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Heterophilic Binding of the Adhesion Molecules Poliovirus Receptor and Immunoglobulin Superfamily 4A in the Interaction Between Mouse Spermatogenic and Sertoli Cells[1]

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ABSTRACT

The cell adhesion protein immunoglobulin superfamily 4A (IGSF4A) is expressed on the surfaces of spermatogenic cells in the mouse testis. During spermatogenesis, IGSF4A is considered to bind to the surface of Sertoli cells in a heterophilic manner. To identify this unknown partner of IGSF4A, we generated rat monoclonal antibodies against the membrane proteins of mouse Sertoli cells grown in primary culture. Using these monoclonal antibodies, we isolated a clone that immunostained Sertoli cells and reacted with the product of immunoprecipitation of the homogenate of mouse testis with anti-IGSF4A antibody. Subsequently, to identify the Sertoli cell membrane protein that is recognized by this monoclonal antibody, we performed expression cloning of a cDNA library from the mouse testis. As a result, we identified poliovirus receptor (PVR), which is another IGSF-type cell adhesion molecule, as the binding partner of IGSF4A. The antibodies raised against PVR and IGSF4A immunoprecipitated both antigens in the homogenate of mouse testis. Immunoreactivity for PVR was present in Sertoli cells but not in spermatogenic cells at all stages of spermatogenesis. Overexpression of PVR in TM4, a mouse Sertoli cell line, increased more than three-fold its capacity to adhere to Tera-2, which is a human cell line that expresses IGSF4A. These findings suggest that the heterophilic binding of PVR to IGSF4A is responsible, at least in part, for the interaction between Sertoli and spermatogenic cells during mouse spermatogenesis.

CD155, Necl-2, Necl-5, SgIGSF, spermatogenesis, testis, TSLC1

INTRODUCTION

Spermatogenesis is the process through which highly differentiated spermatozoa are produced from spermatogenic cells. It is well established that this process is regulated by a variety of hormonal and local humoral factors [1]. Another possible key factor for the regulation of spermatogenesis is direct interaction between spermatogenic and Sertoli cells. The structural junctions formed between these two cell populations include the desmosome-like junction, basal ectoplasmic specialization (ES), and apical ES [2, 3]. The apical ES is formed between elongated spermatids and Sertoli cells near the apical surface of the seminiferous epithelium. It is composed of hexagonally arranged actin filament bundles that are sandwiched between the plasma membrane and flattened cisternae of the endoplasmic reticulum in Sertoli cells [4]. There is evidence that several cell adhesion molecules are involved in the apical ES [5, 6]. Some of these adhesins belong to the immunoglobulin superfamily (IGSF), which is characterized by extracellular regions that contain Ig-like domains [7]. On the other hand, the desmosome-like junction and basal ES attach Sertoli cells to spermatogonia, spermatocytes, and round spermatids, but not to elongating spermatids [8, 9]. The cell adhesion molecules involved in the desmosome-like junction and basal ES are largely unknown.

Previously, we have reported the isolation from the mouse testis of a cell adhesion molecule called spermatogenic immunoglobulin superfamily (SgIGSF) [10]. SgIGSF has been identified as the mouse homolog of IGSF4A, which is a member of the IGSF [11]. IGSF4A is also known as tumor suppressor in lung cancer 1 (TSLC1) [12], RA175 [13], synaptic cell adhesion molecule (SynCAM) [14] and nectin-like molecule-2 (Necl-2) [15]. In the mouse testis, IGSF4A is expressed exclusively on the surfaces of spermatogenic cells and not on Sertoli cells, which suggests that homophilic binding between IGSF4A molecules is not involved in the spermatogenic-Sertoli cell interaction. Furthermore, recombinant IGSF4A can adhere to primary cultured Sertoli cells, which suggests that IGSF4A forms a heterophilic bond with some other molecule expressed on the Sertoli cell surface [16]. Recent studies have demonstrated that male mice that lack the gene for IGSF4A are infertile due to a remarkable decrease in the numbers of mature spermatozoa [17–20], which indicates that the cell-cell interaction involving IGSF4A plays an

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indispensable role in spermatogenesis. These lines of evidence led us to investigate the cell adhesion molecule produced by Sertoli cells that binds to IGSF4A.

In the present study, using rat monoclonal antibodies raised against the membrane proteins of murine Sertoli cells, we identify poliovirus receptor (PVR), a previously known member of the IGSF, as a Sertoli cell-specific binding partner of IGSF4A.

MATERIALS AND METHODS

Animals and Tissue Preparation

Male Slc:ddY mice at 10 wk of age were purchased from Nippon SLC (Hamamatsu, Japan) and female WKY/NCrj Wistar rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). The mice were raised under standard laboratory conditions with a 12L:12D cycle and free access to food and water. All animal experiments were performed according to Guidelines for the Care and Use of Laboratory Animals at Kanazawa University.

Cell Lines

The murine myeloma cell line SP2/O-Ag14, murine Sertoli cell line TM4, and human embryonic carcinoma cell line Tera-2 were obtained from ATCC (Manassas, VA). SP2/O-Ag14 was maintained in Dulbecco modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO) that contained 10% fetal bovine serum (FBS; ICN Biomedicals, Costa Mesa, CA) at 37°C in 5% CO_2. TM4 was maintained in Ham F-12/DMEM (Sigma-Aldrich) that contained 2.5% FBS and 5% horse serum (Invitrogen, Carlsbad, CA) at 37°C in 5% CO_2. Tera-2 was maintained in McCoy 5a medium (Sigma-Aldrich) that contained 1.5 mM L-glutamine and 15% FBS at 37°C in 5% CO_2.

Primary Cultures of Sertoli cells and the Molecular Adhesion Experiment

The isolation and primary culture of Sertoli cells from immature mouse testis were performed as previously described [16]. Sertoli cells cultured on coverslips were fixed with cold 50% methanol and 50% acetone for 10 min, washed in PBS, and incubated with PBS that contained 3% BSA (Sigma-Aldrich), to avoid nonspecific binding of proteins. Preparation of the recombinant protein sIGSF4A-Fc, which comprises the extracellular domain of mouse IGSF4A combined with the Fc of human IgG, has been described previously [21]. After washing PBS, the cells were incubated with sIGSF4A-Fc (50 μg/ml) for 2 h at room temperature. The adhesion of sIGSF4A-Fc to the surfaces of Sertoli cells was visualized by incubating the cells with 1 μg/ml chicken monoclonal anti-SynCAM/TSLC1 (anti-IGSF4A) IgY antibody (clone 3E1; Medical & Biological Laboratories, Nagoya, Japan), which recognizes the extracellular domain of mouse IGSF4A, and then with FITC-labeled goat anti-chicken IgY at 1:100 (Medical & Biological Laboratories) for 1 h at room temperature. For the negative control, the cells were incubated with the Fc of human IgG alone followed by the chicken anti-IGSF4A IgY antibody and either FITC-labeled goat anti-chicken IgY antibody or FITC-labeled goat anti-human Fc antibody. The cells were counterstained in the nucleus with 100 ng/ml Hoechst 33258 and subjected to immunofluorescence microscopy using the BX50/BX-FLA apparatus (Olympus, Tokyo, Japan).

