# 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

expressed by mouse mammary stem cells (86), and is crucial for the survival of the MCF-7 cell line stem cells (87). EPCAM (epithelial cell adhesion molecule) is a transmembrane glycoprotein that mediates homophilic cell–cell adhesion (88). Modulation of *Epcam* activity is thought to affect cell migration, proliferation, and invasion (88, 89) and overexpression of *Epcam* plays a role in cancer development (89–91).

Fluorescence activated cell sorting fractions were analyzed by immunocytochemistry for the human spermatogonial marker SALL4 (55, 80) and human-to-nude mouse xenotransplantation. SALL4 is a member of *sal*-gene family of transcription factors that is highly conserved between species (92–98). SALL4 is expressed by the cells in an early embryo and is important for maintaining pluripotency of embryonic stem cells (99, 100). In addition SALL4 is a conserved marker of undifferentiated spermatogonia (55, 101, 102) and has been implicated in the regulation of spermatogonial differentiation in mice (101).

The MACS fractions were analyzed by human-to-nude mouse xenotransplantation. Analyses of FACS fractions indicated that, similar to the $EPCAM^{dim}$ fraction that we previously described, $ITGA6^{+}$ and $THY1^{dim}$ can be used to effectively isolate and enrich human SSCs from a heterogeneous testis cell suspension. In contrast, only ITGA6 was suitable for sorting human SSCs by MACS, as THY1 and EPCAM provided no enrichment.

## MATERIALS AND METHODS

### Animals

All experiments using animals were approved by the Institutional Animal Care and Use committees of the Magee-Womens Research Institute and the University of Pittsburgh. They were performed in accordance with the National Institutes of Health guidelines for the care and use of animals (assurance A3654-01).

### Preparation of Human Testicular Tissue

Healthy adult human testicular tissue was obtained through the University of Pittsburgh Health Sciences Tissue Bank and Center for Organ Recovery and Education under University of Pittsburgh Institutional Review Board 0506140. After the removal of tissue, it was transported to the laboratory on ice in lactated Ringer's solution. Cells were recovered from human testicular parenchyma using a two-step enzymatic digestion described previously (74, 80, 81). Briefly, testicular tissue was digested with collagenase type IV for 5 minutes at 37°C on the shaker (250 rpm), then shaken vigorously and incubated for another 3 minutes and if necessary 2 additional minutes at 37°C on the shaker. The tubules were then sedimented by centrifugation at 200 × *g* for 5 minutes and washed with Hank's balanced salt solution (HBSS; GIBCO). The tubules were then digested with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and DNase I. The suspension was triturated vigorously three to five times and incubated at 37°C for 5 minutes. The process was repeated in 5-minute increments for up to 15 minutes total. The digestion was stopped by adding 10% fetal bovine serum (FBS) and the cells were strained through 70-$\mu$m strainer (Becton Dickson). The cells were pelleted by centrifugation at 600 × *g* for 15 minutes. Cells were then suspended in minimal essential medium $\alpha$ (MEM$\alpha$) + 10% FBS at a concentration of $40 \times 10^6$ cells/mL and aliquoted in cryovials. An equal volume of cryopreservation medium consisting of MEM$\alpha$ + 20% FBS + 20% dimethylsulfoxide (DMSO) was added drop-wise, making the final concentration $20 \times 10^6$/mL in MEM$\alpha$/15% FBS/10% DMSO). The vials were frozen at a controlled rate using Nalgene freezing containers (Nalgene-Nunc International) or a CryoMed controlled-rate freezer (Thermo Scientific) and then stored in liquid nitrogen. For experiments, the cells were thawed rapidly at 37°C, washed and suspended in MEM$\alpha$ medium containing 10% FBS.

### FACS and MACS

For FACS, human testis cell suspension was stained on ice in Dulbecco's phosphate-buffered saline (D-PBS) containing 10% FBS for 20 minutes with fluorescent-conjugated antibodies (THY1-APC, clone 5E10, 0.5 $\mu$g/$10^6$ cells and ITGA6-PE clone GoH3, 20 $\mu$L/$10^6$ cells; Becton Dickinson). The unbound primary antibody was washed away twice with D-PBS, the cells were filtered through a 35-$\mu$m strainer (Becton Dickinson) and 0.5 $\mu$g/mL propidium iodide (BD Bioscience) was added to distinguish between live and dead cells. The FACS analysis was done using FACSvantage SE (Beckton Dickinson) and the positive staining was identified by comparison with appropriate isotype control to correct for nonspecific binding. Sorting gates were established based on level of marker expression as well as exclusion of dead cells stained with propidium iodide and exclusion of cells exhibiting nonspecific binding or autofluorescence. The MACS protocol was similar to that of FACS, except after fluorescent-conjugated antibody staining (THY1-PE and ITGA6-PE; Becton Dickson; and EPCAM-PE, clone 9C4, 20 $\mu$L/$10^6$ cells; BioLegend) and washes, anti-PE microbeads (2 $\mu$L/$10^6$ cells; Miltenyi Biotec) were used to detect the fluorophore on the primary antibody. The cells were then sorted on a MACS column (Miltenyi Biotec) into positive and negative fractions.

### Immunocytochemistry

Cells from FACS and MACS were spotted on Superfrost slides and fixed with methanol. The cells were then rehydrated with D-PBS and blocked with a buffer containing 3% bovine serum albumin (BSA) and 5% normal goat serum to reduce nonspecific binding. Rabbit anti-SALL4 (1:500; ab29112, Abcam) antibody was added to the cells and incubated for 90 minutes at room temperature. Isotype-matched normal IgG was used as negative control. Primary antibody was detected using goat anti-rabbit AlexaFluor-488 conjugated secondary antibody (1:200; Invitrogen). The slides were mounted with VectaShield (Vector Laboratories) mounting medium containing 6-diamino-2-phenylindole (DAPI) for detection of all nuclei and the staining was observed with a Nikon Eclipse E600 Fluorescence microscope (Nikon) and images captured with MetaView Digital Imaging software.

## Immunofluorescence

Human testicular tissue fragments were fixed with 4% paraformaldehyde overnight, paraffin-embedded and sectioned (5 $\mu$m). The tissue slides were deparaffinized, rehydrated, and incubated for 30 minutes in sodium citrate buffer (10 mM sodium citrate, pH 6.0, 0.05% Tween-20) for antigen retrieval. The tissue was then blocked with a buffer containing 3% bovine serum albumin and 5% normal serum from the host species of the secondary antibody. Subsequently, sections were stained for 90 minutes at room temperature with the following primary antibodies in antibody diluent: mouse anti-UTF1 (1:50; MAB4337, Millipore) goat anti-ZBTB16 (1:50; AF2944, R&D Systems), rabbit anti- KIT; goat anti-KIT (1:400; A4502, DakoCytomation; 1:50; AF332, R&D Systems), rabbit anti-SALL4 (1:500; ab29112, Abcam; 1:40; ab181087, Abcam), mouse anti-ENO2 (1:500, LS-B2890, LSBio), rabbit anti-UCHL1 (1:1,000; 7863-0507, Biogenesis), rabbit anti-EPCAM (1:200; ab71919, Abcam), and rabbit anti-ITGA6 (1:100; ab75737, Abcam). Isotype-matched normal IgG was used as negative control. Primary antibody was detected using AlexaFluor-488 or AlexaFluor-568 conjugated secondary antibodies (1:200; Invitrogen). The slides were mounted with VectaShield mounting medium containing DAPI (Vector Laboratories) for detection of nuclei. Sections were observed with a Nikon Eclipse E600 fluorescence microscope and images captured with MetaView Digital Imaging software. For the quantification of marker overlap, single-positive cells for each marker and double-positive cells were counted in cross-sections of seminiferous tubules. Total stained cell numbers were divided by the number of seminiferous tubule cross-sections (at least 100 per sample $\times$ 3 replicate samples).

## Colorimetric Immunohistochemistry

Human testicular tissue fragments were fixed with 4% paraformaldehyde overnight, paraffin-embedded, and sectioned (5 $\mu$m). The tissue slides were deparaffinized, rehydrated, and incubated for 30 minutes in sodium citrate buffer (10 mM sodium citrate, pH 6.0, 0.05% Tween-20) for antigen retrieval. The tissue was then incubated in peroxidase block for 10 minutes, washed in PBS, and blocked with a buffer containing 3% bovine serum albumin and 5% normal goat serum. Subsequently, sections were stained for 90 minutes at room temperature with rabbit anti-UCHL1 (1:1,000; 7863-0507, Biogenesis). Isotype-matched normal IgG was used as negative control. Primary antibody was detected using goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:200; sc-2054, Santa Cruz Biotechnology) for 30 minutes. Metal-enhanced 3,3′ diaminobenzidine (DAB) substrate kit was used to detect staining (Thermo Scientific). The tissue was then counterstained with periodic acid-Schiff and hematoxylin (Sigma-Aldrich).

