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**MINI-REVIEW** ***Developments in reproductive biology and medicine***

# Human spermatogonial stem cells and their niche in male (in)fertility: novel concepts from single-cell RNA-sequencing

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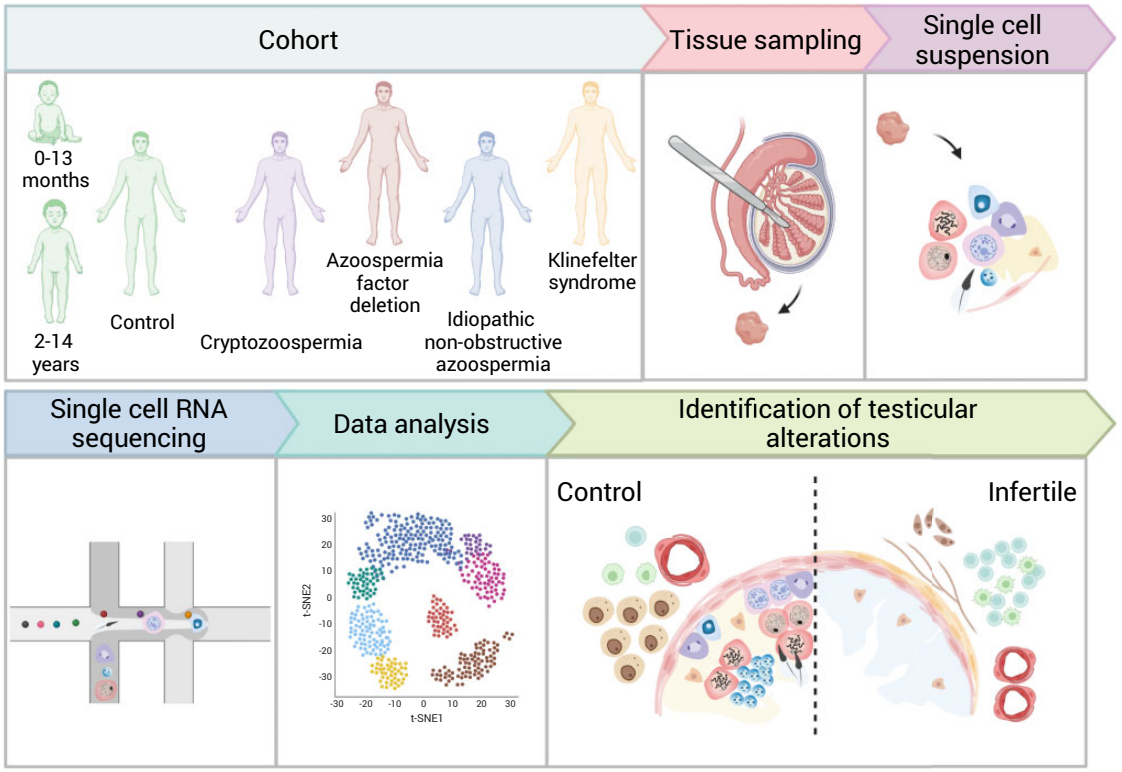
**ABSTRACT:** The amount of single-cell RNA-sequencing (scRNA-seq) data produced in the field of human male reproduction has steadily increased. Transcriptional profiles of thousands of testicular cells have been generated covering the human neonatal, prepubertal, pubertal and adult period as well as different types of male infertility; the latter include non-obstructive azoospermia, cryptozoospermia, Klinefelter syndrome and azoospermia factor deletions. In this review, we provide an overview of transcriptional changes in different testicular subpopulations during postnatal development and in cases of male infertility. Moreover, we review novel concepts regarding the existence of spermatogonial and somatic cell subtypes as well as their crosstalk and provide corresponding marker genes to facilitate their identification. We discuss the potential clinical implications of scRNA-seq findings, the need for spatial information and the necessity to corroborate findings by exploring other levels of regulation, including at the epigenetic or protein level.

**Key words:** single-cell RNA-sequencing / spermatogonial stem cells / spermatogenesis / Sertoli cells / Leydig cells / Klinefelter / azoospermia / AZF deletion / human testis development / male infertility

**GRAPHICAL ABSTRACT**

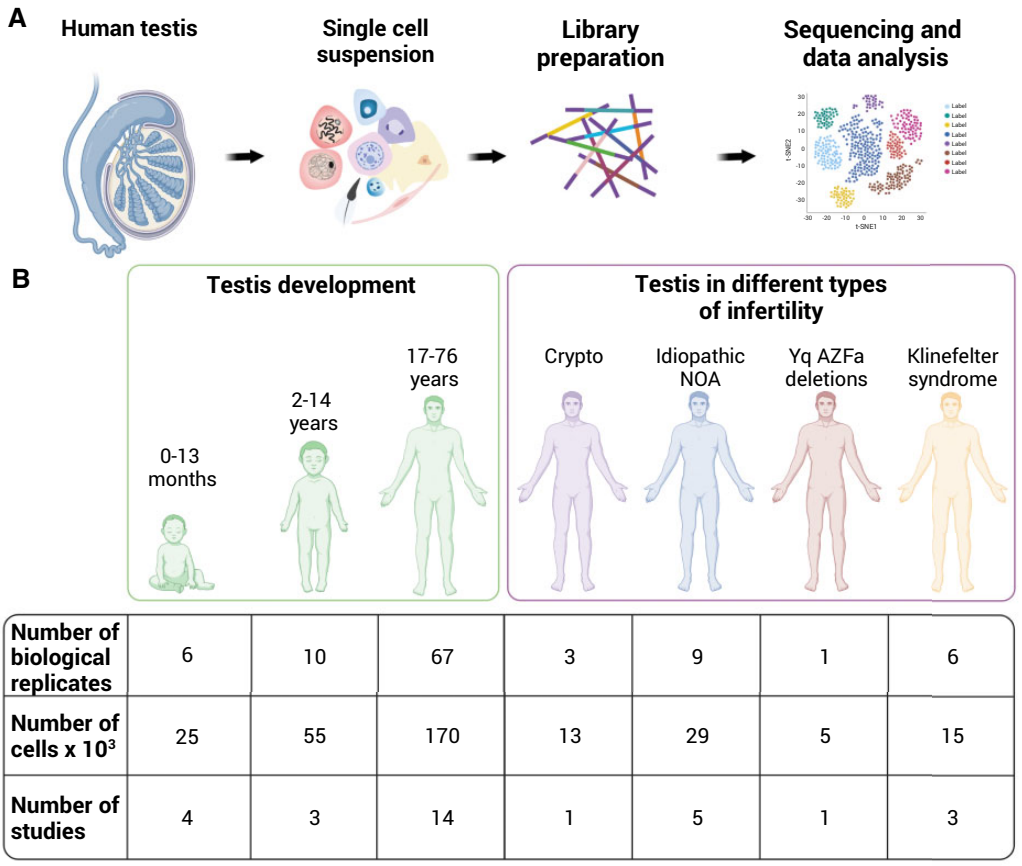



# Introduction

Infertility affects about 15% of couples with half of the cases arising from a male factor (Boivin *et al.*, 2007; Mascarenhas *et al.*, 2012). In the past decades, procedures including IVF and ICSI have been successfully implemented to enable men with very low sperm numbers to father biological children. However, medical approaches to treat the underlying causes of reduced sperm production remain elusive. This is largely because of the limited knowledge of the molecular and cellular interplays responsible for human sperm production and the alterations associated with the failure thereof (Tüttelmann *et al.*, 2018).

