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Identification of side population cells in mouse primordial germ cells and prenatal testis

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ABSTRACT In mammals, the stem cells of spermatogenesis are derived from an embryonic cell population called primordial germ cells (PGCs). Spermatogonial stem cells displaying the "side population" (SP) phenotype have been identified in the immature and adult mouse testis, but noting is known about the expression of the SP phenotype during prenatal development of germ cells. The SP phenotype, defined as the ability of cells to efflux fluorescent dyes such as Hoechst, is common to several stem/progenitor cell types. In the present study, we analyzed and characterized the Hoechst SP via cytofluorimetric analysis of disaggregated gonads at different time points during embryonic development in mice. To directly test the hypothesis that the SP phenotype is a feature of germ cell lineage, experiments were performed on transgenic animals expressing enhanced green fluorescent protein (EGFP) under the control of the *Oct4* promoter, to identify early germ cells up to PGCs. We found that prenatal gonads contain a fraction of SP cells at each stage analyzed, and the percentage of cells in the SP fraction decreases as development proceeds. Surprisingly, more than 50% of the PGCs displayed the SP phenotype at 11.5 dpc (days post coitum). The percentage of germ cells with the SP phenotype decreased steadily with development, to less than 1% at 18.5 dpc. Cytofluorimetric analysis along with immunocytochemistry performed on sorted cells indicated that the SP fraction of prenatal gonads, as in the adult testis, was heterogeneous, being composed of both somatic and germ cells. Both cell types expressed the ABC transporters *Abcg2, Abcb1a, Abcb1b* and *Abcc1*. These findings provide evidence that the SP phenotype is a common feature of PGCs and identifies a subpopulation of fetal testis cells including prospermatogonia whose differentiation fate remains to be investigated.

KEY WORDS: *testis development, spermatogonial stem cell, side population, Oct4-GFP*

In adult mammals, continuous production of spermatozoa is sustained by spermatogonial stem cells (SSCs). SSCs are derived from a population of embryonic precursors called primordial germ cells (PGCs). In the mouse embryo, the progenitors of PGCs appear as Blimp-1 positive cells in the epiblast as early as 6.25 days post coitum (dpc) (Ohinata *et al*., 2005). At about 7.5 dpc, 30-40 PGCs are specified in the extraembryonic mesoderm, in the root of the developing allantois posterior to the primitive streak (Ginsburg *et al*., 1990; Lawson and Hage, 1994). As embryonic development proceeds, PGCs actively migrate to reach and enter the developing gonads. At 11.5 dpc, all PGCs have reached the future gonads and compose a population of around 3000 cells. Around 13.5 dpc, PGCs differentiate according to the genetic sex of the embryo; in females, they differentiate into meiotic oocytes, entering the prophase of meiosis I, whereas in males, they became G0-arrested prospermatogonia (De Felici and McLaren, 1983; Western *et al*., 2008). A few days after birth, germ cells resume proliferation and migrate to the basement membrane of the seminiferous tubules (Hilscher *et al*., 1974; McLaren, 1995). Most of the germ cells will further differentiate during the first wave of spermatogenesis to give rise to mature sperm, while few will become SSCs. A dramatic increase in number of stem cells takes place in the second week of postnatal life, after which time the final size of the stem cell compartment is

Abbreviations used in this paper: GR, gonadal ridge; Ho, Hoechst 33342; PGC, primordial germ cell; SP, side population; SSC spermatogonial stem cell.

