The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage

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Mammalian spermatogenesis is maintained by a continuous supply of differentiating cells from self-renewing stem cells. The stem cell activity resides in a small subset of primitive germ cells, the undifferentiated spermatogonia. However, the relationship between the establishment of this population and the initiation of differentiation in the developing testes remains unclear. In this study, we have investigated this issue by using the unique expression of Ngn3, which is expressed specifically in the undifferentiated spermatogonia, but not in the differentiating spermatogonia or their progenitors, the gonocytes. Our lineage analyses demonstrate that the first round of mouse spermatogenesis initiates directly from gonocytes, without passing through the Ngn3-expressing stage (Ngn3+ lineage). By contrast, the subsequent rounds of spermatogenesis are derived from Ngn3-positive undifferentiated spermatogonia, which are also immediate descendents of the gonocytes and represent the stem cell function (Ngn3+ lineage). Thus, in mouse spermatogenesis, the state of the undifferentiated spermatogonia is not an inevitable step but is a developmental option that ensures continuous sperm production. In addition, the segregation of gonocytes into undifferentiated spermatogonia (Ngn3+ lineage) or differentiating spermatogonia (Ngn3− lineage) is topographically related to the establishment of the seminiferous epithelial cycle, thus suggesting a role of somatic components in the establishment of stem cells.

KEY WORDS: Spermatogenesis, Mouse, Stem cells, Undifferentiated spermatogonia, Seminiferous epithelial cycle, Ngn3, Kit, Galectin 1

INTRODUCTION

In the mammalian testes, numerous spermatozoa are continuously produced throughout the reproductive period. The continuity of spermatogenesis is dependent on stem cells, defined by their self-renewing and differentiating activities. During adult spermatogenesis in mice, the stem cell activity resides in a small, primitive set of spermatogonia referred as the undifferentiated spermatogonia, which correspond to A single, A paired, and A aligned Spermatogonia (de Rooij, 2001; de Rooij and Russell, 2000; Shinohara et al., 2000; Nishimune et al., 1978). Besides self-renewing as a population, undifferentiated spermatogonia generate differentiating spermatogonia [A1 to A4, Intermediate (In), and B spermatogonia], which then differentiate into meiotic spermatocytes, haploid spermatids, and spermatozoa. In the seminiferous tubules, all types of spermatogonia (A single, to B) are localized on the peripheral basement membrane, and the subsequent cell types are arranged in a sequential order towards the lumen (Russell et al., 1990).

The initiation of spermatogenesis and the establishment of stem cells in the developing testes have been a focus of interest. It is commonly accepted that spermatogonia with mature morphology corresponding to adult In and B spermatogonia appear by the beginning of the second week after birth; this is followed by the sequential appearance of the differentiating cells (Bellvé et al., 1977; de Rooij, 1998; Kluij and de Rooij, 1981), whereas others claim that gonocytes give rise to a special type of cell, termed presumptive spermatogonia, and that these cells subsequently generate adult-type spermatogonia (Bellvé et al., 1977; Hilscher et al., 1974; Huckins and Clermont, 1968). In addition, the establishment of stem cells remains unclear because of the difficulties involved in morphologically identifying undifferentiated spermatogonia. It has been shown that transplantable stem cell activity appears around P2 to P4 (McLean et al., 2003). However, the nature of the cells that represent this activity is unknown.

In mature testes, spermatogenesis progresses in a topographically well-coordinated manner, known as the spermatogenic wave (Leblond and Clermont, 1952; Russell et al., 1990). This is a recapitulation of the seminiferous epithelial cycle in the linear layout along the length of the seminiferous tubule. Based on the expression patterns of the seminiferous stage-specific genes in the perinatal immature Sertoli cells, Timmons et al. suggested that the seminiferous epithelial cycle might be pre-patterned in Sertoli cells from the embryonic stage (Timmons et al., 2002). However, the relationship between this presumptive pre-pattern of the cycle and spermatogenesis initiation has been barely investigated. Moreover, it is well known that the first round of spermatogenesis during puberty is less efficient than that of adults, and that it exhibits massive apoptosis (Kluin et al., 1982; Mori et al., 1997). It is also suspected that the first round of spermatogenesis may not produce fertile spermatozoa.

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Gene expression and cell fate analyses may be advantageous regarding the lineage relationship and/or the differentiation of the cells observed. However, such analyses have not been conducted because of the lack of an appropriate set of marker genes. On the one hand, it has been established that the transition of undifferentiated spermatogonia into differentiating spermatogonia coincides with the gain of Kit (also known as c-Kit) expression, a receptor tyrosine kinase. Kit continues to be expressed until meiosis and play essential roles in the survival of the Kit-expressing cells (Schrans-Stassen et al., 1999; Yoshinaga et al., 1991). On the other hand, several genes have been described to be expressed in undifferentiated spermatogonia or similar populations in adult testes, including Ret, Gfrα1, Oct4 (Pou5f1 – Mouse Genome Informatics), and Plzf (Zbtb16 – Mouse Genome Informatics), some of which play crucial roles in the establishment and/or maintenance of stem cell activities (Buas et al., 2004; Costoya et al., 2004; Meng et al., 2000; Pesce et al., 1998). However, these genes are also expressed in gonocytes, and thus cannot differentiate these two populations. Recently, we have identified that neurogenin 3 (Ngn3; Neurog3 – Mouse Genome Informatics), a basic helix-loop-helix transcription factor, is specifically expressed in undifferentiated spermatogonia, and not in gonocytes (Yoshida et al., 2004). The Ngn3-expressing cells fulfill the criteria for the undifferentiated spermatogonia, including their presence throughout the seminiferous epithelial cycle with low frequency, connection of the small number of 2ⁿ cells, survival in cryptoorchid testes and Kit negativity. Upon transition into differentiating spermatogonia, Ngn3 expression is downregulated. Thus, this provides a unique tool with which to elucidate the ontogeny of undifferentiated spermatogonia.