One hurdle in unveiling these molecular pathways is the high complexity of human testicular tissues, consisting of different germ cells as well as somatic cell populations. The advent of high-throughput single-cell RNA-sequencing (scRNA-seq) analyses has opened a new era of reproductive research (Li *et al.*, 2020; Tan and Wilkinson, 2020; Marečková *et al.*, 2022), as this approach and the associated workflow bypass the issue of high tissue complexity and enables the identification of rare and transcriptionally heterogeneous cell types. Multiple scRNA-seq methods have been developed to date, each with specific advantages and disadvantages (Ziegenhain *et al.*, 2017). Overall, the methods all share the following steps in the workflow: the testicular tissue undergoes enzymatic/mechanical digestion to obtain thousands of single cells, which are then subjected to (droplet- or plate-based) scRNA-seq following library preparation; for visualization, sequencing results are compressed into 2D space in which cells are organized in clusters based on their transcriptional profiles, with similar cells plotted in closer proximity (Fig. 1A); the cell identity of individual clusters is then assigned based on the expression of known marker genes, avoiding the isolation of individual cell types prior to analysis.

Taking advantage of scRNA-seq, the transcriptional profiles of thousands of human testicular cells have been generated covering the human neonatal, prepubertal, pubertal and adult period as well as different types of male infertility (Fig. 1B). Based on this growing resource, this review highlights relevant scRNA-seq datasets (Supplementary Table SI) and novel marker genes for different testicular cell types (Table I). What is more, considering transcriptional profiles during development and in (in)fertility, this review provides an overview of novel concepts regarding the existence of subtypes of spermatogonia as well as somatic Sertoli and peritubular myoid cells (PTMs) and grants first insights into their communication pathways in male (in)fertility. Finally, this review discusses the potential clinical implications of the insights gained from scRNA-Seq studies and the limitations associated with them.




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Number of biological replicates** | 6 | 10 | 67 | 3 | 9 | 1 | 6 |
| **Number of cells x $10^3$** | 25 | 55 | 170 | 13 | 29 | 5 | 15 |
| **Number of studies** | 4 | 3 | 14 | 1 | 5 | 1 | 3 |

**Figure 1. Schematic representation of the single-cell RNA-sequencing studies to analyze human testicular tissues.** (**A**) Experimental design, (**B**) samples used. Details are provided in Supplementary Table SI. Studies on each stage of development, and different types of infertility: 0–13 months (Guo *et al.*, 2018, 2021; Sohni *et al.*, 2019; Voigt *et al.*, 2022); 2–14 years (Guo *et al.*, 2020; Zhao *et al.*, 2020; Voigt *et al.*, 2022); 17–76 years (Guo *et al.*, 2017, 2018; Neuhaus *et al.*, 2017; Hermann *et al.*, 2018; Wang *et al.*, 2018; Sohni *et al.*, 2019; Shami *et al.*, 2020; Xia *et al.*, 2020; Zhao *et al.*, 2020; Alfano *et al.*, 2021; Di Persio *et al.*, 2021; Mahyari *et al.*, 2021; Chen *et al.*, 2022; Nie *et al.*, 2022); Crypto (Di Persio *et al.*, 2021); Idiopathic NOA (Wang *et al.*, 2018; Zhao *et al.*, 2020; Alfano *et al.*, 2021; Mahyari *et al.*, 2021; Chen *et al.*, 2022); Yq AZFa deletions (Zhao *et al.*, 2020); Klinefelter syndrome (Laurentino *et al.*, 2019; Zhao *et al.*, 2020; Mahyari *et al.*, 2021). Crypto, cryptozoospermia; NOA, non-obstructive azoospermia; AZF, azoospermia factor.

## A single-cell view of the human spermatogonial compartment in (in)fertile men

Sperm production in men is based on the spermatogonial stem cell (SSC) system. Sustainability of this system requires a balance between stem cell self-renewal and differentiation. SSCs are defined based on these two functional properties and are considered a subpopulation of the entirety of spermatogonia (Sharma *et al.*, 2019). While spermatogonia are expected to hold great potential for the treatment of male infertility (Valli *et al.*, 2014), only recent sequencing advancements have facilitated the analysis of the molecular properties that define spermatogonial subtypes.

To date, different technical angles have been taken to identify subtypes of spermatogonia that may be associated with distinct functions. Traditionally, undifferentiated spermatogonia have been classified in testicular tissue sections based on their nuclear morphology into $A_{dark}$ and $A_{pale}$ spermatogonia (Clermont, 1963). As $A_{dark}$ spermatogonia display highly compacted heterochromatin and low proliferative activity, they are considered to function as reserve stem cells, which are activated in response to injury (Clermont, 1969, 1972; van Alphen et al., 1988; Caldeira-Brant *et al.*, 2020). $A_{pale}$ spermatogonia in contrast, show a higher proliferative activity and are considered the progenitor stem cells (Clermont, 1969, 1972). Despite these distinct functions, transcriptional profiling of laser-capture microdissected $A_{dark}$ and $A_{pale}$ cells from testicular tissue sections revealed highly comparable transcriptional profiles. This finding either implies that the transition between these two spermatogonial types can occur instantaneously (Jan *et al.*, 2017) or that $A_{pale}$/$A_{dark}$ spermatogonia are not different populations, but rather the same population at different stages of the cell cycle (Fayomi and Orwig, 2018).