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation and Western blotting were performed as previously described [16]. Briefly, a cell lysate from a 10-wk-old mouse testis that contained 200 mg of protein was preabsorbed with protein G-agarose (Roche Diagnostics, Mannheim, Germany) for at least 3 h at 4°C. After centrifugation, the supernatant was incubated with 10 μg of rabbit polyclonal anti-IGSF4A antibody [16] or 10 μg of rat monoclonal anti-PVR (clone 8G3) antibody for 2 h, and then incubated with protein G-agarose for at least 3 h at 4°C. After centrifugation, the immunoprecipitated product was washed and resuspended in SDS-PAGE sample buffer for SDS-PAGE.

The cell lysate from the mouse testis and the immunoprecipitated products were subjected to SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The blots were incubated with rabbit polyclonal anti-IGSF4A antibody (1:1000) or rat monoclonal anti-PVR (clone 8G3) antibody (1:2000). After washing, the blots were further incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-rat IgG antibody (1:5000) (DAKO, Glostrup, Denmark). Immunoreactivities were detected with x-ray film after treatment of the blots with the chemiluminescent reagent ECL Plus (Amersham Pharmacia Biotech, Uppsala, Sweden).

Immunohistochemistry

Immunohistochemistry at the light microscopic level was performed as previously described [16, 22, 23]. Briefly, the 4% paraformaldehyde-fixed frozen sections of the mouse testis were first treated with 10% normal goat serum to prevent nonspecific antibody binding, and then incubated overnight at 4°C with the following antibodies: rat monoclonal anti-PVR (clone 8G3) antibody at 1 μg/ml, rabbit polyclonal anti-IGSF4A antibody at 1 μg/ml [16], mouse monoclonal anti-tyrosine α-tubulin antibody (clone TUB-1A2; Sigma-Aldrich) at a 1:2000 dilution [24] or normal rat IgG (DAKO) at 1 μg/ml. After the sections were washed in PBS, the immunoreactivities were visualized by incubating the sections with anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) (1:400) or anti-rat IgG antibody conjugated with Alexa Fluor 594 (1:400) for 1 h at room temperature. The F-actin-binding protein phalloidin conjugated with Alexa Fluor 488 (1:200) was also used to visualize F-actin. The sections were counterstained in the nucleus with 100 ng/ml bisbenzimide H33258 (Hoechst 33258) (Sigma-Aldrich). The sections were then observed with an immunofluorescence microscope (BX50/BX-FLA) or a confocal laser scanning microscope (LSM510; Carl Zeiss, Oberkochen, Germany).

For ultrastructural localization of PVR in the mouse seminiferous epithelium, the postembedding immunoreaction method was performed, as described previously [25, 26]. Ultrathin sections were cut from an LR White (Sigma-Aldrich)-embedded testis block and mounted onto nickel grids. The sections were treated with buffer A (1% normal goat serum, 1% BSA, 0.1% Tween-20, and 0.1% sodium azide in PBS [pH 8.2]) for 30 min, and then incubated with rat monoclonal anti-PVR (clone 8G3) antibody at 1 μg/ml in buffer A for 1 h at room temperature. After washing with buffer A, the sections were subjected to a reaction with colloidal gold (10 nm)-conjugated anti-rat IgG (British BioCell, Cardiff, UK) antibody at a 1:100 dilution in buffer A for 1 h at room temperature. They were then contrasted with uranyl acetate and examined under a JEM-1210 electron microscope (JEOL, Tokyo, Japan).

Production of the Rat Monoclonal Antibody Library

The production of rat monoclonal antibodies was performed according to the rat lymph node method [27]. Isolation of the membrane protein fraction from cultured Sertoli cells was performed as described previously [28]. Three female WKY/NCrj Wistar rats were injected intracutaneously in the hind footpads with 400 μl of an emulsion that contained 1 mg of Sertoli cell membrane proteins in physiological saline and Freund complete adjuvant (Difco, Detroit, MI) mixed at 1:1. Two weeks later, the enlarged medial iliac lymph nodes were dissected from the rats under ether anesthesia and decapsulated in serum-free DMEM to make a lymphocyte suspension. The lymphocytes (1×10^8) and the mouse myeloma SP2/O-Ag14 cells (2×10^7) were fused in serum-free DMEM that contained 50% (w/v) polyethylene glycol 4000 (Merck, Darmstadt, Germany) and 5% dimethyl sulfoxide. The fused cells were resuspended in HAT selection medium, which consists of GIT medium (Wako Pure Chemical Industries, Osaka, Japan), 10% FBS, 10% BM-condimed H1 (Roche, Mannheim, Germany), 10 mM hypoxanthine (H), 0.4 mM aminopterin (A), and 1.6 mM thymidine (T). They were plated in four 96-well tissue culture plates (Becton Dickinson) and cultured at 37°C in 5% CO_2.

The culture medium supernatants were screened in immunohistochemistry and immunoprecipitation assays for antibodies against Sertoli cell membrane proteins that bind to IGSF4A. First, the supernatants were subjected to immunohistochemistry, to examine reactivities with Sertoli cells. The positive supernatants were then examined for reactivities with IGSF4A-immunoprecipitated products of Sertoli cell membrane proteins by Western blotting.

Expression cloning of cDNA

An oligo(dT)-primed TripleEX cDNA library from mouse Sertoli cell poly(A)+ RNA was prepared in *Escherichia coli* XL-1 Blue according to the manufacturers instructions (Clontech, Palo Alto, CA). Expression cloning of cDNA was performed as previously described [29]. A total of 1×10^6 independent plaques was screened for the production of molecules immunoreactive with the supernatant of the hybridoma clones of interest. The positive phages were converted to plasmids in *E. coli* BM25.8 by incubating the bacteria at 31°C in LB plates that contained ampicillin. The plasmids were isolated and subjected to sequencing analysis with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using standard protocols.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR of the total RNA from cultured Tera-2 and TM4 cells was performed as described previously [30]. The following primers were purchased from NIPPON EGT (Toyama, Japan): for PVR, forward 5′-CACAGTTAC-

