Intercellular adhesion molecule-2 is involved in apical ectoplasmic specialization dynamics during spermatogenesis in the rat

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Abstract

In this study, we investigated the role of intercellular adhesion molecule-2 (ICAM2) in the testis. ICAM2 is a cell adhesion protein having important roles in cell migration, especially during inflammation when leukocytes cross the endothelium. Herein, we showed ICAM2 to be expressed by germ and Sertoli cells in the rat testis. When a monospecific antibody was used for immunolocalization experiments, ICAM2 was found to surround the heads of elongating/elongated spermatids in all stages of the seminiferous epithelial cycle. To determine whether ICAM2 is a constituent of apical ectoplasmic specialization (ES), co-immunoprecipitation and dual immunofluorescence staining were performed. Interestingly, ICAM2 was found to associate with β1-integrin, nectin-3, afadin, Src, proline-rich tyrosine kinase 2, annexin II, and actin. Following $CdCl_2$ treatment, ICAM2 was found to be upregulated during restructuring of the seminiferous epithelium, with round spermatids becoming increasingly immunoreactive for ICAM2 by 6–16 h. Interestingly, there was a loss in the binding of ICAM2 to actin during $CdCl_2$-induced germ cell loss, suggesting that a loss of ICAM2–actin interactions might have facilitated junction restructuring. Taken collectively, these results illustrate that ICAM2 plays an important role in apical ES dynamics during spermatogenesis.

Key Words
- ICAM2
- testis
- cadmium chloride
- cell junction

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Introduction

Spermatogenesis is a complex process that culminates in the production and release of step 19 spermatids in the rat, and it involves germ cell development, germ cell adhesion, and germ cell migration (de Kretser & Kerr 1988, O'Donnell *et al*. 2006, 2011). Previous studies from this and other laboratories have described many proteins and signaling cascades that are critical for many aspects of spermatogenesis (Vigodner 2011, Walker 2011, Yeh *et al*. 2011, Cheng & Mruk 2012). For instance, Sertoli–germ cell adhesion is known to be facilitated by several members of the cadherin superfamily, including both classical and desmosomal cadherins (Goossens & van Roy 2005, Lie *et al*. 2011). Intercellular adhesion molecules (ICAMs) comprise another superfamily of adhesion and signaling proteins expressed by different cell types (i.e. endothelial and epithelial cells, platelets, lymphocytes, and monocytes) that are known to mediate homo- and heterophilic interactions. Of these, ICAM2 is a well-studied protein possessing an extracellular domain, a transmembrane domain, and a cytoplasmic

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domain (Staunton *et al*. 1989). Initially described as a receptor for lymphocyte function-associated antigen-1 (LFA1, a β_2 integrin), ICAM2 is known to be critical for cell adhesion and cell movement. Indeed, its loss disrupted leukocyte transmigration *in vitro* and *in vivo* (Gerwin *et al*. 1999, Huang *et al*. 2006, Porter & Hall 2009). Other studies have shown ICAM2 to associate with the actin cytoskeleton by binding various proteins such as α-actinin (Heiska *et al*. 1996), ezrin (Helander *et al*. 1996), radixin (Hamada *et al*. 2001), and moesin (Yonemura *et al*. 1998). Interestingly, ICAM2 clustering was found to trigger tyrosine phosphorylation of ezrin, thereby recruiting phosphoinositide 3-kinase (PI3-K) to the plasma membrane and activating the PI3-K/AKT pathway (Perez *et al*. 2002), which is known to regulate cell proliferation and cell survival (Datta *et al*. 1999, Foukas *et al*. 2010). ICAM2 loss was also found to increase apoptosis in endothelial cells cultured in the absence of serum or in the presence of Fas antibody or staurosporine (Huang *et al*. 2005). Taken collectively, these results illustrate that ICAM2 is a multifunctional protein. Presently, it is not entirely clear whether ICAM2 plays a role in spermatogenesis. A previous study using Sertoli cells isolated from mouse testes has reported ICAM2 to be undetectable under basal conditions and uninducible by cytokines (i.e. interleukin-1, tumor necrosis factor α, and interferon γ) or lipopolysaccharide when assessed by flow cytometry (Riccioli *et al*. 1995). Also, *Icam2* expression was undetectable in 2- and 10-week mouse testes when examined by RT-PCR (Wakayama *et al*. 2009). Herein, we re-examine the presence of ICAM2 in the rat testis, and we describe three key findings. First, we report that *Icam2* is expressed by germ and Sertoli cells, localizing to contact sites between elongating/elongated spermatids and Sertoli cells (i.e. the apical ectoplasmic specialization (ES)). The apical ES is a testis-specific anchoring junction whose function is constituted by several proteins, many of which are normally found within the focal contact such as α6β1 integrin, phosphorylated focal adhesion kinase (FAK), and vinculin (Grove *et al*. 1990, Palombi *et al*. 1992, Siu *et al*. 2003). Secondly, administration of $CdCl_2$, an environmental toxicant, was found to disrupt apical ES-mediated adhesion and increase the steady-state level of ICAM2 in adult rats. Finally, ICAM2 was shown to bind actin in the control testis, an association that was abolished following $CdCl_2$ treatment. Taken collectively, these results illustrate that ICAM2 plays an important role at the apical ES in the seminiferous epithelium of the rat testis.

Materials and methods

Animals

Male Sprague Dawley rats (adults at 300–325 g b.w.; pups at 10–35 days of age) were purchased from Charles River Laboratories (Kingston, NY, USA). Guidelines issued by the Institutional Animal Care and Use Committee of The Rockefeller University were strictly followed throughout this study (protocol numbers 09-016 and 12-506).

Treatment of rats with $CdCl_2$

$CdCl_2$ was used to induce the sloughing of germ cells from the seminiferous epithelium (Setchell & Waites 1970, Wong *et al*. 2004, Siu *et al*. 2009*b*, Elkin *et al*. 2010). In brief, rats (300–325 g b.w.; $n=3$–6 animals per time point) received a single dose of $CdCl_2$ (3 mg/kg b.w., prepared in 0.89% NaCl (w/v)) by i.p. injection. Control rats ($n=3$–6) were left untreated (Grima & Cheng 2000, Wong *et al*. 2004). Upon completion of this experiment, $CdCl_2$-treated animals were treated as biohazard waste and disposed of as directed by The Rockefeller University. The use of $CdCl_2$ was covered by both protocol numbers listed above.

