessels. Leydig cells are the source of testicular androgens which are required for spermatogenesis via signaling within Sertoli cells.[6] Outside seminiferous tubules, peritubular myoid cells are positioned to provide structural support and potentially contribute signals to the spermatogonial niche microenvironment. An orchestrated crosstalk among the testicular soma of Leydig, myoid, and Sertoli cells with spermatogenic germ cells is required for continuous and robust steady-state spermatogenesis, and for regeneration of spermatogenesis following cytotoxic insult.

Development of the spermatogenic lineage begins with primordial germ cells (PGCs) that arise from the inner lining of yolk sac during the pregastrulation embryonic period.[7] After specification, PGCs undergo migration to genital ridges where they coalesce with somatic cells to form undifferentiated gonads. At this point, all cells populating genital ridges have the bipotential capacity to differentiate into either testicular or ovarian tissue. A delicate interplay of molecular signaling occurs within somatic cells of XX and XY embryos to drive the generation of either preSertoli or pregranulosa populations that will form fetal testis or ovary, respectively.[8] In XY embryos, expression of the Y chromosome gene SRY in cells migrating from the mesonephric mesenchyme to genital ridge leads to development of preSertoli cells that create interdigitations around prospermatogonial germ cells to form seminiferous cords. The prospermatogonial population is the fetal precursor to the spermatogenic lineage and arises from PGCs at sex determination. During late fetal period, the prospermatogonial population undergoes waves of proliferation and apoptosis to set a developmentally pristine population that will enter a period of mitotic arrest.[3,9] Prospermatogonia remain in

quiescence until after birth when reentry to the cell cycle occurs over a period that parallels transition to postnatal spermatogonial states.[3] Coincident with morphogenesis of seminiferous cords, interstitial compartment, connective tissues, blood vessels, and Leydig cells also arise during fetal development. Although origins of fetal Leydig cell population are still not fully understood, it is their production of testosterone that drives development of Wolffian ducts and masculinization of external genitalia. All of these embryonic and fetal events occur in orchestration to establish a male gonad that, at birth, is developmentally primed to support proper establishment of a continually cycling spermatogenic lineage during the prepubertal period.

Overview of single cell RNA sequencing technology

The ability to quantify RNAs within cells on a large scale has been game changing for the field of biosciences for more than 2 decades. Beginning with microarray analysis that profiled RNAs of bulk populations of cells and evolving to single cell RNA profiling, the transcriptome of specific cell populations that make up tissues during different developmental time points and circumstances can be defined in a precise way. A limitation of bulk RNA profiling approaches has been an inability to uncover heterogeneity of what is seemingly homogenous populations and difficulty in parsing out transcriptomes of distinct cell populations that comprise a complex tissue. Single cell RNA sequencing (scRNA-seq) was developed to overcome these limitations and provides scientists with a tool to define genes being expressed in individual cells that comprise a lineage or tissue. Outcomes of these studies are leading to groundbreaking discoveries of new cellular states, uncovering of novel molecular mechanisms governing those states, and deepening our understanding disease etiology. A general workflow of scRNA-seq analysis is presented (Figure 1A) and key steps include:

1. Generation of a single cell suspension via disassociation of a tissue using mechanical or enzymatic means.

2. Emulsification of single cell suspension to suspend individual cells into droplets.

3. Lysis of individual cells to liberate mRNAs.

4. Ligation of adapters and reverse transcription to create a cDNA library that carries a molecular barcode that is unique for each cell.

5. Sequencing of cDNA libraries. A raw data matrix is created in which genes constitute row barcodes, samples constitute column barcodes, and values within the matrix are read counts representing the expression of a particular gene in an individual