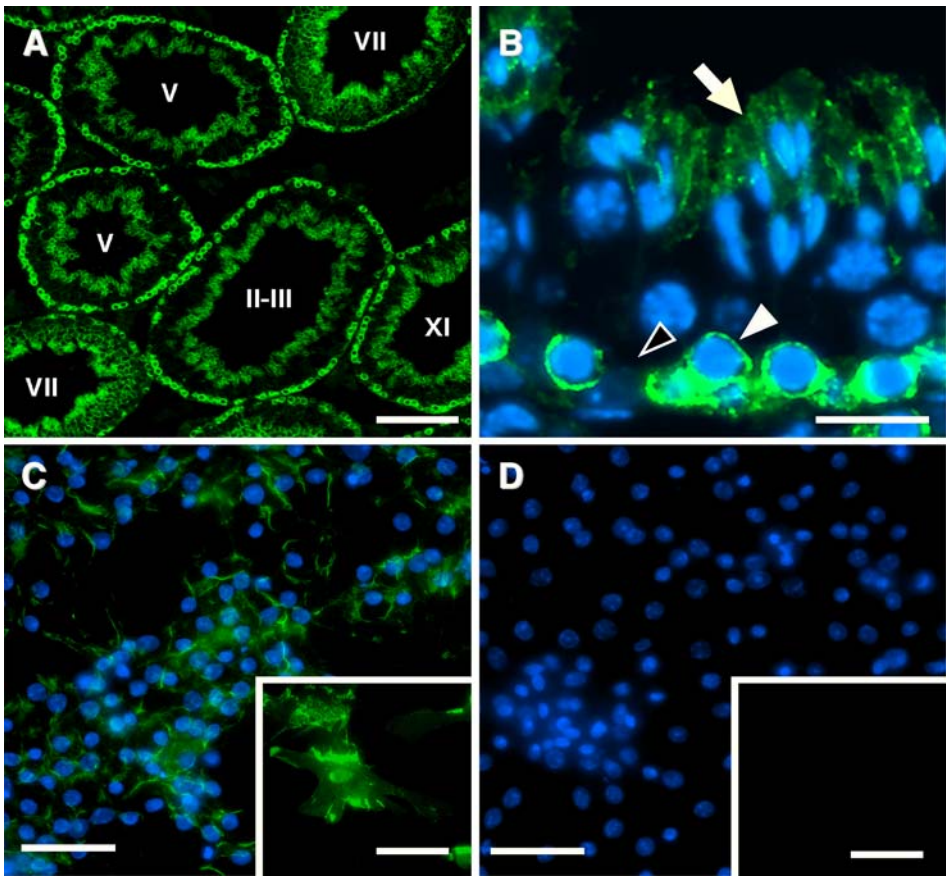

FIG. 1. Fluorescence immunohistochemistry for IGSF4A. **A**) In the adult mouse testis, the pattern of immunoreactivity for IGSF4A (green) in the seminiferous tubules varies with the stage of spermatogenesis. **B**) At stage XI, IGSF4A immunoreactivity is present in the zygotene spermatocytes (white arrowhead) and step 11 spermatids (white arrow). Note that the Sertoli cells (open arrowhead) are devoid of any immunoreactivity. **C**) After the incubation of primary cultured Sertoli cells from a 10-day-old mouse testis with the recombinant sIGSF4A-Fc, the surfaces of the Sertoli cells are immunostained with the anti-IGSF4A antibody (green). The inset shows the immunoreactivity in Sertoli cells at a higher magnification. **D** with inset) After incubation with Fc, Sertoli cells show no immunostaining with the anti-IGSF4A antibody. The same negative result was obtained with the anti-Fc antibody. **B**–**D**) The nuclei are counterstained with Hoechst 33258 (blue), except in the insets. Bar = 100 μm (**A**), 25 μm (**B**), 50 μm (**C**, **D**), and 10 μm (insets).

CAGCTACCTCT-3′ and reverse 5′-GGGTTCCAGTTGCTATGCCT-3′; for IGSF4A, forward 5′-CAGTTGCCAAGTCAATAAGA-3′ and reverse 5′-TCCCGATGGCTTCACATGTT-3′; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCACCACCCTGTTGCTGTA-3′.

Plasmid Construction and Transfection

A cDNA fragment that encompasses the entire coding sequence of mouse PVR was amplified by RT-PCR from the mouse testis cDNA using a forward primer that contains an *Eco*RI restriction site (shown in bold) (5′-**GAATTC**CCACCATGGCTCAACTCGCCCGAGC-3′) and a reverse primer that contains a *Sac*II restriction site (shown in bold) (5′-**CCGCGG**CCTTGTGCTGTTTGGCTCCATGT-3′). The resulting PCR fragment was cloned into the pCRII-TOPO plasmid vector (Invitrogen). This vector was then digested with *Eco*RI and *Sac*II and the cDNA fragment was ligated into the mammalian expression vector pEYFP-N1 (Clontech Laboratories, Mountain View, CA) at the *Eco*RI/*Sac*II sites within the multiple cloning site located downstream of the promoter and enhanced yellow fluorescent protein (EYFP)-coding region. The pPVR/EYFP vector or a vector that contained only EYFP (mock) was transfected using Lipofectamine 2000 reagent (Invitrogen) into TM4 cells that were cultured to subconfluency in 24-well plates. Twenty-four hours after transfection, the confluent cells were used in the cell adhesion experiment.

Cell Adhesion Experiment

Tera-2 cells cultured to subconfluency were dispersed with 0.1% trypsin and 1 mM EGTA. The cells were suspended at 2×10^7 cells in 1 ml of Ham F-12/DMEM and the cell membranes were labeled by the addition of the lipid-binding dye RKH26 (Dainippon Sumitomo Pharma, Osaka, Japan) to a concentration of 4 mM and rotating the tube gently for 5 min. Next, 1×10^3 labeled Tera-2 cells in 1 ml of the same medium were placed onto the confluent cultures of nontransfected, pPVR/EYFP-transfected or mock-transfected TM4 cells in the wells of the 24-well plate. In some wells, recombinant sIGSF4A-Fc or the Fc fragment alone was added at 50 μg/ml to the culture of confluent TM4 cells for 1 h, followed by washing with fresh medium prior to the placement of Tera-2 cells. Furthermore, in some wells, chicken monoclonal anti-IGSF4A IgY antibody or non-immune chicken IgY was added at 10 μg/ml to the suspension cultures of Tera-2 cells for 1 h before their placement onto TM4 cells, and was maintained continuously during the period of coculture. These experiments were performed using three wells for each condition and were repeated three times. After incubation for 2 h, the wells were washed gently with fresh medium and observed under a fluorescent inverted microscope (Axiovert S100; Carl Zeiss). At 10× magnification, three fields were selected randomly in each of the three wells. The number of Tera-2 cells attached to the TM4 cells in each field was counted and expressed as mean ± SD of nine fields. The statistical difference between two out of multiple mean values was examined by one-factor analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. Differences with P values less than 0.05 were considered significant.

RESULTS

Specific Expression of IGSF4A by Spermatogenic Cells in the Mouse Testis

Immunoreactivity for IGSF4A was observed in the epithelia of the seminiferous tubules, at different stages of spermatogenesis (Fig. 1A). Most of the seminiferous tubules were immunostained in the basement membrane and close to the lumen. The immunopositive cells near the basement membrane represent intermediate spermatogonia through to early pachytene spermatocytes, whereas those close to the lumen represent step 7 round spermatids through to the elongated spermatids facing spermiation (Fig. 1B). The immunoreactivities in the spermatogenic cells diminished from middle pachytene spermatocytes through to the step 6 round spermatids. Sertoli cells were also devoid of IGSF4A immunoreactivity. These results are consistent with those in our previous report [16].

