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Identification and characterization of stem cells in prepubertal spermatogenesis in mice☆

Kazuyuki Ohbo,[a,*] Shosei Yoshida,[b,c] Masako Ohmura,[a] Osamu Ohneda,[d] Takehiko Ogawa,[e] Hideaki Tsuchiya,[f] Takashi Kuwana,[f] James Kehler,[g] Kuniya Abe,[h] Hans R. Schöler,[g] and Toshio Suda[a,*]

[a] *The Sakaguchi Laboratory of Developmental Biology, School of Medicine, Keio University, Tokyo, 160-8582, Japan*
[b] *Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Kyoto, 606-8501, Japan*
[c] *"Recognition and Formation", PRESTO, Japan Science and Technology Corporation, Saitama, 332-0012, Japan*
[d] *Department of Anatomy, School of Medicine, University of Tsukuba, Tsukuba, 305-8575, Japan*
[e] *Department of Urology, Yokohama City University, School of Medicine, Yokohama, 236-0004, Japan*
[f] *Department of Pathology, National Institute for Minamata Disease, Minamata, 867-0008, Japan*
[g] *The Center for Animal Transgenesis and Germ Cells Research, University of Pennsylvania, Philadelphia, PA 19348, USA*
[h] *RIKEN Bio-Resource Center, Tsukuba, 305-0074, Japan*

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Abstract

The stem cell properties of gonocytes and prospermatogonia at prepubertal stages are still largely unknown: it is not clear whether gonocytes and prospermatogonia are a special cell type or similar to adult undifferentiated spermatogonia. To characterize these cells, we have established transgenic mice carrying EGFP (enhanced green fluorescence protein) cDNA under control of an Oct4 18-kb genomic fragment containing the minimal promoter and proximal and distal enhancers; Oct4 is reported to be expressed in undifferentiated spermatogonia at prepubertal stages. Generation of transgenic mice enabled us to purify gonocytes and prospermatogonia from the somatic cells of the testis. Transplantation studies of testicular cells so far have been done with a mixture of germ cells and somatic cells. This is the first report that establishes how to purify germ cells from total testicular cells, enabling evaluation of cell-autonomous repopulating activity of a subpopulation of prospermatogonia. We show that prospermatogonia differ markedly from adult spermatogonia in both the size of the KIT-negative population and cell cycle characteristics. The GFP^+ KIT^- fraction of prospermatogonia has much higher repopulating activity than does the GFP^+KIT^+ population in the adult environment. Interestingly, the GFP^+KIT^+ population still exhibits repopulating activity, unlike adult KIT-positive spermatogonia. We also show that ALCAM, activated leukocyte cell adhesion molecule, is expressed transiently in gonocytes. Sertoli cells and myoid cells also express ALCAM at the same stage, suggesting that ALCAM may contribute to gonocyte–Sertoli cell adhesion and migration of gonoyctes toward the basement membrane.

Keywords: Oct4; Prospermatogonia; Prespermatogenesis; KIT; Transgenic mice; GFP; Activated leukocyte cell adhesion molecule

Introduction

Spermatogenesis represents a classic stem cell model. For example, adult male germ stem cells exhibit a quiescent cell cycle, a requirement for stroma cells to regulate germ stem cell behavior and the ability to produce cells of the entire spermatogenic lineage (de Rooij, 1998, 2001). Adult spermatogonia are divided into type A and type B spermatogonia. Type A spermatogonia are further subdivided into A single (As), A paired (Ap), A aligned (Aal), A1, A2, A3, and A4 by shape, numbers of cells connected by intercellular bridges, and amounts of heterochromatin (de Rooij, 1998). However, the biological significance of As, Apr, Aal,

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* Corresponding author. Fax: +81-3-5363-3475.
E-mail addresses: kohbo@sc.itc.keio.jp (K. Ohbo) or sudato@sc.itc.keio.ac.jp (T. Suda).

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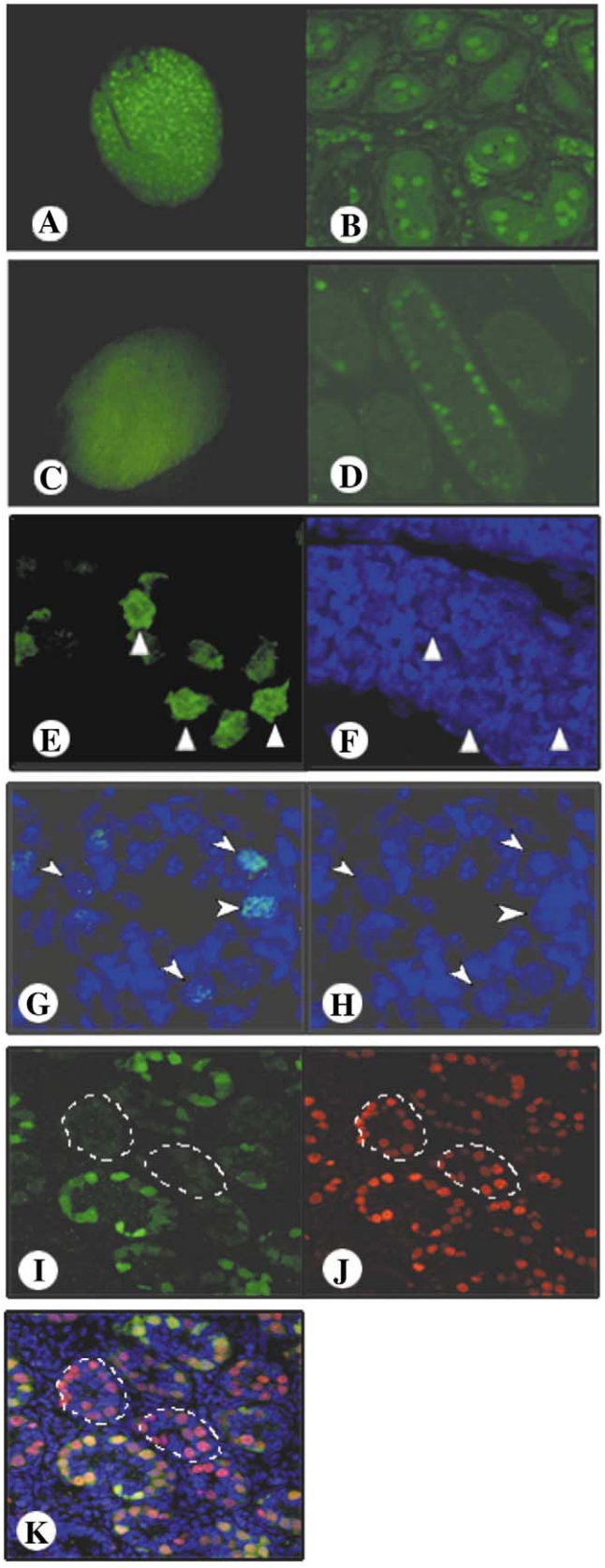
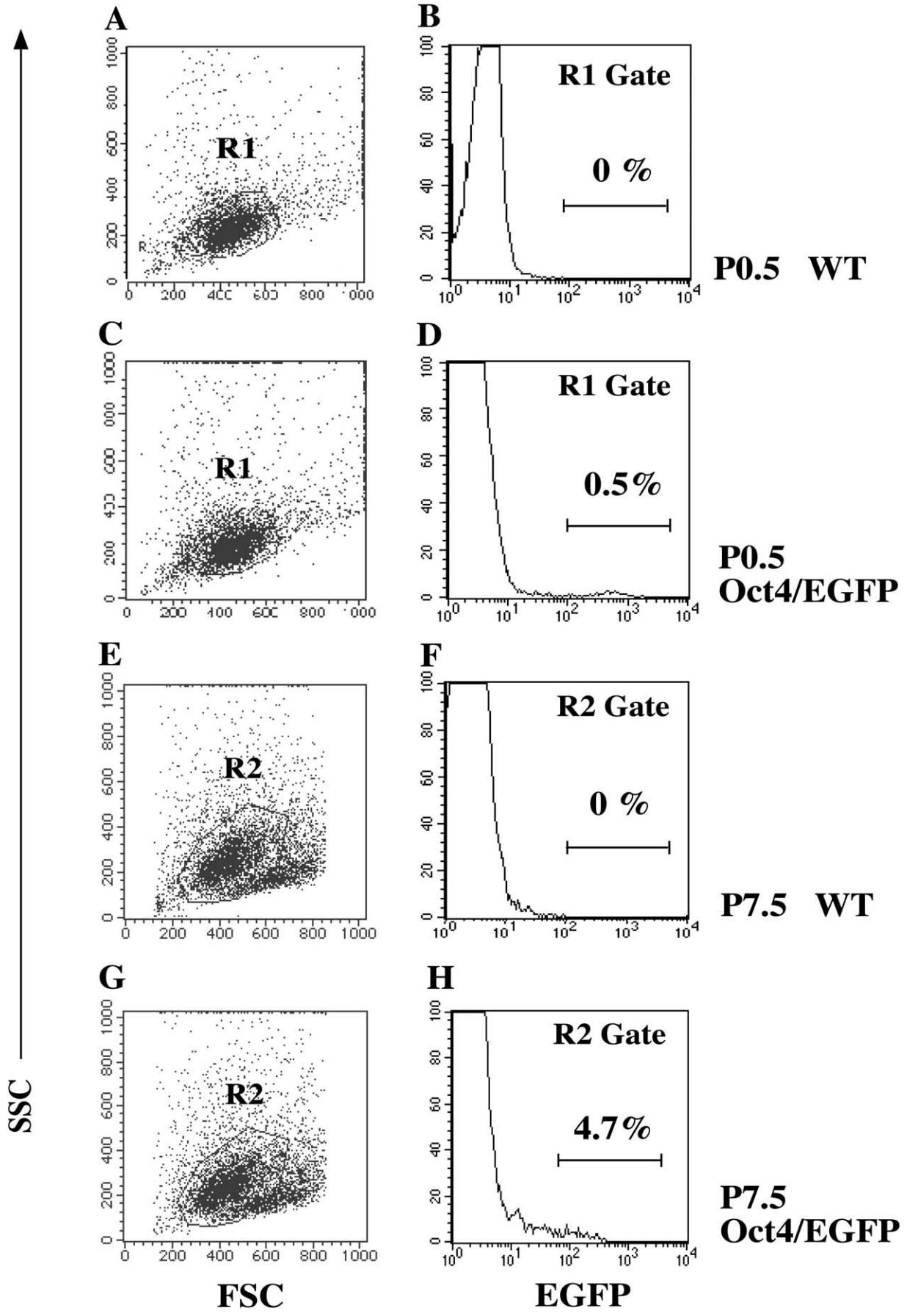


Fig. 1. Fluorescence and immunohistochemical analysis of testis of Oct4/EGFP transgenic mice. Fluorescence stereoscopic view of Oct4/EGFP transgenic mice at P0.5 (A) and P7.5 (C) after peeling off the tunica albuginosa. Section analysis of seminiferous tubules by fluorescence microscopy at P0.5 (B) and P7.5 (D). All tubules contain GFP-positive gonocytes at P0.5 (B). All GFP-positive prospermatogonia localize to the periphery of testis cords at P7.5 (D). Note that only some tubules contain GFP-positive prospermatogonia (D). Fragments of testis cords from P7.5 pups were stained with anti-GFP polyclonal antibody (E, green color-coded) and TOTO3 (F, blue color-coded). Note that most GFP-positive cells have cellular extensions (E, arrowheads). Frozen sections from P7.5 were stained with ant-GFP antibody (light blue color-coded) followed by TOTO3 nuclear staining (G). TOTO3 (blue color-coded) of the same section showed large faintly stained oval nuclei and nuclear vacuoles (H). Arrowheads indicate undifferentiated type prospermatogonia expressing various levels of GFP (G, H). Double-staining of GFP (I), TRA98 (J), and merged GFP and TRA98 (K) at P7.5. Many GFP-positive cells also express TRA98 (I–K). Prospermatogonia in the indicated testis cords (dotted lines) express only TRA98 (K, pink color-coded) but not GFP.

and A1–4 spermatogonia as stem cells has not yet been evaluated, because cell surface markers are not available to purify each type of spermatogonia (de Rooij, 1998). Among spermatogonia, those that express KIT have totally lost repopulating activity (Shinohara et al., 2000). Since “stem cell” is a functional definition, a system to evaluate “stem cell activity” of each type of spermatogonia is desired.

