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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 (FACS), and qRT-PCR for expression of unique germ cell membrane markers. During male fetal development, THY1 and C-Kit are transient markers of gonocytes but not in prespermatogonia and post-natal SSCs. Although C-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, SSEA-4 expression was only detected in the gonocytes, prespermatogonia, SSCs, and Sertoli cells of the fetal and prepubertal testes. After puberty, SSEA-4 expression can only be detected in primitive spermatogonia. Thus, although THY1 and C-Kit are transient markers of gonocytes, SSEA-4 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.

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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 transplantations 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, Sadri-Ardekani *et al.* 2011, Mirzapour *et al.* 2012). Additionally, 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 prior 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, Fujita *et al.* 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, Eildermann *et al.* 2012, Dovey *et al.* 2013, Kossack *et al.* 2013). Although THY1, GFRα1R, GPR125, EPCAM, and SSEA-4 have been reported to be unique membrane markers of adult human SSCs, many of these markers (GFRα1R, 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 SSEA-4 were shown to engraft in mouse seminiferous tubules, SSEA-4 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, prespermatogonia), prior to the onset of spermatogenesis and follow them through puberty (spermatogonia) and adulthood. If a unique membrane marker of primitive germ cells were present through all stages of development it may serve as an important marker of SSCs. Recent studies report 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 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 differentiation of gonocytes (Gkountela *et al.* 2012). While the prespermatogonia expressed VASA, they no longer expressed OCT4A and C-Kit (Pauls *et al.* 2006, Anderson *et al.* 2007, Gkountela *et al.* 2012). Therefore, C-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

Tissues were fixed in 4% paraformaldehyde, embedded in optimal cutting temperature compound (O.T.C) (Sakura Finetek, Torrance, CA), and cryosectioned at 5 μm. Sections were permeabilized with 0.1% Triton-X-100 PBS (Sigma-Aldrich, St. Louis, MO), blocked in 5% BSA-PBS, and incubated overnight at 4°C with the following antibodies: goat anti-VASA (R&D-AF2030 at 1:100 dilution, Minneapolis, MN), anti-THY1 (BD-559869 at 1:50, San Jose, CA, and R&D-AF206 at 1:40), rabbit anti-WT1 (Santa Cruz-SC-192 at 1:75, Dallas, TX), anti-OCT4A (Santa Cruz SC-9081 at 1:75, and SC-8628 at 1:75), mouse anti-SSEA-4 (BD-560308 at 1:50), anti-C-Kit (Santa Cruz SC-5535 at 1:50 and M14 at 1:50), mouse anti-SSEA-1(R&D- FAB2155A at 1:50), mouse anti-TRA-1-81 (BD-560885 at 1:50), mouse anti-TRA-1-60 (BD-560071 at 1:50), goat anti- GFRα-1R (R&D-AF714 at 1:50), rabbit anti-MAGEA4 (Abcam ab76177 at 1:50), rat anti-SSEA-3 (Abcam ab16286 at 1:100), and rabbit anti-GPR125 (Abcam-ab51705 at 1:50, Cambridge, MA). 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) were applied accordingly the following day at 1:200–1:500 dilutions at room temperature for 1 hour. Images were captured using a Leica SP5 AOBS confocal microscope (Leica Microsystems Inc, Buffalo Grove, IL), and analyzed using ImageJ v1.6 (rsbweb.nih.gov).

Testicular Cell Isolation and Fluorescence Activated Cell Sorting (FACS)