Isolation and culture of testicular cells

Seminiferous tubules were isolated from adult rat testes by an enzymatic approach using 0.05% collagenase (w/v) in DMEM/F-12 (Sigma–Aldrich; Zwain & Cheng 1994). Seminiferous tubules were pelleted at 700 ***g***, sonicated in lysis buffer, cleared by centrifugation, and stored at −80 °C until used. Sertoli cells were isolated from testes of 20-day-old rats (Cheng *et al*. 1986, Mruk *et al*. 2003) and cultured in DMEM/F-12 containing 10 μg/ml insulin, 5 μg/ml human transferrin, 2.5 ng/ml epidermal growth factor, and 5 μg/ml bacitracin at high density (0.5×10^6 cells/cm^2) on Matrigel (BD Biosciences, San Jose, CA, USA)-coated 12-well plates. Sertoli cells were then incubated at 35 °C in a humidified atmosphere of 95% (v/v) air and 5% CO_2 (v/v). Two days after being isolated, Sertoli cells were treated with a hypotonic buffer (20 mM Tris, pH 7.4, at 22 °C) for 2.5 min to lyse residual germ cells, yielding Sertoli cells with a purity of ∼98% (Galdieri *et al*. 1981). Sertoli cells were then cultured for an additional 2 days and subsequently terminated for the extraction of RNA or the preparation of cell lysates. Sertoli cell-conditioned medium (SCCM) was also collected and stored at −20 °C until used (Cheng & Bardin 1987, Mruk *et al*. 1998). For Sertoli–germ cell cocultures, Sertoli cells

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were seeded at low density (0.05×10^6 cells/cm^2) on Matrigel-coated glass coverslips or 100 mm dishes and cultured as described above. Four days after plating Sertoli cells, germ cells were isolated from 90-day-old rat testes by a mechanical procedure using sequential filtrations through nylon filters of decreasing pore size (Aravindan *et al*. 1996) and added onto the Sertoli cell epithelium at a Sertoli:germ cell ratio of ~1:3. Cocultures were maintained for up to 2 days in order to facilitate the assembly of stable Sertoli–germ cell junctions in DMEM/F-12 supplemented with 6 mM sodium lactate, 2 mM sodium pyruvate, and the aforementioned factors. Thereafter, cocultures were processed for immunoblotting or immunofluorescence staining. The first time point (i.e. 0 h) was obtained by harvesting Sertoli cells in lysis buffer (10 mM Tris, 0.15 M NaCl, 1% NP-40 (v/v), and 10% glycerol (v/v), pH 7.4, at 22 °C containing protease and phosphatase inhibitor cocktails at a 1:100 dilution; Sigma–Aldrich) and immediately combining Sertoli cells with freshly isolated germ cells so that the ratio of Sertoli:germ cells was ~1:3. The control consisted of culturing Sertoli cells alone without the addition of germ cells.

RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) by following the manufacturer's instructions. RT-PCR was performed as described earlier (Xiao *et al*. 2011). The primer pairs (Gene Link, Hawthorne, NY, USA) used for the amplification of *Icam2* (GenBank accession number NM_001007725) and *S16* (GenBank accession number X17665) were as follows: 5′-TTACTTTGCCATTTCACTTGTTCG-3′ (*Icam2* sense, nucleotides 665–688), 5′-CCATCTGGTTGTCTTGCCTTATTT-3′ (*Icam2* antisense, nucleotides 1047–1070), 5′-TCCGCTGCAGTCCGTTCAAGTCTT-3′ (*S16* sense, nucleotides 67–90), and 5′-GCCAAACTTCTTGGATTCGCAGCG-3′ (*S16* antisense, nucleotides 428–451). PCR was conducted with an initial denaturation at 95 °C for 2 min, followed by 30 cycles with the following parameters: denaturation at 95 °C for 1 min, annealing at 55.9 °C for 1 min, and extension at 72 °C for 1 min. A final extension step at 72 °C for 5 min was also incorporated into PCR cycling conditions. The authenticity of the *Icam2* PCR product was verified by Sanger DNA sequencing (GENEWIZ, South Plainfield, NJ, USA).

Table 1 Antibodies used in this report

Antibody	Host species	Vendor	Catalog number	Application(s)/dilution(s)
ICAM2	Rabbit	Santa Cruz Biotechnology	sc-7933	IB (1:200), IF (1:100), IHC (1:100), IP (2 μg)
	Goat	Santa Cruz Biotechnology	sc-31049	IF (1:100)
Actin	Goat	Santa Cruz Biotechnology	sc-1616	IB (1:200)
Testin	Rabbit	Cheng Lab (Cheng *et al*. 1989)		IB (1:200)
Occludin	Rabbit	Invitrogen	71-1500	IB (1:250)
N-Cadherin	Rabbit	Santa Cruz Biotechnology	sc-7939	IB (1:200)
Nectin-3	Rabbit	Santa Cruz Biotechnology	sc-28637	IB (1:200)
	Goat	Santa Cruz Biotechnology	sc-14806	IF (1:50)
Afadin	Rabbit	Sigma–Aldrich	A0349	IB (1:500)
β1-Integrin	Rabbit	Santa Cruz Biotechnology	sc-8978	IB (1:300), IF (1:100)
Laminin γ3	Rabbit	Cheng Lab (Yan & Cheng 2006)		IB (1:100)
Src	Mouse	Santa Cruz Biotechnology	sc-8056	IB (1:200)
FAK	Rabbit	Millipore	06-543	IB (1:1000)
Pyk2	Rabbit	Santa Cruz Biotechnology	sc-9019	IB (1:200)
Annexin II	Rabbit	Santa Cruz Biotechnology	sc-9061	IB (1:200)
α-Tubulin	Mouse	Santa Cruz Biotechnology	sc-5286	IB (1:200)
ICAM1	Rabbit	Novus Biologicals	NB100-81977	IB (1:500)
Claudin-11	Rabbit	Invitrogen	36-4500	IB (1:250)
JAM-A	Rabbit	Invitrogen	36-1700	IB (1:300)
CAR	Rabbit	Santa Cruz Biotechnology	sc-15405	IB (1:200)
β-Catenin	Rabbit	Invitrogen	71-2700	IB (1:250)
α6-Integrin	Mouse	Santa Cruz Biotechnology	sc-59970	IB (1:300)
Desmoglein-2	Rabbit	Santa Cruz Biotechnology	sc-20115	IB (1:200)
Desmocollin-3	Rabbit	Santa Cruz Biotechnology	sc-48751	IB (1:200)
Connexin 43	Rabbit	Cell Signaling Technology	3512	IB (1:200)
p-FAK-Y397	Rabbit	Invitrogen	44-625G	IB (1:1000)
p-FAK-Y576	Rabbit	Millipore	07-157	IB (1:1000)
p-Src-Y419	Mouse	Millipore	05-677	IB (1:1000)
p-Src-Y530	Rabbit	Abcam	ab4817	IB (1:1000)

IB, immunoblotting; IF, immunofluorescence; IHC, immunohistochemistry; IP, immunoprecipitation.

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Co-immunoprecipitation and immunoblotting

Testis, seminiferous tubule, and Sertoli and germ cell lysates were prepared in lysis buffer. For each reaction, ~800 μg protein was incubated with 2 μg anti-ICAM2 IgG (Table 1), and co-immunoprecipitation (co-IP) was performed as described previously (Xiao *et al*. 2011). Thereafter, immunoprecipitated proteins were separated by SDS–PAGE and transferred onto a nitrocellulose membrane for immunoblotting (Table 1). Proteins were visualized by ECL (Mruk & Cheng 2011*a*), and images were captured with a Fujifilm LAS-4000 mini imaging system as earlier described (Xiao *et al*. 2011). Saturated images were not included in the final statistical analysis.

