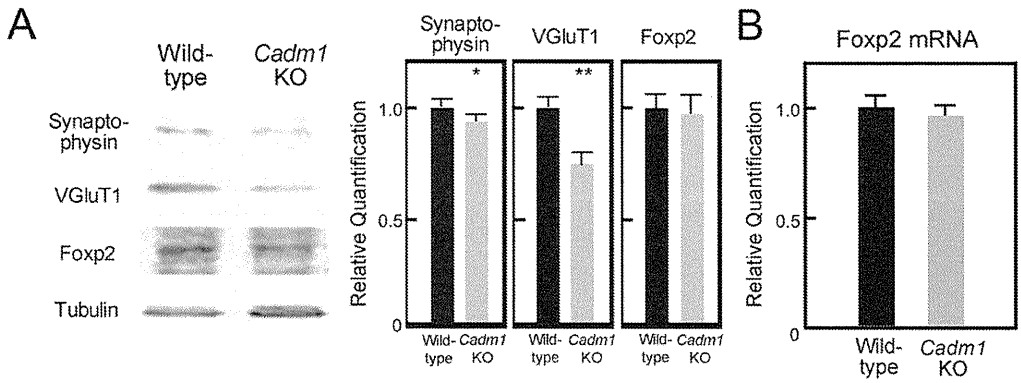

Figure 5. The influence of Cadm1 deficiency on the expression of synaptic proteins and Foxp2 in the cerebellum. (A) Immunoblot analysis of the influence of the deficiency in the cerebellums of *Cadm1* KO and wild-type pups (P10). An example of the typical immunoblotting results is shown. Values are mean±standard error (SEM). Student's *t*-test (*$p<0.05$, **$p<0.01$). Pups: n = 5. All experiments were performed three times. (B) RT-PCR analysis of the influence of the Cadm1 deficiency on the expression of *Foxp2* in the cerebellum of wild-type and *Cadm1* KO pups (P10). Values are mean±standard error (SEM). Pups: n = 5. All experiments were performed three times. A comparison showed no significant difference (Student's *t*-test; $p<0.05$).
doi:10.1371/journal.pone.0030151.g005

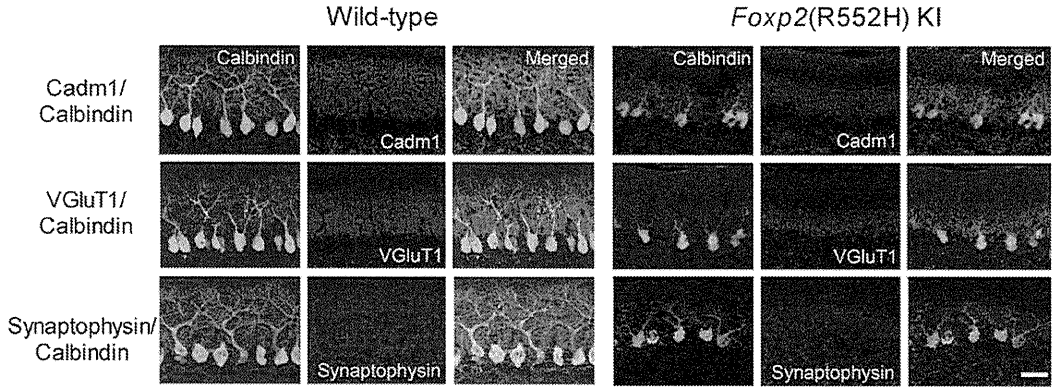

Figure 6. Altered distribution of Cadm1 in the molecular layer of wild-type and *Foxp2*(R552H) KI mice (P11). Cadm1 preferentially distributed in the apical–distal dendritic portion in the molecular layer. The immunoreactivity of Cadm1 as well as that of Synaptophysin and VGluT1, pre-synaptic markers, was decreased in the molecular layer of the *Foxp2*(R552H) KI mice. Green, Calbindin. Red, Cadm1, VGluT1, Synaptophysin. Blue, Hoechst. Bar, 30 μm.
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animals per genotype were examined, and experiments were repeated three times. Quantification of staining intensities was done using LAS AF software (Leica Microsystems). The mean pixel value in the area of interest and in the same size area of the background was calculated. The background level was subtracted from the value found in the area of interest (in the molecular layer). Reported intensities were normalized to control, and the Student's *t*-test was performed for statistical analysis.

Supporting Information

Figure S1 Alteration of Purkinje cells in cerebellum of wild-type and *Foxp2*(R552H) knock-in (*Foxp2* KI) mice, wild-type, and *Cadm1* knockout (*Cadm1* KO) (P11). The immunoreactivity was performed using mouse anti-Calbindin. Bar, 20 μm.
(TIF)

Figure S2 Altered distribution of the Cadm1 of wild-type and *Foxp2*(R552H) KI mice (P11). Values are mean± standard error (SEM). Student's *t*-test (**$p<0.01$). Pups: n = 3. Images: n = 10.
(TIF)

Figure S3 RT-PCR analysis of the expression of *Cadm1* in the cerebellum of wild-type and *Foxp2*(R552H) KI mice (P10). Values are mean±standard error (SEM). Pups: n = 5. All experiments were performed three times. A comparison showed no significant difference (Student's *t*-test; $p<0.05$).
(TIF)

Figure S4 The immunoreactivity (p11) of Synaptophysin (pre-synaptic marker) and PSD-95 (post-synaptic marker). Green, Cadm1. Red, Synaptophysin or PSD-95 (Cell Signaling Technology). Blue, Hoechst. Bar, 30 μm.
(TIF)

Author Contributions

Conceived and designed the experiments: TM MYM BI. Performed the experiments: EF YT. Analyzed the data: EF TM. Contributed reagents/materials/analysis tools: EF TM. Wrote the paper: EF BI TM.

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Cntnap2 expression in the cerebellum of *Foxp2*(R552H) mice, with a mutation related to speech-language disorder

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ABSTRACT

Foxp2(R552H) knock-in (KI) mice carrying a mutation related to human speech-language disorder exhibit impaired ultrasonic vocalization and poor Purkinje cell development. Foxp2 is a forkhead domain-containing transcriptional repressor that associates with its co-repressor CtBP; Foxp2(R552H) displays reduced DNA binding activity. A genetic connection between FOXP2 and *CNTNAP2* has been demonstrated *in vitro*, but not *in vivo*. Here we show that Cntnap2 mRNA levels significantly increased in the cerebellum of *Foxp2*(R552H) KI pups, although the cerebellar population of Foxp2-positive Purkinje cells was very small. Furthermore, Cntnap2 immunofluorescence did not decrease in the poorly developed Purkinje cells of *Foxp2*(R552H) KI pups, although synaptophysin immunofluorescence decreased. Cntnap2 and CtBP were ubiquitously expressed, while Foxp2 co-localized with CtBP only in Purkinje cells. Taken together, these observations suggest that Foxp2 may regulate ultrasonic vocalization by associating with CtBP in Purkinje cells; *Cntnap2* may be a target of this co-repressor.

1. Introduction

Autism spectrum disorder (ASD) is one of the most heritable neurodevelopmental disorders [22]. Mutations in the genes encoding contactin-associated protein 2 (CNTNAP2 and Caspr2) have been identified in patients with ASD and impaired speech-language [2,3]. On the other hand, *FOXP2*, a forkhead box-containing gene, is the causative gene of speech-language disorder [17]; the missense mutation FOXP2 (R553H) co-segregates with members of the KE family affected with speech-language disorder.

