Characterization of human spermatogonial stem cell markers in fetal, pediatric, and adult testicular tissues

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Abstract

Autologous spermatogonial stem cell (SSC) transplantation is a potential therapeutic modality for patients with azoospermia following cancer treatment. For this promise to be realized, definitive membrane markers of prepubertal and adult human SSCs must be characterized in order to permit SSC isolation and subsequent expansion. This study further characterizes the markers of male gonocytes, prespermatogonia, and SSCs in humans. Human fetal, prepubertal, and adult testicular tissues were analyzed by confocal microscopy, fluorescence-activated cell sorting, and qRT-PCR for the expression of unique germ cell membrane markers. During male fetal development, THY1 and KIT (C-Kit) are transient markers of gonocytes but not in prespermatogonia and post-natal SSCs. Although KIT expression is detected in gonocytes, THY1 expression is also detected in the somatic component of the fetal testes in addition to gonocytes. In the third trimester of gestation, THY1 expression shifts exclusively to the somatic cells of the testes where it continues to be detected only in the somatic cells postnatally. In contrast, SSEA4 expression was only detected in the gonocytes, prespermatogonia, SSCs, and Sertoli cells of the fetal and prepubertal testes. After puberty, SSEA4 expression can only be detected in primitive spermatogonia. Thus, although THY1 and KIT are transient markers of gonocytes, SSEA4 is the only common membrane marker of gonocytes, prespermatogonia, and SSCs from fetal through adult human development. This finding is essential for the isolation of prepubertal and adult SSCs, which may someday permit fertility preservation and reversal of azoospermia following cancer treatment.

Introduction

The isolation, expansion, and successful reconstitution of spermatogenesis after spermatogonial stem cell (SSC) transplantation in the murine model have opened new doors for potential human therapeutic applications such as fertility treatments and preservation (Brinster & Avarbock 1994, Brinster & Zimmermann 1994, Kanatsu-Shinohara et al. 2003). Unfortunately, 20 years after the first reports of successful testicular germ cell transplantsations in mice, progress in human SSC research has been limited mainly due to the scarcity of available human testicular tissues for research and the lack of an effective in vivo model fully capable of supporting human spermatogenesis (Brinster & Avarbock 1994, Brinster & Zimmermann 1994, Dym et al. 2009, Cheng & Mruk 2010).

Successful in vitro expansion of prepubertal and adult human SSCs, capable of engrafting in mouse seminiferous tubules in a human murine xenograft model, had been reported using testicular cells from digested seminiferous tubules (Sadri-Ardekani et al. 2009, Liu et al. 2011, Mirzapour et al. 2012). In addition, these in vitro-derived human SSCs were shown to possess pluripotent properties similar to embryonic stem cells (Conrad et al. 2008, Golestaneh et al. 2009, Kossack et al. 2009, Mizrak et al. 2010). However, these in vitro-expanded pluripotent human SSCs, capable of multi-lineage differentiation, have been questioned as recent studies demonstrated that these cells exhibited mesenchymal rather than germ cell properties highlighting the need for further characterization of the SSC population (Ko et al. 2011, Tapia et al. 2011, Chikhovskaya et al. 2012). Most previous human SSC studies used a mixed population of testicular cells (somatic and germ cells combined) from enzymatically digested seminiferous tubules for culture, expansion, and transplantation. Thus, the precise identity of the primitive, pluripotent germ cells within the testicular cell population capable of expansion and engraftment is uncertain.

In order to permit fertility preservation using cryopreservation and subsequent transplantation of autologous...
SSCs in prepubertal boys undergoing sterilizing oncologic treatments, it is vital to develop the ability to isolate SSCs capable of engraftment through the use of unique membrane markers, thus eliminating the risks of malignant cell contamination from the testicular cell population (Fujita et al. 2005, 2006, Hermann et al. 2011, Dovey et al. 2013). While definitive markers of SSCs have been identified in mice, unique membrane markers of primate and human SSCs have yet to be fully characterized (Ebata et al. 2005, Gashaw et al. 2007, Seandel et al. 2007, Conrad et al. 2008, Muller et al. 2008, Dym et al. 2009, Grisanti et al. 2009, Maki et al. 2009, Wu et al. 2009, He et al. 2010, Izadyar et al. 2011, Eldermann et al. 2012, Dovey et al. 2013, Kossack et al. 2013). Although THY1, GFRz1R, GPR125, EPCAM, and SSEA4 have been reported to be unique membrane markers of adult human SSCs, many of these markers (GFRz1R, GPR125, and EPCAM) are also expressed in adult testicular stromal cells, limiting their potential use as markers for SSC isolation (Conrad et al. 2008, Wu et al. 2009, He et al. 2010, Izadyar et al. 2011, Dovey et al. 2013). Although primary adult human testicular cells expressing SSEA4 were shown to engraft in mouse seminiferous tubules, SSEA4 expression was not detected in human SSCs by others (Izadyar et al. 2011, Dovey et al. 2013). Thus, there is a lack of consensus agreement on the unique membrane markers of human SSCs.

