Editorial

Stem Cell INVESTIGATION

Epigenomic and single-cell profiling of human spermatogonial stem cells

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The balance between quiescence and proliferation, self-renewal and differentiation of stem cells is critical for maintaining tissue homeostasis and avoiding premature stem/progenitor cell exhaustion (1,2). Spermatogenesis is a well-characterized stem cell-dependent process. Spermatogonial stem cells (SSCs), which have the ability to self-renew and differentiate to form sperm, are the foundation for subsequent rounds of spermatogenesis in testes (3). The genetic and epigenetic integrity of SSCs are critical for long-term male fertility and the health of offspring.

Infertility has become an increasing problem for (about 15%) human couples worldwide, and many male-related infertility cases result from impaired undifferentiated spermatogonia (4,5). In addition, people from North America, Europe, Australia and New Zealand have significant (about 50%) drop in sperm counts in ejaculated semen (6). Further insights into the cellular and molecular properties of human spermatogonia stem cells (hSSCs) and the mechanisms for the development of functional germ cells are necessary to understand the rising rates of infertility.

Recently, Guo *et al.* (7) from the Cairns laboratory took up this challenge and reported dynamic cell fate commitment during differentiation of hSSCs with comprehensive epigenome and transcriptome analysis. They demonstrated that (I) open chromatin in hSSCs correlates with binding motifs for pioneer factors and hormone receptors; (II) differentiation of hSSCs can be classified into four sequential cellular/developmental states and (III) a key transition involves the cell cycle, transcriptional factors, signaling pathways and the metabolism. These data will not only fill the knowledge gap regarding epigenomic aspects of hSSCs, but will also provide insights into the mechanistic differences between human and mouse spermatogenesis, in which intense mechanistic studies have already been done using genetically modified mouse models.

While it is conceptually straightforward that SSCs refer to the population bearing the abilities for self-renewal, differentiation and shifting into quiescence status, it is difficult to identify a specific population representing SSCs alone. The identity of SSCs in mammals has been debated for decades. It is technically challenging to validate "stemness". The standard method for a functional test in mice is to combine lineage tracing and transplantation of SSC candidates to busulfan treated testis, which then

demonstrate their ability to repopulate the pretreated germ cell free seminiferous tubules and perform full spermatogenesis (8). In humans, xenotransplanting hSSC candidate to busulfan treated mouse testis is the only method. However, the murine SSC niche cannot maintain hSSCs efficiently. Most are lost by differentiation preceded by active proliferation (which is probably associated with differentiation). Although several specific markers enriched in certain populations of undifferentiated spermatogonia have been identified, the heterogeneity and plasticity of undifferentiated spermatogonia presumably underlie the stem cell pool in testes rather than comprising a homogenous cell population with a definitive marker of SSC (9).

Markers for SSC-containing spermatogonia population include THY1, ID4, GFRA1, NANOS2, OCT4, PLZF, and DNMT3L for mice (8,10-14), and SSEA4, GFRA1, BCL6, FGFR3, ID4, SALL4, and ETV5 for humans. Only a portion of THY1+ cells or SALL4+ cells, for example, contain SSC activity (15). These markers are usually enriched in the A-single to A-align undifferentiated spermatogonia based on whole-mount immunostaining of seminiferous tubules, and are expressed in spermatogonia directly attached to the basal membrane of the seminiferous tubules based on immunohistochemical staining of testis sections (8). With the heterogeneous nature of the spermatogonia population, even the best markers identified so far could contain a certain percentage of SSCs, but not every single cell from the population exhibits full SSC function. In addition, the undifferentiated spermatogonia may retain plasticity. While it is generally accepted that A-single spermatogonia have the SSC property, mouse A-paired to A-aligned cells are able to break down to A-single status (16). Furthermore, Ngn3-positive transit amplifying cells have the ability to dedifferentiate into SSCs under an injury-repair based model (17).

One of the most effective ways forward for tackling the detailed property of SSCs would be to take advantage of the single cell techniques to dissect the heterogeneous issue of hSSCs containing the spermatogonia population, as demonstrated by this recent milestone paper from the Cairns laboratory (7). They isolated the SSEA4+ hSSCs and c-KIT+ spermatogonia from the testis of five patients experiencing idiopathic pain, not related to cancer or major inflammation. The choice of SSEA4+ cells to represent hSSCs was based on the following criteria: (I) only a small number of spermatogonia are positive; (II) SSEA4-positive cells exhibit higher telomerase activity; (III) they colonize the murine testes (15), suggesting at least a portion of cells from this population have SSC properties.

