# Fluorescence- and magnetic-activated cell sorting strategies to isolate and enrich human spermatogonial stem cells☆

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**Objective:** To determine the molecular characteristics of human spermatogonia and optimize methods to enrich spermatogonial stem cells (SSCs).
**Design:** Laboratory study using human tissues.
**Setting:** Research institute.
**Patient(s):** Healthy adult human testicular tissue.
**Intervention(s):** Human testicular tissue was fixed or digested with enzymes to produce a cell suspension. Human testis cells were fractionated by fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS).
**Main Outcome Measure(s):** Immunostaining for selected markers, human-to-nude mouse xenotransplantation assay.
**Result(s):** Immunohistochemistry costaining revealed the relative expression patterns of SALL4, UTF1, ZBTB16, UCHL1, and ENO2 in human undifferentiated spermatogonia as well as the extent of overlap with the differentiation marker KIT. Whole mount analyses revealed that human undifferentiated spermatogonia ($UCHL1^{+}$) were typically arranged in clones of one to four cells whereas differentiated spermatogonia ($KIT^{+}$) were typically arranged in clones of eight or more cells. The ratio of undifferentiated-to-differentiated spermatogonia is greater in humans than in rodents. The SSC colonizing activity was enriched in the $THY1^{dim}$ and $ITGA6^{+}$ fractions of human testes sorted by FACS. ITGA6 was effective for sorting human SSCs by MACS; THY1 and EPCAM were not.
**Conclusion(s):** Human spermatogonial differentiation correlates with increased clone size and onset of KIT expression, similar to rodents. The undifferentiated-to-differentiated developmental dynamics in human spermatogonia is different than rodents. THY1, ITGA6, and EPCAM can be used to enrich human SSC colonizing activity by FACS, but only ITGA6 is amenable to high throughput sorting by MACS. (Fertil Steril® 2014;102:566–80. ©2014 by American Society for Reproductive Medicine.)
**Key Words:** Testis, stem cell, FACS, MACS, spermatogonial stem cell, human spermatogonia

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Spermatogenesis is a process that produces millions of sperm each day in postpubertal mammals (1–3). At the foundation of spermatogenesis are spermatogonial stem cells (SSCs) that balance self- renewing divisions with differentiating divisions to maintain the stem cell pool and fuel spermatogenesis, respectively (4–6). Despite their importance to male fertility, there is limited knowledge about the molecular characteristics of the human SSCs, which are typically described as $A_{dark}$ and $A_{pale}$ spermatogonia based on nuclear staining intensity with hematoxylin (6–8).

Most of the information about spermatogonia has been generated using rodent models and although no SSC specific marker has been identified, some markers that are expressed by stem and/or progenitor cells have been described (e.g., GFR$\alpha$1, POU3F1, POU5F1 [OCT4], ZBTB16 [PLZF], NGN3, NANOS2, NANOS3, SOHLH1, SOHLH2, FOXO1, ITGA6 [$\alpha$6-integrin, CD49f], LIN28, ID4, UTF1, CDH1, GPR125, ITGB1 [$\beta$1-integrin, CD29], EPCAM [CD326], CD9, and THY1 [CD90]) (9–38). Rodent SSCs are only definitively identified by their ability to produce spermatogenesis when transplanted into the testes of infertile recipient mice, an assay that was first described by Brinster and Avarbock (39) and Brinster and Zimmermann (40). In the transplant bioassay, each colony of spermatogenesis produced in the recipient testis arises from a single SSC and therefore allows quantification of stem cells (41–44). The combination of the transplant technique with fluorescence activated cell sorting (FACS) has provided insights about additional phenotypic features that can be used to isolate and enrich mouse spermatogonia. Mouse spermatogonia have the phenotype ITGA6$^+$, ITGB1$^+$, THY1$^+$, CD9$^+$, GFR$\alpha$1$^+$, mitochondrial membrane potential$^{high}$, Rhodamine 123 (Rho123)$^{low}$, ITGAV ($\alpha$v-Integrin, CD51)$^-$, KIT (cKIT, CD117) $^-$, MHC-I$^-$, ALDH (aldehyde dehydrogenase) activity$^-$, and CD45$^-$ (16, 25, 27, 45–50). There is a lack of consensus about whether SSC activity can also be recovered in the Hoechst side population fraction of mouse testes (15, 46, 51, 52).