A logical approach to identify the unique membrane markers of human SSCs is to study human male germ cells during development (gonocytes and prespermatogonia), before the onset of spermatogenesis and follow them through puberty (spermatogonia) and adulthood. If a unique membrane marker of primitive germ cells was present through all stages of development, it may serve as an important marker of SSCs. Recent studies have reported two distinct populations of primitive germ cells, gonocytes and prespermatogonia, within the male fetal testes during the first two trimesters of gestation (Pauls et al. 2006, Anderson et al. 2007, Gkountela et al. 2012, Jorgensen et al. 2012). OCT4A and KIT (C-Kit) were expressed in gonocytes during the late-first and early-second trimesters; however, expression of VASA was not found in these gonocytes (Anderson et al. 2007, Gkountela et al. 2012). During the second trimester, prespermatogonia began to appear in the testes, presumably from the differentiation of gonocytes (Gkountela et al. 2012). While the prespermatogonia expressed VASA, they no longer expressed OCT4A and KIT (Pauls et al. 2006, Anderson et al. 2007, Gkountela et al. 2012). Therefore, KIT was the only membrane marker in fetal gonocytes, but such an identifying marker unique to prespermatogonia and spermatogonia remains to be investigated.

Thus, we aim to characterize the expression of human primitive germ cell membrane markers from fetal development (gonocytes and prespermatogonia) through adulthood (spermatogonia and SSCs).

Materials and methods

Testicular tissues

All tissues were obtained after informed consent in accordance with the study protocol approved by the University of California, San Francisco (UCSF) IRB. Human fetal testes (13–24 weeks of gestation) were collected following elective terminations of pregnancy, excluding cases with fetal anomalies (n=33). Gestational age was determined by last menstrual cycle and confirmed with ultrasound and subsequent foot length measurement. Autopsied prepubertal testicular tissues were obtained from deceased subjects, whose death was not related to disorders of their reproductive system (n=3). Adult testicular biopsy samples were collected from patients (n=3) with normal spermatogenesis, who underwent testicular spermatocelectomy, vasovasostomy, and testicular excisional sperm extraction due to anejaculation.

Confocal microscopy

The tissues were fixed in 4% paraformaldehyde, embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA), and cryosectioned at 5 μm. The sections were permeabilized with 0.1% Triton-X-100 PBS (Sigma–Aldrich), blocked in 5% BSA–PBS, and incubated overnight at 4°C with the following antibodies: goat anti-VASA (R&D Systems-AF2030 at 1:100 dilution, Minneapolis, MN, USA), anti-THY1 (BD Biosciences-559869 at 1:50, San Jose, CA, USA, and R&D Systems-AF206 at 1:40), rabbit anti-WT1 (Santa Cruz Biotechnology-SC-192 at 1:75, Dallas, TX, USA), anti-OCT4A (Santa Cruz Biotechnology SC-9081 at 1:75, and SC-8628 at 1:75), mouse anti-SSEA4 (BD Biosciences-560308 at 1:50), anti-C-Kit (Santa Cruz Biotechnology SC-5353 at 1:50 and M14 at 1:50), mouse anti-SSEA1 (R&D Systems-FAB2155A at 1:50), mouse anti-TRA-1-81 (BD Biosciences-560885 at 1:50), mouse anti-TRA-1-60 (BD Biosciences-560071 at 1:50), goat anti- GFRz1R (R&D Systems-AF714 at 1:50), rabbit anti-MAGEA4 (Abcam, Cambridge, MA, USA, ab76177 at 1:50), rat anti-SSEA3 (Abcam ab16286 at 1:100), and rabbit anti-GPR125 (Abcam-ab51705 at 1:50). Primary species-specific isotypes were used for controls. Donkey anti-goat Alexa 488 and 555, donkey anti-sheep Alexa 555, donkey anti-rabbit Alexa 555 and 594, donkey anti-mouse Alexa 488, 555 and 594 (BD Biosciences) were applied accordingly the following day at 1:200–1:500 dilutions at room temperature for 1 h. The images were captured using a Leica SP5 AOBS confocal microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA) and analyzed using ImageJ v1.6 (rsbweb.nih.gov).