**Table I Resource of single-cell RNA-sequencing datasets providing novel marker genes for the different human testicular cell types.**

| Cell type | Cell subtype (Aliases) | Enriched marker genes | Developmental steps at which respective cell types were identified | References |
|---|---|---|---|---|
| **Primordial germ cell like** | PGCL | *NANOG*; *POU5F1* | Neonatal | (Sohni *et al.*, 2019) |
| **Undifferentiated spermatogonia** | State 0 (SSC-1B, SPG-1) | *C19orf84*; *EGR4*; *MAGEA4*; *PIWIL4*; *TSPAN33*; *UTF1* | From birth onwards | (Guo *et al.*, 2018, 2020, 2021; Sohni *et al.*, 2019; Shami *et al.*, 2020) |
| | State 0A (SSC-1A) | *FGFR3*; *MAGEA4*; *UTF1* | From birth onwards | (Sohni *et al.*, 2019; Di Persio *et al.*, 2021) |
| | State 0B (SSC-1C) | *MAGEA4*; *NANOS2*; *UTF1* | From birth onwards | (Sohni *et al.*, 2019; Di Persio *et al.*, 2021) |
| | State 1 (SSC-2) | *GFRA1*; *MAGEA4*; *NANOS3*; *UTF1* | From prepuberty onwards | (Guo *et al.*, 2018, 2020, 2021; Sohni *et al.*, 2019; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| **Differentiating spermatogonia** | State 2 (Diff. SPG) | *DMRT1* *DNMT1*; *KIT*; *MAGEA4*; *MKI67*; *SOHLH2* | From pre/puberty onwards | (Guo *et al.*, 2018, 2020, 2021; Sohni *et al.*, 2019; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| | State 3 (Diff. SPG) | *MAGEA4*; *REC8*; *STRA8* | From pre/puberty onwards | (Guo *et al.*, 2018, 2020, 2021; Sohni *et al.*, 2019; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| **Spermatocyte** | Leptotene | *DMC1*; *DPH7*; *MEIOB*; *RAD51AP2*; *SCML1*; *SPO11*; *SYCP1*; *SYCP2*; *SYCP3*; *TEX12*; *TEX19* | From puberty onwards | (Guo *et al.*, 2018; Hermann *et al.*, 2018; Wang *et al.*, 2018; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| | Zygotene | *DMC1*; *MEIOB*; *MLH3*; *RAD51AP2*; *SCML1*; *SPO11*; *SYCP1*; *SYCP2*; *SYCP3*; *TDRG1*; *TEX12* | From puberty onwards | (Guo *et al.*, 2018; Hermann *et al.*, 2018; Wang *et al.*, 2018; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| | Pachytene | *CCNA1*; *OVOL1*; *OVOL2*; *PIWIL1*; *POU5F2*; *SYCP2*; *SYCP3* | From puberty onwards | (Guo *et al.*, 2018; Hermann *et al.*, 2018; Wang *et al.*, 2018; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| | Diplotene/meiotic divisions | *CCNA1*; *OVOL1*; *OVOL2*; *POU5F2*; *SIRPG*; *SLC26A3* | From puberty onwards | (Guo *et al.*, 2018; Hermann *et al.*, 2018; Wang *et al.*, 2018; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |

(continued)

**Table I Continued**

| Cell type | Cell subtype (Aliases) | Enriched marker genes | Developmental steps at which respective cell types were identified | References |
|---|---|---|---|---|
| **Spermatid** | Early spermatids | *PRM1*; *PRM2*; *SIRT2*; *TEX29*; *TNP1* | From puberty onwards | (Guo *et al.*, 2018; Hermann *et al.*, 2018; Wang *et al.*, 2018; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| | Late spermatids | *PRM1*; *PRM2*; *TNP1* | From puberty onwards | (Guo *et al.*, 2018; Hermann *et al.*, 2018; Wang *et al.*, 2018; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| **Immature Sertoli cells** | Stage a (Immature Sertoli cells 1) | *AMH*; *EGR3* | From infancy onwards | (Guo *et al.*, 2020, 2021; Zhao *et al.*, 2020) |
| | Immature Sertoli cells 2 | *ATP5E*; *TOMM7* | From infancy onwards | (Guo *et al.*, 2020) |
| | Stage b | *S100A13*; *ENO1*; *BEX1* | From infancy onwards | (Zhao *et al.*, 2020) |
| **Mature Sertoli cells** | Stage c (Mature Sertoli cells) | *BEX1*; *CITED1*; *CST9L*; *DEFB119*; *FATE1*; *HOPX*; *SOX9* | From pre/puberty onwards | (Guo *et al.*, 2018, 2020; Wang *et al.*, 2018; Sohni *et al.*, 2019; Zhao *et al.*, 2020; Di Persio *et al.*, 2021) |
| **Leydig/peritubular myoid cells precursor** | Leydig/PTMs (PMCs) precursor | *MAFB; NR4A1* | From birth onwards | (Guo *et al.*, 2020, 2021) |
| **Peritubular myoid cells** | PTMs (PMCs) | *ACTA2*; *DCN*; *MYH11* | From birth onwards | (Guo *et al.*, 2018; Wang *et al.*, 2018; Sohni *et al.*, 2019; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| | Fibrotic PTMs (Fibrotic PMCs) | *CFD*; *CLEC3B*; *DCN* | In adulthood | (Di Persio *et al.*, 2021) |
| **Leydig cells** | Mature Leydig cells | *HSD17B3*; *INSL3*; *STAR* | In adulthood | (Guo *et al.*, 2018; Di Persio *et al.*, 2021) |
| **Endothelial cells** | Endothelial cells | *VWF*; *PECAM1* | From birth onwards | (Guo *et al.*, 2018; Sohni *et al.*, 2019; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| **Pericytes** | Muscular pericytes | *MCAM*; *MUSTN1*; *NOTCH3* | In adulthood | (Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| | Fibroblastic pericytes | *COL4A1*; *NOTCH3* | In adulthood | (Shami *et al.*, 2020) |
| **Immune cells** | T cells | *CD3D*; *CD3E*; *CD52*; *CD69*; *GZMA*; *NKG7*; *TRAC* | In adulthood | (Shami *et al.*, 2020; Di Persio *et al.*, 2021) |
| | Macrophages | *CD14*; *CD68*; *CD163*; *C1QA*; *CSF1R*; *LYZ*; *TYROBP* | From birth onwards | (Guo *et al.*, 2018; Wang *et al.*, 2018; Shami *et al.*, 2020; Di Persio *et al.*, 2021) |

SSC, spermatogonial stem cell.