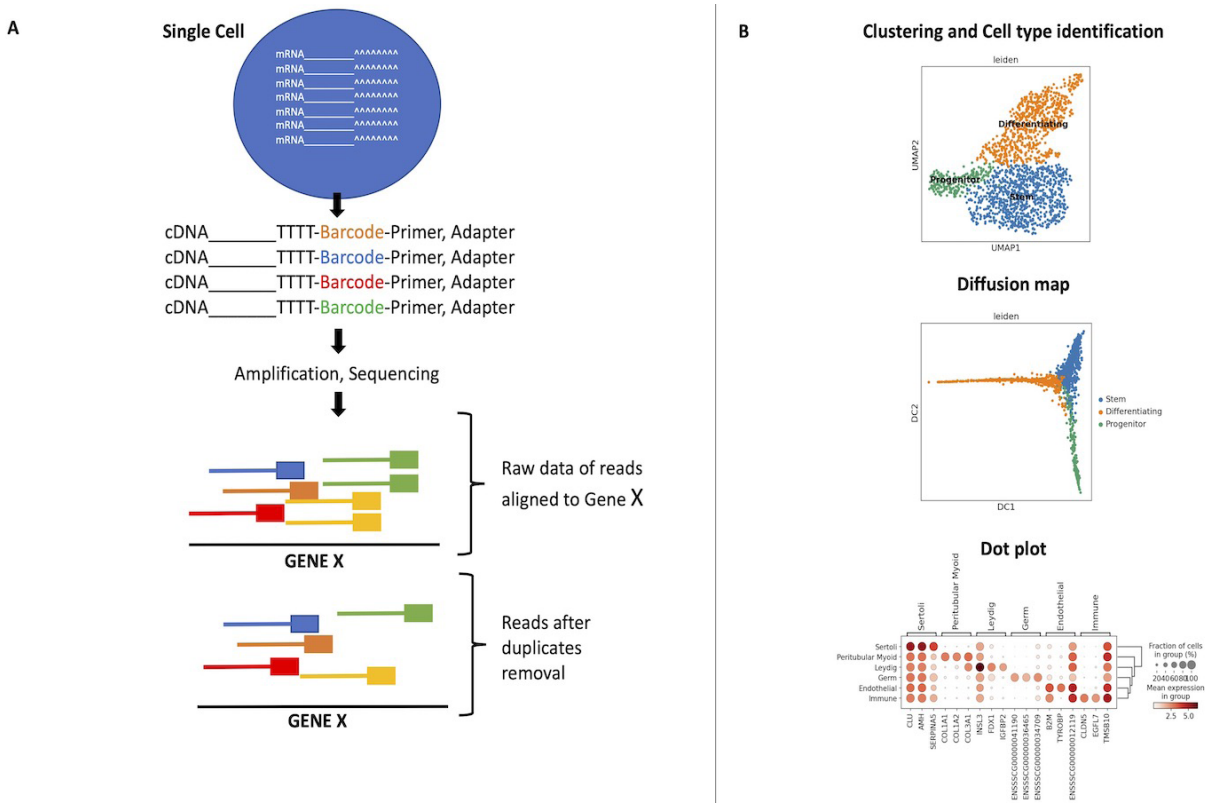

Figure 1. Overview of single cell RNA-sequencing workflow: A. Schematic of mRNA collection, cDNA amplification, barcoding and demultiplexing of reads for single cells. B. Examples of outputs from computational analysis of single cell RNA-sequencing data.

sample. Raw data are then processed for normalization and quality control. Normalization provides scaling of counts to remove technical variations and determine gene expression based on mRNA/cDNA abundance. Quality control measures allow for elimination of libraries derived from nonviable cells.

6. Normalized and quality-controlled data are then analyzed using computational and bioinformatic programs such as Cell Ranger, Seurat, and Monocle. Outputs of these analyses include clustering of cells into populations based on similarities in gene expression profiles, characterizations of cell clusters based on biomarkers or differentially expressed genes, and generation of fate trajectory maps to understand how cells advance along a developmental path from undifferentiated to terminally differentiated states (Figure 1B).

Use of single cell RNA sequencing technology to deepen the understanding of male germ cell biology and testicular function

Over the last few years, scRNA-seq technology was used to generate advances in understanding of molecular pathways that control spermatogenesis and testis development in a variety of mammalian species.[10-14] Through comparison of single cell gene expression profiles of total testicular and germ cell populations from adult mice, monkeys, and humans, conserved and divergent pathways that govern sperm generation have been uncovered.[14] Interestingly, the outcomes of these studies have begun to reveal previously undescribed gene expression characteristics for specific cell types of spermatogenic lineage and testicular support cell populations. These observations suggest that subsets of cells with different functionalities may exist in what have historically been regarded as homogenous populations with similar biological properties. From a human health perspective, understanding the causes of fertility disorders and devising treatment strategies for them may be advanced by comparing single cell gene expression profiles among germ cells and testicular somatic cells of mice, monkeys, and men. Additionally, the integration of information being generated from germ cells of domestic animal species is starting to uncover novel regulators of germ cell function that are conserved across Mammalia.[15] For livestock, utility of single cell gene expression profiling is in the discovery of new pathways regulating spermatogenesis that can be exploited for developing and refining advanced/assisted reproductive technologies.

Recently, we generated scRNA-seq profiles for testicular tissue of prepubertal bulls and boars, including fetal and perinatal male piglets, and integrated this information with mouse and human profiles to compile a comprehensive comparative species database (unpublished). Ongoing exploration of this extensive database is yielding new information about molecules expressed by germ cells that are conserved across species. Importantly, we have identified previously undescribed cells surface molecules and transcription factors that are expressed by germ cells and/or testicular somatic support cells from livestock to man. Currently, we are using these advances in knowledge to devise strategies for purification of germ cells from testicular tissue and optimize conditions to grow livestock spermatogonia in vitro. These strategies are being matched with advanced reproductive technologies such as spermatogonial stem cell transplantation to bolster the utility as a breeding tool in livestock production.

Conflict of interest

JMO is the founder of APYS Biotechnology, LLC, an animal biotechnology company specializing in the commercialization of advanced reproductive technologies and gene editing applications in livestock

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