In the mouse, spermatogonia originate from primordial germ cells (PGCs) that are first specified in extraembryonic mesoderm at embryonic day 7.0 (E7) (Gomperts et al., 1994). PGCs migrate from allantois through the hindgut and settle in the genital ridge by E11.5 (Gomperts et al., 1994). There, PGCs grow continuously until around E16.5, and then enter G0/G1 arrest. While proliferating PGCs are enclosed by Sertoli cells, the fundamental structure of testis cords is established (McLaren, 1998). Mitotically quiescent germ cells, called gonocytes, apparently reenter the cell cycle shortly after birth. At P0.5, gonocytes are uniformly round and separated from the basement membrane (Clermont et al., 1957; Hilscher et al., 1974; Huckins et al., 1968). By several days postpartum, some gonocytes extend cytoplasmic processes peripherally and contact the basement membrane (Clermont et al., 1957). Relocation of gonocytes to the basement membrane is likely critical to ensure survival of these cells, as suggested by the observation that gonocytes remaining in the center of testis cords eventually degenerate (Hasthorpe et al., 1999). Cells relocated at the basement membrane are called “prospermatogonia.”

Spermatogenesis at prepubertal stages, prespermatogenesis, illustrates how adult stem cell systems develop. Prespermatogenesis is more dynamic than adult spermatogenesis. For example, at this stage, germ cells for the first time acquire “stem cell” properties by attaching to the basement membrane (Spradling et al., 2001). Germ cells then physiologically expand their population rapidly like hematopoietic stem cells in fetal liver (Morrison et al., 1995). Although several attempts to establish spermatogonial stem cell lines from adult spermatogonia have been unsuccessful, Feng et al. (2002) reported establishment of germ cell stem


A
B
R1
R1 Gate
0 %
P0.5 WT
C
D
R1
R1 Gate
0.5%
P0.5
Oct4/EGFP
E
F
R2
R2 Gate
0 %
P7.5 WT
G
H
R2
R2 Gate
4.7%
P7.5
Oct4/EGFP
SSC
FSC
EGFP

lines from P6.5 prospermatogonia, but these have not yet been tested in a transplantation assay. It is also reported that testis cords at prepubertal stages create a better environment for stem cells to colonize than adult seminiferous tubules (Shinohara et al., 2001). Accumulating evidence suggests that germ cells at this stage are different from those at adult stages and lead us to focus on analyzing prespermatogenesis.

However, there are many unanswered questions about prespermatogenesis. For example, how gonocytes undergo the transition to adult type spermatogonia is still obscure. Daughter cells of gonocytes may be a special cell type with a potential to give rise to adult type spermatogonia after a few cell divisions (Hilscher et al., 1974). However, evidence for existence of a special type of prospermatogonia is not conclusive, and gonocytes may also give rise directly to adult spermatogonia (Bellve et al., 1977). Therefore, careful characterization of prospermatogonia is necessary to understand how the male germ stem cell system is established and what the differences are between prospermatogonia and adult spermatogonia. Considering that cell surface molecules can be powerful tools to purify and characterize hematopoietic stem cells from mixed cell populations (Nakauchi et al., 1999), analysis of cell surface markers is important for characterization and identification of the stem cell fraction in male germ cells. Furthermore, transplantation assays of candidate stem cell populations are required to demonstrate self-renewing activity.

Several cell surface markers are expressed on both gonocytes and adult undifferentiated spermatogonia. Antigen recognized by the EE-2 antibody (EE-2 antigen which localizes on the cell surface) is expressed on gonocytes and adult spermatogonia (Tanaka et al., 1997). Integrins such as αv, α6, and β1 are also expressed on adult spermatogonia (Shinohara et al., 1999, 2001). However, not only prospermatognia and adult undifferentiated spermatogonia but also somatic cells in the testis express all of these markers. Therefore, identification of germ line-specific markers is critical to characterize germ stem cells and demonstrate self-renewal activity of these cells by transplantation assay.

Expression of Oct4 (also known as Oct3/4), a POU and homeobox transcription factor, is apparently critical for maintenance of a pluripotential phenotype (Brehm et al., 1998). Oct4 is expressed in pluripotential cells such as morulae, the inner cell mass, and the epiblast (Ovitt et al., 1998). By E8.5, its expression is extinguished in derivatives of the embryonic ectoderm and endoderm, Oct4 is then only expressed in PGCs (Ovitt et al., 1998). After birth, expression of Oct4 is restricted to undifferentiated prospermatogonia at prepubertal stages (Pesce et al., 1998). Therefore, Oct4 is a good candidate gene for purification of gonocytes and prospermatogonia. To achieve that goal, we generated transgenic mice carrying EGFP cDNA under control of an Oct4 18-kb genomic fragment, since it is reported that an 18-kb fragment containing the minimal promoter and proximal and distal enhancers is sufficient to reproduce endogenous Oct4 gene expression pattern during embryogenesis (Yeom et al., 1996; Yoshimizu et al., 1999).

In this study, we purified viable gonocytes and prospermatogonia free of contamination by somatic cells from Oct4/EGFP transgenic mice and undertook a functional assay. Using this approach, we screened numerous cell surface markers reportedly expressed on hematopoietic stem cells and progenitor cells, since accumulating evidence about plasticity of stem cells indicates that surface molecules on hematopoietic stem cells would be candidate stem cell markers in gonocytes and prospermatogonia. Among these, we focused on two molecules, KIT and activated leukocyte cell adhesion molecule (ALCAM) (Aruffo et al., 1997; Bowen et al, 1997), because expression profiles of those two molecules dramatically change during embryonic and prepubertal stages. We find that the GFP^+KIT^- population can repopulate more efficiently than the GFP^+KIT^+ population, strongly supporting the idea that the GFP^+KIT^- population contains a greater number of stem cells, the direct precursors of adult undifferentiated spermatogonia. It is notable that the KIT-expressing population in germ cells at prepubertal stages still maintains repopulating activity, although adult KIT-expressing spermatogonia, A1–A4 differentiating spermatogonia, do not have repopulating activity (Shinohara et al., 2000). We also find that ALCAM (CD166), also known as KG-CAM, neurolin, and BEN/DM-Grasp/SC1, is expressed on gonocytes transiently. In addition, the expression pattern of ALCAM and the results of a cell adhesion assay suggest that ALCAM participates in migration and the cell-to-cell adhesion of gonocytes with Sertoli cells and extracellular matrix at the basement membrane.

Materials and methods

Mice

The GOF18-EGFP plasmid was purified and injected into the pronucleus of C57BL/6J fertilized eggs, resulting in generation of six transgenic mouse lines. Four lines expressed GFP proteins. Transgenic mice were backcrossed with C57BL/6J mice. The data presented were obtained from experiments using heterozygous mice. All procedures were performed in accordance with the guidelines of Kuma-

Fig. 2. Flow cytometric analysis of EGFP expression in total testicular cells in Oct4/EGFP transgenic mice at P0.5 and P7.5. Single cell suspensions of testicular cells were prepared and analyzed by FACS. Left column (A, C, E, and G) shows the Forward Scatter (FSC)–the Side Scatter (SSC) profile. The histograms show GFP expression in P0.5 wild type mice (B), P0.5 Oct4/EGFP mice (D), P7.5 wild type mice (F), and P7.5 Oct4/EGFP mice (H) of the gated populations (R1 and R2).