The advent of unbiased scRNA-seq analyses has facilitated an unprecedented sub-classification of spermatogonia based on their transcriptional profiles (Table I). It is of note that all available datasets have separated the spermatogonial compartment into undifferentiated (UTF1$^{+}$/MKI67$^{-}$) and differentiating spermatogonia (KIT$^{+}$/MKI67$^{+}$) (Guo *et al.*, 2018; Hermann *et al.*, 2018; Wang *et al.*, 2018; Sohni *et al.*, 2019; Shami *et al.*, 2020; Di Persio *et al.*, 2021). In particular, the undifferentiated spermatogonia have been further sub-classified

based on gene expression signatures into two transcriptionally distinct spermatogonial subtypes (Table I) termed State 0 (SSC1) and State 1 (SSC2). Two research teams found that the State 0 cells consist of three sub clusters, namely State 0 (SSC1B), State 0A (SSC1A) and State 0B (SSC1C) (Sohni *et al.*, 2019; Tan *et al.*, 2020; Di Persio *et al.*, 2021). Importantly, none of the transcriptional states was defined by a specific cell cycle phase (Guo *et al.*, 2018; Sohni *et al.*, 2019). To date, it is unknown whether the four transcriptional states can also be identified at protein level. To this end, co-localization analyses of state-specific markers in testicular tissue sections and whole mounts need to be performed. In order to assess if the transcriptional states display distinct stem cell potential, sorting and transplantation assays need to be performed. While these functional aspects remain to be addressed, studies from different groups agree with each other in the interpretation that a population of spermatogonia, expressing *PIWIL4*, *EGR4*, *PLPPR3* and *TSPAN33*, among other marker genes, are at the origin of spermatogonial differentiation in the adult human testis (Guo *et al.*, 2018; Hermann *et al.*, 2018; Wang *et al.*, 2018; Sohni *et al.*, 2019; Shami *et al.*, 2020; Di Persio *et al.*, 2021). This theory is corroborated by the fact that the transcriptional profile of this cell state is highly similar to germ cells obtained from testicular tissues of 1-year-old boys (Guo *et al.*, 2018, 2021). Moreover, the stem cell potential of two subpopulations sorted for the State 0 markers TSPAN33$^+$ (Shami *et al.*, 2020) and PLPPR3$^+$ (Tan *et al.*, 2020) was demonstrated by their ability to form spermatogonial colonies following germ cell transplantation. While spermatogonial clusters at the origin of differentiation express *PIWIL4*, the other states of undifferentiated spermatogonia express *FGFR3* (State 0A) and *GFRA1/NANOS3* (State 1), supporting the existence of a transcriptionally heterogeneous stem cell pool (Guo *et al.*, 2018; Sohni *et al.*, 2019; Shami *et al.*, 2020; Di Persio *et al.*, 2021) (Fig. 2A). This setup of transcriptionally distinct spermatogonial states seems to be maintained throughout male reproductive life as comparisons of spermatogonia from young (<22 years) and old (>62 years) men did not reveal differentially expressed genes (Nie *et al.*, 2022).

Importantly, while the different states show highest expression of one or the other marker gene, low expression of these marker genes can also be detected in neighboring states suggesting a gradual transition from one transcriptional state to another, and the ability of these cells to maintain a ready-to-react state, which could allow them to quickly respond to changes in the niche/microenvironment (Fig. 2B). Supporting this hypothesis, RNA velocity analyses of the spermatogonial states from men with intact spermatogenesis have been performed. These analyses infer the short term developmental trajectories of the cells based on the ratio of un-spliced and spliced transcripts, assuming that the cells expressing un-spliced transcripts are earlier in differentiation compared to those expressing the spliced version (Bergen *et al.*, 2020). These analyses revealed that within the same spermatogonial state different cells display distinct velocity vectors, with some cells pointing toward and others against the course of differentiation (Guo *et al.*, 2018; Di Persio *et al.*, 2021).

Intriguingly, evaluation of cells with an $A_{dark}$ morphology by Di Persio *et al.* (2021) revealed that only 3.5% of PIWIL4$^+$ spermatogonia show characteristics of reserve stem cells. What is more, all transcriptionally defined sub-types of undifferentiated spermatogonia (UTF1$^+$, PIWIL4$^+$, FGFR3$^+$, GFRA1$^+$); have the capacity to reserve $A_{dark}$ spermatogonia, although at slightly different proportions ($A_{dark}$ among: UTF1$^+$=78.4%; PIWIL4$^+$=5.8%; FGFR3$^+$=5.7%; GFRA1$^+$=18.1%; NANOS3$^+$=0%) (Di Persio *et al.*, 2021) (Fig. 2A). Importantly, to evaluate whether $A_{dark}$ spermatogonia express multiple marker genes simultaneously further analyses are needed. In sum, the transcriptionally defined origin of germ cell differentiation (PIWIL4$^+$ spermatogonia) is not equivalent to the morphologically defined $A_{dark}$ reserve stem cells (Di Persio *et al.*, 2021). The spermatogonial compartment apparently consists of distinct transcriptional states, supporting the model of a heterogeneous and dynamic stem cell pool (Krieger and Simons, 2015) (Fig. 2A). The molecular profile of the $A_{dark}$–population seems to reflect the transcriptional profiles of the different states, a strategy potentially facilitating a reboot of the heterogeneous stem cell system following gonadotoxic impact. If this assumption is correct, it would call into question the approach of isolating individual spermatogonial subtypes to assess their SSC potential via germ cell transplantation assays. Provided that spermatogonia within the undifferentiated compartment hold this potential, each subpopulation should prospectively re-establish the different transcriptional states following transplantation to re-create an equilibrium within the heterogeneous stem cell pool.

Following the idea of 'failure reveals design', comparative analyses of tissues with complete spermatogenesis and those with drastically reduced sperm production, from cryptozoospermic men, have been performed by Di Persio *et al.* (2021). Unexpectedly, according to evaluations at the RNA and protein levels, the SSC compartment contains significantly more PIWIL4$^+$ spermatogonia in tissues with impaired spermatogenesis than in their control counterparts (Di Persio *et al.*, 2021) (Fig. 3). The following three scenarios can be considered leading to this observation: a failure in the differentiation process, trapping an unusually large fraction of spermatogonia in the most undifferentiated state; an increased proportion of spermatogonia reverting back to the PIWIL4$^+$ state; or a higher proliferation rate of PIWIL4$^+$ spermatogonia (Di Persio *et al.*, 2021). Another revelation from the comparative analyses of normal and cryptozoospermic patient samples was the significantly reduced number of $A_{dark}$ spermatogonia in the latter group. Keeping a larger proportion of spermatogonia in a ready-to-react state ($A_{pale}$) rather than reserving them as $A_{dark}$ spermatogonia can again be considered as a compensatory mechanism to the reduced sperm output but may also place the fertility of cryptozoospermic patients additionally at risk in case of testicular insult (Di Persio *et al.*, 2021) (Figs 2B and 3). To assess whether the described reduction of $A_{dark}$ spermatogonia is a common feature in cases of male infertility, analyses need to be expanded to other patient groups.