In this study, we have investigated the initial steps of spermatogenesis by lineage analyses using an inducible and constitutive Cre recombinase-loxP system, after a detailed profiling of the Ngn3 and Kit expression patterns. Particular attention was given to the small number of 2ⁿ cells, survival in cryptoorchid testes and Kit negativity. Upon transition into differentiating spermatogonia, Ngn3 expression is downregulated. Thus, this provides a unique tool with which to elucidate the ontogeny of undifferentiated spermatogonia.

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MATERIALS AND METHODS

Animals
Ngn3/Cre and CAG-CAT-Z transgenic mice have been described previously (Yoshida et al., 2004; Araki et al., 1995). Ngn3/CreER™ transgenic mice were generated by injecting a DNA construct of CreER™ flanked by the 6.7-kb mouse Ngn3 upstream sequence (Gu et al., 2002) into the pronucleus of a C57BL/6 fertilized egg (Oriental Bio-Service). Ngn3/Cre and Ngn3/CreER™ mice were maintained on a C57BL/6 background, whereas the genetic background of CAG-CAT-Z mice was mixed but immunologically compatible with C57BL/6. The C57BL/6 mice used as the wild type were purchased from Japan SLC and Charles River Japan, and the WBB6F1/W’ (W/W’) mice were purchased from Japan SLC. All the animals were maintained and sacrificed in accordance with the animal experiment guidelines of Kyoto University.

RT-PCR
Poly(A) RNA was prepared from C57BL/6 mouse testes by using a µMACS mRNA isolation kit (Miltenyi). Reverse transcription (RT), with a random primer, and polymerase chain reaction (PCR) were performed using Super Script III (Invitrogen) and LA Taq (Takara) enzymes in accordance with the manufacturers’ recommendations. Primers used for Ngn3, Ret, Oct4 have been described previously (Yoshida et al., 2004) and are as follows: 5’-ACATACAGCGGCACCAACAG-3’ and 5’-TCAAGATTCGAGCATGTACC-3’ for Kit, 5’-CTCTTTGATGTCGCGACGACGATTTC-3’ and 5’-GTTGCGCCCTCTAGGCCACAA-3’ for β-actin.

In situ hybridization (ISH) on sections
Under anesthesia with avertin, mice were perfused fixed with 4% paraformaldehyde (Nakalai) in PBS, and their testes were excised. Mice less than 2 weeks of age were anesthetized with isoflurane, and their testes were excised without perfusion. After removal of the tunica albuginea, the testes were immersed overnight in the same fixative, embedded in paraffin wax and sectioned. ISH was performed as described previously (Yoshida et al., 2001); the detailed protocol is available upon request. For double-staining ISH, a fluorescein-labeled probe (synthesized using a Fluorescein RNA Labeling mix) was hybridized simultaneously with a digoxigenin (DIG)-labeled probe. DIG was visualized by using an AP-conjugated anti-DIG antibody and BM purple substrate, and fluorescein was visualized by using an AP-conjugated anti-Fluorescein antibody and the HNPP Fluorescent Detection Set. The second colorization was performed after detection of the first label and inactivation of AP in PBS at 75°C for 30 minutes. For Ngn3 (DIG) and Kit (fluorescein) double staining, fluorescein was visualized first; for Ngn3 or Kit (DIG) and galectin 1 (fluorescein) staining, DIG was detected first. All the reagents were obtained from Roche. Specimens were counterstained with Nuclear Fast Red, Hoechst 33258, or propidium iodide (PI), as appropriate. Galectin 1 probes were prepared from the EST clone IMAGE 5712148 (Invitrogen). Templates for other probes have been described previously (Yoshida et al., 2001; Yoshida et al., 2004). None of the sense probes yielded any signal. The specimens were photographed using a DMRBE fluorescence microscope (Leica) equipped with an Axioacam digital camera (Zeiss Vision), and the images were processed with Adobe PhotoShop.

Whole-mount ISH
Whole-mount ISH was performed on untangled P4 seminiferous tubules attached to APS-coated glass slides (Matsumani). Slides were then fixed with 4% paraformaldehyde in PBS (4°C, 2 hours) and dehydrated-rehydrated through a methanol series. The subsequent hybridization procedure and reagents used were based on those described previously (Hogan et al., 1994), with modifications adapted for samples on slides; hybridization buffer was 50% formamide, 5x SSC (pH 4.5), 1% SDS, 50 µg/ml RNAse and 50µg/ml heparin. The detailed protocol is available upon request. The antibody detection and colorization was carried out as described above, using an AP-conjugated anti-DIG antibody and BM purple AP substrate (Roche).