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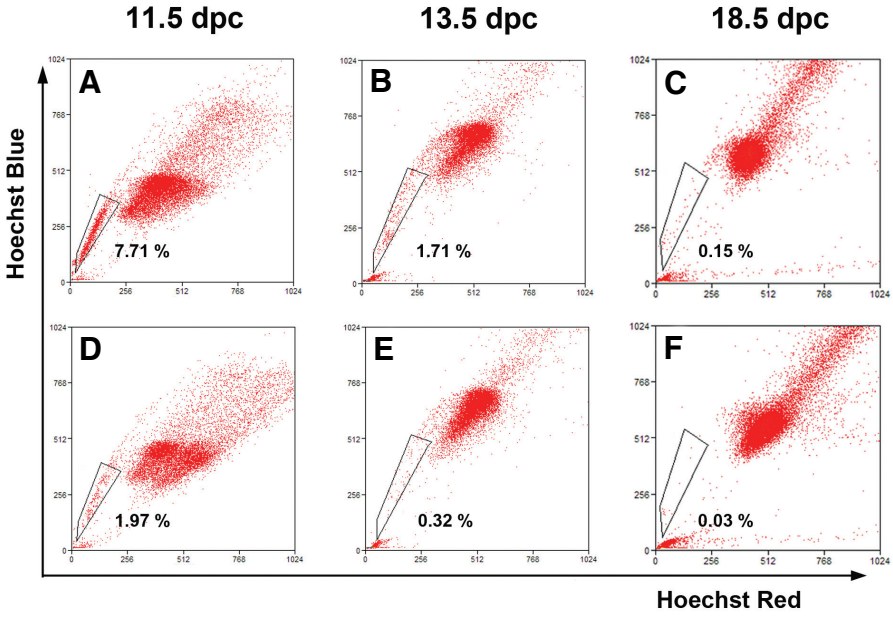
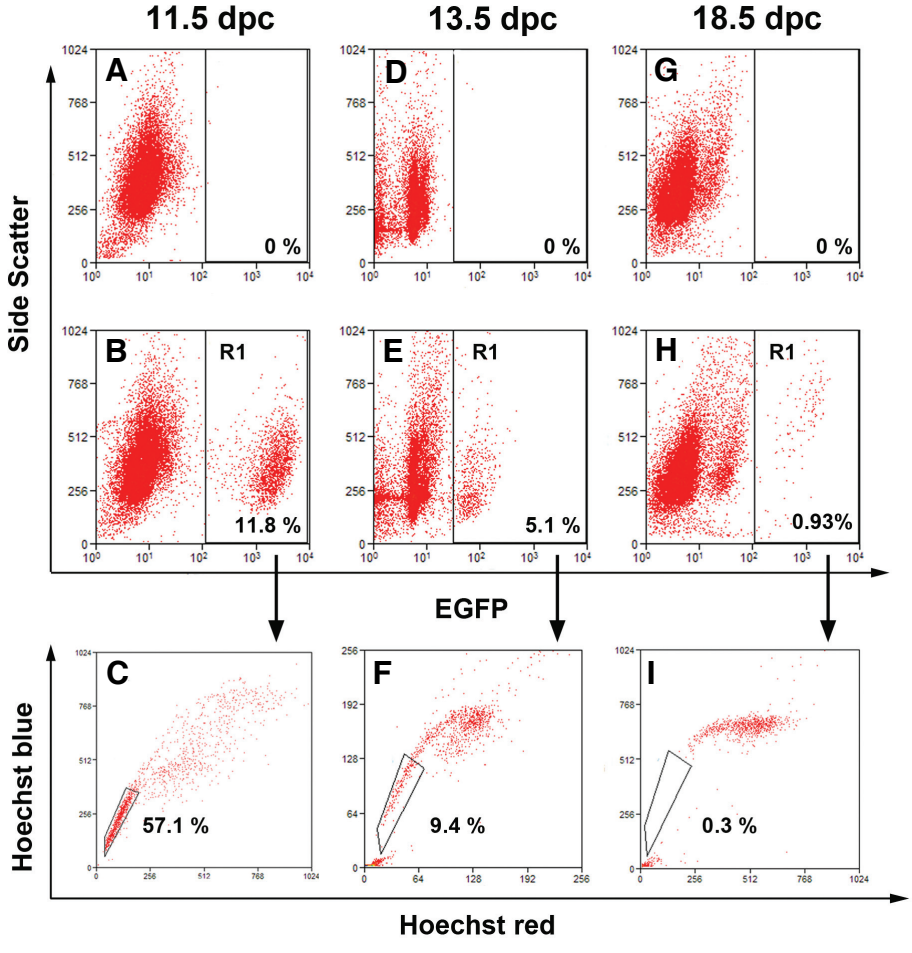


Fig. 1 (Above). In the embryonic mice testis, a subset of cells displays a side population (SP) phenotype. *FACS profiles of wild-type embryonic testis cells stained with Ho* **(A,B,C)** *or with Ho in the presence of verapamil* **(D,E,F)**. *Percentages of cells in the boxed SP regions are indicated. The presence of verapamil during the supravital Ho staining reduced SP fractions. A representative experiment is shown (n = 3).*



set (McLean *et al.*, 2003). It has been recently suggested that adult SSCs are already selected among PGCs before 13.5 dpc (Ueno *et al.*, 2009), but the molecular mechanisms underlying SSC fate commitment remain obscure.

