

Marker expression reveals heterogeneity of spermatogonia in the neonatal mouse testis

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Abstract

Prospermatogonia transition to type A spermatogonia, which provide the source for the spermatogonial stem cell (SSC) pool. A percentage of these type A spermatogonia then differentiate to enter meiosis as spermatocytes by ~P10. It is currently unclear as to when these distinct populations are initially formed in the neonatal testis, and when the expression of markers both characteristic of and required for the adult undifferentiated and differentiating states is established. In this study, we compared expression of known spermatogonial cell fate markers during normal development and in response to the differentiation signal provided by retinoic acid (RA). We found that some markers for the undifferentiated state (ZBTB16/PLZF and CDH1) were expressed in nearly all spermatogonia from P1 through P7. In contrast, differentiation markers (STRA8 and KIT) appeared in a subset of spermatogonia at P4, coincident with the onset of RA signaling. GFRA1, which was present in nearly all prospermatogonia at P1, was only retained in STRA8/KIT – spermatogonia. From P4 through P10, there was a great deal of heterogeneity in the male germ cell population in terms of expression of markers, as markers characteristic of the undifferentiated (except GFRA1) and differentiating states were co-expressed through this interval. After P10, these fate markers diverged to mark distinct populations of undifferentiated and differentiating spermatogonia, and this pattern was maintained in juvenile (P18) and adult (P > 60) testes. Taken together, these results reveal that the spermatogonia population is heterogeneous during the first wave of spermatogenesis, and indicate that neonatal spermatogonia may not serve as an ideal substitute for studying the function of adult spermatogonia.

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Introduction

In the mouse, prospermatogonia (also called gonocytes and, less commonly, prespermatogonia) proliferate briefly after sex determination in the fetal testis, and then enter a prolonged quiescent period from approximately embryonic (E) day 14.5 until postnatal (P) days 1–2 (Vergouwen *et al.* 1991, Western *et al.* 2008). At that point, neonatal prospermatogonia begin to move to the periphery of the testis cords and resume mitosis as spermatogonia, marking the initiation of spermatogenesis (Nagano *et al.* 2000, Drumond *et al.* 2011). Spermatogonia become flanked by Sertoli cells within the cord and myoid cells outside the cord, and respond to juxtacrine and paracrine signals from these somatic cells in this ‘niche’ to either remain undifferentiated (A_{undiff}) or differentiate (A_{diff}) to ultimately enter meiosis by ~P10 (de Rooij 2001). This is termed the first wave of spermatogenesis, and it does not rely upon stem cell function, indicating that the first complement of sperm develop directly from this first group of spermatogonia (Yoshida *et al.* 2006). In subsequent waves, the spermatogonial stem cell (SSC) population provides a

consistent source of progenitor spermatogonia that differentiate to ensure fertility for the remainder of the male reproductive lifespan.

A_{undiff} and A_{diff} spermatogonia are further characterized based on their topology; the most well-accepted current model predicts that stem cell potential predominates in individual A_s spermatogonia, and this potential progressively diminishes as they divide into clones with retained intercellular bridges ($A_{pr-A_{al}}$; Yang & Oatley 2014). Although spermatogonial development begins shortly after birth in the mouse, it is unclear how similar development during the first wave of spermatogenesis is to that during steady-state spermatogenesis in the adult. This is an important point, as numerous studies utilize isolated spermatogonia from neonatal mice (especially at P7, where there are a relatively high percentage of the testicular cell population) as a substitute for adult spermatogonia. Recent studies have suggested that functional differences exist between neonatal and adult spermatogonia. Replication-dependent viruses more readily transduced spermatogonia from neonatal mice compared with their adult counterparts, suggesting that these spermatogonia divide more

rapidly (Nagano *et al.* 2001, 2002). In support of this concept, de Rooij *et al.* demonstrated that spermatogonial development is accelerated in juvenile rats and hamsters (van Haaster & de Rooij 1993). A comparison of germ cell transplantation results from neonatal (P6) and adult mice revealed that the recolonization index for pup SSCs was half that of the adult, indicating delayed proliferation/expansion after transplantation and suggesting a difference in the ability of these spermatogonia to self-renew and differentiate (Ebata *et al.* 2007).

It is unclear whether prospermatogonia transition to SSCs, which then differentiate, or whether both populations arise directly from prospermatogonia. In addition, little is currently known regarding the cell fate decisions that take place in the neonatal testis, which result in creation of a functional stem cell population. In the adult testis, type A spermatogonia are classified functionally as stem cell and progenitor (both undifferentiated and differentiating). These adult populations are distinguishable at the histological level by their morphology and the stage of the seminiferous epithelium in which they reside. In addition, a number of protein markers have been identified that are differentially expressed in spermatogonia that are undifferentiated (e.g., ZBTB16/PLZF, ID4, GFRA1, RET, and *Neurog3/Ngn3*) and differentiating (e.g., STRA8, KIT, SOHLH1, and SOHLH2) (reviewed by Yang & Oatley (2014)). Immunostaining results from several studies using adult tissues revealed that there is heterogeneity among the spermatogonial population (Nakagawa *et al.* 2007, Grisanti *et al.* 2009, Suzuki *et al.* 2009). However, it is currently unclear whether this has phenotypic consequence or whether heterogeneity is observed as the spermatogonial population is created in the neonatal testis.