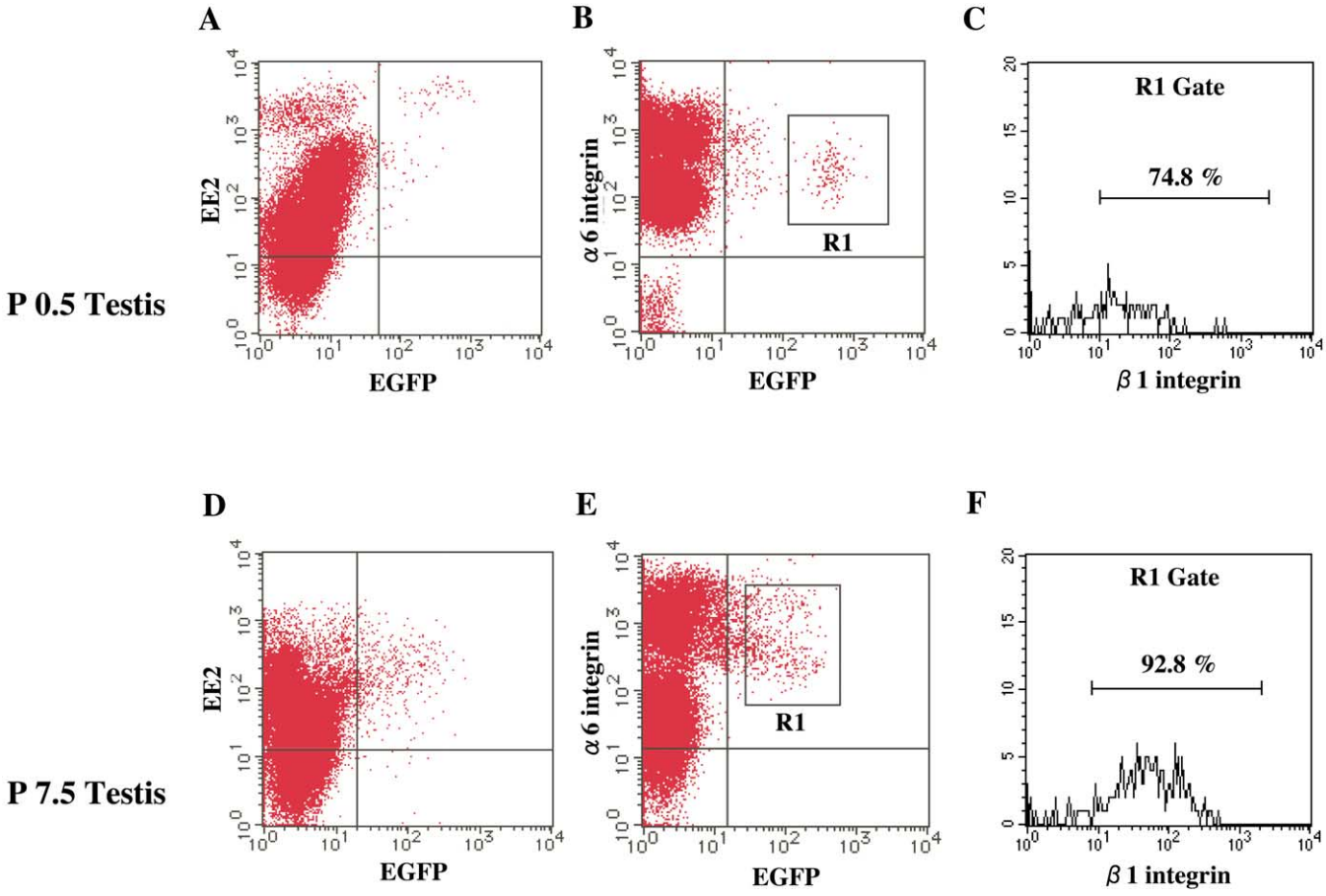


Fig. 3. Flow cytometric analysis of EE-2 antigen, α6 integrin, and β1 integrin. Single cell suspensions were prepared from P0.5 testes (A–C) and P7.5 (D–F), and stained with purified EE-2 mAb (A, D), RPE-anti-α6 integrin mAb (B, E), and biotin-anti-β1 integrin mAb (C, F). The second antibodies, RPE-anti-rat IgG Ab and streptavidin-ECD, were used for purified EE-2 mAb and biotin-anti-β1 integrin mAb, respectively. (C, F) β1 integrin expression on testicular cell populations gated by EGFP and α6 integrin expression. RPE, R-phycoerythin: ECD, Texas Red.

moto University, Kumamoto, Japan. For timed pregnant mice, the date the vaginal plug was defined as E0.5.

Antibodies

Commercially available antibodies used for fluorescence-activated cell sorting analysis (FACS) were as follows: monoclonal RPE (R-phycoerythrin)-labeled anti-α6 integrin antibody (Ab) (BD Pharmingen, San Jose, CA), monoclonal biotin-anti-β1 integrin Ab (BD Pharmingen), monoclonal anti-CD16/CD32 Ab (Fc block) clone 2.4G2 (BD Pharmingen), monoclonal APC (allophycocyanin)-labeled anti-KIT (CD117) Ab (clone 2B6, BD Pharmingen). The 2B8 Ab does not interfere with ligand binding. Monoclonal purified anti-KIT (CD117) Ab clone ACK2 was used for immunohistochemistry (BD Pharmingen). Monoclonal purified anti-TRA98 Ab and anti-EE2 Ab were kindly provided by Dr. Nishimune (Osaka University) (Koshimizu et al., 1995; Tanaka et al., 1997). The anti-GFP antibody, (MBL, Nagoya, Japan), was used for immunohistochemistry. Purified and biotin anti-mouse ALCAM antibodies have been described previously (Ohneda et al., 2001). RPE-anti-rat IgG (H+L) Ab (Southern Biotechnology Associates Inc, San Jose, CA), Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes, Eugene, OR) and Alexa Fluor 546 anti-rat IgG (H+L) (Molecular Probes) were used for purified first antibodies. Streptavidin-ECD (Texas Red) (Immunotech, Marseille, France) and streptavidin-Alexa Fluor 488 (Molecular Probes) were used for the biotin-labeled first antibody. TOTO3 iodine (642/660) (Molecular Probes) was used for nuclear staining.

Cell culture and transfection

OP9 cells were maintained in αMEM supplemented with 20% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) and 50 U/ml Penicillin and 50 μg/ml Streptomycin. OP9 cells were transfected with 0.9 μg of pRK5-Grasp, a mouse ALCAM expression vector, and 0.1 μg of pCAG-neo by using Effectene Transfection Reagent (QIAGEN GmbH, Hildern, Germany) according to the manufacturer's instructions (Ohneda et al., 2001). For controls, the vector

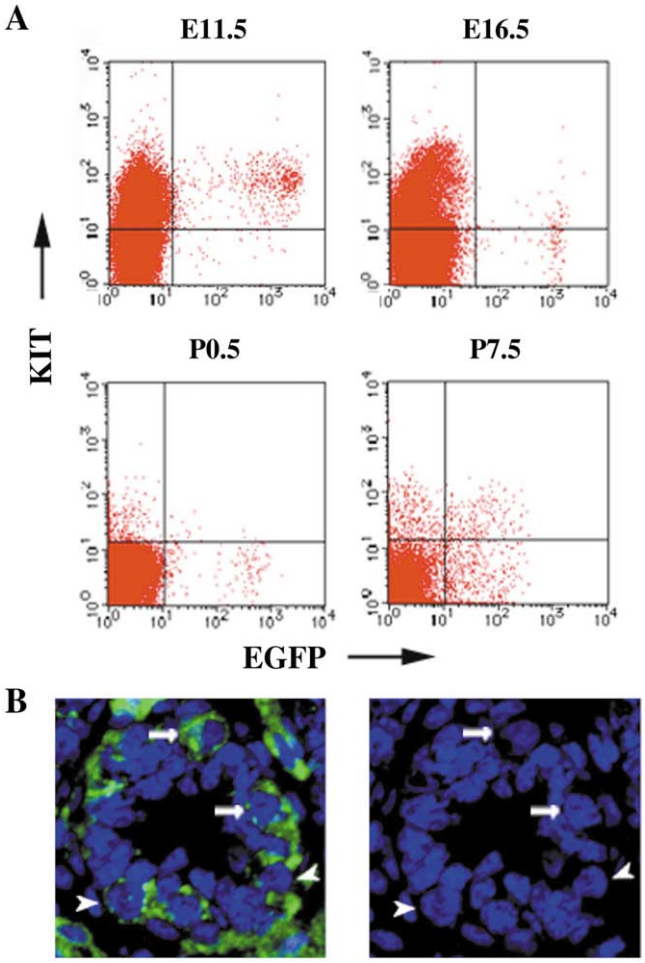


Fig. 4. KIT expression on GFP-positive germ cells during mouse development. Male gonads were dissected at indicated periods and treated with collagenase. After making single cell suspensions, cells were stained with APC-labeled anti-KIT mAb and analyzed by FACS (A). All GFP-positive primordial germ cells express KIT at E11.5 (A). KIT expression is downregulated starting at E16.5 (A). No GFP^+ gonocytes express KIT at P0.5 (A). GFP^+ prospermatogonia contain both KIT^- and KIT^+ populations at P7.5 (A). APC, allophycocyanin. Immunohistochemical analysis of KIT-expressing germ cells. Expression of KIT protein was detected by TSA Biotin system with TOTO3 nuclear staining as described in Materials and methods. Specimens from P7.5 testes were analyzed by confocal laser microscope. The left figure in (B) shows merged KIT (green color-coded) and TOTO3 (blue color-coded) double staining. The right figure in (B) shows TOTO3 staining (blue color-coded). TOTO3 nuclear staining shows that KIT expressing cells have a large oval nucleus that is faintly stained and nuclear vacuoles (B, arrows) as do KIT weak positive and negative cells (B, arrowheads).

pRK5 was transfected instead of pRK5-Grasp. Cells stably transfected with ALCAM were selected in media containing G418 (500 μg/ml) (WAKO Pure Chemical Industries Ltd., Osaka, Japan). Isolated colonies were analyzed for ALCAM expression by both RT-PCR and fluorescence-activated cell sorting (FACS) analysis using the anti-ALCAM mAb. Stable cell lines expressing ALCAM were maintained in αMEM supplemented with 20% FBS and 300 μg/ml G418.

FACS and LSC analysis

Encapsulated testes were dissected and incubated with PBS containing 1 mM EDTA for 15 min at room temperature, pipetted in PBS plus 1 mg/ml collagenase (Sigma, St. Louis, MO) and 100 U DNaseI (Invitrogen Corp., Carlsbad, CA), and incubated for 20 min at 37°C. Cell suspensions were preincubated first with Fc-block and then with antibodies, namely, anti-KIT, anti-EE-2, anti-α6 integrin, and anti-β1 integrin. The samples were analyzed by FACS Calibur (Becton Dickinson, San Jose, CA). For cell sorting experiments, cells were suspended in PBS containing 4% FBS. Cell sorting and analysis were performed by using a FACS Vantage flow cytometer/cell sorter equipped with CELL Quest software (Becton Dickinson). Sorted cells in DMEM medium containing 10% FBS were washed once with the medium and prepared for further analysis. Cell cycle kinetics were measured by propidium iodide (Sigma) staining of sorted specimens. Cytospin smears of the sorted cells were made and incubated with propidium iodide with 100 μg/ml RNaseA (Sigma). The specimens were analyzed by using Laser Scan Cytometer (LSC2; Olympus, Tokyo, Japan). Experiments of cell cycle kinetics were done three times and are reported as an average with standard deviation.

Direct fluorescence histochemistory

To analyze the GFP signal, testes were treated with PBS containing 2% PFA (Nacalai Tesque, Kyoto, Japan) overnight at 4°C and washed with cold PBS twice. Testes were further incubated in PBS containing 6.8% sucrose (Nacalai Tesque) for 10 h at 4°C and treated with 100% acetone (WAKO) for 1 h at room temperature. Testes were then reacted with Technobit 8100 solution (Heraeus Kulzer GmbH, Wehrheim, Germany) according to the manufacturer's protocol. After embedding into Technobit solution, testes were sectioned at 7 μm and analyzed by fluorescence microscope (IX70 system; Olympus, Tokyo, Japan).

Immunohistochemistry

To stain GFP and TRA98, testes were dissected and fixed with 4% periodate-lysine-paraformaldehyde (PLP) for 60 min. Then, the testes were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan), sectioned at 10 μm, and mounted onto Silan-coated slides (Matsunami, Tokyo, Japan). The specimens were incubated with anti-GFP polyclonal Ab and anti-TRA98 mAb overnight at 4°C. After washing with PBS, Alexa Fluor 488 goat anti-rabbit IgG (1:100) and Alexa Fluor 546 anti-rat IgG (1:100) were incubated for 60 min at room temperature (R.T.). For staining KIT, the testes were embedded in Tissue-Tek O.C.T. compound without fixation. The sectioned specimens were fixed with acetone for 10 min. Then, TSA Biotin

system (PerkinElmer, Boston, MA) was used according to the manufacturer's protocol. After overnight incubation in anti-KIT mAb, slides were incubated with biotinylate-secondary antibody. After reacting with streptavidin-HRP, the specimens were incubated with the biotinyl tyramide amplification reagent. Then, the specimens were reacted with streptavidin-Alexa Fluor 488. For nuclear staining, the specimens were treated with TOTO3 (1:500; Molecular Probes). For whole-mount immunohistochemistry, testes were fixed with PBS containing 4% paraformaldehyde (PFA) and sequentially dehydrated with PBS containing 25, 50, and 75% ethanol. The dissected tubules for immunostaining were rehydrated at R.T. and washed with PBS. The specimens were incubated with blocking buffer (PBS containing 0.5% BSA) at R.T. for 30 min. Then, the tubules were stained with anti-GFP Ab in blocking buffer overnight at 4°C. After washing with PBS, the tubules were incubated with Alexa Fluor 488 goat anti-rabbit IgG(H+L) (1:100) in blocking buffer. After washing with PBS, tubules were stained with TOTO3. Images of GFP, TRA98, KIT, and nucleus stained by TOTO3 were obtained by using a confocal laser scanning system (Carl Zeiss LSM 510 system; Carl Zeiss, Oberkochen, Germany). The immunostaining of ALCAM was performed as described previously (Ohneda et al., 2001). Briefly, testes were treated with PBS containing 2% PFA overnight and embedded in polyester wax (BDH Laboratory Supplies, Poole, England). The specimens were sectioned at 7 μm and mounted onto MAS-coated slides (Matsunami). The first antibody was developed with HRP-conjugated anti-rat Ig antibody (Biosource, Camarillo, CA). Diaminobenzidine (DAB; Dojin Chem., Kumamoto, Japan) with NiCl was used for the HRP color reaction.

