Mice depleted of the coxsackievirus and adenovirus receptor display normal spermatogenesis and an intact blood–testis barrier

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Abstract

The coxsackievirus and adenovirus receptor (CXADR (CAR)) is a cell adhesion molecule expressed mainly in epithelial cells. Numerous evidence indicate that CXADR has an important role in testis development and function of the blood–testis barrier (BTB) *in vitro*. The role of CXADR in testis physiology *in vivo* has, however, not been addressed. We therefore constructed a conditional CXADR knockout (cKO) mouse model in which CXADR can be depleted at any chosen timepoint by the administration of tamoxifen. We report for the first time that testicular depletion of CXADR in adult and pubertal mice does not alter BTB permeability or germ cell migration across the BTB during spermatogenesis. Adult cKO mice display normal junctional ultra-structure and localization of the junctional proteins claudin-3, occludin, junction-associated molecule-A (JAM-A), and ZO1. The BTB was intact with no leakage of biotin and lanthanum tracers into the tubular lumen. Adult CXADR cKO mice were fertile with normal sperm parameters and litter size. Breeding experiments and genotyping of the pups demonstrated that CXADR-negative sperm could fertilize WT eggs. In addition, knocking down CXADR from postnatal day 9 (P9) does not affect testicular development and BTB formation. These cKO mice were analyzed at P49 and P90 and display an intact barrier and uncompromised fertility. We conclude that CXADR possesses no direct role in testicular physiology *in vivo*.

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Introduction

The coxsackievirus and adenovirus receptor (CXADR (CAR)), first recognized as a receptor for coxsackievirus and adenovirus, is a junctional protein expressed in the epithelia of various tissues including testis (Raschperger *et al.* 2006). A number of expression studies and *in vitro* experiments have indicated an essential role of CXADR in the testes (Mirza *et al.* 2006, 2007, Wang & Cheng 2007, Wang *et al.* 2007, Su *et al.* 2012). No *in vivo* studies addressing these findings have, however, been reported.

CXADR belongs to the cortical thymocyte marker of the *Xenopus* (CTX) family. In addition to CXADR, also the junction-associated molecules (JAM-A, JAM-B, and JAM-C) and junctional adhesion-like molecule (JAML) belong to this family (Chretien *et al.* 1998, Moog-Lutz *et al.* 2003). Some CTX family members play a critical role in transepithelial migration of circulating lymphocytes and leukocytes during inflammatory conditions, and some have also been suggested to facilitate transepithelial migration of male germ cells across the

blood–testis barrier (BTB; Chavakis *et al.* 2004, Wang & Cheng 2007, Verdino & Wilson 2011). CTX proteins have also been reported to have other functions in the testis, such as regulation of sperm motility and spermatid differentiation (Gliki *et al.* 2004, Mirza *et al.* 2006, Sakaguchi *et al.* 2006, Shao *et al.* 2008).

In rodents, CXADR is highly expressed in both Sertoli and germ cells during perinatal and postnatal development up to the age of 17 days. On completion of BTB, at postnatal day 21 (P21) a sharp decrease in both mRNA expression and CXADR protein was observed (Mirza *et al.* 2007, Wang *et al.* 2007). In adult mice, CXADR expression becomes restricted and stage dependent and is predominantly localized to migrating preleptotene cells at stages VII and VIII enclosed in an intermediate compartment flanked by the tight junction marker occludin (Mirza *et al.* 2007). In addition, CXADR and claudin-3 were shown to localize to similar subcellular positions. Claudin-3 was previously reported to associate transiently with newly formed tight junctions

at the time germ cells move across the BTB (Russell 1977, Mruk & Cheng 2004, Mirza *et al.* 2007). These results suggested that CXADR might be involved in germ cell migration. Further support for this hypothesis was provided by the finding that Sertoli cells express JAML, a protein that was previously reported to form a functional complex with CXADR that supported transepithelial migration of leukocytes (Moog-Lutz *et al.* 2003, Zen *et al.* 2005). We therefore hypothesized that the CXADR–JAML interaction facilitated migrating of preleptotene spermatocytes across the BTB via a similar mechanism.

On the basis of the presence of CXADR in the acrosome region of mature spermatozoa, CXADR has also been implicated to have a role in fertilization (Shaw *et al.* 2004, Mirza *et al.* 2006).

JAM-C, a protein absolutely required for proper differentiation of male germ cells, forms a complex with CXADR (Gliki *et al.* 2004, Mirza *et al.* 2006). In addition, CXADR partially co-localize with the junction markers ZO1, JAM-A and JAM-B as well as with occludin in the tubules that harbor migrating spermatocytes (Mirza *et al.* 2006, Wang & Cheng 2007, Wang *et al.* 2007). These results suggest that CXADR is part of large protein complex localized at or near the BTB. Moreover, a recent *in vitro* study has revealed that CXADR disruption by siRNA resulted in mislocalized ZO1 and altered Sertoli cell barrier permeability (Su *et al.* 2012). These results point toward the role of CXADR in BTB maintenance.

