

## LY6A/E (SCA-1) Expression in the Mouse Testis

Maaïke P.A. van Bragt,<sup>1,2</sup> Nadia Ciliberti,<sup>3</sup> William L. Stanford,<sup>3</sup> Dirk G. de Rooij,<sup>2,4</sup> and Ans M.M. van Pelt<sup>2</sup>

Department of Endocrinology,<sup>2</sup> Faculty of Biology, Utrecht University, 3584 CH Utrecht, The Netherlands  
Institute of Biomaterials and Bioengineering,<sup>3</sup> Department of Chemical Engineering and Applied Chemistry,  
Institute of Medical Science, University of Toronto, Toronto, Canada M5S 1A8  
Department of Cell Biology,<sup>4</sup> University Medical Center Utrecht, 3584 CH Utrecht, The Netherlands

### ABSTRACT

Recently, it was found by two research groups that LY6A, known widely in the stem cell community as stem cell antigen-1 or SCA-1, is expressed on testicular side population (SP) cells. Whether these SP cells are spermatogonial stem cells is a point of disagreement and, therefore, the identity of the LY6A-positive cells as well. We studied the expression pattern of LY6A in testis by immunohistochemistry and found it to be expressed in the interstitial tissue on peritubular myoid, endothelial, and spherical-shaped peritubular mesenchymal cells. To address the question whether LY6A has a function in spermatogenesis or testis development, we studied the testis of *Ly6a*<sup>-/-</sup> mice (allele *Ly6a*<sup>tm1Pmf</sup>). We found no morphological abnormalities or differences in numbers of spermatogonia, spermatocytes, Leydig cells, or macrophages in relation to the number of Sertoli cells. Therefore, we conclude that LY6A expression does not influence testis development or spermatogenesis and that spermatogonial stem cells are LY6A negative.

interstitial cells, Leydig cells, spermatogenesis, testis

### INTRODUCTION

Spermatogenesis, the process during which spermatozoa are formed after several steps of mitosis, meiosis, and differentiation, begins with the spermatogonial stem cells. Spermatogonial stem cells must self-renew to keep spermatogenesis going throughout life, and, on the other hand, must give rise to differentiating cells to ultimately produce spermatozoa [1]. The mechanisms by which stem cell fate is regulated are still largely unknown. Research in this field is hampered by the fact that there are only 35 000 spermatogonial stem cells in a mouse testis [2] and by the fact that spermatogonial stem cells cannot yet be isolated from the testis as a pure population.

Attempts to isolate spermatogonial stem cells were made and considerable enrichment was reached by using surface markers, such as integrin alpha 6, KIT, and integrin alpha v [3]. Progress in the purification protocol has been possible by the use of the spermatogonial stem cell transplantation technique as an assay for these cells. Only true spermatogonial stem cells will be able to colonize and repopulate depleted seminiferous tubules of recipient mice [4].

Goodell et al. [5] were the first to discover that a small, distinct subset of bone-marrow cells can easily be isolated with fluorescence activated cell sorting (FACS) when making use of the fact that these cells are able to quickly expel the fluorescent dye, Hoechst 33342 (Hoechst). The isolated cells were called side population (SP) cells, and they were also found in other tissues, i.e., skeletal muscle [6], mammary gland [7], brain [8], and liver [9]. It was suggested that the SP cells are the most primitive and true stem cells in each of these tissues.

The testis also contains SP cells [10–13]. Kubota et al. [10] were the first to report the existence of SP cells in the testis. Intriguingly, these authors did not find colonization of recipient mouse testes with SP cells from adult cryptorchid mice. The surface markers they found on the SP cells were also different from those present on spermatogonial stem cells. In contrast, Lassalle et al. [11] reported that their isolated testicular SP cells were able to colonize recipient mouse testes upon transplantation and hence were spermatogonial stem cells. In addition, they showed that the SP cells expressed *Stra8* and integrin alpha 6, two germinal cell markers [3, 14]. Similar results were obtained by Falciatori et al. [12] by isolating SP cells from 20-day-old mice. Interestingly, Lo et al. [13] found restoration of Leydig cell numbers and testosterone levels in LH receptor-knockout (*LhrKO*) recipients when SP cells were transplanted into the interstitial tissue of the testis. *LhrKO* mice are infertile due to targeted disruption of LH/hCG [15]. Restoration of Leydig cell numbers and testosterone levels in *LhrKO* mice after transplantation with SP cells indicates that testicular SP cells include progenitors of Leydig cells.

