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—Original Article—

CD2 is a surface marker for mouse and rat spermatogonial stem cells

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Abstract. The spermatogonial stem cell (SSC) population in testis is small, and the lack of SSC markers has severely handicapped research on these cells. During our attempt to identify genes involved in SSC aging, we found that CD2 is expressed in cultured SSCs. Flow cytometric analysis and spermatogonial transplantation experiments showed that CD2 is expressed in SSCs from mature adult mouse testes. Cultured SSCs transfected with short hairpin RNAs (shRNAs) against CD2 proliferated poorly and showed an increased frequency of apoptosis. Moreover, functional analysis of transfected cells revealed impairment of SSC activity. Fluorescence activated cell sorting and spermatogonial transplantation experiments showed that CD2 is expressed not only in mouse but also in rat SSCs. The results indicate that CD2 is a novel SSC surface marker conserved between mouse and rat SSCs.

Key words: CD2, Spermatogonial stem cells, Transplantation

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Spermatogenesis is a biologically unique process of producing gametes from spermatogonial stem cells (SSCs) [1, 2]. SSCs reside on the basement membrane of the seminiferous tubules, and slowly divide to reproduce; during that process, they produce committed progenitors that undergo a limited number of divisions before entering meiosis. Spermatocytes migrate through the blood-testis barrier and form haploid cells in the adluminal compartment. Spermatozoa are eventually released from the testes during ejaculation. Single SSCs produce a large number of spermatozoa, and SSCs in the testis comprise 0.02–0.03% of the total male germ cells in mice [2, 3]. Because SSCs are morphologically indistinguishable from committed progenitor spermatogonia, identification of SSC markers would facilitate the analysis of this unique cell population. In particular, cell surface markers enable use of fluorescence-activated cell sorting (FACS) to collect candidate cell populations from any donor testes for spermatogonial transplantation, which is the most reliable functional assay for SSCs [4].

More than 10 SSC markers have been identified in mice [5]. Although attempts to find SSC-specific markers have not been successful, combining these markers by multiparameter cell sorting by FACS has enabled significant enrichment of SSCs. Initial studies used heat-induced germ cell degeneration and cryptorchid mice, which only have undifferentiated spermatogonia [6]; these germ cells are kept in a physiologically abnormal environment, which may influence their phenotype and function. However, it is now possible to collect a cell population from mature wild-type mice that is significantly enriched in SSCs. By exploiting MCAM (melanoma cell adhesion molecule) expression, we recovered one SSC for every five cells [7]. Although complete mouse SSC purification has not been achieved, SSC enrichment provides a variety of experimental opportunities, including with respect to molecular characterization or genetic manipulation.

While mouse SSCs have been extensively characterized, little is known about SSCs in other animal species. Although rat SSCs can undergo spermatogenesis in mouse testis [8], their biological characteristics are significantly different from those of mice. For example, because rat testis is very vulnerable to high body temperature, so the cryptorchid technique cannot be used to collect SSCs [9]. Moreover, their growth characteristics are still unknown. Rat SSCs proliferate more rapidly than mouse SSCs in the mouse seminiferous tubules [10]. Rat donor-derived colonies expanded by an average of 85.8 μm/day compared to 31.2 μm/day for mouse colonies. However, rat SSCs respond only weakly to SSC self-renewal factors (GDNF and FGF2) *in vitro*, and their doubling time is about ~2 weeks, while that of mouse SSCs is 2.5 days [11, 12]. Characterization of rat SSCs is limited by the paucity of cell surface markers. To date, only EPCAM, THY-1, and CD9 have been reported to be expressed on rat SSCs [13, 14]. Both THY-1 and CD9 are expressed on testicular somatic cells. EPCAM is better than these markers in terms of specificity, but it is relatively widely expressed in germ cell populations [15]. More effort is needed to elucidate the differences in SSC characteristics among different species.

In this study, we report that CD2 is a marker for mouse and rat SSCs. While studying the molecular mechanism of SSC aging, we identified CD2 on SSCs. CD2 belongs to the immunoglobulin superfamily and mediates cell adhesion [16]. It is normally found on T cells and natural killer cells, but not on B cells. It interacts with CD48, an immunoglobulin superfamily protein with a glycophosphatidylinositol anchor [17]. CD2-expressing cells were collected from mouse and rat testes and its expression on SSCs was confirmed by spermatogonial transplantation.

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Materials and Methods

Animals and cell culture

Germline stem (GS) cells used in the present study were derived from the transgenic C57BL/6 Tg14(act-EGFP)OsbY01 mouse line (designated green) [18]. Where indicated, we also used GS cell lines derived from green mice on a DBA/2 background (a gift from Dr M Okabe of Osaka University, Osaka, Japan) [12,19]. For SSC enrichment studies, testis cells were collected from the transgenic mouse line B6,129-TgR(ROSA26)26SOR (designated ROSA26; Jackson Laboratory, Bar Harbor, ME, USA). For rat experiments, we used the transgenic rat line TgN(act-EGFP)Osb4 (Japan SLC, Shizuoka, Japan). For short hairpin RNA (shRNA)-mediated gene knockdown (KD), all KD vectors were purchased from Open Biosystems (Huntsville, AL, USA) (Supplementary Table 1: online only), and a mixture of lentiviral particles was used for transfection; pLKO1-Scramble shRNA was used as the control (Addgene, Cambridge, MA, USA). Preparation of virus particles was described previously [20]. Virus concentration was measured using a Lenti-X p24 Rapid Titer Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. The multiplicity of infection (moi) was adjusted to 15.0.