Transplantation assay

R1 (GFP^+KIT^+) and R2 (GFP^+KIT^-) populations were sorted and resuspended in DMEM containing 2% FBS. Donor mice, immunologically compatible C57BL/6 mice at 8 weeks of age, were treated with busulfan (50 mg/ml, 500 mg/kg body) to deplete intrinsic germ cells. Each cell suspension was transplanted into the testis of donor mice. Approximately 10 μl of cell suspension containing 10% (v/v) of a 0.4% Trypan Blue solution (Gibco BRL) were injected into the seminiferous tubules of mice following procedures described previously (Ogawa et al., 2000).

Analysis of transplanted germ cells by fluorescent and confocal microscopy

Fluorescence of the transplanted germ cells in recipient testes was analyzed by a fluorescent microcopy (IX70 system). After the indicated period, recipient testes were dissected and the length of the GFP-positive region was analyzed by the fluorescent microscopy. The length of GFP-positive seminiferous tubules was evaluated by using Viewfinder Lite and Lumina vision software (Mitani Corporation, Kyoto, Japan). To compare repopulating activity of transplanted cells, the length of the GFP-positive region was divided by the number of transplanted cells. To obtain confocal images of transplanted germ cells, an Olympus Fluoview FV500 system was used (Olympus, Tokyo, Japan).

Whole-mount analysis by confocal microscopy

Fluorescence of prospermatogonia in whole-mount staining specimens was analyzed by a confocal laser scanning system (Carl Zeiss LSM 510 system). In order to observe the three-dimensional architecture of the spermatogonial networks on the basement membrane, laser confocal pictures were taken sequentially every 2 μm parallel to the longitudinal axis of the seminiferous tubules with objective lens of $\times$40 magnification and zoom $\times$1.0. The data from each point of focal plane that are collected sequentially by scanning across the field were built up to a three-dimensional image.

RT-PCR analysis

Total RNA of ALCAM cDNA-transfected OP9 cells and mock-transfected OP9 cells were prepared by using the RNeasy mini kit (Qiagen) according to the company's instruction. Total RNA was treated with DNaseI (RQ1 RNase-Free; Promega, Madison, WI). Reverse transcriptase and PCRs were performed by using the Advantage-RT-for-PCR kit (BD Biosciences Clontech, Palo Alto, CA). PCR was performed by using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA). The thermocycling parameters and sequences of PCR primers are as follows: for ALCAM, 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with sense primer 5′-CGA ACC CTG CCT GTG TCA TGC ACA ATA-3′ and antisense primer 5′-TAT CGT CTG CCT CAT CGT GCT CTG GAA T-3′; for GAPDH, 30 cycles at 94°C for 30s, 60°C for 30s, and 72°C for 30s with sense primer 5′-AAG CCC ATC ACC ATC TTC CA-3′ and antisense primer 5′-CAG GGT TTC TTA CTC CTG-3′. Ten microliters of PCR product was electrophoresed on a 2.0% agarose gel.

Cell to cell adhesion assay

Procedure for cell-to-cell adhesion assay was described previously (Takakura et al., 1998). The ALCAM-negative population was sorted from mock-transfected OP9 cells and the ALCAM-high-positive population was sorted from ALCAM cDNA transfected OP9 cells. The sorted ACLAM-negative OP9 cells and ALCAM-positive OP9 cells were seeded in 96-well plates, respectively. To interfere with

homotypic binding between ALCAM on OP9 cells and that on gonocytes, the sorted GFP-positive gonocytes from P0.5 testes (3000 cells per well) were preincubated with ALCAM-Fc protein. As control, CD4-Fc protein was used. Then, the preincubated gonocytes were seeded on the sorted ALCAM-negative and-positive OP9 cells, respectively. After incubation for 60 min at 37°C, plates were shaken vigorously. Supernatants are harvested and the number of nonadherent (unbound) cells was determined. Experiments were done in triplicate.

Results

Characterization of GFP-positive cells in the testis of Oct4/EGFP mice

To investigate function of germ stem cells at prepubertal stages, it is essential to purify gonocytes and prospermatogonia from differentiating germ cells. Oct4 is a candidate gene to purify gonocytes because endogenous Oct4 is expressed in undifferentiated prospermatogonia at prepubertal stages. As an 18-kb genomic fragment of the Oct4 gene has been reported to faithfully reproduce the endogenous gene expression pattern during embryogenesis (Yeom et al., 1996), we established transgenic mice carrying EGFP cDNA under the control of the Oct4 18-kb genomic fragment. At P0.5 and P7.5, the testes of transgenic mice were clearly labeled by GFP, respectively (Fig. 1A and C). Analysis of tissue sections showed round GFP-positive cells located separately from the basement membrane in most testis cords at P0.5 (Fig. 1B). Sections of P7.5 testis cords revealed that only cells attached to the basement membrane expressed GFP, although GFP-negative cells remained at the center of testis cords (Fig. 1D). At P7.5, the strength of the GFP signal varied, ranging from bright cells to dark cells within a testis cord (Fig. 1D). In addition, unlike P0.5 testes, some testis cords contained GFP-positive cells but others did not, although there were prospermatogonia in the testis cords (Fig. 1D). Signals seen outside the testis cords in Fig. 1B and D were due to autofluorescence of steroids and lipids in Leydig cells and blood cells in blood vessels (see Supplementary Fig. 1). We also performed immunohistochemical analysis of testis cords using an anti-GFP polyclonal antibody (Fig. 1E–H). Whole-mount immunohistochemical analysis showed that most GFP-positive cells have cellular extensions similar to pseudopodia. Intercellular bridges, typical of Aal spermatogonia, were not observed (Fig. 1E and F). In section specimens, undifferentiated prospermatogonia classified by TOTO3 nuclear staining express different levels of GFP (arrowheads in Fig. 1G and H). Also, some undifferentiated type prosermatogonia do not express GFP. GFP-positive cells only localized in testis cords, and neither Sertoli cells nor Leydig cells, including other somatic cells, give any signals in immunohistochemical studies (data not shown). Next, we performed double staining of GFP and TRA98 to observe whether GFP-expressing cells also express TRA98, a nuclear protein expressed in gonocytes and adult spermatogonia (Koshimizu et al., 1995). TRA98 was expressed in almost all GFP-positive prospermatogonia at P7.5 (Fig. 1J). Double staining of TRA98 (red color-coded) and GFP (green color-coded) clearly showed the existence of GFP-negative prospermatogonia in TRA98-positive prospermatogonia (pinkish cells in areas surrounded by dotted lines in Fig. 1K). Altogether, these results strongly suggest that prospermatogonia at P7.5 are specifically labeled by GFP protein and a mixture of cells which are morphologically indistinguishable express different levels of Oct4. Although the GFP expression level is homogeneous in P0.5 testis cords, its expression level becomes heterogeneous by P7.5. Because Oct4 plays an important role in maintaining undifferentiated status (Pesce et al., 2000), the observation of different levels of GFP expression suggests that those cells are at different maturational states.

FACS analysis of GFP-positive testicular cells in Oct4/EGFP mice

To characterize GFP-positive cells, we made single cell suspensions of testicular cells from Oct4/EGFP mice and analyzed GFP expression by FACS. The autofluorescence signals observed in the fluorescence microscopic study were eliminated in FACS analysis by using band-path filters and compensation of signals (see Supplementary Fig. 2). We defined the gate that contains spermatogonia on the basis of size (Forward Scatter, FSC) and complexity (Side Scatter, SSC) (R1 in Fig. 2A and C, and R2 in Fig. 2E and G). We detected a clear GFP-positive population in P0.5 testes compared with wild type littermates (Fig. 2B and D). GFP-positive cells constituted approximately 0.5% of total testicular cells (Fig. 2D). At P3.5, the proportion of GFP-positive cells slightly increased (data not shown), and approximately 4.7% of testicular cells were GFP-positive at P7.5 (Fig. 2H). However, the strength of the GFP signal was downregulated by P14.5 (data not shown). The absolute number of GFP-positive cells at P7.5 increased approximately 20 times per testis over that seen at P0.5 (data not shown). At P7.5, the strength of GFP signals varied, in agreement with the analysis of tissue sections seen in Fig. 1D (Fig. 2H). In addition, we observed expression of membrane-type stem cell factor (SCF) to confirm that Sertoli cells do not express GFP in testis at P7.5, because the major population of mSCF-positive cells is Sertoli cells. All GFP-positive cells were mSCF-negative in P7.5 testes (data not shown), suggesting that GFP is not expressed in Sertoli cells, which express mSCF. Both histological and FACS studies suggest that GFP expression in Oct4/EGFP mice is similar to the endogenous Oct4 expression pattern reported