Scoring Kit- and Ngn3-positive spermatogonia in the seminiferous tubule sections with various galectin 1 mRNA levels
Testicular sections of young mice were double stained for Kit or Ngn3 and galectin 1. All the tubule cross sections were photographed under a fluorescence microscope and categorized according to their galectin 1 signal strength (high, medium or low). The same specimens were independently examined under bright-field illumination for the number of Kit- or Ngn3-positive spermatogonia contained in individual tubule sections. Then, expected positive cell numbers were calculated assuming their non-biased distributions, and the ‘preferences’ (actual positive cell number/expected positive cell number) were determined; these were statistically evaluated by χ² test between the actual and expected cell numbers within or outside of the particular categories of the segments. For each data point, more than 600 seminiferous tubule cross-sections in five or six testicular slices were examined.

The data were then analyzed to determine whether the Ngn3- and Kit-positive spermatogonia distributions have some correlation with the categories based on the galectin 1 mRNA levels in seminiferous tubule segments. Statistical evaluations were performed as explained below, using the data of the Ngn3-positive cells at P5 as an example (see Tables S2-S6 in the supplementary material).

The five P5 testes specimens used for analyses contained 741 cross-sections of the seminiferous tubules in total, and all Ngn3-positive cells were observed (see Table S2 in the supplementary material). These 137 cells were classified according to the galectin 1 level of the seminiferous tubule segments. Statistical evaluations were performed as explained below, using the data of the Ngn3-positive cells at P5 as an example (see Tables S2-S6 in the supplementary material). The five P5 testes specimens used for analyses contained 741 cross-sections of the seminiferous tubules in total, and all Ngn3-positive cells were observed (see Table S2 in the supplementary material). These 137 cells were classified according to the galectin 1 level of the seminiferous tubule segments. Statistical evaluations were performed as explained below, using the data of the Ngn3-positive cells at P5 as an example (see Tables S2-S6 in the supplementary material).
Initiation of mouse spermatogenesis

DEVELOPMENT

Pulse labeling of Ngn3-positive spermatogonia and transplantation

Tamoxifen [40 mg/kg; Calbiochem, solubilized in Sesame oil (Nakalai) at 10 mg/ml] was administered intraperitoneally at P5 and P6 to double-transgenic male mice obtained from crossing Ngn3/CreERTM males with CAG-CAT-Z females. The control injection did not contain tamoxifen. For transplantation, double-transgenic male injected with tamoxifen at P5 and P6 were sacrificed at P8. A single-cell suspension was prepared from their testes, and was transplanted into the seminiferous tubules of W/Wv mice previously transplanted with CAG-CAT-Z/galactosidase (β-gal) has been described previously (Yoshida et al., 2004). After being photographed, specimens were re-fixed with 10% formalin, embedded in paraffin wax, sectioned, and stained with Nuclear Fast Red or Hematoxylin and Eosin.

Analysis of offspring from Ngn3/Cre:CAG-CAT-Z male mice

The Ngn3/Cre:CAG-CAT-Z double transgenic male mice were weaned around P28 and housed with C57BL/6 female mice in isolated cages for natural mating. Offspring were analyzed after birth or after the dissection of the pregnant females. The date of fertilization was determined based on the date of birth or the developmental stage of the embryos. Using tail or limbs, the offspring were genotyped by PCR using the primers described previously (Araki et al., 1995). The presence of the CAG-CAT-Z reporter gene was tested with Z3 and Z1 primers, and approximately 50% of the offspring tested were positive. Intact and recombined transgenes were distinguished using AG2 and Z3, which flank the CAT gene and loxp sequences. All offspring demonstrated the presence of either the intact or the recombined transgene. Consistent with this, samples showing the recombined pattern never retained the CAT gene (using CAT2 and CAT3).

RESULTS

Appearance of Ngn3- and Kit-positive spermatogonia in early spermatogenesis

As a first step to evaluate spermatogenesis initiation, the expression of Ngn3 and Kit was examined by RT-PCR and in situ hybridization (ISH) during the first postnatal week (Fig. 1). In neonates, when germ cells are in the gonocyte stage, Ngn3 expression is below the detection level of ISH and is barely detectable by RT-PCR. Detection of Ngn3 expression by ISH starts around P3 to P4 in a tiny portion of nascent spermatogonia located on the basement membrane, followed by an increase in the number of Ngn3-positive spermatogonia through P5 to P7 (Fig. 1B, parts a-f). This profile is reflected by the results from RT-PCR using whole testis RNA (Fig. 1A). This is in contrast to Ret and Oct4, which also exhibit limited expression in undifferentiated spermatogonia, or similar populations, in the adult testes (Meng et al., 2000; Pesce et al., 1998). These genes are readily detected in neonatal gonocytes (P1-P2) and nascent spermatogonia (around P3-P4), and they are then downregulated in subsets of spermatogonia, although expression remains persistent in another subset up until P7. RT-PCR gives consistent results (Fig. 1A,B, parts m-x). Kit is not expressed in gonocytes at P1, although interstitial cells express this gene throughout (Manova et al., 1990) (red arrowheads, Fig. 1B, parts g-l). The germ line expression of Kit starts at a similar or slightly (approximately 1 day) earlier time when compared with that of Ngn3 (blue arrowheads). The number of Kit-positive spermatogonia was constantly greater than that of Ngn3-positive spermatogonia (Fig. 1B, parts a-l). Both interstitial and germ cell Kit expression contribute to the RT-PCR result.