## Whole Mount Immunohistochemistry

Human testicular tissue was teased apart using Collagenase type IV (1 mg/mL) and DNase I (1 mg/mL) in D-PBS. The tissue was then fixed overnight with 4% paraformaldehyde. The tubules were permeabilized using PBS and 0.1% Triton-X, blocked with a blotto milk solution in D-PBS (D-PBS + 0.02 mg/mL blotto dry milk powder + 5% Triton-X), and stained with a rabbit anti-UCHL1 (1:500; 7863-0507, Biogenesis) and goat anti-KIT (1:50; AF332, R&D Systems) primary antibodies overnight at 4°C. The primary antibodies were detected with donkey anti-rabbit IgG AlexaFluor568 and donkey anti-goat IgG AlexaFluor488 (1:200; Invitrogen). Finally, the seminiferous tubules were mounted with VectaShield mounting media containing DAPI (Vector Laboratories) with raised coverslips and imaged with fluorescent microscopy.

## Xenotransplantation and Whole Mount Immunofluorescent Quantification of Human SSC Colonizing Activity in Mouse Seminiferous Tubules

The human-to-nude mouse xenotransplantation was performed as a biological assay to investigate colonizing activity of putative human SSCs. After FACS and MACS, unsorted and sorted testicular cell fractions were transplanted into the testes of busulfan-treated (40 mg/kg; Sigma, at 5–6 weeks of age), immune-deficient nude mice (NCr nu/nu; Taconic) as previously described (74, 80–82). Briefly, xenotransplantation was performed 5 weeks after busulfan treatment by injecting cell suspensions containing 10% trypan blue (Invitrogen) into the seminiferous tubules of recipient mouse testes through the efferent ducts. Approximately 7 $\mu$L of cell suspension was injected per testis. For quantitative analysis of colonization by human donor spermatogonia, the testes were recovered 8 weeks after transplantation, the tunica was removed, and the intact seminiferous tubules were dispersed gently with Collagenase IV (1 mg/mL) and DNase I (1 mg/mL) in D-PBS. The tubules were fixed for 4 hours in 4% paraformaldehyde and the whole mount immunofluorescence was carried out by dehydrating samples in a graded series of methanol dilutions before incubating in MeOH:DMSO:$H_2O_2$ (4:1:1) solution for 3 hours. The tubules were then rehydrated, blocked with a blotto milk solution in D-PBS (D-PBS + 0.02 mg/mL blotto dry milk powder + 5% Triton-X), and stained with a rabbit anti-primate testis cell primary antibody (81) at a 1:800 dilution overnight at 4°C. The primary antibody was detected with goat anti-rabbit IgG AlexaFluor488 (1:200; Invitrogen). Finally, the seminiferous tubules were mounted with VectaShield mounting media containing DAPI (Vector Laboratories) with raised coverslips and imaged with fluorescent microscopy. Spermatogonial colonies were counted based on the following criteria: at least four cells exhibiting spermatogonial morphology (ovoid shape with high nuclear-to-cytoplasmic ratio) and located on the basement membrane in a continuous area of recipient seminiferous tubule ($\leq$100 $\mu$m between cells).

## Statistical Analysis

We analyzed the data using linear mixed effect models, and performed Tukey's tests, as described in Hothorn et al. (103), to compare differences among the percent of $SALL4^+$ cells in unsorted versus sorted cell fractions in the immunocytochemistry experiments and colonizing activity in the human-to-nude mouse xenotransplant bioassay.

# RESULTS

## Acquisition of Human Testicular Tissue

Testicular tissues used in this study were obtained from a total of 12 postpubertal organ donors (aged 14–50 years). Testes weighed 11.3–26.0 g and produced a theoretical yield (after correcting for tissue removed for pathology and immunofluorescence studies) of $1.4 \times 10^9 \pm 0.14 \times 10^9$ cells per donor. All human testis cell suspensions used in this study were cryopreserved as described previously and thawed at a later date for experimentation. Human testicular cells used in this study were frozen for 1–15 months.

## Immunohistochemical Staining of Human Testicular Sections

Immunohistochemical costaining analysis was done to investigate the coexpression of known mouse and/or non-human primate spermatogonia markers in adult human testis. ZBTB16 and SALL4, which mark most stem and progenitor spermatogonia in rodents (102), were expressed in cells located on the basement membrane. Roughly 89% of ZBTB16$^+$ cells were also positive for SALL4 (Fig. 1A–1D), but also a small population of ZBTB16$^+$ cells (11%) did not express SALL4. A subpopulation of SALL4$^+$ cells also did not express ZBTB16 (11%) (Fig. 1D). Costaining with SALL4 and KIT showed limited overlap between the two markers (Fig. 1E–1H). UTF1 expression was also restricted to cells on the seminiferous tubule basement membrane (Fig. 1I–1L). Costaining with UTF1 and SALL4 indicated that 65% of the SALL4$^+$ cells express both markers, whereas 35% of expressed SALL4 only. Seventeen percent of UTF1$^+$ cells express UTF1 only (Fig. 1L). To confirm that UTF1 is not expressed by differentiating spermatogonia, we costained UTF1 with a differentiation marker KIT (Fig. 1M–1P) and found that there is almost no overlap between these two markers. Based on these results, we believe that UTF1 is a more restricted marker of stem and progenitor spermatogonia than SALL4. This interpretation is consistent with results of van Bragt and colleagues (24) who concluded that UTF1 is restricted to $A_{single}$, $A_{paired}$, and $A_{aligned4}$ spermatogonia in rats. Similar to SALL4, UCHL1 expression is less restricted than UTF1 (Fig. 1Q–1T) with 75% of UCHL1$^+$ cells coexpressing both markers and 25% expressing UCHL1 only. UTF1$^+$ cells were UCHL1$^+$ 87% of the time and UTF1$^+$ only 13% (Fig. 1T). Costaining with KIT, confirms that although UCHL1 is less restricted than UTF1, it is not expressed by differentiating cells, demonstrated by limited costaining with KIT (Fig. 1U–1X). We also analyzed the expression pattern of a novel marker ENO2, which exhibited nearly complete overlap of expression with UCHL1 (Fig. 1Y–1BB). By transitive logic, ENO2 is a marker of undifferentiated spermatogonia in humans because it exhibits nearly complete overlap with UCHL1, which has very little overlap with KIT. The overlap between ENO2 and SALL4 is less complete, with 78% of the ENO2+ cells expressing SALL4 and 12% expressing ENO2 only (Fig. 1CC–1FF). These results indicate that ENO2 expression is slightly broader than SALL4 expression in human undifferentiated spermatogonia. Sections stained with isotype control IgG are shown in Supplemetal Figure 1. Supplemental Figure 2 (available online) summarizes our interpretation of these results in terms of the order and breadth of marker expression by human spermatogonia.

## Correlation of Spermatogonial Markers with Dark and Pale Descriptions of Nuclear Morphology and Clone Size

To correlate molecular markers of human spermatogonia described in this study with classic descriptions of nuclear staining intensity ($A_{dark}$ and $A_{pale}$), we performed colorimetric immunohistochemistry for UCHL1 followed by periodic acid-Schiff and hematoxylin counterstaining. The results in Figure 2A confirm that UCHL1 is expressed by $A_{dark}$ and $A_{pale}$ spermatogonia, which are considered the reserve and active stem cells of the human testis, respectively (104, 105). To correlate UCHL1 expression with clone size, we performed immunofluorescent analysis of UCHL1 expression in whole mount preparations of human seminiferous tubules. UCHL1 was expressed by cells located on the basement membrane of the seminiferous tubules and arranged as single cells and clones of two, four, and sometimes eight interconnected cells. In contrast, KIT-expressing cells were typically arranged in clones of 4, 8 and sometimes 16 interconnected cells (Fig. 2B–2F). The density of undifferentiated spermatogonia on the basement membrane of human seminiferous tubules appears more than in rodents (cf. Fig. 2B to previous reports for mouse) (17, 19, 30, 102), whereas KIT$^+$ differentiating spermatogonia are considerably less dense in human tubules than in mouse (cf. Fig. 2C to previous reports for mouse) (17, 30, 102).