Both Kubota et al. [10] and Falciatori et al. [12] looked for expression of surface markers on testicular SP cells. The marker they both found to be present on testicular SP cells was the glycosyl phosphatidylinositol-linked cell-surface glycoprotein, LY6A (also known as SCA-1). LY6A is frequently used for isolation of hematopoietic stem cells. Sorting with the specific E13-161.7 antibody yields a 100-fold enrichment of hematopoietic stem cells out of bone marrow [16]. Expression of LY6A is not restricted to hematopoietic stem cells, it is also found on skeletal muscle stem cells, mammary epithelium stem cells, kidney epithelial cells, osteoblasts, and vasculature of brain, heart, and liver [6, 7, 17, 18]. LY6A is encoded by the prototypic member of the *Ly-6* gene family, *Ly6a*, encoding two alleles (*Ly6a.1* or *Ly6e* and *Ly6a.2*) in a mouse strain-specific manner [17]. Thus, LY6A has also been referred to as Ly6A/E. In addition to differing by two amino acids, the two alleles demonstrate differential tissue distribution due to differential transcriptional or posttranscriptional regulation [19, 20]. In *Ly6a.2* mouse strains, including C57BL/6, essentially all

<sup>1</sup>Correspondence: Maaïke van Bragt, Department of Endocrinology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.  
FAX: 31 030 253 2837; e-mail: m.vanbragt@bio.uu.nl

Received: 24 January 2005.

First decision: 17 February 2005.

Accepted: 25 May 2005.

© 2005 by the Society for the Study of Reproduction, Inc.

ISSN: 0006-3363. <http://www.biolreprod.org>

bone marrow-repopulating cells are LY6A-positive compared with 25% in *Ly6a.1/Ly6e* strains, such as BALB/c [21]. Also, the number of LY6A-positive thymocytes and peripheral T lymphocytes is different between different mouse strains [21, 22]. In *Ly6a*<sup>-/-</sup> mice, LY6A was found to have important functions in regulating the repopulating capacity of hematopoietic stem cells and the development of committed progenitor cells, megakaryocytes, and platelets [23]. *Ly6a*<sup>-/-</sup> mice also exhibit defects in T cell signaling [24] and self-renewal capacity of early mesenchymal precursors [25], that give rise to adipocytes, osteoblasts, chondrocytes, and muscle cells [26, 27].

In this paper, for the first time, the localization of LY6A in the testis is described.

Furthermore, to determine a possible role of LY6A in spermatogenesis or testis development, we studied the morphology and numbers of various testicular cell types in *Ly6a*<sup>-/-</sup> mice.

## MATERIALS AND METHODS

### Animals

*Ly6a*<sup>-/-</sup> mice (allele *Ly6a*<sup>tm1Pmf</sup>) on the BALB/c background and BALB/c wild-type littermates were generated as described before and maintained at Mount Sinai Hospital, Toronto [24, 25]. FVB and C57BL/6 mice were used and maintained according to regulations provided by the animal ethical committee of the University of Utrecht, which also approved the experiments.

### Immunohistochemistry

For immunolocalization of LY6A, testes from adult mice of different strains, FVB, C57BL/6, and BALB/c, were fixed in Bouin fluid. Five-micrometer paraffin sections were mounted on 3-aminopropyl triethoxysilane (TESPA, Sigma, St. Louis, MO)-coated glass slides and dried overnight at 37°C. Endogenous peroxidase was blocked with 0.35% H<sub>2</sub>O<sub>2</sub> in PBS for 15 min. After blocking in 5% normal rabbit serum (Vector Laboratories, Burlingame, CA), slides were incubated overnight at 4°C with 1:200 LY6A/E antibody (E13.161.7 no. 553333 or D7 no. 557403; BD Biosciences, Franklin Lakes, NJ) in 1% BSA in PBS (Sigma, St. Louis, MO). Secondary biotinylated rabbit anti-rat antibody (BA-4000, Vector Laboratories) was used 1:200 in 1% BSA in PBS for 1 h at room temperature. Horseradish peroxidase avidin-biotin complex reaction was performed according to the manufacturer's protocol (Vector Laboratories). Antibody was finally detected by diaminobenzidine (DAB; Sigma) in 50 mM Tris-HCl, pH 7.6. The reaction was amplified with cobalt-chloride (0.025%) and nickel ammonium sulfate (0.02%). Sections were counterstained with nuclear fast red, dehydrated, and mounted with Pertex (Cellpath Ltd., Hemel Hempstead, U.K.). For positive and negative controls, Bouin-fixed kidney [28] and *Ly6a*<sup>-/-</sup> mice testis were used, respectively.