Immunohistochemistry and immunofluorescence staining

Seven micrometer-thick frozen cross sections were obtained from adult rat testes, mounted onto poly-L-lysine-coated microscope slides (Polysciences, Warrington, PA, USA), and fixed in either Bouin's fixative or 4% paraformaldehyde (w/v) in PBS (10 mM NaH_2PO_4, 0.15 M NaCl, pH 7.4, at 22 °C) as described previously (Xiao *et al*. 2011). Immunohistochemistry was performed on frozen sections using the SuperPicTure Polymer Detection kit (Invitrogen) and by following the manufacturer's instructions (Table 1). Sections were permeabilized with 0.1% Triton-X100 (v/v), blocked with 10% normal donkey serum (v/v) in PBS, and incubated with primary antibody (Table 1). The color reaction was developed using 3-amino-9-ethylcarbazole, which yielded a brownish immunoreactive signal. Immunofluorescence staining was performed on frozen sections as described previously (Xiao *et al*. 2011). An Alexa Fluor 555 secondary antibody (donkey anti-rabbit IgG; Invitrogen) was used to detect ICAM2, and an Alexa Fluor 488 secondary antibody (donkey anti-goat IgG; Invitrogen) was used to detect β1-integrin and nectin-3. All sections processed for immunofluorescence staining were mounted with ProLong Gold antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). Images were captured with an Olympus BX61 microscope and MicroSuite Five software (V1224; Olympus America, Melville, NY, USA). Images were analyzed with Photoshop CS3 Extended software (Adobe Systems).

General methods

Protein concentration was determined using a D_C protein assay kit (Bio-Rad Laboratories) and microplate reader (model 680, Bio-Rad Laboratories) with BSA as a standard. F-actin was stained using frozen testis cross sections as described previously (Sarkar *et al*. 2008, Kopera *et al*. 2009, Mruk & Lau 2009).

Statistical analyses

All experiments were conducted in triplicate, and each experiment was repeated at least three times. Statistical analyses were performed with GB-STAT software (V7.0, Dynamic Microsystems, Silver Spring, MD, USA). $P<0.05$ was taken as statistically significant.

Results

Level of ICAM2 during testis development and its expression by germ and Sertoli cells

To set the stage for this study, the steady-state level of ICAM2 was examined in developing testes by immunoblotting (Fig. 1A). When data points were individually compared against the level of ICAM2 in the 10-day postnatal testis, significant decreases were noted from 12 to 90 days of age (Fig. 1B). Thereafter, *Icam2* expression in the adult rat testis (90 days of age) and germ and Sertoli cells was examined by RT-PCR (Fig. 1C) and immunoblotting (Fig. 1D and Table 1). By both methods, ICAM2 was present in the adult testis, germ (isolated from 90-day-old testes and harvested immediately), and Sertoli (isolated from 20-day-old testes and cultured for 4 days) cells. The purity of germ cells was assessed by immunoblotting to determine whether these cells were immunoreactive for testin, a Sertoli and Leydig cell protein (Cheng *et al*. 1989, Zong *et al*. 1992). Testin was present in testis and Sertoli cell lysates, as well as in SCCM, but not in germ cell lysate as previously reported (Fig. 1D; Cheng *et al*. 1989, Zong *et al*. 1992). This illustrated that germ cell isolations were of negligible Sertoli and Leydig cell contamination. Based on these results, *Icam2* expression was higher in Sertoli vs germ cells (Fig. 1E). We emphasize that cells were isolated from testes at two different developmental stages. The reason for this is that it is difficult to isolate highly pure Sertoli cells (relative purity ~85%) from the adult rat testis (Li *et al*. 2001, Anway *et al*. 2003, Lui *et al*. 2003), and it is equally difficult to isolate highly pure germ cells from 20-day-old testes. Nevertheless, Sertoli cells isolated from 20-day-old testes were included in this analysis because they had ceased to divide and were differentiated (Orth 1982). They also mimicked Sertoli cells isolated from adult rat testes both morphologically and functionally (Li *et al*. 2001, Lui *et al*. 2003).

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We also emphasize that freshly isolated germ cells were used in this analysis. This is because germ cell viability cannot be extended in culture. Next, the localization of ICAM2 was investigated in the adult testis. ICAM2 localized largely to sites adjacent to elongating spermatids (Fig. 1F). In early and late stages of the seminiferous epithelial cycle, ICAM2 staining was diffuse, surrounding both concave and convex sides of spermatid heads.