Tissues were subjected to a two-step enzymatic digestion with collagenase IV (1mg/ml) (Sigma-Aldrich) in DMEM/F12 + Glutamax (Invitrogen, Grand Island, NY) 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. Cells were incubated with the following antibodies: anti-SSEA-4 FITC (BD-560308), anti-THY-1 APC (BD-559869), anti-c-KIT PE (R&D-FAB332P), anti-TRA-1-81 PE (BD-560885) and anti-SSEA-1 APC (R&D-FAB2155A), in 1% bovine serum albumin (BSA) for 30 min at 37°C. Cell sorting was performed on a BD FACS Aria Flow Cytometer and analyzed using FlowJo v9.6 (Ashland, OR). 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, Valencia, CA) and cDNA was synthesized using qScript cDNA Super Mix (Quanta Biosciences, Gaithersburg, MD). Each qPCR amplification was performed in triplicate at 250 cells/reaction using FastStart Universal SYBR Green Master Mix with ROX (Roche, Pleasanton, CA) and Applied Biosystem 7500 Real-time PCR System (Carlsbad, CA). Please see supplemental table for list of primers and sequences. Gene expression was analyzed using the $2^{-(\Delta C(t))}$ and $2^{-(\Delta\Delta C(t))}$ methods. ANOVA were used for statistical analyses.

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, GFRα1R, SSEA-1, SSEA-4, C-Kit, and THY1), expression of embryonic stem cell membrane markers (TRA1-60 and TRA1-81) were also evaluated in both gonocytes and prespermatogonia. GPR125, GFRα1R, TRA1-60, TRA1-81, and SSEA-1 expression was not detected in either early or late second trimester testes by confocal microscopy and flow cytometry (data not shown). 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 10 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 MAGEA further confirming that they are indeed primitive germ cells (Fig. 1C). Given the prior finding of OCT4A expression in VASA negative gonocytes, 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.

SSEA-4 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 SSEA-4 (Fig. 1E). Both $VASA_D$ and $VASA_B$ cells expressed SSEA-4 at similar levels indicating SSEA-4 to be a common marker for both gonocytes and prespermatogonia. However, SSEA-4 expression was also detected in the remaining VASA negative cells making up the cord. WT1 expression was then evaluated to determine whether these SSEA-4+/VASA− cells were Sertoli cells. WT1 was expressed exclusively in all SSEA-4+/VASA− cells (Fig. 1F) confirming that all the non-germ cells expressing SSEA-4+ in the seminiferous cords were Sertoli cells.

Gonocytes transiently express C-Kit and THY1

C-Kit and THY1 expression in gonocytes, prespermatogonia, and Sertoli cells was also evaluated. Gonocytes expressing OCT4A were found to co-express C-Kit, in addition to $VASA_D$ (Fig. 2A). In contrast to SSEA-4, 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 SSEA-4+/THY1+ cells are primitive gonocytes (Fig. 2C). Within the cords, THY1+ cells co-expressed OCT4A further confirming that SSEA-4, THY1, and C-Kit are membrane markers on gonocytes (Fig. 2D).

To confirm that SSEA-4+/THY1+ cells were in fact gonocytes, male fetal testes were digested, FACS sorted and individually analyzed for SSEA-4 expression. SSEA-4+ and SSEA-4− cells were individually assessed for THY1 expression (Fig. 3A). At 19 weeks of gestation, ~10% of the total SSEA-4+ cells were THY1+ (gonocytes) (Fig. 3A). The remaining ~90% of the SSEA-4+ cells were THY1 negative (prespermatogonia and Sertoli cells). In contrast, >90% of SSEA-4− cells expressed THY1. Similar to confocal microscopy observations that the number of $VASA_D$ cells decreased with advancing gestation (Fig. 1A–B), the number of gonocytes (SSEA-4+/THY1+) decreased to ~6% at 23 weeks of gestation (Fig. 3A).

These findings were confirmed at the molecular level by qPCR analysis. SSEA-4+/THY1+ (gonocytes) expressed high levels of *VASA* (139-fold) and *OCT4A* (13-fold) (Fig 3B). Although, *VASA* and *OCT4A* were also detected in SSEA-4+/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 the SSEA-4+/THY1− population than the SSEA-4+/Thy1+ population providing further support for the presence of Sertoli cells (Fig. 3C). Lastly, *VIM* expression was significantly higher in the somatic SSEA-4− population in comparison to both SSEA-4+/THY1+ and SSEA-4+THY1− population (Fig. 3D).

After 32 weeks of gestation, all cells (gonocytes, prespermatogonia, and Sertoli cells) within the seminiferous cord continued to express SSEA-4 (data not shown). The ratio of $VASA_D$/$VASA_B$ cells decreased to less than 1/5 when all VASA+ cells from 10 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, C-Kit and OCT4A expression were no longer detected in either $VASA_D$ or $VASA_B$ cells (data not shown).