### Immunofluorescence

Immunolocalization of LY6A in adult mice testis was performed on Bouin-fixed testes of different mice strains, FVB, C57BL/6, and BALB/c. Five-micrometer paraffin sections were mounted on TESP-coated glass slides and dried overnight at 37°C. Slides were incubated overnight at 4°C with 1:50 LY6A/E antibody (E13.161.7 no. 553333 or D7 no. 557403; BD Biosciences) in 5% normal rabbit serum in Coons buffer (10 mM 5,5-diethylbarbituric acid sodium salt, 145 mM NaCl, pH 7.4, 0.01% BSA, 0.2% Triton X-100). Second, rabbit anti-rat (BA-4000; Vector Laboratories), and third antibody, goat anti-rabbit Texas red, were used 1:100 in coons and incubated for 1 h at room temperature. Sections were mounted with VECTASHIELD (Vector Laboratories) and viewed on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal laser scanning microscope (Leica, Heidelberg, Germany). Images were recorded digitally. For positive and negative controls, Bouin-fixed kidney and *Ly6a*<sup>-/-</sup> mice testis were used, respectively.

### Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine 5-Triphosphate-Biotin Nick End Labeling on *Ly6a*<sup>-/-</sup> Mice Testis

Detection of apoptotic cells was performed on 10- and 19-wk-old *Ly6a*<sup>-/-</sup> mice and wild-type littermates. Five-micrometer Bouin-fixed, paraffin-embedded testis sections were boiled for 5 min in 10 mM citric buffer (pH 6.0) at 98°C and slowly cooled to room temperature. Endogenous peroxidase was blocked with 3% H<sub>2</sub>O<sub>2</sub> in MilliQ (MQ) for 5 min. Sections were washed three times with PBS before a 60-min incubation in 5-triphosphate-biotin nick end labeling (TUNEL) mix at 37°C. TUNEL mix consists of 0.3 U/μl calf thymus terminal deoxynucleotidyl transferase (Amersham Biosciences, Freiburg, Germany), 6.66 μM/μl biotin dUTP (Roche, Basel, Switzerland) in terminal transferase buffer (Amersham Biosciences). TUNEL reaction was stopped by incubation in 300 mM NaCl, 30 mM sodium citrate in MQ for 15 min at room temperature. After washing with PBS, sections were blocked with 2% BSA (Sigma) in PBS at room temperature for 10 min. Sections were treated for 30 min at 37°C in a moist chamber with a 1:20 dilution of ExtrAvidin peroxidase antibody. After three washes in PBS, detection was performed with DAB+ (Dako, Glostrup, Denmark). Sections were counterstained with Mayer hematoxylin, dehydrated, and mounted with Pertex (Cellpath Ltd., Hemel Hempstead, U.K.). The number of TUNEL-positive cells was counted in four and three testes of 10- and 19-wk old-animals, respectively, and calculated as number per 100 tubule cross sections. At least 100 tubules were counted. Numbers were expressed as mean ± SEM, and statistical analysis was performed using the unpaired *t*-test.

### Cell Counts

Five-micrometer sections were stained with periodic acid Schiff (PAS) and counterstained with hematoxylin. Numbers of A spermatogonia, preleptotene spermatocytes, Leydig cells, and macrophages were counted in 10- and 19-wk-old *Ly6a*<sup>-/-</sup> and wild-type littermate testes. For 10- and 19-wk-old animals, four and three testes of either genotype were counted, respectively. Cell numbers were expressed per 1000 Sertoli cells according to the method of Heller et al. [29, 30]. Cell counts were performed until at least 300 Sertoli cells were scored. All cell numbers were expressed in mean ± SEM, and statistical analysis was performed using the unpaired *t*-test.

## RESULTS

### Immunolocalization of LY6A in the Testis

To investigate the localization of LY6A in the testis, we performed both immunohistochemical and immunofluorescence studies with the monoclonal antibody, E13.161.7, that recognizes LY6A in both *Ly6a* and *Ly6e* mouse strains. With both techniques, a similar expression pattern was observed. In C57BL/6 (not shown) and FVB (Fig. 1, A, D, and G) wild-type mice, the cell membrane of most peritubular myoid cells as well as endothelial cells (Fig. 1G) were stained. In BALB/c mice some peritubular myoid, but no endothelial cells were stained (Fig. 1, B, E, and H). In addition, staining was often observed in spindle-shaped peritubular mesenchymal cells (Fig. 1H) in the interstitial tissue of all three mouse strains. No staining was present in seminiferous tubules in any of the strains tested. Also, in Leydig cells and macrophages, no staining could be observed in any of the strains. As a negative control, testes of *Ly6a*<sup>-/-</sup> mice were used. No staining was observed in these control sections (Fig. 1, C and F). For positive control, we showed expression of LY6A in the renal vessels of the kidney (Fig. 1I). Furthermore, using the D7 monoclonal antibody for both immunohistochemistry and immunofluorescence, the same expression pattern was observed as with the E13.161.7 antibody (data not shown).