Spatial separation of the newly born Ngn3- and Kit-positive spermatogonia

As is apparent in Fig. 1B, Ngn3- or Kit-positive spermatogonia showed a highly biased localization within the seminiferous tubule segments; some segments contain many positive cells, whereas others are devoid of these cells. Double-staining ISH (Fig. 2A-D) clarified that Ngn3 and Kit signals are not only found in distinct cells but are also spatially separated in different seminiferous tubule segments. A comparison between adjacent specimens revealed that the Ngn3 and Kit signals rarely overlap within a single seminiferous tubule segment (Fig. 2A-D,E-G; see also Table S1 in the supplementary material). These data indicate that the Ngn3-positive spermatogonia and Kit-positive spermatogonia are generated in a spatially separated manner along the length of the seminiferous tubules at this early stage.

Emergence of the Ngn3- and Kit-positive spermatogonia, and the seminiferous epithelial cycle pre-pattern

In mature testes, the seminiferous epithelium repeats a cyclical program, known as the seminiferous epithelial cycle (Leblond and Clermont, 1952; Russell et al., 1990). In mice, an entire cycle takes ~8.6 days and is divided into stages I to XII, defined by particular combinations of the different stages of spermatogenic cells, such as spermatids and spermatocytes. Areas of seminiferous epithelium at any stage occupy a cylindrical segment, and such segments are arranged along the tubule length in their chronological order (I, II, III, IV...). As a result, mouse spermatogenesis exhibits a wave-like progression along the tubule length, which is known as the spermatogenic wave.

Although the seminiferous epithelial cycle is defined by the differentiation steps of germ cells, several genes expressed in Sertoli cells also represent stage specificities, indicating that germ cells and Sertoli cells are well coordinated in the seminiferous epithelial cycle. Timmons et al. demonstrated that immature Sertoli cells of perinatal stages have a similar gene expression profile to that observed in adult seminiferous tubules (Timmons et al., 2002). Among a number of genes examined, galecitin 1 (Lgals1) has been shown to be a stage-restricted gene in the seminiferous tubules. In neonates, galecitin 1 expression was detectable by RT-PCR (Fig. 1A), whereas in adults, galecitin 1 transcription was barely detectable by RT-PCR (Fig. 1A)
occur as a single pulse in each cycle; it initiates between stages VIII and IX, has the highest accumulation of transcripts at stages X-XII, and then becomes weaker before regaining strong expression at stage IX (Timmons et al., 2002). These authors also demonstrated that the cyclic expression of galectin 1 and other genes can be observed in the XXSxr* testes, which are devoid of germ cells. Based on these observations, they suggested that, in the perinatal testis cord, Sertoli cells possess an intrinsic cyclic program, which may be a seminiferous epithelial cycle pre-pattern, and subsequently become coordinated with the differentiation of germ cells.

To further evaluate pre-pubertal galectin 1 mRNA expression, we investigated its topographical pattern by whole-mount ISH (see Fig. S1A in the supplementary material). In P4 seminiferous tubules, galectin 1 mRNA was detected in an essentially identical pattern to that observed in the adult; segmental expressions of high, medium and low level of galectin 1 mRNA are arranged along the tubule length in this order. As is shown in Fig. S1B,C in the supplementary material, galectin 1 expression was also variable among the tubule segments at P14 and P21. At these immature stages, the relationship of galectin 1 levels with the types of spermatogenic cells included in each segment was identical to that observed in the mature testes. Thus, seminiferous stage-specific galectin 1 expression in mature testes can be traced back to pre-pubertal stages. It has also been suggested that the galectin 1 expression domain moves so that it is always associated with a particular stage of spermatogenic cells. This is in agreement with the interpretation by Timmons et al. (Timmons et al., 2002) that pre-pubertal galectin 1 expression is a pre-pattern of the adult cycle. We also examined the adult testes of W/Wv mice and cryptorchid testes, both of which lack differentiating spermatogenic cells. In these testes, galectin 1 mRNA in Sertoli cells represented variable levels among the tubule sections, similar to as in normal adult testes (see Fig. S1D-F in the supplementary material), supporting the idea that the galectin 1 cycle can occur independently of the synchronous spermatogenic differentiation (Timmons et al., 2002).

We were, therefore, prompted to investigate the possible link between the emergence of Ngn3- and Kit-positive spermatogonia and the presumptive seminiferous epithelial cycle pre-pattern represented by galectin 1 mRNA (Fig. 3). The intensities of the galectin 1 signals in the Sertoli cell cytoplasm at the center of the tubules largely differ among segments (Fig. 3A-H). At P3 and P4, when Kit-positive spermatogonia begin to be detected, they
showed a strong preference for segments with a high level of galectin 1 (Fig. 3A,B,I; Table S2 in the supplementary material). This preference continues as the number of Kit-positive spermatogonia increases at P5, when they become detectable also in segments with a medium level of galectin 1 (Fig. 3E,F,I). Assuming the wave-like progression of the seminiferous epithelial cycle, these data suggest that Kit-positive spermatogonia are born at stages with a high level of galectin 1, and, as the cycle progresses, their distribution extends to regions with a lower level of galectin 1 (schematically represented in Fig. 3J). Ngn3-positive spermatogonia prefer segments with medium level of galectin 1 (Fig. 3C,D,G-J).