## Immunohistochemical Evaluation of Cell Surface Markers in Adult Human Testes

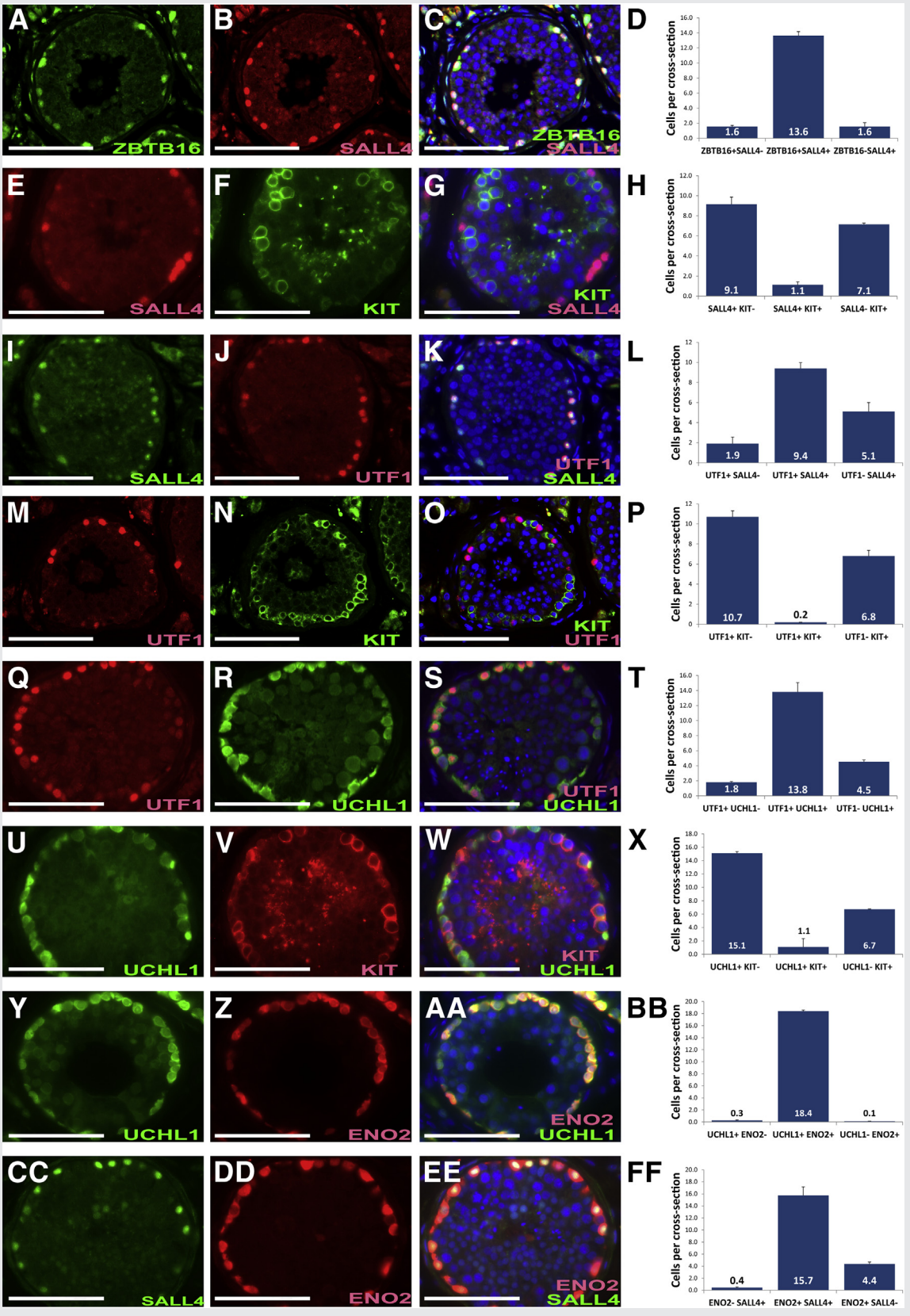
THY1, ITGA6, and EPCAM are cell surface markers that have each been used to isolate and enrich SSCs in other species (15, 16, 25, 74). Previous studies indicated that these cell surface markers are conserved in human testes (59, 75, 80, 83) and we hypothesized that each could be used to isolate and enrich human SSCs by FACS and MACS. We were not able to confirm the expression of THY1 in adult human testes by immunohistochemistry in the present study. However, other investigators (59, 75, 83) have reported that this marker is expressed in human testes.

Immunohistochemical analysis of ITGA6 expression in healthy adult human testis sections indicated that this antigen is expressed by many germ cells, including cells located on the basement membrane of seminiferous tubules (Supplemental Fig. 3A–3C, available online) and that EPCAM is expressed by cells on the basement membrane of the seminiferous tubules, as well as a few cells located more toward the lumen (Supplemental Fig. 3D–3F).

## Expression of THY1 in Adult Human Testicular Cell Suspensions

THY1 is a marker of mouse, rat, and non-human primate SSCs (15, 25, 74), as well as a marker for mouse and human hematopoietic stem cells (106–108). Therefore, we hypothesized that THY1 is a marker for human SSCs and analyzed the expression on adult human testicular cells using

## FIGURE 1




*Valli. Sorting spermatogonia from human testes. Fertil Steril 2014.*

## FIGURE 1 Continued

Expression of ZBTB16, UTF1, SALL4, UCHL1, ENO2, and KIT in human seminiferous epithelium. Immunofluorescence costaining for SALL4 and ZBTB16 (A–D), SALL4 and KIT (E–H), UTF1 and SALL4 (I–L), UTF1 and KIT (M–P), UTF1 and UCHL1 (Q–T), UCHL1 and KIT (U–X), UCHL1 and ENO2 (Y–BB), and SALL4 and ENO2 (CC–FF) in adult human testis. 6-Diamino-2-phenylindole (DAPI) staining (*blue*) identifies all the nuclei. The *bar graphs* show quantification and relative proportion of each costaining. The quantification is shown as the mean number of positive cells per cross-section of a seminiferous tubule. At least 100 seminiferous tubules were counted from three different organ donors. *Bar graphs* in D, H, L, P, T, X, and BB indicate the mean number of marker positive cells per tubule. Error bars represent SEM. Scale bars = 100 $\mu$m.

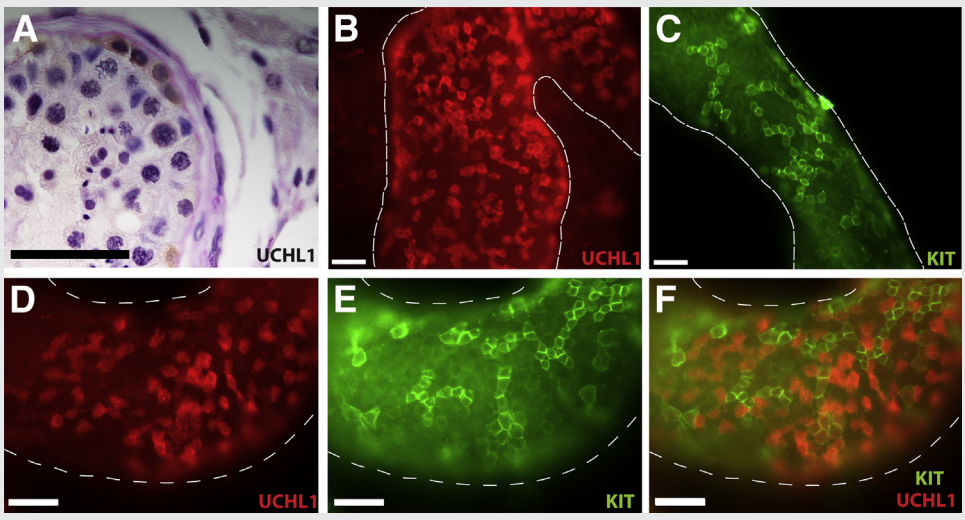
*Valli. Sorting spermatogonia from human testes. Fertil Steril 2014.*

FACS and MACS. Adult human testis cell suspensions stained with THY1 showed three populations of cells based on their level of fluorescence and on negative PE axis, which helps us plot the cells on a multicolor histogram and therefore eliminate autofluorescence. As indicated in Figure 3A, the three distinct populations were THY1$^{bright}$, THY1$^{dim}$, and THY1$^{-}$. The negative gate was established based on staining with isotype control IgG (Supplemental Fig. 4A). The THY1 bright, dim and negative fractions represented 12.2% ± 4.2%, 19.0% ± 4.0%, and 46.5% ± 7.0% of the live cells, respectively. Immunofluorescence staining revealed that 6.8% ± 0.1% of unsorted human testicular cells express human spermatogonia marker SALL4, compared with 7.2% ± 0.3% in the THY1$^{-}$ fraction ($P<.01$), 15.5% ± 0.9% in the THY1$^{dim}$ fraction ($P<.01$), and only 0.4% ± 0 in the THY1$^{bright}$ fraction ($P<.01$) (Fig. 3B and 3D–3G). To confirm the immunocytochemistry results and to functionally correlate THY1 expression in adult human testis to SSC colonizing activity, the human-to-nude mouse xenotransplantation assay was performed. The transplant results confirm that SSC colonizing activity was depleted from THY1$^{bright}$ fraction (0.57 colonies/$10^5$ cells; $P<.01$ compared with the unsorted controls). Most of SSC colonizing activity was recovered in the THY1$^{dim}$ fraction (48.2 colonies/$10^5$ cells; $P<.01$ compared with the unsorted controls), compared with 9.03 and 9.67 colonies/$10^5$ cells in unsorted and THY1$^{-}$ fractions, respectively (Fig. 3C). Based on these results, there is roughly a fivefold enrichment of SSC colonizing activity in the THY1$^{dim}$ fraction of human testis cells. Immunohistochemical assessment of human colonizing events in recipient mouse testes indicate that colonizing cells are located on the basement membrane of seminiferous tubules and contain ENO2$^{+}$ undifferentiated human spermatogonia as well as ENO2$^{-}$ human cells that are presumably more differentiated germ cells (Supplemental Fig. 5, available online). We previously reported that all cells in human colonizing events express the germ cell marker VASA (80).