Adhesion of IGSF4A to the Sertoli Cell Surface

To determine if Sertoli cells express any cell adhesion molecule that binds to IGSF4A, a molecular adhesion

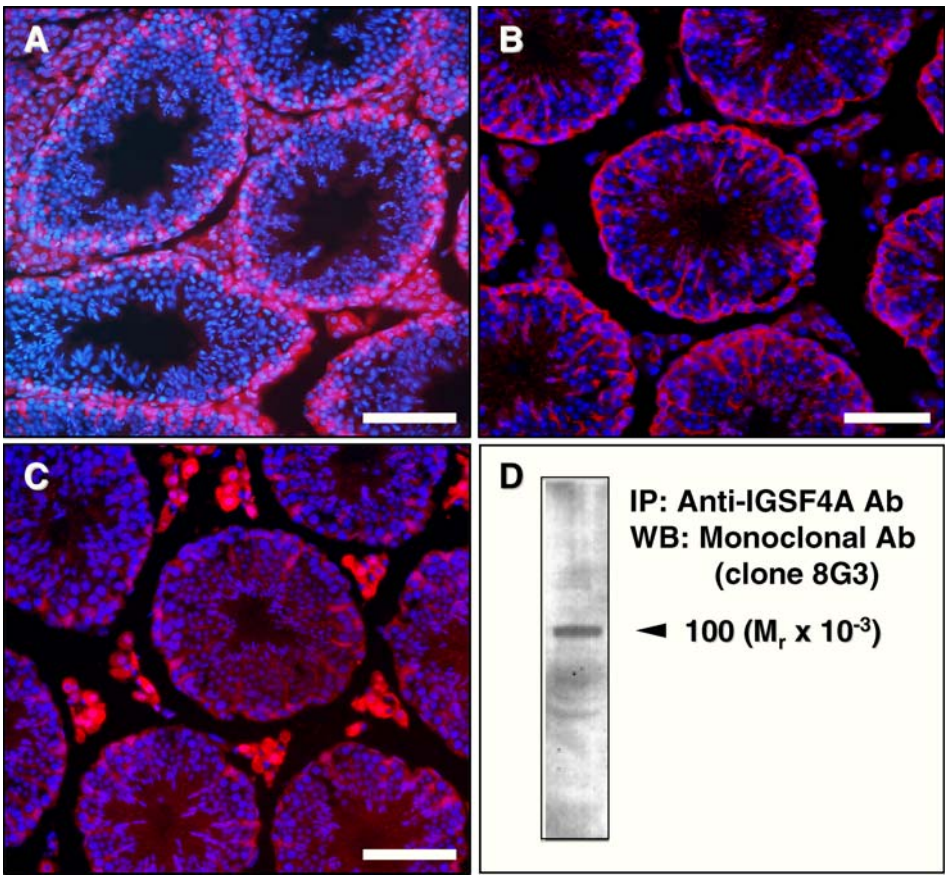

FIG. 2. Screening of different rat monoclonal antibodies against the membrane proteins of Sertoli cells. **A**–**C**) Fluorescence immunohistochemistry of mouse seminiferous tubules with monoclonal antibodies reactive with Sertoli cells (**A**, clone 3A2; **B**, clone 11B6; **C**, clone 8G3). Bar = 100 μm. (**D**) Western blot analysis showing that the monoclonal antibody clone 8G3 reacts with the product of immunoprecipitation (IP) of the mouse testis homogenate with the anti-IGSF4A antibody. Note the single strong protein band of approximately 100 kDa, which represents PVR. Several other monoclonal antibody clones reactive with Sertoli cells also formed single protein bands of similar molecular mass (data not shown).

experiment was performed using a primary culture of Sertoli cells and the recombinant protein sIGSF4A-Fc, which consists of the extracellular domain of IGSF4A and the Fc fragment of human IgG. SIGSF4A-Fc adhered to the surface of Sertoli cells, as detected by immunofluorescence of the antibody against the extracellular domain of IGSF4A (Fig. 1C and inset). In contrast, when the cells were incubated with the Fc fragment alone, neither the same antibody nor the antibody against the Fc fragment showed any immunoreactivity on the cell surface, which indicates that Sertoli cells do not express IGSF4A, and that the Fc portion of sIGSF4A-Fc does not adhere to Sertoli cells (Fig. 1D and inset). These results suggest that IGSF4A binds to certain cell adhesion molecules on the surface of Sertoli cells in a heterophilic manner.

Cloning of PVR as a Candidate Heterophilic Binding Partner of IGSF4A

To identify the cell adhesion molecule expressed by Sertoli cells that can specifically bind to IGSF4A, we prepared a library of rat monoclonal antibodies against the membrane proteins of Sertoli cells grown in primary culture. In total, 1152 wells that contained hybridoma clones that were obtained in three separate fusion experiments were analyzed, and the culture supernatants from 156 wells were shown by immunohistochemistry to react with Sertoli cells. For the second screening, we examined whether these clones reacted with the anti-IGSF4A-immunoprecipitated products of the mouse testis lysate by Western blotting the culture supernatants using the limiting dilution method. Among the 156 clones that reacted with Sertoli cells in immunohistochemistry, five clones (3A2, 3H9, 5C10, 8G3, and 11B6) were found to be reactive with the immunoprecipitation products. These hybridoma clones were confirmed to react specifically with the candidate Sertoli cell surface-binding partner of IGSF4A by both immunohistochemistry and Western blotting (Fig. 2, A–D).

To identify the Sertoli cell proteins recognized by the monoclonal antibodies produced by these selected hybridoma clones, we screened a library of mouse testis cDNA that was introduced on an expression vector. One cDNA clone with a protein product that was reactive with the monoclonal antibody clone 8G3 was isolated. Sequencing analysis of this cDNA clone revealed that it was identical to the cDNA of a known cell adhesion molecule, poliovirus receptor (PVR) [31], which is also known as nectin-like molecule-5 (Necl-5) [32], CD155 [33] and Tage4 [34] (GenBank accession no. BC032283). The other four monoclonal antibodies failed to react with the products of the mouse testis cDNA library in the present screening.

Expression and Cellular Localization of PVR in the Mouse Testis

The expression and cellular localization of PVR in the mouse testis were examined by immunohistochemistry using the rat monoclonal anti-PVR (clone 8G3) antibody (Fig. 3). The immunoreactivity was distributed in a radial pattern in the epithelia of the seminiferous tubules and was also detected in the cells of the interstitial tissue (Fig. 3A). However, the latter immunoreactivity seemed to be nonspecific, since incubation with normal rat IgG also stained the cells of the interstitial tissue (Fig. 3B). In the seminiferous epithelium, PVR immunoreactivity was distributed diffusely from the basal to the apical portions of the epithelium, regardless of the stage of spermatogenesis, and appeared as finger-like processes or thin