Altogether, it is reasonable to suppose that a particular subset of gonocytes that localizes to the galectin 1-high segments of the seminiferous tubules directly gives rise to Kit-positive spermatogonia without Ngn3 expression. This is in contrast to the situation in adults, where essentially all of the Kit-positive spermatogonia are descendants of Ngn3-positive spermatogonia (Yoshida et al., 2004). Ngn3-positive spermatogonia preferentially appear at segments with medium levels of galectin 1.

Self-renewing stem cell activities of nascent Ngn3-positive spermatogonia

We then analyzed the fates of the pre-pubertal spermatogonia subpopulations to evaluate their differentiation characteristics. To determine the fate of Ngn3-positive spermatogonia, transgenic mice expressing tamoxifen-inducible Cre recombinase (CreERT²) (Hayashi and McMahon, 2002) in Ngn3-positive spermatogonia were generated using the Ngn3 regulatory sequence (Fig. 4B,C). The CreERT² protein is activated transiently after tamoxifen administration and recombines the target loxP sites in expressing cells (Fig. 4A). In double-transgenic mice possessing the CAG-CAT-Z reporter (Araki et al., 1995), Ngn3-positive spermatogonia were successfully labeled for β-gal expression (encoded by lacZ) in a tamoxifen-dependent manner (Fig. 4E,F). Three months after labeling, many patches of labeled cells with complete spermatogenesis were observed (Fig. 4G,H). Given that the completion of the spermatogenic process requires approximately 1 month (Russell et al., 1990) and that the activity of tamoxifen does not persist beyond several days after its administration (T.N. and S.Y., unpublished) (Gu et al., 2002), this result indicates that the cells that expressed Ngn3 at the time of tamoxifen administration continue to self-renew, as well as to generate differentiating cells. In addition, when the labeled pre-pubertal testes were dissociated and transplanted into germ cell-depleted seminiferous tubules (Brinster, 2002), a number of spermatogenic colonies of labeled cells were generated after 3 months (Fig. 4G-I), indicating a repopulating activity of the pre-pubertal Ngn3-positive spermatogonia. Thus, the pre-pubertal Ngn3-positive spermatogonia already possess the essential traits of the adult-type undifferentiated spermatogonia. It is noteworthy that the emergence of Ngn3-positive spermatogonia parallels the appearance of transplantable stem cell activity at around P2-P4 (McLean et al., 2003).

Contribution of spermatogonia that express or do not express Ngn3 to spermatogenesis

As discussed earlier, a particular subset of gonocytes appeared to develop into Kit-positive spermatogonia without passing through the Ngn3-positive undifferentiated spermatogonia stage. Ohbo et al. reported that the prepubertal Kit-positive spermatogonia barely show stem cell activity (Ohbo et al., 2003). However, their other characteristics are as yet unknown; they might either differentiate further or degenerate. Therefore, we developed a transgenic system in which cells that have undergone a Ngn3-positive stage (Ngn3⁺ lineage) and those that have never expressed this gene (Ngn3⁻ lineage) can be identified by differential labeling (Fig. 5A). For this purpose, we used transgenic mice that express constitutively active
Cre recombinase controlled by the Ngn3 regulatory sequence (Ngn3/Cre) (Yoshida et al., 2004). In double-transgenic mice possessing the CAG-CAT-Z reporter (Araki et al., 1995), the Ngn3+ lineage can be labeled for β-gal expression as a result of an irreversible recombination, whereas the Ngn3− lineage retains the intact form of the reporter gene and expresses the CAT (chloramphenicol acetyltransferase) gene instead. In these mice, β-gal is not detected in neonatal testes, reflecting the absence of Ngn3 expression in the germ line until birth (Yoshida et al., 2004). Active Cre recombinase begins to be expressed in Ngn3-positive cells as early as P3, and promptly causes the recombination that allows the expression of lacZ (Fig. 5B-D). In P5 testes, more Kit-positive spermatogonia were detected than lacZ-positive spermatogonia (Fig. 5E,F), indicating that the majority of these prepubertal Kit-positive spermatogonia were devoid of lacZ expression, which is in agreement with the direct generation of Kit-positive spermatogonia without Ngn3 expression.

Furthermore, the contributions of the Ngn3− (lacZ-negative/CAT-positive) and Ngn3+ (lacZ-positive/CAT-negative) lineages of cells were examined during the maturation of the testes. At P21, when round spermatids first appear, the germ cells of the Ngn3− lineage are localized almost exclusively at the center of the seminiferous tubules, i.e. in the most advanced cells (Fig. 5G-I). At P28, the germ cells of the Ngn3+ lineage contribute to the innermost layer of the spermatogenic cells (Fig. 5J-L). At this age, the degree of maturation varies among the seminiferous tubule segments. The most advanced segments contain elongated spermatids, which consist mostly of cells of the Ngn3+ lineage (e.g. tubule 1 in Fig. 5K). The contribution of the Ngn3− lineage is prominent in the second advanced group of segments (e.g. tubule 2). In the least matured segments, essentially no cells of the Ngn3− lineage were observed (see tubule 3). Thus, cells of Ngn3− lineage specifically contribute to the leading edge of pubertal spermatogenesis, which gives rise to the first spermatozoa that are released around P35 (Kluin et al., 1982). In the fully mature testes,