### Expression of ITGA6 in Adult Human Testicular Cell Suspension

To determine whether ITGA6 is expressed on human spermatogonia and could be used as a positive selection marker to enrich human SSCs, adult human testicular cell suspensions were stained with a PE-conjugated antibody against ITGA6 and sorted by FACS. Two distinct populations of cells were gated–ITGA6$^{-}$ and ITGA6$^{+}$ (Fig. 3H), which represented 27.6% ± 7.6% and 11.6% ± 3.0% of the live cells, respectively. The negative gate was established based on staining with isotype control IgG (Supplemental Fig. 4B). Immunocytochemistry of the ITGA6 sorted fractions and

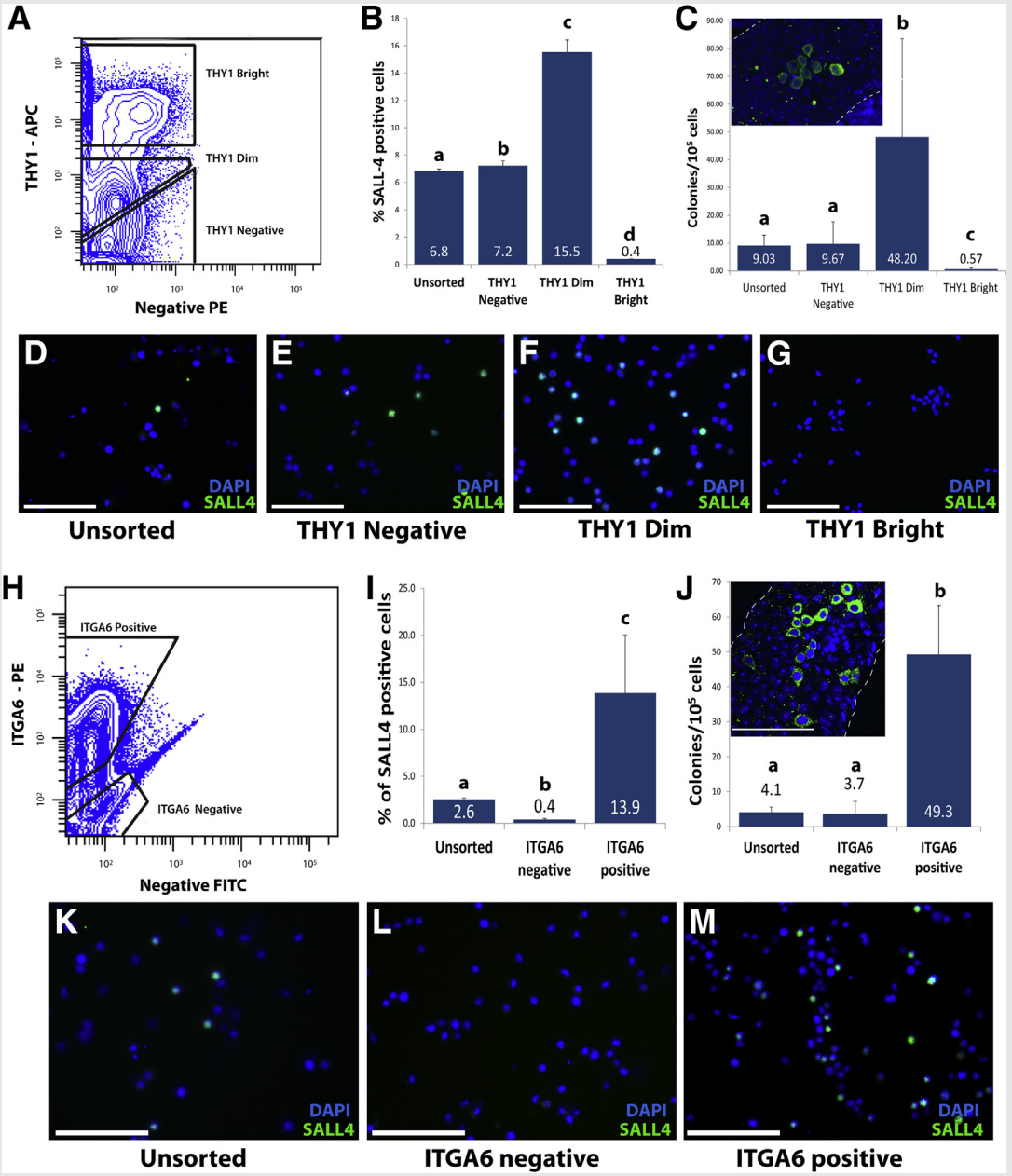
## FIGURE 2




UCHL1 expression in adult human testis. (A) UCHL1 staining in periodic acid-Schiff and hematoxylin-stained adult human testis section. UCHL1 is expressed by $A_{dark}$ and $A_{pale}$ spermatogonia. (B, D) UCHL1 and (C, E) KIT staining in whole mount staining of adult human testis. (F) UCHL1 clones are smaller (mostly 1–4 cells), whereas KIT clones tend to be bigger (>8 cells). Scale bar = 50 $\mu$m.

*Valli. Sorting spermatogonia from human testes. Fertil Steril 2014.*

## FIGURE 3




Expression of THY1 and ITGA6 in adult human testis. (A) Fluorescence-activated cell sorting was used to characterize and sort human testicular cells based on the level of THY1 expression. Based on THY1-APC staining intensity and negative PE autofluorescence, three populations were identified—THY1$^{bight}$, THY1$^{dim}$, and THY1$^{neg}$. Negative gates were defined by analysis of human testis cells stained using APC-conjugated isotype control antibodies (Supplemental Fig. 4A). (B) After the sort, all sorted fractions, as well as the unsorted cells, were fixed and immunocytochemistry (ICC) for SALL4 was performed. SALL4$^{+}$ cells were enriched in the THY1$^{dim}$ fraction compared with the unsorted cells. (C) To confirm the ICC results, human-to-nude mouse xenotransplants were also performed. Two months after transplant, colonies of human spermatogonia were identified in mouse recipient testes. (C, J insets) Examples of colonies of human spermatogonia in whole mount preparations of recipient mouse seminiferous tubules stained with the rabbit anti-primate antibody. Colonies in each recipient testis were counted and normalized to $10^5$ viable cells transplanted per testis. (D–G) Representative images of SALL4 staining from each sorted fraction and unsorted cells. (H) Fluorescence-activated cell sorting for ITGA6 in human testis resulted in two different populations based on ITGA6 -PE staining intensity and negative FITC autofluorescence—ITGA6$^{+}$ and ITGA6$^{-}$. Negative gates were defined by analysis of human testis cells stained using PE-conjugated isotype control antibodies (Supplemental Fig. 4B). (I) After the sort, all sorted fractions, as well as the unsorted cells, were fixed and ICC for SALL4 was performed. SALL4$^{+}$ cells were enriched in the ITGA6$^{+}$ fraction compared with the unsorted cells. (J) To confirm the ICC results, human-to-nude mouse xenotransplants were also done. Two months after transplant, colonies of human spermatogonia were identified in mouse recipient testes. Colonies in each recipient testis were counted and normalized to $10^5$ viable cells transplanted per testis. (K–M) Representative images of SALL4 staining from each sorted fraction and unsorted cells. At least 10 views were counted from each fraction based on 6-diamino-2-phenylindole (DAPI) staining and SALL-4 staining. Different letter indicate $P<.01$; same letters indicate $P>.05$. *Bar graphs* in B, C, I, and J are presented as mean ± SEM. Scale bar = 100 μm.

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unsorted cells revealed that 13.8% ± 6.2% of cells in the ITGA6$^+$ fraction were SALL4$^+$ (Fig. 3I and 3M), compared with 2.6% ± 0.2% in the unsorted cell population ($P<.01$) (Fig. 3I and 3K). SALL4$^+$ cells were depleted from the ITGA6$^-$ fraction (0.38% ± 0.1%; $P<.01$ compared with the unsorted controls; Fig. 3I and 3L). To confirm the immunocytochemistry results, colonizing activity in ITGA6 sorted and unsorted cells was assessed by xenotransplantation into nude mouse testes. On average, 49.3 colonies/$10^5$ cells were found in mice transplanted with cells from the ITGA6$^+$ fraction ($P<.01$ compared with the unsorted controls), whereas only 4.1 colonies/$10^5$ cells and 3.7 colonies/$10^5$ cells were observed from mice transplanted with unsorted and ITGA6$^-$ cells, respectively (Fig. 3J). Thus, SSC colonizing activity resides predominantly in the ITGA6$^+$ fraction of human testis cells and is enriched approximately 12-fold compared with the unsorted population.