## A single-cell view of testicular somatic cells in (in)fertile men

Application of scRNA-seq technology is helping to reveal communication pathways between germ and somatic cells in the normal situation and, moreover, to shed light on the molecular remodeling of somatic cells in cases of male infertility. Single-cell studies reported transcriptional profiles of the functionally distinct somatic cell types: Sertoli, Leydig and PTM cells. In addition to these testis-specific somatic lineages, the testicular interstitium is enriched with blood vessels and immune cells, also captured by scRNA-seq-analyses. Considering these diverse somatic cell types, which potentially impact spermatogonial

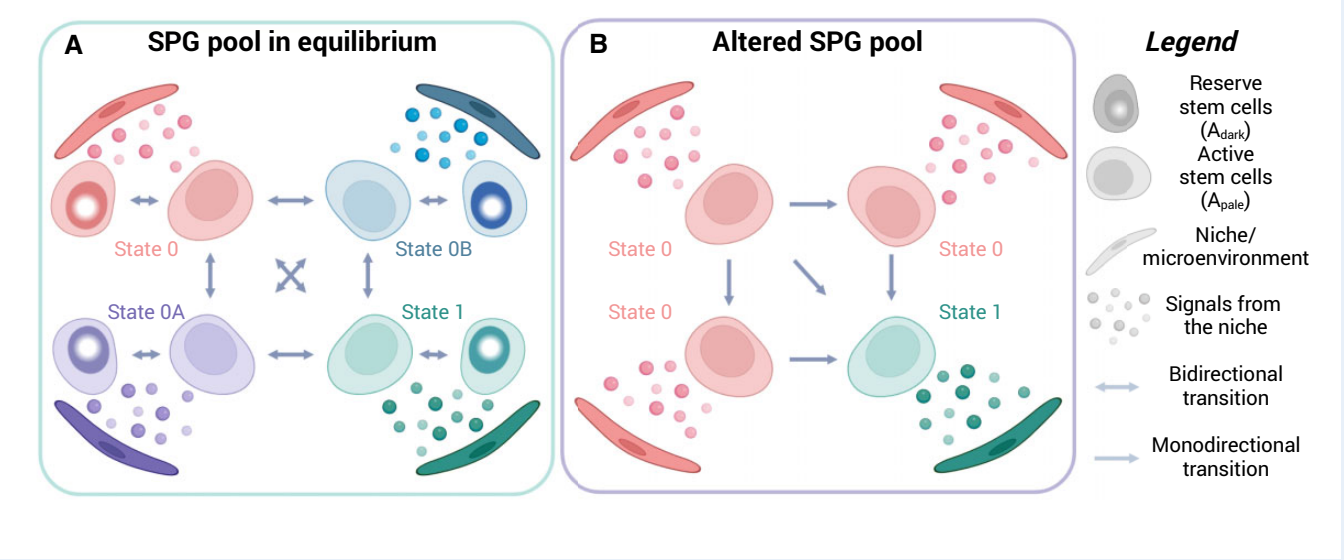



**Figure 2. Model of the undifferentiated human spermatogonial pool dynamics in homeostasis and following an alteration of the microenvironment.** (**A**) During tissue homeostasis, the adult pool of spermatogonia (SPG) is highly heterogeneous in terms of transcriptional and morphological profiles. The pool is highly dynamic and the cells may transfer reversibly from one transcriptional or morphological state to the other based on different signals associated with the microenvironment. By 'microenvironment' we refer here to all the testicular cells exchanging signals with the stem cell pool including all the testicular somatic as well as germ cells. This flexibility may allow the cells to respond to different stimuli while still maintaining their stem cell properties. (**B**) In case of reduced sperm production, we propose a model according to which the altered microenvironment results in an altered crosstalk with the SPG pool. Based on data obtained from men with cryptozoospermia, the SPG pool polarizes in this altered microenvironment by reducing its morphological (reduction of $A_{dark}$) and transcriptional heterogeneity and placing more spermatogonia in a ready-to react state, potentially in a futile attempt to counterbalance the limited number of sperm being produced. It remains to be elucidated whether: the observed alterations of the SPG pool are a cause or consequence of male infertility phenotypes; and SPG can re-establish the heterogeneous stem cell pool if placed back into an intact microenvironment.

function, scRNA-seq datasets are providing novel insights regarding the communication pathways between spermatogonia and their niche in germ cell function and dysfunction.

## The molecular profile of testis-specific Sertoli cells in male (in)fertility

Sertoli cells are considered the nurse cells of the testis as they provide physical and nutritional support to germ cells within the seminiferous tubules. Moreover, they form the blood–testis barrier, which provides an immune-privileged environment for germ cells (França *et al.*, 2016). Owing to their size and complex cellular architecture, the analysis of adult human Sertoli cells in testicular tissues with full spermatogenesis has proven particularly challenging. As a consequence, a low representation of this cell type arising from the single-cell encapsulation process has been reported (Guo *et al.*, 2018; Sohni *et al.*, 2019). Despite this limitation, multiple studies have identified clusters of cells expressing known Sertoli cell marker genes, such as *AMH*, *SOX9* and *DMRT1*, thereby facilitating identification of novel marker genes including *BEX1*, *CITED1* and *FATE1* (Table I) (Guo *et al.*, 2018; Wang *et al.*, 2018; Sohni *et al.*, 2019). What is more, analyses of testicular tissues from neonatal, prepubertal, pubertal and adult have revealed transcriptionally distinct subtypes of Sertoli cells (Guo *et al.*, 2020; Zhao *et al.*, 2020). Zhao *et al.* (2020) identified three subtypes, which are referred to as stages a, b and c. While stages a and b are regarded as immature Sertoli cell stages, stage c is considered the mature Sertoli cell stage (Table I). During human development, the number of stage a and stage b Sertoli cells decreases until pre-puberty, while the mature Sertoli cell stage c is emerging.

At transcriptional level, immature/stage a Sertoli cells show a high mRNA expression level of mitotic and metabolism related genes, in particular those associated with oxidative phosphorylation. In contrast, mature Sertoli cells (stage c) show low expression levels of mitotic and metabolism-related genes but higher levels related to the glycolytic pathway. Stage b shows an intermediate molecular signature. Overall, these results suggest that Sertoli cells pass three distinct developmental stages during which they become quiescent, reduce their metabolic activity and switch from an oxidative-phosphorylation based to a glycolysis-based metabolism. Furthermore, few immature Sertoli cells of both stage a and stage b are present in the adult testis (Zhao *et al.*, 2020). However, the presence of the described human Sertoli cell subtypes and their developmental path remains a matter of debate, as an independent study by Guo *et al.* (2020) identified also two distinct immature Sertoli cell stages (immature Sertoli cells 1 and 2, Table I) but suggest that they converge into the mature form at the time of puberty and are not present in adult testicular tissues. Further experiments will be necessary to confirm the postnatal developmental path of human Sertoli cells.

