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Single cell transcriptome sequencing: A new approach for the study of mammalian sex determination

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ABSTRACT

Mammalian sex determination is a highly complex developmental process that is particularly difficult to study due to the limited number of gonadal cells present at the bipotential stage, the large cellular heterogeneity in both testis and ovaries and the rapid sex-dependent differentiation processes. Single-cell RNA-sequencing (scRNA-seq) circumvents the averaging artifacts associated with methods traditionally used to profile bulk populations of cells. It is a powerful tool that allows the identification and classification of cell populations in a comprehensive and unbiased manner. In particular, scRNA-seq enables the tracing of cells along developmental trajectories and characterization of the transcriptional dynamics controlling their differentiation. In this review, we describe the current state-of-the-art experimental methods used for scRNA-seq and discuss their strengths and limitations. Additionally, we summarize the multiple key insights that scRNA-seq has provided to the understanding of mammalian sex determination. Finally, we briefly discuss the future of this technology, as well as complementary applications in single cell -omics in the context of mammalian sex determination.

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1. Introduction

Gonad differentiation into testis or ovaries represents a unique model for the study of cell fate decisions during mammalian organ development. The indifferent gonadal primordium is composed of bipotential progenitor cells that include primordial germ cells, and the supporting and steroidogenic cell lineages. The fate of these somatic and germ cell lineages depends on the genetic sex and is controlled by antagonistic genetic programs that lead to commitment, propagation, and maintenance of testis or ovary fate. For more in-depth information about gonadal differentiation and the genetics of mammalian sex determination, see the following reviews (Carré and Greenfield, 2014; Greenfield, 2015; Lin and Capel, 2015; Spiller et al., 2017; Svingen and Koopman, 2013; Wilhelm et al., 2015; Windley and Wilhelm, 2016). There are many outstanding questions regarding the identity of gonadal progenitors and the transcriptional events that drive cell lineage specification. Classical transcriptomic approaches – such as RNA-Seq and microarrays – have provided valuable information about the changes at play during sex determination and sexual differentiation (Jameson et al., 2012; McClelland et al., 2015; Munger et al., 2013; Nef et al., 2005). Nevertheless, these techniques require a substantial number of cells and result in the measurement of gene expression levels that are averaged over a cell population. Without *a priori* knowledge about specific cell populations, classical transcriptomic analyses do not allow the discovery of new cell types, or the deciphering of the chronology of genetic programs responsible for cell differentiation.

With the development of single-cell RNA sequencing (scRNA-seq) techniques, we are now able to obtain unique insights into gene expression at the level of the smallest and most fundamental unit of an organism: the single cell. By profiling the transcriptome of individual cells, scRNA-seq has shown great effectiveness in assessing cell heterogeneity in any given tissue (Burns et al., 2015; Chen et al., 2017; Scialdone et al., 2016; Usoskin et al., 2014; Villani et al., 2017). It has also helped to reconstruct developmental trajectories and model transcriptional dynamics in numerous developmental processes including that of the lung (Treutlein et al., 2014), brain (Tasic et al., 2016), retina (Macosko et al., 2015), pancreas (Baron et al., 2016), of immune cells (Björklund et al., 2016), early embryonic development (Petropoulos et al., 2016) and hematopoiesis (Wilson et al., 2015).

In this review, we describe the applications of scRNA-seq in the field of gonadal development and differentiation, the different methodologies and their current limitations. We will summarize the first findings and how this technology is anticipated to revolutionize our understanding of gonadal differentiation by identifying progenitor cells, mapping lineage progression and cell type diversification, and also allowing the reconstruction of the genetic programs controlling testicular differentiation. Finally, we will briefly discuss the future of methodologies and complementary applications in single cell –omics, relevant for the study of mammalian sex determination.

2. How does scRNA-seq benefit the understanding of sex determination?

Since the discovery of the testis-determining gene *Sry* (Sinclair et al., 1990), the genetic program of sex determination has been intensively studied, using various different transcript profiling technologies, from gene by gene Southern blot and RT-PCR, to more comprehensive techniques such as microarrays (Jameson et al., 2012; Munger et al., 2013; Nef et al., 2005), NanoString (Pitetti et al., 2013), and RNA-Seq (Inoue et al., 2015; Maatouk et al., 2017; McClelland et al., 2015). All of these techniques share a common requirement for a considerable amount of material – from hundreds of thousands to millions of cells – the availability of which can be limiting, especially at early developmental stages or with small populations of a given cell type. Recent developments in low-input RNA extraction protocols have significantly reduced the minimum necessary material, leading to the first scRNA-seq experiment on a single mouse blastomere (Tang et al., 2009). Today, scRNA-seq enables the study of small tissues and rare cell populations at an unprecedented resolution.

The scarcity of specific marker genes for differentiating cell populations limits our understanding of the developing gonads. In particular, there are no known marker genes for discriminating between the supporting and steroidogenic cell lineages before sex determination. Moreover, the origin of the peritubular myoid cells, as well as that of adult Leydig cells, is unresolved. By deconstructing the tissue one cell at a time, it is now possible to record cellular composition without *a priori* knowledge. ScRNA-seq has demonstrated its ability to identify sub-cell types in as complex tissues as the brain, and to identify new cell type-specific signatures based on hundreds of genes (Macosko et al., 2015).

Performing time series scRNA-seq experiments not only resolves stage-by-stage cell composition, but also rebuilds cell lineage trajectories and models transcriptional dynamics during cell differentiation. As cells differentiate in a non-synchronous manner, scRNA-seq provides a snapshot of different cell transcriptional states, that can be used to infer the sequence of events driving cell differentiation.

While major questions remains regarding gonadal cell composition and the transcriptional events that drive cell lineage specification, traditional experimental approaches including population-averaged measurements (e.g. bulk RNA-Seq) are technically difficult and tedious due to the limited number of progenitor cells available. Data from bulk analysis masks the inherent variability of individual cells and lineage types, leading to averaging artifacts in its interpretation. scRNA-seq can give access to individual transcriptomes of gonadal cells, and therefore represents a precious tool to unravel the molecular basis of sex determination.

3. Basic framework of single-cell RNA-sequencing technology

All scRNA-seq protocols follow a similar basic strategy: single cells are isolated and lysed, their mRNA content is reverse transcribed to cDNA, and the resulting minute amount of cDNA is pre-amplified and prepared for sequencing. It has been estimated that the proportion of transcripts that are successfully reversed transcribed in most of the existing scRNA-seq protocols is around 10% of the total amount of RNA present in a cell, thus the detection of low abundance transcripts is unreliable (Liu and Trapnell, 2016). To date, a number of different manual and automatic scRNA-seq methods have been developed, and about 20 different protocols

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