In single cell transcriptome analysis of SSEA4+ and c-KIT+ cells, potential intermediate/transitional states were detected between SSEA4+ and c-KIT+ cells, and a "pseudotime model" of hSSCs differentiation, based on the deduced sequential gene expression gathered from individual cells, has been proposed (7). The development from SSEA4+ hSSCs to c-KIT+ spermatogonia can be divided into four states. For state 1, the quiescent state, the SSEA4+ cells express the genes from cluster A (enriched in RNAs encoding transcription factors) and cluster B (enriched in stem cell signaling factors and zinc finger transcription factors). In state 2, cells leaving the quiescent state, cluster A genes are down regulated and cluster D (enriched in genes promoting cell-cycle, replication, and DNA repair factors) are upregulated. In state 3, when cells transit into the differentiation and proliferative state, cluster B genes are down regulated and cluster C (enriched in transcription factors associated with spermatogonial differentiation, signaling receptors, and mitochondrial factors/regulators) are upregulated. In state 4, when cells proceed to the differentiation and proliferative state, they constantly express cluster C and D genes. Many novel hSSCs markers correlating to the quiescent hSSCs can be used in the future to isolate or label this specific subtype of hSSCs for the next round of epigenomic and single cell transcriptomic studies, as well as real-time imaging from explant cultures of human seminiferous tubules. These potential follow-up studies could shed significant light on the enigmatic population critical for long-term male fertility.

Furthermore, key pathways during spermatogonial transition were identified. For example, INTEGRIN/TSPAN and NOTCH/HES1 pathway are highly enriched in hSSCs. By contrast, NMD, meiosis, and DNA recombination-related gene are highly expressed in KIT+ cells. Chromatin factors including the PRC1 complex, which regulates expression of germline genes in mouse spermatogonia (18), are also upregulated in hSSCs. However, unlike mouse SSCs, POU5F1 (OCT4) expression was not detected in hSSCs, and the Pou5f1 promoter was highly methylated in hSSCs. Interestingly, while two core pluripotent genes (OCT4/POU5F1 and NANOG) are repressed in both DNA methylation and chromatin levels in hSSCs, other pluripotency factors KLF4, SALL4, TCF3, MBD3, STAT3, and KLF2, are consistently expressed in hSSCs. Hypomethylation and open chromatin in germline-expressed genes (DDX4 and DAZL) confirm the germ cell epigenetic/transcription status of SSEA4+ hSSCs. It seems that hSSCs suppress core pluripotent factors,

OCT4 and *NANOG*, to maintain unipotent germ cell identity while keeping other pluripotent factors active or poised, in preparation to acquire totipotency after fertilization. Another angle to this phenomenon would be that these hSSCs' active pluripotent genes, KLF4, SALL4, TCF3, etc., have other necessary functions in hSSCs, similar but not identical to their role in pluripotent stem cells. For example, the deregulation of SALL4A and SALL4B in *Dnmt3l* KO mice has been associated with failed maintenance of sufficient quiescent SSCs that eventually leads to a germ line exhaustion phenotype (14).

Together, Guo *et al.* (7) demonstrated novel signaling pathways and potential positive and negative feedback loops among a few stage dependently expressed gene clusters during hSSC transition from quiescent to proliferative and from self-renewal to differentiation, based on the single cell transcriptome analysis. One of the most effective ways to functionally validate these findings would be to perform hypothesis driven genetic modifications on cultured hSSCs differentiating *in vitro*. However, while mouse SSCs have been successfully cultured *in vitro* (19-21), this feat has not been achieved yet for hSSCs, due to the difference in the microenvironment needed for maintaining stemness of hSSCs. The hSSC-specific signaling pathways identified in the quiescent and proliferative hSSC population may provide critical insights to facilitate the hSSC culture *in vitro*.

Guo *et al.* (7) further investigated chromatin status in SSCs, which plays a key role in modulating the transition of gene expression determining the process of spermatogenic differentiation. To elucidate these changes, Guo *et al.* profiled DNA methylation [via whole-genome bisulfite sequencing (WGBS)], chromatin accessibility [via ATAC sequencing (ATAC-seq)], and transcriptome (via RNA-seq) from the isolated bulk population of SSEA4+ hSSCs and c-KIT+ spermatogonia. This study is the first global profiling of these features of the cell population containing hSSCs. While hSSCs may not be solely determined by the marker SSEA4, this study is still an important milestone for future studies on human spermatogenesis.