### Morphology and Cell Counting in *Ly6a*<sup>-/-</sup> Mice Testes

To address the question whether LY6A has a function in spermatogenesis, we studied testes of 10- and 19-wk-old

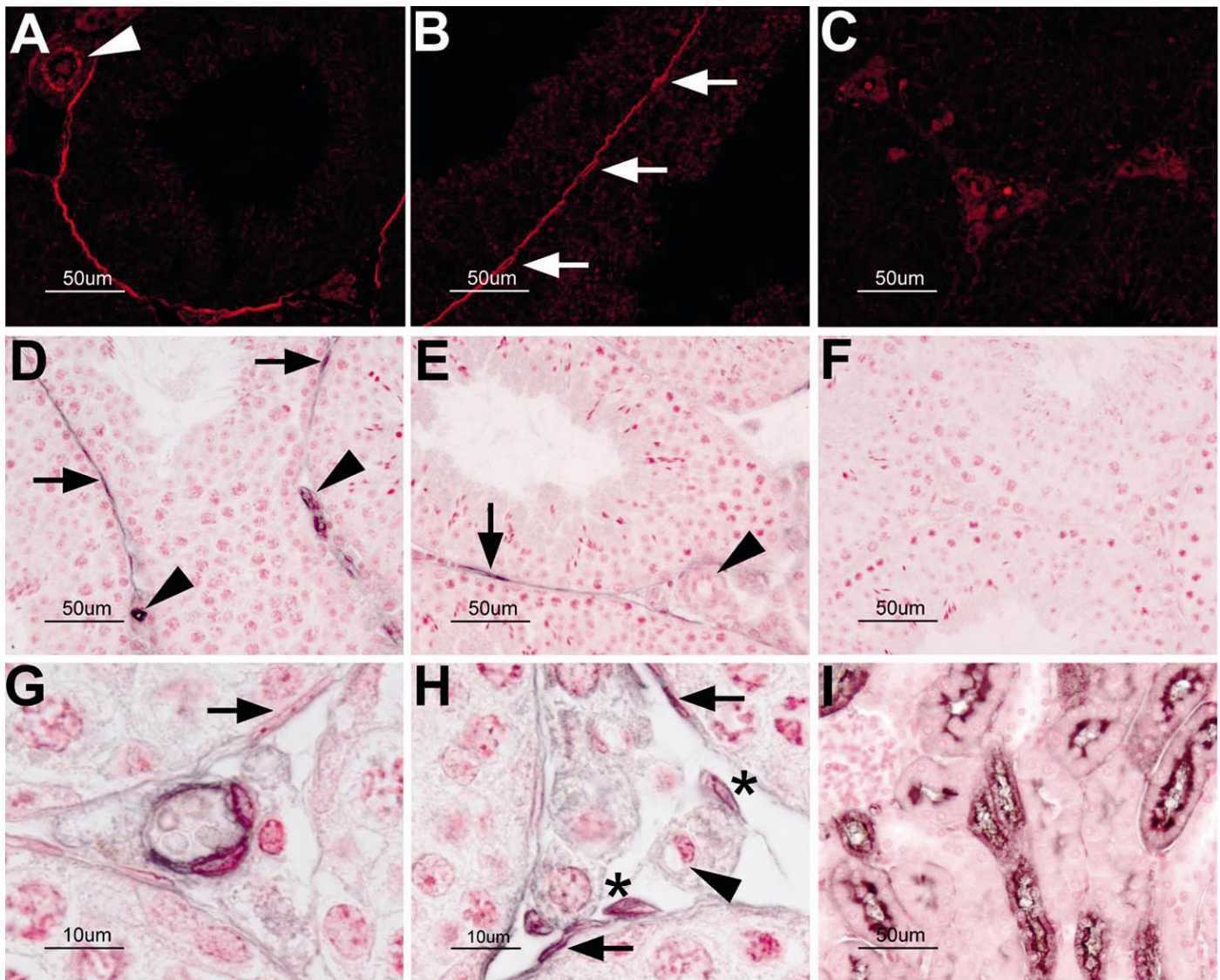


FIG. 1. Immunolocalization of LY6A in the testis of different mice strains. A–C) immunofluorescence staining of LY6A in FVB (A), BALB/c (B), and *Ly6a*<sup>-/-</sup> mouse testis (C). D–F) Immunohistochemical staining of LY6A in FVB (D), BALB/c (E), and *Ly6a*<sup>-/-</sup> mouse testis (F). G–H) Higher magnification of FVB (G) and BALB/c testis (H). Indicated are endothelial cells (arrowheads), peritubular myoid cells (arrow), and spherical-shaped peritubular mesenchymal cells (asterisk). No staining is observed in the Leydig cells nor in the seminiferous tubules. I) Kidney; staining is observed in the renal vessels [28].

*Ly6a*<sup>-/-</sup> mice and wild-type littermates. PAS-stained tubules cross sections of *Ly6a*<sup>-/-</sup> mice appear normal, i.e., a lumen is formed and all spermatogenic cell types are present (Fig. 2A). To study this in more detail, we counted the numbers of A spermatogonia and preleptotene spermatocytes in epithelial stages VII and VIII in 10- and 19-wk-old animals and found no significant differences between *Ly6a*<sup>-/-</sup> and their wild-type littermates (Table 1). In the interstitial tissue, we counted the numbers of Leydig cells and macrophages in 10- and 19-wk-old mice. No significant