We undertook this study to answer several important questions that have not been addressed in the neonatal testis. First, using established adult spermatogonial cell fate markers, are there homogenous or heterogeneous populations during the first wave of spermatogenesis? Secondly, when do markers of cell fate diverge during the first wave of spermatogenesis? Thirdly, how do neonatal spermatogonia respond to a differentiation signal (retinoic acid (RA)) by modifying expression of cell fate markers? In this study, we report that spermatogonia constitutively express certain markers of undifferentiated fate (CDH1 and ZBTB16) through ~P10, but express the receptors GFRA1 or KIT (or neither) during this interval. This suggests that there are three subpopulations of spermatogonia in the neonatal testis: GFRA1+/KIT− (undifferentiated, can respond to GDNF), GFRA1−/KIT+ (differentiating, can respond to KITL), or GFRA1−/KIT− (potentially uncommitted to a specific cell fate). This third subpopulation progressively decreases as the KIT+ population increases, suggesting that these spermatogonia are

differentiating at later points to generate the asynchrony observed in the adult testis.

Materials and methods

Animal care

CD-1 mice were used for all analyses except where indicated otherwise. In particular, the *Id4*-GFP mice were backcrossed onto a C57Bl/6 background (Chan *et al.* 2014). Animal procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of East Carolina University (AUP #A178a). RA injections were administered as reported previously, and mice were killed by decapitation (P0–P10) or by CO₂ asphyxiation followed by thoracotomy (P18 and P>60) (Busada *et al.* 2014).

Tissue processing for histological analysis

Testes for histological analysis were fixed for 1 h to overnight in Bouin's solution (Ricca Chemical, Arlington, TX, USA) at 4 °C, washed in multiple changes of 1 × PBS, dehydrated through an ethanol series, and processed for paraffin embedding. Sections of 5 μm thickness were cut and stained with hematoxylin and eosin (H&E) using standard methods. Images were taken using an Axio Observer A1 microscope (Carl Zeiss Microscopy, LLC) equipped with a XL16 digital camera and Exponent version 1.3 Software (Dage-MTI, Michigan City, IN, USA).

Immunostaining frozen sections

Testes were fixed for 2 h to overnight in 4% paraformaldehyde (PFA), washed multiple times in 1 × PBS, transferred to 30% sucrose, embedded in O.C.T, and stored at −80 °C. Sections of 5 μm thickness were cut and placed on positively charged slides and stored at −20 °C before use. Immunolabeling was performed by standard methods. Briefly, blocking and antibody incubations were carried out at room temperature in 1 × PBS containing 3% BSA + 0.1% Triton X-100, and stringency washes were performed with 1 × PBS + 0.1% Triton X-100. Sections were blocked for 1 h before incubation with a primary antibody for 1 h (see Table 1). Secondary antibodies (1:500, Alexa Fluor-488 or -555, Invitrogen) plus phalloidin-635 (1:500, Life Technologies) were incubated for 1 h at room temperature. Primary antibody was omitted as a negative control. Cover slips were mounted with Vectastain containing DAPI (Vector Laboratories, Burlingame, CA, USA), and images obtained using a Fluoview FV1000 confocal laser-scanning microscope (Olympus America). Immunolabeling was performed in triplicate on testes from at least two different animals. Images used for quantitation were assessed using ImageJ (U. S. National Institutes of Health, Bethesda, MD, USA). Between 21 and 30 cords from at least two different animals were counted. Each marker was co-stained with DDX4 to label all germ cells. Cells were counted as positive for a marker when selected by the threshold tool using the default algorithm. Thresholds for each marker counted were as follows: STRA8 = 70–255, KIT = 70–255, DDX4 = 100–255, ZBTB16 = 50–255, CDH1 = 100–255, and GFRA1 = 50–255.

Table 1 Primary antibodies.

Protein	Spermatogonia expression	References	Antibody
STRA8 KIT	A_{diff} (A_1-) A_{diff} (A_1-)	Zhou <i>et al.</i> (2008a) Mithraprabhu & Loveland (2009)	Abcam rabbit polyclonal (49602), 1:3000 Santa Cruz goat polyclonal (sc-1494), 1:1000 Cell Signaling rabbit polyclonal (3074S), 1:1000 Santa Cruz rabbit polyclonal (sc-22839), 1:500
ZBTB16/PLZF	A_{undiff} (A_s, A_{pr}, A_{al})	Buaas <i>et al.</i> (2004) and Costoya <i>et al.</i> (2004)	Abcam rabbit polyclonal (ab-13840), 1:250 R&D Systems goat polyclonal (AF2030), 1:800 Cell Signaling rabbit polyclonal (3195P), 1:200 R&D Systems goat polyclonal (AF560), 1:200
DDX4/VASA/MVH	A_{undiff} and A_{diff}		
CDH1/ECAD GFRA1	A_{undiff} (A_s, A_{pr}, A_{al}) A_{undiff} (A_s, A_{pr})	Tokuda <i>et al.</i> (2007) Naughton <i>et al.</i> (2006) and Grasso <i>et al.</i> (2012)	

Whole-mount immunostaining of testis cords

Testes were first decapsulated, and then cords were mechanically separated. Cords were fixed for 1 h in 4% paraformaldehyde and washed multiple times in $1\times$ PBS. Individual cords were transferred to slides and IIF performed by standard methods. Briefly, blocking and antibody incubations were carried out at room temperature in $1\times$ PBS containing 3% BSA+0.1% Triton X-100, and stringency washes were performed with $1\times$ PBS+0.1% Triton X-100. Secondary antibodies (1:500, Alexa Fluor-488 or -555, Life Technologies) were incubated for 1 h at room temperature. Primary antibody was omitted as a negative control, and fluorescent images were obtained as described earlier in this study.