The available *in vivo* knockout (KO) models of various junctional proteins have helped to understand their physiological role in the testis, and they reveal varying phenotypes in mice. These include no apparent phenotype in JAM-B^{-/-} mice (Sakaguchi *et al.* 2006) and cingulin (Guillemot *et al.* 2004), slowly degenerative, as for occludin^{-/-} mice (Saitou *et al.* 2000), subfertility in JAM-A^{-/-} mice (Cooke *et al.* 2006), and sterility in claudin-11^{-/-} and JAM-C^{-/-} mice (Gow *et al.* 1999, Gliki *et al.* 2004). The JAM-A^{-/-} phenotype was attributable to the role of JAM-A in the regulation of spermatozoal motility (Shao *et al.* 2008). No *in vivo* KO model has so far addressed the role of CXADR in the testis.

This study is the first *in vivo* model that explores the physiological role of CXADR in testis by using a conditional CXADR KO (cKO) mouse model. CXADR-depleted animals were generated by a tamoxifen-inducible Cre system, in which the *Cxadr* gene could be excised at any chosen timepoint (Pazirandeh *et al.* 2011). We report a detailed characterization of testicular structure and function in the absence of CXADR.

Materials and methods

Animal care and handling

Mice were housed with unlimited access to food and water and exposure to 12 h light:12 h darkness cycles and all animal experimentation was conducted in accordance with the

accepted standards of humane animal care, and was approved by the Local Animal Ethical Committee at Karolinska Institutet, Stockholm (N179/08).

CXADR cKO mice

The CXADR cKO mice were generated using Cre/lox P technology published before (Pazirandeh *et al.* 2011), where the *Cxadr* gene can be disrupted in all tissues at any chosen timepoint by tamoxifen-regulated Cre-mediated recombination *in vivo*. Mice with a floxed *Cxadr* allele was generated at the MCI/ICS (Mouse Clinical Institute, Institut Clinique de la Souris, Illkirch, France). Briefly, mice were generated on a C57BL6 (+/+) background with lox P site inserted around exon 2 of the *Cxadr* gene (F/F mice). Further breeding with C57BL/6 mice expressing a universal CMV-driven tamoxifen-inducible Cre-*Ertm* gene (+/+;Cre mice) created mice harboring both the floxed *Cxadr* gene and the inducible CRE protein (F/F;Cre mice). Tamoxifen treatment activates CRE that results in the excision of the *Cxadr* gene and consequently a depletion of the CXADR protein. Littermates with genotypes F/F or +/+;Cre were used as a control for the cKO animals as indicated in the text.

Genotyping was carried out by PCR on genomic DNA from tail using primer pairs described previously (Pazirandeh *et al.* 2011). In the PCR that uses a primer pair that encompasses one of the lox P sites, the DNA fragment of 211 bp represents the WT allele, whereas the 261 bp fragment represents the lox P-flanked allele. In DNA from cKO animals, in which the two lox P sites have recombined, this PCR does not give rise to any PCR product.

Tamoxifen gavage for the generation of adult and prepubertal CXADR cKO

Adult animals (minimal age 4–6 weeks) of genotype F/F;Cre and F/F were gavaged with 0.25 mg/g body weight tamoxifen (Sigma, T5648) once a day for 5 consecutive days according to reported procedure (Young *et al.* 2008). Mice were killed and analyzed 3 weeks ('standard protocol', Pazirandeh *et al.* (2011)), 6 weeks, or 12 weeks following the first dose of tamoxifen. For fertility analysis, the male CXADR cKO mice and their control littermates were kept for 6 weeks following the first dose of tamoxifen in order to flush out all CXADR positive sperm before they were mated with WT females. For generation of prepubertal cKO, the pups received at P8 a single dose of 0.5 mg tamoxifen as reported previously (Young *et al.* 2008). These mice were killed at P9, P24, P49, and P90. The efficiency of Cre-mediated excision was analyzed by PCR on genomic DNA isolated from the tail as described previously (Pazirandeh *et al.* 2011), and CXADR protein downregulation was examined by western blotting.

Histological and transmission electron microscopic analysis

For histological analysis, testes were fixed either in Bouin's solution for at least 16 h and stored in 70% ethanol at 4 °C, or in 4% paraformaldehyde–PBS (pH 7.2) overnight and dehydrated in a graded series of ethanol. The samples were paraffin embedded and 5 µm-thick sections were stained with the

periodic acid–Schiff–hematoxylin technique (PAS) and hematoxylin staining using standard protocols.