Previous studies in the mouse have shown that SSCs can be identified by their ability to efflux dyes such as Hoechst 33342 (Falciatori *et al.*, 2004; Lassalle *et al.*, 2004). This ability, defined as the "side-population" (SP) phenotype, is common to several stem/progenitor cell types (Challen and Little, 2006) and relies on the activity of ATP-binding cassette (ABC) transporter superfamily, including multidrug resistance 1 (Mdr1a/1b, mouse; MDR1, human) and breast cancer resistance protein 1 (Bcrp1, mouse; ABCG2 human) (Bunting *et al.*, 2000; Zhou *et al.*, 2001). The activity of these transporters can be blocked by several inhibitors (verapamil, reserpin, Ko 123, and others). Inclusion of these inhibitors during dye incubation results in loss of the SP fraction. Although the precise function of the SP phenotype in stem cell physiology remains to be elucidated, it has proven valuable as a marker for identification of multipotent stem cells from a variety of tissues. SSCs displaying the SP phenotype have been identified in the immature and adult mouse testis (Falciatori *et al.*, 2004, Lassalle *et al.*, 2004) but no data have been available on SP expression in the prenatal testis to date. To investigate whether the SP phenotype is expressed earlier during testis development, we analyzed and characterized Hoechst staining via cytofluorimetric analysis of disaggregated gonads at different time points of prenatal development, up to sex-indifferent gonadal ridges. To directly test the hypothesis that SP phenotype is a feature of germ cell lineage, cytofluorimetric analyses were performed on transgenic animals expressing

Fig. 2. A subset of primordial germ cells displays an SP phenotype in the embryonic mice testis. *FACS profile of cells stained with Ho isolated from wild-type embryonic testes* **(A,D,G)** *and age-matched Oct4-GFP embryonic testes* **(B,C,E,F,H,I)**. *Wild-type testes were included in the analysis as negative controls to set gates for EGPF fluorescence. The fraction of EGFP-positive cells (germ cells) is indicated as R1 at each age analyzed* **(B,E,H)**. *Only a subset of germ cells fell in the SP region* **(C,F,I)**. *The percentage of SP cells in the EGFP-expressing population is indicated for each age. A representative experiment is shown (n = 3).*

EGFP under the control of the *Oct4* promoter, in order to identify male germ cells up to PGCs.

Results

To determine whether the SP phenotype is expressed in early testis development, we searched for SP cells in the gonads of the developing mouse embryo. Cells were disaggregated from 11.5 dpc gonadal ridges (GRs) and 13.5 and 18.5 dpc testes and subjected to Hoechst 33342 (Ho) vital staining using a protocol for detection of SP in immature testis (Falciatori *et al*., 2004). A small Ho-effluxing SP fraction was readily identified by cytometric analysis at all stages analyzed (Fig. 1 A,B,C). The percentage of cells in the SP fraction was highest in 11.5 dpc GRs (mean±SEM, 8.15 ± 0.26), when gonads are at sex indifferent stage, and decreased thereafter in 13.5 and 18.5 dpc testes (1.44 ± 0.07 and 0.11 ± 0.02, respectively). To assess whether Ho efflux could be blocked by inhibiting ABC transporter activity, cells were stained with Ho in the presence of 50 μm of verapamil. Cytofluorimetric analysis showed that, under these conditions, the SP fraction was markedly reduced at all stages (Fig. 1 D,E,F).