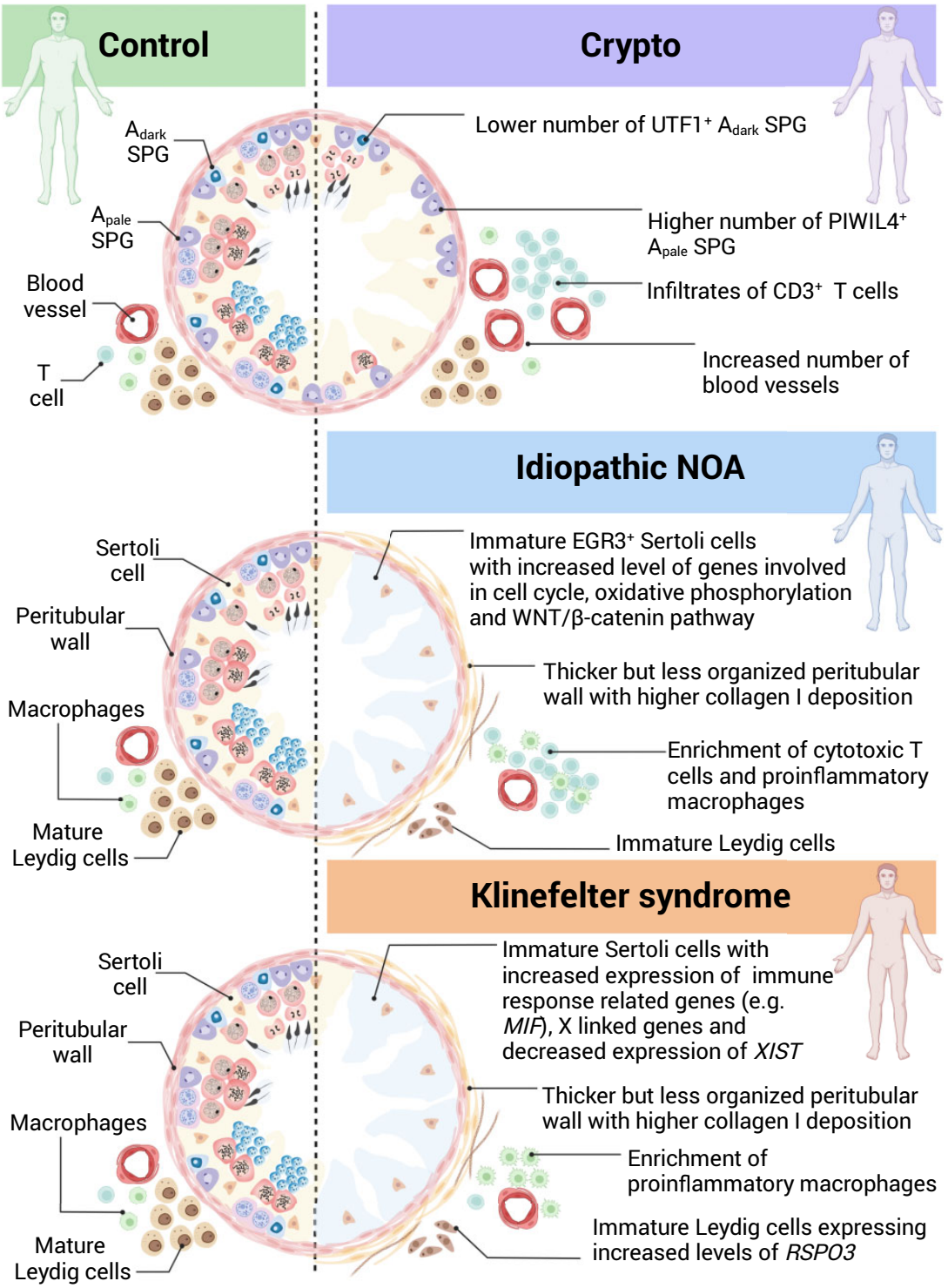



**Figure 3. Schematic summary of the cellular and molecular alterations identified using single-cell RNA-sequencing of human testicular single-cell suspensions in three types of male infertility.** Crypto, cryptozoospermia; NOA, non-obstructive azoospermia; AZF, azoospermia factor; SPG, spermatogonia; UTF1, undifferentiated embryonic cell transcription factor 1; PIWIL4, Piwi-like RNA-mediated gene silencing 4; CD3, cluster of differentiation 3; EGR3, early growth response 3; WNT, Wingless/Integrated; MIF, macrophage migration inhibitory factor; XIST, X-inactive specific transcript; RSPO3, R-spondin 3.

To gain insight into transcriptional changes in Sertoli cells associated with male infertility, comparative analyses have been performed. Intriguingly, analysis of the transcriptional Sertoli cell profiles performed in two independent studies in patients with idiopathic non-obstructive azoospermia (iNOA) revealed signs of maturation arrest (Fig. 3). Specifically, cells showed higher expression of cell cycle associated genes and genes involved in the oxidative phosphorylation metabolism when compared with mature Sertoli cells isolated from patients with obstructive azoospermia and normal spermatogenesis (Zhao *et al.*, 2020; Alfano *et al.*, 2021). Moreover, Zhao *et al.* (2020) also identified high expression levels of early immature Sertoli cell marker genes, including *EGR3* and *FOSB*, and low expression levels of adult marker genes such as *HOPX* and *TSC22D1*. Zhao *et al.* (2020) suggested that this de-differentiation of Sertoli cells is regulated via the WNT/β-catenin signaling pathway, which is corroborated by data showing that the inhibition of this pathway *in vitro* blocked Sertoli cell proliferation, decreased the expression of immature marker genes (e.g. *AMH*, *INHA*, *EGR3* and *JUN*) and increased expression of adult Sertoli cell marker genes (e.g. *HOPX* and *TSC22D1*). Independent experiments are needed to confirm this finding and to explore the interplay of the WNT pathway with other signaling pathways relevant for germ and somatic cell function. ScRNA-seq has also been used to investigate the transcriptional profile of Sertoli cells from patients carrying azoospermia factor a deletions or presenting with Klinefelter syndrome (Laurentino *et al.*, 2019; Zhao *et al.*, 2020; Mahyari *et al.*, 2021) (Fig. 3). In both cases, Sertoli cells showed similar signs of maturation arrest as the cells in men with iNOA. Additionally, two independent groups showed that the Klinefelter Sertoli cells upregulate immune response related genes (e.g. *MIF*), suggesting that aberrant immune cell activation might be a universal pathogenic process resulting from sex chromosomal dysfunction (Zhao *et al.*, 2020; Mahyari *et al.*, 2021). Moreover, Klinefelter Sertoli cells showed higher expression of X-linked genes (Zhao *et al.*, 2020; Mahyari *et al.*, 2021), although not to the expected extent. Also, the finding of a reduced expression of *XIST*, a long non-coding RNA involved in chromosome X inactivation, suggests that some Klinefelter Sertoli cells may have an euploid XY karyotype (Mahyari *et al.*, 2021).

