'Side Population' cells in adult mouse testis express *Bcrp1* gene and are enriched in spermatogonia and germinal stem cells

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Accepted 13 October 2003

Development 131, 479-487
Published by The Company of Biologists 2004
doi:10.1242/dev.00918

Summary

Stem cells in various somatic tissues (bone marrow, skeletal muscle) can be identified by the 'Side Population' marker based on Hoechst 33342 efflux. We show that mouse testicular cells also display a 'Side Population' that express *Bcrp1* mRNA, the ABC transporter responsible for Hoechst efflux in hematopoietic cells. Inhibition of Hoechst efflux by specific BCRP1 inhibitor Ko143 show that germinal 'Side Population' phenotype is dependent on BCRP1 activity. Analysis of two well-defined models of altered spermatogenesis (*W/W^v* mutants and cryptorchid male mice) and RNA expression studies of differentiation markers demonstrate that germinal 'Side Population' contains spermatogonial cells. In addition, α_6-integrin and Stra8 germinal stem cell markers, are expressed in the 'Side Population'. In vivo repopulation assay clearly establishes that testis 'Side Population' in adult mice is highly enriched in male germ stem cells.

Key words: Spermatogenesis, Stem cells, BCRP1/ABCG2, Transplantation

Introduction

Spermatogenesis is a step-wise process that requires coordination of mitosis, meiosis and differentiation. Numerous knockout and spontaneously mutated murine models exhibit altered spermatogenesis, underscoring the complexity of homeostatic control in this lineage (reviewed by Escalier, 2001). Inactivating genes involved in DNA repair, proliferation, apoptosis or cell-cell interactions generally results in a block of germ cell differentiation. In addition, functional spermatogenesis requires the support of somatic cells, especially Leydig cells (located between seminiferous tubules) and Sertoli cells [3% of the cells inside tubules (Bellve, 1993)]. Although in adult mice the main subpopulation is set up by haploid cells in terminal maturation (spermatids), the events necessary to spermatogenesis completion (i.e. germ cell expansion, meiosis entry) involve fewer than 40% of germ cells. This population is highly heterogeneous and includes both pre-meiotic and meiotic cells. Spermatogenesis initiates with the mitotic expansion of spermatogonia, the various differentiation stages (at least 7) of which can be distinguished by morphological criteria (Chiarini-Garcia and Russell, 2001). The transition from diploid B spermatogonia to tetraploid primary spermatocytes characterizes meiosis entry. Following the pachytene step, spermatocytes I undergo a reduction to diploid spermatocytes II, which are themselves reduced to spermatids. In adult rodents, germ cell differentiation is continuous and each step is concomitantly present inside the seminiferous tubules (Russell et al., 1990).

The presence of stem cells – as for all self-renewing tissues – among spermatogonia is already clearly established and several markers have been recently identified. The location of undifferentiated spermatogonia close to the basement membrane of seminiferous tubules suggests that extracellular matrix receptors may be expressed on stem cells. Using functional assays, the ability of the candidate stem cells – positive for receptor expression – to recolonize in vivo sterile testis has been established. Screening for the integrin receptors of laminin has demonstrated that the cell populations expressing β_1 or α_6 chains include stem cells and that the α_6-integrin positive cell population is more highly enriched in stem cells (Shinohara et al., 1999; Shinohara et al., 2000b). These subunits are also stem cell markers in some other epithelia [hepatocytes (Suzuki et al., 2000), keratinocytes (reviewed by Watt, 2002)]. Based on α_v expression, the $\alpha_6^{hi}SSC^{lo}\alpha_v^-$ fraction of cryptorchid mouse testicular cells is greatly enriched in germ stem cells (Shinohara et al., 2000b). Among the genes expressed early during spermatogenesis, another stem cell marker has been characterized: the *Stra8* gene is expressed in spermatogonia (Oulad-Abdelghani et al., 1996) and the activity of its regulatory sequences enables the purification of germinal stem cells in transgenic mice (Giuili et al., 2002).

In different lineages [hematopoietic (Goodell et al., 1996), skeletal muscle (Jackson et al., 1999; Gussoni et al., 1999), neural system (Hulspas and Quesenberry, 2000)], stem cells share a phenotype based on the efflux of vital fluorescent dyes. The use of DNA-dye staining [i.e. *bis*-benzimide Hoechst

33342 (Ho)] coupled to flow cytometry analysis on these lineages revealed a population exhibiting a low Ho fluorescence and highly enriched in stem cells: the 'Side Population' (SP). Some members (MDR1, BCRP1/ABCG2) of the ATP-binding cassette (ABC) transporter family contribute to the dye efflux component of the SP phenotype (reviewed by Gottesman et al., 2002; Bunting, 2002). BCRP1 (Abcg2 Unigene cluster Mm. 196728) was recently reported to be responsible for Ho efflux, at least in hematopoietic stem cells, as described in *Bcrp1*$^{-/-}$ mice (Zhou et al., 2002).

Evidence that stem cells of different somatic lineages share similar molecular properties (Lowell, 2000) led us to address the question concerning the occurrence of SP in the male germinal lineage of adult mice. We have demonstrated that testicular cells also display an SP population and that the expression of markers of germinal stem cells is, partly (α_6-integrin) or even totally (Stra8), restricted to SP cells. In addition, transplantation of testicular SP cells demonstrated that spermatogonial stem cells are present in testicular SP of adult mice.

Materials and methods

Mice

Adult C57BL/6 males (Iffa Credo) and *W/W*^{v} males (Brinster and Zimmermann, 1994) – all parent strains issued from The Jackson Laboratory – were raised in our animal facilities. Experimental cryptorchid males were produced by suturing the testis fat pad to the abdominal wall at 5 weeks of age and analyzed 2-3 months after surgery (Nishimune et al., 1978; Shinohara et al., 2000a). EGFP transgenic mice (C57BL/6-TgN(beta-act-EGFP)01Osb) were used to detect donor-derived spermatogenesis after germ cell transplantation (Okabe et al., 1997). In the testis of EGFP transgenic mice, germ cells are labeled with EGFP except elongated spermatids and spermatozoa. All animal procedures reported in this paper were carried out in accordance with French Government regulations (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture).