In several tissues and organs the SP fraction is heterogeneous in terms of cell types. In order to identify germ cells in the SP fraction, we took advantage of a transgenic mice model expressing EGFP under the control of the *Oct4* promoter. Because *Oct4* is expressed exclusively in the germline in the postimplantation mouse embryo (Yeom *et al*., 1996), this animal model has been widely used to identify and analyze germ cells during embryonic development (Anderson *et al*., 1999; Yoshimizu *et al*., 1999). Disaggregated cells from age-matched wild-type and transgenic gonads were vital stained with Ho and analyzed for both Ho and EGFP fluorescence levels (Fig. 2). Cytofluorimetric analyses of wild-type gonads were performed as a control to set gates for EGFP fluorescence (Fig. 2 A, D and G). In the GRs of the transgenic mice, around 10% of total cells were EGFP^{pos}. FACS analysis showed that virtually all EGFP^{pos} cells were positive for TG-1 (SSEA-1) antigen confirming their PGC identity (not shown). The percentage of EGFP^{pos} cells dropped to 5% at 13.5 dpc and to less than 1% in 18.5-dpc testes (Fig. 2 B, E and H). Since during this period nearly all male germ cells express the Oct4-EGFP transgene (Ohbo *et al*., 2003; Yoshimizu *et al*., 1999), we assume that such a decrease was due to the expansion of the testicular somatic cells in comparison to germ cells, which undergo G0 arrest around 13.5 dpc (Western *et al*., 2008). To assess the percentage of germ cells displaying the SP phenotype at each stage, we analyzed the Ho profile of EGFP^{pos} cells. Surprisingly, around 60% of EGFP^{pos} cells displayed the SP phenotype at 11.5 dpc. The percentage of EGFP^{pos} germ cells with the SP phenotype then steadily decreased with age, reaching less than 1% at 18.5 dpc (Fig. 2 C, F and I). Cytofluorimetric analysis indicated that EGFP^{pos} represented more than 90% of total SP cells at 11.5 dpc, less than 15% at 13.5 dpc, and it became undetectable at 18.5 dpc (not shown) suggesting that SP fraction in fetal testes is heterogeneous in terms of cell types. To further characterize the SP fraction, SP and non-SP cells were sorted from 13.5 dpc testis and analyzed for the expression of VASA, a specific marker of germ cells (Tanaka *et al*., 2000) (Fig. 3A). As expected, we found that VASA-positive cells represented a fraction of total SP cells approximately in the range of the EGFP^{pos} cells (see above) (Fig. 3B). These data indicate that, while a large percentage of PGCs express the SP phenotype in sex-indifferent GRs, this marker is restricted to a small prospermatogonia subpopulation and to unidentified gonadal somatic cell type/s by 13,5 dpc.