Results

Heterogeneity in neonatal germ cell morphology

Male germ cell development in neonatal mice (\sim P0–P7) has been described largely based on changes in cellular morphology. Some studies have suggested that homogenous populations exist during this time (e.g., Bellve *et al.* 1977), while others reported type A spermatogonia with a distinct morphology (e.g., Kluin & de Rooij 1981). We carefully examined the morphology and nuclear diameter of germ cells in CD-1 mice, which we have used for our recent studies. Germ cells are readily identified based on established morphological characteristics (Kluin & de Rooij 1981), and identification can be verified by immunostaining for ZBTB16/PLZF (Supplementary Figure S1A, B, and C, see section on supplementary data given at the end of this article), which was detectable in all germ cells in the neonatal testis, as we have reported previously (Busada *et al.* 2014). The prospermatogonia population at P1 appeared homogenous, with germ cells sharing similar nuclear diameters and chromatin morphologies. In contrast, at P4, the spermatogonia population appeared heterogeneous, with differing nuclear diameters and chromatin morphology (Fig. 1), which supports the potential for multiple spermatogonial types as early as P4.

Cell fate marker expression in neonatal prospermatogonia (P1)

There have been no published comprehensive analyses of neonatal spermatogonia development using established adult fate markers. Therefore, it is not clear when specific spermatogonial cell types appear in the neonatal testis, as it is difficult to distinguish undifferentiated and differentiating type A spermatogonia based solely on morphological criteria. Shortly after birth (P1), the testis cords contain peripherally located dividing Sertoli cells and centrally located quiescent prospermatogonia (de Rooij & Griswold 2012). To determine as to what percentage of prospermatogonia express certain spermatogonial fate markers, we co-immunolabeled with antibodies against CDH1, ZBTB16, and GFRA1 (undifferentiation markers) and KIT and STRA8 (differentiation markers) along with an antibody against the pan germ cell marker DDX4, which enabled us to determine the percentage of germ cells expressing various cell fate markers. Approximately 100% of P1 prospermatogonia had detectable levels of CDH1, ZBTB16, and GFRA1, but almost none had detectable expression of the differentiation markers KIT or STRA8 (Fig. 2 and Supplementary Figure S2, see section on supplementary data given at the end of this article).

Cell fate marker expression in P4–P10 spermatogonia

We then examined marker expression at P4, a time when the testis contains morphologically distinct populations of spermatogonia (Kluin & de Rooij 1981; Fig. 1). We hypothesized that the presence of morphological differences indicates various degrees of spermatogonial differentiation. To examine this, we co-localized ZBTB16, CDH1, and STRA8 with GFRA1 and KIT, canonical markers of undifferentiated and differentiating spermatogonia respectively (Oulad-Abdelghani *et al.* 1996, Buaas *et al.* 2004, Costoya *et al.* 2004, Bugeau *et al.* 2005, Prabhu *et al.* 2006, Tokuda *et al.* 2007, Zhou *et al.* 2008a, Grisanti *et al.* 2009, Mithraprabhu & Loveland 2009, Grasso *et al.* 2012). At P4, there were largely distinct subpopulations of spermatogonia that

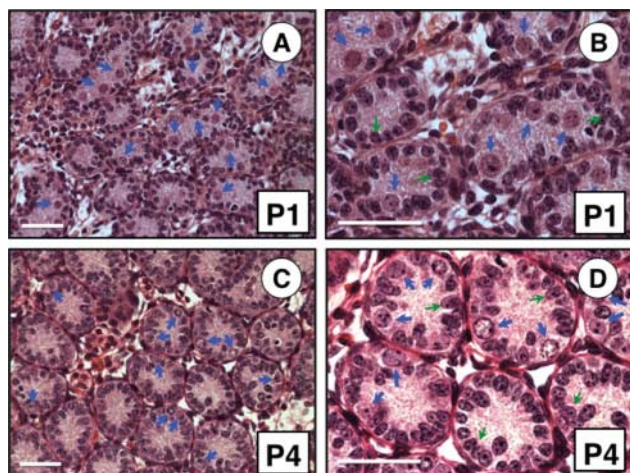


Figure 1 Spermatogonia exhibit morphological heterogeneity by P4. Sections from Bouin's solution-fixed testes from mice at P1 (A and B) and P4 (C and D) were stained with H&E. Blue arrows identify prospermatogonia (A and B) and spermatogonia, the latter of which exhibited a number of different nuclear sizes and morphologies (C and D). Green arrows (B and D) indicate representative Sertoli cells. Scale bars = 60 μ m.

were either GFRA1+ (60%) or KIT+ (35%), and <10% of the population were GFRA1+/KIT+ (Fig. 3 and Supplementary Figures S2, S3 and S4, see section on supplementary data given at the end of this article). All spermatogonia stained positive for both ZBTB16 and CDH1, accepted markers of the undifferentiated fate. In addition, nearly all KIT+ spermatogonia were STRA8+ (Busada *et al.* 2015), indicating that these cells had been exposed to RA. Considering only these markers, it is clear that at least two unique populations of spermatogonia

exist in the testis at P4 (GFRA1+/KIT-/CDH1+/ZBTB16+/STRA8- and GFRA1-/KIT+/CDH1+/ZBTB16+/STRA8+). In addition, based on germ cell identification by DAPI staining, it is apparent that small populations of spermatogonia are KIT-/GFRA1-.