## Enrichment of Human Spermatogonia Using MACS

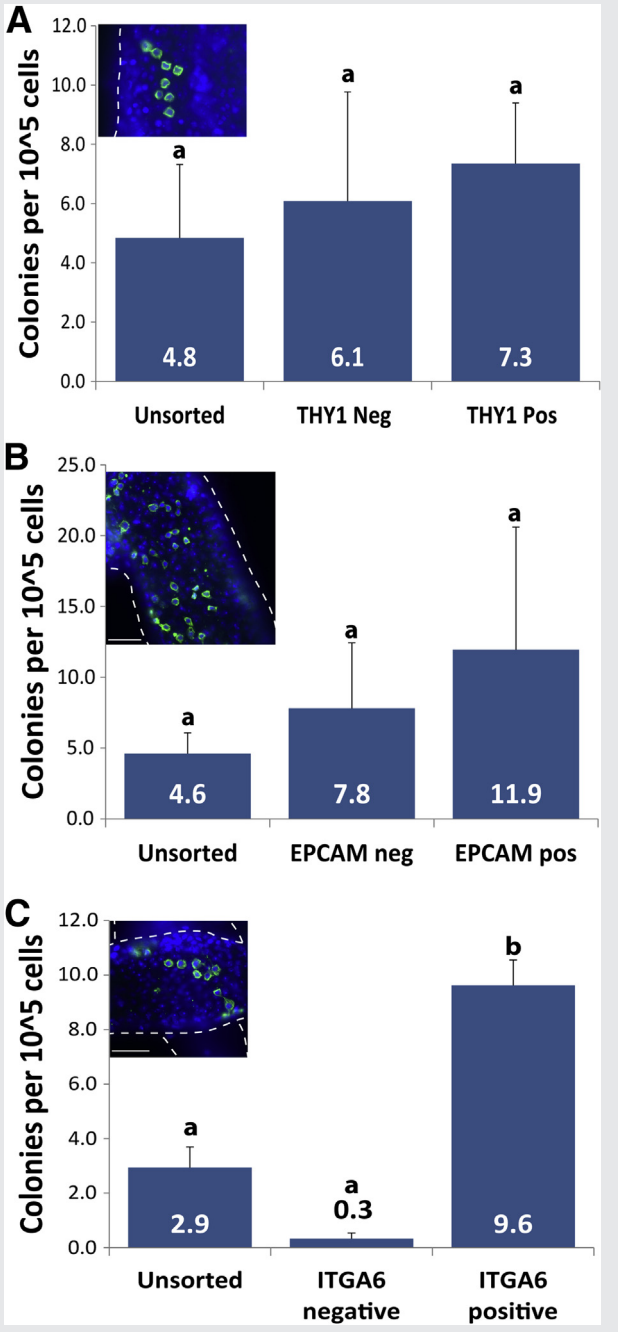
Analysis of FACS indicated that ITGA6 and THY1 can be used to effectively isolate and enrich human SSCs from a heterogeneous testis cell suspension. However, the FACS sorting approach has limited throughput (~30 × $10^6$ cells/d). Therefore, we decided to evaluate a higher throughput sorting approach (MACS) to maximize the use of human testicular cells and compare the results with FACS. We evaluated the fractionation of human testis cells by THY1 MACS where there is no option to distinguish between bright and dim expression of THY1. The cells were sorted into THY1 positive and negative fractions using MACS and then transplanted into nude mouse testes to analyze SSC colonizing activity relative to unsorted human testis cells. Unsorted cells produced 4.8 ± 2.5 colonies/$10^5$ cells, compared with 6.1± 2.0 and 7.3 ± 3.7 colonies/$10^5$ cells in THY1$^-$ and THY1$^+$ fractions, respectively ($P>.05$, compared with unsorted and each other), indicating that MACS did not effectively fractionate SSC colonizing activity based on THY1 expression (Fig. 4A). Similar to the THY1 FACS results in this study, we previously reported the SSC colonizing activity is enriched in the EPCAM$^{dim}$ fraction of human testis cells (80). Therefore, it is not surprising that MACS did not effectively fractionate SSC colonizing activity from human testis cells based on EPCAM expression (Fig. 4B).

In contrast, MACS was effective for isolation and enrichment of human SSC colonizing activity based on ITGA6 expression (Fig. 4C). The SSC colonizing activity in the ITGA6$^+$ MACS fraction was enriched more than threefold (9.6 ± 0.9 colonies/$10^5$ cells) compared with the unsorted fraction (2.9 colonies/$10^5$ cells; $P<.05$; Fig. 4C). The SSC colonizing activity was nearly depleted in the ITGA6$^-$ fraction, which produced only 0.3 ± 0.2 colonies/$10^5$ cells, indicating that almost all SSCs were recovered in the ITGA6$^+$ fraction.

## DISCUSSION

In rodents, SSCs are defined by their ability to establish and maintain spermatogenesis when transplanted into infertile mouse testes (39, 40, 109, 110). Although there is no

**FIGURE 4**




Magnetic-activated cell sorting (MACS) of human testicular cells for THY1, EPCAM, and ITGA6. Human testicular cells were MACS sorted into two fractions—negative and positive. Both positive and negative fractions from MACS, as well as unsorted cells, were transplanted into nude mouse testis. (Inset A–C) Two months after transplant, colonies of human spermatogonia were identified in whole mount preparations of recipient mouse seminiferous tubules using the rabbit anti-primate antibody. Colonies in each recipient testis were counted and normalized to $10^5$ viable cells transplanted per testis. (A, B) For THY1 and EPCAM, no significant difference was found between the unsorted cells and the sorted fractions ($P>.05$). (C) ITGA6$^+$ fraction was enriched roughly threefold compared with unsorted cells ($P<.05$). *Bar graphs* are presented as mean ± SEM. Scale bar = 100 μm. Different letter indicate $P<.05$, same letters indicate $P>.05$.

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specific molecular marker of rodent SSCs (except possibly ID4 [18]), stem and progenitor spermatogonia can be described collectively by expression of some or all of the following markers: GFR$\alpha$1, POU3F1, POU5F1, ZBTB16, NGN3, NANOS2, NANOS3, SOHLH1, SOHLH2, FOXO1, ITGA6, LIN28, ID4, UTF1, CDH1, GPR125, ITGB1, EPCAM, CD9, and THY1 (9–38, 45, 111), and by their clonal arrangement on the basement membrane of seminiferous tubules ($A_{single}$, $A_{paired}$, $A_{aligned}$; 112). In humans, stem spermatogonia are described primarily as $A_{dark}$ and $A_{pale}$ based on the intensity of nuclear staining with hematoxylin (6–8). There is limited information about how dark and pale descriptions of nuclear morphology correlate with transplantation potential, molecular markers, or clone size, although recent progress on the molecular details has been published by von Kopylow and colleagues (56, 57) and also Lim and colleagues (58).

In the present study, we show that spermatogonia on the basement membrane of human seminiferous tubules have the phenotype of SALL4$^+$, ZBTB16$^+$, UTF$^+$, UCHL1$^+$, and ENO2$^+$ (Fig. 1). The expression of SALL4, ZBTB16, UTF1, and UCHL1 in human testes has been reported previously (22, 53–55, 59, 63). ENO2 is a gene that was identified by Oatley and co-workers because it is up-regulated in ID4-GFP+ spermatogonia (personal communications). This is the first study to demonstrate that ENO2 is expressed by human spermatogonia and coexpressed with established markers of human stem and progenitor spermatogonia (i.e., UCHL1 and SALL4) (55, 59). This is also the first study to quantify the expression of these markers at the cellular level and describe their expression relative to other stem and progenitor markers by costaining. We believe this systematic molecular profiling will identify subpopulations of cells (e.g., putative stem, progenitor and differentiating cells) that will become the subject of future investigations.

Most cells that express SALL4, ZBTB16, UTF1, UCHL1, and ENO2 do not express the differentiation marker KIT, as demonstrated by direct costaining (i.e., UCHL1/KIT, SALL4/KIT, and UTF1/KIT) or transitive logic (UCHL1/ENO2; Fig. 1). These results suggest that SALL4, ZBTB16, UTF1, UCHL1, and ENO2 mark human undifferentiated spermatogonia and immunohistochemical analysis confirms that UCHL1 is expressed by $A_{dark}$ and $A_{pale}$ spermatogonia, the putative SSCs in human testes (Fig. 2). Examination of these markers in whole mount preparations of seminiferous tubules provides novel insights into human spermatogenic lineage development. Our results indicate that UCHL1 tended to be expressed by smaller clones (1–4 cells), whereas KIT is expressed in larger clones (usually $\geq$8 cells). Collectively, these results indicate that several markers of rodent stem and progenitor spermatogonia are conserved in humans and that spermatogonial differentiation in humans is correlated with increased clone size and initiation of KIT expression, similar to rodents (17, 102).