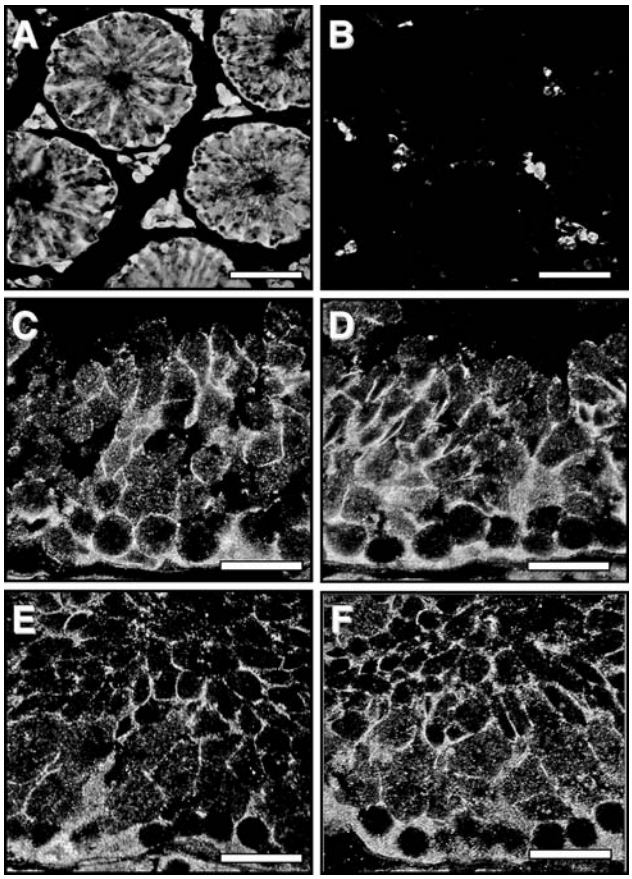

FIG. 3. Light microscopic immunohistochemistry for PVR in the adult mouse testis. Frozen sections of 10-wk-old testes were immunostained with the rat monoclonal anti-PVR antibody (clone 8G3) (**A**, **C**–**F**) or normal rat IgG (**B**). **A**) Immunoreactivity for PVR is present in the seminiferous epithelium and also in the cells of the interstitial tissue. **B**) No immunoreactivity is detected within the seminiferous tubules using normal rat IgG as control. Some interstitial cells are stained nonspecifically. **C**–**F**) At stages I–III (**C**), IV–VI (**D**), IX (**E**) and X–XII (**F**) immunoreactivity is present throughout the seminiferous epithelium. Bar = 100 μm (**A**, **B**) and 25 μm (**C**–**F**).

sheets. These characteristics of PVR immunoreactivity were indicative of Sertoli cells (Fig. 3, A and C–F).

To confirm the cellular localization of PVR in the seminiferous epithelium, we performed double-immunofluorescence microscopy using a combination of the rat monoclonal anti-PVR antibody (clone 8G3) and the mouse monoclonal antibody against tyrosine α-tubulin, which is a Sertoli cell marker [24], or phalloidin, which binds to F-actin, an ES marker [5]. The immunoreactivities for PVR and tyrosine α-tubulin colocalized in the seminiferous epithelium (Fig. 4, A–C). In addition, the fluorescence for phalloidin partially overlapped with the immunoreactivity for PVR (Fig. 4, D–F). None of these antibodies immunostained spermatogenic cells. These results confirm that PVR is expressed specifically by Sertoli cells but not by spermatogenic cells.

Ultrastructural localization of PVR in the seminiferous tubules was performed by postembedding immunoelectron microscopy (Fig. 5). The gold particles representing PVR immunoreactivity were located exclusively in the Sertoli cells. The Sertoli cells were characterized by a large irregular-shaped nucleus with a tripartite nucleolus. In the apical portions of the seminiferous epithelium, where Sertoli cells are in contact with elongated spermatids, the gold particles lined up along the cell membranes of the Sertoli cells (Fig. 5, A and B). In the basal portions of the epithelium, where Sertoli cells are in contact with earlier spermatogenic cells, the gold particles were located in both the cell membrane and cytoplasm of the Sertoli cells (Fig. 5, C and D). No focal enrichment of PVR immunoreactivity was noted in the membranes of the Sertoli cells.

Interaction Between PVR and IGSF4A in the Mouse Testis

To examine whether PVR interacts with IGSF4A in vivo, each antigen was immunoprecipitated in the mouse testis lysate with the corresponding antibody and then examined with the other antibody by Western blotting. When the testis lysate was immunoprecipitated with rat monoclonal anti-PVR or rabbit polyclonal anti-IGSF4A antibody, both the anti-PVR and anti-IGSF4A antibodies detected the corresponding protein bands of 100 kDa and 130 kDa, respectively, in either immunoprecipitation product (Fig. 6, A and B). The control immunoprecipitation products without the testis cell lysate showed no immunoreactive protein bands for either antibody. These

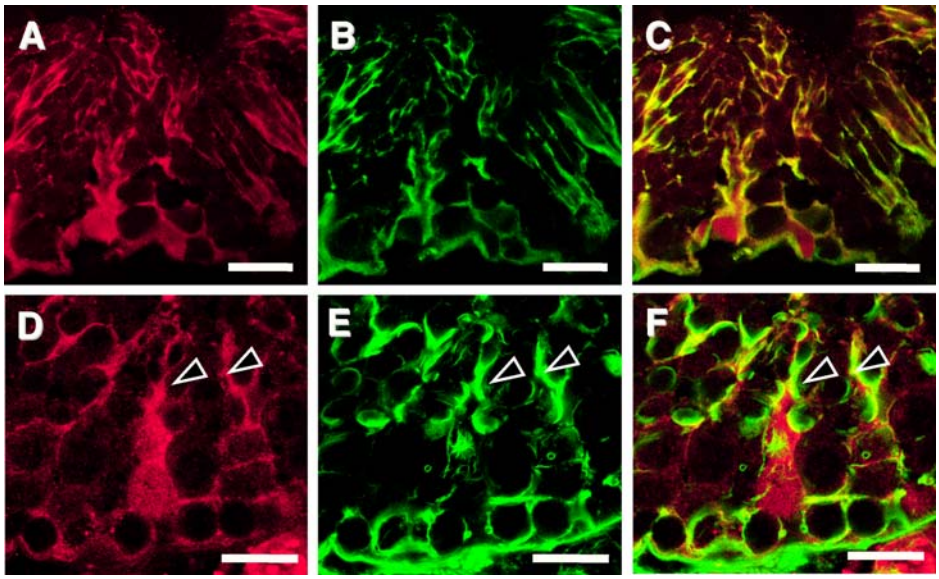

FIG. 4. Double immunostaining of the seminiferous epithelium of adult mouse testis with rat monoclonal anti-PVR antibody and mouse monoclonal anti-tyrosine α-tubulin antibody or phalloidin. **A**–**C**) The immunoreactivities for PVR (red, **A**) and for tyrosine α-tubulin (green, **B**), a marker of Sertoli cells, mostly overlap when the photographs are merged (yellow, **C**). **D**–**F**) Fluorescence for phalloidin, which represents the actin filaments in the apical and basal ectoplasmic specializations (green, **E**), overlaps with the corresponding portions of the immunoreactivity for PVR (red, **D**) when the photographs are merged (yellow, **F**). **A**–**F**) Open arrowheads represent apical ES. Bar = 25 μm.