cytes in epithelial stages VII and VIII in 10- and 19-wk-old animals and found no significant differences between *Ly6a*<sup>-/-</sup> and their wild-type littermates (Table 1). In the interstitial tissue, we counted the numbers of Leydig cells and macrophages in 10- and 19-wk-old mice. No significant

FIG. 2. Morphology and apoptosis in *Ly6a*<sup>-/-</sup> testis. A) *Ly6a*<sup>-/-</sup> mice testis. B) The number of TUNEL-positive germ cells  $\pm$  SEM per 100 tubular cross sections of *Ly6a*<sup>-/-</sup> mice and wild-type littermates testis of 10 and 19 weeks.

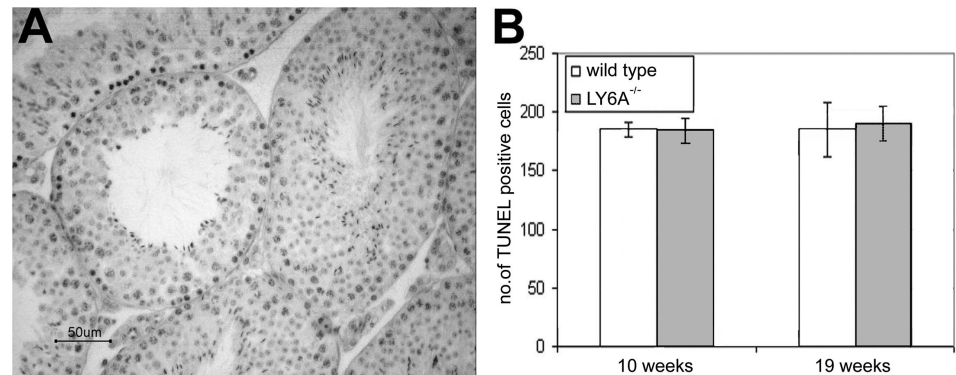


TABLE 1. Number of A spermatogonia, spermatocytes, Leydig cells, and macrophages in *Ly6a*<sup>-/-</sup> and littermates of 10 and 19 wk.<sup>a</sup>

Cell type	10 wk		19 wk	
	Wild-type	<i>Ly6a</i> <sup>-/-</sup>	Wild-type	<i>Ly6a</i> <sup>-/-</sup>
A spermatogonia	343 ± 14	337 ± 37	363 ± 13	387 ± 60
Preleptotene spermatocytes	5478 ± 217	5963 ± 196	5603 ± 420	5857 ± 89
Leydig cells	1148 ± 15	1079 ± 32	1197 ± 34	1059 ± 62
Macrophages	115 ± 14	117 ± 3	158 ± 30	161 ± 11

<sup>a</sup> Numbers are expressed as mean ± SEM number per 1000 Sertoli cells.

differences in the numbers of Leydig cells or macrophages were observed between *Ly6a*<sup>-/-</sup> mice and their wild-type littermates (Table 1).