Testicular cell isolation and fluorescence-activated cell sorting

The tissues were subjected to a two-step enzymatic digestion with collagenase IV (1 mg/ml; Sigma–Aldrich) in DMEM/F12 + Glutamax (Invitrogen) for 20 min at 37°C, followed by trypsin EDTA 0.25% (UCSF Cell Culture Facility) and DNase I (50 μg/ml; Sigma–Aldrich) for 20 min, and filtered through a 70 μm cell strainer. The cells were incubated with the following antibodies: anti-SSEA4 FITC (BD-560308), anti-THY1 APC via free access

(BD-559869), anti-c-KIT PE (R&D-FAB332P), anti-TRA1-81 PE (BD-560885), and anti-SSEA1 APC (R&D-FAB2155A), in 1% BSA for 30 min at 37 °C. Cell sorting was carried out on a BD fluorescence-activated cell sorting (FACS) Aria Flow Cytometer and analyzed using Flowjo v9.6 (Flowjo, Ashland, OR, USA); 50 000–200 000 events were acquired for analyses.

Molecular analyses
Subpopulations of testicular cells were sorted directly into RNA lysis buffer. Total RNA was isolated using the RNeasy Micro Kit (Qiagen) and cDNA was synthesized using qScript cDNA Super Mix (Quanta Biosciences, Gaithersburg, MD, USA). Each qPCR amplification was carried out in triplicate at 250 cells/reaction using FastStart Universal SYBR Green Master Mix with ROX (Roche) and Applied Biosystem 7500 Real-time PCR System. The list of primers and sequences is provided in Supplementary Table, see section on supplementary data.

Results
Male fetal testes contain two populations of germ cells defined by the relative expression of VASA
In addition to published membrane markers of human and mouse SSCs (GPR125, GFRz1R, SSEA1, SSEA4, KIT, and THY1), expression of embryonic stem cell membrane markers (TRA1-60 and TRA1-81) was also evaluated in both gonocytes and prespermatogonia. The expression of GPR125, GFRz1R, TRA160, TRA1-81, and SSEA1 was not detected in either early- or late-second trimester testes by confocal microscopy and flow cytometry (data not shown). The germ cells expressing VASA were first detected at 13 weeks of gestation (the earliest time point examined) and continued to increase in number throughout gestation. At 13 weeks of gestation, two populations of germ cells were identified based on relative expression of VASA, VASA dim (VASA_D), and VASA bright (VASA_B) (Fig. 1A). Moreover, the number of VASA_B cells increased with advancing gestation. At gestational week 13 and 24, the ratio of VASA_D/VASA_B germ cells decreased from 3/2 (Fig. 1A) to 1/3 when all VASA+ cells from ten cords were counted (Fig. 1B), respectively, suggesting that the VASA_B population represent the prespermatogonia population as the number of VASA_B cells increased with gestation. In contrast, the VASA_D population may represent the rare gonocyte population. Both VASA_D and VASA_B cells co-expressed MAGEA4 further confirming that they are indeed primitive germ cells (Fig. 1C). Given the previous finding of OCT4A expression in VASA-negative gonoocytes, we evaluated VASA_D expression in these cells. Rare OCT4A-positive cells were detected only in cells expressing low levels of VASA (Fig. 1D), demonstrating that VASA_D cells are indeed gonocytes.