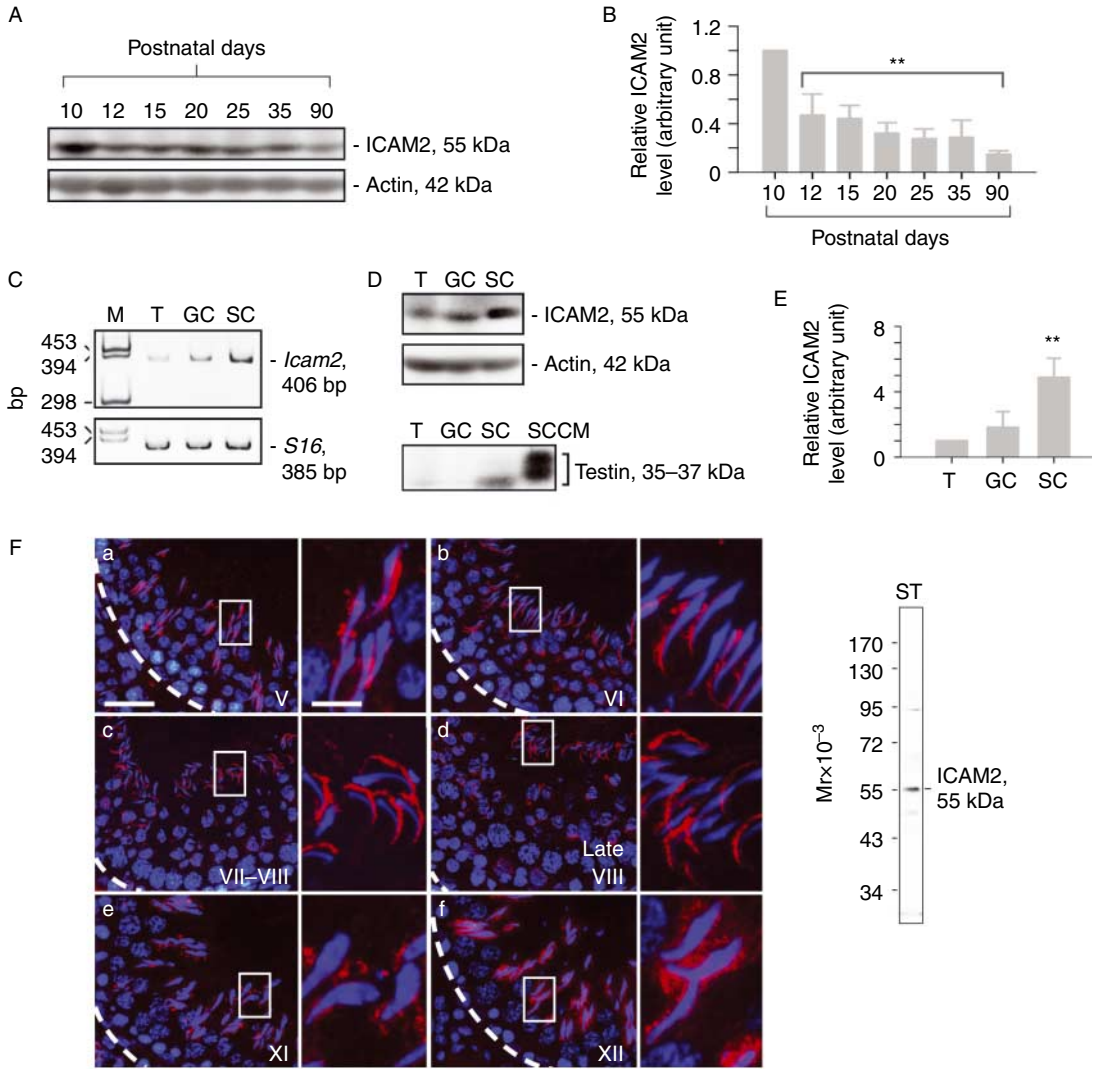

Figure 1

Steady-state ICAM2 level decreases in the developing rat testis and its presence in germ and Sertoli cells. Lysates obtained from 10-, 12-, 15-, 20-, 25-, 35-, and 90-day-old testes were used for immunoblotting experiments (A, ~50 μg protein/lane). Actin was used as an internal control. Histogram (B) summarizing immunoblotting results. Each ICAM2 data point was normalized against its corresponding actin data point and then against 'postnatal day 10' which was arbitrarily set at 1. Each bar represents mean±S.D. of three independent experiments. $^{**}P<0.01$ (Student's *t*-test). RT-PCR (C) and immunoblotting (D, ~50 μg protein/lane) experiments showing ICAM2 in the 90-day-old testis (T), germ (GC), and Sertoli cells (SC). *S16* and actin were used as internal controls. Germ cell purity was assessed using testin as a marker for an immunoblotting experiment (D). SCCM was used as a positive control. Histogram (E) summarizing immunoblotting results. Each ICAM2 data point was normalized against its corresponding actin data point and then against 'testis', which was arbitrarily set at 1. Each bar represents mean±S.D. of at least three independent experiments. $^{**}P<0.01$ (Student's *t*-test). M, DNA molecular weight marker VI (Roche). Immunofluorescence staining was performed on 7 μm-thick frozen testis cross sections with anti-ICAM2 IgG (F, Table 1). Boxed areas (F, a, b, c, d, e, and f) correspond to magnified images that are shown to the right of each low magnification image. Dashed lines (F, a, b, c, d, e, and f) mark the periphery of seminiferous tubules. Stages of the seminiferous epithelial cycle are denoted as Roman numerals (F). DAPI was used to visualize nuclei (F, a, b, c, d, e, and f). Bar (F, a; also applies to b, c, d, e, and f)=40 μm; bar (F, first upper left inset; also applies to all other insets)=10 μm. An immunoblotting experiment showing the monospecificity of the ICAM2 antibody in seminiferous tubule (ST, ~100 μg protein) lysate (F, far-right panel). Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0434.

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At some stages such as at stages XI and XII, ICAM2 was far removed from the proximity of the spermatid head, illustrating that ICAM2 was present within Sertoli cells. Before spermiation, however, ICAM2 staining was very discrete, concentrating largely to the convex side of spermatid heads (Fig. 1F). A weak ICAM2 immunoreactive signal was also found to associate with round spermatids in some stages, but no immunoreactive signal was found at the blood–testis barrier (BTB) whose function is constituted by tight junctions (TJs), basal ESs, desmosomes, and gap junctions (GJs; Mruk & Cheng 2010, Cheng & Mruk 2012). Finally, the monospecificity of

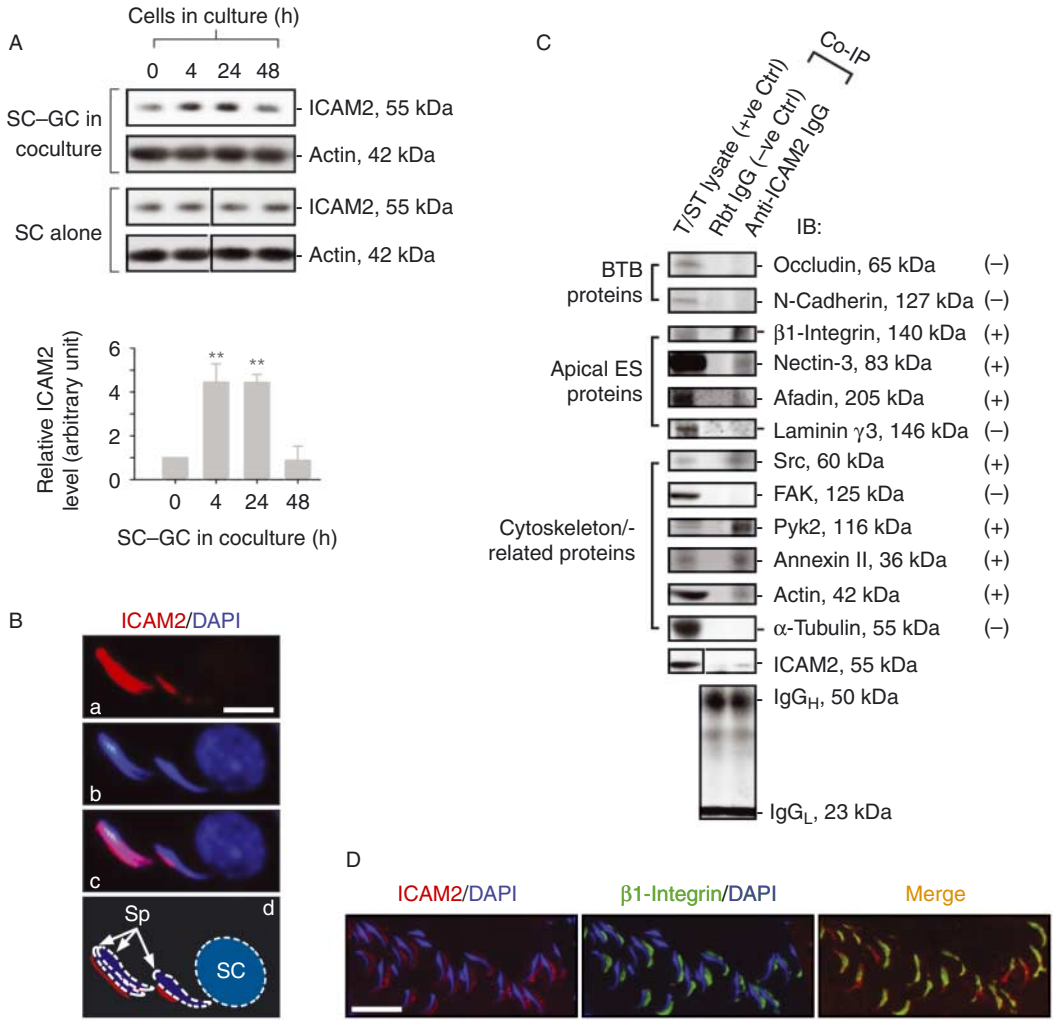

Figure 2

ICAM2 is a constituent protein of the apical ES. Sertoli–germ cell cocultures were terminated at different time points, and lysates were used for immunoblotting experiments (A, upper panel). The control consisted of culturing Sertoli cells alone without the addition of germ cells (A, bottom of top panel). Actin was used as an internal control. Histogram (A, bottom panel) summarizing immunoblotting results from Sertoli–germ cell cocultures. Each ICAM2 data point was normalized against its corresponding actin data point and then against '0 h', which was arbitrarily set at 1. Each bar represents mean ± S.D. of three independent experiments. $^{**}P<0.01$ (Student's *t*-test). Immunofluorescence staining was performed using Sertoli–germ cell cocultures (B, a, b, and c). Schematic illustration (B, d) summarizing results. Bar (B, a; also applies to b and c) = 10 μm. SC, Sertoli cell; Sp, spermatid. Co-IP and immunoblotting (IB) experiments showing structural interactions with ICAM2 when testis (T) or seminiferous tubule (ST) lysate and anti-ICAM2 IgG were used (C, Table 1). For the positive control (+ve Ctrl), T or ST lysate was used for immunoblotting only. For the negative control (−ve Ctrl), rabbit IgG was used in place of anti-ICAM2 IgG. +, positive co-IP result; −, negative co-IP result. IgG heavy (IgG_H, 50 kDa) and light (IgG_L, 23 kDa) chains served as indicators of equal protein processing. The ability of anti-ICAM2 IgG to pull down interacting proteins was confirmed using anti-ICAM2 IgG for both co-IP and immunoblotting. Dual immunofluorescence staining was performed on 7 μm-thick frozen testis cross sections with anti-ICAM2 and anti-β1-integrin IgGs (D, Table 1). DAPI was used to visualize nuclei (D). Bar (D, far-left panel; also applies to middle and far-right panels) = 20 μm. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0434.