Testicular single-cell suspensions

Cells were isolated from 3-month-old male mice according to a modification of the protocol of Vincent et al. (Vincent et al., 1998). The albuginea was removed and the seminiferous tubules were dissociated using enzymatic digestion by collagenase type I at 100 U/ml for 25 minutes at 32°C in Hanks' balanced salt solution (HBSS) supplemented with 20 mM HEPES pH 7.2, 1.2 mM $MgSO_47H_2O$, 1.3 mM $CaCl_22H_2O$, 6.6 mM sodium pyruvate, 0.05% lactate. After an HBSS wash and centrifugation, the pelleted tubules were further incubated in Cell Dissociation Buffer (In Vitrogen) for 25 minutes at 32°C. The resulting whole cell suspension was successively filtered through a 40 μm nylon mesh and through a 20 μm nylon mesh to remove cell clumps. After an HBSS wash, the cell pellet was resuspended in incubation buffer (HBSS supplemented with 20 mM HEPES pH 7.2, 1.2 mM $MgSO_47H_2O$, 1.3 mM $CaCl_22H_2O$, 6.6 mM sodium pyruvate, 0.05% lactate, glutamine and 1% fetal calf serum) and further incubated at 32°C in a water bath. Cell concentrations were estimated using Trypan Blue staining (>95% viable cells).

To discard the interstitial cells, a filtration step with a 40 μm nylon mesh was added after the collagenase digest, and the seminiferous tubules were subsequently treated as previously described.

Flow cytometry analysis and cell sorting

Two million cells were diluted in 2 ml incubation buffer and stained with Hoechst 33342 (5 μg/ml) for 1 hour at 32°C. Before analysis, propidium iodide (PI at 2 μg/ml) was added to exclude dead cells. For analysis of CD45 expression, cells were incubated 20 minutes at 4°C with 1 μg of Cychrome anti-mouse CD45 clone 30-F11 (Pharmingen) in incubation buffer. Cells were then washed, resuspended in incubation buffer with PI and maintained at 4°C before analysis. Cychrome rat IgG2b (Pharmingen) isotype was used as control.

Analysis and cell sorting were performed on a dual-laser FACStar Plus flow cytometer (Becton Dickinson) equipped with a 360 nm UV argon laser and a 488 nm argon laser. Hoechst blue and red fluorescence emissions were collected using a combination of 400 nm long pass and 505 nm short pass filters, and a 630/30 band pass filter. Cychrome fluorescence was collected with 695/35 band pass filter. EGFP and TruCount microbeads fluorescences were collected using a short pass 560 nm dichroic mirror, and 530/30 and 695/35 band pass filters.

α_6-integrin magnetic cell sorting (MACS)

This procedure was modified from a previously published method (Shinohara et al., 2000b). To purify α_6-integrin positive cells, 40×10^6 testicular cells were suspended in 60 μl of incubation buffer and labeled with 40 μl of PE conjugated anti-α_6-integrin antibodies (GoH3, BD Biosciences) for 15 minutes at 4°C. Cells were washed and resuspended in HBSS supplemented with 1 mM HEPES pH 7.2 and 0.5% bovine serum albumin (HBSS/HEPES/BSA). Labeled cells were then sorted with anti-PE antibody microbeads (Miltenyi Biotech) according to the manufacturer's procedure. The α_6-integrin-positive and negative cellular fractions were collected and resuspended at 10^6 cells/ml in incubation medium. For transplantation, SP cells were sorted in tubes coated with 3% BSA containing DMEM supplemented with 10% FBS.

Hoechst efflux inhibition

Hoechst efflux inhibition was performed by preincubating α_6-integrin-positive cells (10^6 cells/ml) for 30 minutes at 32°C in incubation medium supplemented either with 2-deoxyglucose and sodium azide (Sigma) at 50 mM and 15 mM final concentrations respectively, or verapamil (Sigma) at 25 and 75 μg/ml (Scharenberg et al., 2002), or specific BCRP1 inhibitor Ko143 at 200 nM (van der Pol et al., 2003). Cells were further stained with Ho in the presence of the inhibitor as previously described. They were then washed in ice-cold incubation buffer and resuspended in cold incubation medium containing 2 μg/ml PI before flow cytometry analysis.

RNA extraction and RT-PCR

For RNA purification, from 50,000 to 100,000 cells were sorted in silicone treated tubes containing PBS and 20 U recombinant RNasin® ribonuclease inhibitor (Promega). After centrifugation of the cells, total RNA was purified using the RNeasy® Mini Kit according to the manufacturer's instructions (Qiagen). RNA concentrations were quantified using the RiboGreen® RNA quantification kit (Molecular Probes). The first strand of cDNA was synthesized from 100 ng of total RNA, in 6.7 mM $MgCl_2$, 67 mM Tris-HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$ with 5 μM pdN_6 and 1.25 mM each dNTP. After 5 minutes denaturation at 70°C, 200 U of M-MLV RT were added and the reaction mix (20 μl final) was further incubated for 45 minutes at 42°C (Ory et al., 2001). The cDNA was then diluted to 150 μl and 10 μl were used in PCR (5 μl for β-actin). All PCR reactions were classically performed using the primers shown in Table 1.

RT-PCR assays were performed twice on each subpopulation and in addition on populations purified from two independent sorting experiments. Equal amounts of RNA were tested in parallel for the absence of genomic DNA.