## The molecular profile of testis-specific Leydig and peritubular myoid cells in male (in)fertility

Adult Leydig cells are located in the interstitial space between seminiferous tubules and produce testosterone following stimulation by LH. In accordance with this, Leydig cells have high expression levels of genes involved in the steroidogenic pathway including *STAR* and *HSD17B3* (Table I) (Guo *et al.*, 2018; Wang *et al.*, 2018; Sohni *et al.*, 2019). Distinct from this are the PTMs, which surround the seminiferous tubules in multiple layers and create the peristaltic movements necessary to move spermatozoa toward the epididymis. Moreover, the human PTMs secrete GDNF, relevant for SSC proliferation (Spinnler *et al.*, 2010), as well as a plethora of molecules involved in other signaling pathways including *PDGFRA*, *EGFR*, *PTCH1* and *PPARA* (Guo *et al.*, 2018; Sohni *et al.*, 2019; Shami *et al.*, 2020). PTMs are also of crucial importance for secretion of extracellular matrix (ECM) components, relevant for the communication between germ cells and somatic cells (Mayerhofer, 2013). In line with this, independent single-cell datasets have demonstrated expression of *ACTA2* or *MYH11*, as genes related to muscle contraction (Table I) (Guo *et al.*, 2018; Wang *et al.*, 2018; Sohni *et al.*, 2019). Furthermore, single-cell data from Di Persio *et al.* (2021) identified two subtypes of adult PTMs, with likely distinct functions. While one subpopulation is expressing the aforementioned muscle contraction related genes, the other subpopulation is possibly involved in ECM deposition as it displays high expression levels of genes relevant for ECM components (*DCN*, *CFD* or *CLEC3B*, Table I) as well as low expression levels of steroidogenic markers (i.e. *HSD17B3* and *STAR*) compared to the cluster of Leydig cells (Di Persio *et al.*, 2021); these two functionally distinct adult PTMs (PTM ACTA2$^{+}$ and fibrotic PTM DCN$^{+}$, respectively) were suggested to be located in different layers surrounding the human seminiferous tubules (Mayerhofer, 2013). Interestingly, other single-cell studies (Guo *et al.*, 2018; Sohni *et al.*, 2019; Shami *et al.*, 2020) have not found these cells in adult human testis but rather a population of immature Leydig cells expressing genes coding for ECM components as well as *DLK1*, which is expressed in fetal Leydig cells and in spindle-shaped cells located adjacent to the peritubular cells of the seminiferous tubules in the adult human testis (Lottrup *et al.*, 2014). We speculate that these immature Leydig cells may be the same cell population as fibrotic PTMs that have been labeled differently. Further studies are therefore needed to evaluate and clarify the nature and function of these cells in the adult human testis. Interestingly, developmental scRNA-seq studies from Guo *et al.* (2020, 2021) show the existence of a common Leydig-PTM progenitor. These cells persist in the testis until puberty and express *MAFB* and *NR4A1* (Guo *et al.*, 2020), which have been identified as Leydig–PTM progenitor marker genes also in the mouse (DeFalco *et al.*, 2011; Wen *et al.*, 2016). After puberty, the two lineages segregate and acquire a cell type-specific transcriptional profile, specifically *DLK1* and *IGF1* for the Leydig and *ACTA2* and *MYH11* for the PTMs (Guo *et al.*, 2020). Independent evaluations and an increased sample size will be needed to validate the existence of this common progenitor, as well as the pathways and the signals involved in its differentiation into Leydig, PTM and fibrotic PTM cells.

The study of Leydig and PTM cells in male infertility has helped to uncover intriguing insights regarding their molecular alterations. Investigating the transcriptional profile of Leydig cells in men with iNOA, Alfano *et al.* (2021) showed a reduced expression of mature Leydig cell marker genes (*INSL3*, *HSD17B3*, *IGF2*) and increased expression of the immature Leydig cell marker genes *DLK1 and EGR1*, and suggested a delayed or arrested Leydig cell maturation in these patients. Furthermore, PTMs in testes from men with iNOA displayed increased expression of genes involved in ECM deposition and collagen organization (Alfano *et al.*, 2021), in line with previous studies reporting increased levels of fibrosis in iNOA testicular tissues (Adam *et al.*, 2012). A similar dysregulation of ECM-related genes was reported in testicular tissues from cryptozoospermic patients (Di Persio *et al.*, 2021). In-depth histological evaluation of the molecular composition of the ECM layers surrounding the seminiferous tubules of patients with iNOA showed an enrichment of collagen I and absence of collagen IV, which resulted into tubules with a thicker but less organized peritubular wall compared to controls (Alfano *et al.*, 2021) (Fig. 3). One scRNA-seq study using testicular tissues from Klinefelter men revealed an increased proportion of immature Leydig cells and increased expression of genes involved in ECM deposition and regulation of the inflammatory response (Mahyari *et al.*, 2021). Interestingly, Leydig cells

in Klinefelter tissues expressed increased levels of *RSPO3*, a potentiator of the WNT signaling pathway whose receptors are downregulated in Klinefelter Sertoli cells (Mahyari *et al.*, 2021) (Fig. 3). These findings support the theory suggested by Zhao and colleagues, that the WNT/β-catenin signaling pathway may have a role in controlling Sertoli cell maturation and that the Leydig cells may be involved in the regulation of this process.

### Blood vessels and immune cells in the interstitial space

In addition to the testis-specific cell types, scRNA-seq of testicular tissues from men with normal spermatogenesis has also captured transcriptional profiles of endothelial cells, muscular and fibroblastic pericytes, macrophages and T lymphocytes (Shami *et al.*, 2020; Di Persio *et al.*, 2021) (Table I). Evaluating the transcriptional profile of testicular macrophages showed high expression levels of anti-inflammatory factor (*IL10*) and low levels of inflammatory factors (*IL1A*, *IL17C*, *IL12A*, *IL22*, *IL11* and *IL6*), suggesting an enrichment of M2 macrophages in men with normal spermatogenesis. Interestingly, these cells show high expression levels of the inflammatory factor receptors *IL13RA1* and *IL1R2* suggesting the potential to quickly respond to inflammatory stimuli (Zheng *et al.*, 2021). ScRNA-seq analyses of testicular tissues from men with iNOA, cryptozoospermic and Klinefelter syndrome all showed similar signs of inflammation. Men with iNOA showed an enrichment of cytotoxic T cells ($CD8^+$), which express granzyme K and M (*GZMK*, *GZMM*) but also proinflammatory chemokines (*CCL4*, *CCL5*) and *IL-32* (Alfano *et al.*, 2021). A similar T cell enrichment has been reported in cryptozoospermic patients together with an increase in the number of blood vessels per $mm^2$ of testicular tissue (Di Persio *et al.*, 2021). Furthermore, macrophages in iNOA tissues displayed an overexpression of genes involved in proinflammatory pathways, which was also found in testicular tissues of patients with Klinefelter syndrome (Mahyari *et al.*, 2021). Overall, the increased vascularization, the pro-inflammatory macrophage polarization and the enrichment of cytotoxic T lymphocytes are all signs of chronic local inflammation of testicular tissue, a feature shared by different infertility types and identified by multiple groups. This altered testicular microenvironment, which may impact spermatogenesis, is an aspect that remains to be further elucidated.