During the past few years, several laboratories have started to describe the molecular characteristics of human SSCs. A number of SSC markers are conserved from mice to non-human primates and humans (Supplemental Table 1, available online). Based on immunofluorescence and colorimetric staining of adult human testicular sections, human spermatogonia on the basement membrane of the seminiferous tubules express UTF1, SALL4, ZBTB16, GFR$\alpha$1, UCHL1, GPR125, LIN28, EXOSC10, FGFR3, DSG2, CBL, SSX2, and OCT2 (22, 53–63). Less is known about cell surface markers that could be used to isolate and enrich human SSCs. THY1, a glycophosphatidylinositol-anchored cell surface protein that belongs to the immunoglobulin-like superfamily of genes (64), has been shown to be expressed by neuronal cells, CD34$^+$ hematopoietic stem cells, fibroblasts, and endothelial cells (65–71). THY1 is involved in diverse processes, including cell migration, cell–cell/cell–matrix interactions (72), and T-cell activation (73). In the testis, THY1 has been shown with transplantation assay to be a conserved SSC marker in rodents (15) and non-human primates (74). However, the expression of THY1 in human spermatogonia has been contradictory. He et al. (59) showed that THY1 expression is limited to a few rare cells on the basement membrane of seminiferous tubules, whereas Izadyar et al. (75) showed staining in the germ cells located toward the lumen of the tubule and also in peritubular and interstitial cells. Both of these reports are based on immunofluorescence staining and no transplants were performed. Human to human transplants are not possible as a routine bioassay, but xenotransplants into the testes of infertile nude mice has emerged as a quantitative assay for human and non-human primate spermatogonia (22, 61, 74–82).

A few studies have reported enrichment of putative human SSCs by sorting based on cell surface marker expression (GPR125, SSEA4, EPCAM, ITGA6, and CD9) (59, 61, 75, 80, 83), but currently only three studies have confirmed their results by demonstrating SSC colonizing activity in the xenotransplant assay. Magnetic activated cell sorting (MACS) revealed enrichment of SSC colonizing activity in the SSEA4$^+$ and CD9$^+$ fractions of human testis cells (61, 75) and FACS for EPCAM resulted in a sixfold enrichment of colonizing activity in the EPCAM$^{dim}$ fraction (80). At present, no human data are available regarding whether spermatogonial markers used in FACS are also appropriate for MACS and vice versa. The choice of whether to use FACS or MACS depends on the desired output. Fluorescence activated cell sorting has limited throughput ($\sim 30 \times 10^6$ cells/d). It is fairly time consuming and requires specialized equipment and a skilled operator, but it allows high resolution selection of sorting gates. Magnetic activated cell sorting has a lower resolving power, but is generally a faster and a higher throughput sorting strategy that can be performed on the laboratory bench and does not require specialized equipment. A single adult human testis that can be obtained for research through an organ donor program can contain more than 1 billion cells, which is far beyond the typical sorting capacity of FACS. Magnetic activated cell sorting can easily be scaled to accommodate this number of cells and maximize the use of this valuable human tissue resource for fundamental research. In addition, MACS is technically accessible and affordable, which will facilitate application for enriching SSCs in the clinical setting.

Therefore, in this study, we evaluated FACS and MACS to isolate and enrich human SSCs based on cell surface marker expression of THY1 (CD90), ITGA6 (CD49f) (FACS and MACS), and EPCAM (MACS only; we previously reported FACS for EPCAM) (80). ITGA6 is the integrin alpha chain 6. Integrins are cell surface proteins that are made up of an alpha chain and a beta chain and they provide a link between extracellular matrix proteins and the cytoskeleton (84). ITGA6 has been shown to regulate glioblastoma stem cells (85), is