SSEA4 is a common membrane marker for gonocytes, prespermatogonia, and Sertoli cells
All of the cells within the seminiferous cord, during the second and third trimesters, expressed SSEA4 (Fig. 1E). Both VASA_D and VASA_B cells expressed SSEA4 at similar levels, indicating SSEA4 to be a common marker for both gonocytes and prespermatogonia. However, SSEA4 expression was also detected in the remaining VASA-negative cells making up the cord. WT1 expression was then evaluated to determine whether these SSEA4+/VASA− cells were Sertoli cells. WT1 was expressed exclusively in all SSEA4+/VASA− cells (Fig. 1F), confirming that all the non-germ cells expressing SSEA4+ in the seminiferous cords were Sertoli cells.

Gonocytes transiently express KIT and THY1
KIT and THY1 expression in gonocytes, prespermatogonia, and Sertoli cells was also evaluated. Gonocytes expressing OCT4A were found to co-express KIT, in addition to VASA_D (Fig. 2A). In contrast to SSEA4, the majority of THY1 expression was detected on cells outside of the seminiferous cords with the exception of a few cell clusters within the cords during the second trimester (Fig. 2B). Within the seminiferous cord, THY1+ cells were arranged in small clusters of 3–6 cells and expressed low levels of VASA (VASA_D) (Fig. 2C). In contrast, VASA_B cells never expressed THY1. Furthermore, WT1 expression was never detected in THY1+ cells, indicating that SSEA4+/THY1+ cells are primitive gonocytes (Fig. 2C). Within the cords, THY1+ cells co-expressed OCT4A further confirming that SSEA4, THY1, and KIT are membrane markers on gonocytes (Fig. 2D).

To confirm that SSEA4+/THY1+ cells were in fact gonocytes, male fetal testes were digested, FACS sorted, and individually analyzed for SSEA4 expression. SSEA4+ and SSEA− cells were individually assessed for THY1 expression (Fig. 3A). At 19 weeks of gestation, ~10% of the total SSEA4+ cells were THY1+ (gonocytes; Fig. 3A). The remaining ~90% of the SSEA4+ cells were THY1− (prespermatogonia and Sertoli cells). In contrast, >90% of SSEA− cells expressed THY1. Similar to confocal microscopy observations that the number of VASA_D cells decreased with advancing gestation (Fig. 1A and B), the number of gonocytes (SSEA4+/THY1+) decreased to ~6% at 23 weeks of gestation (Fig. 3A).

These findings were confirmed at the molecular level by qPCR analysis. SSEA4+/THY1+ (gonocytes) expressed high levels of VASA (139-fold) and OCT4A (13-fold; Fig. 3B). Although, VASA and OCT4A were also detected in SSEA4+/THY1− (prespermatogonia and Sertoli cells), their levels were significantly lower than the pure gonocyte population, confirming that this population contains both prespermatogonia and Sertoli cells (Fig. 3B). Both AMH and SOX9 were more highly expressed in VASA+B cells.

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Figure 1 The dynamic of VASA, OCT4A, and SSEA4 expression in male testes at 13–24 weeks of gestation. (A) Two populations of germ cells were observed based on relative expression of VASA, VASA dim (VASAD) vs VASA bright (VASAB), shown here at 13 weeks of gestation. Ratio of VASAD/VASAB germ cells ~ 3/2. (B) The ratio of VASAD/VASAB germ cells (~ 1/3) decreased with advancing gestation, shown here at 24 weeks of gestation. (C) All VASA− cells co-expressed MAGEA4, shown at 20 weeks of gestation. (D) Only VASAD germ cells expressed OCT4A during the second trimester, shown here at 21 weeks of gestation. (E) Both VASAD and VASAB germ cells expressed similar levels of SSEA4 shown at 20 weeks of gestation. (E and F) Fetal Sertoli cells also expressed SSEA4. Arrow head, arrow, and asterisk indicate VASAD, VASAB, and Sertoli cells respectively. Ratio of VASAD/VASAB cells was determined by counting all VASA− cells from ten cords. Donkey anti-goat Alexa 488 (VASA), anti-mouse Alexa 594 (MAGEA4), anti-rabbit Alexa 594 (OCT4A, WT1), and anti-mouse Alexa 555 (SSEA4) were used. *indicates somatic cells outside of the cords.
expressed in the SSEA4+/THY1− population than the SSEA4+/Thy1+ population, providing further support for the presence of Sertoli cells (Fig. 3C). Lastly, VIM expression was significantly higher in the somatic SSEA4− population in comparison with both SSEA4+/THY1+ and SSEA4+/THY1− population (Fig. 3D).