Spermatogenesis is an extremely productive system that produces millions of sperm per gram of testicular tissue each day in rodents and humans (1–3). However, our results suggest that the dynamics of spermatogenic lineage development in humans may be different than rodents. In rodents, rare undifferentiated spermatogonia are heavily outnumbered by transit-amplifying differentiated spermatogonia (113). In contrast, we found that the number of undifferentiated spermatogonia in human testes was greater than the number of KIT$^+$ differentiated spermatogonia (Fig. 2; Supplemental Fig. 2). Thus, it appears that the highly productive spermatogenic system in rodents depends on a small pool of stem and progenitor spermatogonia and a large pool of transit-amplifying cells, whereas the human spermatogenic lineage is characterized by a relatively larger pool of stem and progenitor cells and a smaller pool of transit-amplifying cells.

Fluorescence-activated cell sorting is suitable for characterizing relatively small cell populations ($\leq 30 \times 10^6$) and can be used to achieve significant enrichment of SSCs (15, 25, 45, 50, 74, 80, 114–117). When coupled with molecular marker screening (using markers that are restricted to stem and progenitor spermatogonia) and the stem cell transplant assay to validate sorted fractions, FACS can be a powerful tool for dissecting the molecular phenotype of SSCs. In the current study, we used SALL4 immunocytochemistry to screen sorted cell populations. We considered SALL4 an excellent marker for screening human stem and progenitor spermatogonia because it is conserved in mice (55, 101, 102), rats (Gassei and Orwig, unpublished), monkeys (55), and humans (55), including expression by human $A_{dark}$ and $A_{pale}$ spermatogonia (55). SALL4 immunocytochemistry provided a rapid assessment of sorted fractions and was an excellent predictor of the results from human-to-nude mouse SSC xenotransplantation, which has an inherent 2-month delay to analysis. Based on the data presented, we believe that UTF1, ZBTB16, UCHL1, and ENO2 would also be good markers to rapidly screen for human stem and progenitor spermatogonia.

Spermatogonial stem cell transplantation is the experimental "gold standard" for assaying SSCs (118, 119). Spermatogonial stem cell transplantation in humans may someday be feasible in the clinical setting (120), but cannot be used as a routine bioassay. However, Nagano et al. (77) demonstrated that human SSCs can engraft the testes of infertile, immune compromised mice. Human SSCs do not produce complete spermatogenesis in mouse seminiferous tubules, but they do execute several functions that are consistent with the activity of SSCs: [1] they migrate to the basement membrane of seminiferous tubules without being phagocytosed by mouse Sertoli cells; [2] they proliferate to produce characteristic chains and networks of spermatogonia; and [3] they persist for several months. Human-to-nude mouse xenotransplantation is becoming a routine bioassay for human SSCs (22, 61, 75, 77–80).

Studies using FACS followed by transplantation of sorted fractions have established that ITGA6, THY1, and EPCAM are markers of SSCs in rodents (15, 16, 25). Similar methodology with FACS or MACS followed by human-to-mouse xenotransplantation has been used to demonstrate that EPCAM, CD9, and SSEA4 are markers of human SSCs (61, 75, 80). Human testis cells have also been fractionated by MACS based on expression of GPR125, THY1, and ITGA6 (59, 83, 121), but stem cell activity in sorted fractions was not tested by transplantation.

Flow cytometry analyses in the current study identified two distinct THY1$^{+}$ populations in the human testis that we designated dim and bright. SALL4 staining as well as xenotransplant results suggested that most of the SSCs were in the THY1$^{dim}$ fraction and SSC colonizing activity in that fraction was enriched approximately fivefold compared with unsorted human testis cells (Fig. 3C). Almost no SSCs are found in the THY1$^{bright}$ fraction. Similar to the THY1 results reported in the present study, we previously reported that SSC colonizing activity is recovered in the EPCAM$^{dim}$ fraction of human testis cells and depleted in the EPCAM$^{bright}$ and EPCAM$^{-}$ fractions (80). Interestingly, neither of these markers could be used to effectively fractionate and enrich SSC colonizing activity from the human testes using MACS. The SSC colonizing activity was recovered in both the bound and flowthrough fractions and colonizing activity in each fraction was similar to unsorted controls (Fig. 4A and B). Perhaps this result can be attributed to the low expression level of these two antigens in human SSCs. Considering our MACS results, it is noteworthy that THY1 MACS is routinely used to sort SSCs from mouse testes (116, 122–126). These results may indicate that there are species-specific differences in the level of THY1 expression. Alternatively, these results may indicate technical differences between direct labeling with bead-conjugated THY1 primary antibodies (mouse) and indirect labeling using bead conjugated secondary antibodies (current study). The bead conjugated anti-mouse THY1 antibodies did not cross-react with the human THY1 antigen (data not shown). Flow cytometric analysis of ITGA6 in human testis cells revealed only two distinct populations, positive and negative, and most of SSC colonizing activity was recovered in the ITGA6$^{+}$ fraction, which was enriched 12-fold compared with unsorted controls (Fig. 4J). In contrast to THY1 and EPCAM, cells with SSC colonizing activity could be effectively isolated and enriched from heterogeneous human testis cell suspensions using ITGA6 MACS. However, the level of enrichment achieved by ITGA6 MACS (3.3-fold) was less than ITGA6 FACS (12-fold). Sorting resolution by FACS is typically more than MACS because FACS allows for gating of cell populations based on simultaneous evaluation of several parameters, including viability (PI$^{-}$), cell size (forward scatter of incident light), cell complexity (side scatter of incident light), and specific immunoreactivity (autofluorescent$^{-}$, nonspecific binding$^{-}$).

We identified several proteins with expression limited primarily to undifferentiated spermatogonia (KIT$^{-}$ cells) located on the basement membrane of seminiferous tubules in human testes. These markers may provide insights into the molecular mechanisms that regulate the function of human SSCs and can be used to screen human cell populations or tissues for putative SSCs. In addition, they can be used to validate newly discovered markers of human stem and progenitor spermatogonia using costaining approaches similar to those used in the current study to validate the expression of ENO2 in human undifferentiated spermatogonia. In the present study and a previous study (80), we demonstrated that human SSCs have the cell surface phenotype THY1$^{dim}$, EPCAM$^{dim}$, and ITGA6$^{+}$. SSEA4 and CD9 are also cell surface markers of human SSCs that have been validated by human-to-mouse xenotransplantation (61, 75). These markers can now be used alone or in combination to achieve significant enrichment of human SSCs for downstream studies. Magnetic-activated cell sorting can also be used for isolation and enrichment of SSCs before initiation of SSC cultures, as previously described for mice (116, 127). ITGA6 (current study), CD9 (61), and SSEA4 (75) are also amenable to immunomagnetic sorting, which has virtually unlimited cell sorting capacity and will facilitate isolation of SSCs from human testes that can contain more than one billion cells.