Testis cell transplantation and analysis of recipient mice

Total unstained testicular single-cell suspensions, used as control, and sorted testis SP cells both obtained from EGFP transgenic males were resuspended in Dulbecco's modified Eagle's medium containing 10%

Table 1. Primers used

Target genes	Upper primers	Lower primers
Dazla (Wang et al., 2001)	TTCAGGCATATCCTCCTTATC	ATGCTTCGGTCCACAGACTTC
Kit	AATGGAAGGTTGTCGAGGA	CAAATCATCCAGGTCCAGAG
Stra8 (Oulad-Abdelghani et al., 1996)	GCCAGAATGTATTCCGAGAA	CTCACTCTTGTCCAGGAAAC
β actin	TCGTGCGTGACATCAAAGAGA	GAACCGCTCGTTGCCAATAGT
Crem τ	TGCCTGGTATTCCCAAGATTG	CGGCTGATTGTGCTGCATA
Hsp 70-2	CGCCTCACCCAACTAGATATCA	GCTTCATATCGGACTGCACTGT
H1t	CAGTTTCCAAGCTGATTCCTG	AAACTCACTTCCTCCCTGCTG
Bcrp1 (Zhou et al., 2001)	CCATAGCCACAGGCCAAAGT	GGGCCACATGATTCTTCCAC

fetal bovine serum, and 100 μg/ml DNAse Type I (Sigma) and 0.5% Trypan Blue, and maintained at 4°C throughout the procedure. Cell concentrations were adjusted ranging from 10 to 50×10^6 cells/ml and 1.6 to 2.5×10^6 cells/ml for total and SP sorted cell populations, respectively. Donor testis cell populations were transplanted into immunologically compatible C57BL6 recipient mice that were previously treated with busulfan (40 mg/kg, Sigma) to destroy endogenous spermatogenesis (Brinster and Zimmermann, 1994; Ohta et al., 2000). Busulfan was intraperitoneally injected at 4-6 weeks of age and the mice were used as recipients 4 weeks later. In addition recipient mice received a single 7.6 mg/kg i.p. injection of leuprolide (gonadotropin-releasing hormone agonist, Sigma) prior transplantation; treatment is known to enhance colonization after spermatogonial transplantation (Ogawa et al., 1998). Recipient mice were anesthetized by Avertin injection (640 mg/kg, i.p.). A volume of 10 μl of donor cell suspension was introduced in each recipient testis via the efferent ductules according to the method of Ogawa et al. (Ogawa et al., 1997). Approximately 70-90% of seminiferous tubules were filled with the donor cell suspension as monitored by Trypan Blue staining.

Analysis of transplanted testis was performed 10 weeks after donor cell transplantation. Recipient mice were sacrificed and the removed testes were observed under an Olympus epifluorescent microscope to detect the presence of EGFP fluorescent seminiferous tubules. Single cell suspension from recipient testis were prepared and stained with Ho. To determine the repopulation efficiency, the EGFP-positive cell number per recipient testis was measured by flow cytometry using the TruCount™ (BD Biosciences) methodology on testicular cell suspension according to manufacturer's instructions. Briefly, recipient testis cellular suspension (0.5 ml) with 2 μg/ml PI was added into BD TruCount tube. Microbeads lyophilized pellet dissolved, releasing a known number of fluorescent beads. After flow analysis, EGFP-positive cell number per testis could be calculated by dividing the number of EGFP cells by the number of fluorescent beads, then multiplying by the bead concentration, dilution factor and cellular suspension volume. To compare repopulation efficiency of SP and total cells, EGFP-positive cell number per testis was normalized to 10^5 cell injected. Statistical analysis was performed by Student's *t*-test.

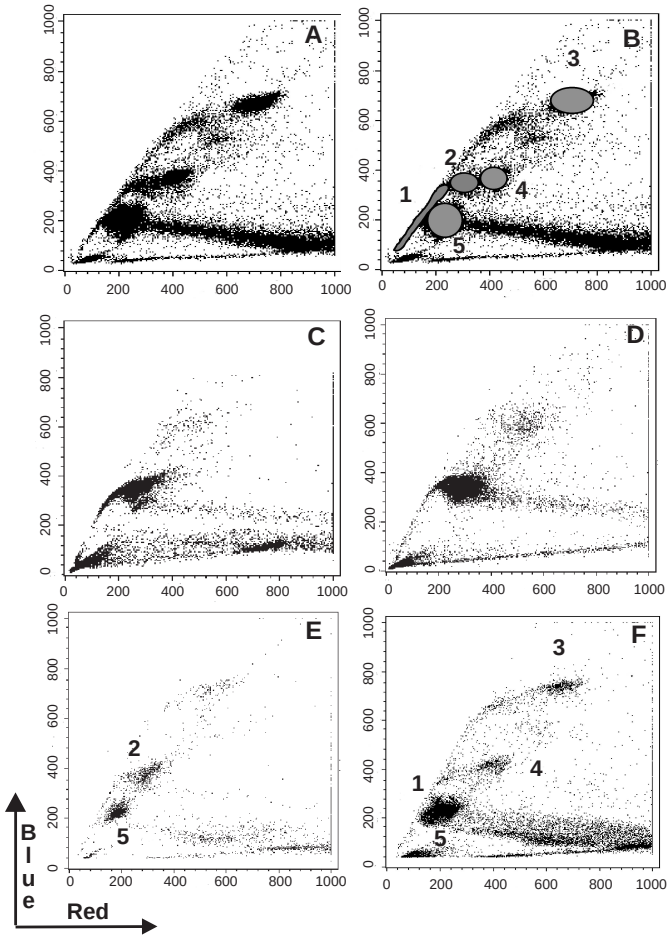

Fig. 1. Flow cytometric analysis of Hoechst 33342 fluorescence. (A) C57BL/6 adult murine testicular cells. (B) Definition of the five main subpopulations and respective percentages of total testicular viable cells: 1 (1%), 2 (2.4%), 3 (8%), 4 (7%), 5 (70%). (C,D) Comparison with two infertile murine models: *W/W^v* mutant (C) and cryptorchid C57BL/6 male (D). (E,F) Identification of somatic interstitial cells: interstitial cell-enriched fraction (E) compared with the fraction enriched in seminiferous tubules (F). Vertical axis shows blue (Ho) fluorescence, horizontal axis red (Ho/PI) fluorescence.