For transmission electron microscopy (TEM) analysis, the testes were immediately processed in 2% glutaraldehyde+0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose and 3 mM CaCl₂, pH 7.4 according to the published procedure (Pazirandeh *et al.* 2011). Semi-thin sections (0.5 µm) were stained with toluidine blue and used for light microscopic analysis. Ultrathin sections (~40–50 nm) were contrasted with uranyl acetate (Merck) and examined under a Leo 906 TEM at 80 kV (Carl Zeiss SMT, Oberkochen, Germany). Digital images were taken by using a Morada digital camera (Soft Imaging System GmbH, Münster, Germany).

Indirect double immunofluorescence and western blot analysis

The method of protein extract preparation for western blot, tissue storage, and fixation for immunofluorescence was as reported previously (Sollerbrant *et al.* 2003, Mirza *et al.* 2006, 2007). A Leica confocal fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) was used for immunofluorescence analyses.

Antibodies

Rabbit, polyclonal anti-CXADR antibodies (IG1, RP1284, and RP291) and the antibody toward Calnexin have been described (Andersson & Pettersson 1998, Raschperger *et al.* 2006). MAB toward rat occludin (MOC37) was a kind gift from Dr M Furuse, Kobe University, Japan. Rat anti-ZO1 is a MAB purchased from Chemicon, Billerica, MA, USA (MAB1520). Rabbit anti-occludin (ABT146), rabbit anti-ZO1 (AB2272), and rabbit anti-claudin-3 (ABT43) were from Zymed/Invitrogen (Life Technologies). Goat anti-JAM-A was from R&D system (AF1077, Minneapolis, MN, USA). The antibodies were used in concentrations reported previously (Mirza *et al.* 2006, 2007). Secondary antibodies were goat anti-rabbit Alexa Fluor 488, donkey anti-rat Alexa Fluor 594 (A-11008 and A-21209, Molecular Probes, Burlington, ON, USA), and HRP labeled anti-rabbit IgG (RPN4301, GE Healthcare, Stockholm, Sweden, UK). Antibodies to acrosome marker PNA labeled with Alexa Fluor 488 (L21409) and secondary antibodies such as goat anti-rabbit Alexa-Fluor 546 antiserum (A11035) and Alexa-Fluor-488 Streptavidin (S32354) were purchased from Molecular Probes.

Qualitative assessment of BTB permeability

Lanthanum immersion fixation and electron microscopic evaluation

Lanthanum immersion method was used for functional investigation of the BTB in adult CXADR cKO. Testes tissues were prepared according to reported method (Neaves 1973, Willems *et al.* 2010). Briefly, adult CXADR cKO (F/F;Cre mice) and littermate controls (F/F) were killed 3 weeks after the first tamoxifen treatment and the testes tissues were immediately pre-fixed for 5 min in the fixative containing 1% lanthanum and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.3 to allow it to harden the tissue. After that, testis were cut into

small pieces (2–3 mm²) and immersed in the same fixative, for 5 h, at room temperature. After immersion, tissue samples were rinsed in the same buffered lanthanum solution but without glutaraldehyde at 4 °C until processed. The tissues were then post fixed for 8 h in 1% O₃O₄ in 0.1 M containing 1% of the same lanthanum solution at pH 7.2 at 4 °C. After overnight staining in 0.5% aqueous uranyl acetate, the samples were dehydrated in alcohol and embedded in Epon 812-Araldite. Ultrathin section (~40–50 nm) were cut and contrasted with uranyl acetate (Merck) and examined in a Leo 906 TEM at 80 kV.

Biotin tracer studies

The integrity of BTB was analyzed in both adult CXADR cKO mice and sexually mature mice KO at P9 by intra-testicular infusion of biotin tracer according to reported methods (Meng *et al.* 2005). Briefly, adult mice from both treatment groups were anesthetized with pentobarbitone (Ayrton Saunders Ltd, Liverpool, UK) diluted 1:10 in 0.9% NaCl. The animals received 0.1 ml of the diluted stock/10 g body weight by i.p. injections. Testes were injected with either the qualitative tight junction (TJ) functional tracer biotin (10 mg/ml, EZ-Link Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL, USA) in a volume of 10% testis weight, or the biotin vehicle, PBS (pH 7.4), and 1 mM CaCl₂. Testes were removed and directly fixed in optimum cutting temperature (OCT) and frozen after 30 min. The penetration of the biotin tracer in tissue cryosections was visualized directly under fluorescence microscopy using Alexa-488 streptavidin (Molecular Probes). For co-localization studies with occludin, the sections were incubated with rabbit anti-occludin antibodies (1:200) for 1 h, washed, and incubated with Streptavidin Alexa Fluor along with the goat anti-rabbit Alexa-546 secondary antiserum (Molecular Probes).