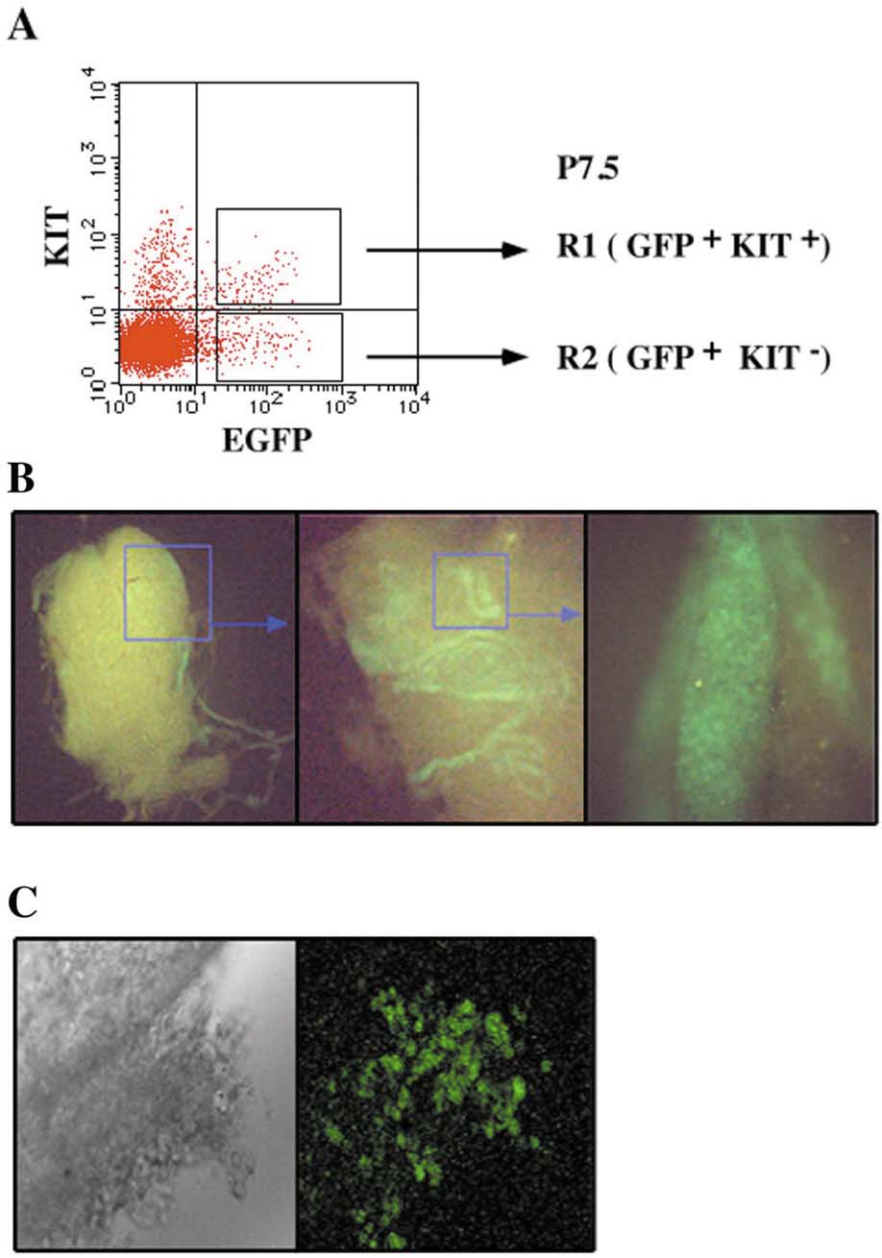
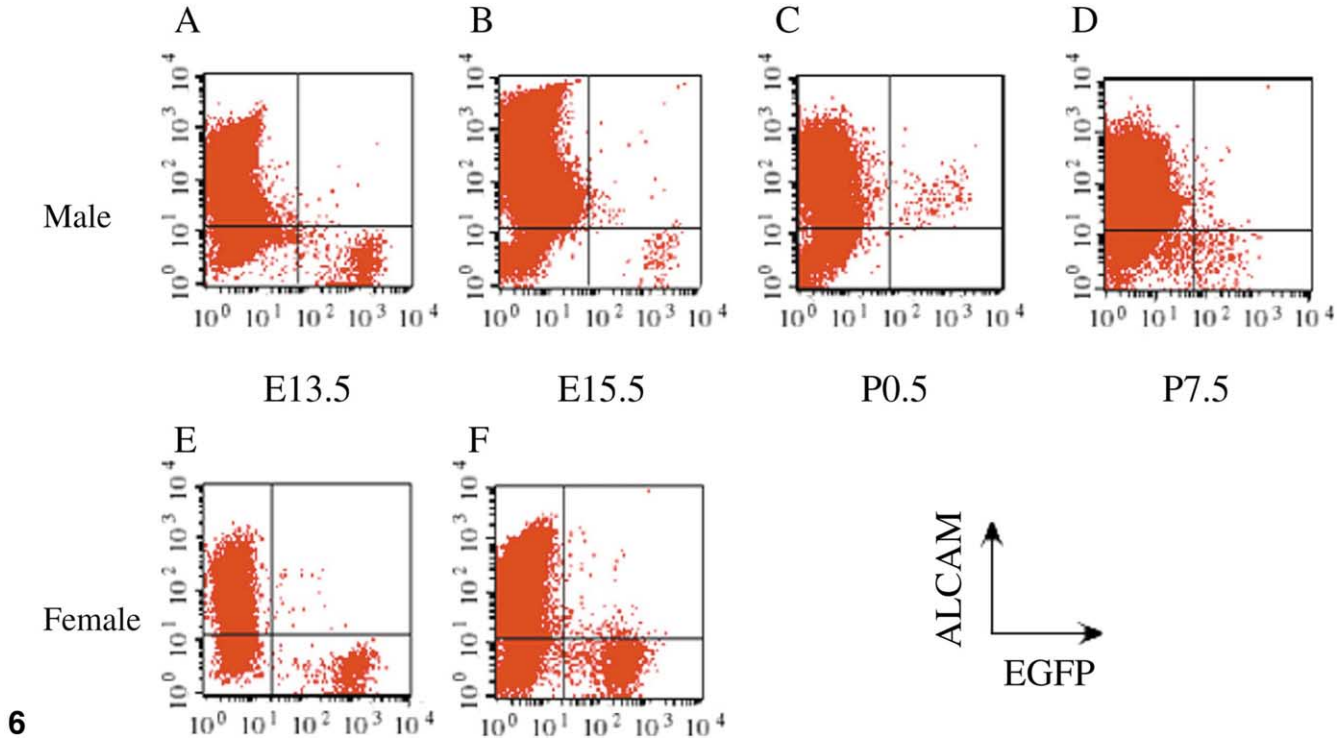


Fig. 5. Transplantation analysis of GFP^+KIT^- and GFP^+KIT^+ populations. Pattern of flow cytometric analysis of sorted populations (A). APC-labeled anti-KIT mAb clone 2B8 that does not interfere ligand binding was used. The GFP^+KIT^+ population (R1) and GFP^+KIT^- population (R2) from P7.5 pups were sorted and transplanted to testes of busulfan-treated C57/BL6 mice. Fluorescein stereoscopic views of testis and testis cords from transplanted R2 (GFP^+KIT^-) cells (B). The middle figure shows high magnification of box in the left figure, and the right figure shows high magnification of box in the middle figure (B). Confocal analysis of GFP^+ cells in testis cord from transplanted R2 (GFP^+KIT^-) cells (C). APC, allophycocyanin.

previously in testis cords at the first week postpartum (Pesce et al., 1998), and that transgenic mice would therefore be useful for enriching viable germ stem cells.

Expression of EE-2 antigens and α6 β1 integrin

We also stained GFP-labeled germ cells with cell surface markers, EE-2 antigen, α6 integrin, and β1 integrin, all reportedly expressed in adult spermatogonia (Koshimizu et al., 1995; Shinohara et al, 2001). The EE-2 antigen was also reported to be expressed on prenatal mouse testis on E17 (Koshimizu et al., 1995). As shown in Fig. 3A and D, all GFP-positive cells were also EE-2 positive on both P0.5 and P7.5. We also observed expression of α6 integrin and β1 integrin at the first week postpartum. Most GFP-positive cells (99%) also expressed α6 integrin at both P0.5 and P7.5. The GFP^+ α6 $integrin^+$ population (R1 in Fig. 3B and E) was analyzed for expression of β1 integrin. At P0.5 and



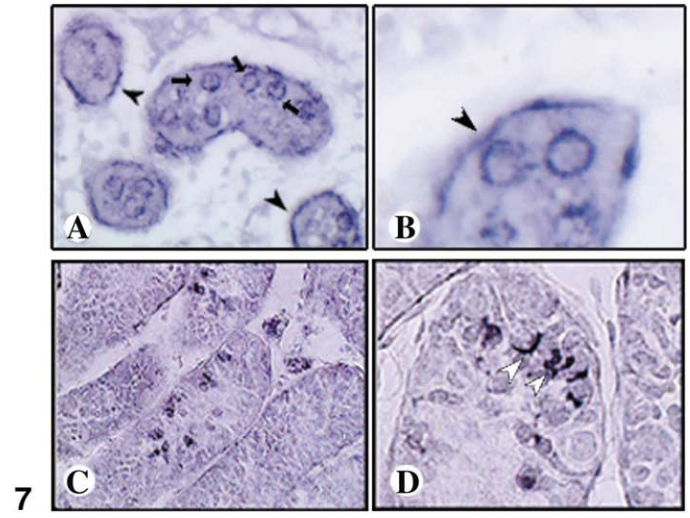


Fig. 6. Flow cytometric analysis of ALCAM expression on GFP-positive germ cells during mouse development. Single cell suspensions of total gonadal cells were stained with biotin-anti-ALCAM mAb followed by incubation with RPE-anti-rat IgG Ab. The samples were then analyzed by FACS. There was no GFP^+ALCAM^+ population at E13.5 (A, E) and E15.5 (B, F) in males (A, B) and females (E, F). One hundred percent of GFP^+ cells coexpress ALCAM at P0.5 (C). One percent of cells express both GFP and ALCAM at P7.5 (D).

Fig. 7. Immunohistochemical staining of seminiferous tubes using anti-ALCAM mAb. P0.5 (A, B) and P7.5 (C, D) testes were fixed and stained with purified anti-ALCAM mAb. The first antibody was developed with HRP-conjugated anti-rat Ig antibody. DAB with NiCl was used for the HRP color reaction. Punctate signals were observed on gonocytes (arrows) and myoid cells (arrowheads) at the basement membrane at P0.5 (A). ALCAM protein accumulated at the side facing the basement membrane (arrowhead in B). ALCAM protein localizes to the interface between cells (arrowheads in D) located at the center of testis cords but not at the periphery at P7.5 (C, D) The original magnification was 200×.

P7.5, 74.8 and 92.8% of the α6 integrin-positive population expressed β1 integrin, respectively (Fig. 3C and F). In addition, the expression of glial-derived neurotrophic factor receptor α subunit (GFR α) was confirmed by RT-PCR analysis (data not shown). Together with the results of anatomical and histological analysis, these results again suggest that germ cells are specifically marked by GFP. In addition, because EE2 antigen and $\alpha_6\beta_1$ integrin were also expressed on primordial germ cells (PGCs) at embryonic stages (Anderson et al., 1999; unpublished data), those

Table 1
Spermatogenic potential of GFP^+KIT^- and GFP^+KIT^+ testis cells

Fraction	No.	Number of transplanted cells	Length of GFP positive region (mm)	Index (length/10,000 cells)	Weight (mg)
GFP^+KIT^-	1	33.000	16.5	5.00	20.2
	2	30.000	19.7	6.57	20.4
	3	30.000	22.4	7.47	34.2
	4	30.000	27.7	9.23	28.4
GFP^+KIT^+	1	30.000	0.9	0.30	28.3
	2	30.000	0	0	20.0
	3	30.000	2.5	0.83	29.2
	4	30.000	3.1	1.03	24.9

Note. GFP^+Kit^- cells and GFP^+Kit^+ cells were sorted and transplanted to busulfan-treated adult mouse testes. After 3 months, the length of the GFP-positive region was measured and calculated per transplanted 10,000 cells.

markers are expressed throughout all the germ cell stages from embryo to adult.