Cntnap2 is an adhesion molecule required for the formation of axoglial paranodal junctions surrounding the nodes of Ranvier in myelinated axons [21,23]. In the human brain, CNTNAP2 is also expressed in the circuits important for language development [2]. Cntnap2 is localized to the synaptic plasma membrane fraction [3] and to membrane traffic vesicles [4,20]. The C-terminal region of Cntnap2 contains the PDZ binding domain EYFI [14], which is similar to that of Cadm1, a synaptic adhesion molecule related to ASD [11,19,26]. Cntnap2 associates in *cis* with the contactin family, the glycosyl-phosphatidyl inositol-anchored neural cell adhesion molecule contactin/F3, TAG-1, NB-1, and NB-2, molecules required for the transport of Cntnap2 to the cell surface [9]. Cntnap2 also acts as a negative regulator of neurite outgrowth [8].

The relationship between speech-language disorder and ASD is not clear, although impaired social communication, including speech-language, is one of the phenotypes of ASD patients. FOXP2 has DNA-binding activity and exhibits repressor activity via its interaction with co-repressors such as C-terminal binding protein (CtBP) [18]. A genetic connection between FOXP2 and *CNTNAP2* has been shown *in vitro* [25]; FOXP2 binds the CAAATT motif in an intron of *CNTNAP2* and negatively regulates the expression of CNTNAP2 mRNA, but FOXP2(R553H) does not.

Infant rodents emit ultrasonic vocalizations (USVs), whistle-like sounds of frequencies between 40 and 100 kHz, that play an important communicative role in mother–offspring interactions when the pups are isolated from their mother and littermates [5]. *Foxp2* knock-out (KO) pups and knock-in (KI) pups harboring the *Foxp2*(R552H) mutation that corresponds to the human *FOXP2*(R553H) mutation exhibit severe USV impairment, suggesting that human speech and mouse USVs have a common molecular basis in the brain [15,24]. *Foxp2*(R552H) KI mice also display poor Purkinje cell development in the cerebellum [15]. Microarray analysis of the cerebellar gene expression of *Foxp2*(R552H) KI pups (P10) suggests that Foxp2 regulates USV-related gene expression in Purkinje cells.

To provide additional insight into the relationship between impairment of speech-language and ASD, we examined co-localization of CtBP and Foxp2 as well as Cntnap2 expression in the cerebellum of *Foxp2*(R552H) KI mice with impaired USV. Our

Abbreviations: Cntnap2, contactin-associated protein 2; CtBP, C-terminal binding protein; USV, ultrasonic vocalization; ASD, autism spectrum disorder.

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observations demonstrate the *in vivo* genetic connection between Foxp2 and *Cntnap2*.

2. Materials and methods

We followed the fundamental guidelines for proper conduct of animal experiment and related activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, and all of the protocols for animal handling and treatment were reviewed and approved by the Animal Care and Use Committee of Jichi University (approval numbers, H22-179; 10-179) and International University of Health and Welfare (approval numbers, D1008; 10118). Wild-type and *Foxp2*(R552H) KI mice (129/sv) [15] (male mice) were used for experiments.

Mouse brains were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C overnight. Frozen sections (10 μm thick) were cut on a cryostat and immunostained with mouse anti-calbindin (Sigma), rabbit anti-calbindin (Sigma), mouse anti-synaptophysin (Sigma), mouse anti-CTBP (Santa Cruz Biotech.), rabbit anti-caspr2 (Cntnap2; Abcam), and rabbit anti-Foxp2 (Abcam). Alexa Fluor 488 and Alexa Fluor 568 conjugated secondary antibodies against mouse and rabbit IgG were purchased from Molecular Probes. Nuclei were detected by Hoechst 33342 (Molecular Probes). The reactivity was viewed using a Leica AF6000 immunofluorescence microscope or a Leica SP5 confocal microscope (Leica Microsystems). At least three different animals per genotype and experiments were repeated three times.

Total RNA was prepared from brains of wild-type and *Cadm1* KO [13] and *Foxp2*(R552H) KI male mice [15] by RNeasy mini kit (Qiagen) according to the manufacturer's specifications. Complementary DNAs were synthesized from total RNA (1 μg) using reverse transcriptase (Invitrogen) as described previously [12]. Quantitative real-time (RT) PCR analysis was performed by Applied Biosystems 7500 fast real-time PCR system (Applied Biosystems) using the TaqMan Gene Expression Assays (Applied Biosystems) based on published sequences for genes encoding the respective mouse *Cntnap2*, and VIC-labeled mouse Gapd (VIC-labeled MGD probe; Applied Biosystems) as endogenous control. For each sample, the 20 μl total volume consisted of 10 μl TaqMan Fast Universal PCR Master Mix (2×; Applied Biosystems), 1 μl TaqMan Gene Expression Assays, and 5 μl of each first-strand cDNA sample. The real-time PCR fragments were amplified as follows: 1 cycle at 95 °C for 20 s, 60 cycles at 95 °C for 3 s and 60 °C for 30 s. Results were analysed by Student's *t*-test ($p < 0.05$ was considered statistically significant).

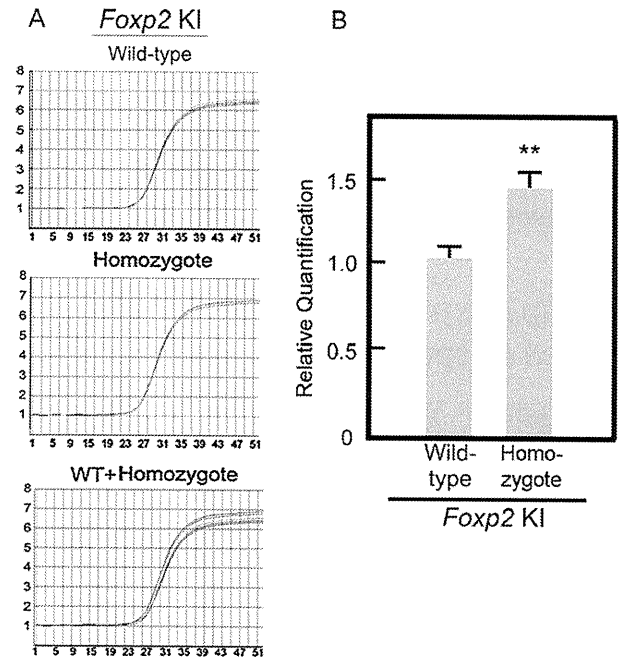

Fig. 1. Increased expression of Cntnap2 in the cerebellum of *Foxp2*(R552H) KI mice (P10). Quantitative real-time PCR analysis of the expression of Cntnap2 mRNA in the cerebellum of *Foxp2*(R552H) KI mice (A and B). The expression of Cntnap2 mRNA in *Foxp2*(R552H) KI mice was compared with that in wild-type mice. Values are mean ± SEM for at least three independent determinations. Student's *t*-test (**$p < 0.01$).