By P7, all differentiating (KIT+) spermatogonia remained CDH1+ and 80% were still ZBTB16+, and there was almost no overlap in GFRA1 and KIT expression (17 and 42% of DDX4+ cells respectively). We observed this same relationship in spermatogonia in C57Bl/6 testes at P6 (Supplementary Figure S3). However, a correlation between staining intensity became apparent in a subset of spermatogonia (e.g., strong KIT+ with weak ZBTB16+). In addition, there was less observed STRA8 and KIT co-expression observed in P4 spermatogonia, which resulted in populations that were KIT+/STRA8+, KIT+/STRA8-, and KIT-/STRA8+ (Fig. 4). Analysis of these markers alone indicated that multiple distinct populations of germ cells exist at P7 (GFRA1+/KIT-/CDH1+/ZBTB16+/STRA8-, GFRA1-/KIT+/CDH1+/ZBTB16+/STRA8+, GFRA1-/KIT-/CDH1+/ZBTB16+/STRA8+, and GFRA1-/KIT-/CDH1+/ZBTB16-/STRA8+ and GFRA1-/KIT+/CDH1+/ZBTB16-/STRA8-).

By P10, GFRA1 and KIT were still detectable in distinct populations (8 and 75% of DDX4+ cells respectively), but there was a stark divergence in the expression of undifferentiated markers ZBTB16 and CDH1. All spermatogonia that were GFRA1+ were CDH1+/ZBTB16+, but most KIT+ cells were now CDH1- (Fig. 5). In addition, the staining intensity began to correlate with presumed cell fate; for example, KIT+

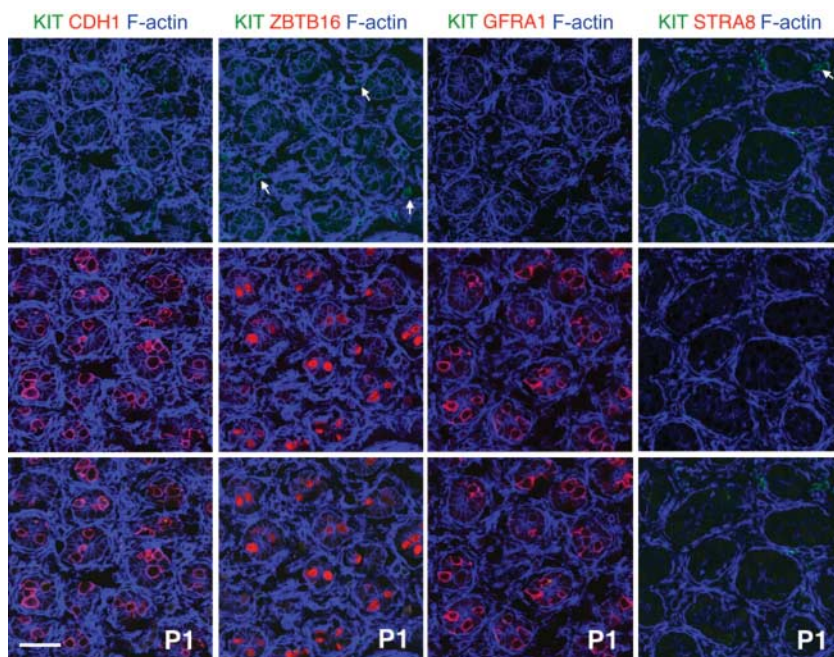


Figure 2 Fate marker detection in germ cells of the neonatal testis at P1. Phalloidin was used to label F-actin (blue) in all images. Each column contains one representative section labeled with antibodies against the proteins listed at the top. The first row is green+blue channels, the second is red+blue, and the bottom row is a merged image of green+red+blue. White arrows indicate KIT+ interstitial cells, which are present throughout testis development (Rothschild *et al.* 2003). Scale bar = 60 μ m.

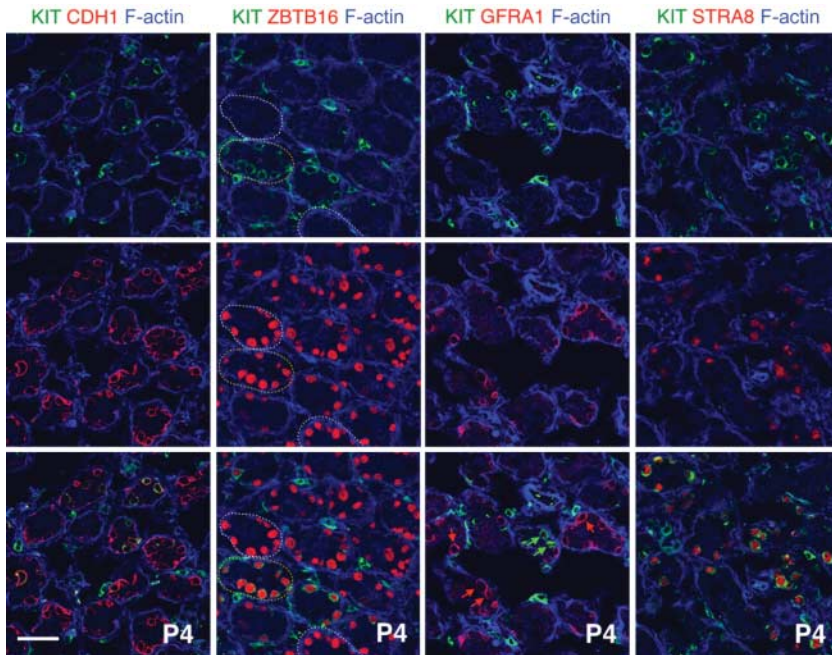


Figure 3 Fate marker detection in germ cells of the neonatal testis at P4. Phalloidin was used to label F-actin (blue) in all images. Each column contains one representative section labeled with antibodies against the proteins listed at the top. The first row is green + blue channels, the second is red + blue, and the bottom row is a merged image of green + red + blue. Cords containing ZBTB16+ spermatogonia were either KIT+ (yellow dashed lines) or KIT- (white dashed lines). Green arrows indicate KIT+ spermatogonia and red arrows point to GFRA1+ spermatogonia. Scale bar=60 μ m.