**Fig. 3.** Distribution of the newly-born Ngn3- and Kit-positive spermatogonia, and the seminiferous epithelial cycle prepattern. (A-H) Double-staining ISH of testes sections at the indicated ages with the indicated genes. Overlaid images of Kit or Ngn3 (purple, bright field) and galectin 1 (red fluorescence) are shown in A,C,E,G; galectin 1 signals in the same field are shown in B,D,F,H. Arrows in A and C indicate the Kit- and Ngn3-positive spermatogonia, respectively. E,F and G,H are adjacent sections. Note the variable intensities of galectin 1 signals within the seminiferous tubule cross-sections and their relationship with Kit- or Ngn3-positive spermatogonia (see Results). Asterisks (E-H) represent typical segments with a high galectin 1 mRNA signal; Kit-positive, but not Ngn3-positive, spermatogonia preferentially localize to these segments. Segments marked by hearts show lower galectin 1 signals and exhibit a preference for Ngn3-positive spermatogonia. Scale bars: 100 μm, in D for A-D, in H for E-H. (I) Quantification of Kit- or Ngn3-positive spermatogonia localized in tubule segments categorized by different levels of galectin 1 mRNA (high, medium or low). Tubule cross-sections marked with H, M or L in B and D are examples of segments with high, medium and low levels of galectin 1 mRNA, respectively. Data are represented as ‘preference’ to each category of tubule segment. a and b represent significant deviation with P-values of <0.0001 and <0.05, respectively. See Materials and methods and Tables S2-S7 in the supplementary material. The number of Ngn3-positive spermatogonia at P3 and P4 was too low for statistical analyses. (J) Model for the generation of spermatogonia subpopulations. One area of seminiferous tubules is represented to align tubules with increasing age. The galectin 1 mRNA level is shown by the gradient: black, highest; white, lowest. As galectin 1 expression increases and reduces in cycles (black to gray to white to black, etc.), its expression domain shifts leftward in a wave-like manner. Kit-positive spermatogonia (red ovals) appear specifically at the galectin 1-high segments (red bands). By contrast, Ngn3-positive spermatogonia (green ovals) are generated separately from Kit-positive spermatogonia around the segments of medium level of galectin 1 expression (green bands).
germ cells of the Ngn3− lineage are absent, and spermatogenesis is completely dependent on germ cells of the Ngn3+ lineage, while somatic cells, including Sertoli, myoid and interstitial cells, remain CAT positive (arrows) (Fig. 5M,N) (Yoshida et al., 2004).

It is theoretically possible that the CAT-positive/β-gal-negative cells are the result of incomplete recombination in cells with weak and short Ngn3 expression. However, in our double-transgenic mice, the recombination by Cre is particularly efficient, as determined by the quick and accurate recombination (Fig. 5B-D), and by the essentially complete recombination in the mature testes (Fig. 5M,N, Fig. 6) (Yoshida et al., 2004). The pattern of the CAT-positive cell contribution is highly reproducible among individuals from independent Ngn3/Cre transgenic lines. Therefore, considering the tight relationship between the pre-pubertal Kit-positive (Ngn3-negative) spermatogonia subpopulation and the seminiferous epithelial cycle, it is probable that at least a major part of the CAT-positive cells found in the first round of spermatogenesis represents a particular subpopulation of germ cells: those produced from a distinct program that lacks the Ngn3-positive, undifferentiated spermatogonia stage.

Fertilizing ability of the Ngn3− lineage of spermatogenesis
The pubertal, so-called first wave of spermatogenesis includes massive apoptosis (Kluin et al., 1982; Mori et al., 1997) and it is sometimes suspected to not produce fertile spermatozoa. We attempted to elucidate whether this inefficiency is related to the Ngn3− and Ngn3+ lineages. As shown in Fig. 5J-L, the germ cells of the Ngn3− lineage do survive and differentiate into morphologically mature sperm. We also found that germ cells of both the Ngn3− and Ngn3+ lineages exhibit cell death with no clear preferences (data not shown). Next, the fertility of the spermatozoa that were generated in the first round of spermatogenesis was tested as follows: Ngn3/Cre:CAG-CAT-Z male double-transgenic mice were mated with non-transgenic females, and each of the offspring were tested to determine whether they carried the recombined or the intact form of the reporter gene in tissues without Ngn3 expression (Fig. 6A). Offspring with an intact reporter gene are derived by fertilization with spermatozoa of the Ngn3− lineage, whereas those with the recombined reporter gene are derived from the Ngn3+ lineage. In our experiment, the youngest paternal age at fertilization was P40. Spermatozoa with an intact reporter gene exclusively contributed to the offspring obtained from the mating at P40 and P41 (Fig. 6B), reflecting fertilization by the first-released spermatozoa after epididymal passage. By contrast, offspring obtained by mating older mice derived from the recombined spermatozoa. It is noteworthy that males that produced offspring with the intact reporter gene at their first matings, later produced offspring with only the recombined reporter gene. This shows that, the first round of spermatogenesis can produce functional spermatozoa regardless of the distinct differentiation program. This is in agreement with the observation that full-term embryonic development can be supported by at least some of the round spermatids in the first round of spermatogenesis after microinsemination by injecting into oocytes (Miki et al., 2004).