#### Apoptosis in *Ly6a*<sup>-/-</sup> Mice Testes

To determine if LY6A deficiency causes abnormal apoptosis of germ cells, we performed a TUNEL assay on *Ly6a*<sup>-/-</sup> mice and wild-type testes. No significant differences were found between the numbers of apoptotic germ cells in *Ly6a*<sup>-/-</sup> mice and wild-type littermates (Fig. 2B). In the interstitial tissue of both *Ly6a*<sup>-/-</sup> and wild-type testes, we sporadically observed a TUNEL-positive Leydig cell. No TUNEL staining was observed in the peritubular myoid, endothelial, or peritubular mesenchymal cells.

#### DISCUSSION

The present data, for the first time, show that, in the testis, LY6A is solely expressed in the interstitial tissue and not in seminiferous tubules. In the three mouse strains tested, we found LY6A to be expressed in peritubular myoid cells and in spindle-shaped peritubular mesenchymal cells. Furthermore, we found in both FVB and C57BL/6 but not in BALB/c mice testis, LY6A to be expressed in endothelial cells. Difference in expression pattern of LY6A between different mouse strains is a known phenomenon. For both lymphocytes and hematopoietic stem cells, LY6A expression was found to differ between haplotypes [21, 22]. There are two allelic variants of *Ly6a*, *Ly6e*, and *Ly6a.2*, which are under distinct genetic control, resulting in two haplotypes, Ly6<sup>a</sup> and Ly6<sup>b</sup>, respectively. BALB/c encodes the Ly6<sup>a</sup> haplotype, while C57BL/6 encodes the Ly6<sup>b</sup> haplotype [21]. This may well explain the difference in expression of LY6A in endothelial cells in C57BL/6 versus BALB/c mice. Expression of LY6A was shown before in vasculature of heart, brain, and liver in C57BL/6 mice [17]. The haplotype of FVB mice is not known, but our results would now suggest that FVB mice have the Ly6<sup>b</sup> haplotype.

Testicular peritubular myoid [31], endothelial [32], and peritubular mesenchymal cells [31] all originate from the mesonephros. It was shown that the aorta-gonads-mesonephros (AGM) region is largely LY6A positive [33]. The hematopoietic stem cells as well as endothelial cells also arise from the AGM region [34, 35] and show LY6A expression [16, 17], suggesting a relationship between LY6A expression and the embryonic origin. However, there are also LY6A-positive cells that may not arise from the AGM region.

The germ cells and thus the spermatogonial stem cells originate from extraembryonic regions [36]. In this article, we show that LY6A expression is absent in all germ cells, including the spermatogonial stem cells. This is surprising because stem cells of many other tissues are found to be LY6A positive. However, satellite cells of striated muscle

tissue are also LY6A negative [37]. Furthermore, it was already suggested by Kubota et al. [10] that spermatogonial stem cells do not express LY6A on their surface.

LY6A is known to be expressed on SP cells of many tissues, including hematopoietic system [16], mammary gland [7], and skeletal muscle [6, 37]. Recently, four groups showed the presence of SP cells in the testis [10–13]. While Kubota et al. [10] found SP cells not be able to colonize the testis of busulfan-treated recipient mice, the opposite was found by Falciatori et al. [12] and Lassalle et al. [11], indicating disagreement about the question whether or not testicular SP cells are spermatogonial stem cells. The differences between these findings may have various causes. First, the groups use different types of donor mice, adult versus immature, and cryptorchid versus noncryptorchid. Second, for the isolation of SP cells, different protocols were used with respect to tissue dissociation, Hoechst concentration, time and temperature of Hoechst incubation, and stringency in the selection of SP cells (FACS gating). Recently, it was described that these parameters should be determined for each tissue tested because these factors all affect yield, viability, and homogeneity of SP cells and differ per tissue [38]. Therefore, different cell populations can be isolated by using different isolation protocols. Further research should be performed to optimize the SP protocol and to investigate whether there is a testicular SP that harbors spermatogonial stem cells.

Nevertheless Kubota et al. [10] and Lassalle et al. [11] agree that testicular SP cells express LY6A brightly and therefore it was important to identify the LY6A-positive cells to unravel the confusion about the identity of testicular SP cells.

Our results support the findings of Kubota et al. [10] that LY6A-positive SP cells are not spermatogonial stem cells because no staining for LY6A was observed in the seminiferous tubules, including spermatogonial stem cells. Interestingly, the present data support and extend the results obtained by Lo et al. [13, 39], who showed restoration of Leydig cell numbers and testosterone levels in *Lhr*KO mice after transplantation of SP cells into the interstitial tissue. They conclude that Leydig cell progenitors are among the SP cells. The present results indicate that spindle-shaped peritubular mesenchymal cells, which are thought to be progenitors of Leydig cells [40], express LY6A on their cell membranes.