cells had weak staining for ZBTB16, and the opposite was also true (strong ZBTB16 correlated with weak or no staining for KIT). As at P7, there were three distinct populations of differentiating cells that were KIT+/STRA8+, KIT+/STRA8-, and KIT-/STRA8+. The latter population was centrally located in the testis cords, and probably are preleptotene spermatocytes, which have the highest levels of STRA8 (Zhou *et al.* 2008a). We co-immunostained testis cords with ZBTB16 and GFRA1 or KIT to determine whether GFRA1 or KIT staining correlated with spermatogonial chain length.

As expected, GFRA1 was primarily detected in A_s and A_{pr} spermatogonia, while KIT was detected in longer chains (Supplementary Figure S4).

It has been recently demonstrated that ID4 was enriched in A_s SSCs, and that transgenic mice faithfully expressed GFP downstream of an *Id4* promoter in SSCs (Oatley *et al.* 2011, Chan *et al.* 2014). We obtained testes from *Id4*-GFP mice at P6 and co-localized GFP with GFRA1 and KIT, as these largely marked unique populations of spermatogonia. We found that, in general, GFRA1 signal was strongest in spermatogonia

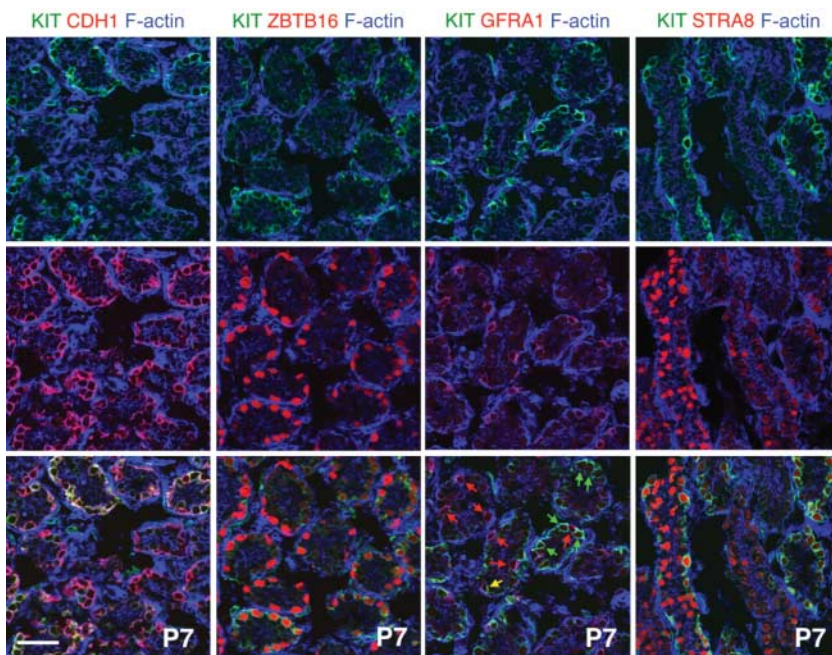


Figure 4 Fate marker detection in germ cells of the neonatal testis at P7. Phalloidin was used to label F-actin (blue) in all images. Each column contains one representative section labeled with antibodies against the proteins listed at the top. The first row is green + blue channels, the second is red + blue, and the bottom row is a merged image of green + red + blue. Red arrows indicate GFRA1+/KIT- spermatogonia, green arrows indicate GFRA1-/KIT+ spermatogonia, and the yellow arrow points to a rare GFRA1+/KIT+ spermatogonium. Scale bar=60 μ m.

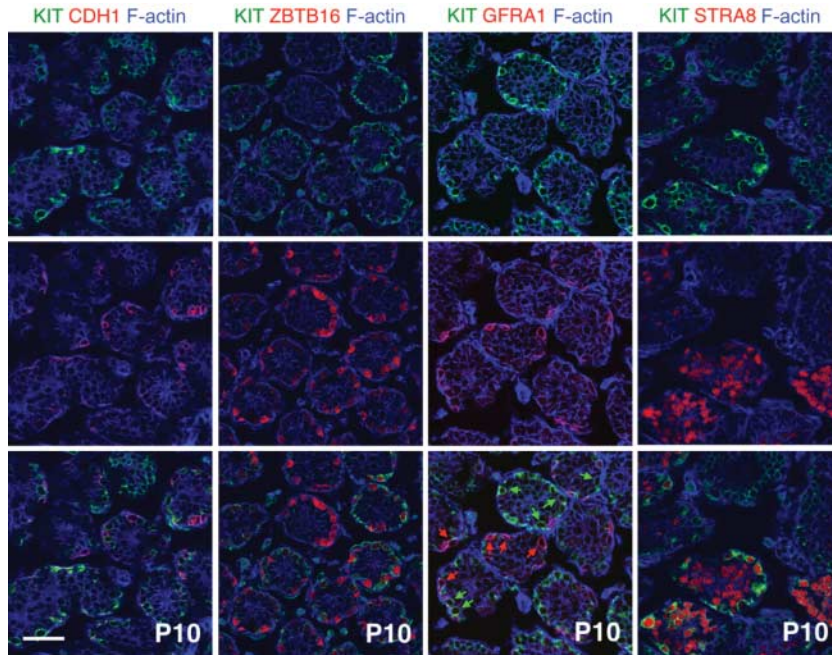


Figure 5 Fate marker detection in germ cells of the neonatal testis at P10. Phalloidin was used to label F-actin (blue) in all images. Each column contains one representative section labeled with antibodies against the proteins listed at the top. The first row is green+blue channels, the second is red+blue, and the bottom row is a merged image of green+red+blue. Red arrows indicate GFRA1+/KIT- spermatogonia and green arrows indicate GFRA1-/KIT+ spermatogonia. Scale bar=60 μ m.

with bright GFP, and that KIT signal was strongest in spermatogonia with dim or absent GFP signal (Supplementary Figure S5, see section on supplementary data given at the end of this article).