3. Results

We quantitatively measured the level of Cntnap2 mRNA in the cerebella of wild-type and *Foxp2*(R552H) KI pups (P10). The level of Cntnap2 mRNA increased 1.6-fold ($p < 0.05$) in the cerebellum of *Foxp2*(R552H) KI mice (Fig. 1A and B). However, the mRNA levels of other genes, including *Cadm1*, were unchanged (Supplementary Fig. S1). Thus, the expression of *Cntnap2* appears to be under the control of Foxp2 in the cerebellum.

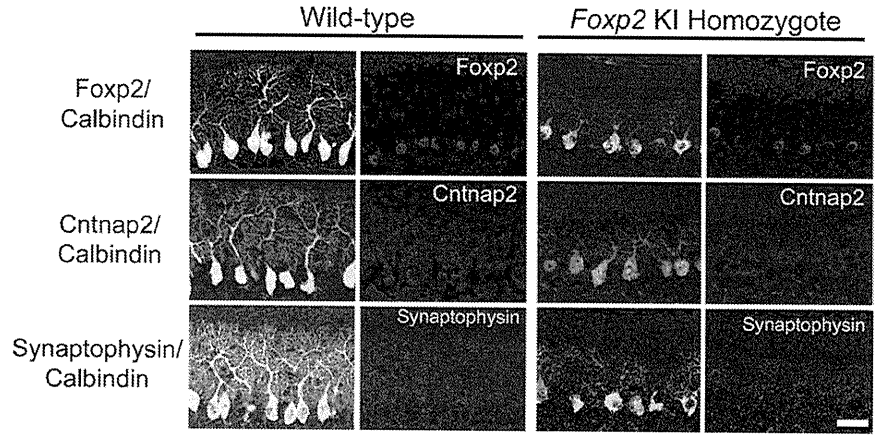

Fig. 2. Cntnap2 localization in the cerebellum of *Foxp2*(R552H) KI pups. Cntnap2 distribution was examined in the cerebellum of wild-type and *Foxp2*(R552H) KI pups (P10) by immunostaining. The Cntnap2 intensity preferentially distributed in the proximal dendritic portion. In contrast with synaptophysin, intensity of Cntnap2 immunoreactivity was not decreased in the *Foxp2*(R552H) KI pups. Green; calbindin. Red; Foxp2, Cntnap2 or synaptophysin. Blue; Hoechst. Bar indicates 30 μm.

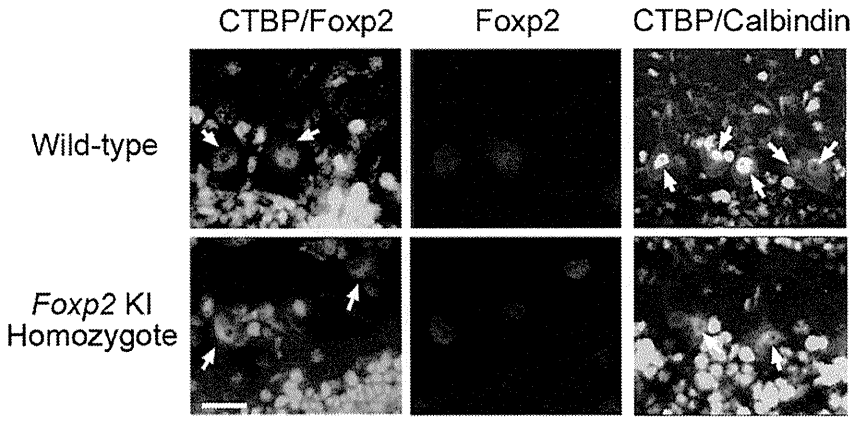

Fig. 3. Localization and distribution of CtBP in the cerebellum of wild-type and *Foxp2*(R552H) KI mice (P10). The CtBP co-localized with Foxp2 (left panel). The CtBP localized both in the Purkinje cells and granular cells (right panel). As Foxp2 localized in the nuclei of Purkinje cells (see Fig. 2), the CtBP co-localized with Foxp2 in the nuclei of Purkinje cells. Green; CTBP. Red; Foxp2 or calbindin. Blue; Hoechst. Bar indicates 30 μm.

To elucidate the role of Cntnap2 in USV, we examined Cntnap2 localization in the cerebella of wild-type and *Foxp2*(R552H) KI mice. Cntnap2 immunoreactivity was preferentially located in the soma and proximal dendritic portion of Purkinje cells, as well as in the granular cells of the wild-type P10 pups (Fig. 2). *Foxp2*(R552H) KI pups exhibited poor development of Purkinje cell dendrites with reduced synaptophysin immunoreactivity [15]. In contrast, the intensity of Cntnap2 immunofluorescence was apparently unchanged (Fig. 2). However, given the poor development and decreased number (20% reduction) of Purkinje cells in the *Foxp2*(R552H) KI pups, the intensity of Cntnap2 immunofluorescence seems to be relatively increased in the Purkinje cells.

Foxp2 associates with its co-repressor CtBP and acts as a transcriptional repressor [18]. In the cerebellum, CtBP localized to the nuclei of granular cells and Purkinje cells (Fig. 3, right), while Foxp2 localized to the nuclei of Purkinje cells but not granular cells (Fig. 2). CtBP co-localized with Foxp2 and Foxp2(R552H) in the Purkinje cells of wild-type and *Foxp2*(R552H) KI mice, respectively (Fig. 3, left).

4. Discussion

Foxp2(R552H) KI mice exhibit abnormal cerebellar development and poor dendrite development [15]. In addition, the selective expression of FOXP2 in the Purkinje cells of *Foxp2*(R552H) KI mice partially improves their USVs (unpublished observation), suggesting that Purkinje cells are an essential component of the Foxp2-mediated USV network. We observed a statistically significant increase in Cntnap2 mRNA levels in *Foxp2*(R552H) KI mice. Expression of Cntnap2 and its co-repressor CtBP was ubiquitous in granular cells and Purkinje cells, but Foxp2 expression was restricted to the Purkinje cells. Given the ratio of granular cells to Purkinje cells in the cerebellum, Cntnap2 mRNA must be up-regulated in the Purkinje cells of the *Foxp2*(R552H) KI mice more than 1.6 fold. Thus, our observations suggest that Cntnap2 is a Foxp2 target in Purkinje cells, and that the Foxp2 and CtBP complex acts as a transcriptional repressor.

The apparently unchanged intensity of Cntnap2 immunofluorescence in *Foxp2*(R552H) KI mice, which display poor Purkinje cell dendrite development and decreased synaptophysin immunofluorescence, suggests that Cntnap2 expression is relatively increased in the decreased number of synapses on the dendrites. Cntnap2 must be up-regulated and degraded, whereas synaptophysin may not be up-regulated but is degraded.

Human speech and mouse USVs have a common molecular basis in the brain that is mediated by Foxp2 [15,24], and the cerebellum is related to the network engaged in the production of spoken human language [1,6]. Functional magnetic resonance imaging has suggested the existence of two cerebro-cerebellar networks for human verbal working memory; cerebellar abnormality with Purkinje cell loss has been observed in several autopsy samples from ASD patients [10]. A *Cntnap2* mutation occurs in ASD patients with speech-language disorder [2,3], suggesting that Cntnap2 is an essential factor for speech-language. However, Cntnap2 is relatively up-regulated in the Purkinje cells of *Foxp2*(R552H) KI pups (Fig. 1) with impaired USV. Cntnap2 is a negative regulator of neurite outgrowth [8], suggesting that Cntnap2 may be a negative regulator in the network involved in Foxp2-mediated USV and/or speech-language. However, it is presently unclear why Cntnap2-mediated negative regulation is necessary for Foxp2-mediated USV. This unresolved question will be an important issue for future study; USV and speech-language function may be regulated by a dual positive and negative synaptic system.