Sperm collection and sperm parameter analysis

The sperm parameters were carried out as reported previously (Bjorndahl *et al.* 2002). Briefly, for motility analysis, the minced caudal epididymis was suspended in pre-warmed (37 °C) sperm preparation medium (SU-medium) constituting Earle's balanced salt solution (E 2888; Gibco; Life Technologies), supplemented with sodium pyruvate (P4562; Gibco; Life Technologies), 1.1 mg/ml; Penicillin-G (P 4687; Gibco; Life Technologies), 10 000 IE/ml, and 1% BSA (A8918; Gibco; Life Technologies). Duplicate samples of 5 µl each were used to estimate the number of motile, live immotile, and dead immotile sperms. For sperm count, 50 µl of the above sperm suspension was diluted with 950 µl 0.4% formalin (in distilled water containing 5% NaHCO₃). Each of the two chambers in an improved Neubauer chamber was filled, and after sedimentation, spermatozoa were counted according to NAFA ESHRE manual for semen analyses (World Health Organization 2010). The morphology was analyzed by examining sperm under light microscopy and also by staining with papanicolau stain. Three age-matched cKO and three littermate controls were used, and both sperm counts and sperm motility procedures were carried out twice for each sample and then averaged.

Fertility assay

Adult CXADR cKO male mice and control littermates at the age of 6 weeks were treated with tamoxifen as described earlier.

They were then mated with 6-week-old WT C57BL6 females. Each mating pair was monitored over a 3-month period to examine the number of litters and the number of pups per litter. Prepubertal mice treated with tamoxifen at P9 were analyzed in the same way and control littermates at the age of 8 weeks. Western blot analysis was carried out at the completion of the fertility experiment to analyze CXADR expression in the testis.

Statistical analysis

Student's *t*-test with 'Shapiro-Wilk' normality test was used to compare averages between two different experimental groups (SigmaPlot 11.0; Systat Software, Inc., Erkrath, Germany). Number of pups per litter is expressed as mean $\pm$ s.d. *P* value of ≤ 0.05 was considered statistically significant.

Results

CXADR is efficiently downregulated in the testes of adult cKO mice

We previously reported that tamoxifen-induced Cre-mediated deletion of the *Cxadr* gene in adult mice results in an efficient downregulation of the CXADR protein in several tissues (Pazirandeh *et al.* 2011). In this study, we extend this finding to include also the male reproductive system. Western blot analysis of whole testes extract from tamoxifen-treated adult mice demonstrated a significant decrease in CXADR protein levels in the testes of cKO animals compared with littermate controls (Fig. 1A). Calnexin was used as a loading control. Indirect immunostaining using a CXADR-specific antibody confirmed this result because the stage-dependent CXADR staining we reported previously was absent in the cKO mice (Mirza *et al.* 2007; Fig. 1B). CXADR remained downregulated for at least 3 months (length of experiment, Pazirandeh *et al.* (2011) and data not shown). CXADR was also efficiently downregulated in the vas deferens, seminal vesicles, and epididymis of tamoxifen-treated cKO animals (data not shown). CXADR was not expressed in Leydig cells in neither cKO animals nor controls (Fig. 1B).

We previously reported CXADR expression in male germ cells (Mirza *et al.* 2006). To analyze whether CXADR was downregulated in the mature sperm of cKO males, western blots were carried out on epididymal sperm extracts. The results demonstrate that the amount of CXADR protein detected in the sperm from cKO males is significantly lower compared with the littermate controls (Fig. 1C). Similarly, immunofluorescence analysis of isolated sperm revealed that 90% of the sperm cells lacked any detectable CXADR protein (Fig. 1D and data not shown). The remaining 10% displayed faint CXADR staining which was not surprising considering the fact that tamoxifen-dependent, Cre-mediated, excision is normally not taking place in 100% of the target cells (Hayashi & McMahon 2002).