Immunostaining procedure

For the staining of GS cells, cells were dissociated into single cells by incubation in trypsin (Sigma, St. Louis, MO, USA). The cells were concentrated on glass slides by centrifugation using a Cytospin 4 unit (Thermo Electron, Cheshire, UK). The slides were then fixed in 4% paraformaldehyde for 10 min on ice and treated with 0.1% Triton-X and 4.7% sodium citrate in phosphate-buffered saline (PBS).

For immunostaining of testes and spleens, samples were collected from 6-week-old C57BL/6 (B6) mice, and fixed in 4% paraformaldehyde for 2 h at 4°C before embedding in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) for cryosectioning. For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, cells were stained with an In Situ Cell Death Detection Kit: TMR Red (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Hoechst 33342 was used for counterstaining. The antibodies used for immunostaining are listed in Supplementary Table 2 (online only).

Magnetic cell sorting (MACS)

Testis cells were dissociated by collagenase type II digestion (1 mg/ml; Sigma), as described previously [21]. For enrichment of mouse SSCs, the dissociated testis cells were incubated with an anti-CDH1 antibody (ECCD2; gift from Dr M Takeichi from RIKEN CDB, Kobe, Japan), and cells were isolated using magnetic beads, as described previously (Miltenyi Biotec, Gladbach, Germany) [14]. For enrichment of rat SSCs, we used an anti-CD9 antibody (RPM.7; BD Biosciences, Franklin Lakes, CA, USA).

Flow cytometry and FACS

For characterization by flow cytometry, we dissociated GS cells by incubation in Cell Dissociation Buffer (Thermo Fisher Scientific, Waltham, MA, USA). The cells were analyzed by FACSCalibur (BD Bioscience). In experiments using thymocytes, thymi were dissociated by mechanical dissociation using glass slides. For FACS, CDH1 (mouse) or CD9 (rat)-selected cells were incubated with an allophycocyanin (APC)-conjugated anti-mouse CD2 (RM2-5) or phycoerythrin (PE)-conjugated anti-rat CD2 antibody (OX-34; both from BioLegend, San Diego, CA, USA), respectively. For CD109 selection, we used PE-conjugated mouse anti-human CD109 antibody (496920; R&D Systems, Minneapolis, MN, USA). The cells were sorted using a FACSAria III (BD Biosciences). Forward scatter values and propidium iodide were used to exclude dead cells. The antibodies used for flow cytometry are listed in Supplementary Table 2.

Polymerase chain reaction (PCR) analyses

Total RNA was recovered using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was produced using a Verso cDNA synthesis kit for reverse transcription (Thermo Fisher Scientific). For real-time PCR, the CFX Connect™ Real Time System (Bio-Rad, Hercules, CA, USA) and FastStart Universal SYBR Green Master were used according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Transcript levels were normalized to that of *Hprt.* The PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The PCR primer sequences are listed in Supplementary Table 3 (online only).

Spermatogonial transplantation

For transplantation of mouse donor cells, 4-week-old B6 × DBA/2 F1 (BDF1) recipient mice received intraperitoneal injection of busulfan (44 mg/kg). For transplantation of rat donor cells, we treated 4-week-old KSN nude mice with busulfan (44 mg/kg) and used them for transplantation to prevent immunological rejection (Japan SLC). To avoid bone marrow failure, KSN nude mice were transplanted with homologous bone marrow cells 2–5 days after busulfan treatment. At least 1 month after busulfan injection, single cell suspensions were microinjected into the seminiferous tubules of busulfan-treated mice via the efferent duct, as described previously [22]. Each injection filled 75–85% of the seminiferous tubules. The Institutional Animal Care and Use Committee of Kyoto University approved all animal experimentation protocols.

Analysis of the recipient testes

Recipient mice were sacrificed 2 (mouse donor) or 3 months (rat donor) post-transplantation. In experiments using ROSA26 mice, testes were incubated with 5-bromo-4-chloro-3-indolyl β-D-galactoside after fixation with 4% paraformaldehyde for 2 h, as described previously [23]. In experiments using green mouse or rat derived cells, donor cell colonies were counted under UV light. Donor germ cell clusters were defined as colonies if the entire basal surface of the tubule was occupied and the colonies were at least 0.1 mm in length [23].

Statistical analysis

Results are presented as the mean ± SEM. Data were analyzed by Student's *t*-test. Multiple comparisons were conducted by analysis of variance followed by Tukey's HSD test.

Results

CD109 and CD2 expression in SSCs

We recently reported that GS cells cultured for 60 months (60M-GS cells) proliferate more actively than those cultured for 5 months (5M-GS cells) [19]. Because the 60M-GS cells showed markers of differentiating spermatogonia more strongly, we examined whether 5M-GS cells exhibit markers more specific for SSCs. We particularly focused on cell surface markers because they facilitate cell collection and function as receptors for ligands. We analyzed previous microarray data and found that CD109 and CD2 are more strongly expressed in young GS cells. Aged GS cells showed more significant downregulation of CD109 (from 539.2 to 94.1) compared to CD2 (from 680.1 to 176.5)[19]. Because these data were based on RNA levels, we examined the expression of CD109 and CD2 in GS cells by flow cytometry. Consistent with the RNA analyses, 5M-GS cells expressed CD109 more strongly than did 60M-GS cells. However, only one of the 60M-GS cell lines showed downregulation of CD2 (Fig. 1A); the other line did not show a significant change in CD2 expression levels, suggesting that CD2 is not associated with aging.