LY6A is not only useful as a surface marker, it also plays an important role in the regulation of hematopoietic stem cells [23] and mesenchymal progenitors [25]. *Ly6a*<sup>-/-</sup> mice exhibit an age-dependent osteoporosis phenotype due to a primary defect in the self-renewal capacity of mesenchymal progenitors [25]. Furthermore, they have defects in the repopulating ability of hematopoietic stem cells upon competitive and serial transplantation of hematopoietic stem cells in lethally irradiated mice [23]. In contrast, we found that development and morphological appearance of the

*Ly6a*<sup>-/-</sup> testes were normal in 10- or 19-wk-old animals. Also, the numbers of spermatogonia and preleptotene spermatocytes in stages VII/VIII of the epithelial cycle were similar to those in wild-type mice. This suggests that spermatogonial proliferation in wild-type and *Ly6a*<sup>-/-</sup> mice is similar with respect to kinetics as well as the numbers of spermatocytes produced. Furthermore, Leydig cell numbers were not significantly different in 10- or 19-wk-old *Ly6a*<sup>-/-</sup> mice and age-matched wild-type mice, indicating that Leydig cells are normally formed and do not deplete until at least 19 wk of age. However, turnover of interstitial cells, including progenitors as well as adult Leydig cells, is low [41]. Therefore, there might be depletion of peritubular mesenchymal progenitors and Leydig cells in older mice. Challenging of the *Ly6a*<sup>-/-</sup> peritubular mesenchymal progenitors, by transplanting them into the interstitium of *Lhr*KO recipients testis, might, however, reveal a function of LY6A in the repopulating capacity of these cells.

Based on our results, we can conclude that LY6A-positive cells in the testis are not spermatogonial stem cells but do include cells with morphological appearance of Leydig cell progenitors. Furthermore, LY6A deficiency does not influence testis development, spermatogenesis, or peritubular mesenchymal cell and Leydig cell development. The present results also indicate the importance of performing localization analysis *in situ* rather than just relying on cell sorting and FACS analysis.