Cntnap2 may cooperate or compete with synaptic adhesion molecules, including Cadm1, in Foxp2-mediated USV. Mutations in *CADM1* and *NLGN-3* occur in ASD patients [7,26]. *Cadm1* KO, as well as *Nlgn-3*- and *Nlgn-4*-deficient mice, exhibit impaired USV [16], and Cadm1 localizes to the molecular layer of the dendritic arbor of Purkinje cells, with reduced distribution in *Foxp2*(R552H) KI pups (Fujita et al., submitted). Cntnap2 localized not only to the proximal dendrites, but also to the soma of Purkinje cells (Fig. 2) and to membrane traffic vesicles [4,20], indicating that Cntnap2 is involved in the membrane trafficking of synaptic molecules to the synapse. Since the cytoplasmic tail of Cntnap2 carries a PDZ binding motif identical to the one in Cadm1, and since its *cis*-binding partners are members of the contactin family of the immunoglobulin superfamily, it may be possible that the contactin-2(TAG-1)-Cntnap2 complex and Cadm1 cooperatively or competitively participate in speech-language and USV activity in the molecular layer.

In conclusion, *Cntanp2* expression is repressed by Foxp2 in Purkinje cells. Cntnap2 may cooperate or compete with synaptic molecules such as Cadm1 in Foxp2-mediated USV and speech-language.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2011.11.022.

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ORIGINAL ARTICLE

Localisation of RA175 (Cadm1), a cell adhesion molecule of the immunoglobulin superfamily, in the mouse testis, and analysis of male infertility in the RA175-deficient mouse

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Summary

RA175, a member of the immunoglobulin superfamily, plays an important role in cell adhesion, and *RA175* gene-deficient mice ($RA175^{-/-}$) show oligoasthenoteratozoospermia. To understand the function of RA175, location in the testis and the morphological features of its spermatogenic cells in $RA175^{-/-}$ mice were investigated. Immunohistochemical studies revealed that RA175 immunoreactivity was observed on the cell surface of the spermatogenic cells at specific stages. A strong reaction was detected from type A spermatogonia to pachytene spermatocytes at stage IV and from step 6 to step 16 spermatids during spermatogenesis. From pachytene spermatocytes at stage VI to step 4 spermatids, the reaction was not detected by the enzyme-labelled antibody method and was faintly detected by the indirect immunofluorescence method. Abnormal vacuoles in the seminiferous epithelium, showing exfoliation of germ cells, and ultrastructural abnormality of the elongate spermatids were revealed in the $RA175^{-/-}$ testes. Other members of the immunoglobulin superfamily such as basigin, nectin-2 and nectin-3, which have an important role in spermatogenesis, were immunohistochemically detected in the $RA175^{-/-}$ testis. These observations indicate a unique expression pattern of RA175 in the testis and provide clues regarding the mechanism of male infertility in the testis.

Introduction

Immunoglobulin superfamily (IgSF) molecules serve as cell adhesion receptors, and are implicated in diverse cellular phenomena such as cell shape and polarisation, cytoskeletal organisation, cell motility, proliferation, survival and differentiation (Holness & Simmons, 1994; Hynes, 1999). It is known that the IgSF members such as basigin and nectins are expressed in the testis and have a crucial function in spermatogenesis (Toshimori *et al.*, 2006). For example, basigin (CD147/EMMPRIN, Maekawa *et al.*, 1998; Toyama *et al.*, 1999), nectins (Bouchard *et al.*, 2000; Inagaki *et al.*, 2006) and Jam-C/Jam3 (Gliki *et al.*, 2004; Fujita *et al.*, 2007) are expressed on the testicular cells, and their gene-deficient mice show male sterility.

Another IgSF member, RA175 (Cadm1), was first characterised as one of the genes preferentially expressed during the differentiation of P19 mouse embryonal carcinoma cells induced by retinoic acid (Urase *et al.*, 2001; Fujita *et al.*, 2003). The same molecule has been characterised independently and is called by different names: tumour suppressor of lung cancer 1 (TSLC1) (Kuramochi *et al.*, 2001); immunoglobulin superfamily member 4 (IGSF4) (Gomyo *et al.*, 1999); spermatogenic immunoglobulin superfamily member (SgIGSF) (Wakayama *et al.*, 2001); synaptic cell adhesion molecule (SynCAM) (Biederer *et al.*, 2002) and nectin-like molecule 2 (Necl2) (Shingai *et al.*, 2003). These facts indicate the diverse functions of the molecule RA175. RA175 has three immunoglobulin domains in its extracellular region that are involved in cell–cell adhesion via a homophilic or heterophilic interaction (Masuda *et al.*, 2002; Fujita *et al.*, 2003; Wakayama & Iseki, 2009). In order to elucidate the functions of RA175, we have generated mice lacking the gene and found that the resulting $RA175^{-/-}$ male mice were infertile, showing oligoasthenoteratozoospermia

(Fujita *et al.*, 2006). *RA175*$^{-/-}$ females and heterozygous mice were fertile and no obvious defects were observed. Mice deficient in this gene were subsequently reported by other groups (Surace *et al.*, 2006; van der Weyden *et al.*, 2006; Yamada *et al.*, 2006), and their results agreed with our previous report (Fujita *et al.*, 2006).

In this study, we investigated the location of RA175 in the testis in detail and the characteristics of *RA175*$^{-/-}$ mice to understand the function of RA175. Furthermore, the location of other IgSF members, such as basigin, nectin-2 and nectin-3, which play important roles in spermatogenesis, was examined in the *RA175*$^{-/-}$ testis.

Materials and methods

Animals and antibodies

The generation of mice with the targeted disruption of the *RA175* gene has been previously reported (Fujita *et al.*, 2006). Animal handling was approved by the Animal Research Committee of Chiba University. More than 10 *RA175*$^{-/-}$ and *RA175* $^{+/+}$ mice (3–6 months old) were examined in this study, and more than five sections were analysed per mouse by immunohistochemistry and indirect immunofluorescence. Anti-RA175 antibody was raised against a peptide corresponding to the C-terminal region of RA175, as described previously (Fujita *et al.*, 2003). Goat anti-basigin antibody (sc-9757) and goat anti-nectin-2 antibody (sc-14799) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and rat monoclonal antibody against nectin-3 was obtained from Abcam (Cambridge, UK). The rat monoclonal antibody TRA98 was a gift from Dr Nishimune of Osaka University.

Immunohistochemistry

Wild-type mice were anaesthetised with pentobarbital and fixed with Bouin's fluid by perfusion through the left ventricle. Testes were removed, embedded in paraffin, and cut at a thickness of 3 μm. These sections were immunostained with either anti-RA175 antibody, anti-basigin antibody or anti-nectin-2 antibody. Non-specific binding of the antibody was blocked by placing the sections in phosphate buffered saline (PBS) containing 5% normal goat serum or foetal bovine serum for 30 min at room temperature (RT). The sections were then incubated with either rabbit anti-RA175 antibody, goat anti-basigin antibody or goat anti-nectin-2 antibody at 4 °C overnight. After washing with PBS, the samples were incubated with biotinylated goat anti-rabbit IgG (Dako Japan Company, Kyoto, Japan) or biotinylated rabbit anti-goat IgG (Dako) for 1 h, followed by incubation with streptavidin–biotin peroxidase complex solution (Dako) for 30 min. Immunohistochemical reactions were visualised using 3,3′-diaminobenzidine and H_2O_2. Several adjacent sections were stained with periodic acid Schiff (PAS) and counterstained with haematoxylin to determine the stages of the seminiferous epithelium.