Figure 1 Downregulation of CXADR in adult testis following tamoxifen administration. (A and C) Western blot analyses of extracts from testis (A) and sperm (C) isolated from tamoxifen-treated cKO mice (F/F;Cre) and littermate controls (Ctrl) (F/F). Calnexin (Clnx) was used as a loading control. 50 μ g protein was loaded in each lane. (B) Indirect immunofluorescence staining of testicular cross-sections using a CXADR-specific antibody (RP291) showing that CXADR was efficiently and specifically downregulated in tamoxifen-treated adult CXADR cKO mice at P90 in stage VIII tubules. Owing to the different fixation methods required to visualize CXADR in the epithelium and in the spermatids, CXADR staining is not very prominent in the spermatids in the control panel in this figure. An antibody toward occludin was included to visualize the BTB, and DAPI was included to facilitate identification of germ cells. (D) Indirect immunofluorescence staining of sperm from P140 tamoxifen-treated cKO (F/F;Cre) and littermate controls (Ctrl) (F/F). A fluorescence-labeled lectin (PNA) and DAPI were used to visualize the acrosome region and nucleus respectively. The results shown are representative of three independent experiments. Scale bar: 40 μ m.

CXADR protein levels in tamoxifen-treated $+/+$; Cre control animals were indistinguishable from WT animals (Pazirandeh *et al.* (2011) and data not shown). Together these results demonstrated that Cre-mediated excision of the *Cxadr* gene was efficient in the testis, and that neither tamoxifen treatment itself nor the presence of the CRE protein alone can disrupt expression from the *Cxadr* gene.

CXADR-depleted testis display normal morphology and sperm parameter

Testicular size and weight were normal in adult cKO mice analyzed between 4 weeks and 4 months after the administration of tamoxifen (data not shown).

Histological analysis of PAS-stained testis sections from cKO animals showed normal tubular lumen size and presence of all differentiated stages of germ cells (Fig. 2A). Also the cellular organization in the seminiferous tubules, given by the Sertoli and germ cells, and the numbers and appearance of the interstitial cells of cKO animals appeared normal (Fig. 2A). Analysis by high-resolution light microscopy of PAS-stained epididymal spermatozoa displayed morphologically normal cells with evenly stained acrosome regions and tails, indistinguishable from the littermate controls (Fig. 2B). In addition, the analysis of sperm from cKO mice at P140

Figure 2 Gross morphology of testis and sperm was normal in adult tamoxifen-treated cKO animals. (A) PAS staining of testicular sections from mice at P50 control (Ctrl) (F/F) and CXADR cKO mice (F/F;Cre) showed apparently normal tubules with sperm in various stages of development. Images represent 20× and 100× resolution. Scale bars: 100 and 20 µm. (B) Sperm morphology was analyzed after papanicolaou staining. Scale bars: 20 µm (B). The results shown are representative of three independent experiments.

showed normal sperm titers ($1.98 \pm 0.09 \times 10^6/\text{ml}$) and motility (60.3 ± 3.78) when compared with littermate controls which had an average sperm count of $3.14 \pm 0.31 \times 10^6/\text{ml}$ and motility 56.3 ± 1.11 . The cKO sperm titer and motility were not significantly different from the littermate controls with *P* values of 0.1 and 0.4 respectively ($P > 0.05$). Each value corresponds to the average of three age-matched males.

In addition, also the morphology of the vas deferens, seminal vesicles, and epididymis appeared normal (data not shown).

Adult cKO mice have structurally normal cellular junctions at the BTB

A detailed electron microscopic analysis of tight junctions and ectoplasmic specializations in the testes revealed no major differences in the Sertoli–Sertoli and Sertoli–germ cells junctions, respectively, between control and cKO mice (Fig. 3A). In addition, the subcellular distribution of the junctional proteins ZO1, occludin, JAM-A, and claudin-3 appeared normal in testicular sections from adult cKO when analyzed by indirect immunofluorescence (Fig. 3B).

The same results were obtained when mice were analyzed up to the age of 8 months (length of experiment and data not shown). We conclude that the structure and protein composition of the cellular junctions at the BTB is unaffected in the absence of CXADR.

Absence of CXADR does not affect the BTB integrity and function

The integrity of BTB was analyzed in the adult cKO mice at P49 and P90 by immersion fixation of the freshly

Figure 3 The structure of cellular junctions in CXADR-depleted adult mice is maintained. (A) Transmission electron microscopy (TEM) images of testes from P78 tamoxifen-treated cKO mice (F/F;Cre) and littermate controls (Ctrl) (F/F). No significant difference is seen at the Sertoli–Sertoli cell and Sertoli–germ cell interfaces (arrow). High-resolution images are shown on the left and right side of the overview pictures. Scale bars: 2 µm and 500 nm. (B) Indirect immunofluorescence staining on frozen testicular sections derived from tamoxifen-treated cKO mice (F/F;Cre) and littermate controls (Ctrl) (F/F). Specific antibodies toward ZO1, occludin, JAM-A, and claudin-3 (Cld 3) were used. DAPI was included to visualize nuclei. Scale bar: 40 µm. The results shown are representative of three independent experiments.