Fig. 4. Pulse labeling of Ngn3-positive spermatogonia using tamoxifen-inducible Cre transgenic mice, and their stem cell activities. (A) Scheme for the tamoxifen-dependent recombination by CreERTM, resulting in the labeling of cells with β-gal expression. (B) The experiment schedule. (C,D) ISH on adjacent sections from a P8 Ngn3/CreERTM transgenic mouse testis detecting Ngn3 and CreERTM expression and indicating their overlapped expression (arrowheads). (E,F) Whole-mount X-Gal staining of a seminiferous tubule of a P8 Ngn3/CreERTM, CAG-CAT-Z double-transgenic mouse with (E) and without (F) tamoxifen administration (+tam). Inset (E) is at higher magnification. Note the tamoxifen-dependent appearance of β-gal-positive spermatogonia. Mice with only the CAG-CAT-Z transgene do not exhibit positive staining after tamoxifen administration (data not shown). (G) Double-transgenic mice were injected with tamoxifen at P5 and P6, and their seminiferous tubules were subjected to X-gal staining at the age of 3 months. Many β-gal-positive cells persist as distinct segments (arrowheads). (H) Cross section of a β-gal-positive segment containing a complete set of spermatogenic cells stained with β-gal (nuclear counterstaining in red). (I-K) Stem cell activity of Ngn3-positive spermatogonia after microinsemination by injecting into oocytes (Miki et al., 2004).
Fig. 5. Chase of cells of Ngn3+ and Ngn3− lineages during the maturation of testes using Ngn3/Cre;CAG-CAT-Z double-transgenic mice. (A) Experimental design. In Ngn3-positive cells of Ngn3/Cre;CAG-CAT-Z mice, the reporter gene is irreversibly recombined between loxP sites by Cre recombinase driven by the Ngn3 regulatory sequence, thereby labeling their progenies with β-gal (encoded by lacZ), while CAT expression is lost (Ngn3− lineage). The reporter gene remains intact in cells that have never expressed Ngn3; these cells express CAT, but not lacZ (Ngn3− lineage). (B-D) Serial sections of P3 testes probed for Ngn3, Cre and lacZ, representing the overlapping expression of these genes in a single seminiferous tubule (arrowheads). (E,F) A pair of adjacent sections of a P5 testis, which were hybridized for Kit and lacZ expression. The Kit-positive spermatogonia apparently outnumber the lacZ-positive ones. (G-I) P21 testis probed for CAT. H shows a higher magnification of a part of G; I shows a PI-stained fluorescence image of H. CAT signals are preferentially detected at the center of the seminiferous tubules (arrowheads). Arrows indicate CAT signals in the interstitial cells. (J-L) P28 testis probed for CAT and lacZ. (K) Higher magnification of a part of J; (L) the same location but in the next section. CAT-positive and lacZ-negative (Ngn3− lineage) spermatogenic cells are preferentially found in the innermost layer (arrowheads). Seminiferous tubules marked 1-3 are examples of the different degrees of maturation (see Results). (M,N) P56 testis probed for CAT and lacZ. All germ cells are CAT negative and lacZ positive. Arrows indicate somatic cells positive for CAT (Sertoli and interstitial cells). Scale bars: 100 μm.

Fig. 6. Contribution of cells of the Ngn3+ and Ngn3− lineages to functional spermatozoa. (A) Experimental design. Ngn3/Cre;CAG-CAT-Z double-transgenic male mice were mated with non-transgenic females, and the offspring genotyped to determine whether the contributed spermatozoa carried an intact (CAG-CAT-Z) or recombined (CAG-Z) form of the reporter gene. (B) Frequency of offspring with the intact (gray) and recombined (white) forms of the reporter gene, classified according to the paternal age at fertilization.
The fragility of pubertal spermatogenesis may be a result of cell-extrinsic factors, such as hormonal conditions, as described previously (Russell et al., 1987).

**DISCUSSION**

On the basis of our study, we have proposed a model for the initiation of spermatogenesis (Fig. 7). In the pre-pubertal testis, a particular subset of gonocytes directly gives rise to Kit-positive differentiating spermatogonia in the seminiferous tubule segments with a high level of galectin 1 mRNA; these spermatogonia have not passed through the Ngn3-positive undifferentiated stage. This population (Ngn3− lineage) differentiates promptly in the first round of spermatogenesis, resulting in the production of fertile spermatooza. Contrastingly, another set of gonocytes become Ngn3-positive spermatogonia in distinct segments with a medium level of galectin 1. This population (Ngn3+ lineage) is capable of self-renewal and the generation of differentiating cells, and supports the continuity of steady-state spermatogenesis. In Ngn3-expressing undifferentiated spermatogonia, the expression of Plzf, Ret or Oct4, genes that also characterize the PGC and/or gonocytes, is retained. Gain of Ngn3 expression thus reflects a cell-type switch from precursors to self-renewing cells.