Indirect immunofluorescence (IIF)

Wild-type and *RA175*$^{-/-}$ mice were anaesthetised with pentobarbital, and perfused through the heart with 4% paraformaldehyde in PBS. The testes and epididymides were removed and immersed in a fixative for an additional 4 h. They were embedded in OCT compound and cut at a thickness of 20 μm on a cryostat. After blocking the sections for 1 h at RT, the testicular sections were incubated with rabbit anti-RA175 antibody or rat anti-nectin-3 antibody, and the sections from the caput epididymidis were incubated with rat monoclonal antibody TRA98 at 4 °C overnight. The slides were then incubated with Alexa Fluor 488 goat anti-rabbit IgG (0.5 μg ml^{-1}; Invitrogen, Carlsbad, CA, USA) or goat anti-rat IgG (0.5 μg ml^{-1}; Invitrogen) together with propidium iodide (1 μg ml^{-1}; Sigma, St. Louis, MO, USA) and RNase (10 μg ml^{-1}; Sigma) in the blocking solution for 1 h at RT. After washing with PBS, the samples were mounted with PermaFluor (Immunon, Pittsburgh, PA, USA) and observed using a confocal laser scanning microscope (LSM510; Carl Zeiss, Oberkohen, Germany). To stain the actin filaments and nuclei in the testis, the frozen sections were incubated with 0.83 μg ml^{-1} FITC-labelled phalloidin (Sigma), 1 μg ml^{-1} propidium iodide (Sigma) and 10 μg ml^{-1} RNase (Sigma) for 1 h, and observed under a confocal laser scanning microscope.

Transmission electron microscopy

The animals were anaesthetised with pentobarbital and perfused transcardially with 3% glutaraldehyde in HEPES buffer. The testes and epididymides were excised and immersed in the same fixative for 2 h at RT. After post-fixation with 1% OsO_4, the tissues were embedded in epoxy resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed under an electron microscope (JEM 1200EX, JEOL, Tokyo, Japan).

Results

Immunohistochemical localisation of RA175 in the mouse testis

The immunohistochemical localisation of RA175 in the wild-type mouse testis was determined by the enzyme-labelled antibody method (Fig. 1). Paraffin sections of the

testis were immunostained with rabbit anti-RA175 antibody. Adjacent sections were stained with PAS and haematoxylin to identify the stages of the seminiferous tubules (data not shown). The results revealed the stage-specific localisation of RA175 in the testicular germ cells. RA175 immunoreactivity was detected on the cell surface of spermatogonia and spermatocytes until stage IV, although all type A spermatogonia did not exhibit a positive reaction. The immunoreactivity became weak in the pachytene spermatocytes at stage V and was not detected in the pachytene spermatocytes at stage VI through step 4 round spermatids at stage IV. Interestingly, the positive staining reappeared on the cell surface of step 5 spermatids. Staining around the head became weak in step 13 spermatids and then disappeared in step 14 spermatids, whereas immunoreactivity around the cytoplasm was detected. Just before spermiation, immunoreactivity was detected only in the residual bodies. Mature sperm in the cauda epididymidis did not exhibit an immunopositive reaction (data not shown). A detailed examination revealed that the immunopositive reaction was confined to the apical surface of type A spermatogonia and was not detected in the basal surface which is in contact with the basal lamina (Fig. 1g). These results are summarised in Fig. 1h. Control experiments using *RA175*$^{-/-}$ testes revealed no RA175 immunoreaction in the spermatogenic cells.

Localisation of RA175 in the testis detected by IIF

Localisation of RA175 in the testis was also detected by IIF (Fig. 2), and positive reactions was observed in the germ cells within the seminiferous tubules. Germ cells immunostained by the enzyme-labelled antibody method (Fig. 1) were also IIF positive (Fig. 2). In addition, late pachytene spermatocytes to early round spermatids, which did not show a positive reaction by the

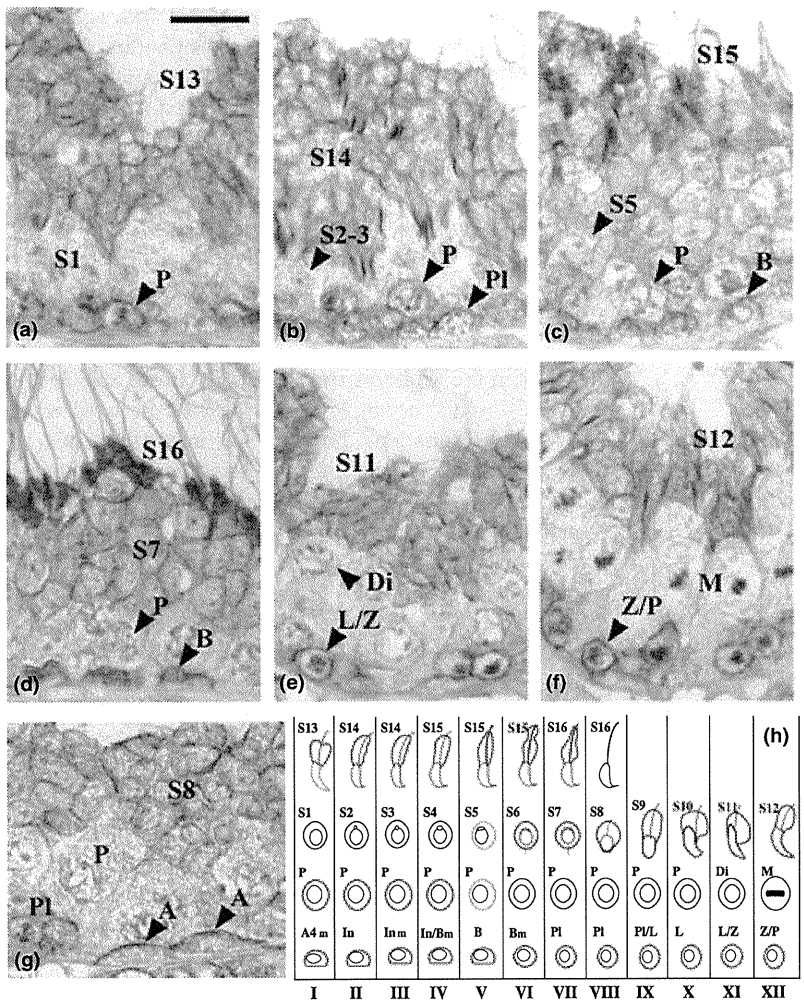

Fig. 1 Immunohistochemical localisation of RA175 in the mouse seminiferous epithelium. (a–g) Paraffin sections of wild-type mouse testes were immunostained with an anti-RA175 antibody (brown) by the enzyme-labelled antibody method and were counterstained with haematoxylin (blue). (a) Stage I, (b) Stage II–III, (c) Stage V, (d) Stage VII, (e) Stage XI, (f) Stage XII. (g) Stage VIII. Bar = 20 μm. (h) Summary of the immunolocalisation of RA175 in the testis. Positive immunostaining is shown in red. A, type A spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocyte; L/Z; leptotene/zygotene spermatocyte; Z/P, zygotene/pachytene spermatocyte; P, pachytene spermatocyte; Di, diplotene spermatocyte; M, metaphase of the spermatocyte; S1, S2–3, S5, S7, S8, S11, S13, S14, S15 and S16, step 1, 2–3, 5, 7, 8, 11, 13, 14, 15 and 16 spermatid respectively.