SSEA-4 continues to be the membrane marker for spermatogonial stem cells (SSCs) postnatally

Both SSCs and Sertoli cells from 4-month and 4-year old boys continued to express SSEA-4 within the seminiferous cord, similar to the fetal testes in the third trimester (Fig. 4C). Neither THY1 nor C-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 $VASA_D$ postnatally (Fig. 4D).

When adult seminiferous tubules were examined, two populations of germ cells, $VASA_D$ and $VASA_B$, were also detected. $VASA_D$ germ cells were seen in the basement membrane, where primitive spermatogonia and SSCs are located (Fig. 4E). In contrast, $VASA_B$ germ cells were seen nearer toward the lumen consistent with mature spermatocytes. Interestingly, SSEA-4 expression was detected only in $VASA_D$ germ cells indicating that SSEA-4 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 summarizes 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 spermatogonial stem cells from 13 weeks of gestation through adulthood. We report dynamic changes in the expression of known germ cell markers THY1, C-Kit, OCT4A, and VASA and identified SSEA-4 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 (Fujiwara *et al.* 1994, Tanaka *et al.* 2000). Prior 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 demonstrates that human gonocytes co-expressed OCT4A, C-Kit, and VASA during the first trimester (7–11 weeks of gestation) (Gkountela *et al.* 2012). Similarly, there was also an uncoupling of OCT4A+/C-Kit+/VASA+ gonocytes into OCT4A+/C-Kit+/VASA− gonocytes and OCT4A−/C-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 $VASA_D$ and $VASA_B$ 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 populations of male human germ cells (C-Kit+/VASA−, and C-Kit−/VASA+) in second trimester testes, we did not detect any C-Kit+/VASA− gonocytes at any time points in our studies. Since the same anti-VASA antibody was used, 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 C-Kit), all $VASA_D$ germ cells co-expressed both OCT4A and C-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 C-Kit + and OCT4A+ gonocytes seen previous studies, the number of OCT4A+/C-Kit+/$VASA_D$ 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 C-Kit, THY1, and SSEA4 are markers of human gonocytes. Specifically, C-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, Ko *et al.* 2011, Tapia *et al.* 2011, Chikhovskaya *et al.* 2012, Eildermann *et al.* 2012). We 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 SSEA-4 (Yango *et al.* 2013). 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, Yango *et al.* 2013).

SSEA-4 is also a known marker of undifferentiated pluripotent human embryonic stem cells, cleavage to blastocyst stage embryos, and bone marrow derived mesenchymal stem cells (MSCs) (Henderson *et al.* 2002, Rosu-Myles *et al.* 2013). Although associated with undifferentiated cells, the function of SSEA-4 is currently unknown and remained to be investigated (Brimble *et al.* 2007). We demonstrated that SSEA-4 was the common marker of human gonocytes, prespermatogonia, and primitive spermatogonia starting at 13 weeks of gestation through post-puberty, in contrast to the transient expression of THY1 and C-Kit seen in gonocytes. Although restricted to the seminiferous cord, SSEA-4 expression was not exclusively expressed in the germ cell compartment within the fetal and prepubertal testes. In addition to gonocytes and prespermatogonia, SSEA-4 was also found to be a marker of human Sertoli cells prior to puberty as demonstrated by confocal microscopy and confirmed by molecular analyses of subpopulations of SSEA-4 expressing cells. However, there was a significant change in SSEA-4 expression within the seminiferous tubules after puberty. Whereas SSEA-4 expression continued to be restricted to the primitive spermatogonia in adult, Sertoli cells no longer expressed SSEA-4. Our findings are consistent with recent reports that SSEA-4 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 SSEA-4 antibody, we also evaluated for SSEA-1 and SSEA-3 expression by FACS and confocal microscopy and found that neither SSEA-1 and SSEA-3 was expressed in human testicular tissues (data not shown), consistent with previous studies in primates (Muller *et al.* 2008).