Cell fate marker expression in the P18 and adult ($P > 60$) testis

By P18 in the mouse, the blood–testis barrier was formed, Sertoli cells no longer divide, lumina have appeared, and the seminiferous epithelia contain spermatogonia and spermatocytes at various stages of progression through

meiosis, but haploid round spermatids are not yet present (Griswold 1998, Hess & Renato de Franca 2008). The examination of fate markers revealed that KIT staining was weak or absent in nearly all ZBTB16+, CDH1+, and GFRA1+ spermatogonia (Fig. 6). There were STRA8+/KIT+, STRA8+/KIT-, and STRA8-/KIT+ germ cells, but these populations were present in separate tubules (Fig. 6). Therefore, there was significant separation of the classic spermatogonial cell fate markers by this time, and this continued into adulthood (Supplementary Figure S6, see section on supplementary data given at the end of this article).

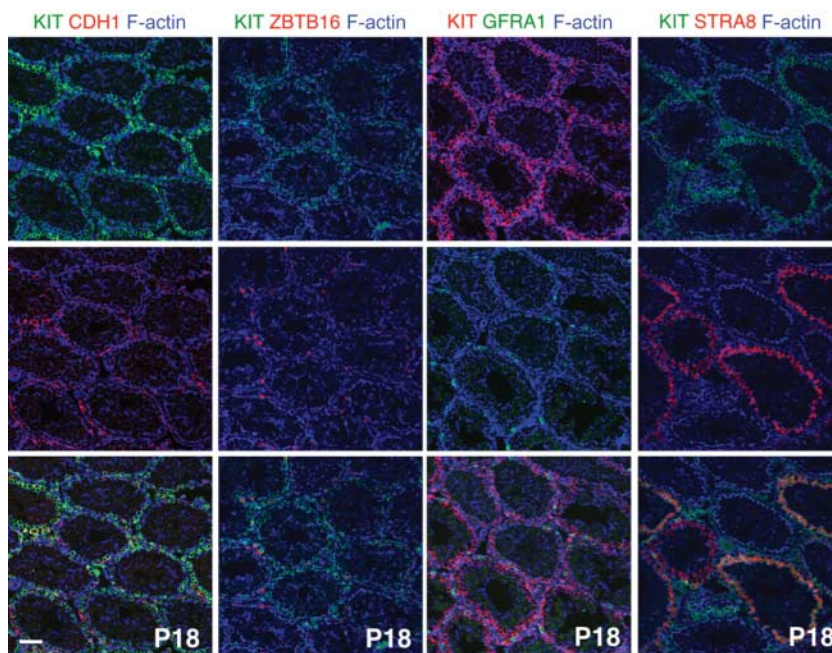


Figure 6 Fate marker detection in germ cells of the neonatal testis at P18. Phalloidin was used to label F-actin (blue) in all images. Each column contains one representative section labeled with antibodies against the proteins listed at the top. The first row is green+blue channels, the second is red+blue, and the bottom row is a merged image of green+red+blue. Scale bar=60 μ m.

How does marker expression change in response to a differentiation signal?

Spermatogonia differentiate in response to RA, beginning in the neonatal testis at P3–P4 and then throughout the remainder of the male reproductive lifespan (van Pelt & de Rooij 1991, Gaemers *et al.* 1998, Zhou *et al.* 2008a, Snyder *et al.* 2011, Busada *et al.* 2014). We utilized an *in vivo* model of spermatogonial differentiation, in which we administered exogenous RA at P1, 2–3 days before their normal exposure (Zhou *et al.* 2008a, Snyder *et al.* 2011, Busada *et al.* 2014). As shown previously (Busada *et al.* 2014), exogenous RA induced the precocious expression of differentiation markers STRA8 and KIT by 24 h (Fig. 7A). However, mRNA levels also transiently increased for *Cyp26a1* and *Cyp26b1* (Busada *et al.* 2014), which encode two RA-degrading enzymes, and by 48 h after treatment, STRA8 and KIT were absent (Fig. 7B and C), presumably due to loss of all testicular RA signaling. The *Cyp26a1* and *Cyp26b1* levels began to normalize by 72 h after RA exposure, suggesting that the testicular RA environment returned to normal, and germ cells eventually entered meiosis in similar numbers in RA-exposed animals, albeit 2 days later than vehicle-treated controls (Busada *et al.* 2014). Results from that experiment indicated that expression of STRA8 and KIT did not result in an irreversible commitment to differentiation, but rather that spermatogonia must receive multiple consistent signals to differentiate. To assess this, we followed changes in expression of spermatogonial cell fate markers over the 144 h following RA exposure at P1.