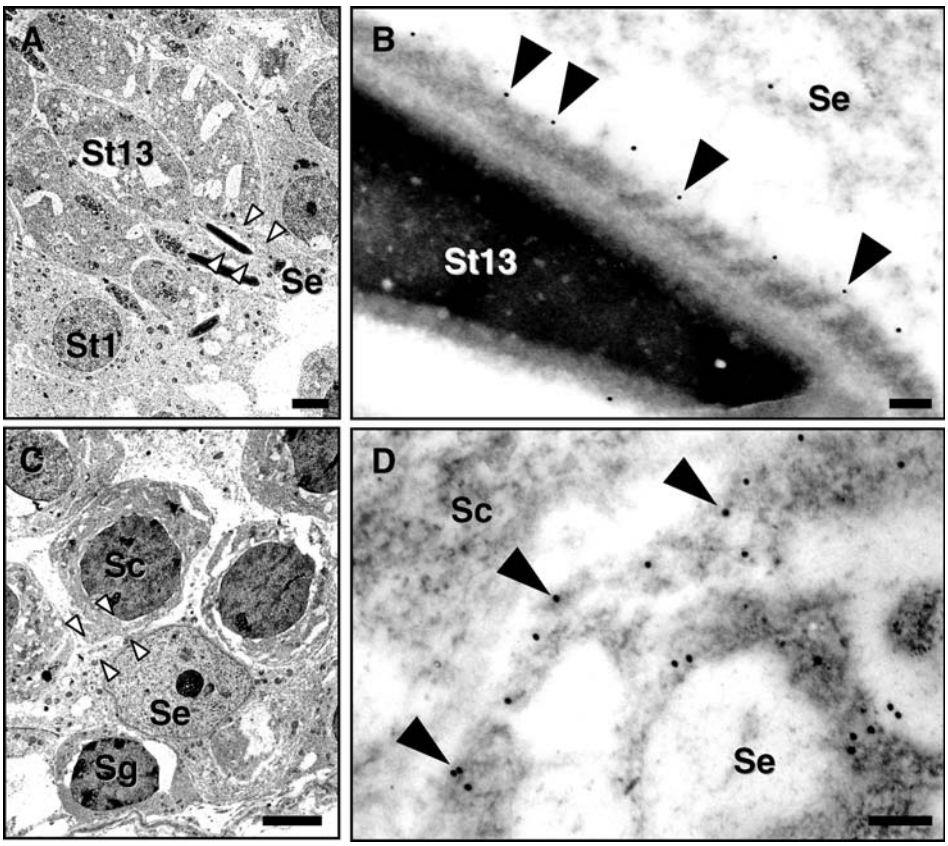

FIG. 5. Electron microscopic immunocytochemistry showing the localization of PVR in the seminiferous epithelium of adult mouse testis using the postembedding method. **A**) In the stage I tubule, the step 1 (St1) and step 13 (St13) spermatids, as well as Sertoli cells (Se) are visible. **B**) Higher magnification of the area labeled with white arrowheads in **A**. The gold particles representing immunoreactivity for PVR are located in the portions of the Sertoli cell (Se) membrane (black arrowheads) that are in contact with the head of the spermatid (St), which is immunonegative. **C**) In the stage IV tubule, type B spermatogonia (Sg), pachytene spermatocytes (Sc), and Sertoli cells (Se) are visible. **D**) Higher magnification of the area labeled with white arrowheads in **C**. The gold particles representing the immunoreactivity for PVR are located mainly on the cell membrane (black arrowheads) but are also present in the cytoplasm of Sertoli cells (Se). The neighboring spermatocyte (Sc) is immunonegative. Bar = 2 μm (**A**, **B**) and 100 nm (**C**, **D**).

results suggest that IGSF4A on spermatogenic cells and PVR on Sertoli cells form relatively strong bonds in vivo.

The cellular localization of PVR was compared with that of IGSF4A by double-immunofluorescence microscopy (Fig. 7). As already demonstrated, PVR immunoreactivity was distributed throughout the membranes of Sertoli cells, whereas IGSF4A immunoreactivity was restricted to two spermatogenic cell populations that were located basally and apically in the seminiferous epithelium. Consequently, the two immunoreactivities colocalized only at the portions of the Sertoli cell membrane that were in contact with IGSF4A-immunopositive spermatogenic cells.

Adhesion of Spermatogenic and Sertoli Cells Through the Interaction of IGSF4A and PVR

To investigate whether the heterophilic interaction between PVR and IGSF4A is responsible for cell-to-cell adhesion, we cocultured the murine Sertoli cell line TM4 and the human embryonic carcinoma cell line Tera-2. TM4 cells are characterized by a large irregular shape with many projections, similar to primary cultured Sertoli cells [35], whereas Tera-2 cells are derived from a lung metastasis of testicular embryonic carcinoma cells [36]. RT-PCR revealed that TM4 expressed PVR but not IGSF4A, whereas Tera-2 expressed IGSF4A but not PVR (Fig. 8A). The cell membranes of the Tera-2 cells were labeled in advance with a fluorescent dye and examined for ability to adhere to a confluent culture of TM4 cells in the presence and absence of PVR overexpression. The results of a representative experiment are shown (Fig. 8, B and C). When TM4 cells were transfected with mouse PVR cDNA, the average number of adhered Tera-2 cells per field increased 3.1-fold ($P < 0.01$) compared to nontransfected or mock-transfected TM4 cells. In contrast, preincubation of PVR-transfected TM4 cells with the recombinant protein sIGSF4A-Fc, which represents the extracellular domain of IGSF4A, reduced the number of adhered Tera-2 cells by 82% ($P < 0.01$) compared with preincubation of PVR-transfected TM4 cells with the Fc, or by 45% ($P < 0.05$) compared with the mock-transfected TM4 cells without preincubation. Furthermore, the addition to the culture medium of chicken monoclonal anti-IGSF4A IgY antibody, which binds to the extracellular domain of IGSF4A, reduced the numbers of Tera-2 cells that adhered to PVR-transfected TM4 cells by 80% ($P < 0.01$) or that adhered to mock-transfected TM4 cells by 40% ($P < 0.05$). The experiment was repeated three times with similar results. These results indicate that PVR overexpression in naturally PVR-expressing cells promotes their capacity to adhere to IGSF4A-expressing cells, and this effect is abolished by inhibiting the binding between PVR and IGSF4A, which suggests that the heterophilic interaction between PVR and IGSF4A is responsible for cell-to-cell adhesion.

DISCUSSION

Using rat monoclonal antibodies against the membrane proteins of mouse Sertoli cells and expression cloning, we have identified PVR [31] as a heterophilic binding partner of IGSF4A in the mouse testis. PVR and IGSF4A belong to a subclass of IGSF adhesion molecules that are known as the nectins and nectin-like molecules (necls) [37]. PVR and IGSF4A are also called Necl-5 and Necl-2, respectively. Members of this subclass are composed of three extracellular Ig-like domains, a single transmembrane region, and a cytoplasmic domain [37]. Through the interactions of their Ig-like domains, these adhesion molecules form *cis*-dimers on the same cell membrane and *trans*-dimers on the opposing cell membranes. A distinction between the nectins and necls is the ability of the former to interact with the cytoplasmic actin-

binding protein afadin [37]. The binding activity of PVR is exclusively heterophilic, whereas the binding activities of other nectins and necls are both homophilic and heterophilic [38]. Furthermore, while other nectins and necls mediate only cell-to-cell contact, PVR is also involved in the contact between cells and the extracellular matrix component vitronectin [39].

PVR is expressed by many cancer cells and cultured cell lines [40]. In normal animal tissues, PVR is known to be expressed abundantly by immunocompetent cells in the tonsils and Peyer patches [39, 41]. The present report is the first to characterize the expression and localization of PVR in the mouse seminiferous epithelium. From immunohistochemistry at both the light and electron microscopic levels, PVR appears to be distributed diffusely on the Sertoli cell membrane throughout the thickness of the seminiferous epithelium, without focal enrichment in any specific areas. A similar diffuse membrane distribution has been observed for IGSF4A in the apical elongated spermatids and basal earlier spermatogenic cells [16]. Although it was difficult in the present study to recognize the apical and basal ES or desmosome-like junction morphologically, the diffuse distributions of PVR and IGSF4A suggest that the site of interaction between these adhesion molecules are not restricted to specific morphological structures formed between spermatogenic and Sertoli cells. Since the portions of Sertoli cell membrane in contact with round spermatids, which do not express IGSF4A, also showed intense PVR immunoreactivity, it is possible that round spermatids express some other IGSF molecule as a binding partner of Sertoli cell PVR. In some cultured cell systems, PVR has been reported to bind heterophilically to IGSF molecules, such as poliovirus receptor-related (PVRL) 3 [42], CD226 [43], and CD44 [44], although the physiological roles of combinations of these molecules and PVR have not been documented. The heterophilic binding of PVR to IGSF4A, as revealed in the present study in the mouse seminiferous epithelium, is sufficiently strong to be detected by coimmunoprecipitation. Furthermore, the present cell adhesion experiment in a cultured system shows that the binding between PVR and IGSF4A can indeed mediate cell-to-cell adhesion.