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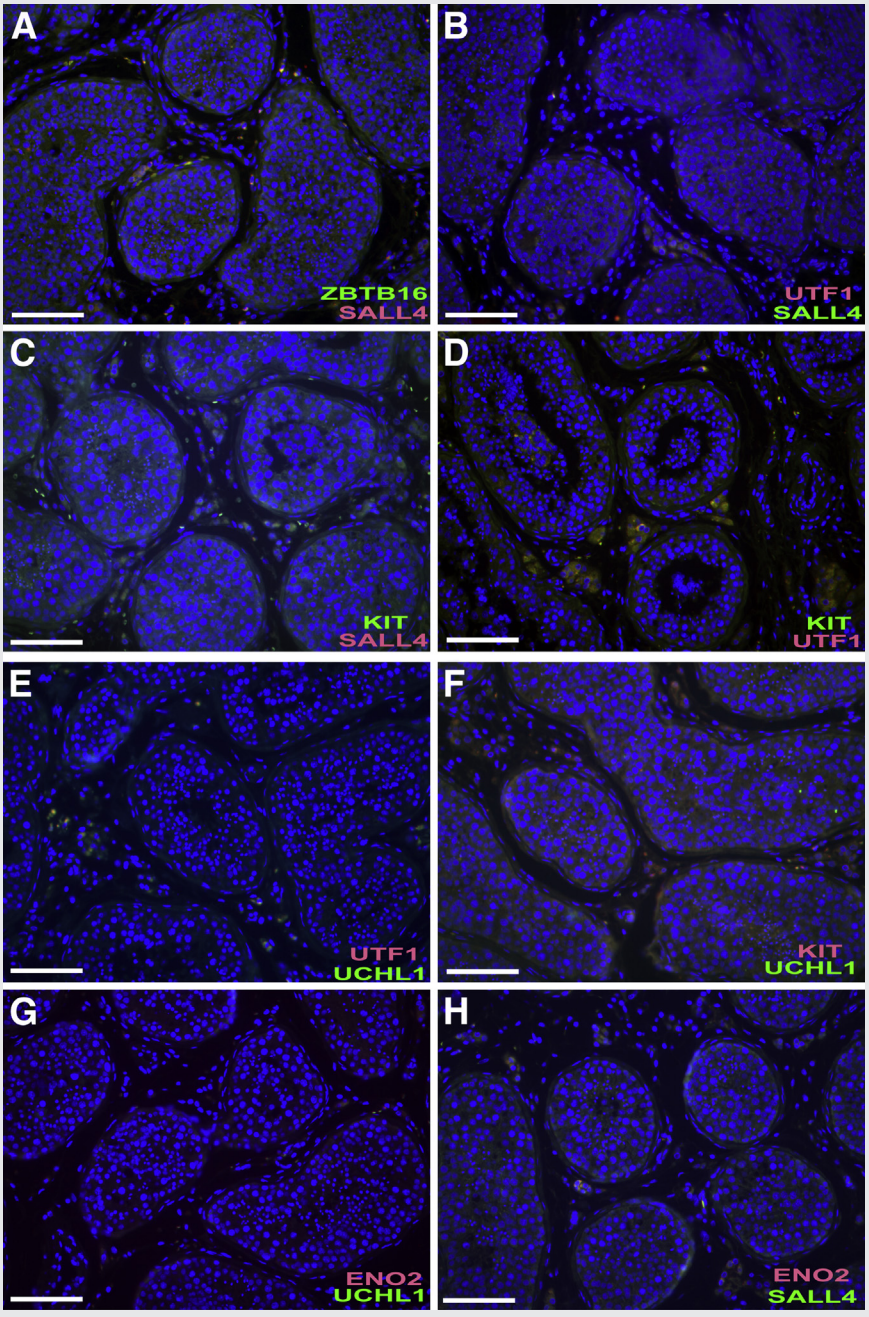
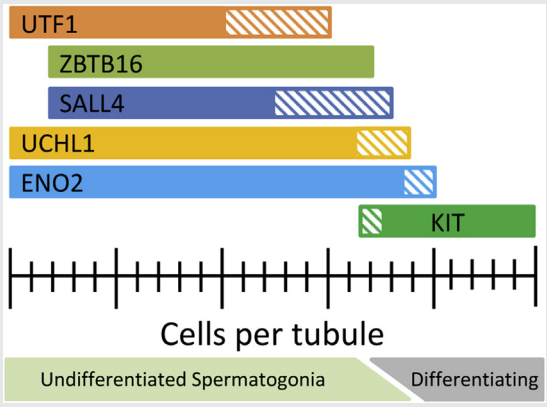
## SUPPLEMENTAL FIGURE 1




Isotype controls for (A) ZBTB16 and SALL4, (B) UTF1 and SALL4, (C) SALL4 and KIT, (D) UTF1 and KIT, (E) UTF1 and UCHL1, (F) KIT and UCHL1, (G) ENO2 and UCHL1, (H) ENO2 and SALL4. Scale bars = 100 $\mu$m.

*Valli. Sorting spermatogonia from human testes. Fertil Steril 2014.*

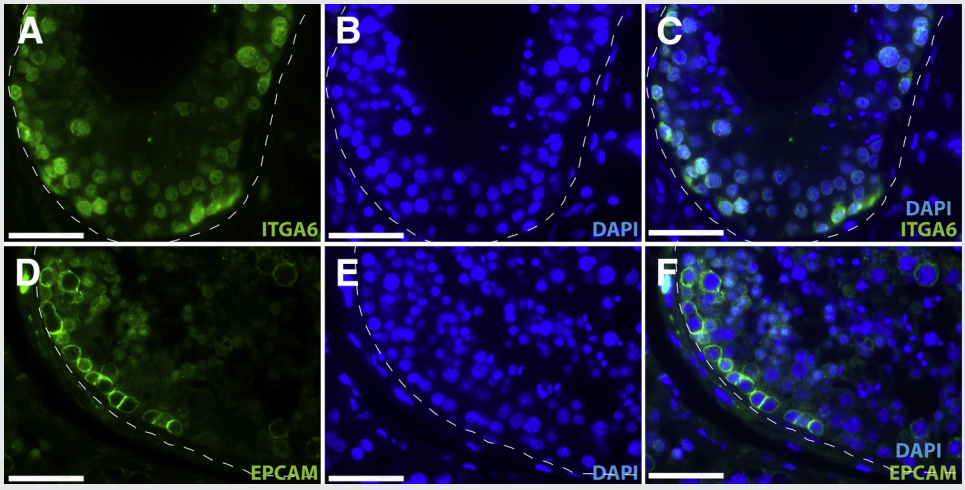
## SUPPLEMENTAL FIGURE 2




Summary of marker expression in adult human testis. *Colored bars* indicate the overlap of markers based on data from Figure 1. *Striped* area indicates range in data. UTF1 seems to be the most restricted marker of human spermatogonia, followed by ZBTB16 and SALL4. There is also almost no overlap between these markers and differentiation marker KIT. UCHL1 and ENO2 are more widely expressed on the basement membrane of the seminiferous tubule and have slightly more overlap with KIT.

*Valli. Sorting spermatogonia from human testes. Fertil Steril 2014.*

## SUPPLEMENTAL FIGURE 3




ITGA6 and EPCAM expression in adult human testis sections. Immunofluorescence staining for ITGA6 (A, C) and EPCAM (D, F) in adult human testis. 6-Diamino-2-phenylindole (DAPI) staining (*blue*) (B, E) identifies all the nuclei. Scale bars = 50 $\mu$m.

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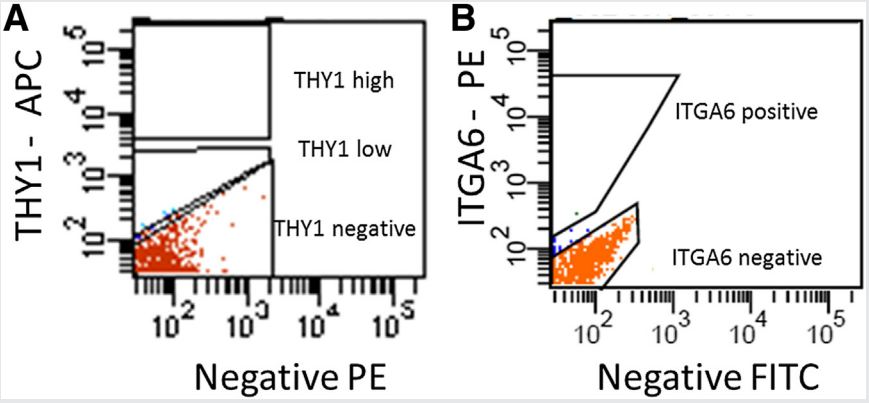
## SUPPLEMENTAL FIGURE 4




Isotype controls for (A) THY1 and (B) ITGA1 fluorescence-activated cell sorting. Negative gates were defined by analysis of human testis cells stained using (A) APC-conjugated and (B) PE-conjugated isotype control antibodies.

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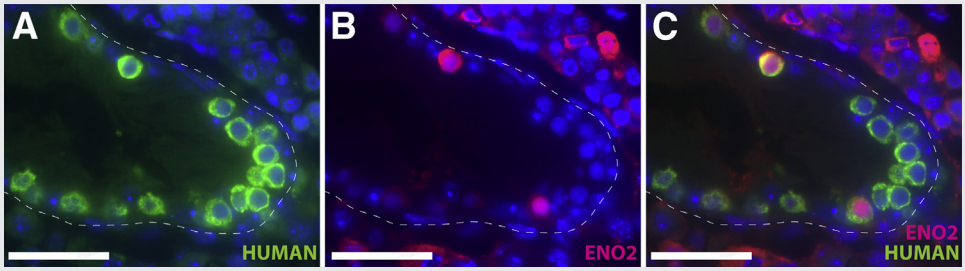
## SUPPLEMENTAL FIGURE 5




Rabbit anti-primate antibody and ENO2 costaining of recipient mouse testes xenotransplanted with human testis cells. Immunofluorescence costaining for the primate antibody (A, C) and ENO2 (B, C) in human-to-nude mouse xenotransplants testis. 6-Diamino-2-phenylindole (DAPI) staining (*blue*) identifies all the nuclei. Scale bars = 50 $\mu$m.