Identification of antigens of hematopoietic progenitor cells on neonate spermatogonia: KIT

Hematopoiesis is a well-characterized stem cell system (Osawa et al., 1996). Observations of stem cell plasticity and transdifferentiation suggest that common molecules maintaining stem cell character could be expressed in both the germ cells and hematopoietic cells (Fuchs et al., 2000; Weissman, 2000a,b). Therefore, we isolated GFP-positive germ cells from Oct4/EGFP mice and assayed for expression of cell surface molecules reported to play important roles in hematopoietic stem cells (HSCs) and progenitor cells by either RT-PCR or FACS analysis. FACS analysis identified ALCAM, KIT, and endoglin on GFP-positive gonoyctes, while PCR identified CD34 in GFP-positive gonocytes. Here, we analyzed ALCAM and KIT since the expression levels of both mRNAs and proteins dramatically changed during germ cell development.

In the germ line, undifferentiated spermatogonia do not express KIT in adults (Yoshinaga et al., 1991; Shinohara et al. 1999); whether KIT is expressed in gonocytes and prospermatogonia has, however, been controversial (Yoshinaga et al., 1991; Hasthorpe et al., 1999). Differences have been reported in expression of cell surface markers critical for purification of HSCs, such as CD34, between embryonic HSCs and adult HSCs (Dzierzak, 1999). Therefore, we analyzed expression of KIT on GFP-positive prospermatogonia at embryonic and neonatal stages. From E11.5 to E13.5, GFP-positive PGCs highly expressed KIT (Fig. 4A, and data not shown). However, the expression level was downregulated around E16.5 (Fig. 4A). In P0.5 testes, all GFP-positive gonocytes were negative for KIT (Fig. 4A). The expression of KIT was upregulated in GFP-positive gonocytes and 57% of the GFP-positive population expressed KIT at P7.5 (Fig. 4A). Even at P3.5, the stage before KIT-positive cells such as differentiating type A and type B spermatogonia emerge, we detected GFP and KIT double-positive cells. Therefore, we asked whether KIT-expressing cells in P7.5 testis cords are undifferentiated. KIT-expressing cells attached to the basement membrane exhibited a homogeneous nucleus with nuclear vacuoles, suggesting that they are undifferentiated type prospermatogonia (arrows in Fig. 4B) that are morphologically indistinguishable from KIT-negative cells (arrowheads in Fig. 4B).

During embryonic stages and just after birth, FACS analysis revealed that the signals of both GFP and KIT showed compact distribution that became broad at p7.5. These results might imply that germ cells might be a homogeneous population during embryonic stages. However, after birth, prospermatogonia that are morphologically identical are proceeding through different cell fates.

Different repopulating activity between the GFP^+KIT^- population and the GFP^+KIT^+ population

Although we identified two different cell populations, GFP^+KIT^- and GFP^+KIT^+, in P7.5 testes, there was no direct evidence that these two populations could give rise to adult male germ cells, or that the two populations might exhibit different repopulating activity of adult type germ cells. To evaluate the repopulating activity of the two populations, we sorted both populations, R1 (GFP^+KIT^+) and R2 (GFP^+KIT^-), and transplanted them to busulfan-treated adult mouse testis (Fig. 5A). After 3 months, we observed GFP-positive seminiferous tubules using fluorescence and confocal microscopy (Fig. 5B,C). To evaluate and compare repopulating activity of R1 and R2 populations, we measured the length of the GFP-positive testis cord and calculated the length of the GFP-positive region per transplanted 10,000 cells. As shown in Table 1, the GFP^+KIT^- population has a higher repopulating activity than the GFP^+KIT^+ population. The possible explanations of these results are (1) the GFP^+KIT^- population contains more stem cells, (2) the GFP^+KIT^- population has higher hom-

ing activity, or (3) the GFP^+KIT^- population has higher cell proliferating activity.

Therefore, we observed the cell cycle kinetics of these two populations by propidium iodide staining after sorting each population. The GFP^+KIT^- population showed 74.3 ± 2.3% of the cells in G0/G1 phase, 7.6 ± 1.5% in S phase, and 17.9 ± 2.7% in G2/M phase. The GFP^+Kit^+ population showed 63.9 ± 3.6% in G0/G1 phase, 9.1 ± 1.2% in S phase, and 25.1 ± 4.7% in G2/M phase. The cell cycle kinetics of the GFP^+KIT^- population is slightly slower than that of the GFP^+KIT^+ population, suggesting that higher repopulating activity in GFP^+KIT^- population observed in transplantation experiments is not due to differences of proliferating activity of GFP^+KIT^- population but more likely due to containing more stem cells and/or higher homing activity than the GFP^+KIT^+ population.

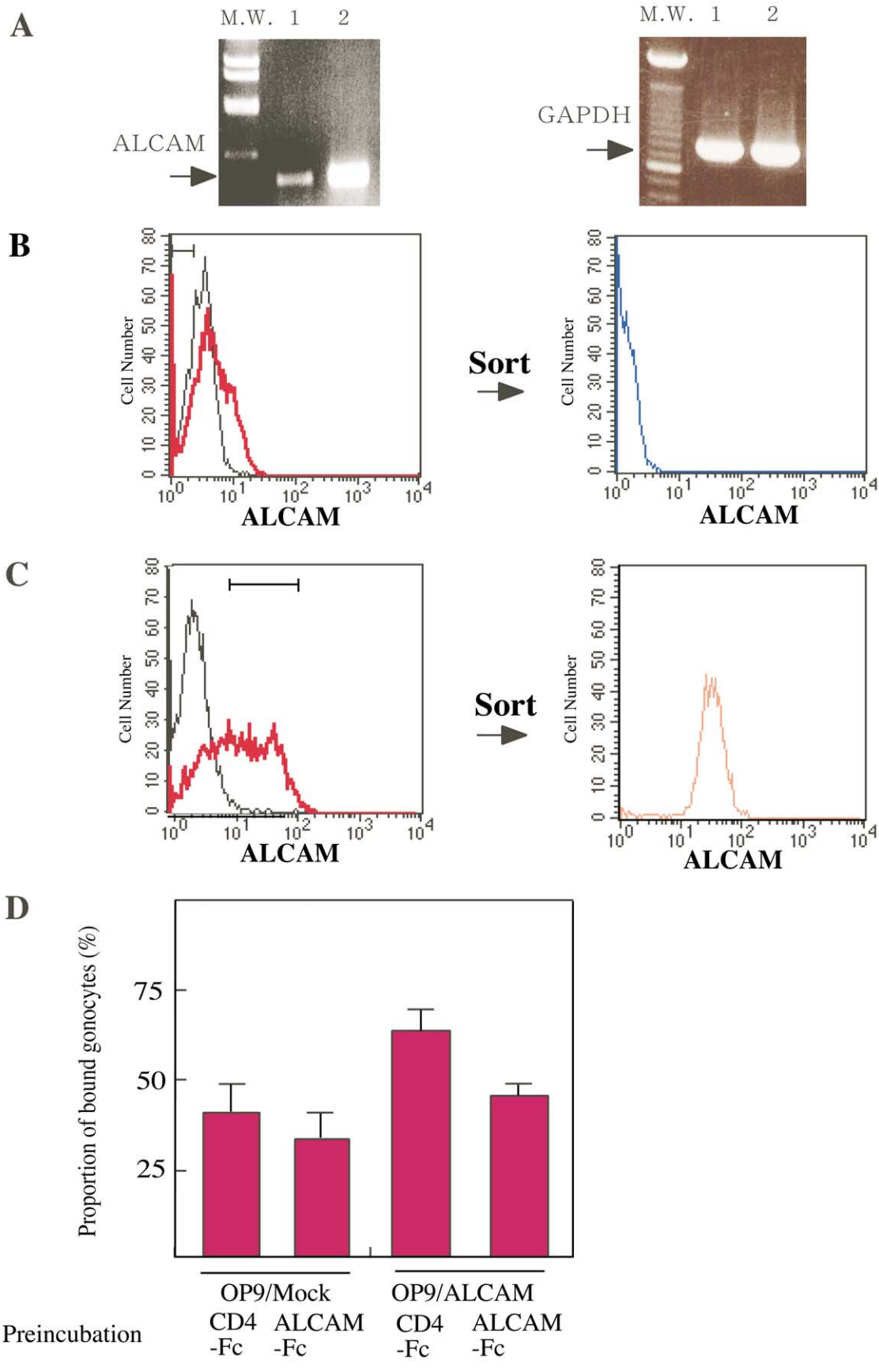
Identification of antigens of hematopoietic progenitor cells on neonate spermatogonia: ALCAM

FACS analysis showed that ALCAM, that expresses on HSCs, bone marrow stroma cells surrounding HSCs, and mesenchymal stem cells, was expressed only on neonate gonocytes (Fig. 6A and B). ALCAM is a cell surface immunogloblin superfamily that is involved with homophilic adhesion as well as CD6 (Aruffo et al., 1997; Bowen et al., 1997; Nelissen et al., 2000). Neither male nor female PGCs at E13.5 and E15.5 express ALCAM (Fig. 6). We detected strong expression of ALCAM on most GFP-positive gonocytes (99%) at P0.5 (Fig. 6C). At P3.5, ALCAM was also detected on GFP-positive gonocytes immediately before migration toward the basement membrane (data not shown). However, only a small population of GFP-positive cells expressed ALCAM at P7.5 (Fig. 6D). ALCAM expression was observed in both the Sertoli cell lines (15P1 and M15) and the GFP^-SCF^+ fraction of cells, which contains Sertoli cells, although the expression of ALCAM was weaker than that seen in gonocytes at P0.5 (data not shown). Next, we examined ALCAM expression in testis by immunohistochemistry. At P0.5, we detected punctate signals in gonocytes (arrows) and myoid cells (arrowheads) in many testis cords (Fig. 7A). Some gonocytes showed ALCAM protein accumulated at the side facing the basement membrane (arrowhead in Fig. 7B). At P7.5, neither gonocytes nor myoid cells expressed ALCAM but some cells located away from the basement membrane strongly expressed ALCAM (Fig. 7C). ALCAM protein localized at the interface of adherent cells (arrowheads in Fig. 7D). Isotype control antibody gave no signals in either P0.5 or P7.5 specimens (data not shown). These results suggest that ALCAM may play a role in migration toward the basement membrane at an early stage of prespermatogenesis, but may not be required once prospermatogonia migrated to the designated locations on the basement membrane.

Cell-to-cell adhesion assay

To confirm that ALCAM functions as a cell adhesion molecule on gonocytes, we undertook a cell-to-cell adhesion assay. We first stably introduced ALCAM cDNA into OP9 stroma cells derived from M-CSF-deficient mice, which weakly express ALCAM, by transfection with pRK5-Grasp (Kodama et al., 1994). Overexpression of ALCAM was confirmed by both RT-PCR and FACS analysis (Fig. 8A, data not shown). We sorted an ALCAM-negative population from mock-transfected OP9 cells (Fig. 8B) and an ALCAM-high positive population from OP9/ ALCAM cells (Fig. 8C) and plated them on 96-well plates. Three hours after incubation with either ALCAM-Fc protein to interfere homotypic binding between ALCAM on OP9 cells and gonocytes, or control CD4-Fc protein, sorted GFP-positive gonocytes from P0.5 testes were seeded on ALCAM-positive and -negative OP9 cells, respectively. Then, a cell adhesion assay was performed as described previously (Takakura, et al., 1998). The results indicated that 67% of gonocytes preincubated with CD4-Fc bind to the sorted ALCAM-positive OP9 cells, but the preincubation of gonocytes with ALCAM-Fc protein reduced binding with ALCAM-positive OP9 cells to 47% (Fig. 8D). As control, 31 and 40% of the gonocytes preincubated with either ALCAM-Fc or CD4-Fc, respectively, bind to ALCAM-negative OP9 cells. These results suggest that ALCAM functions as a cell adhesion molecule in germ cells at an initial step of migration around P0.5.