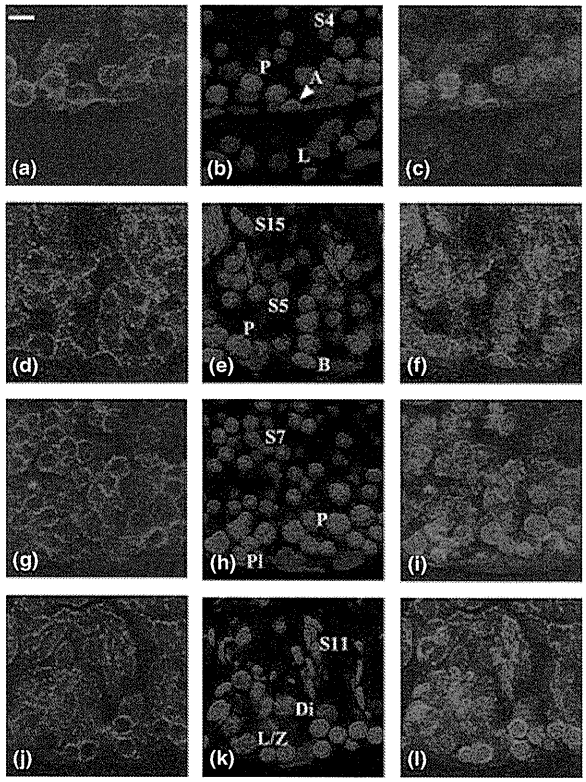

Fig. 2 Immunolocalisation of RA175 in the mouse seminiferous epithelium revealed by IIF. Frozen sections of wild-type testes were immunostained with anti-RA175 antibody (green; a, d, g) and counterstained with propidium iodide for nuclei (red; b, e, h). c, f and I shows merged images. (a–c) Stage IV, (d–f) stage V, (g–i) stage VII, (j–l) stage XI. Bar = 10 μm.

enzyme-labelled antibody method (Fig. 1), were found to be IIF positive. Although reactivity on the cell surface was less distinctive, a diffuse staining in the cytoplasm was revealed by IIF staining (Fig. 2).

Morphological characterisation of the testis and epididymis from *RA175*$^{-/-}$ mouse

Testes from *RA175*$^{-/-}$ mice frequently exhibited abnormal vacuoles in the seminiferous epithelium (Fig. 3a, b). Elongate spermatids were obviously reduced in number in the *RA175*$^{-/-}$ testis. Electron micrographs revealed elongate spermatids with deformed nuclei and/or acrosomes, which were observed frequently in the *RA175*$^{-/-}$ testis (Fig. 3e). An ectopic manchette (Fig. 3f) was often observed; 16 spermatids had irregular manchette out of 44 spermatids. Although the marginal rings of the acroplaxome appeared normal (Fig. 3g), apical ectoplasmic specialisations of the Sertoli cells were separated from the spermatids, and small vacuoles were formed around the spermatids (Fig. 3g, h). This is probably caused by the dissociation of the elongate spermatids from the Sertoli cells through lack of RA175.

Many cells, but not spermatozoa, were found in the epididymis of the *RA175*$^{-/-}$ mouse (Fig. 4). An indirect immunofluorescence study using TRA98 revealed that many cells in the epididymal duct were immunopositive. As the TRA98 antibody recognises germ cells at various stages of differentiation from spermatogonia to spermatids (Tanaka *et al.*, 1997), the positive cells were identified as immature spermatogenic cells and not as spermatozoa (Fig. 4b, d). Round spermatids such as step 3, 5 and 7 spermatids were detected in the epididymal duct by electron microscopy (Fig. 4e, f).

Localisation of basigin, nectin-2 and nectin-3 in the *RA175*$^{-/-}$ testis

To examine the expression of other IgSF members, testes from *RA175*$^{-/-}$ mice were immunostained with anti-basigin, anti-nectin-2 and anti-nectin-3 antibodies (Fig. 5). Basigin was detected in the cytoplasm of round spermatids as well as in the periphery of spermatocytes and spermatids in the wild-type testes (Fig. 5b). In the *RA175*$^{-/-}$ testes, the localisation of basigin was similar to that in the wild-type testes (Fig. 5a). Nectin-2 was localised at the ectoplasmic specialisations of the Sertoli cells, which locates both in the Sertoli–Sertoli junctions of the basal regions (basal ectoplasmic specialisation) and in the Sertoli–spermatid junctions of the adluminal regions (apical ectoplasmic specialisation) of the seminiferous tubules in the wild-type testes (Fig. 5d). In the case of the *RA175*$^{-/-}$ testis, nectin-2 was detected at the basal ectoplasmic specialisations, however, the immunoreaction at the apical ectoplasmic specialisations was not conspicuous, partly because the number of normal elongate spermatids was considerably decreased (Fig. 5c). Nectin-3 was localised in the spermatids adjoining the apical ectoplasmic specialisations in the wild-type testis (Fig. 5f). The spermatids at step 8–9 in the *RA175*$^{-/-}$ testis exhibited a similar nectin-3-immunopositive reaction to the wild-type testis (data not shown). However, at stage XII, when the abnormal morphology of the elongate spermatids at step 12 became noticeable in the *RA175*$^{-/-}$ testis, nectin-3 immunoreactivity was reduced in comparison with that in the wild-type testis (Fig. 5e, f).

An abnormal distribution of actin filaments in the *RA175*$^{-/-}$ testis was revealed by phalloidin staining (Fig. 5g, h). Actin filaments were not observed around the malformed heads of elongate spermatids, and an accumulation of the actin filaments was found in the seminiferous tubules.