Results

An SP population is detected in testicular cells stained with Hoechst 33342

In order to determine whether the SP phenotype was also conserved in the spermatogenic lineage, single cell suspensions of adult mice testis were analyzed using flow cytometry after Ho staining (Goodell et al., 1996). Populations were separated using a two-window discrimination of red and blue fluorescence emissions along the ordinate and abscissa axis respectively (Fig. 1). The PI uptake allowed dead cells to be grouped along the ordinate axis; cellular debris were localized near the origin.

On the complex fluorescence profile (Fig. 1A), whole testicular cells could be resolved into four distinct major populations (annotated in Fig. 1B): haploid (number 5), diploid (numbers 2 and 4) and tetraploid (number 3). An additional subpopulation (number 1) exhibited low Ho staining correlated with the fluorescence feature of SP cells, therefore suggesting an active Hoechst efflux.

The SP population includes spermatogonial cells

To identify further the different subpopulations, the Ho staining pattern of normal cells was compared to those of two well-defined models of altered spermatogenesis: the *W/W*^{v} mutant and the cryptorchid male. Infertile *W/W*^{v} adult males display an early block in spermatogenesis, owing to mutations in the *Kit* gene resulting in a defective Kit/Kit ligand pathway. The cryptorchidism induces by heat shock a depletion in differentiating germ cells. In addition to somatic cells, the seminiferous tubules of *W/W*^{v} mutants therefore contain rare spermatogonia (Brinster and Zimmermann, 1994), and proliferating early spermatogonia are present in the cryptorchid testis (de Rooij et al., 1999).

Analysis of single cell suspensions purified from *W/W*^{v} and cryptorchid whole testis gave similar fluorescence profiles (Fig. 1C,D, respectively). Compared with the analysis of normal cells (Fig. 1A), subpopulations 3, 4 and 5 were totally absent while subpopulations 1 and 2 were conserved in the two models. This suggests that on the normal pattern the subpopulations 3 (DNA content 4n) and 4 (2n) were meiotic, and population 5 (n) was postmeiotic corresponding to spermatids. The SP fraction (1) and the diploid population (2), common to the three profiles, might include premeiotic cells and somatic cells.

To assign somatic interstitial cells to subpopulations 1 and/or 2, a filtration step was added (see Materials and methods) to collect on the one hand a fraction enriched in interstitial cells and on the other hand a tubular and germinal cell suspension. The comparison of the two Ho profiles (Fig. 1E,F) strongly suggested that the diploid subpopulation 2 was mainly composed of interstitial cells. Some SP cells were detected in the interstitial cell-enriched fraction (Fig. 1E). This fraction contained 76% cells positive for CD45 marker (data not shown), demonstrating that they were hematopoietic cells. A contamination by haploid cells was present in the interstitial cell-enriched fraction. In the seminiferous tubules fraction (Fig. 1F), the SP population was present as well as subpopulations 3, 4 and 5. In this fraction, 93% of SP cells were negative for the CD45 marker and confirmed that this population contained premeiotic spermatogonial cells.

In conclusion, in the Ho profile of whole testicular cells (Fig. 1B) were clearly defined: the diploid interstitial population (2), the SP fraction including spermatogonial cells (1), the meiotic populations – tetraploid spermatocytes I (3) and diploid population 4 (probably spermatocytes II) – and the postmeiotic spermatids (5).

RT-PCR analysis of differentiation markers on flow-sorted subpopulations

Further characterization was achieved by analysis of differentiation marker expression on sorted cell populations. A *W/W*^{v} cell suspension was used as a control because few spermatogonia are present and it is also enriched in SP cells (Fig. 1C).

Each subpopulation was tested for the expression of genes transcribed before the first meiotic division (Fig. 2): *Dazl* and *Kit* genes (Ruggiu et al., 1997; Vincent et al., 1998). Both genes were expressed only in the SP cells and in the tetraploid cell population (4n), confirming that these subpopulations corresponded to spermatogonia and spermatocytes I, respectively. This result was emphasized by *H1t* RNA analysis, whose level of expression was higher in spermatocytes I (4n) than in premeiotic SP cells (Drabent et al., 1998). The *Kit* gene mutations have little effect on the levels of *Kit* RNA in *W/W*^{v} cells (Nocka et al., 1990).

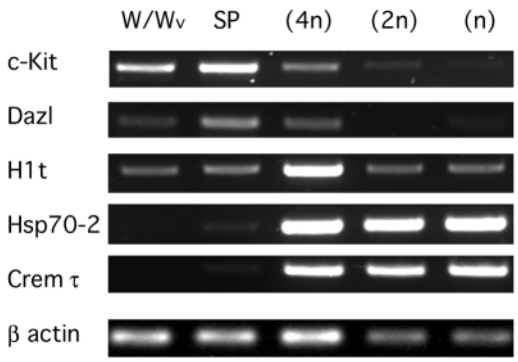

Fig. 2. Analysis of differentiation marker expression on the sorted cell subpopulations. RNA from whole testis cell suspension of mutant *W/W*^{v} males was analyzed in parallel with a sorted cell population (according to Fig. 1B) from normal adult males: population 1 (SP), population 3 (4n), upper diploid population 4 (2n) and population 5 (n). RT-PCR was performed for *Kit* (35 cycles), *Dazl* (30 cycles), *H1t* (30 cycles), *Hsp70-2* (30 cycles) and *Crem* τ (30 cycles) genes. β-actin (30 cycles) served as a normalization control.