Figure 4 The integrity of BTB is not affected by downregulation of CXADR. Leakage across the BTB was analyzed in P90 mice by two different methods. (A) An intact BTB in both adult tamoxifen-treated cKO mice (F/F;Cre) and littermate controls (Ctrl) (F/F) prevented the penetration of the electron-dense lanthanum tracer beyond the tight junctions (arrows). Scale bars: 2 μm and 500 nm. (B) The fluorescent biotin tracer was restricted to the basal compartment of seminiferous tubules 30 min after injection into the interstitial space in the testes. The tight junctions were stained with an antibody toward occludin. DAPI was used to visualize nuclei. Scale bars: 10 and 40 μm (low and high magnifications). At least three mice were used in each experiment.

excised testes in electron-dense lanthanum tracer grains, and by intra-testicular injection of fluorescent biotin tracer. Both of these chemicals are restricted to enter seminiferous tubules if the BTB is intact. When analyzed by TEM, the lanthanum tracer was restricted at the Sertoli cell barrier preventing its passage into the lumen of seminiferous tubular in both cKO animals and littermate controls (Fig. 4A). This phenomenon was common to all the seminiferous tubules irrespective of stages. Similarly, the biotin tracer was not able to pass the BTB and was instead restricted to the interstitial space and the basal compartment in both cKO and control testis when analyzed by immunofluorescence microscopy (Fig. 4B).

The same results were obtained when mice were analyzed at the age of 8 months (length of experiment and data not shown). Immunofluorescence staining using an occludin-specific antibody was used as a marker for tight junctions. These results demonstrate that the integrity and function of the BTB is maintained in the absence of CXADR.

CXADR-negative sperm can fertilize WT eggs

For the fertility assessment, cKO males and their littermate controls were mated with WT females 6 weeks after the last tamoxifen treatment. The cKO mice gave rise to similar number of pups per litter as the control mice (Fig. 5A). Genotyping of the pups demonstrated that 95% of the pups showed the presence of a single WT band and the rest of 5% pups had heterozygous band (Fig. 5B). This data suggests that CXADR-negative sperm could fertilize a WT egg. Interestingly, the genotype of the pups from above mating of CXADR cKO mice showed that 90% of cKO pups displayed DNA fragments of 211 bp for the WT allele from WT egg and the absence of lox P-flanked allele (261 bp) from CXADR cKO sperm. The F/F controls showed heterozygote bands showing the presence of both lox P-flanked from F/F male mice and WT allele from WT female mice (Fig. 5B).

Barrier formation and function is not affected by depletion of CXADR during the prepubertal period

Owing to the strong and widespread expression of CXADR during the prepubertal period, CXADR has been claimed to have role in the establishment of the BTB. In order to

Figure 5 CXADR-negative sperm can fertilize WT eggs. (A) Adult mice at P80, tamoxifen-treated CXADR cKO mice (F/F;Cre) ($n=8$), and littermate controls (Ctrl) (F/F) ($n=7$) were mated with WT females ($n=16$). Mating cages were monitored for a period of 3 months to determine the average number of pups per litter. No significant difference between the two groups was observed ($P>0.05$; Student's t -test; P value=0.58). The graphs represent the mean number of pups per litter in each group, and error bars indicate the s.d. (B) Pups were genotyped by PCR on tail DNA isolated from pups from representative litters of tamoxifen-treated cKO mice (F/F;Cre) ($n=5$) and littermate controls (Ctrl) (F/F) mice ($n=5$) mated with 6-week-old WT females as described in 'Materials and methods' section. The DNA fragment of 211 bp represents the WT allele whereas the 261 bp fragment represents the lox P-flanked allele. In DNA from cKO animals, in which the two lox P sites have recombined, this PCR does not give rise to any PCR product. M, size marker.

analyze the role of CXADR in this process, cKO pups were created by injecting tamoxifen at P8, that is before the BTB is formed in mice (Nagano & Suzuki 1976, Meng *et al.* 2005). CXADR was efficiently and irreversibly downregulated in the cKO pups 1–2 days after the first tamoxifen treatment as analyzed by PCR analysis. Western blot analysis of testicular extracts from P24, P49, and P90 mice showed CXADR downregulation (Pazirandeh *et al.* 2011; Fig. 6A and data not shown).

The cKO pups and littermate controls were analyzed for morphology, BTB integrity, and fertility at P49 and adult age. There were no histological differences in the seminiferous tubules in cKO compared with the littermate controls, and all animals displayed a normal repertoire of germ cells in the tubules (Fig. 6B). The BTB was intact as determined by the restricted distribution of biotin tracer outside of the seminiferous tubules (Fig. 6C). Immunofluorescence staining using an occludin-specific antibody was used as a marker for tight junctions. Finally, mating of the cKO mice with WT females resulted in comparable number of pups as compared with their littermate controls (Fig. 6D).