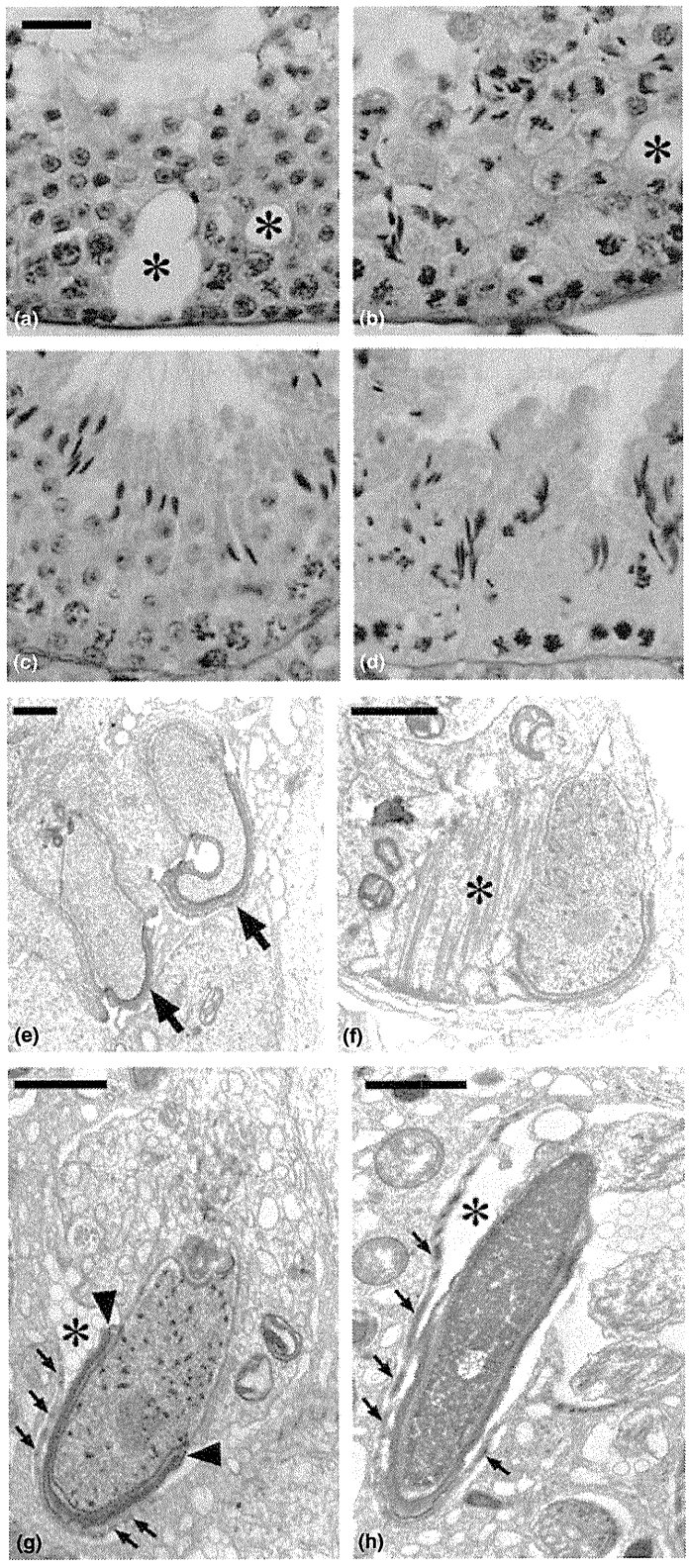

Fig. 3 (a–d) Seminiferous epithelia at stage VI (a, c) and XII (b, d) from *RA175*$^{-/-}$ (a, b) and wild-type mice (c, d) stained with PAS-haematoxylin. The number of elongate spermatids was markedly decreased and vacuoles (*) in the seminiferous tubules were observed in the *RA175*$^{-/-}$ testes (a, b). Bar = 20 μm. (e–h) Electron microphotographs of *RA175*$^{-/-}$ testis. Step 9 spermatids with deformed nuclei and acrosomes (e; arrows) and with ectopic manchette (f; asterisk). Small vacuoles (g; asterisk) and dissociation (h; asterisk) between the acrosome of the spermatid and ectoplasmic specialisation (g, h; small arrows) in the Sertoli cell are observed. The marginal rings of the acroplaxome (g; arrowheads) appear to be normal. Bar = 1 μm.

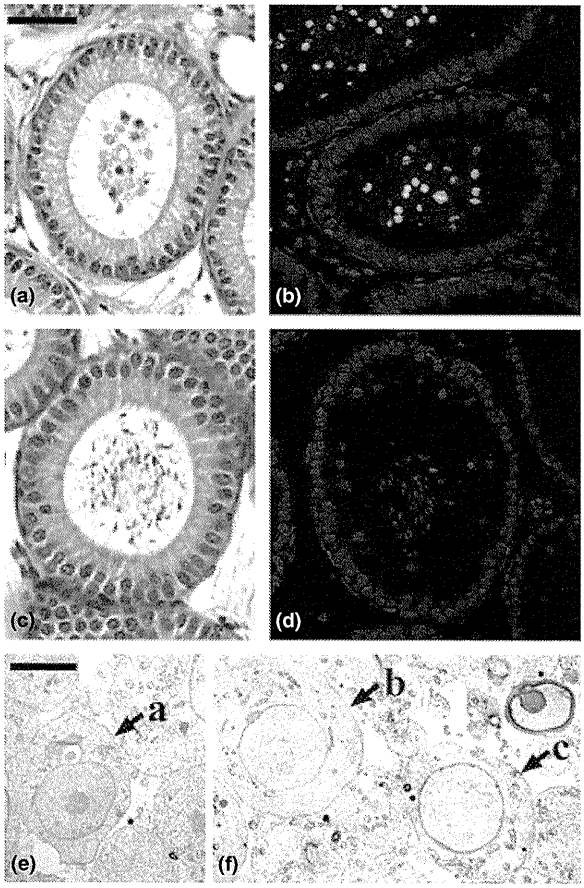

Fig. 4 Caput epididymidis from *RA175*$^{-/-}$ (a, b, e, f) and wild-type mice (c, d). (a, c) Stained with haematoxylin and eosin. (b, d) Immunostained with TRA98 antibody (green) and propidium iodide (red). Bar = 50 μm. (e, f) Electron micrographs of the lumen of the caput epididymidis from *RA175*$^{-/-}$ mouse. Step 3 (a), step 5 (b) and step 7 (c) spermatids were observed in the lumen. Bar = 5 μm.

Discussion

To clarify the function of RA175 during spermatogenesis, it is essential to detect the precise location of this molecule in the testis. Our results in this report (Fig. 1h) revealed a few differences from the previous study (Wakayama *et al.*, 2003; Yamada *et al.*, 2006), which reported that the RA175 protein was detected in spermatogenic cells from intermediate spermatogonia to early pachytene spermatocytes and from step 7 spermatids to the residual bodies of step 16 spermatids. First, we found that several type A spermatogonia were immunopositive for RA175. Spermatogonia are classified either as type A, intermediate, or type B. Type A spermatogonia are the most primitive spermatogonia and are subdivided into undifferentiated (As, Apr and Aal) and differentiating (A1, A2, A3 and A4) spermatogonia (de Rooij & Grootegoed, 1998). As immunonegative type A spermatogonia were also detected, all subpopulations of type A spermatogonia do not express RA175. At present, it is not yet known which subtype(s) of type A spermatogonia express the RA175 protein, however, RA175 can be used as a marker for the differentiation of type A spermatogonia. Secondly, we found RA175 immunoreactivity until pachytene spermatocytes at stage IV, and the reaction diminished thereafter and was not detected from pachytene spermatocytes at stage IV through step 4 spermatids. Subsequently, the activity reappeared in step 5–6 spermatids. Furthermore, by the IIF method, positive reactions for RA175 were detected to some extent in the cytoplasm of mid- to late-pachytene spermatocytes and in round spermatids, which showed no positive staining by the enzyme-labelled antibody method. These differences might be caused by the experimental systems, including the mice strains and antibodies used. However, our IIF results might reflect the intrinsic distribution of RA175, which does not completely disappear during the mid-pachytene spermatocyte to the step 4 spermatid stage.