Although GS cells are enriched in SSCs, the frequency of SSCs is very low (1–2%). Therefore, we carried out spermatogonial

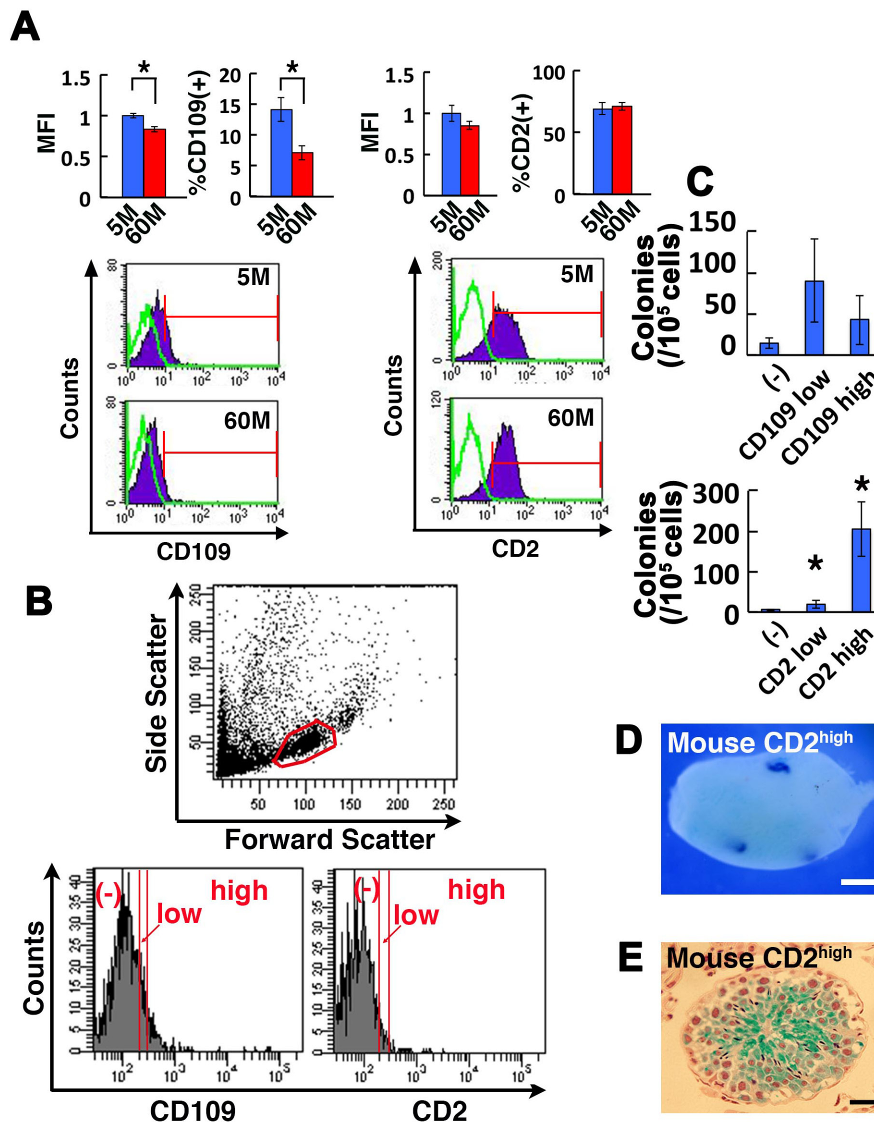

Fig. 1. CD2 expression in mouse spermatogonial stem cells (SSCs). (A) Flow cytometric analysis of CD109 and CD2 expression in germline stem (GS) cells cultured for 5 months (5M-GS) and GS cells cultured for 60 months (60M-GS cells) (n = 4). Green lines indicate the control. (B) Flow cytometric analysis of wild-type mouse testis cells based on CD109 and CD2 after magnetic cell sorting (MACS) selection with an anti-CDH1 antibody. (C) Colony count (n = 6 for CD109; n = 12 for CD2). (D) Macroscopic appearance of recipient testis transplanted with CD2^{high} ROSA testis cells. (E) Histological appearance of recipient testis. Bar = 1 mm (D), 50 µm (E). Asterisk indicates statistical significance ($P < 0.05$). MFI, mean fluorescence intensity.

transplantation to confirm CD109 expression on SSCs. We collected undifferentiated spermatogonia by MACS using an antibody against CDH1; 0.5 ± 0.2% (mean ± SEM; n = 3) of the total testis cells were recovered. We carried out two sets of spermatogonial transplantation experiments using these cells (Fig. 1B). In the first set of experiments, we used an anti-CD109 antibody and collected cells with different levels of CD109 expression. $CD109^+$ cells comprised 2.3 ± 0.9% (mean ± SEM; n = 3) of the CDH1-selected cells, suggesting that only a small proportion of undifferentiated spermatogonia express this molecule. We divided the $CD109^+$ cells into two equal-sized populations. The cells were then transplanted into the seminiferous tubules of infertile mice. Two months after transplantation, the recipient mice were euthanized, and their testes were stained with X-gal to detect donor cell-derived colonies. Quantification of the number of colonies showed that $CD109^{high}$, $CD109^{low}$, and $CD109^-$ cells produced 42.6, 89.9, and 14.5 colonies per 10^5 cells (n = 6; Fig. 1C). Although the number of colonies was slightly higher for $CD109^{low}$ cells, there was no significant difference between this population and control cells (0.7 per 10^5 transplanted cells).