SSEA-4 is a conserved extracellular marker of primitive male human germ cells through all stages of development as described here. Recent studies also report fibroblast growth factor receptor 3 (FGFR3) as a potential conserved membrane maker of human primitive spermatogonia (von Kopylow *et al.* 2010, von Kopylow *et al.* 2012, Kossack *et al.* 2013). However, additional studies are still needed to further characterize this population (von Kopylow *et al.* 2010, von Kopylow *et al.* 2012). In contrast to prior studies, we did not detect GPR125 and GFRα1R expression in the fetal testes (Wu *et al.* 2009, He *et al.* 2010, Dovey *et al.* 2013). Additionally, GPR125, GFRα1R, 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 GFRα1R may be expressed in human fetal testes at a defined gestational window that we may not have evaluated, as mouse studies have demonstrated that Gfrα1 mRNA is detected in the testes up to dpc 14 and undetectable thereafter (Golden *et al.* 1999).

Recent studies demonstrated that highly purified sorted adult human testicular SSEA-4+ cells are germ cells that have not entered meiosis and can give rise to SSC colonies capable of expansion *in vitro* (Yango *et al.* 2013). Enriched adult testicular SSEA-4+ cells were able to colonize mouse seminiferous tubules after transplantation confirming that the SSEA-4+ population is highly enriched for SSCs (Izadyar *et al.* 2011). Thus, current evidence supports the use of SSEA-4 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 pre-pubertal 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, SSEA-4 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 Material

Refer to Web version on PubMed Central for supplementary material.

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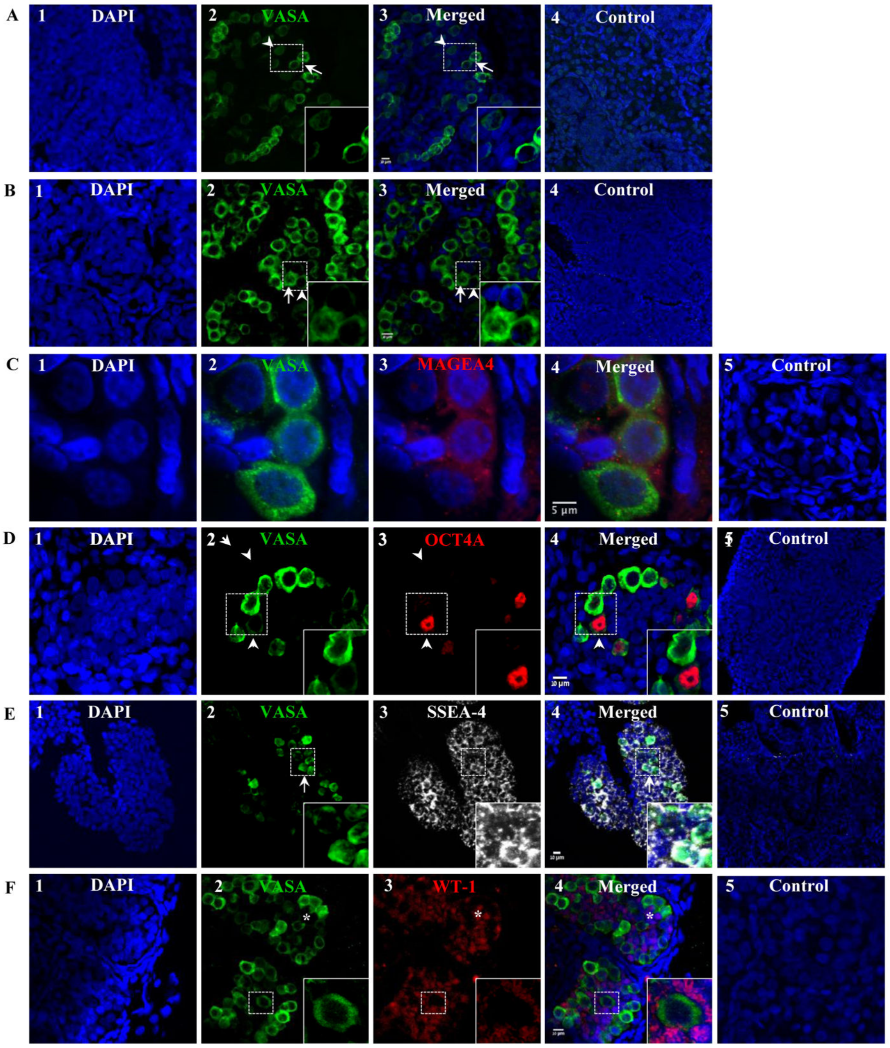

Figure 1.