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the ICAM2 antibody was assessed by immunoblotting (Fig. 1F, right). A predominant 55 kDa protein was observed in seminiferous tubule lysate by SDS–PAGE, and these results are in agreement with the previously published reports on human and murine ICAM2 (Nortamo *et al*. 1991, Xu *et al*. 1996).

ICAM2 is a constituent protein of the apical ES, co-immunoprecipitating and co-localizing with β1-integrin, nectin-3, and F-actin

Sertoli–germ cell cocultures were subsequently used for immunoblotting (Fig. 2A) and immunofluorescence staining (Fig. 2B). When compared with Sertoli cells cultured alone, the steady-state level of ICAM2 increased 4–24 h after the addition of germ cells to Sertoli cells (Fig. 2A), and its localization concentrated to the convex side of spermatid heads (Fig. 2B). These data, together with Fig. 1, suggested that ICAM2 may be a constituent protein of the apical ES, which is the only anchoring device present between elongating/elongated spermatids and Sertoli cells (Russell 1977, Mruk & Cheng 2004, Vogl *et al*. 2008). To investigate this, co-IP experiments were performed to determine whether ICAM2 interacts structurally with apical ES proteins (Fig. 2C). ICAM2 was shown to associate with β1-integrin, nectin-3, and afadin but not with laminin γ3 (apical ES proteins). In addition, ICAM2 did not associate with occludin (a TJ protein) and N-cadherin (a basal ES protein), and these results are in agreement with the lack of ICAM2 immunoreactivity at the BTB (Fig. 1). These experiments also showed ICAM2 to associate with Src and proline-rich tyrosine kinase 2 (Pyk2), nonreceptor tyrosine kinases (Fig. 2C). Moreover, previous studies have shown ICAM2 to associate with several actin-binding proteins (Heiska *et al*. 1996, 1998, Yonemura *et al*. 1998, Yoon *et al*. 2008). Here, we showed ICAM2 to interact with annexin II and actin (Fig. 2C). Annexin II is a Ca^{2+}-dependent phospholipid binding protein with functions in membrane stabilization, junction dynamics, and cytoskeletal organization (Gerke & Moss 2002). IgG heavy and light chains served as indicators of equal protein processing. The association of ICAM2 with β1-integrin was confirmed by dual immunofluorescence staining when these two proteins co-localized to the convex side of spermatid heads during stage VII of the seminiferous epithelial cycle (Fig. 2D).

To corroborate results shown in Figs 1 and 2, ICAM2 was also co-stained with either nectin-3 or F-actin (Fig. 3). Nectin-3 is a Ca^{2+}-independent immunoglobulin-like molecule that facilitates apical ES-based adhesion. It is expressed by elongating/elongated spermatids where

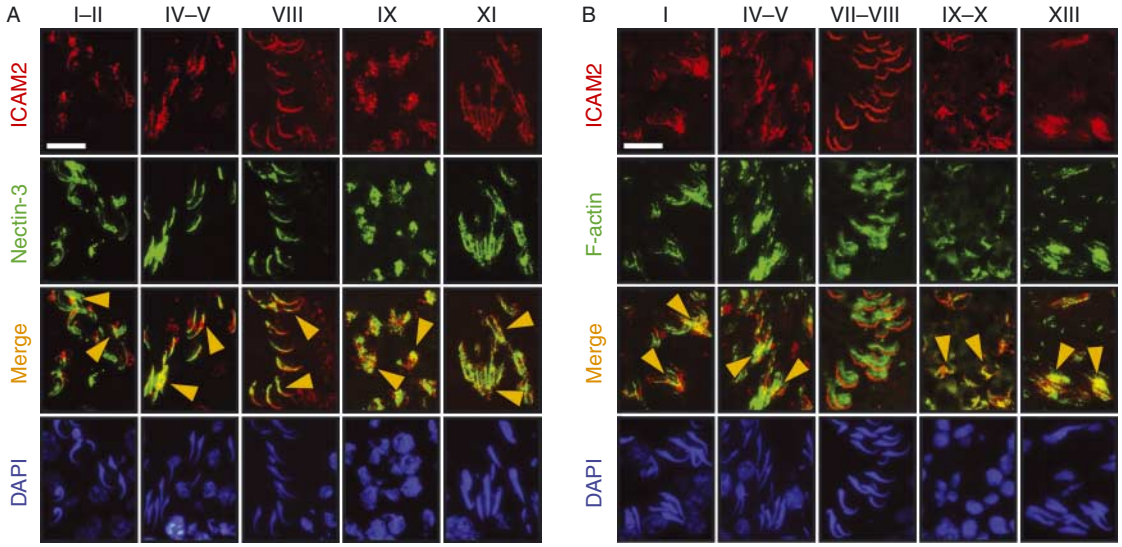

Figure 3
ICAM2 co-localizes with nectin-3 and F-actin in the seminiferous epithelium of the rat testis. Dual immunofluorescence staining was performed on 7 μm-thick frozen testis cross sections with anti-ICAM2 and anti-nectin-3 IgGs (A) or with anti-ICAM2 IgG and Oregon Green 488 phalloidin (B). Orange arrowheads point to areas of co-localization (A and B). Stages of the seminiferous epithelial cycle are denoted as Roman numerals (A and B). DAPI was used to visualize nuclei (A and B). Bar (A, upper left panel; also applies to all other panels) = 20 μm; bar (B, upper left panel; also applies to all other panels) = 20 μm. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0434.