Fig. 8. Cell adhesion analysis by ALCAM. RT-PCR analysis of ALCAM expression was performed (A, left panel): RNA was prepared from mock-transfected OP9 cells (A, lane 1) and ALCAM cDNA-transfected OP9 cells (A, lane 2). RT-PCR was performed as described in Material and methods. RT-PCR analysis of GAPDH expression was also performed as a control (A, right panel). Sorting profiles of ALCAM-negative OP9 cells (B). Mock-transfected OP9 cells were stained with an anti-ALCAM mAb. The ALCAM-negative population (bar, left panel) was sorted. Right histogram shows FACS profile after sorting. No expression of ALCAM was detected. Sorting profiles for OP9 cells highly expressing ALCAM (C). ALCAM-transfected OP9 cells were stained with an anti-ALCAM mAb. The ALCAM-positive population (bar, left panel) was sorted. Right histogram shows ALCAM expression (red line) on the sorted cells used for cell–cell adhesion assay. Cell-to-cell adhesion assay (D). After sorting, ALCAM-negative OP9 cells and ALCAM-positive OP9 cells were incubated with sorted gonocytes from P0.5 that had been incubated with either CD4-Fc or ALCAM-Fc protein. Gonocytes highly expressing ALCAM bind with the sorted ALCAM-positive OP9 cells more efficiently than ALCAM-negative OP9 cells. Preincubation of gonocytes with ALCAM-Fc protein reduce adhesion between gonocytes and OP9 cells. All experiments represent results from triplicate assays. A representative result from two independent experiments is shown.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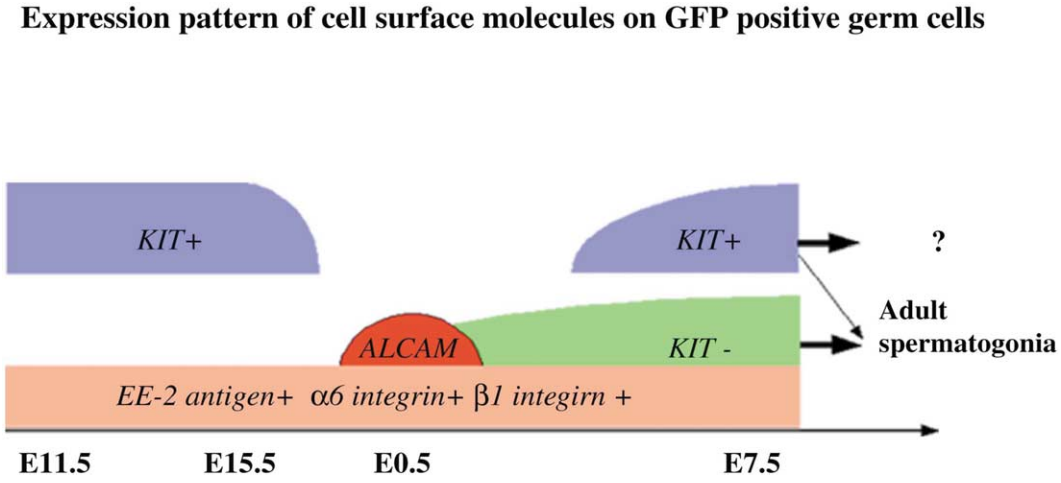

Fig. 9. Summary of the expression pattern of cell surface molecules on GFP^+ germ cells. EE2 antigens, α6 integrin, and β1 integrin are expressed on GFP^+ germ cells continuously during embryonic and neonatal stages. GFP^+ cells are uniformly positive for KIT until E15.5 at which time KIT is downreguated. No GFP^+ gonocytes express KIT at P0.5, two populations (GFP^+KIT^- and GFP^+KIT^+) exist with increasing numbers of cells in both populations. Based on results of the transplantation assay, the GFP^+KIT^- population gives rise to adult spermatogonia, while it is not clear how the GFP^+KIT^+ population, which may retain the potential to contribute to adult spermatogenesis, contributes to adult spermatogenesis. ALCAM is expressed transiently just before and after birth on GFP expressing gonocytes. The fate of $ALCAM^+$ cells existing at center of testis cords at P7.5 is unclear.

Discussion

The aim of this study was to characterize biological properties of germ stem cells at prepubertal stages, separate them from mixed populations, and demonstrate self-renewal activity of the isolated cells in transplantation assays. The present study demonstrates that germ cells are specifically marked by EGFP protein at prepubertal stages in Oct4/EGFP mice. Our findings indicate that prospermatogonia are further subdivided into subpopulation in terms of stem cell activity, GFP^+KIT^- and GFP^+KIT^+ population. The GFP^+KIT^+ population is unique in that it retains immature properties while expressing differentiation markers such as KIT. Summary of the expression pattern of cell surface molecules on GFP-positive germ cells including KIT as shown in Fig. 9.

Characterization of precursor cells of adult spermatogonia

We have shown that the GFP^+KIT^- population is able to repopulate an adult environment more efficiently than the GFP^+KIT^+ population, and that even after 4 months, donor-derived GFP-positive testicular cells were seen in recipient mice. Given that one spermatogenic cycle is approximately 35 days in mice, the transplanted population contains stem cells that mature in an adult environment (Kiger et al., 2001). This is the first evidence that undifferentiated prospermatogonia at prepubertal stages consist of two groups that have different repopulating activity.

The GFP^+KIT^+ population might be a special type of cells appearing only at prepubertal stages. The architecture of the nucleus of the GFP^+KIT^+ cells, i.e., a homogeneous nuclear staining pattern with nuclear vacuolation, is indistinguishable from that of the GFP^+KIT^- cells, while morphology of adult KIT-positive spermatogonia are different from those of adult KIT-negative spermatogonia. In addition, although the adult KIT-positive cells have lost self-renewal activity (Shinohara et al., 2000), we have shown that the GFP^+KIT^+ cells at prepubertal stages retain stem cell activity. One possible explanation accounting for this discrepancy is that the GFP^+KIT^+ population in neonatal spermatogonia contains stem cells. Alternatively, some cells of the GFP^+KIT^+ population could retain the ability to return to the GFP^+KIT^- population, which may be the only population with self-renewal activity. At least if any, there is a possibility that some of the KIT-positive population might give rise to new stem cells at prepubertal stages. This view may be supported by the result that a small number of A4 cells are still capable of producing new stem cells (Clermont and Bustons-Obregon, 1968; Dym and Clermont, 1970).

In the adult, most evidence suggests that germ line stem cells are KIT-negative and their population size is extremely small, i.e., only 0.01% of testicular cells (Meistrich et al., 1993; Tegelenbosch et al., 1993). However, at prepubertal stages, the GFP^+KIT^- population constitutes at least 2.7% of testicular cells (Fig. 2F), a proportion significantly higher than that seen in adults. Moreover, cell cycle kinetics of both the GFP^+KIT^- cells and the GFP^+KIT^+ cells is also quite different from that seen at adult stages. These characteristics, high proportion of GFP-positive prespermatogonia among total testicular cells, and their high proliferating activity at this stage, might be reasons why introduction of exogenous genes by retrovirus-mediated gene transfer is easier at prepubertal stages than at adult. Actually, a re-

porter retrovirus vector was introduced into 2–20% of short-term-cultured P5–P7 prospermatogonia, and transgenic mice were efficiently produced from the cultured prospermatogonia (Nagano et al., 2001). The one reported germ cell stem line, created with mouse telomere reverse transcriptase by using retrovirus vector, was also established from P6 prospermatogonia (Feng et al., 2002).

Regional variation in the level of Oct 4 expression at P7.5 testis cords

Sertoli cells, a compartment of seminiferous epithelium, play an important role as stroma cells in the germ cell system (Schlatt et al., 1997). However, the extent of heterogeneity of germ cells and seminiferous epithelium at prepubertal stages has not been elucidated. In the present study, expression of GFP and Oct4 showed a regional variation at P7.5 (Fig. 1, and unpublished data). Xenograft transplantation studies revealed that heterogeneity of the seminiferous epithelium is controlled by signals from germ cells (de Rooij, 1998). Therefore, seminiferous epithelium containing GFP-positive prospermatogonia might have stroma activity different from that containing GFP-negative prospermatogonia. Recently, regional variation in gene expression in prepubertal Sertoli cells has been reported for *Amh*, *Rbp1*, *galectin1*, *Cts1*, and *Sgp2*, suggesting the existence of heterogeneity of seminiferous epithelium (Timmons et al., 2002). The authors showed that there are at least two different types of seminiferous epithelium at P7.5; one expressing *Amh* and *Rbp1* and the other expressing *galectin1*, *Cts1*, and *Sgp2*. It would be of interest to observe whether one of those gene expression pattern correlate with GFP expression in Oct4/EGFP mice.

ALCAM and cell adhesion

We examined the expression of cell surface molecules expressed on hematopoietic progenitor cells in prospermatogonia because transdifferentiation studies suggest that there are common mechanisms maintaining stem cells from different lineages. In the present study, we showed that one receptor, ALCAM, is expressed on gonocytes. ALCAM is a member of the immunoglobulin superfamily with a short cytoplasmic tail and mediates homotypic ALCAM–ALCAM interactions (Aruffo et al., 1997; Bowen et al., 1997; Nelissen et al., 2000). In the hematopoietic system, ALCAM apparently mediates adhesion between $CD34^{+}$ hematopoietic stem cells and the surrounding bone marrow stroma cells (Cortes et al., 1997; Ohneda et al., 2001; Arai et al., 2002).

In the testes of the neonatal mice, migration of gonocytes is crucial for subsequent development of germ cells (McGuinness et al., 1992). Matrix proteins such as laminin and NCAM are candidate molecules mediating the gonocyte migration (Orth et al., 1995; 1998). Because ALCAM is expressed not only on germ cells but also on Sertoli cells around P0.5, it also may contribute to gonocyte–Sertoli cell interaction and initiation of migration toward the basement membrane. An approach using $ALCAM^{-/-}$ gonocytes would address how ALCAM contributes to cell migration and stem cell activity. Since gonocytes may acquire stem cell activity after attachment to Sertoli cells and basement membrane, the analysis of relocation and adhesive mechanisms will provide important clues to understanding how gonocytes acquire stem cell activity.