The dynamic of VASA, OCT4A, and SSEA-4 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 ($VASA_D$) vs. VASA bright ($VASA_B$), shown here at 13 weeks of gestation. Ratio of $VASA_D/VASA_B$ germ cells ~3/2. (B) The ratio of $VASA_D/VASA_B$ 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 $VASA_D$ germ cells expressed OCT4A during the second trimester, shown here at 21 weeks of gestation. (E) Both $VASA_D$ and $VASA_B$ germ cells expressed similar levels of SSEA-4 shown at 20 weeks of gestation. (E–F) Fetal Sertoli cells also expressed SSEA-4. Arrow head, arrow, and asterisk indicate $VASA_d$, $VASA_b$, and Sertoli cells, respectively. Ratio of $VASA_D/VASA_B$ cells was determined by counting all VASA+ cells from 10 cords. Donkey anti-goat Alexa 488 (VASA), anti-mouse Alexa 594 (MAGEA4), anti-rabbit Alexa 594 (OCT4A, WT-1), and anti-mouse Alexa 555 (SSEA-4) were used.

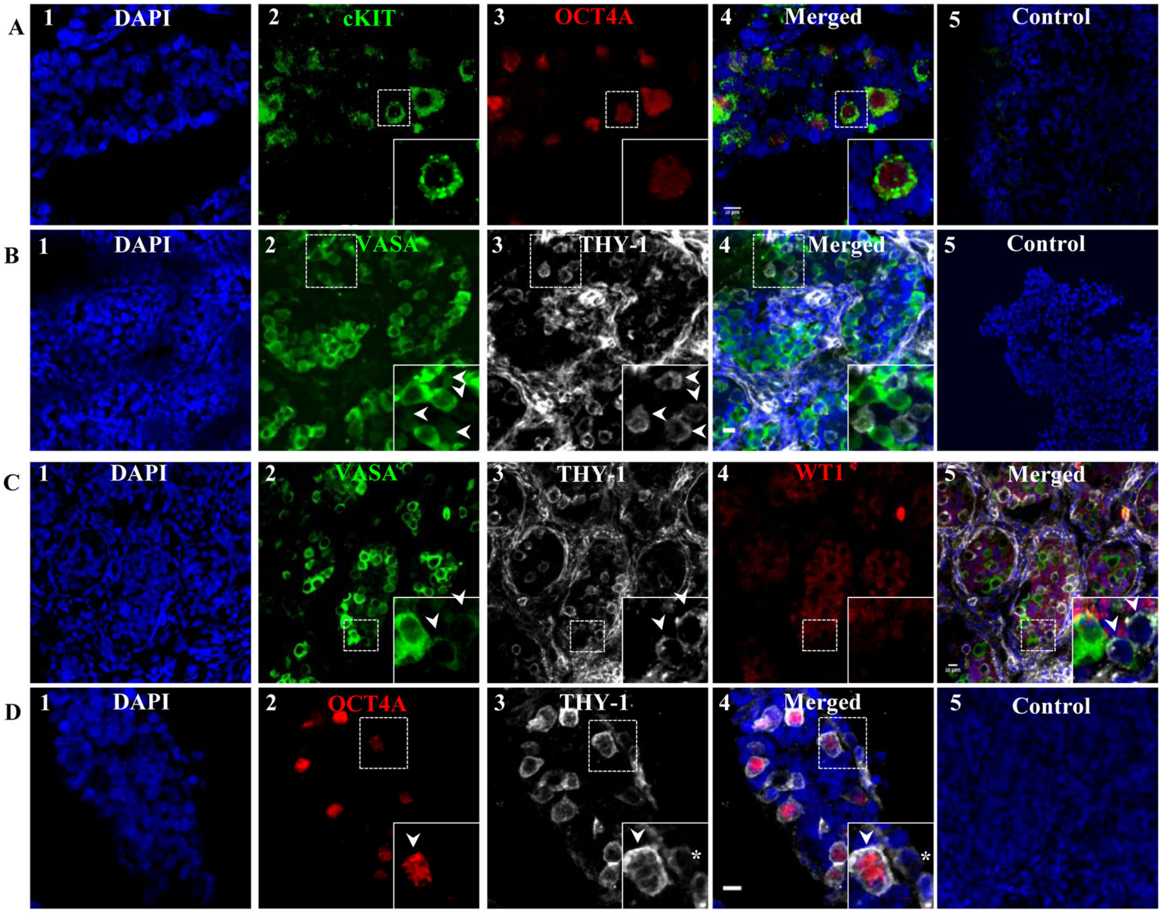

Figure 2.

Extracellular membrane markers of fetal male gonocytes. Gonocytes also co-expressed C-Kit (A) and THY1 (B), shown at 15 and 19 weeks of gestation, respectively. While SSEA-4+ is a general marker for all germ and Sertoli cells in the fetal testes, THY1 expression within the SSEA-4 population was restricted only to gonocytes that expressed low level of VASA (VASA$_D$) (C) and OCT4A (D), shown at 21 and 18 weeks of gestation, respectively. Arrow heads indicate VASA$_D$ cells (gonocytes). Donkey anti-goat Alexa 488 (VASA), anti-mouse Alexa 488 (C-Kit), anti-rabbit Alexa 594 (OCT4A), and anti-mouse Alexa 555 (THY1) were used.