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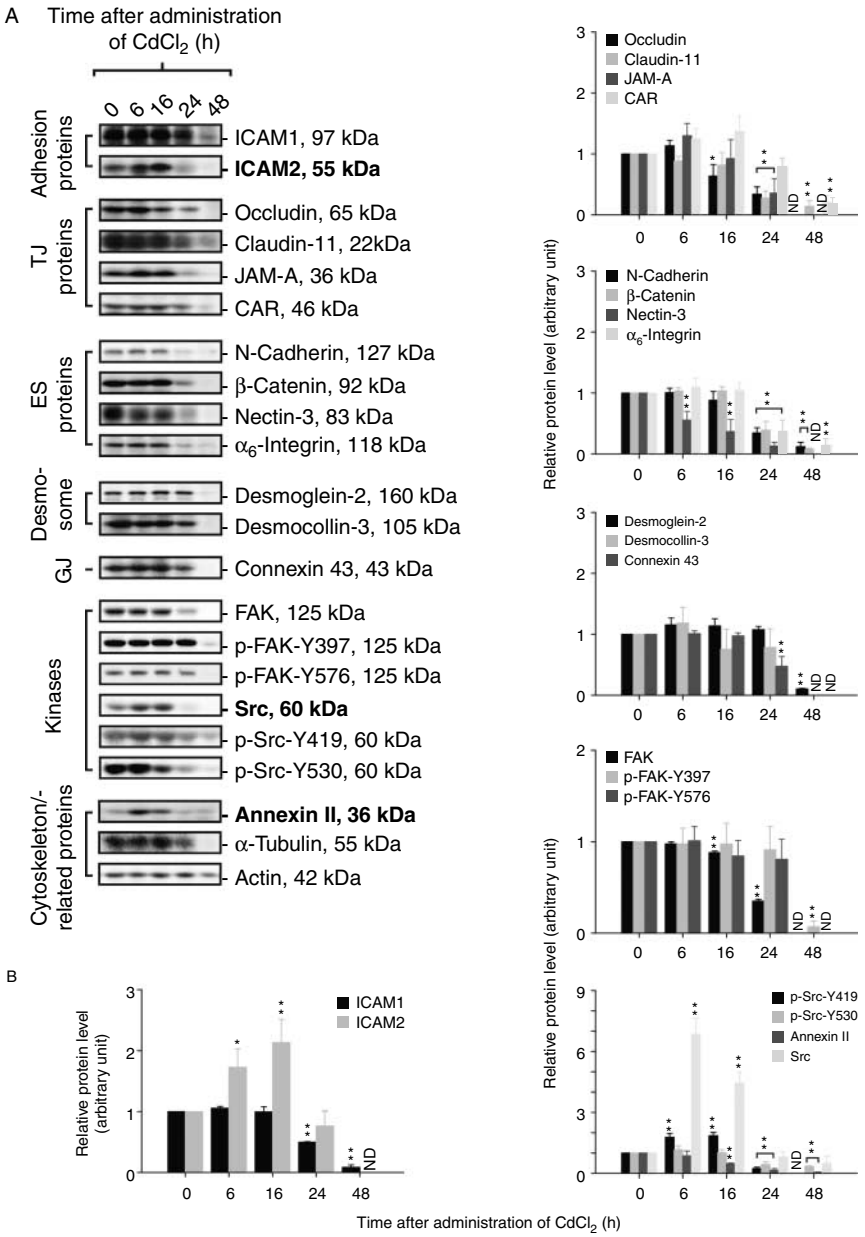

Figure 4

ICAM2, Src, and annexin II increase during $CdCl_2$-induced testis damage. Immunoblotting experiments showing changes in the levels of several proteins (A). Proteins whose levels increased following $CdCl_2$ treatment are labeled as bold. Actin was used as an internal control. Histograms (B) summarizing immunoblotting results. Each data point was normalized against its corresponding actin data point and then against '0 h', which was arbitrarily set at 1. Each bar represents mean ± S.D. of $n = 3$–6 rats. $^*P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test). ND, not detected

it associates heterotypically with Sertoli cell nectin-2 (Ozaki-Kuroda *et al*. 2002). Interestingly, ICAM2 was shown to co-localize partially with nectin-3 in spermatids (Fig. 3A). However, there were more areas where co-localization between ICAM2 and nectin-3 was not evident, and this staining likely corresponded to the presence of ICAM2 within Sertoli cells. ICAM2 also co-localized partially with F-actin throughout the seminiferous epithelial cycle, except at stages VII and VIII when red (ICAM2) and green (F-actin) signals did not merge into an orange sigal (Fig. 3B). DAPI was used to assist in the staging of seminiferous tubules. Taken collectively, these data demonstrate that ICAM2 is an apical ES protein.

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ICAM2 is upregulated during $CdCl_2$-induced germ cell loss

Previous studies have shown $CdCl_2$ to induce germ cell sloughing from the seminiferous epithelium, as well as BTB disruption (Setchell & Waites 1970, Wong *et al*. 2004, Siu *et al*. 2009*b*, Elkin *et al*. 2010). Moreover, there was evidence of interstitial edema and hemorrhage, as well as testicular weight loss, necrosis, atrophy, and calcification (Chiquone 1964, Zielinska-Psuja *et al*. 1976, Selypes *et al*. 1992). Following i.p. injection of a single dose of $CdCl_2$ to adult rats, immunoblotting was used to examine the levels of several proteins that are representative of TJ, ES, desmosome, and GJ function (Fig. 4). Interestingly, the levels of only three proteins increased within 6–16 h of $CdCl_2$ treatment, which coincided with germ cell sloughing from the seminiferous epithelium. These proteins were ICAM2, annexin II, and Src with annexin II exhibiting the highest fold changes. The levels of virtually all proteins decreased by 48 h, including the levels of ICAM2, annexin II, and Src (Fig. 4). In this regard, it is worth noting that many of these proteins are expressed by both Sertoli and germ cells. Thus, their downregulation at 48 h when testes were devoid of most germ cells may be