Discussion

Numerous indirect evidence indicate that the junction protein CXADR has an important role in testis development and physiology. Despite this, in this study, we demonstrate that CXADR is dispensable for normal testis development and function in mice.

Histological analysis of testicular sections from adult CXADR cKO mice revealed no obvious difference in tubular size and a normal distribution of germ cells in all stages of differentiation. Both migration of preleptotene/leptotene cells across the BTB and germ cell differentiation therefore appear normal in the absence of CXADR. This result was surprising because the finding that CXADR is expressed in a stage-dependent manner in the BTB and also interacts with proteins important for testis function led us to hypothesize a role for CXADR in spermatogenesis (Cohen *et al.* 2001, Zen *et al.* 2005, Mirza *et al.* 2006, 2007). The results instead imply that the interaction between CXADR and JAM1, previously suggested to confer transepithelial migration of cells in other epithelia, appears not to have the same role in the BTB. Similarly, the mechanism by which JAM-C control germ cell differentiation does not appear to involve CXADR, because disruption of the interaction between these two proteins does not affect germ cell differentiation.

Expression of CXADR was previously shown to correlate with epithelial permeability both *in vivo* and *in vitro*, and loss of CXADR results in mislocalization of the tight junction proteins occludin and ZO1 at the BTB in Sertoli cells cultured *in vitro* (Cohen *et al.* 2001, Raschperger *et al.* 2006, Su *et al.* 2012). However, our ultrastructural analysis of the cellular junctions in the testis failed to reveal any differences between CXADR cKO and control mice, and the BTB was intact with no

Figure 6 The formation of the BTB and fertility were normal in prepubertal CXADR-depleted mice. CXADR depletion of prepubertal mice at P9 was generated by a single dose of tamoxifen at P8. cKO (F/F;Cre) ($n=7$) and control (Ctrl) (F/F) littermate ($n=7$). Mice at P49 were used for CXADR protein expression, morphological analysis, and barrier integrity (A, B and C). (A) Western analysis of testicular extract demonstrated an efficient downregulation of CXADR in cKO mice at P49. Calnexin (Clnx) was used as a loading control. (B) Hematoxylin staining of testicular sections failed to reveal any difference between the two groups in gross morphology and germ cell differentiation at P49. Images are representative of three independent experiments. Scale bar: 50 μ m. (C) A biotin permeation experiment showed that a well-established BTB barrier restricted the passage of biotin from the interstitium to the adluminal compartment in both groups. An antibody toward occludin was used as a marker for tight junctions, and the nuclei were counterstained with DAPI. Images are representative of three independent experiments. Scale bars: 20 and 40 μ m. (D) A fertility experiment was conducted with prepubertal (P9), tamoxifen-treated cKO mice (F/F;Cre) ($n=5$), and control littermates (Ctrl) ($n=5$) at the age of 8 weeks. The two groups of mice were mated with WT C57BL/6 females ($n=16$). Each pair was monitored for 3 months. No statistically significant difference between the number of pups per litter was observed (P value=0.4).

leakage of biotin or lanthanum tracers into the tubular lumen. In addition, analysis of the tight junction proteins ZO1, occludin, JAM-A, and claudin-3 failed to reveal any change in amount or subcellular localization in the

cKO mice further supporting that the BTB was intact. The reason for the discrepancy is not known but likely reflects the differences between *in vitro* and *in vivo* model systems. We conclude that CXADR is dispensable for the integrity and maintenance of the BTB *in vivo*.

We previously reported the presence of CXADR on the surface of acrosome-reacted spermatozoa, indicating its involvement in events succeeding the acrosome reaction, such as passage through zona pellucida or interaction with the egg plasma membrane (Mirza *et al.* 2006). Surprisingly, the acrosome structure was intact in the CXADR cKO mice. Furthermore, the absence of CXADR in the sperm did not interfere with fertilization, as CXADR cKO male mice were fertile and able to transfer the deleted *Cxadr* allele to the offspring. In fact 90% of the pups in each litter carried the deleted *Cxadr* allele, which correlated well with the percentage of sperm found to lack CXADR expression.