Finally, we used RT-PCR to identify which ABC transporter/s

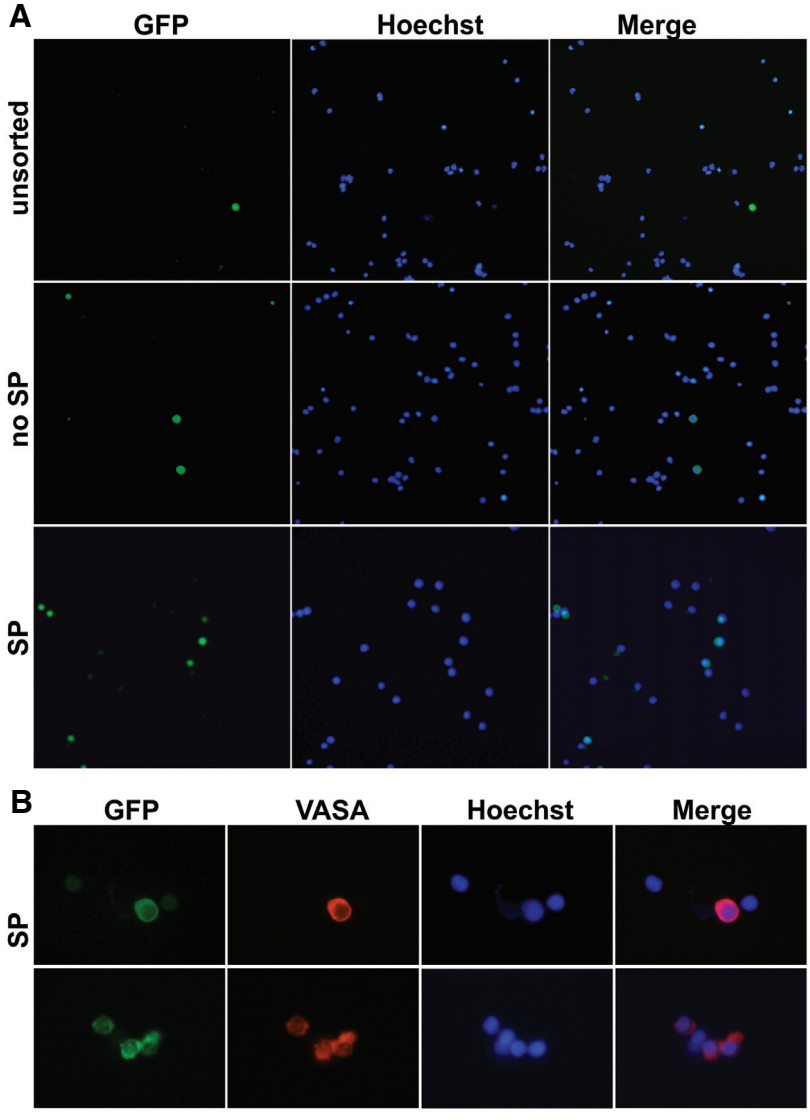


Fig. 3. Immunofluorescence for the germ cell specific VASA protein on Oct4-EGFP cells isolated from 13.5 dpc testes of CD-1 x OG2-F1 mice. (A) *Oct4-EGFP^{pos} cells in unsorted cells, sorted no SP and sorted SP cells.* **(B)** *Immunofluorescence for VASA protein on SP cells. Pictures were acquired with an Zeiss Axyoplan II fluorescence microscope using a 20X (A) or a 60X (B) objectives.*

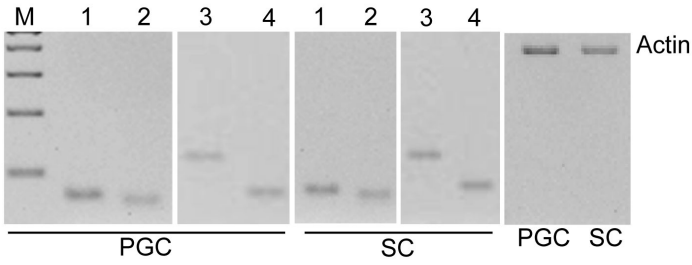


Fig.4. Representative RT-PCR analysis of ABC transporter gene expression in primordial germ cells (PGC) and somatic cells (SC) purified from 11.5 dpc gonadal ridges. *Lanes represent:* **(1)** Abcg2*;* **(2)** Abcb1a*;* **(3)** Abcb1b*;* **(4)** Abcc1*. Actin is shown as loading control.*

involved in Ho efflux are expressed in isolated PGCs and somatic cells. GRs at 11.5 dpc were disaggregated, and PGCs and somatic cells were purified for RNA extraction (Pesce and De Felici, 1995). Semi-quantitative-PCR was then performed to analyze *Abcg2* (encoding for BCRP1), *Abcb1a* (encoding for MDR1a), *Abcb1b* (encoding MDR1b), and *Abcc1* (encoding for MRP1) transcripts (Fig. 4). All transcripts analyzed were expressed in both PGCs and somatic cells.

Discussion

In the present study, we carried out an analysis of SP phenotype expression during prenatal development of the testis, up to the sex indifferent gonadal ridge stage. Using cytofluorimetry of Hoechst-stained gonadal cells, we found that, at 11.5 dpc, the embryonic stage at which gonads are still sex indifferent, the SP fraction represents a larger percentage of total viable cells (around 8%) when compared to the percentage of SP cells in the postnatal testis (range 0.1-1.3 %(Falciatori *et al.*, 2004). The number of cells in the SP fraction strongly decreased with time over the course of development, and by 18.5 dpc, the percentage of SP cells was equal to that of postnatal testes (Falciatori *et al.*, 2004). By using transgenic mice in which the prenatal germ cells express EGFP, we also found that a large proportion of 11.5 dpc PGCs (around 60%) expressed the SP phenotype; at 18.5 dpc, however, less than 1% of $EGFP^{pos}$ prospermatogonia were found within the SP gate. Between 11.5 and 13.5 dpc, the percentage of the EGFP-positive cells in the gonads decreased from 12% to 5% (about a 2-fold reduction) while, in the same time frame, the percentage of EGFP-positive cells in the SP gate decreased from about 60% to 10% (a 6-fold reduction). This indicates that the decrease in the percentage of SP cells in the testis is not only due to the high proliferation rate of non-SP somatic and germ cells (Schmahl *et al.*, 2000), but also to down-regulation of the SP phenotype in the male germ cell population.