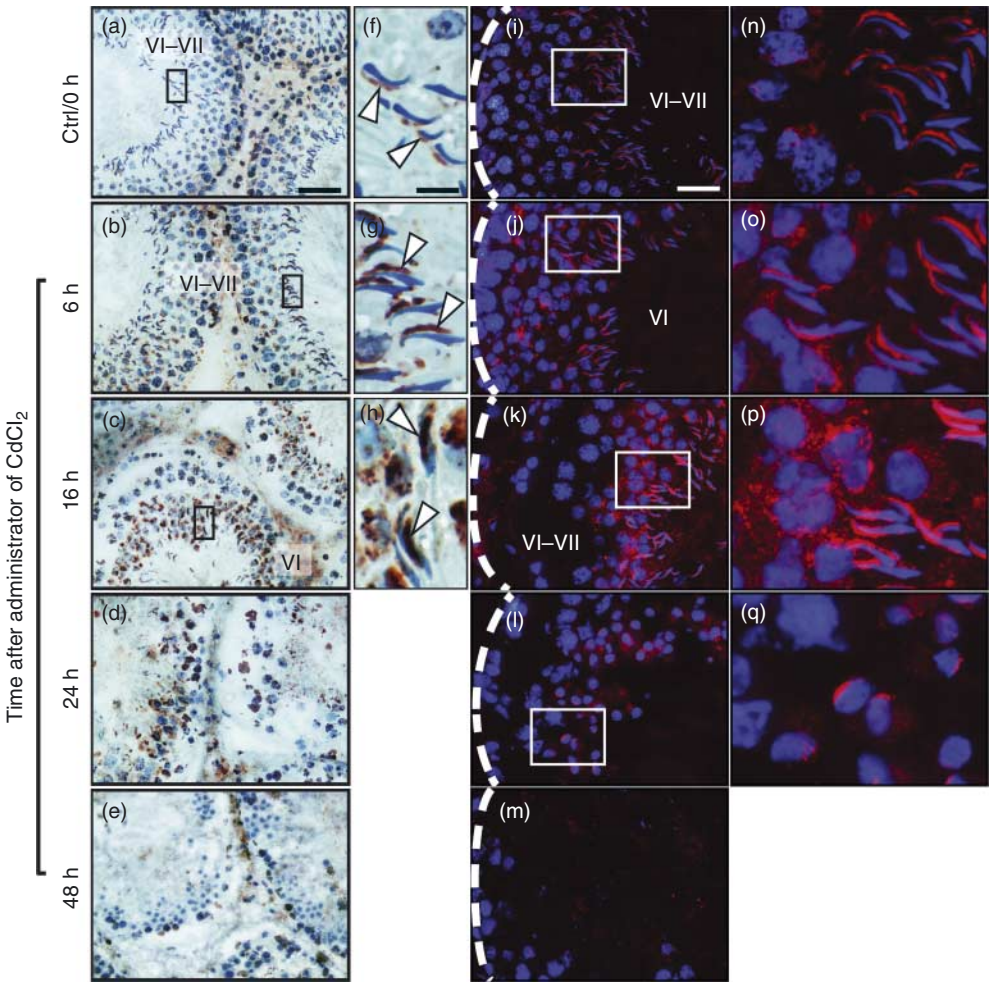

Figure 5
ICAM2 is upregulated in germ cells following $CdCl_2$ treatment. Immunohistochemistry (a, b, c, d, e, f, g, and h) and immunofluorescence staining (i, j, k, l, m, n, o, p, and q) were performed on 7 μm-thick frozen testis cross sections obtained from control (Ctrl) and $CdCl_2$-treated rats with anti-ICAM2 IgG. Boxed areas (a, b, c, i, j, k, and l) correspond to magnified images that are shown to the right of each low magnification image. White arrowheads (f, g, and h) point to immunoreactive ICAM2 at the apical ES. Dashed lines (i, j, k, l, and m) mark the periphery of seminiferous tubules. Stages of the seminiferous epithelial cycle are denoted as Roman numerals (a, b, c, i, j, and k). DAPI was used to visualize nuclei (i, j, k, l, m, n, o, p, and q). Bar (a, also applies to b, c, d, and e) = 70 μm; bar (f, also applies to g, h, n, o, p, and q) = 10 μm; bar (i, also applies to j, k, l, and m) = 40 μm. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0434.

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the result of changes in cell-to-cell ratios within the seminiferous epithelium.

Because ICAM2 was one of the few proteins to increase following administration of $CdCl_2$, its localization was investigated in testes from treated and untreated rats. The goal of this experiment was to determine whether there were any changes in the localization of ICAM2 during $CdCl_2$-induced restructuring of the seminiferous epithelium. In line with the results shown in Fig. 1, ICAM2 localized to elongating/elongated spermatids in the untreated testis (Fig. 5). However, an increase in immunoreactive ICAM2 was noted by 6–16 h of $CdCl_2$ treatment. It is also worth noting that round spermatids became increasingly immunoreactive for ICAM2 by 6–16 h of $CdCl_2$ treatment when compared with the untreated testis, which may have contributed to the increase in ICAM2 detected by immunoblotting (Fig. 4). By 24–48 h, the seminiferous epithelium was depleted of most germ cells, and ICAM2 immunoreactivity was nearly lost (Fig. 5).

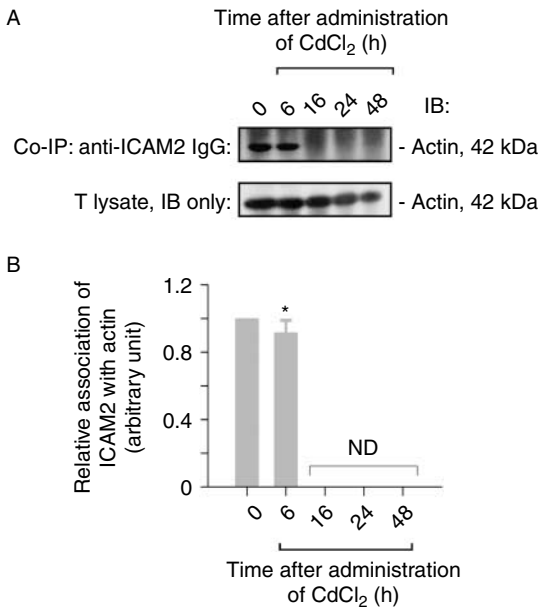

Figure 6
$CdCl_2$-induced testis restructuring disrupts ICAM2–actin interactions. For co-IP experiments (A), ~800 μg testis (T) lysate from $CdCl_2$-untreated and treated rats was incubated with anti-ICAM2 IgG, followed by immunoblotting (IB) with an actin antibody (Table 1). Actin was used as an internal control. Histogram (B) summarizing co-IP and immunoblotting results. The relative association of ICAM2 with actin at 0 h was arbitrarily set at 1. Each bar represents mean ± S.D. of three independent experiments. *$P<0.01$ (Student's *t*-test). ND, not detected.

$CdCl_2$-induced restructuring of the seminiferous epithelium results in the loss of ICAM2–actin interactions

As shown in Fig. 3B, ICAM2 co-localized partially with F-actin at the apical ES throughout the seminiferous epithelial cycle, except at stages VII and VIII when red (ICAM2) and green (F-actin) signals did not merge into an orange signal. These results suggested that dissociation of ICAM2 from actin may be critical for the restructuring of cell junctions and for the subsequent release of spermatozoa at spermiation. As $CdCl_2$ is known to trigger germ cell sloughing from the seminiferous epithelium, we aimed to assess ICAM2–actin interactions with this model as well. Interestingly, there was a loss in the binding of ICAM2 to actin by 16–48 h when anti-ICAM2 IgG was used for co-IP (Fig. 6). These results are significant because the steady-state level of ICAM2 increased following $CdCl_2$ treatment (Fig. 4), illustrating that the loss in protein–protein interactions was not likely the result of changes in cell–cell ratios.

Discussion

In this study, we describe three key findings. First, we report that germ and Sertoli cells expressed *Icam2* and that ICAM2 localized to elongating/elongated spermatid and Sertoli cell contact sites known as the apical ES, a testis-specific cell junction (Fig. 7). Based on co-IP and immunofluorescence results, ICAM2 was concluded not to be a constituent protein of the BTB. Secondly, we report that ICAM2 was upregulated following administration of $CdCl_2$ and that round spermatids became increasingly immunoreactive for ICAM2 during restructuring of the seminiferous epithelium. Finally, we report that $CdCl_2$-induced restructuring of the seminiferous epithelium involved a loss of ICAM2–actin interactions. Based on immunofluorescence results, this loss in ICAM2–actin interactions may also facilitate spermiation in the normal testis. Previous studies using other *in vitro* and *in vivo* models have shown ICAM2, an integral membrane protein, to play an important role in cell adhesion and cell movement (Li *et al*. 1993, Woodfin *et al*. 2009). In the seminiferous epithelium, ICAM2 staining surrounded the heads of elongating/elongated spermatids. Before spermiation, however, ICAM2 staining became very discrete, concentrating to the convex side of spermatid heads at the site of the apical ES. While this staining pattern may be related to the adhesion of germ cells to Sertoli cells, it may also be related to the restructuring of the apical ES, which occurs before