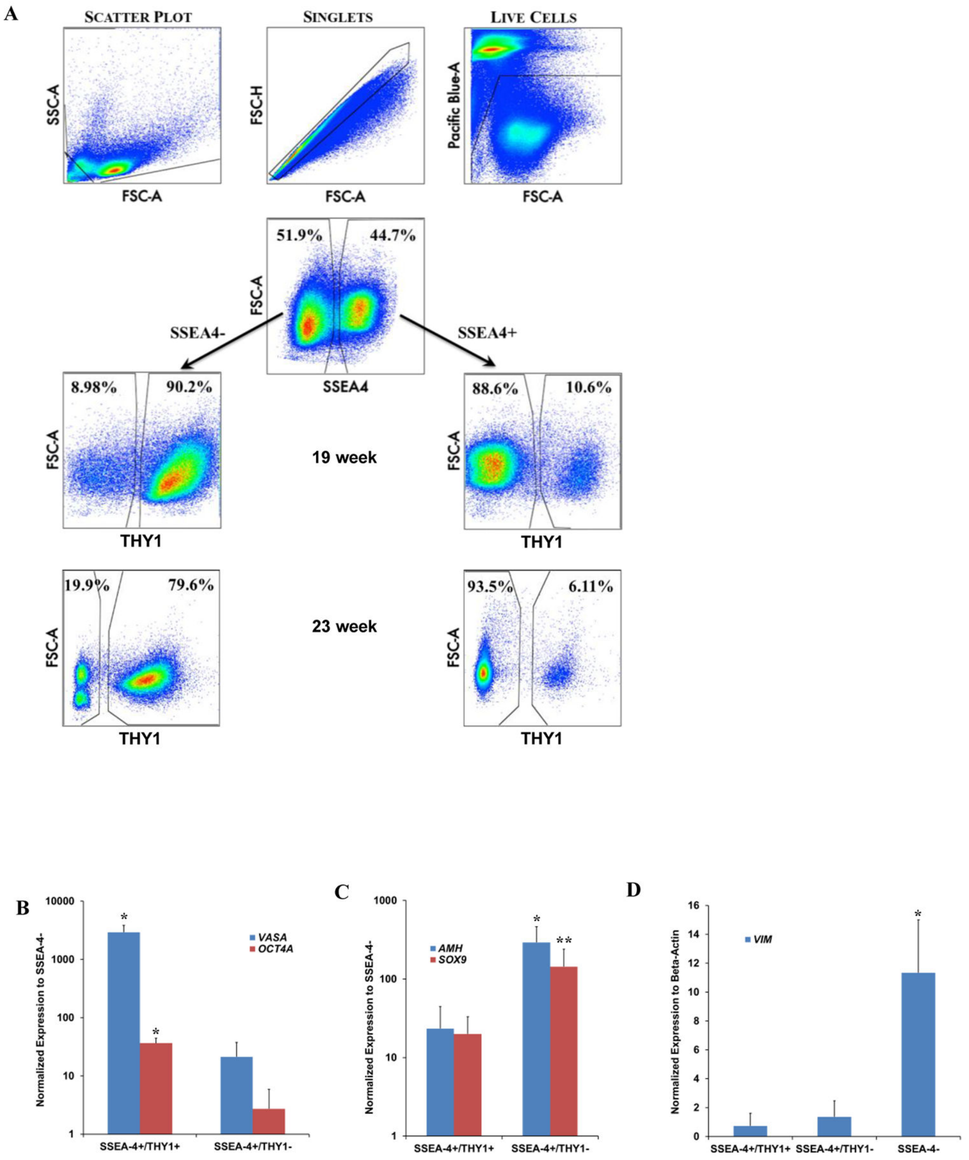

Figure 3.

Molecular analyses of fetal gonocytes, prespermatogonia, and Sertoli cells. (A) SSEA-4 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 prior to sorting. SSEA-4+ and SSEA-4− cells were evaluated individually for THY1 expression. Consistent with confocal microscopy findings, the ratio of fetal gonocytes to prespermatogonia (VASA_D/VASA_B) declined with advancing gestation as demonstrated here between 19 and 23 weeks of gestation. (B) SSEA-4+/THY1+ cells (gonocytes) expressed *VASA* and *OCT4A* at significantly higher levels than SSEA-4+/THY1− (prespermatogonia/Sertoli cells) cells. (C) SSEA-4+/THY1− cells expressed significantly higher levels of genes (*AMH*) specific to Sertoli cells. *SOX9* expression was not evaluated statistically for significance because only 2 biological samples were analyzed in the SSEA-4+/THY1− population. (D) Very low level of stromal marker *VIM* was detected in the SSEA-4+ populations. All qPCR reactions were ran in triplicates with 3 biological samples per group at 19 weeks of gestation except for one condition in which only 2 biological samples were analyzed indicated as **. * indicates statistical significant with p<0.01.