The *Hsp70-2* and *Crem* τ genes are transcribed from spermatocytes I to spermatids (Zakeri et al., 1988; Foulkes et al., 1993; Dix et al., 1996). In the same way (Fig. 2), their expression was detected in pure subpopulations 4n, 2n and n, confirming in particular that the upper diploid population (4) was meiotic.

Spermatogonial stem cell markers are expressed in SP cells

As the SP fraction was a phenotype common to normal, *W/W*^{v} and cryptorchid testicular cells (Fig. 1), it might include undifferentiated spermatogonia. The expression of α_6-integrin and *Stra8* markers of germinal stem cells was analyzed (Shinohara et al., 1999; Giuili et al., 2002).

First, as previously mentioned, the α_6-integrin-positive population is highly enriched in stem cells. We took advantage of this property to enrich for α_6-integrin-positive cells by immunomagnetic purification prior to DNA staining and FACS analysis. The selected α_6-integrin-positive cells (Fig. 3A), representing 2-4% of the total population, was principally composed of SP cells and spermatids compared with the α_6-integrin negative cell fraction (Fig. 3B). Nine percent of α_6-integrin-positive cells displayed an SP phenotype. Excluding the contaminating haploid spermatids (subpopulation 5) from analysis, the SP population represented 60% of α_6-integrin-positive cells, showing that the stem cell-enriched α_6-integrin-positive fraction was highly enriched in SP cells.

Second, the expression of the *Stra8* gene was tested in the pure sorted subpopulations (Fig. 3C). *Stra8* gene expression was restricted to the SP population of normal mice and was also detected in the *W/W*^{v} mutant cell suspension which is consistent with the presence of spermatogonial stem cells in the SP fraction.

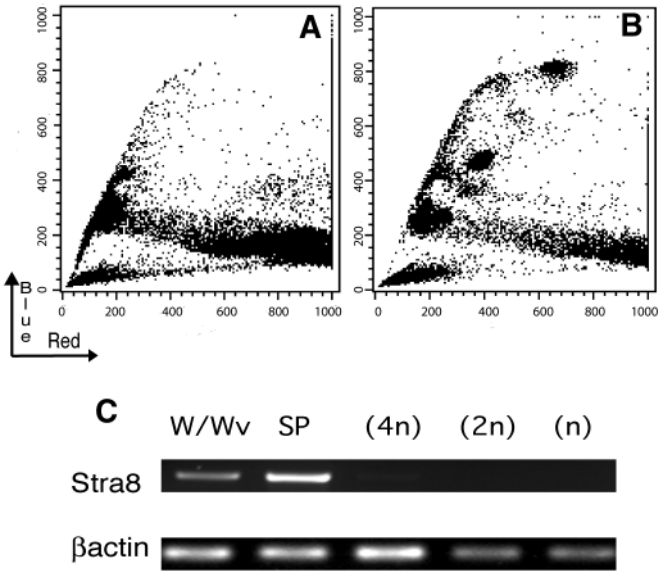

Fig. 3. Analysis of the SP population for the expression of α_6-integrin and *Stra8* germinal stem cell markers. Ho fluorescence flow cytometric analyses of α_6-integrin-positive cell fraction (A) and α_6-integrin-negative cell fraction (B) after immunomagnetic sorting. (C) RT-PCR analysis of *Stra8* gene expression (35 cycles) on *W/W*^{v}, and sorted normal populations: SP cells (SP), tetraploid population (4n), upper diploid population (2n) and haploid population (n).

The germinal SP phenotype depends on ABC transporter activity and SP cells express *Bcrp1/Abcg2* gene

The SP phenotype is caused by an active Ho efflux via members of the ABC transporter superfamily. This mechanism can be inhibited either by ATP depletion or specifically using ABC transporter inhibitors (Zhou et al., 2001; Scharenberg et al., 2002). To determine whether ABC transporter activity was involved in the SP phenotype of spermatogonial cells, the effects of sodium azide and deoxyglucose, and verapamil, were assessed in the α_6-integrin-positive fraction. The SP population was markedly reduced in the presence of sodium azide and deoxyglucose (Fig. 4B, 3.4%) compared with controls (Fig. 4A, 11.5%) showing that this phenotype in germinal cells was energy-dependent. Verapamil – an inhibitor of PgP – at 25 μg/ml moderately inhibited the Ho efflux (Fig. 4C, 8.8%) suggesting that PgP was not the major ABC transporter involved in this process. However, an increase in the efflux inhibition (Fig. 4D, 3.6%) was observed at higher verapamil concentration (75 μg/ml), as already described for the SP population in bone marrow or carcinoma cell line (Goodell et al., 1996; Scharenberg et al., 2002).

The effect of the MDR1 inhibitor verapamil was not complete on the germinal SP fraction and the study of *Bcrp1*$^{-/-}$ mice demonstrates a major role for BCRP1/ABCG2 protein – rather than P-gp/MDR1- in the SP phenotype (Zhou et al., 2002). *Bcrp1* gene expression was therefore tested in each purified subpopulation (Fig. 4E). *Bcrp1* RNA was detected in the SP cells of normal mice and also in *W/W*^{v} mutants. Although RNA was not detected in tetraploid meiotic cells, it appears that *Bcrp1* gene transcription was resumed at the end of meiosis because RNA levels were markedly increased in spermatids. Using the specific BCRP1 inhibitor Ko143 (Allen et al., 2002), SP phenotype was markedly reduced (Fig. 4F,G). Hoechst efflux inhibition by Ko143 shows that the BCRP1 transporter activity is involved in the germinal SP phenotype.