Heterogeneity of prospermatogonia

One important question, which should be addressed in the future, is whether germ stem cells characterized by morphological criteria are functionally heterogeneous in terms of stem cell activity. In adult, undifferentiated spermatogonia identified by morphological criteria are supposed to be isolated cells randomly spaced along the tubules (Huckins, 1971). However, Tadokoro et al. (2002) recently showed that only 35% of adult undifferentiated type spermatogonia were Oct4-positive, suggesting that adult undifferentiated spermatogonia seem to be heterogeneous in terms of Oct4 expression. In prepubertal stages, prospermatogonia are also randomly distributed, but only a portion of them is GFP-positive as same as adult. In both adult and neonate, elucidation of the difference of stem cell activity between an Oct4-positive and an Oct4-negative fraction is necessary and the study is in progress. In order to purify the definitive stem cell fraction, it will be important to identify markers that are differentially expressed along the germ stem cell population, purify the germ stem cells by the expression profile of the markers, and confirm stem cell activity of the each fraction by transplantation assays.

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References

Anderson, R., Fassler, R., Georges-Labouesse, E., Hynes, R.O., Bader, B.L., Kreidberg, J.A., Schaible, K., Heasman, J., Wylie, C., 1999. Mouse primordial germ cells lacking beta1 integrins enter the germline but fail to migrate normally to the gonads. Development 126, 1655–1664.

Arai, F., Ohneda, O., Miyamoto, T., Zhang, X.Q., Suda, T., 2002. Mesenchymal stem cells in perichondrium express activated leukocyte cell adhesion molecule and participate in bone marrow formation. J. Exp. Med. 195, 1549–1563.

Aruffo, A., Bowen, M.A., Patel, D.D., Haynes, B.F., Starling, G.C., Gebe, J.A., Bajorath, J., 1997. CD6-ligand interactions: a paradigm for SRCR domain function? Immunol. Today 18, 498–504.

Bellve, AR., Cavicchia, J.C., Millette, C.F., O'Brien, D.A., Bhatnagar, Y.M., Dym, M., 1977. Spermatogenic cells of the prepubertal mouse. Isolation and morphological characterization. J. Cell Biol. 74, 68–85.

Bowen, M.A., Bajorath, J., D'Egidio, M., Whitney, G.S., Palmer, D., Kobarg, J., Starling, G.C., Siadak, A.W., Aruffo, A., 1997. Characterization of mouse ALCAM (CD166): the CD6-binding domain is conserved in different homologs and mediates cross-species binding. Eur. J. Immunol. 27, 1469–1478.

Brehm, A., Ovitt, C.E., Scholer, H.R., 1998. Oct-4: more than just a POUerful marker of the mammalian germline? APMIS 106, 114–124.

Clermont, Y., Perrey, B., 1957. Quantative study of the cell population of the seminiferous tubules in immature rats. Am. J. Anat. 100, 241–268.

Clermont, Y., Bustons-Obregon, E., 1968. Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "in toto." Am. J. Anat. 122, 237–247.

Cortes, F., Deschaseaux, F., Uchida, N., Labastie, M.C., Friera, A.M., He, D., Charbord, P., Peault, B., 1997. HCA, an immunoglobulin-like adhesion molecule present on the earliest human hematopoietic precursor cells, is also expressed by stromal cells in blood-forming tissues. Blood 89, 2706–2716.

de Rooij, D.G., 1998. Stem cells in the testis. Int. J. Exp. Pathol. 79, 67–80.

de Rooij, D.G., 2001. Proliferation and differentiation of spermatognonial stem cells. Reproduction 121, 347–354.

Dym, M., Clermont, Y., 1970. Role of spermatogonia in the repair of the seminiferous epithelium following x-irradiation of the rat testis. Am. J. Anat. 128, 265–282.

Dzierzak, E., 1999. Embryonic beginnings of definitive hematopoietic stem cells. Ann. N. Y. Acad. Sci. 872, 256–262.

Feng, L.X., Chen, Y., Dettin, L., Reijo Pera, R.A., Herr, J.C., Goldberg, E., Dym, M., 2002. Generation and in vitro differentiation of a spermatogonial cell line. Science 297, 392–395.

Fuchs, E., Segre, J.A., 2000. Stem cells: a new lease on life. Cell 100, 143–155.

Gomperts, M., Wylie, C., Heasman, J., 1994. Primordial germ cell migration. Ciba Found. Symp. 182, 121–134.

Hasthorpe, S., Barbic, S., Farmer, P.J., Hutson, J.M., 1999. Neonatal mouse gonocytes proliferation assayed by an in vitro clonogenic method. J. Reprod. Fertil. 116, 335–344.

Hilscher, B., Hilscher, W., Bulthoff-Ohnolz, B., Kramen, U., Birke, A., Pelzer, H., Gauss, G., 1974. Kinetics of gametogenesis. I. Comparative histological and autoradiographic studies of oocytes and transitional prospermatogonia during oogenesis and prespermatogenesis. Cell Tissue Res. 154, 443–470.

Huckins, C., Clermont, Y., 1968. Evolution of gonocytes in the rat testis during late embryonic and post-natal life. Arch. Anat. Hist. Embryol. 51, 343–354.

Huckins, C., 1971. The spermatogonial stem cell population in adult rats. II. A radioautographic analysis of their cell cycle properties. Cell Tissue Kinet. 4, 313–334.

Kiger, A.A., Fuller, M.T., 2001. Male germ-line cells, in: Marshak, D.R., Gardner, R.L., Gottlieb, D. (Eds.), Stem Cell Biology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 149–187.

Kodama, H., Nose, M., Niida, S., Nishikawa, S., Nishikawa, S., 1994. Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stroma cells. Exp. Hematol. 22, 979–984.

Koshimizu, U., Nishioka, H., Watanabe, D., Dohmae, K., Nishimune, Y., 1995. Characterization of a novel spermatogenic cell antigen specific for early stages of germ cells in mouse testis. Mol. Reprod. Dev. 40, 221–227.

McGuinness, M.P., Orth, J.M., 1992. Gonocytes of male rats resume migratory activity postnatally. Eur. J. Cell Biol. 59, 196–210.

McLaren, A., 1998. Gonad development: Assembling the mammalian testis. Curr. Biol. 8, R175–R177.

Meistrich, M.L., van Beek, M.E.A.B., 1993. In: Cell and Molecular Biology of the Testis, Desjardins, S., Ewing, L.L., (Eds.), Oxford Univ. Press, New York, pp. 251–257.

Morrison, S.J., Hemmati, H.D., Wandycz, A.M., Weissman, I.L., 1995. The purification and characterization of fetal liver hematopoietic stem cells. Proc. Natl. Acad. Sci. USA 92, 10302–10306.

Nagano, M., Brinster, C.J., Orwig, K.E., Ryu, B.Y., Avarbock, M.R., Brinster, R.L., 2001. Transgenic mice produced by retroviral transduction of male germ-line stem cells. Proc. Natl. Acad. Sci. USA 98, 13090–13095.

Nakauchi, H., Takano, H., Ema, H., Osawa, M., 1999. Further characterization of CD34-low/negative mouse hematopoietic stem cells. Ann. N. Y. Acad. Sci. 872, 57–6.

Nelissen, J.M.D.T., Peters, I.M., de Grooth, B.G., van Kooyk, Y., Figdor, C.G., 2000. Dynamic regulation of activated leukocyte cell adhesion molecule-mediated homotypic cell adhesion through the actin cytoskeleton. Mol. Biol. Cell 11, 2057–2068.

Ogawa, T., Dobrinski, I., Avarbock, M.R., Brinster, R., 2000. Transplantation of male germ line stem cells restores fertility in infertile mice. Nat. Med. 6, 29–34.

Ohneda, O., Ohneda, K., Arai, F., Lee, J., Miyamoto, T., Fukushima, Y., Dowbenko, D., Lasky, L.A., Suda, T., 2001. ALCAM (CD166): its role in hematopoietic and endothelial development. Blood 98, 2134–2142.

Orth, J.M., Jester, W.F., 1995. NCAM mediates adhesion between gonocytes and Sertoli cells in cocultures from testes of neonatal rats. J. Androl. 16, 389–399.

Orth, J.M., 1998. Gonocytes-Sertoli cell interaction in testes of neonatal rats, in: Zirkein, B.R. (Ed.), Germ Cell Development, Division, Disruption and Death, Springer-Verlag, New York, 11–18.

Osawa, M., Hanada, K., Hamada, H., Nakauchi, H., 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273, 242–245.

Ovitt, C.A., Scholer, H.R., 1998. The molecular biology of Oct4 in the early embryo. Mol. Hum. Reprod. 4, 1021–1031.

Pesce, M., Wang, X., Wolgemuth, D.J., Scholer, H.R., 1998. Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. Mech. Dev. 71, 89–98.

Pesce, M., Scholer, H.R., 2000. Oct-4: control of totipotency and germline determination. Mol. Reprod. Dev. 4, 452–457.

Schlatt, S., Meinhardt, A., Nieschlag, E., 1997. Paracrine regulation of cellular interaction in the testis: factors in search of a function. Eur. J. Endocrinol. 137, 107–117.

Shinohara, T., Avarbock, M.R., Brinster, R.L., 1999. β1- and α6-integrin are surface markers on mouse spermatogonial stem cells. Proc. Natl. Acad. Sci. USA 96, 5504–5509.

Shinohara, T., Orwig, K.E., Avarbock, M.R., Brinster, R.L., 2000. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc. Natl. Acad. Sci. USA 97, 8346–8351.

Shinohara, T., Orwig, KE., Avarbock, MR., Brinster, RL., 2001. Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc. Natl. Acad. Sci. USA 98, 6186–6191.

Spradling, A., Drummond-Barbosa, D., Toshie, K., 2001. Stem cells find their niche. Nature 414, 98–104.

Tadokoro, Y., Yomogida, K., Ohta, H., Tohda, A., Nishimune, Y., 2002. Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. Mech. Dev. 113, 29–39.

Takakura, N., Huang, X.L., Naruse, T., Hamaguchi, I., Dumont, D.J., Yancopoulos, G.D., Suda, T., 1998. Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. Immunity 9, 677–686.

Tanaka, H., Pereira, L.A., Nozaki, M., Tsuchida, J., Sawada, K., Mori, H., Nishimune, Y., 1997. A germ cell-specific nuclear antigen recognized by a monoclonal antibody raised against mouse testicular germ cells. Int. J. Androl. 20, 361–366.

Tegelengosch, R.A., de Rooji, D.G., 1993. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat. Res. 290, 193–200.

Timmons, P.M., Rigby, P.W.J., Poirier, F., 2002. The murine seminiferous epithelial cycle is pre-figured in the Sertoli cells of the embryonic testis. Development 129, 635–647.

Yeom, Y.I., Fuhrmann, G., Ovitt, C.E., Brehm, A., Ohbo, K., Gross, M., Hubner, K., Scholer, H.R., 1996. Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development 122, 881–894.

Yoshimizu, T., Sugiyama, N., De Felice, M., Yeom, Y.I., Ohbo, K., Masuko, K., Obinata, M., Abe, K., Scholer, H.R., Matsui, Y., 1999. Germline-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in mice. Dev. Growth. Differ. 41, 675–684.

Yoshinaga, K., Nishikawa, S., Ogawa, M., Hayashi, S., Kunisada, T., Fujimoto, T., Nishikawa, S., 1991. Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. Development 113, 689–699.

Weissman, I.L., 2000a. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science 287, 1442–1446.

Weissman, I.L., 2000b. Stem cells: units of development, units of regeneration, and units in evolution. Cell 100, 157–168.