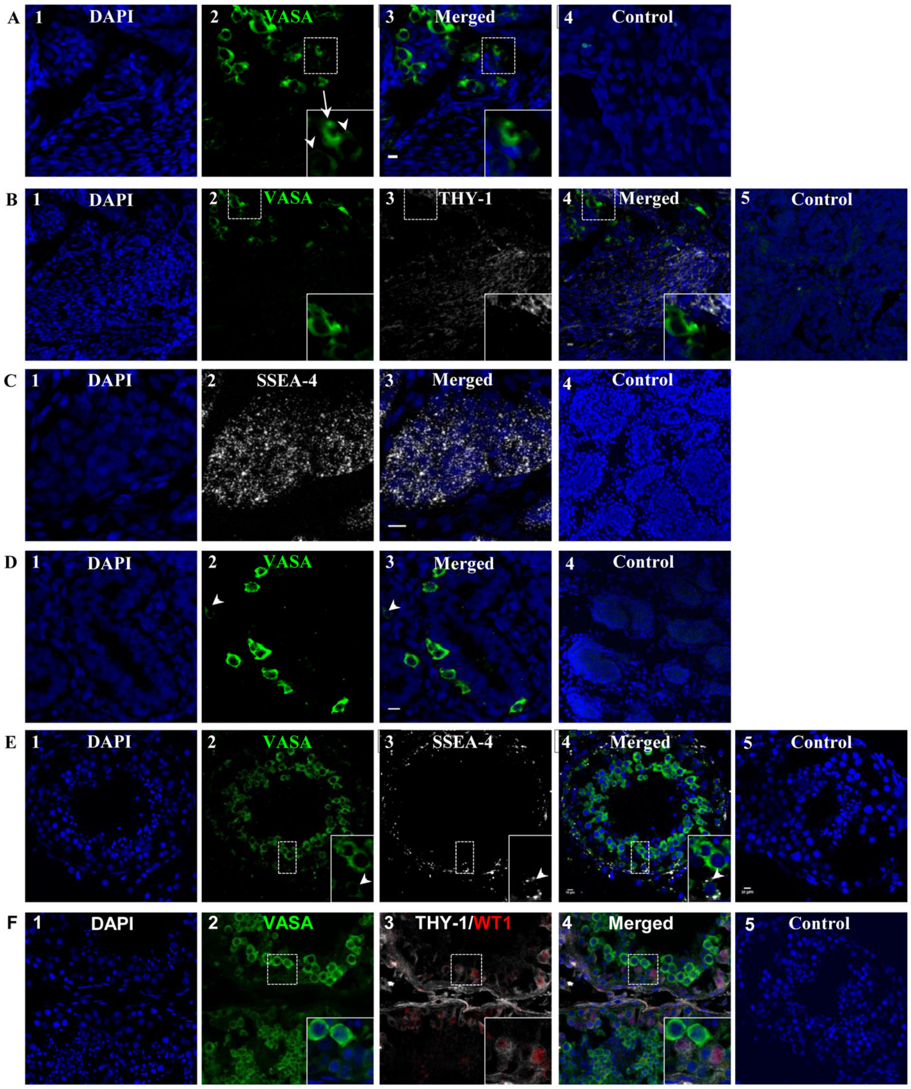

Figure 4.

Changes in germ cell marker expression in the fetal testes after 24 weeks of gestation and after birth. (A) The number of gonocytes (VASA$_D$ cells) per testicular cord continued to decrease with advancing gestation. The ratio of VASA$_D$/VASA$_B$ cells was <20% at 32 weeks of gestation as shown here. (B) Although THY-1 continued to be expressed in the somatic cells outside of the seminiferous cords, all germ cells ceased to express THY-1, shown at 37 weeks of gestation. (C) In contrast, SSEA-4 continued to be expressed in all cells within the seminiferous cord postnatally, shown here at 4 years of age. (D) The ratio of VASA$_D$/VASA$_B$ 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$_D$) whereas differentiating