After 32 weeks of gestation, all cells (gonocytes, prespermatogonia, and Sertoli cells) within the seminiferous cord continued to express SSEA4 (data not shown). The ratio of VASA−/VASAB cells decreased to <1/5 when all VASA+ cells from ten cords were counted, consistent with the continuous differentiation of gonocytes to prespermatogonia during fetal development (Fig. 4A). After the second trimester, THY1 expression was detected exclusively in somatic cells outside of the seminiferous cords (Fig. 4B), while KIT and OCT4A expression was no longer detected in either VASAα or VASAβ cells (data not shown).

**SSEA4 continues to be the membrane marker for SSCs postnatally**

Both SSCs and Sertoli cells from 4-month and 4-year-old boys continued to express SSEA4 within the seminiferous cord, similar to the fetal testes in the third trimester (Fig. 4C). Neither THY1 nor KIT expression was detected within the seminiferous cord of these prepubertal testes (data not shown). However, in contrast to the fetal testes, <10% of spermatogonia were VASAα postnatally (Fig. 4D).

When adult seminiferous tubules were examined, two populations of germ cells, VASAα and VASAβ, were also detected. VASAα germ cells were seen in the basement membrane, where primitive spermatogonia and SSCs are located (Fig. 4E). In contrast, VASAβ germ cells were seen nearer toward the lumen consistent with mature spermatocytes. Interestingly, SSEA4 expression was detected only in VASAα germ cells, indicating that...
SSEA4 remained to be the membrane marker of primitive spermatogonia (Fig. 4E). Although THY1 expression had been found in early-fetal gonocytes, THY1 expression was restricted solely to somatic cells (Sertoli cells, peritubular interstitial cells, and cells making up the lamina propria) in adult men (Fig. 4F). Figure 5 shows the membrane markers of gonocytes and primitive spermatogonia (SSCs) during fetal and postnatal development.

Discussion

We conducted a comprehensive in vitro characterization of germ cell membrane markers in human gonocytes, prespermatogonia, and SSCs from 13 weeks of gestation through adulthood. We report dynamic changes in the expression of known germ cell markers THY1, KIT, OCT-4A, and VASA, and identified SSEA4 as a conserved extracellular membrane marker of male primitive germ cells during human male germ cell development.

In murine studies, VASA and OCT4 are co-expressed in primordial germ cells during their migration to the gonadal ridge (Fujiiwara et al. 1994, Tanaka et al. 2000). Previous human studies reported that male germ cells do not express VASA until after 14 weeks of gestation (Anderson et al. 2007). Although OCT4A was described as the quintessential marker of human gonocytes during the first trimester, there was an uncoupling of OCT4A-expressing gonocytes during the second trimester, as most gonocytes ceased to express OCT4A and differentiated into VASA expressing prespermatogonia (Pauls et al. 2006, Anderson et al. 2007). However, a recent study has demonstrated that human gonocytes co-expressed OCT4A, KIT, and VASA during the first trimester (7–11 weeks of gestation; Gkountela et al. 2012). Similarly, there was also an uncoupling of OCT4A+/KIT+/VASA+ gonocytes into OCT4A+/KIT+/VASA− gonocytes and OCT4A−/KIT−/VASA+ prespermatogonia during the second trimester (Anderson et al. 2007, Gkountela et al. 2012). Due to limited sample availability, we focused our studies on male testes at 13 weeks of gestation and beyond. In the present study, we described VASAD and VASAB cells as two temporally and spatially distinct populations of germ cells that persisted through the second and third trimester. Our findings suggest a similar uncoupling of gonocytes and prespermatogonia to that previously reported in humans (Pauls et al. 2006, Anderson et al. 2007). While Gkountela and colleagues described two major distinct