DNA methylation analysis in SSEA4+ hSSCs by WGBS demonstrates that DNA methylation does not markedly change between hSSCs and mature sperm (7), consistent with the study in mice (22). Although these results represent overall features of DNA methylation in human and mouse spermatogenesis, further investigation of DNA methylation in mice identified differentially methylated regions during spermatogonial differentiation that correlates with developmental programs of spermatogenesis (23,24). Therefore, it is possible that DNA methylation is precisely regulated in each stage of human spermatogenesis at specific loci. Starting from this study in hSSCs, detailed investigation on the stage specific regulation of DNA methylation in human spermatogenesis is warranted in future studies.

By ATAC-seq analysis in hSSCs, Guo *et al.* identified a specific motif with high chromatin accessibility in 36,048 hSSCs. They also detected pioneer factor (CTCFL/BORIS, DMRT1, NFYA/B) and hormone receptor (HRE, GR, AR) binding motifs at these regions. Although another study on ATAC-seq analysis in mouse spermatogenesis identified common sites in accessible chromatin (25), the stage of enrichment is different. For example, NFYA/B sites appear in accessible chromatin in meiotic spermatocytes in mice, but not in undifferentiating spermatogonia (25). Therefore, it would be intriguing to speculate that the gene expression programs are distinct between human and mouse spermatogenesis.

Guo *et al.* further identified unique features of repeat elements and demonstrated that the Satellite elements were hypomethylated in hSSCs (7), unlike ES cells and other somatic lineages. Expression of ACRO1 satellites were significantly increased from fertilization onward, providing an interpretation that hypomethylation of ACRO1 satellites in hSSCs poises the ACRO1 satellites to be activated after fertilization. Also, some LTR elements (LTR12C, LTR12D and LTR12E) show moderate chromatin opening in hSSCs that is enriched with NFYA/B motif, but not in ES cells. These elements belong to a superfamily of endogenous retrovirus, the ERV1 superfamily. In mice, transcription of another superfamily of endogenous retrovirus ERVL was associated with germline expression during spermatogenesis and oogenesis (26,27). However, association of ERV1 in gametogenesis has not been reported. Therefore, the study presents a novel feature of repeat elements in the regulation of human gametogenesis distinct from that in mice.

The discoveries and resources from Guo *et al.* shed significant light on the current understanding of states and properties of hSSCs and their derivatives. More importantly, it provides a solid foundation for future experiments. For example, combined with the novel "Abseq" single cell protein profiling methods by barcoding antibodies (28), one can validate the single cell RNA-seq results and further study the protein dynamics of various hSSC subtypes in ultrahigh-throughput fashion. The ultimate goal would be to compile imaging technology and the outcome of the single cell transcriptome/protein profiling result.

The contribution from this recent publication and potential follow up studies from the Carines laboratory can be applied in a stepwise manner: (I) one can take advantage

of the novel markers identified in various spermatogonia states to isolate or label subpopulations for more detailed transcriptomic/proteomic profiling and visualization and to further characterize heterogeneity of hSSCs and their derivatives, as described in more detail above. Molecular profiling of the specific subtypes of somatic niche cells also provide important information; (II) one can establish a novel *in vitro* culture microenvironment with the signaling factors deduced from Guo *et al.* to facilitate functional manipulation and drug screening. The *in vitro* culture system has taught us a great deal about mouse SSC properties. However, as the current culture system constantly selects for faster proliferating cells, it usually under-represents the quiescent population of SSCs, which could be the most critical cell population for long-term male fertility. For example, the DNMT3L+ quiescent SSCs population that compose around a quarter of freshly isolated THY1+ cells in mice (8,14) cannot be found in cultured SSCs. On the other hand, applying 3D culture/printing technology that merges biomaterial with germ cells or even somatic cells to reconstruct the hSSC niche may provide new hope, since certain extracellular matrix components and biomaterials tend to guide stem cells back into a quiescent status (29,30). With the sperm count for a significant portion of the human population decreasing, there is a need to mitigate infertility issues. A successful hSSC culture system would not only be applicable for revealing more basic knowledge, but would also provide a platform for drug screening to secure human fertility. In contrast, driving hSSCs into reversible quiescent status, although a long shot, can also be a potential alternative direction for birth control. The knowledge for guiding SSCs in and out of quiescent status may further be applied to manage farm animals or endangered species with seasonal breeding traits.