## REFERENCES

- De Rooij DG. Stem cells in the testis. *Int J Exp Pathol* 1998; 79:67–80.
- Tegelenbosch RA, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutat Res* 1993; 290:193–200.
- Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *Proc Natl Acad Sci U S A* 2000; 97:8346–8351.
- Ogawa T, Arechaga JM, Avarbock MR, Brinster RL. Transplantation of testis germinal cells into mouse seminiferous tubules. *Int J Dev Biol* 1997; 41:111–122.
- Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J Exp Med* 1996; 183:1797–1806.
- Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 1999; 401:390–394.
- Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM, Goodell MA. Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. *Dev Biol* 2002; 245:42–56.
- Murayama A, Matsuzaki Y, Kawaguchi A, Shimazaki T, Okano H. Flow cytometric analysis of neural stem cells in the developing and adult mouse brain. *J Neurosci Res* 2002; 69:837–847.
- Shimano K, Satake M, Okaya A, Kitanaka J, Kitanaka N, Takemura M, Sakagami M, Terada N, Tsujimura T. Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1. *Am J Pathol* 2003; 163:3–9.
- Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proc Natl Acad Sci U S A* 2003; 100:6487–6492.
- Lassalle B, Bastos H, Louis JP, Riou L, Testart J, Dutrillaux B, Fouchet P, Allemand I. 'Side population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. *Development* 2004; 131:479–487.
- Falciatori I, Borsellino G, Haliassos N, Boitani C, Corallini S, Battistini L, Bernardi G, Stefanini M, Vicini E. Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. *FASEB J* 2004; 18:376–378.
- Lo KC, Lei Z, Rao CV, Beck J, Lamb DJ. De novo testosterone production in luteinizing hormone receptor knockout mice after transplantation of Leydig stem cells. *Endocrinology* 2004; 145:4011–4015.
- Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P, Bronner S, Lutz Y, Chambon P. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by *Stra8*, a novel retinoic acid-responsive gene. *J Cell Biol* 1996; 135:469–477.
- Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV. Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. *Mol Endocrinol* 2001; 15:184–200.
- Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science* 1988; 241:58–62.
- van de Rijn M, Heimfeld S, Spangrude GJ, Weissman IL. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. *Proc Natl Acad Sci U S A* 1989; 86:4634–4638.
- Van Vlasselaer P, Falla N, Snoeck H, Mathieu E. Characterization and purification of osteogenic cells from murine bone marrow by two-color cell sorting using anti-Sca-1 monoclonal antibody and wheat germ agglutinin. *Blood* 1994; 84:753–763.
- Reiser H, Coligan J, Palmer E, Benacerraf B, Rock KL. Cloning and expression of a cDNA for the T-cell-activating protein TAP. *Proc Natl Acad Sci U S A* 1988; 85:2255–2259.
- LeClair KP, Palfree RG, Flood PM, Hammerling U, Bothwell A. Isolation of a murine Ly-6 cDNA reveals a new multigene family. *EMBO J* 1986; 5:3227–3234.
- Spangrude GJ, Brooks DM. Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells. *Blood* 1993; 82:3327–3332.
- Codias EK, Cray C, Baler RD, Levy RB, Malek TR. Expression of Ly-6A/E alloantigens in thymocyte and T-lymphocyte subsets: variability related to the Ly-6a and Ly-6b haplotypes. *Immunogenetics* 1989; 29:98–107.
- Ito CY, Li CY, Bernstein A, Dick JE, Stanford WL. Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. *Blood* 2003; 101:517–523.
- Stanford WL, Haque S, Alexander R, Liu X, Latour AM, Snodgrass HR, Koller BH, Flood PM. Altered proliferative response by T lymphocytes of Ly-6A (Sca-1) null mice. *J Exp Med* 1997; 186:705–717.
- Bonyadi M, Waldman SD, Liu D, Aubin JE, Grynbas MD, Stanford WL. Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. *Proc Natl Acad Sci U S A* 2003; 100:5840–5845.
- Bellows CG, Wang YH, Heersche JN, Aubin JE. 1,25-Dihydroxyvitamin D3 stimulates adipocyte differentiation in cultures of fetal rat calvaria cells: comparison with the effects of dexamethasone. *Endocrinology* 1994; 134:2221–2229.
- Aubin JE. Bone stem cells. *J Cell Biochem Suppl* 1998; 30–31:73–82.
- Kotton DN, Summer RS, Sun X, Ma BY, Fine A. Stem cell antigen-1 expression in the pulmonary vascular endothelium. *Am J Physiol Lung Cell Mol Physiol* 2003; 284:L990–996.
- Heller CG, Lalli MF, Pearson JE, Leach DR. A method for the quantification of Leydig cells in man. *J Reprod Fertil* 1971; 25:177–184.
- Teerds KJ, Closset J, Rommerts FF, de Rooij DG, Stocco DM, Colenbrander B, Wensing CJ, Hennen G. Effects of pure FSH and LH preparations on the number and function of Leydig cells in immature hypophysectomized rats. *J Endocrinol* 1989; 120:97–106.
- Buehr M, Gu S, McLaren A. Mesonephric contribution to testis differentiation in the fetal mouse. *Development* 1993; 117:273–281.
- Martineau J, Nordqvist K, Tilmann C, Lovell-Badge R, Capel B. Male-specific cell migration into the developing gonad. *Curr Biol* 1997; 7:958–968.
- de Bruijn MF, Ma X, Robin C, Ottersbach K, Sanchez MJ, Dzierzak E. Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* 2002; 16:673–683.
- Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* 1996; 86:897–906.
- Sainio K, Raatikainen-Ahokas A. Mesonephric kidney—a stem cell factory? *Int J Dev Biol* 1999; 43:435–439.
- Ginsburg M, Snow MH, McLaren A. Primordial germ cells in the mouse embryo during gastrulation. *Development* 1990; 110:521–528.
- Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA. Myogenic specification of side population cells in skeletal muscle. *J Cell Biol* 2002; 159:123–134.
- Montanaro F, Liadaki K, Schienda J, Flint A, Gussoni E, Kunkel LM. Demystifying SP cell purification: viability, yield, and phenotype are defined by isolation parameters. *Exp Cell Res* 2004; 298:144–154.
- De Rooij DG, Van Bragt MP. Leydig cells: testicular side population harbors transplantable Leydig stem cells. *Endocrinology* 2004; 145:4009–4010.
- Mendis-Handagama SM, Ariyaratne HB. Differentiation of the adult Leydig cell population in the postnatal testis. *Biol Reprod* 2001; 65:660–671.
- Teerds KJ, De Rooij DG, Rommerts FF, van der Tweel I, Wensing CJ. Turnover time of Leydig cells and other interstitial cells in testes of adult rats. *Arch Androl* 1989; 23:105–111.