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## SUPPLEMENTAL TABLE 1

**Germ cell markers in rodents, non-human primates, and humans.**

| | Rodents | | | Non-human primates | | | Humans | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | RT-PCR | IHC ICC | TR | RT-PCR | IHC ICC | TR | RT-PCR | IHC ICC | TR | Comments | References |
| Germ cell[a] | | | | | | | | | | | |
| ID4 | X | IHC-XS<br>IHC-WM | X | | | | | | | $A_s$ | (18) |
| EXOSC10 | | | | | | | X | IHC-XS | | $A_d$ | (56) |
| OCT2 | | | | | | | | IHC-XS | | $A_d$ | (58) |
| FGFR3 | | | | | | | X | IHC-XS | | $A_d$, $A_p$ | (57) |
| EGR3 | | IHC-WM | | | | | | | | $A_s$, $A_{pr}$ | (128) |
| NANOS2 | X | IHC-XS<br>IHC-WM | X | | | | | IHC-XS | | $A_s$, $A_{pr}$, $A_d$,$A_p$, B-RS | (30–32, 129, 130) |
| GFRa1 | | IHC-XS<br>IHC-WM | X | | IHC-XS | | | IHC-XS | | $A_s$-$A_{al4}$, $A_d$, $A_p$, $B_1$, $B_2$ | (49, 59–61, 74, 111, 114, 131) |
| UTF1 | X | IHC-XS | | | | | X | IHC-XS | | $A_s$-$A_{al4}$, $A_d$, $A_p$, BM | (24, 54) |
| ZBTB16 | | IHC-XS<br>IHC-WM | X | | IHC-XS | | X | IHC-XS | | $A_s$-Aal, Ad, $A_p$, $B_1$, $B_2$, BM | (13, 14, 22, 59, 60, 74, 101, 102, 132) |
| SALL4 | | IHC-XS<br>IHC-WM | | | IHC-XS | | X | IHC-XS | | $A_s$-Aal, $A_d$, $A_p$, B | (55, 101, 102, 132) |
| POU5F1 (OCT4) | | IHC-XS<br>IHC-WM | X | | IHC-XS | | | | | $A_s$-Aal, BM | (133–135) |
| POU3F1 | | IHC-XS | X | | | | | | | Spermatogonia on the BM | (37) |
| RET | | IHC-XS | | | | | | | | $A_s$-Aal | (9) |
| BCLB6 | | IHC-XS | X | | | | | | | Rare cells on the BM. RS in stage 7 | (125, 136) |
| LHX1 | | IHC-XS | X | | | | | | | Rare cells on the BM. RS in stage 7 | (125) |
| ETV5 | | IHC-XS | X | | | | | | | On the BM | (125) |
| NANOS3 | X | IHC-XS<br>IHC-WM | | | | | | | | $A_s$-A1 | (30–33) |
| SOX3 | | IHC-XS | | | | | | | | $A_s$-Aal | (137) |
| GPR125 | | IHC-XS<br>IHC-WM | X | | IHC-XS | | X | IHC-XS<br>ICC | | $A_s$-Aal, rare cells on the BM | (20–22, 59, 132) |
| LIN28 (TEX17) | | IHC-XS<br>IHC-WM | | | IHC-XS | | | IHC-XS | | $A_s$-Aal, rare cells on the BM | (19, 62) |
| UCHL1 (UCHL1) | | IHC-XS | | | IHC-XS | | X | IHC-XS | | Spermatogonia, cells on the BM | (22, 59, 132, 138) |
| NGN3 | | IHC-XS<br>IHC-WM | X | | IHC-XS | | | | | $A_s$-Aal, $A_p$, $B_{1-4}$, PS | (28, 29, 74, 137) |
| SOHLH1 | X | IHC-XS<br>IHC-WM | | | | | | | | GFR$\alpha$1$^-$ spermatogonia | (34, 139) |
| SOHLH2 | X | IHC-XS<br>IHC-WM | | | | | | | | GFR$\alpha$1$^-$ spermatogonia | (34–36, 139) |
| CBL | | | | | | | X | IHC-XS | | BM | (53) |
| DSG2 | | | | | | | X | IHC-XS | | BM | (53) |
| SAGE1 | | | | | | | | IHC-XS | | B | (58) |
| TRA-1-81 | | | | | IHC-XS | | | | | Rare cells on the BM | (132, 140) |
| MAGEA4 | | | | | IHC-XS | | | IHC-XS | | BM, some spc | (59, 61, 132, 133, 141) |
| SNAP91 | | | | | | | X | IHC-XS | | BM, some spc | (53) |

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## SUPPLEMENTAL TABLE 1

**Continued.**

| | Rodents | | | Non-human primates | | | Humans | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | RT-PCR | IHC ICC | TR | RT-PCR | IHC ICC | TR | RT-PCR | IHC ICC | TR | Comments | References |
| RBM | X | IHC-XS | | | | | | IHC-XS | | $A_s$-A4<br>$A_d$, $A_p$, B-RS | (142–146) |
| RARγ | X | IHC-XS<br>IHC-WM | | | | | | | | $A_{al}$-A1 | (147) |
| KIT | X | IHC-XS<br>IHC-WM | X | X | IHC-XS | | X | IHC-XS | | $A_{al}$-RS<br>$A_p$, $B_{1-4}$, PS, BM | (45, 59, 74, 75, 102, 114, 148–152), current publication |
| STRA8 | X | IHC-XS<br>IHC-WM | X | | | | | | | $A_s$-RS | (153–155) |
| TAF4b | | IHC-XS | X | | | | | | | $A_s$-Spc | (156) |
| DAZL | X | IHC-XS | | | IHC-XS | | X | IHC-XS | | $A_s$-Spc | (22, 81, 157, 158) |
| VASA | | IHC-XS | | | IHC-XS | | X | | | $A_s$-RS | (22, 114, 132, 159, 160) |
| mRNA-221/222 | | IHC-XS | X | | | | | | | On the BM | (122) |
| mRNA-146 | X | | | | | | | | | Undifferentiated spermatogonia | (161) |
| mRNA-20 | X | IHC-XS | X | | | | | | | Undifferentiated spermatogonia | (162) |
| mRNA-106a | X | IHC-XS | X | | | | | | | Undifferentiated spermatogonia | (162) |
| miRlet7 | | IHC-XS | | | | | | | | On the BM | (163) |
| Cell surface[b] | | | | | | | | | | | |
| GFRa1 | | IHC-XS<br>IHC-WM | X | | IHC-XS | | | IHC-XS | | $A_s$-$A_{al4}$, $A_d$, $A_p$, $B_1$, $B_2$ | (49, 59–61, 74, 111, 114, 131) |
| KIT | X | IHC-XS<br>IHC-WM | X | X | IHC-XS | | X | IHC-XS | | $A_{al}$-RS<br>$A_p$, $B_{1-4}$, PS, BM | (45, 59, 74, 75, 102, 114, 148–152) |
| THY1 (CD90) | | | X | | | X | | IHC-XS | X | | (15, 25, 59, 74, 75, 83, 114), current publication |
| CDH1 (CD324) | | IHC-XS<br>IHC-WM | X | | | | | | | $A_s$-$A_{al}$ | (17, 164) |
| CD9 | | IHC-XS | X | | | | | IHC-XS | X | BM | (26, 27, 61, 117) |
| CD29 (β1-integrin) | | | X | | | | X | IHC-XS | | BM | (16, 23, 148) |
| CD49f (ITGA6) | | | X | | IHC-XS | | X | IHC-XS<br>ICC | X | BM | (16, 45, 59, 75, 83, 114, 121, 148), current publication |
| EPCAM | | IHC-XS | X | | | | X | | X | | (22, 25, 26, 80, 165), current publication |
| SSEA4 | | | | | IHC-XS | X | | IHC-XS | X | | (75, 114, 132, 140, 166) |
| GPR125 | | IHC-XS<br>IHC-WM | X | | IHC-XS | | X | IHC-XS<br>ICC | | $A_s$-Aal, rare cells on the BM | (20–22, 59, 132) |

*Note:* Aal = A aligned spermatogonia; Ad = A dark spermatogonia; Ap = A pale spematogonia; Apr = A paired spermatogonia; As = A single spermatogonia; B = type B spermatogonia; BM = basement membrane; ICC = immunocytochemistry; IHC-WM = immunohistochemistry whole-mount; IHC-XS = immunohistochemistry cross-section; mRNA = messenger RNA; PS = pachytene spermatocytes; RS = round spermatids; RT-PCR = reverse transcriptase–polymerase chain reaction; Spc = spermatocytes; TR = transplant.

[a] We attempted to sort the germ cell markers in the order of their expression from undifferentiated to differentiating spermatogonia.

[b] Examined with flow cytometry or fluorescence-activated cell sorting experiments. FGFR3 and TRA-1-81 are also cell surface markers but not included here because they have not been studied in the context of flow cytometry or fluorescence-activated cell sorting.

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