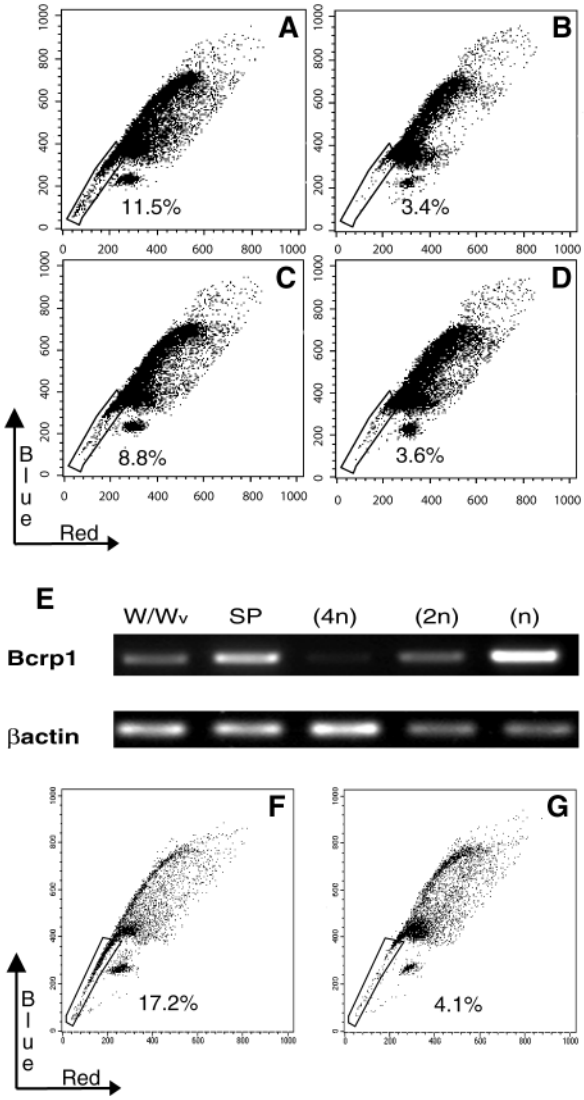

Fig. 4. The germinal SP phenotype is related to ABC transporter activity. α_6-integrin-positive cell fraction stained with Ho without inhibitor (A), with deoxyglucose plus sodium azide (B), 25 μg/ml (C) or 75 μg/ml of verapamil (D). (E) *Bcrp1* expression analysis (35 cycles) in testicular subpopulations: SP cells (SP), tetraploid population (4n), upper diploid population (2n) and haploid population (n). α_6-integrin-positive cell fraction stained with Ho without inhibitor (F) and with specific BCRP1 inhibitor Ko143 (G).

In vivo repopulation assay in busulfan-treated recipient mice show that the testis SP is enriched for stem cell activity

To determine stem cell activity, donor EGFP germ cells were used for transplantation into seminiferous tubules of busulfan-treated recipient normal mice. EGFP germ cells were obtained from EGFP transgenic mice whose spermatogenesis is normal

(Okabe et al., 1997). As we have shown that α_6-integrin-positive fraction is highly enriched in SP cells, testis cells were firstly enriched for α_6-integrin-positive cells by immunomagnetic purification. The testis EGFP-positive SP was then purified by flow sorting (Fig. 5A) and transplanted into recipient testis.

Ten weeks later, the SP EGFP-positive cells have colonized the seminiferous tubules of recipient testis (Fig. 5B). Testicular single cell suspensions from recipient testis were prepared and analyzed by flow cytometry according to EGFP and Ho fluorescences. The EGFP-positive cells were detected among the negative recipient cells (Fig. 5C). Flow analysis showed that 49±4% (mean±s.e.m.) of EGFP cells were spermatids (haploid DNA content) and that EGFP cells displayed the Ho profile typical of normal spermatogenesis (Fig. 5D), demonstrating that transplanted testis SP cells were able to proliferate and differentiate. To analyze the efficiency of repopulation, the number of EGFP-positive cells per recipient testis was determined by flow cytometry using TruCount^{TM} methodology (Fig. 5E). As shown by repopulation assay, the testis SP was 15-fold enriched in stem cell activity compared with total cells (Fig. 5F). Those data confirm that the testis SP cells in adult mice contain male germinal stem cells.

Discussion

Like other self-renewing processes in adult tissues, spermatogenesis originates from a small pool of stem cells. The molecular mechanisms governing germinal stem cell renewal and initiation of differentiation remain barely understood. Their understanding would be facilitated by purification of the stem cell population. It is already well established that complex self-renewing cell lineages can be analyzed and cells of interest purified using vital DNA dye as Ho, combined with flow cytometry analysis. Red and blue fluorescence emissions of Ho result from three cellular properties: (1) ploidy, (2) structure and accessibility of chromatin, and (3) Ho efflux caused by ABC transporter activity. In agreement with these criteria, our results show that whole testicular cells are distributed in five main subpopulations in adult mice. Four of them are defined by their DNA content: tetraploid (population 3), diploid (populations 2 and 4) and haploid (population 5). The fifth population (1) exhibits an SP phenotype because of Ho efflux.

The nature of each subpopulation has been identified. The lower diploid one (2) is present only in whole cell suspensions and is composed of somatic interstitial cells. This population may include Leydig cells, endothelial and blood cells which are released during collagenase treatment (Bellve, 1993). The meiotic origin of the tetraploid (3) and upper diploid (4) populations was assigned by comparison with two models of early blocked spermatogenesis –*W/W^v* mutants and cryptorchid males – in which these cells are completely absent. This is confirmed by RT-PCR studies: these two cell populations express meiotic differentiation markers (*Crem* τ and *Hsp70-2*) highly but express spermatogonial markers weakly (*Kit* and *Dazl*) or not at all (*Stra8*). Consequently, these populations can be identified as spermatocytes I (tetraploid cells) and spermatocytes II (diploid cells). The red Hoechst fluorescence shift of the spermatocytes II (upper diploid) versus interstitial cells (lower diploid) might result from differences in their chromatin structure. Finally, spermatids are identified, as population 5, by their haploid DNA content and their expression of *Crem* τ and *Hsp70-2* genes.