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Footnote

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References

1. Wabik A, Jones PH. Switching roles: the functional plasticity of adult tissue stem cells. EMBO J 2015;34:1164-79.
2. Xin T, Greco V, Myung P. Hardwiring Stem Cell Communication through Tissue Structure. Cell 2016;164:1212-25.
3. Oatley JM, Brinster RL. Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 2008;24:263-86.
4. Boivin J, Bunting L, Collins JA, et al. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. Hum Reprod 2007;22:1506-12.
5. Matzuk MM, Lamb DJ. The biology of infertility: research advances and clinical challenges. Nat Med 2008;14:1197-213.
6. Levine H, Jørgensen N, Martino-Andrade A, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis. Hum Reprod Update 2017;23:646-59.
7. Guo J, Grow EJ, Yi C, et al. Chromatin and Single-Cell RNA-Seq Profiling Reveal Dynamic Signaling and Metabolic Transitions during Human Spermatogonial Stem Cell Development. Cell Stem Cell 2017;21:533-46.e6.
8. Tseng YT, Liao HF, Yu CY, et al. Epigenetic factors in the regulation of prospermatogonia and spermatogonial stem cells. Reproduction 2015;150:R77-91.
9. Yoshida S. Elucidating the identity and behavior of spermatogenic stem cells in the mouse testis. Reproduction 2012;144:293-302.
10. Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A 2003;100:6487-92.
11. Buaas FW, Kirsh AL, Sharma M, et al. Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 2004;36:647-52.
12. Sada A, Suzuki A, Suzuki H, et al. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science 2009;325:1394-8.
13. Shoji M, Tanaka T, Hosokawa M, et al. The TDRD9-MIWI2 Complex Is Essential for piRNA-Mediated Retrotransposon Silencing in the Mouse Male Germline. Dev Cell 2009;17:775-87.
14. Liao HF, Chen WS, Chen YH, et al. DNMT3L promotes quiescence in postnatal spermatogonial progenitor cells.

Development 2014;141:2402-13.
15. Izadyar F, Wong J, Maki C, et al. Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. Hum Reprod 2011;26:1296-306.
16. Nakagawa T, Sharma M, Nabeshima Y, et al. Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 2010;328:62-7.
17. Zhang T, Oatley J, Bardwell VJ, et al. DMRT1 is required for mouse spermatogonial stem cell maintenance and replenishment. PLoS Genet 2016;12:e1006293.
18. Maezawa S, Hasegawa K, Yukawa M, et al. Polycomb directs timely activation of germline genes in spermatogenesis. Genes Dev 2017;31:1693-703.
19. Kanatsu-Shinohara M, Ogonuki N, Matoba S, et al. Improved serum- and feeder-free culture of mouse germline stem cells. Biol Reprod 2014;91:88.
20. Huang YH, Chin CC, Ho HN, et al. Pluripotency of mouse spermatogonial stem cells maintained by IGF-1 dependent signaling pathway. FASEB J 2009;23:2076-87.
21. Guan K, Nayernia K, Maier LS, et al. Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 2006;440:1199-203.
22. Hammoud SS, Low DH, Yi C, et al. Chromatin and transcription transitions of mammalian adult germline stem cells and spermatogenesis. Cell Stem Cell 2014;15:239-53.
23. Hammoud SS, Low DH, Yi C, et al. Transcription and imprinting dynamics in developing postnatal male germline stem cells. Genes Dev 2015;29:2312-24.
24. Kubo N, Toh H, Shirane K, et al. DNA methylation and gene expression dynamics during spermatogonial stem cell differentiation in the early postnatal mouse testis. BMC Genomics 2015;16:624.
25. Maezawa S, Yukawa M, Alavattam KG, et al. Dynamic reorganization of open chromatin underlies diverse transcriptomes during spermatogenesis. Nucleic Acids Res 2018;46:593-608.
26. Davis MP, Carrieri C, Saini HK, et al. Transposon-driven transcription is a conserved feature of vertebrate spermatogenesis and transcript evolution. EMBO Rep 2017;18:1231-47.
27. Franke V, Ganesh S, Karlic R, et al. Long terminal repeats power evolution of genes and gene expression programs in mammalian oocytes and zygotes. Genome Res 2017;27:1384-94.
28. Shahi P, Kim SC, Haliburton JR, et al. Abseq: Ultrahigh-throughput single cell protein profiling with droplet microfluidic barcoding. Sci Rep 2017;7:44447.
29. Lee MK, Lin SP, HuangFu WC, et al. Endothelial-derived extracellular matrix ameliorate the stemness deprivation during ex vivo expansion of mouse bone marrow-derived mesenchymal stem cells. PLoS One 2017;12:e0184111.
30. Wong TY, Chang CH, Yu CH, et al. Hyaluronan keeps mesenchymal stem cells quiescent and maintains the differentiation potential over time. Aging Cell 2017;16:451-60.

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