The fact that the first round of spermatogenesis produces fertile spermatooza even though they are not derived from self-renewing undifferentiated spermatogonia supports the idea that the stem cell step is not ‘the gateway’ that all progenitor cells must enter and from which all differentiating cells must originate. Rather, as shown in Fig. 7, the stem cell step can be considered to be a ‘developmental option’ that branches out of the process of spermatogenic differentiation and ensures continuous spermatooza production. This idea might also be true for other stem cell systems. It has been recently shown that, in the *Drosophila* germ line, some primordial germ cells do not develop into self-renewing stem cells but directly enter into the differentiation process (Asaoka and Lin, 2004). In mammalian yolk sac hematopoiesis, although the first appearance of the hematopoietic stem cells is not completely understood, the first round of differentiation of blood cells occurs prior to the appearance of detectable hematopoietic stem cell activity (Palis and Yoder, 2001).

The determination of the mechanisms underlying the emergence of the two spermatogonial subpopulations from gonocytes at distinct segments of seminiferous tubule is an important issue. Although this study still leaves this question unsolved, several possibilities can be discussed. If we simply extend the interpretation of Timmons et al. (Timmons et al., 2002) that the somatic components have their intrinsic pre-pattern of the seminiferous epithelial cycle, germ cell differentiation would be under their downstream control. Therefore, one possibility is that gonocytes might be a homogeneous population in which individual cells are bipotential and select their fates in response to the local controls by somatic cells upon transition into spermatogonia. A second possibility is that gonocytes might be heterogeneous and that a particular subpopulation is committed to contributing to the self-renewing component. Compatible with this idea, Orwig et al. (Orwig et al., 2002) reported that neonatal rat gonocytes exhibit a morphological heterogeneity that is closely related to their transplatable stem cell activities. At a molecular level, neonatal mouse gonocytes represent some heterogeneity in terms of Oct4 expression level (Ohmura et al., 2004), raising the possibility that Oct4 high-expressers might be committed to self-renewal. Other possibilities include that commitment might occur under the control of somatic cells at the gonocyte stage or earlier. However, we cannot rule out the possibility that germ cells might decide their own fate independently from the somatic cells, and that coordination is brought about by germ-soma interactions, including the cooperation of the Sertoli cell cycle by germ cells, or sorting of the germ cell subpopulations according to somatic cell-derived environments.

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**Fig. 7. Model for the two lineages in the mouse spermatogenesis.** In the first postnatal week, gonocytes directly give rise to Kit-positive differentiating spermatogonia and Ngn3-positive undifferentiated spermatogonia in parallel. This process is closely related to the presumptive seminiferous epithelial cycle pre-pattern, which initiates before birth. Kit-positive spermatogonia are specifically generated in the galectin 1-high segments (pink arrow 1). These cells do not pass through a Ngn3-positive, undifferentiated spermatogonia stage and differentiate in the first round of spermatogenesis, resulting in the formation of fertile spermatooza (Ngn3− lineage). By contrast, Ngn3-positive undifferentiated spermatogonia are generated preferably at galectin 1-medium segments (green arrow). They subsequently act as a self-renewing stem cell population, while also providing cells that transform into differentiating spermatogonia. Thus, these cells support steady-state spermatogenesis following the first round of spermatogenesis (Ngn3+ lineage). The transformation of undifferentiated spermatogonia into differentiating spermatogonia is tightly related to the seminiferous epithelial cycle, and Kit-positive differentiating spermatogonia are established in stages of high galectin 1 expression (stage IX–X), indicated by pink arrow 2. See Results for more details. PGC, primordial germ cells.
It is noteworthy that the direct generation of differentiating spermatagonia from gonocytes occurs in the seminiferous segments with a high level of galectin 1 mRNA (Fig. 7), because this is likely to reflect the common features of pre-pubertal and adult spermatogenesis, and, therefore, their continuity. In the adult, Kit-positive differentiating spermatagonia are established around stages IX-X (Schrans-Stassen et al., 1999). These stages show the highest level of galectin 1 (Timmons et al., 2002) (Fig. 7). We believe this coincidence would strengthen the assumption by Timmons et al. that the seminiferous epithelial cycle is pre-figured already in the pre-pubertal stage (Timmons et al., 2002). As a result, the establishment of the spermatogenic wave may accompany the completion of the first round of spermatogenesis, thus ensuring a constant release of the mature spermatocytes following puberty.

The direct derivation of the first differentiating spermatagonia from gonocytes is in agreement with the hypothesis of de Rooij and colleagues, which was based on morphology, and on the backwards extrapolation of the progressive appearance of differentiating cell types (de Rooij, 1998; Kluin and de Rooij, 1981). The present study not only provides evidence for their hypothesis by means of gene expression and lineage analyses, but also extends it with regard to the fate of the first differentiating spermatagonia. Although previous studies could not define the fate of the first differentiating spermatagonia because of the fragility of pubertal spermatogenesis, our study provides evidence that the first differentiating spermatagonia that appear give rise to functional spermatozoa. We have also demonstrated that, immediately after the gonocyte-spermatagonia transition, adult-type undifferentiated spermatagonia appear as Ngn3-positive cells, which already exhibit stem cell characteristics (i.e., self-renewal and the generation of differentiating cells). Accordingly, we do not believe it necessary to consider the existence of the special type of ‘pre-spermatagonia’ between gonocytes and adult-type spermatagonia (Bellev et al., 1977; Hilscher et al., 1974; Huckins and Clermont, 1968). Finally, this study has shown the close relationship between the genesis of the spermatogenic subpopulations and the establishment of the seminiferous epithelial cycle.

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