Since the percentage of SP cells in a variety of cell populations is very low, usually less than 1% (Rochon *et al.*, 2006; Zhou *et al.*, 2001) the high percentage of PGCs displaying SP phenotype is somewhat surprisingly. Even in ES cells, which have a number of similarities with PGCs, the proportion of SP cells is below 5% (Vieyra *et al.*, 2009). Considering the lack of information about the physiological function of ABC transporters in PGCs, we can only speculate regarding the reason for the high percentage of SP cells within the PGC population. Analogous to their possible functions in the stem/progenitor cells of adult tissues (Zhou *et al.*, 2002), the activity of the ABC transporters in PGCs might provide protection from toxic substrates. Interestingly, ABCG2 activity prevents the accumulation of porphyrin, a constituent of heme, and ROS-induced DNA damage in ES cells. Moreover, inhibition of ABCG2 results in down-regulation of *Nanog* and *Oct4* and therefore can impact the self-renewal of ES cells (Susanto *et al.*, 2008). Although PGCs are germ line-committed cells under certain *in vivo* and *in vitro* conditions, they can actually transform into embryonal carcinoma (EC) and embryonal germ (EG) stem cell lines (De Felici *et al.*, 2009; Donovan and De Miguel, 2003). Therefore, among the stemness characteristics usually associated with possible SP functions, differentiation and pluripotency could also be modulated by ABC transporters in PGCs.

Taking into account the percentage of the SP cells in the whole gonad and the relative contribution of the $EGFP^{pos}$ germ cells to this fraction, it is evident that this subpopulation is composed of both germ and somatic cells. Detection of VASA-positive germ cells and VASA-negative somatic cells in sorted SP cells from 13.5 dpc gonads further confirmed these findings. Consistently, our data indicated that both germ and somatic cells express all the ABC transporters analyzed, particularly ABGC2, a molecular determinant of the Hoechst SP phenotype (Zhou *et al.*, 2001). A previous study indicated that, in the adult testis, the SP fraction also contains stem/progenitors for androgen-producing Leydig cells in addition to SSCs (Lo *et al.*, 2004). It would be interesting to test whether embryonic Leydig progenitor cells are also present in the SP fraction from prenatal testes.

Ohumura and colleagues recently showed that a nucleolar protein called nucleostemin, found at high levels in several types of stem cells, identifies germ cells with undifferentiated status and long-term testis repopulating capability in prenatal gonads (Ohmura *et al.*, 2008). Interestingly, the expression pattern of this protein closely resembles that of the SP phenotype described here. Since SP phenotype is expressed by immature (Falciatori *et al.*, 2004) and adult SSCs (Lassalle *et al.*, 2004) it would be interesting to further investigate the cell lineage that links embryonic to adult SP germ cells.

In conclusion, we found that while SP phenotype is a common feature of PGCs in undifferentiated gonads and it becomes restricted in fetal testis to a cell population containing a small subset of prospermatogonia whose fate remains to be established.

Materials and Methods

Animals

CD-1 mice were obtained from our colony at the Department of Public Health and Cell Biology, University of Rome Tor Vergata. Transgenic mice (OG2) (Boiani *et al.*, 2002; Yeom *et al.*, 1996) expressing EGFP driven by the germ-cell specific promoter/enhancer region of the Oct4 gene (GOF-18/deltaPE) were kindly provided by K. John McLaughlin. Female CD-1 mice were mated with male OG2 mice to produce (CD-1 x OG2) F1 hybrids. Animals were housed in a standard animal facility with free access to food and water, in accordance with the guidelines for animal care at the University of Rome Tor Vergata.