In the second set of experiments, we used an anti-CD2 antibody for FACS. $CD2^+$ cells comprised 0.9 ± 0.3% (mean ± SEM; n = 3) of the CDH1-selected cells (Fig. 1B). We fractionated the $CDH1^+$ cells using an anti-CD2 antibody in the same manner as for CD109, and carried out spermatogonial transplantation to determine the SSC activity of individual populations. Analysis of recipient testes showed that $CD2^{high}$ cells produced the largest number of colonies (Fig. 1C, D). The number of colonies generated by $CD2^{high}$, $CD2^{low}$, and $CD2^-$ cells generated 204.3, 18.4, and 5.2 colonies per 10^5 transplanted cells, respectively (n = 12). Only the $CD2^{high}$ population showed significant enrichment of SSCs compared to the unfractionated control testis cells (0.7 per 10^5 transplanted cells). Histological analysis of the recipient testes showed normal appearing spermatogenesis generated from the $CD2^{high}$ cells (Fig. 1E). These results showed that CD2 is expressed in SSCs.

Expression of CD2 in GS cells and testis

Although the data suggested that SSCs express CD2, CD2 expression was not detected by RNA sequencing analysis of fresh spermatogonia in a previous study [24]. Because these experiments were conducted using B6 mice, we assessed the expression of CD2 in B6 GS cells. Flow cytometric analysis showed that CD2 is expressed similarly in GS cells in B6 and DBA backgrounds at comparable levels (Fig. 2A). The lack of CD2 expression revealed by RNA-sequencing of spermatogonia in B6 mice suggested that CD2 is upregulated during in vitro culture. As expected, GS cells exhibited significantly increased CD2 expression following cytokine starvation and self-renewal factor (FGF2 and GDNF) supplementation (Fig. 2B). These results suggested that CD2 expression is influenced by the extent of self-renewal factor stimulation.

Because SSCs in fresh testis-cell suspension showed the expression of CD2, we carried out immunostaining of the testis. Although we were able to find CD2 expression by flow cytometry, we were not able to find CD2 expression in germ cells (Fig. 2C). Because CD2 expression was increased after self-renewal factor stimulation, we also stained recipient testes transplanted with donor germ cells. However, no signal was detected in germ cells in the recipient testes (Fig. 2D). Instead, CD2 was expressed in interstitial areas of the testis. We also stained the spleen as a positive control, and this staining clearly showed that CD2 is expressed in spleen.

In mice, CD2 binds to CD48. CD48 is a member of the CD2 family and is expressed in lymphocytes, dendritic cells, and endothelial cells. Because CD2 was found in SSCs, we examined its expression in testis. Immunostaining revealed that CD48 is expressed in both $CDH1^+$ undifferentiated spermatogonia and KIT^+ differentiating spermatogonia (Fig. 2E, F). CD48 expression was weak in $SYCP3^+$ spermatocytes but was not detected at all in somatic cells. Because CD48 is expressed in germ cells in vivo, we subjected GS cells to flow cytometry. Although CD48 expression on thymocytes was detected, GS cells did not show CD48 expression (Fig. 2G). Therefore, CD2 expressed by SSCs may receive signals from CD48 on adjacent germ cells.

Phenotypic analysis of GS cells after shRNA transfection

To examine the impact of CD2 expression in SSCs, we carried out shRNA transfection experiments. GS cells transfected with *Cd2* shRNAs showed less proliferation than control cells (Fig. 3A–C). While control cells proliferated by ~6.3-fold over 7 days, GS cells transfected with *Cd2* KD cells proliferated by ~2.3-fold during the same period (Fig. 3C). Because immunostaining using an anti-MKI67 antibody (a marker for cell proliferation) showed no significant difference in the number of $MKI67^+$ cells (Fig. 3D), we examined whether the low proliferation rate was due to increased apoptosis and carried out TUNEL assay. As expected, GS cells transfected with *Cd2* shRNAs showed an increased frequency of apoptosis. While 19.3% were $TUNEL^+$ in control cells, 83.5% of GS cells transfected with *Cd2* shRNAs were $TUNEL^+$ (Fig. 3E).

To examine the impact of CD2 on the SSC phenotype, we carried out flow cytometry. We found that *Cd2* KD significantly reduced the number of GS cells showing GFRA1 or CDH1 expression, suggesting an impact on undifferentiated spermatogonia (Fig. 3F). Real-time PCR also indicated downregulation of *Nanos2* and *Nanos3* by *Cd2* depletion (Fig. 3G). *Neurog3*, *Foxo1* and *Etv5* genes were also downregulated, albeit not significantly. Because GFRA1 is a receptor component of GDNF and *Nanos2* is expressed in A_{single} or A_{paired} undifferentiated spermatogonia, these results suggested a role for CD2 in SSCs.

Functional analysis of GS cells after shRNA transfection

To determine the impact of CD2 on SSC activity in a functional manner, we carried out spermatogonial transplantation experiments. B6 GS cells were transfected with shRNAs against *Cd2*, and the cells were transplanted into the seminiferous tubules of busulfan-treated mice. The number of colonies generated by *Cd2* KD and control cells were 1.8 and 4.6 per 10^5 transplanted cells, respectively (n = 12; Fig. 4A, B); the difference was statistically significant. While control cells could reinitiate spermatogenesis, poor colonization was seen in recipients of GS cells transfected with *Cd2* shRNAs (Fig. 4C). These results indicate that *Cd2* depletion compromises SSC function.