Previous studies have indicated that adhesion molecules other than IGSF4A and PVR are expressed in the seminiferous epithelium. These include N-cadherin, junctional adhesion molecule (JAM) 2, JAM3, PVRL2 (formerly called Nectin-2), PVRL3 (Nectin-3), integrin $\alpha 6\beta 1$, and laminin $\gamma 3$ [5, 45–47]. N-cadherin, which is a member of the classical calcium-dependent cadherin family, is found in association with all the morphological adhesion structures formed between Sertoli cells and between Sertoli and spermatogenic cells, including the adherens junction, desmosome, desmosome-like junction, apical ES, and basal ES. In these structures, N-cadherin is localized to the cell membranes of both sides, to facilitate homophilic binding [48, 49]. On the other hand, JAMs and nectins are members of the IGSF and are found primarily associated with the apical ES formed between Sertoli and elongated spermatids. In this position, JAM2 and PVRL2 located on the sides of the Sertoli cells bind heterophilically to JAM3 and PVRL3, respectively, which are located on the sides of the elongated spermatids. However, as revealed in the present study, the combination of PVR and IGSF4A is unique, in that it is involved in adhesion not only between Sertoli cells and elongated spermatids located apically but also between Sertoli cells and the early spermatogenic cells that are located basally in the seminiferous epithelium.

Recently, the IGSF4A-deficient mouse was generated and shown to have disruption of spermatogenesis resulting in male infertility, which indicates that IGSF4A is indispensable for spermatogenesis [17–20]. In the seminiferous tubules of adult IGSF4A-deficient mice, many elongating spermatids with distorted shapes are found to have detached from the epithelium and accumulated in the lumen. Many apoptotic spermatogenic cells are found throughout the epithelium, whereas almost no mature motile spermatozoa are found in the lumen. In view of these observations, the present results suggest that the heterophilic binding between IGSF4A and PVR accounts, at least partly, for the interaction between spermatogenic and Sertoli cells, which is indispensable for spermatogenesis.

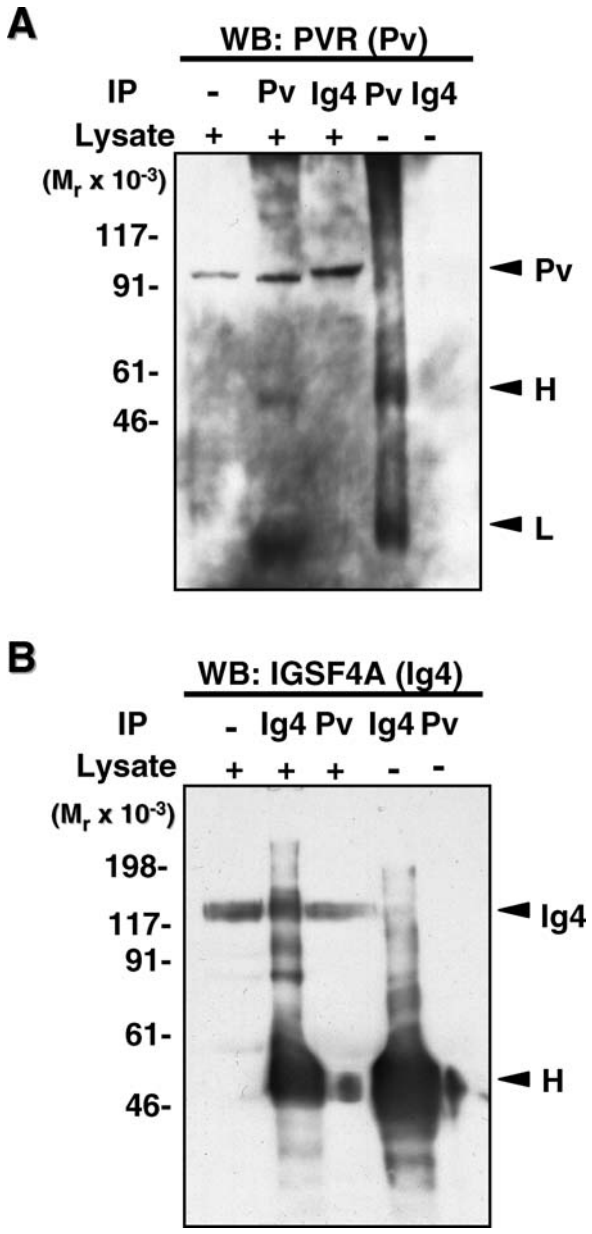

FIG. 6. Western blot analysis (WB) showing the interaction between PVR and IGSF4A in the adult mouse testis. **A**, **B**) Immunoprecipitation (IP) was performed in the presence or absence of cell lysate from the 10-wk-old mouse testis using the antibody against PVR (Pv) or IGSF4A (Ig4). The cell lysate and products of IP were electrophoresed, blotted, and probed with rat monoclonal anti-PVR antibody (**A**) or rabbit polyclonal anti-IGSF4A antibody (**B**). The molecular masses of the immunoreactive bands for PVR (100 kDa) and IGSF4A (130 kDa) are indicated. H and L represent the bands for the coprecipitated heavy and light chains of the IgG, respectively.

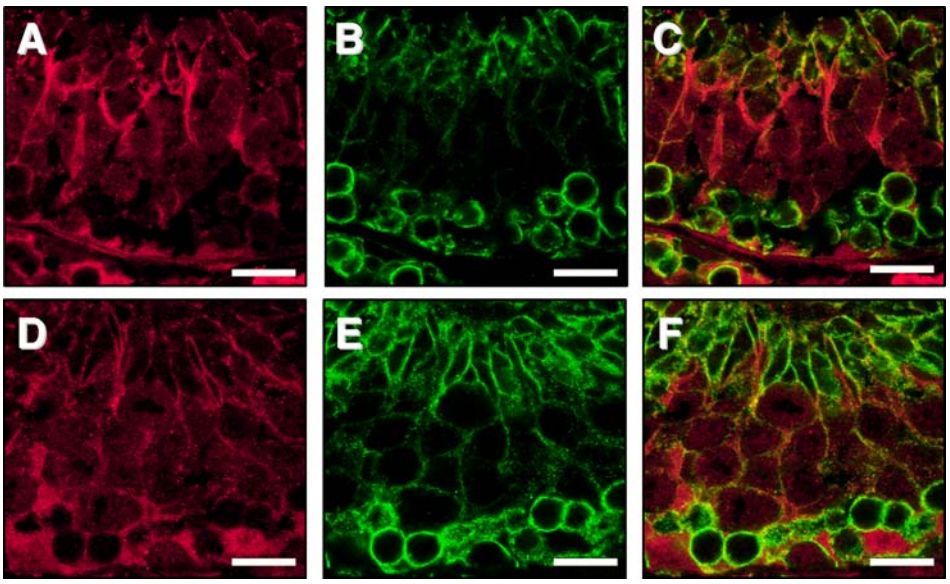

FIG. 7. Double immunostaining of the adult mouse testis with rat monoclonal anti-PVR and rabbit polyclonal anti-IGSF4A antibodies. At stages IV–VI (**A**–**C**) and XII (**D**–**F**), the immunoreactivity for PVR is distributed across the entire Sertoli cell surface (red; **A**, **D**), whereas that for IGSF4A is present on the surface of a subpopulation of spermatogenic cells located in the apical and basal regions of the seminiferous epithelium (green; **B**, **E**). These two immunoreactivities overlap where the corresponding cell populations adhere to each other (yellow; **C**, **F**). Bar = 25 μm.