Cell preparation

Gonadal ridges (GRs) and testes were collected as previously described (De Felici, 1998) from embryos at 11.5, 13.5 and 18.5 day post coitus (dpc). The day on which a vaginal plug was detected was designated as 0.5 dpc. To obtain a single cell suspension, GRs (11.5 dpc) or

testes (13.5 and 18.5 dpc) were collected in Hepes-MEM (GIBCO/BRL) with 10% BSA, dissected from mesonophroi, and incubated in trypsin-EDTA solution for 5 min at room temperature, followed by gentle pipetting. Where indicated, PGCs were isolated from somatic cells by MiniMACS as previously described (Pesce and De Felici, 1995).

Hoechst 33342 staining, flow cytometry analysis and cell sorting

Hoechst 33342 staining and cytofluorimetric analysis was performed as described previously (Falciatori *et al*., 2004). Typically, cell suspensions (about 10^6 cells/ml) obtained from 50-60 11.5 dpc GRs, 35-40 13.5 dpc testes or 25-38 18.5dpc testes, were stained in MEM containing 5 μg/ml Hoechst 33342 (Sigma, Milano) with or without 50 μM verapamil (Sigma) at 37°C for 90 min. After the incubation, cells were spun down, resuspended in PBS containing 1% fetal bovine serum and analyzed in a Vantage SE (Becton Dickinson) equipped with three lasers. Dead cells and debris were excluded from the plots by means of propidium iodide (PI) staining (2 μg/ml). Hoechst dye was excited at 351 nm, and the resulting fluorescence was split through a 640/LP filter and detected at two wavelengths using 424/44 (Hoechst blue) and 660/20 (Hoechst far red) filters. Cells labeled in the presence of verapamil were used in all experiments to set the SP gate. In experiments with OG2 mouse cells, cytofluorimetric analyses of wild-type testes were performed as control to set gates for GFP fluorescence. In some experiments, cells obtained from 11.5 dpc GRs of OG2 mice were FACS analysed after staining with TG-1 (SSEA-1) antibody (a kind gift from P. Donovan, University of Florida, USA), a specific marker of PGCs within the GRs (Solter *et al*., 1978) as reported in Cory *et al*. (2009).

Cell sorting was performed as previously described (Grisanti *et al*., 2009). Data were analyzed using Summit Software v. 4.3.1 (Cytomation). At least three experiments were performed for each testis developmental stage.

Immunocytochemistry

Immunostaining on sorted cells was performed as previously described (Pesce *et al*., 1995). Briefly, cells were attached to poly-l-lysine-coated slides and fixed in 4% paraformaldehyde at 4°C for 10 min. Staining was performed using goat anti-VASA (1:400; AbCam) antibody at room temperature for 1 hour. After washing, slides were incubated with donkey anti-goat Cy3-conjugated antibody (Molecular Probes) at room temperature for 1 hour. For negative control, the primary antibody was omitted from the staining procedure.

RNA extraction and RT-PCR assay

Total RNA was extracted from purified PGCs and somatic cells with RNeasy microkit (Qiagen) in accordance with the manufacturer's instructions. First-strand cDNA synthesis was performed as follows: 50 ng of total RNA was reverse transcribed by 50 U of Superscript™II (Invitrogen) using 50 ng of random hexamers, in the presence of 0.5 μM deoxynucleotide triphosphates in a final volume of 20 μl. The reaction mixture was incubated for 1 h at 42°C and then heat denatured for 15 min at 75°C. DNA contamination or PCR carryover controls were performed omitting reverse transcriptase during reverse transcription step. Four microliters of cDNA was amplified using the following PCR conditions: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 30 cycles. The following primers were used:

Abcg2 (Gene Bank accession: NM_011920)
forward 5'-AAATGGAGCACCTCAACCTG-3'
reverse 5'-CCCATCACAACGTCATCTTG-3'
Abcb1a (Gene Bank accession NM_011076.2)
forward 5'-ACGGACAGGACATCAGAACC-3'
reverse 5'-CCTGACTCACCACACCAATG-3'
Abcb1b (Gene Bank accession NM_011075)
forward 5'-TTGGCACAACAACTCAT-3'
reverse 5'-GGCTTTCGCATAGTCAGGAG-3'
Abcc1 (Gene Bank accession NM_008576)
forward 5'-CACATGTTGGGAAGCACATC-3'
reverse 5'-GACCAGGATCCGTGTCTTGT-3'.

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