Figure 3 Molecular analyses of fetal gonocytes, prespermatogonia and Sertoli cells. (A) SSEA4 and THY1 can be used as markers for separating gonocytes from prespermatogonia/Sertoli cells by FACS. Cellular debris clumps and dead cells were gated out before sorting. SSEA4+ and SSEA4− cells were evaluated individually for THY1 expression. Consistent with confocal microscopy findings, the ratio of fetal gonocytes to prespermatogonia (VASA+/VASA−) declined with advancing gestation as demonstrated here between 19 and 23 weeks of gestation. (B) SSEA4+/THY1− cells (gonocytes) expressed VASA and OCT-4A at significantly higher levels than SSEA4−/THY1− (prespermatogonia/Sertoli cells) cells. (C) SSEA4+/THY1− cells expressed significantly higher levels of genes (AMH specific to Sertoli cells, SOX9 expression was not evaluated statistically for significance because only two biological samples were analyzed in the SSEA4+/THY1− population. (D) Very low level of stromal marker VIM was detected in the SSEA4+ populations. All qPCR reactions were run in triplicates with three biological samples per group at 19 weeks of gestation except for one condition, in which only two biological samples were analyzed indicated as **. * statistical significant with P<0.01.
Figure 4 Changes in germ cell marker expression in the fetal testes after 24 weeks of gestation and after birth. (A) The number of gonocytes (VASA\textsubscript{D} cells) per testicular cord continued to decrease with advancing gestation. The ratio of VASA\textsubscript{D}/VASA\textsubscript{B} cells was <20% at 32 weeks of gestation as shown here. (B) Although THY1 continued to be expressed in the somatic cells outside of the seminiferous cords, all germ cells ceased to express THY1, shown at 37 weeks of gestation. (C) In contrast, SSEA4 continued to be expressed in all cells within the seminiferous cord postnatally, shown here at 4 years of age. (D) The ratio of VASA\textsubscript{D}/VASA\textsubscript{B} germ cells (<10%) continued to decline postnatally, shown here at 4 months of age. (E) Seminiferous tubules matured and formed lumen post pubertally as shown here from a normal adult sample. Primitive spermatogonia, located at the basement membrane, expressed low level of VASA (VASA\textsubscript{D}) whereas differentiating spermatocytes expressed high level of VASA (VASA\textsubscript{B}). While SSEA4 continued to be expressed in VASA\textsubscript{D} spermatogonia, it was no longer expressed in Sertoli cells. (F) THY1 continued to be the marker of somatic cells within the seminiferous tubules. In addition, as Sertoli cells ceased to express SSEA4 after puberty, they began to express THY1 as shown with co-expression of WT1. Arrow heads and arrows indicate VASA\textsubscript{D} and VASA\textsubscript{B} cells respectively. Donkey anti-goat Alexa 488 (VASA), anti-rabbit Alexa 594 (WT1), and anti-mouse Alexa 555 (SSEA4) were used.
populations of male human germ cells (KIT^+/VASA^- and KIT^-/VASA^+) in second trimester testes, we did not detect any KIT^+/VASA^- gonocytes at any time points in our studies. As we used the same anti-VASA antibody, it is possible that differences in tissue processing and our use of confocal microscopy may account for the discrepancy in the relative detection of VASA expression between studies. Although none of the VASA_B germ cells in our study expressed markers associated with gonocytes (OCT4A and KIT), all VASA_D germ cells co-expressed both OCT4A and KIT, suggesting that they were the same population of primitive gonocytes previously reported (Pauls et al. 2006, Gkountela et al. 2012). Similar to the decline in KIT^+ and OCT4A^+ gonocytes seen in previous studies, the number of OCT4A^+/KIT^+/VASA_B gonocytes also declined with advancing gestation in our studies (Pauls et al. 2006, Anderson et al. 2007, Gkountela et al. 2012).

Comprehensive screening of previously reported extracellular membrane markers of SSCs and embryonic stem cells revealed that KIT, THY1, and SSEA4 are the markers of human gonocytes. Specifically, KIT was found to be a transient marker of gonocytes during the second trimester; thereafter, its expression was not detected within the primitive germ cell compartment thereafter, consistent with previous human studies (Pauls et al. 2006, Gkountela et al. 2012). While THY1 was shown to be a marker of mouse SSCs, its role as marker of primate and adult human SSCs is controversial (Kubota et al. 2003, Conrad et al. 2008, Ko et al. 2010, 2011, Tapia et al. 2011, Chikhovskaya et al. 2012, Eildermann et al. 2012). We have recently demonstrated, using highly purified population of adult human testicular THY1^+ cells for analyses, that THY1^+ is a marker of adult testicular somatic cells, rather than SSCs which expressed SSEA4 (Smith et al. 2014). The findings of transient THY1 expression within the gonocyte population during the second trimester of gestation confirm that THY1 is not a marker of human SSCs postnatally (Ko et al. 2011, Tapia et al. 2011, Chikhovskaya et al. 2012, Smith et al. 2014).