Expression of CD2 in rat SSCs

Because only a few SSC markers were identified in rat SSCs, we examined the expression of CD2 in rat SSCs. We used green rats,

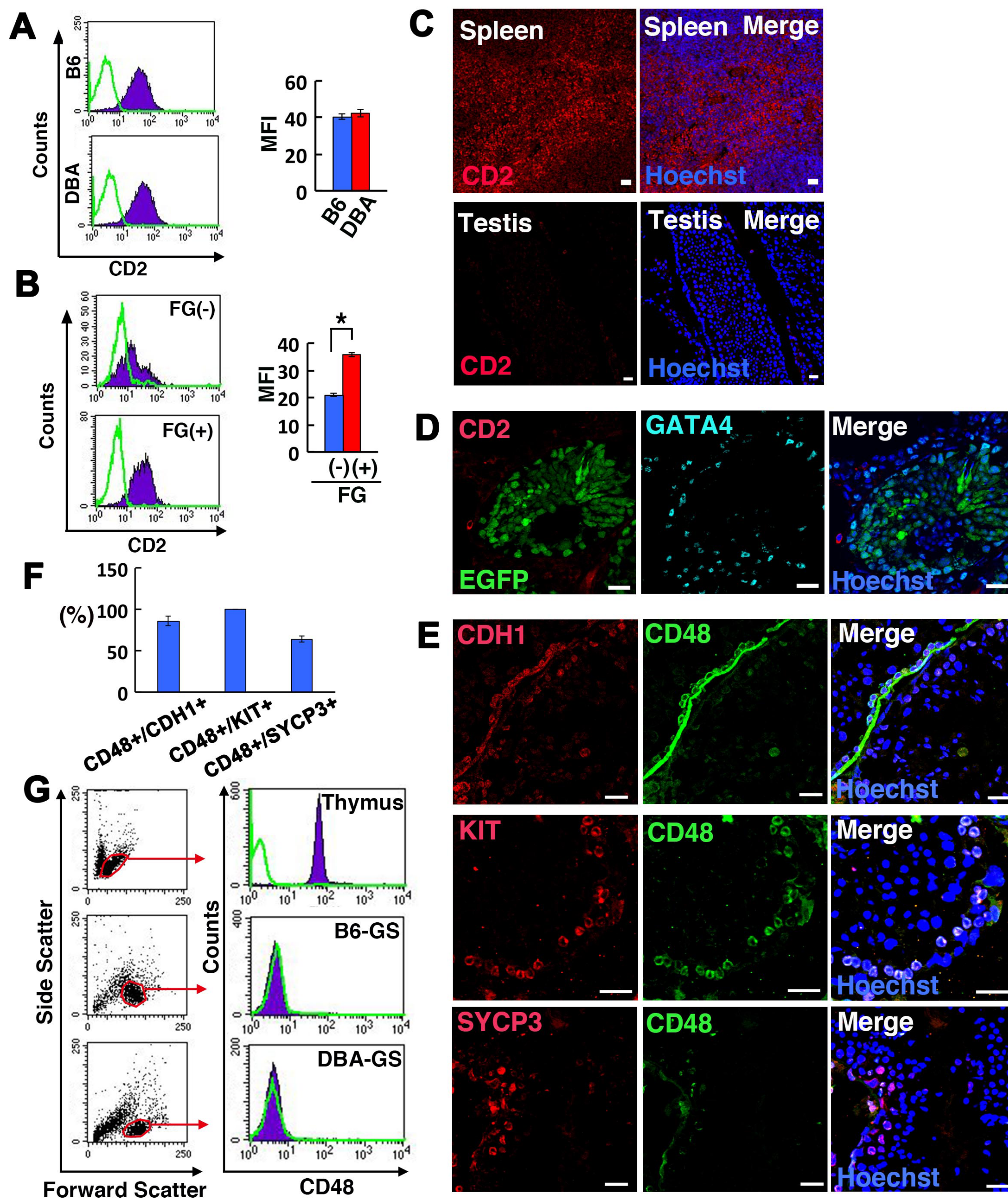

Fig. 2. CD2 expression in germline stem (GS) cells and testis. (A) Flow cytometric analysis of CD2 expression in B6- and DBA-GS cells (n = 4). Green lines indicate the control. (B) Flow cytometric analysis of CD2 induction by self-renewal factors (n = 4). Following cytokine withdrawal for 1 day, GS cells were stimulated with FGF2 and GDNF (FG), and analyzed 3 days later. (C, D) Immunostaining of CD2 in wild-type (C) or recipient (D) mouse testes. Spleen was used as the positive control (C). (E) Immunostaining of CD48 in wild-type mouse testis. (F) Quantification of $CD48^+$ germ cells in seminiferous tubules (n = 39–44 tubules). (G) Flow cytometric analysis of CD48 expression in GS cells. Thymocytes were used as the positive control. Bar = 20 µm (C–E). Asterisk indicates statistical significance ($P < 0.05$). MFI, mean fluorescence intensity.

which express *Egfp* gene ubiquitously, including spermatogenic cells [14]. We collected testes from ~3–4-week-old rats and carried out immunostaining for CD2 (Fig. 5A). Like mouse testes, we were unable to detect CD2 in rat spermatogenic cells; but a relatively strong signal was found in cells outside of the seminiferous tubules.