## Germ-soma communication in testicular tissues of (in)fertile men

Interactions between germ and somatic cells as well as among somatic cell types are essential for the development and function of human testicular tissues. Multiple scRNA-seq studies highlighted signaling pathways, including NOTCH, KIT, HEDGEHOG, WNT, retinoic acid, activin/inhibin/TGFβ/BMP, GDNF and FGF, as the most relevant in testicular tissues with normal spermatogenesis (Sohni *et al.*, 2019; Guo *et al.*, 2020; Shami *et al.*, 2020; Zhao *et al.*, 2020; Di Persio *et al.*, 2021). Interestingly, the highest inferred number of ligand-receptor based interactions in human was found between spermatogonia and somatic cells, suggesting a high degree of signal exchange between these two compartments (Shami *et al.*, 2020). As an example, the activin/Inhibin pathway has been shown to be of relevance during puberty. While Sertoli cells downregulate *INHA* (subunit of inhibin A and B) as they mature, Leydig cells increase the expression of *INHBA* (subunit of activin A and AB) after puberty, suggesting an increased activin and decreased inhibin activity in the adult (Guo *et al.*, 2020; Zhao *et al.*, 2020). Importantly, human spermatogonia express activin receptors (*ACVR1B*, *BMPR1B*, and *ACVR2B*). However, only the most undifferentiated spermatogonial states (State 0 and 1) express key inhibitors of activin signaling (*FST*, *BAMBI* and *NOG*) (Guo *et al.*, 2020; Zhao *et al.*, 2020). The activin pathway may, therefore, have a role in regulating the transition from undifferentiated to differentiating spermatogonia in the human (Guo *et al.*, 2020). Interestingly, in the mouse this transition has been shown to be regulated by the retinoic acid pathway via induction of STRA8 expression, while the expression of *STRA8* in the human is confined to the preleptotene stage, limiting its role to inducing the meiotic entry and suggesting species-specific regulatory mechanisms (Guo *et al.*, 2020; Shami *et al.*, 2020). The TGFβ/activin pathway together with the steroidogenic and the WNT/β-catenin pathways have been shown to play an important role also during Sertoli cell maturation at puberty and maturation reversion in cases of infertility (Guo *et al.*, 2020; Zhao *et al.*, 2020; Alfano *et al.*, 2021; Mahyari *et al.*, 2021). Moreover, scRNA-seq analyses of the testicular microenvironment in cases of infertility have also reflected signs of fibrosis and inflammation, and an altered composition of the somatic niche. Cell-cell interaction analyses in cryptozoospermic patients comparing to controls have revealed a stronger interaction between chemokines secreted by blood vessel cells, macrophages and T lymphocytes (*CCL2-3-4-5-14* and *CCL3L1*) and their receptor (*ACKR2*) expressed by undifferentiated spermatogonia, suggesting a direct impact of the pro-inflammatory microenvironment on spermatogonial function (Di Persio *et al.*, 2021). In conclusion, multiple groups have considered the influence of signals from the somatic microenvironment on germ cell populations. However, the potential relevance of ligands produced by germ cells and their impact on somatic cell maturation remains underexplored. This bi-directional interplay between soma and germline may be relevant during development as well as in cases of male infertility, considering the alterations in somatic cell transcriptional profiles outlined above. Further experiments are needed to close this knowledge gap.

## Conclusion

In this review, we demonstrate that scRNA-seq of human testicular tissues has tremendously expanded our knowledge regarding the SSC compartment and the testicular somatic cells. This approach allowed the identification of novel germ and somatic cell transcriptional states and their alterations in male infertility, and it provided first insights into the molecular interplay between the germ and the somatic cell compartment. It remains a key research question how alterations of this interplay lead to male infertility.

The impact of this approach could be even further optimized if a common ‘pipeline’ would be implemented including sample preparation, cluster assignment and a common nomenclature. Thereby, data reproducibility could be further increased and the interpretation of results among different groups could be homogenized. Such a pipeline

would allow to further increase the impact of this approach also for translational research.

We consider the following clinical implications of understanding the molecular changes unveiled by scRNA-seq studies. First, the identification of pathways controlling human germ and somatic cell differentiation is a necessity for the development of protocols for human *in vitro* spermatogenesis, which would be an important aspect, i.e. for fertility preservation programs. Second, novel marker genes identified in the frame of scRNA-seq studies can be considered promising candidate genes for male infertility and may be included in genetic screening in the emerging field of reproductive genetics. Finally, and prospectively, the discovery of specific dysfunctional molecular pathways can pave the way for the design of new treatment strategies, which specifically target the altered signal in order to revert the patient phenotype. However, despite the clear advantages of high-throughput scRNA-seq, the following limitations need to be considered for interpretation of respective datasets and with regard to possible future use in the clinic. First, the process to obtain a single-cell suspension requires the dissolution of 3D tissue structures. While the microenvironments of SSC crucially impact their function (Krieger and Simons, 2015), available scRNA-seq datasets are inherently missing this layer of spatial information. Spatial transcriptomics (Chen *et al.*, 2021; Marx, 2021) may prospectively help to fill this information gap in particular as the resolution is continuously improving. Moreover, ultra-high content imaging approaches are required to determine whether the findings at RNA level, in particular those regarding the existence of cellular subtypes, are corroborated by protein data. A second limitation lies in the poor representation of selected testicular cell types in single-cell datasets. Sertoli cells, which are characterized by their complex 3D structure and comparatively large cellular size in adult testicular tissues with full spermatogenesis, are not well represented in respective datasets (Sohni *et al.*, 2019; Guo *et al.*, 2020). Finally, while the resource of scRNA-seq datasets has been continuously growing in recent years, distinct phases of testicular development and male infertility phenotypes are represented only by individual samples. An expansion of available scRNA-seq datasets and integration with additional levels of information (i.e. whole genome sequencing, whole genome bisulfite sequencing, assay for transposase-accessible chromatin (ATAC) sequencing), will prospectively enable an even more complete picture of the molecular dynamics associated with testicular development and (dys)function.

## Supplementary data

Supplementary data are available at *Human Reproduction* online.

## Data availability

No new data were generated or analyzed in support of this research.

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## Authors' roles

S.D.P. and N.N.: literature search and review, writing and revising of text and tables.

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## Conflict of interest

The authors declare no competing interests.

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