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 CdCl2 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 CdCl2-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

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 Kreter & 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
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 CdCl2

CdCl2 was used to induce the sloughing of germ cells from the seminiferous epithelium (Setchell & Waites 1970, Wong et al. 2004, Siu et al. 2009b, Elkin et al. 2010). In brief, rats (300–325 g b.w.; n=3–6 animals per time point) received a single dose of CdCl2 (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, CdCl2-treated animals were treated as biohazard waste and disposed of as directed by The Rockefeller University. The use of CdCl2 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 pelleted at 80 × g, cultured for an additional 2 days and subsequently terminated for the extraction of RNA or the preparation of cell lysates. Sertoli 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
were seeded at low density (0.05 × 10⁶ cells/cm²) 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'-TTACTTTGCATTT-CACTTGTTTCG-3' (ICAM2 sense, nucleotides 665–688), 5'-CCATCTGGTTGTCTTGGCGCTAATT-3' (ICAM2 antisense, nucleotides 1047–1070), 5'-TCGCTGACGTCCCGTCGTCCTTT-3' (S16 sense, nucleotides 57–90), and 5'-GCCAGCTTGGGATCGCGCGC-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 61 °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) |
| Actin    | Goat        | Santa Cruz Biotechnology | sc-31049 | IF (1:100) |
| Testin   | Rabbit      | Cheng Lab (Cheng et al. 1989) | sc-1616 | IB (1:200) |
| Occludin | Rabbit      | Invitrogen | 71-1500 | IB (1:200) |
| N-Cadherin | Rabbit   | Santa Cruz Biotechnology | sc-7939 | IB (1:200) |
| Nectin-3 | Rabbit      | Santa Cruz Biotechnology | sc-28637 | IB (1:200) |
| Afadin   | Rabbit      | Sigma–Aldrich | sc-14806 | IF (1:50) |
| β1-Integrin | Rabbit    | Santa Cruz Biotechnology | A0349 | IB (1:500) |
| Laminin γ3 | Rabbit  | Santa Cruz Biotechnology | sc-8978 | IB (1:300), IF (1:100) |
| Src      | Rabbit      | Sigma–Aldrich | sc-8056 | IB (1:100) |
| 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 (1:250) |
| Claudin-11 | Rabbit | Invitrogen | 36-4500 | IB (1:250) |
| JAM-A    | Rabbit      | Invitrogen | 36-1700 | IB (1:300) |
| CAR      | Rabbit      | Invitrogen | 31-1505 | 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 | 07-677 | IB (1:1000) |
| p-Src-Y530 | Rabbit | Abcam | ab4817 | IB (1:1000) |

IB, immunoblotting; IF, immunofluorescence; IHC, immunohistochemistry; IP, immunoprecipitation.
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). Immunohistochemistry and immunofluorescence staining were 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 DC 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).
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). 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) represents 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.
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 (IgGH, 50 kDa) and light (IgGL, 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 https://dx.doi.org/10.1530/JOE-12-0434.
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 Ca2+-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 Ca2+-independent immunoglobulin-like molecule that facilitates apical ES-based adhesion. It is expressed by elongating/elongated spermatids where
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 signal (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.

Figure 4
ICAM2, Src, and annexin II increase during CdCl2-induced testis damage. Immunoblotting experiments showing changes in the levels of several proteins (A). Proteins whose levels increased following CdCl2 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.o. of n = 3–6 rats. *P < 0.05; **P < 0.01 (Student’s t-test). ND, not detected.
ICAM2 is upregulated during CdCl₂-induced germ cell loss

Previous studies have shown CdCl₂ to induce germ cell sloughing from the seminiferous epithelium, as well as BTB disruption (Setchell & Waites 1970, Wong et al. 2004, Siu et al. 2009b, 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 CdCl₂ 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 CdCl₂ 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 CdCl₂ 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 CdCl₂-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 (l, 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.
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 CdCl₂, 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 CdCl₂-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 CdCl₂ treatment. It is also worth noting that round spermatids became increasingly immunoreactive for ICAM2 by 6–16 h of CdCl₂ 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).

**CdCl₂-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 spermatids at spermiation. As CdCl₂ 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 CdCl₂ 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 CdCl₂ and that round spermatids became increasingly immunoreactive for ICAM2 during restructuring of the seminiferous epithelium. Finally, we report that CdCl₂-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
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 lipids.
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. 2009b, Elkin et al. 2010). This in vivo model was used because environmental toxicants can affect spermatogenesis and contribute to subfertility/infertility (Siu et al. 2009a, Mruk & Cheng 2011b, 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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