Although these results suggested a lack of CD2 expression in rat SSCs, we analyzed CD2 expression by FACS because CD2 was also not detected in mouse germ cells by immunostaining of histological sections. Testis cells were stained with an antibody against CD9 antigen, which is conserved in mouse and rat SSCs [14]. MACS

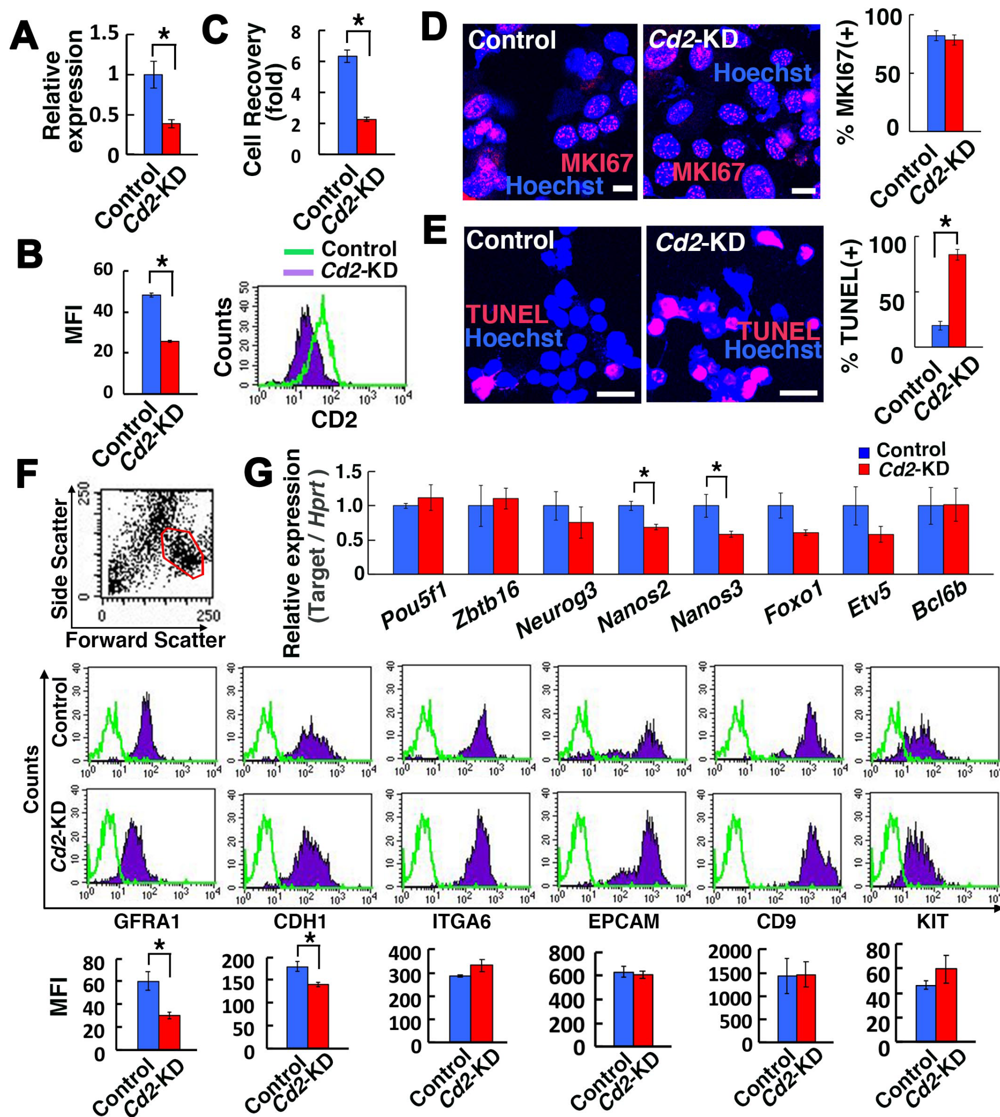

Fig. 3. Phenotype of germline stem (GS) cells following transfection of short hairpin RNA (shRNA) against *Cd2*. (A) Real-time PCR analysis of *Cd2* expression following transfection of shRNAs against *Cd2* 3 days after transfection (n = 4). (B) Flow cytometric analysis of CD2 expression 3 days after *Cd2* knockdown (KD) (n = 3). (C) Proliferation of GS cells after *Cd2* KD (n = 4). GS cells were transfected with shRNA and passaged at 7 days. Cell recovery was determined 7 days after the passage. (D) Immunostaining of MKI67 3 days after *Cd2* KD (n = 4). (E) Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining of GS cells 3 days after *Cd2* KD (n = 4). (F) Flow cytometric analysis of spermatogonia markers 3 days after *Cd2* KD (n = 3). (G) Real-time PCR analysis of spermatogonia marker genes 3 days after *Cd2* KD (n = 3). Bar = 20 µm (D, E). Asterisk indicates statistical significance (P < 0.05). MFI, mean fluorescence intensity.

selection was next performed to enrich CD9-expressing cells. After MACS selection, 1.3 ± 0.3% (mean ± SEM; n = 3) of total cells were recovered. Flow cytometry showed that 91.0 ± 12.5% (mean ± SEM; n = 3) of CD9-selected cells expressed CD2 (Fig. 5B).

We next carried out spermatogonial transplantation. We fractionated the MACS-selected $CD9^+$ cells into three fractions according to the CD2 expression level. The cells with a relatively strong CD2 signal were separately collected as $CD2^{high}$ cells. The other CD2-expressing cells were collected as $CD2^{low}$ cells. The cells were transplanted into busulfan-treated nude mice. Analysis of recipient mice at 3 months after transplantation revealed that $CD2^{low}$ cells are significantly enriched in SSCs (Fig. 5C). The number of colonies generated from $CD2^-$, $CD2^{low}$, $CD2^{high}$ and control cells was 0, 95.0, and 62.5 per 10^5 transplanted cells, respectively (n = 10; Fig. 5D). Only $CD2^{low}$ cells showed significant enrichment compared to control cells (3.0 per 10^5 transplanted cells). Immunostaining of recipient testes showed that $CD2^{low}$ cells regenerated normal-appearing spermatogenesis (Fig. 5E). These result show that rat SSCs express CD2.