spermatocytes expressed high level of VASA (VASA$_B$). While SSEA-4 continued to be expressed in VASA$_D$ spermatogonia, it was no longer expressed in Sertoli cells. (F) THY-1 continued to be the marker of somatic cells within the seminiferous tubules. Additionally, as Sertoli cells ceased to express SSEA-4 after puberty, they began to express THY-1 as shown with co-expression of WT1. Arrow heads and arrows indicate $VASA_D$ and $VASA_B$ cells, respectively. Donkey anti-goat Alexa 488 (VASA), anti-rabbit Alexa 594 (WT-1), and anti-mouse Alexa 555 (SSEA-4) were used.

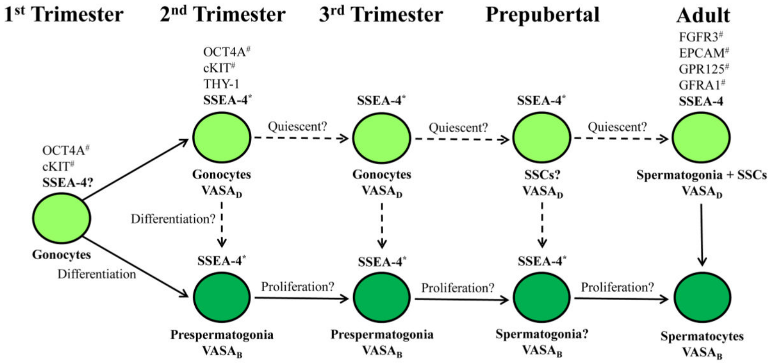

Figure 5.

Markers of human gonocytes, pre-spermatogonia, and spermatogonia during development.

[*] 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