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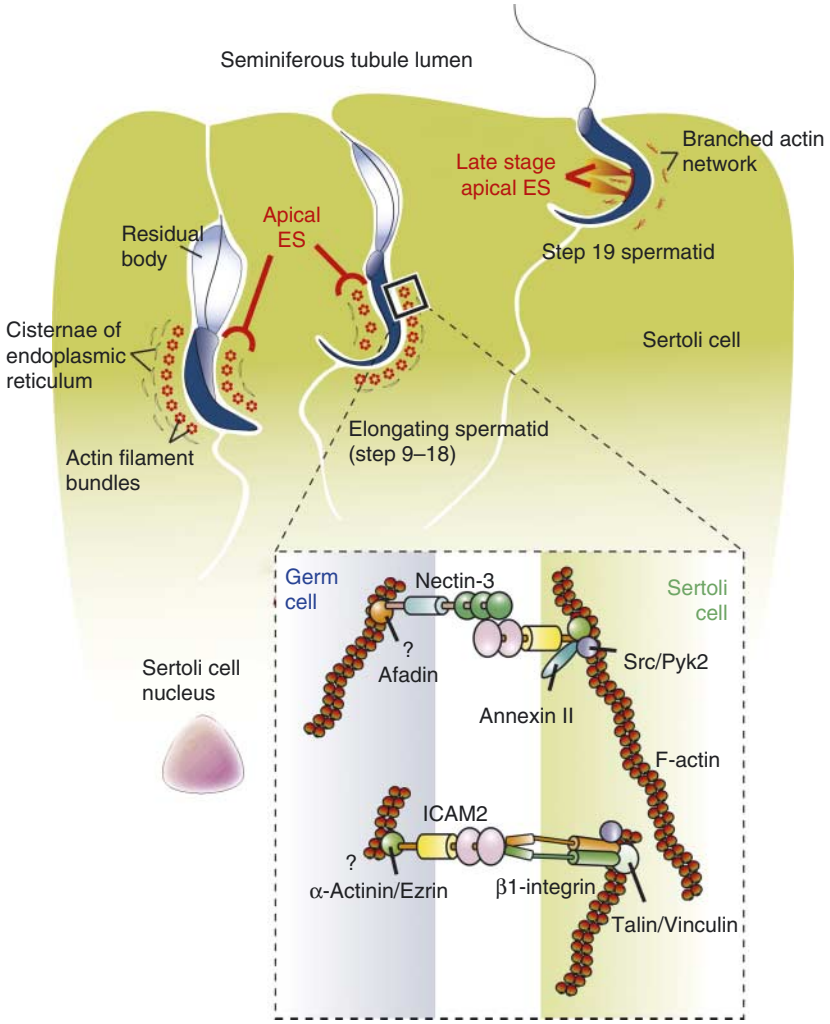

Figure 7

Schematic illustration summarizing ICAM2 interactions at the apical ES in the seminiferous epithelium of the rat testis. During spermatogenesis, elongating/elongated spermatids (blue) remain attached to Sertoli cells (green) via apical ESs. The apical ES is a tripartite adhesive structure composed of many structural (e.g. β1-integrin, nectin-3), scaffolding (e.g. afadin), and signaling (e.g. Src) proteins, supported by bundles of F-actin and outlined by cisternae of endoplasmic reticulum. Based on co-IP and/or dual immunofluorescence staining results (Figs 2, 3, and 6), we concluded ICAM2 to be a constituent protein of the apical ES, associating with β1-integrin, nectin-3, afadin, Src, Pyk2, annexin II, and actin. Moreover, ICAM2 failed to co-localize with F-actin at stages VII and VIII of the seminiferous epithelial cycle (Fig. 3), suggesting that disruption of ICAM2–actin interactions may facilitate restructuring of the apical ES. For the sake of simplicity, we depict within a single dimensional plane three spermatids at different stages of development, although this does not accurately represent the organization of the seminiferous epithelium *in vivo*. Also, only proteins interacting with ICAM2 are shown in this schematic illustration; other apical ES proteins are not shown. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0434.

spermiation (Russell 1977, 1993, Russell & Peterson 1985). These results were corroborated by dual immunofluorescence staining when ICAM2 was found to co-localize partially with β1-integrin and nectin-3, as well as with F-actin. In the testis, β1-integrin is present both at the apical ES and at the BTB, whereas nectin-3 is present only at the apical ES (Ozaki-Kuroda *et al*. 2002, Cheng *et al*. 2011). Furthermore, nectin-3 is expressed only by spermatids (Ozaki-Kuroda *et al*. 2002, Takai & Nakanishi 2003). Taken together with results from co-IP experiments, which showed ICAM2 to bind β1-integrin and nectin-3, ICAM2 appears to function in the adhesion of late-stage spermatids to Sertoli cells, and its role as a cell adhesion protein is in agreement with other studies.

In this study, ICAM2 was also found to bind annexin II. Annexin II, a member of the annexin family of proteins, is known to bind negatively charged phospholipids such as phosphatidylinositol 4,5-bisphosphate found in cellular

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membranes in a Ca^{2+}-dependent manner (Gerke & Moss 2002, Gerke *et al*. 2005). Annexin II has diverse roles in different epithelial and endothelial cells, and it is known to be present in Sertoli cells (Dreier *et al*. 1998). For instance, it can function in membrane domain stabilization, ion transport, endocytosis, cell proliferation, signal transduction, junction dynamics, and cytoskeletal organization (Gerke & Moss 2002, Gerke *et al*. 2005). In each of these Ca^{2+}-regulated processes, annexins are believed to bring together or 'bridge' several cytoplasmic proteins, thereby assembling multi-protein complexes that are required for normal cell function. Thus, the adhesive role of ICAM2 is further supported by its interaction with annexin II. It is possible that annexin II is helping to bridge ICAM2 to actin-binding proteins such as ezrin in the control testis. It is also possible that Src is phosphorylating proteins within this protein complex as annexin II is known to be a substrate of v-Src (Haynes & Moss 2009). This may result in clustering of ICAM2 and in additional changes in protein–protein interactions, thereby triggering signaling cascades that control cell adhesion and cell movement in the testis.

Herein, we show that ICAM2 was upregulated and that ICAM2 associated strongly with round spermatids following administration of $CdCl_2$, an environmental toxicant known to induce germ cell sloughing from the seminiferous epithelium (Setchell & Waites 1970, Wong *et al*. 2004, Siu *et al*. 2009*b*, Elkin *et al*. 2010). This *in vivo* model was used because environmental toxicants can affect spermatogenesis and contribute to subfertility/infertility (Siu *et al*. 2009*a*, Mruk & Cheng 2011*b*, Lagos-Cabre & Moreno 2012). Annexin II and Src, which were shown to bind ICAM2, also increased following administration of $CdCl_2$. As annexin II is known to link the cytoskeleton to the plasma membrane and to be involved in membrane dynamics, its role may be to facilitate the displacement of ICAM2 away from the cell surface (i.e. endocytosis) and to contribute to junction restructuring. It is also possible that these three proteins increased because they assemble into a functional multi-protein complex. Nevertheless, ICAM2 may prove to be an excellent marker of testicular dysfunction in future studies because any deviation from its normal expression and/or localization pattern appears to disrupt cell–cell interactions.

Interestingly, ICAM2 did not co-localize with F-actin at stages VI and VII of the seminiferous epithelial cycle, suggesting that dissociation of ICAM2 from actin may be critical for the restructuring of cell junctions and for the subsequent release of spermatozoa at spermiation. These results were corroborated when testis lysates were used from $CdCl_2$-treated rats for co-IP experiments. This mechanism of junction restructuring may also involve protein endocytosis as ICAM2 would no longer be linked to its scaffold. At this point, it is worth noting that ICAM2 is not the only ICAM expressed by the testis. ICAM1 is also expressed by this organ but its primary role is in BTB dynamics (Xiao *et al*. 2012). Regardless, ICAM1 was also found to localize to the apical ES, suggesting that it too may be important in Sertoli–spermatid adhesion. While *Icam2* null mice were found to be fertile (Gerwin *et al*. 1999), it is possible that other proteins such as ICAM1 may have compensated for its loss of function or that ICAM2 is simply dispensable for Sertoli–spermatid adhesion. It is also possible that a loss of *Icam2* may not have significantly affected existing protein–protein interactions at the apical ES so that germ cell adhesion and spermatogenesis remained unaffected. Future studies will likely provide further insight on the role of ICAM2 in the testis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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