SSEA4 is also a known marker of undifferentiated pluripotent human embryonic stem cells, cleavage to blastocyst stage embryos, and bone marrow-derived mesenchymal stem cells (Henderson et al. 2002, Rosu-Myles et al. 2013). Although associated with undifferentiated cells, the function of SSEA4 is currently unknown and remained to be investigated (Brimble et al. 2007). We demonstrated that SSEA4 was the common marker of human gonocytes, prespermatogonia, and primitive spermatogonia starting at 13 weeks of gestation through postpuberty, in contrast to the transient expression of THY1 and KIT seen in gonocytes. Although restricted to the seminiferous cord, SSEA4 expression was not exclusively expressed in the germ cell compartment within the fetal and prepubertal testes. In addition to gonocytes and prespermatogonia, SSEA4 was also found to be a marker of human Sertoli cells before puberty as demonstrated by confocal microscopy and confirmed by molecular analyses of subpopulations of SSEA4 expressing cells. However, there was a significant change in SSEA4 expression within the seminiferous

![Figure 5](https://www.reproduction-online.org)

**Figure 5** Markers of human gonocytes, prespermatogonia, and spermatogonia during development.

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*Indicates also a marker of sertoli cells
#Previously reported as markers of gonocytes and adult human SSCs.
VASA_D, VASA dim; VASA_B, VASA bright.
tubules after puberty. Whereas SSEA4 expression continued to be restricted to the primitive spermatogonia in adult, Sertoli cells no longer expressed SSEA4. Our findings are consistent with recent reports that SSEA4 expression is restricted exclusively to primitive spermatogonia within the adult primate and human seminiferous tubules (Muller et al. 2008, Maki et al. 2009, Izadyar et al. 2011, Pacchiarotti et al. 2013). To assess the specificity of the SSEA4 antibody, we also evaluated for the expression of SSEA1 and SSEA3 by FACS and confocal microscopy and found that neither SSEA1 nor SSEA3 was expressed in human testicular tissues (data not shown), consistent with previous studies in primates (Muller et al. 2008).

SSEA4 is a conserved extracellular marker of primitive male human germ cells through all stages of development as described herein. Recent studies have also reported fibroblast growth factor receptor 3 (FGFR3) as a potential conserved membrane maker of human primitive spermatogonia (von Kopylow et al. 2010, 2012, Kossack et al. 2013). However, additional studies are still needed to further characterize this population (von Kopylow et al. 2010, 2012). In contrast to previous studies, we did not detect GPR125 and GFRz1R expression in the fetal testes (Wu et al. 2009, He et al. 2010, Dovey et al. 2013). In addition, GPR125, GFRz1R, and EPCAM expression does not appear to be specific to germ cells (Wu et al. 2009, He et al. 2010, Dovey et al. 2013). We recognize that GFRz1R may be expressed in human fetal testes at a defined gestational window that we may not have evaluated, as mouse studies have demonstrated that Gfra1 mRNA is detected in the testes up to dpc 14 and undetectable thereafter (Golden et al. 1999).

Recent studies have demonstrated that highly purified sorted adult human testicular SSEA4+ cells are germ cells that have not entered meiosis and can give rise to SSC colonies capable of expansion in vitro (Smith et al. 2014). Enriched adult testicular SSEA4+ cells were able to colonize mouse seminiferous tubules after transplantation confirming that the SSEA4+ population is highly enriched for SSCs (Izadyar et al. 2011). Thus, current evidence supports the use of SSEA4 as a membrane marker to isolate human primitive spermatogonia postnatally for in vitro expansion and differentiation. Currently, only one study demonstrated the ability to expand human SSCs in vitro by culture of unpurified prepubertal testicular tissue; however, the membrane markers of these prepubertal SSCs were not evaluated (Sadri-Ardekani et al. 2011).

In summary, we have described and characterized the dynamic changes in the expression of extracellular membrane markers of human male primitive germ cells from 13 weeks of gestation through adult. Specifically, SSEA4 was shown to be a unique ontogenically conserved marker of human spermatogonia through all stages of development. This finding contributes to the knowledge gap of identifying primitive spermatogonia for future transplantation studies.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-14-0123.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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