RA175 immunoreactivity was found at the apical site and not at the basal site of the spermatogonia. This implies that RA175 is localised at the germ cell surface which contacts the Sertoli cells. Thus, it is important to detect the binding partner of RA175 which probably exists in the Sertoli cells. Wakayama *et al.* (2007) identified a poliovirus receptor (PVR) as a binding partner of RA175, which was present in the Sertoli cells of the mouse testis. This finding implies a heterophilic binding between RA175 on germ cells and PVR on the Sertoli cells in the testis.

In *RA175*$^{-/-}$ mice, the number of elongate spermatids was remarkably decreased in the testes, and the remaining elongate spermatids had abnormally shaped heads and acrosomes. Vacuoles were frequently found in the seminiferous epithelium of the *RA175*$^{-/-}$ testis, implying that a cluster of germ cells was exfoliated from the seminiferous epithelium. These observations agreed with those of previous reports (Fujita *et al.*, 2006; van der Weyden *et al.*, 2006; Yamada *et al.*, 2006) and demonstrated that RA175 is responsible for the attachment of germ cells to the seminiferous epithelium and spermatid morphogenesis in the testis. The exfoliated germ cells are partly phagocytosed by Sertoli cells (Fujita *et al.*, 2006), and other cells accumulate in the epididymal duct. It is notable that all germ cells showing RA175-immunopositive reaction were not necessarily exfoliated from the seminiferous epithelium in the *RA175*$^{-/-}$ testis. Other factors probably compensate in part for the function of RA175. Basigin, another IgSF member, exists in these germ cell stages. The molecule was also detected in the

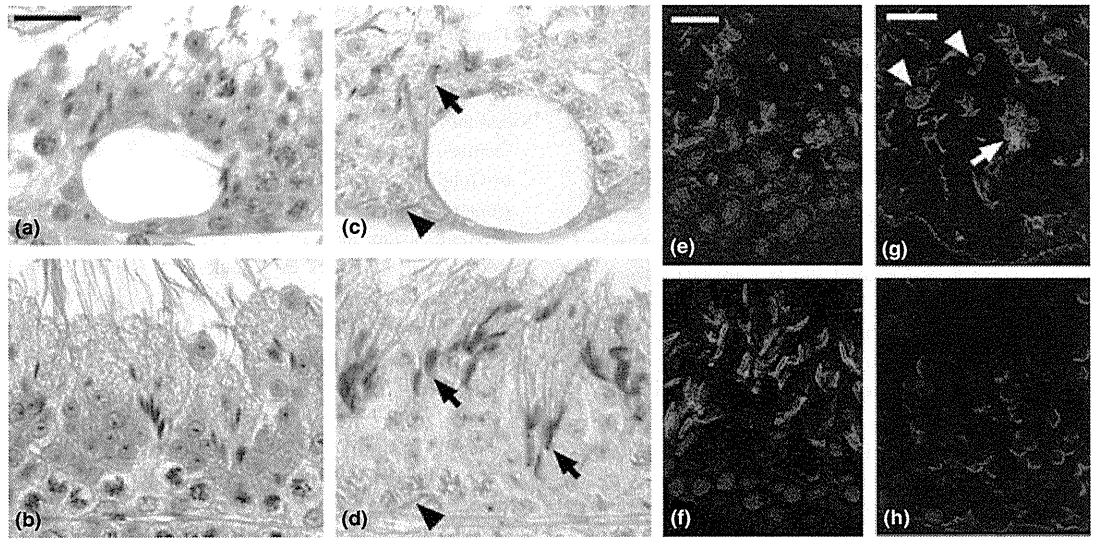

Fig. 5 Immunohistochemical localisation of basigin (a, b), nectin-2 (c, d) and nectin-3 (e, f), and distribution of actin filaments (g, h) in the testis. Testes from *RA175*$^{-/-}$ (a, c, e, g) and wild-type mice (b, d, f, h) were stained with anti-basigin antibody (a, b), anti-nectin-2 antibody (c, d), anti-nectin-3 antibody (e, f), or FITC-labelled phalloidin (g, h), and counterstained with haematoxylin (a–d) or propidium iodide (red; e–h). Arrowheads in (c), (d); basal ectoplasmic specialisations. Arrows in (c), (d); apical ectoplasmic specialisations. (g, h) In the *RA175*$^{-/-}$ testes, actin filaments were not observed around the malformed head of the elongate spermatids (arrowheads in g), and were observed to accumulate in the seminiferous tubules (arrow in g). Bar = 20 μm.

RA175$^{-/-}$ testis and is one of the candidates for such factors.

An ultrastructural investigation of elongate spermatids in the *RA175*$^{-/-}$ testis demonstrated abnormal morphology of the manchette, a microtubular structure around the nucleus of the elongate spermatid. The ectopic manchette of the spermatid has been reported in other gene-deficient mice showing male infertility, such as *GOPC*$^{-/-}$ (Ito *et al.*, 2004) and *Hrb*$^{-/-}$ (Kierszenbaum *et al.*, 2004) mice. The manchette is involved in the nuclear elongation and condensation of the spermatid, and spermatozoa from *GOPC*$^{-/-}$ and *Hrb*$^{-/-}$ mice have round heads without acrosomes, which are different from the *RA175*$^{-/-}$ phenotype. Another structure termed the acroplaxome, an F-actin/keratin 5-containing cytoskeletal plate anchored to the spermatid nucleus (Kierszenbaum *et al.*, 2003), is also important in determining the shape of the spermatid head. The acroplaxomes appeared to be normal in *RA175*$^{-/-}$ mice, and the abnormal shapes of the heads and acrosomes of the elongate spermatids in the *RA175*$^{-/-}$ testis were not triggered by the acroplaxome abnormality, and could be a secondary effect of *RA175* deficiency.

An unusual accumulation of actin filaments near the lumen of the seminiferous tubules was observed in the *RA175*$^{-/-}$ testis. These actin filaments were components of the apical ectoplasmic specialisations between Sertoli cells and spermatids, and were formed from the discarded ectoplasmic specialisations of exfoliated spermatids. Such structures are frequently found in seminiferous tubules lacking spermatids (Hosoi *et al.*, 2002).

Nectin-2 and nectin-3 are associated with the actin cytoskeleton through afadin, and nectin-2 on Sertoli cells and nectin-3 on spermatids interact at the apical ectoplasmic specialisations (Ozaki-Kuroda *et al.*, 2002). We examined the immunolocalisation of nectin-2 and nectin-3 in the *RA175*$^{-/-}$ testis, and their signals were decreased or lost around the malformed heads of the elongate spermatids, indicating the detachment of spermatids from the Sertoli cells in the seminiferous epithelium. However, a small number of elongate spermatids that survived and appeared to have normal morphology showed positive immunoreactions for nectins. Although nectin-3 is reported to interact with RA175 (necl-2) (Takai *et al.*, 2003), their expression sites were differed in the testis, and the roles of RA175 and nectins are different in spermatogenesis as discussed in the previous paper (Fujita *et al.*, 2006).

In this report, we revealed the characteristic pattern of RA175 expression in the testis, and demonstrated that basigin, nectin-2 and nectin-3, the other members of IgSF, were also expressed in the *RA175*$^{-/-}$ testis. Many IgSF members are necessary to achieve normal spermatogenesis in the testis, and RA175 plays an important role as an adherent molecule between the germ cells and Sertoli cells in the testis. Further studies will provide

important clues regarding the regulation of the mechanism of the cell adhesion system in the testis and in understanding human infertility.

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