Exogenous RA induced the precocious appearance of STRA8+/KIT+ spermatogonia by 24 h (Fig. 7A; Busada *et al.* 2014) but, by 48 and 72 h post-RA injection, STRA8

and KIT both became undetectable in spermatogonia (Fig. 7B and C). There were no apparent changes in the expression of the undifferentiated markers ZBTB16 and CDH1 over this interval (Fig. 7E, F, G, H, I, J, K, and L), suggesting that the spermatogonia population had returned to an undifferentiated state following the single exposure to RA. At 144 h after RA injection, STRA8 and KIT again became detectable in spermatogonia, indicating a resumption of RA signaling (Fig. 7D). Although specific testis cords were either KIT+ or KIT– in vehicle-treated controls, as in WT (Supplementary Figure S7, see section on supplementary data given at the end of this article), this was largely lost in testes of RA-treated mice (Fig. 7D, H, and L). This resulting similarity in the expression of fate markers suggests homogeneity in differentiation status, and provides an explanation for the widespread synchronization of the seminiferous epithelial cycle observed in adult mice treated with RA as neonates (Snyder *et al.* 2011, Hogarth *et al.* 2013).

Discussion

In this study, we examined the expression of established markers of spermatogonial cell fate in spermatogonia during the first wave of spermatogenesis in the mouse. Importantly, we found that the receptors GFRA1 and KIT, which mark undifferentiated and differentiating spermatogonia, were predominantly localized to distinct cells from P4 onward. This is in contrast to ZBTB16 and CDH1, markers for undifferentiated spermatogonia in the adult, which were present in nearly all germ cells from P1 through P7. These were also co-localized with the differentiation markers STRA8 and KIT in a subset of spermatogonia at P4, concomitant with the onset of RA signaling. This co-localization of undifferentiated

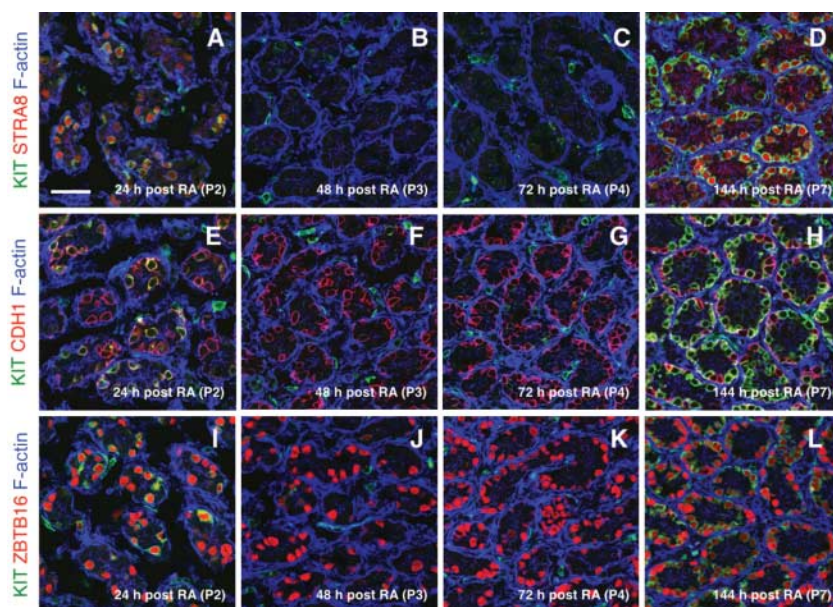


Figure 7 Fate marker changes in germ cells of the neonatal testis following RA-induced differentiation. Phalloidin was used to label F-actin (blue) in all images. Each row contains a temporal detection of RA-induced changes in testis sections. KIT is labeled green in all images, while STRA8 (top row, A, B, C, and D), CDH1 (middle row, E, F, G, and H), and ZBTB16 (bottom row, I, J, K, and L) are labeled in red. The hours after RA injection are indicated on each image, and the actual age is in parentheses. Scale bar = 60 μ m.

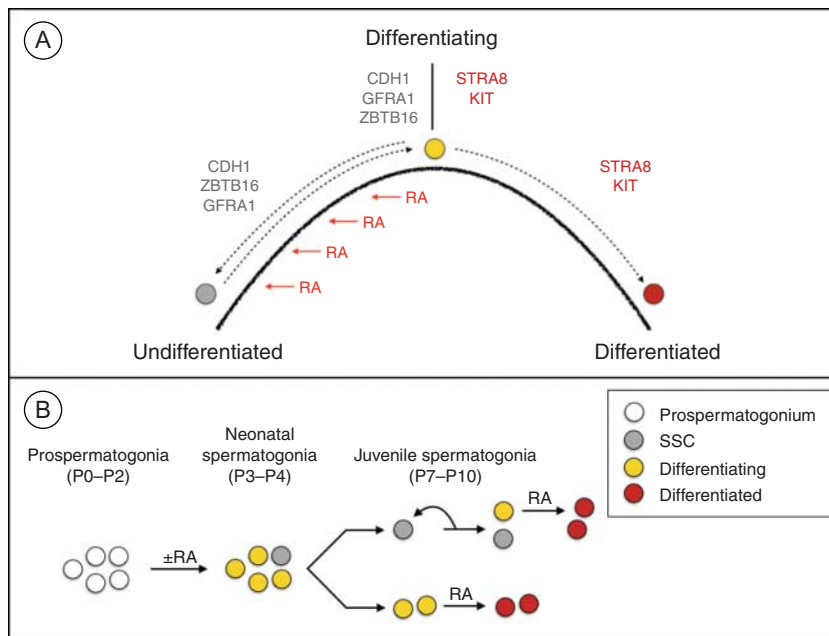


Figure 8 Model for spermatogonial development in the neonatal testis. (A) At P1, prospermatogonia express undifferentiated markers such as CDH1, ZBTB16, and GFRA1. Following continuous or multiple exposures to RA, differentiating spermatogonia express STRA8 and KIT; a single dosage of RA is not sufficient for differentiation, and spermatogonia appear to revert to the undifferentiated state. (B) Prospermatogonia (white) transition to a heterogeneous population of differentiating and undifferentiated (putative stem cell) spermatogonia. Populations of differentiating spermatogonia enter meiosis, while the SSCs remain undifferentiated to provide a consistent source of differentiated spermatogonia after the first wave of spermatogenesis.