The SP phenotype after Ho staining is one of the molecular

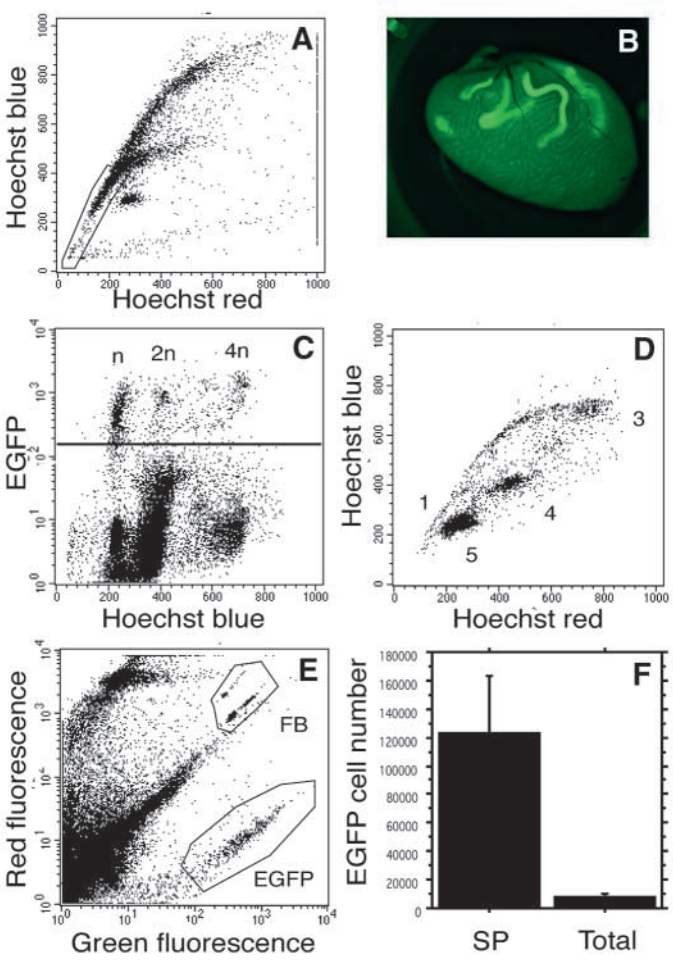

Fig. 5. In vivo repopulation assay of the testis SP EGFP-positive in busulfan-treated recipient analysed 10 weeks after transplantation. (A) Ho fluorescence flow cytometric analyses of α_6-integrin-positive cell fraction from EGFP donor cells. Sorting gate of the testis SP is enclosed in box. (B) Detection of fluorescent seminiferous tubules in a recipient testis transplanted with the EGFP testis SP as determined by fluorescence microscopic analysis of whole testis. (C) Flow cytometric analysis of EGFP and Ho blue fluorescences of testicular single cell suspensions prepared from recipient testis transplanted with the EGFP testis SP and (D) Ho fluorescence flow cytometric (red/blue Ho fluorescence) analysis of EGFP-positive cells in recipient testis gated from Fig. 5C. (E) Green and red fluorescence analysis of testicular cell suspension from recipient testis in order to determine EGFP-positive cell number per recipient testis by flow cytometry using TruCount^{TM} (BD Biosciences) methodology (see Materials and methods). EGFP-positive cells (EGFP) and TruCount fluorescent beads (FB) are boxed. (F) Enhanced colonization of recipient testis by the transplanted testis SP (n=8 testis) compared with total population (n=8 testis). EGFP-positive cell number per recipient testis was normalized to 10^5 cells injected. The values are mean±s.e.m. Difference between SP and total cells is significant (P<0.03). Results were obtained from three independent experiments.

properties shared by a number of somatic cells [hematopoietic, muscle and neuronal cells, as well as ES cell lines (for a review, see Bunting, 2002)] in various species (mouse, pig, monkey and human) (Goodell et al., 1997). Our results clearly show the presence of an SP fraction among adult male germ cells. This SP fraction represents 1% of the total viable cells (or 0.6% of total testicular cells), which is on the order of magnitude of somatic SP population sizes (Goodell et al., 1996; Zhou et al., 2002). In addition, the germinal SP fraction described here exhibits properties of somatic SP populations (Zhou et al., 2001; Scharenberg et al., 2002): (1) the SP phenotype corresponds to an active Ho exclusion that can be inhibited by ATP depletion; and (2) the SP phenotype is specifically sensitive to inhibitors of ABC pumps. The SP population includes spermatogonial cells as confirmed by the RNA expression of differentiation markers, in particular *Kit* and *Dazl*. Their expression is known to initiate in the A spermatogonia the *Kit* gene (Manova et al., 1990) and in the more differentiated B spermatogonia the *Dazl* gene (Niederberger et al., 1997), which is consistent with our RT-PCR data.

The screening of two markers of germinal stem cells strongly suggests that the SP population also includes the spermatogonial stem cells. The α_6-integrin-positive cells are enriched in stem cells and their Ho profile demonstrates that the majority of α_6-integrin-positive cells exhibit a SP phenotype. In addition, *Stra8* gene expression is restricted to the SP population, confirming the presence of spermatogonia and stem cells. We have also observed that the experimental cryptorchid mouse model, highly enriched in spermatogonial stem cells, is also highly enriched in SP population.