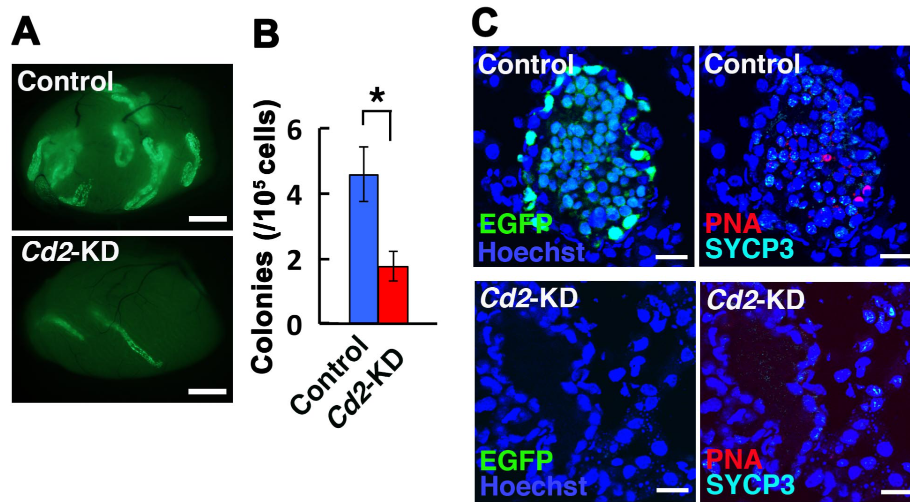

Fig. 4. Functional analysis of CD2 by spermatogonial transplantation. (A) Macroscopic appearance of recipient testis 2 months after transplantation of B6 germline stem (GS) cells with *Cd2* short hairpin RNAs (shRNAs). (B) Colony count (n = 12). (C) Histological appearance of recipient testis. Bar = 1 mm (A), 20 µm (C). Asterisk indicates statistical significance (P < 0.05).

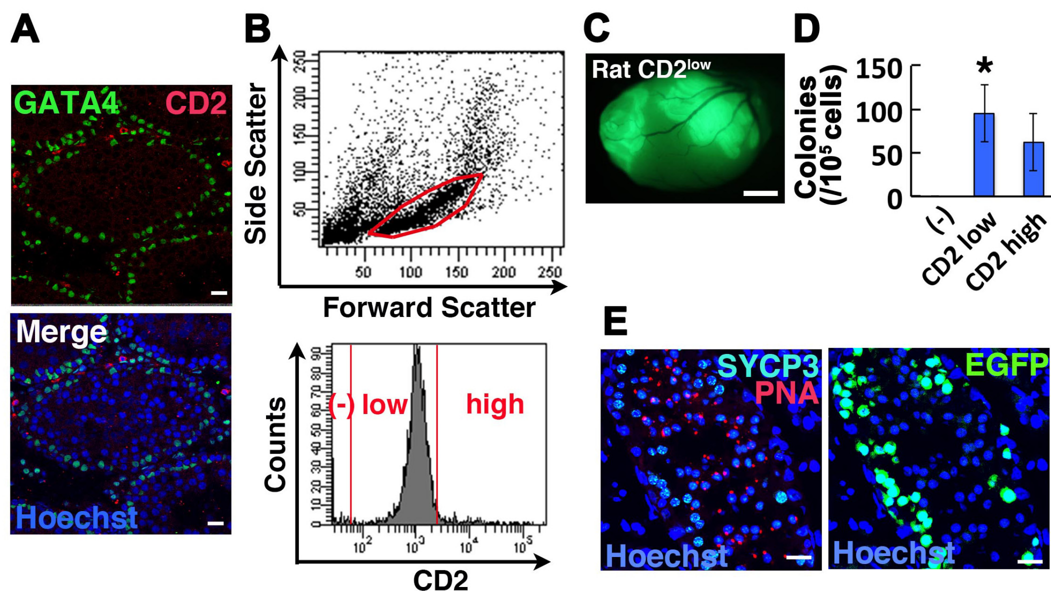

Fig. 5. CD2 expression in rat spermatogonial stem cells (SSCs). (A) Immunostaining of CD2 in rat testis. (B) Flow cytometric analysis of wild-type rat testis cells after magnetic cell sorting (MACS) selection with an anti-CD9 antibody. Note that the percentage of CD2-expressing cells represents proportion of $CD2^+$ cells in the forward scatter/side scatter chart in three separate experiments. (C) Macroscopic appearance of a recipient testis. (D) Colony count (n = 10). (E) Immunostaining of recipient testis. Bar = 1 mm (C), 20 µm (A, E). Asterisk indicates statistical significance (P < 0.05).

Discussion

Because of the rarity of SSCs, the identification of SSC markers is an indispensable step in SSC research. We have identified several cell surface markers, but these are insufficient for obtaining a pure population of SSCs, even in mice [5]. Few markers are available in other animal species, which has hampered our understanding of the difference between the mouse and other animals. Appropriate use of SSC markers is also critical for molecular characterization of SSCs because some markers, such as ITGA6 or THY1, are expressed not only in germ cells but also in somatic cells, which complicates the interpretation. Although these markers are used for collecting and analyzing the gene expression of SSCs, contamination by somatic cells is inevitable. Although the use of several markers in combi-

nation can increase the purity of SSCs, cell recovery is typically significantly reduced due to the multiple steps involved in selection. Thus, identification of new SSC markers with a higher specificity is urgently needed in SSC research.