and differentiated markers persisted through P10, after which fate marker divergence was observed at P18 and in the adult testis. Taken together, our findings reveal that neonatal spermatogonia are a heterogeneous population as early as P4, and exhibit significant differences in marker expression in comparison with juvenile and adult spermatogonia. These data reveal molecular differences between neonatal and adult spermatogonia that may in part explain functional differences in terms of colonization ability and cell cycle length. In addition, data from this study reveal that caution should be exercised when making conclusions about spermatogonial behavior when utilizing neonatal spermatogonia as a substitute for adult spermatogonia.

The neonatal transition from prospermatogonia to spermatogonia is critical for establishment of spermatogenesis and lifelong male fertility. However, it was unclear when male germ cells begin to show signs of heterogeneity in terms of cell fate markers. We did not detect significant heterogeneity in cell morphology or the expression of cell fate markers in prospermatogonia at P1. Almost none of the prospermatogonia exhibited detectable levels of the differentiation markers STRA8 and KIT, while nearly all prospermatogonia had detectable GFRA1, ZBTB16, and CDH1. This is in agreement with some reports (e.g., ZBTB16 and CDH1 (Costoya *et al.* 2004, Tokuda *et al.* 2007)) but in contrast with others (e.g., ~50% RET+ or GFRA1+ at P0, although data not shown for GFRA1 (Naughton *et al.* 2006)). It is possible that, in the latter study, overnight fixation or the harsh tissue processing steps preceding paraffin embedding compromised some of the epitopes for GFRA1 and prevented their detection.

Our data support a model (see Fig. 8A) by which spermatogonia constitutively express undifferentiated

markers, and the differentiation program is initiated in response to RA in a subset of cells. Except for GFRA1, these undifferentiated markers persist until at least P7, after which there is significant separation. A similar pattern was observed for SALL4, which is detectable in all spermatogonia through P7, but then diverges to be expressed in undifferentiated spermatogonia in the adult (Gassei & Orwig 2013). After exposure to RA in this study, nearly all spermatogonia became STRA8+/KIT+. However, by 48 h, these differentiation markers were lost, and only undifferentiated markers were constitutively expressed during this period. This suggests that the program of differentiation is initiated in undifferentiated spermatogonia, and that perhaps the most important aspect of commitment to a stem cell fate is avoiding exposure to a differentiation signal such as RA. Indeed, nearly all prospermatogonia exposed to RA (either by culture in excess RA or deletion of the RA-degrading enzyme *Cyp26b1*) prematurely expressed differentiation markers such as *Stra8*, *Dmc1*, *Sycp3*, *Sohlh1*, and *Kit* and attempt to enter meiosis (MacLean *et al.* 2007, Trautmann *et al.* 2008, Zhou *et al.* 2008a,b, Li *et al.* 2009, Busada *et al.* 2014). However, we have recently found that the signal provided by a one-time bolus of RA was not sufficient to maintain the differentiated state; by 48 h after RA injection, all of the neonatal spermatogonia that were previously STRA8+/KIT+ had lost expression of both markers and only had detectable undifferentiated markers for at least 3 more days (Busada *et al.* 2014). As spermatogonia express both undifferentiated and differentiating markers simultaneously, it is reasonable to predict that these cells have not yet committed to the differentiated fate (Fig. 8B). Our results corroborate the concept that multiple, or continuous,

exposure(s) to RA is necessary to maintain STRA8 and KIT expression.

Beginning at P4, there are at least two distinct populations of spermatogonia that express either the KIT or GFRA1 receptor. The separate expression of these two receptors provides these distinct populations of spermatogonia with the ability to respond differentially to the ligands GDNF and KITL, which are provided by neighboring Sertoli and peritubular myoid cells (Hofmann 2008, Chen *et al.* 2014). GDNF binds to the GFRA1 and RET co-receptors to regulate SSC maintenance both *in vitro* and *in vivo* (Meng *et al.* 2000, 2001, Viglietto *et al.* 2000, Kubota *et al.* 2004, 2011, He *et al.* 2007). In contrast, KITL binding to KIT is required for spermatogonial differentiation and their subsequent entry into meiosis (Besmer *et al.* 1993, Kissel *et al.* 2000, Mithraprabhu & Loveland 2009). It is potentially the gain of these cell surface receptors, which informs a spermatogonium to remain undifferentiated or to differentiate to eventually enter meiosis. While binding of either ligand to their cognate receptor(s) activates the PI3K/AKT signaling pathway, the distinct outcomes indicate the involvement of different components of this complex pathway.

Taken together, our results indicate that prospermatogonia transition to an initial heterogeneous population of undifferentiated and differentiating spermatogonia by ~P4. This supports the model whereby prospermatogonia directly give rise to the foundational pool of SSCs (which function to ensure lifelong fertility) as well as the undifferentiated progenitors and differentiating spermatogonia that become spermatozoa during the first wave of spermatogenesis.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0653>.

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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