To confirm that the stem cell population is present in the testis SP, its functional activity was studied by an in vivo assay, namely the establishment of a spermatogenesis after transplantation in sterile testis (Brinster and Zimmermann, 1994). Donor EGFP germ cells from adult males were used for transplantation into seminiferous tubules of busulfan-treated recipient mice. In order to improve SP cell sorting, testis cells were first enriched for α_6-integrin-positive cells, before flow sorting of the testis SP and transplantation into recipient testis. Results showed that this testis SP fraction colonized seminiferous tubules of recipient testis as observed 10 weeks after transplantation. Testis SP cells can proliferate and differentiate. In addition, the testis SP was 15-fold enriched in stem cell activity compared with total cells, demonstrating that the testis SP in adult mouse contain male germinal stem cells. A recent study reported that, although the SP phenotype was detected in testis, the SP fraction did not display spermatogonial stem cell activity by transplantation assay (Kubota et al., 2003). Those data, which are in contradiction with our results, were obtained using the cryptorchid mouse model as a SP donor – a model in which spermatogenesis is early blocked by heat shock. By contrast, our experiments involve normal transgenic testicular cells as a SP donor. The apparent discrepancy might result from the use of those two different models. Heat shock could modify the regulation of ABC transporter gene expression. Consequently the cellular composition of the testis SP could be modified between both models, although SP is observed. Additional works need to be carried out to investigate this discrepancy.

At least in hematopoietic lineages, Ho exclusion is directly caused by BCRP1/ABCG2 protein activity as demonstrated in *Bcrp1*-null mice (Zhou et al., 2002). We have shown that *Bcrp1* RNA is detected in the germinal SP population cells and that Hoechst efflux can be inhibited by BCRP1 specific inhibitor. Thus, BRCP1 activity is a determinant of the germinal SP phenotype reported here. The *Bcrp1/Abcg2* gene is a member of the superfamily of the ABC transporters that are key elements of the blood-testis and blood-brain barriers. The blood-testis barrier is composed of capillary endothelial cells and Leydig cells, and by the myoid layer of the seminiferous tubules and Sertoli cells. Those somatic cells express specific ABC transporters, such as P-gP/MDR1 (endothelial and myoid cells), and MRP1 (Sertoli and Leydig cells) (Wijnholds et al., 1998; Bart et al., 2002). Therefore, we cannot exclude that somatic cells might be present in the SP fraction, especially for myoid cells expressing P-gP. In the germinal lineage, P-gP/MDR1 expression has been recently detected in postmeiotic cells (late spermatids) (Melaine et al., 2002). As demonstrated here, *Bcrp1* RNA is also present in spermatids, but Hoechst efflux was not observed for spermatids, suggesting an RNA storage process (Kleene, 2001). Concerning spermatogonial and male germinal stem cells, BRCP1 is the first ABC transporter detected in these cells with an active efflux phenotype. Such a pattern of *Bcrp1* transcription regulation has been described in the hematopoietic differentiation process. The *Bcrp1* gene is expressed in hematopoietic stem cells, downregulated during differentiation commitment and finally reinduced in specific lineages (Scharenberg et al., 2002). BCRP1 has been reported to confer on hematopoietic stem cells a resistance to mitoxantrone, a chemotherapeutic drug (Miyake et al., 1999; Allen et al., 1999), suggesting that its physiological role is to protect stem cells from cytotoxic substrates. In this context, it is not surprising that germinal stem cells express such ABC transporters as these cells are involved in the integrity and in the good transmission of genetic material to progeny. This self-protection mechanism may constitute the last barrier against genotoxic stress. If stem cells were hit by mutagenesis, hereditary risk would become continuous during the lifespan of the individual. In this way it is important to note that mitoxantrone – a specific substrate of ABCG2 – is a potentially mutagenic DNA intercalating drug.

Whether Bcrp1 is a common marker of stem cells in various tissues raises the question about the significance of ABC transporters in stem cell biology and their presumed role in the regulation of stem cell self-renewal and differentiation processes. Steady-state hematopoiesis is functionally normal in *Bcpr1*-null mice, despite the absence of SP phenotype, and Bcrp1 does not appear to play a major role in the hematopoietic differentiation process. The same observations were also made in *Mdr1a/Mdr1b*-null mice (Zhou et al., 2002). In the same way, *Bcrp1*-null mice are fertile and spermatogenesis appears normal (Zhou et al., 2002; Jonker et al., 2002). These results suggest that ABCG2 is not essential for spermatogenesis, especially for spermatogonial and stem cell differentiation in testis. Those observations confirm the idea of the protective role against genotoxicity. However, redundancy between different ABC transporter pathways in stem cell biology cannot be ruled out, which could explain the lack of impairment in hematopoiesis and spermatogenesis in *Bcrp1*-null mice.

The testis SP in male adult mice was confirmed to be highly enriched in male germ stem cells by specific germ stem cell

marker analysis and by in vivo repopulation assay after transplantation. Hence, the SP phenotype should be a common marker of germinal and somatic stem cells, although somatic and germinal lineages are separated with distinct fates very early in the development of the mouse embryo (from 6-7 days post-coitum onwards) (Zhao and Garbers, 2002). SP sorting constitutes an alternative strategy to isolate a highly enriched germinal stem cell population. It might be possible to enrich SP cells using a specific antibody-based procedure – Bcrp1 being a cell surface marker. The SP phenotype has been described in hematopoietic stem cells of various species (mouse, monkey, human) (Goodell et al., 1997), suggesting that this phenotypic marker is highly conserved between species in stem cell from somatic lineages. In the same way, we suggest that the SP germinal male stem cell phenotype could be conserved between different species. It would constitute an interesting strategy to isolate highly enriched germinal stem cell populations without having to know their stem cell-specific surface markers.

We thank Dr Okabe for the generous gift of the EGFP transgenic mice. We thank Dr Schinkel for the generous gift of the BCRP1 inhibitor Ko143. We thank S. Chevillard and K. Ory for helpful discussion on RT-PCR analysis. We thank P. Flament and W. Heliere for their technical assistance in animal facilities. This work was supported in part by grant from Electricité De France, and The Nuclear Toxicology program from the Commissariat à l'Energie Atomique.

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