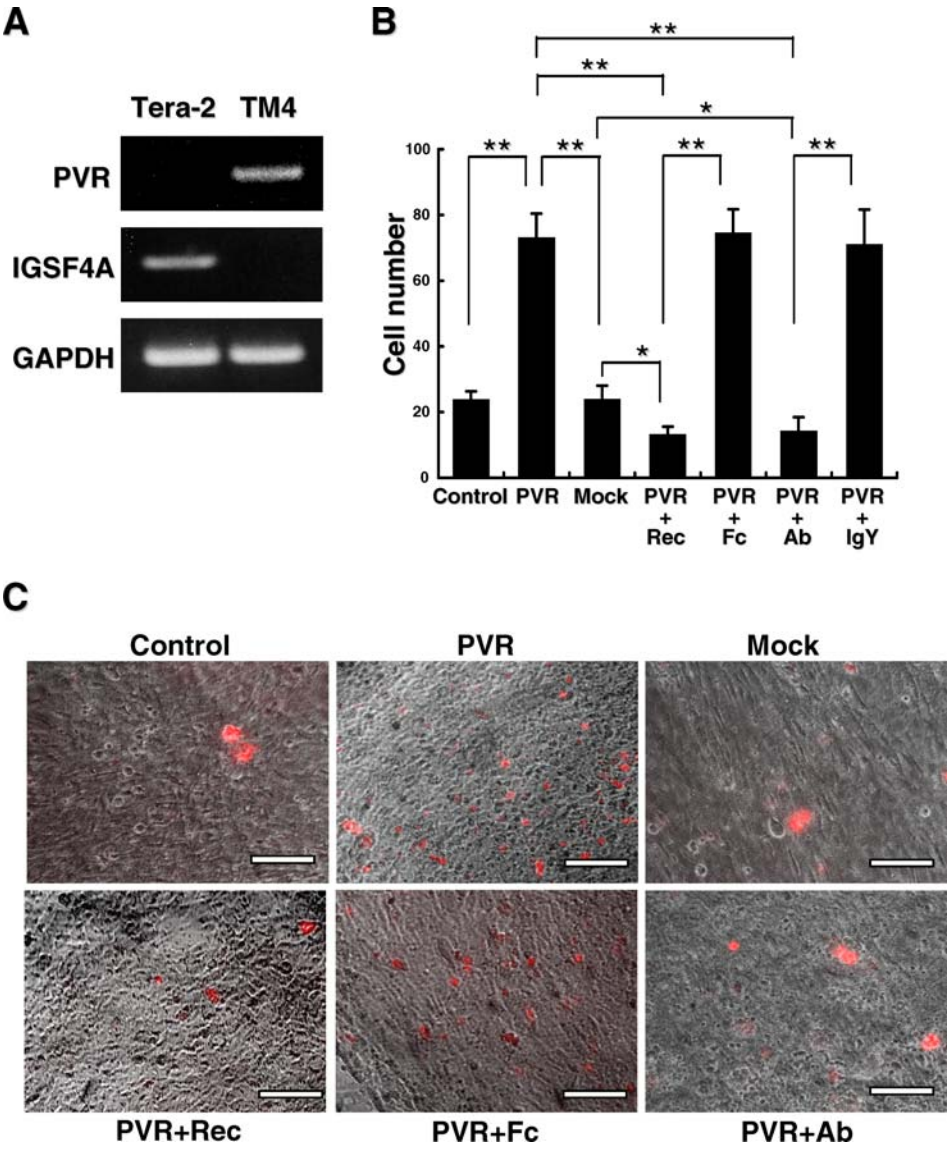

FIG. 8. Adhesion between PVR- and IGSF4A-expressing cells. **A**) Expression of PVR and IGSF4A in TM4 and Tera-2 cells, respectively, as detected by RT-PCR. GAPDH represents the positive control gene. **B**, **C**) TM4 cells were cultured to confluency in 24-well plates without (Control) or with transfection with PVR (PVR) or the vector alone (Mock), and the PVR-transfected cells were preincubated for 1 h without or with recombinant sIGSF4A-Fc (PVR+Rec) or Fc (PVR+Fc) in the culture medium. The fluorescently labeled Tera-2 cells were then placed on the TM4 cells and cultured for 1 h in the absence or presence of chicken monoclonal anti-IGSF4A IgY antibody (PVR+Ab) or non-immune chicken IgY (PVR+IgY). The average number of Tera-2 cells attached per field is plotted with SD (**B**, graph; **C**, picture). The PVR-transfected TM4 cells adhere 3.1-fold more Tera-2 cells than do the nontransfected or mock-transfected TM4 cells. Preincubation of PVR-transfected TM4 cells with sIGSF4A-Fc or the presence of anti-IGSF4A antibody in the culture medium reduces significantly the number of adhered cells. *, $P < 0.05$; **, $P < 0.01$; n = 6–9. Bar = 50 μm.

The mechanism by which the adhesion between spermatogenic and Sertoli cells regulates the process of spermatogenesis is largely unknown and will be the subject of future study. In the case of E-cadherin-mediated cell-to-cell adhesion at the adherens junctions of epithelial cells, it is well documented that the homophilic binding between cadherins regulates intracellular signaling mechanisms through the cytoplasmic proteins catenin α and β, which function as adapters between the cytoplasmic domains of cadherins and actin filaments. Furthermore, the cytoplasmic domains of nectins bind to afadin that is associated with actin filaments [37]. In the cultured cell systems, PVR binds through its cytoplasmic protein 4.1-binding motif to Tctex-1, which is a dynein light chain [35], and simultaneously binds through its PDZ-binding motif to the l1B subunit of the clathrin adaptor complex [50]. IGSF4A is known to bind through its protein 4.1-binding motif to erythrocyte membrane protein band 4.1-like 3 (also known as Dal-1/protein 4.1B), which is an actin-binding protein [51], and through its PDZ-binding motif to calcium/calmodulin-dependent serine protein kinase (CASK) [14] and syntenin [14] in cultured neurons, or to the membrane protein palmitoylated 6 (MPP6) (also known as Pals2) [15] and MPP3 (also known as Dlg3) [52] in cultured HEK293 cells. However, whether these potential signaling molecules are involved in the interaction of PVR with IGSF4A in the testis and function in spermatogenesis is unknown and warrants further investigation.

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