We initiated this study to find markers that can distinguish young and aged SSCs. In our recent study, we found that 60M-GS cells proliferate more actively than 5M-GS cells. In particular, we focused on cell surface molecules because they facilitate cell collection. Our gene expression analysis showed that CD109 and CD2 are strongly expressed on 5M-GS cells. We carried out spermatogonial transplantation experiments, and found that CD2, but not CD109, is expressed in SSCs. Although flow cytometry revealed that CD2 is not associated with aging of GS cells, its expression in SSCs led us to test its characteristics and function.

CD2 is a cell adhesion molecule and is 55–66 kDa protein [16]. It is glycosylated type I transmembrane protein and belongs to the immunoglobulin superfamily. CD2 is expressed on T cells and natural killer cells and interacts with other adhesion molecules, such as CD48. CD48 transduces signals through CD2, and CD2-CD48 interactions promote T cell activation and immunoglobulin class switching to IgG2a in B cells [25]. The binding of CD2 to CD48 lowers the threshold for T cell activation by specific antigens and facilitates T cell adhesion to antigen presenting cells. CD48 is also expressed by hematopoietic progenitor cells, but not by hematopoietic stem cells (HSCs). It influences HSCs by altering the bone marrow cytokine milieu [26]. Several signal transducing enzymes and adapter proteins have been shown to interact with the intracellular portion of CD2, including CD2AP [27].

Although we were unable to identify CD2 expression in germ cells by immunostaining, flow cytometry showed its expression in $CDH1^{+}$ spermatogonia and FACS experiments confirmed its expression on SSCs. We think that our failure to detect CD2 by immunostaining is due to its relatively poor sensitivity compared to flow cytometry. Quantitative analysis of recipient testes showed that $CD2^{high}$ cells were 291.9-fold enriched for SSCs. Assuming a colonization efficiency of 10% [23], this result suggested that SSCs accounts for 1 in every 48.9 $CD2^{high}$ cells. This value is comparable to that of $EPHA2^{high}$ cells, which are present in a small population of undifferentiated spermatogonia. We recently reported that transplanting this fraction led to approximately 257.2-fold enrichment of SSCs [28]. Therefore, although we did not detect CD2 expression by immunostaining, the FACS results from our FACS studies and downregulation of markers of undifferentiated spermatogonia by *Cd2* depletion strongly suggest that CD2 is expressed in a small proportion of undifferentiated spermatogonia.

We also demonstrated CD2 expression in rats. Rat spermatogenesis is similar to mouse spermatogenesis, but rat SSCs have not been characterized extensively, in part because few markers are available. Only three antigens (EPCAM, THY-1, and CD9) have been identified on rat SSCs [13, 14]. Flow cytomeric analysis showed that the majority of CD9-selected cells express CD2 (Fig. 5B). This is probably because we used relatively young adults in this experiment. Results of our transplantation experiments add CD2 to the list of antigens expressed on rat SSCs, and use of these markers in combination may increase the purity of SSCs. Assuming a 10% colonization efficiency, the degree of rat SSC enrichment was 31.6-fold compared to unfractionated control total testis cells. $CD2^{low}$ cells harbor 1 SSCs for every 105.2 cells. Although this is modest compared to mice, it should be noted here that the current rat transplantation experiments were carried out using relatively immature testes. Therefore, the degree of enrichment could be increased if fully mature rat testes were used as donor cells.

Depletion of *Cd2* by shRNA caused significant downregulation of GFRA1. Moreover, transplantation assay revealed a significant reduction in SSC activity. The function of CD2 was previously analyzed by gene targeting in mice [29]. The homozygous KO mice did not show apparent changes in spermatogenesis. Because it was significantly upregulated by self-renewal factor stimulation, it is possible that CD2 might modify signaling pathways during proliferation of SSCs after damage caused by, for instance, radiation or chemicals. However, spermatogonia in busulfan-treated testes without neighboring germ cells proliferate more actively than those in normal testes [23], which suggests that CD48 expression does not provide strong influence on CD2-mediated proliferation. Because there is also no reported phenotype in *Cd48* KO mice and increased apoptosis occurred after *Cd2* depletion, we speculate that CD2 may function in CD48-independent manner. Although *Cd2* and *Cd48* KO mice failed to show reproductive phenotype, *Cd2ap* KO mice showed significantly impaired spermatogenesis [27]. The KO mice gradually lost spermatogenesis and became infertile, suggesting that CD2AP plays a role in SSCs. CD2AP was originally discovered as a protein that is involved in CD2 clustering, facilitates receptor patterning in the contact area by linking specific adhesion receptors to the actin cytoskeleton [30]. In testes, its expression was thought to be restricted to endothelial cells, Leydig cells and cells in the basal compartment of seminiferous tubules [27]. Because a database search showed that it is strongly expressed in GS cells, we speculate that a complex comprising CD2 and CD2AP is critical for sustaining SSCs. Future functional analysis is required to understand the relationship between CD2 and CD2AP and their role in self-renewal.

It will be interesting to determine whether CD2 expression is conserved in SSCs of other animal species. Because transplantation of xenogeneic SSCs, including of primates and humans, into immunodeficient nude mouse testes resulted in stable colonization and proliferation despite the large genetical distance [31, 32], SSCs of different animal species likely exhibit common surface properties in terms of cell adhesion and the response to cytokines. However, only a few antigens are known despite extensive research. Because spermatogonial transplantation is easily carried out in mice and the mouse spermatogenesis cycle is relatively shorter (35 days) than that of other animal species, it seems reasonable to use mouse SSCs to identify new surface antigens and extrapolate the results to other animal species. Non-rodent SSCs are poorly characterized due to the inefficiency of spermatogonial transplantation and lack of long-term culture systems. Analysis of combination of cell surface markers by FACS, as demonstrated herein in mouse, will be useful for characterizing this cell population.

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