CXADR is highly expressed in both Sertoli and germ cells during perinatal and postnatal development, followed by a rapid downregulation of both mRNA and protein levels (Mirza *et al.* 2007). The expression of CXADR therefore coincides with the formation of the BTB which occurs around days 11–15 in rodents (Nagano & Suzuki 1976). CXADR is also a downstream target of follicle-stimulating hormone (Mirza *et al.* 2007). These results, together with the reports that CXADR affects the formation and maintenance of functional tight junctions *in vitro* (Cohen *et al.* 2001, Su *et al.* 2012), led us to believe that CXADR might be important for the formation of the BTB in the developing testis. Knockdown of CXADR in postnatal mice, however, failed to reveal any effects on BTB integrity and fertility, and the testis of these cKO mice had normal appearance of differentiating spermatocytes at P24 and P49 according to criteria described by Russell (1990). CXADR therefore appears to be dispensable for testis development and formation of the BTB.

To our knowledge, there are no studies analyzing the effects of tamoxifen on the BTB. However, there are reports indicating that tamoxifen can have both positive and negative effects on spermatogenesis in a context in which tamoxifen was administered immediately before analysis (Carreau & Hess 2010). In our model, tamoxifen is only delivered during a very limited time period (once a day for 5 consecutive days). This treatment is enough to permanently inactivate the *Cxadr* gene (this manuscript and Pazirandeh *et al.* (2011)). Tamoxifen has a half-life of 5–7-day in humans, and probably shorter in mice due to faster metabolism. For these reasons, we feel it highly unlikely that tamoxifen would mask any loss of CXADR function in the mice many months after completing the tamoxifen treatments.

Gene KO and knockdown of integral tight junction proteins and tight junction-associated proteins have revealed a plethora of functions for these proteins in different epithelial barriers *in vivo* (Furuse 2009). It is becoming increasingly clear that tight junction proteins not only constitute mechanical barriers but also mediate signaling

and regulatory roles, which highlights the complexity of epithelial barriers. Each epithelium is structurally and functionally very different, and the phenotype of a particular gene KO or knockdown depends on the combination of proteins present in the large protein complex that makes up the different barriers. It is therefore not surprising that CXADR, despite expressed in most epithelial barriers in the body and the devastating effects of CXADR KO and knockdown on epithelial barriers and cellular junctions in other tissues (Lim *et al.* 2008, Pazirandeh *et al.* 2011, Mirza *et al.* 2012), does not seem to have a role in the testis. Redundant functions may operate and proteins compensating for the loss of CXADR might ensure function of this important organ to secure survival of the species.

The present study concludes that CXADR alone is not essential for testis development, spermatogenesis, BTB function, or fertility in mice *in vivo*.

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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References

- Andersson AM & Pettersson RF 1998 Targeting of a short peptide derived from the cytoplasmic tail of the G1 membrane glycoprotein of Uukuniemi virus (Bunyaviridae) to the Golgi complex. *Journal of Virology* **72** 9585–9596.
- Björndahl L, Barratt CL, Fraser LR, Kvist U & Mortimer D 2002 ESHRE basic semen analysis courses 1995–1999: immediate beneficial effects of standardized training. *Human Reproduction* **17** 1299–1305. (doi:10.1093/humrep/17.5.1299)
- Carreau S & Hess RA 2010 Oestrogens and spermatogenesis. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **365** 1517–1535. (doi:10.1098/rstb.2009.0235)
- Chavakis T, Keiper T, Matz-Westphal R, Hersemeyer K, Sachs UJ, Nawroth PP, Preissner KT & Santos S 2004 The junctional adhesion

- molecule-C promotes neutrophil transendothelial migration *in vitro* and *in vivo*. *Journal of Biological Chemistry* **279** 55602–55608. (doi:10.1074/jbc.M404676200)
- Chretien I, Marcuz A, Courtet M, Katevuo K, Vainio O, Heath JK, White SJ & Du Pasquier L 1998 CTX, a *Xenopus* thymocyte receptor, defines a molecular family conserved throughout vertebrates. *European Journal of Immunology* **28** 4094–4104. (doi:10.1002/(SICI)1521-4141(199812)28:12<4094::AID-IMMU4094>3.0.CO;2-2)
- Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT & Bergelson JM 2001 The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *PNAS* **98** 15191–15196. (doi:10.1073/pnas.261452898)
- Cooke VG, Naik MU & Naik UP 2006 Fibroblast growth factor-2 failed to induce angiogenesis in junctional adhesion molecule-A-deficient mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26** 2005–2011. (doi:10.1161/01.ATV.0000234923.79173.99)
- Furuse M 2009 Knockout animals and natural mutations as experimental and diagnostic tool for studying tight junction functions *in vivo*. *Biochimica et Biophysica Acta* **1788** 813–819. (doi:10.1016/j.bbame.2008.07.017)
- Glikli G, Ebnet K, Aurrand-Lions M, Imhof BA & Adams RH 2004 Spermatid differentiation requires the assembly of a cell polarity complex downstream of junctional adhesion molecule-C. *Nature* **431** 320–324. (doi:10.1038/nature02877)
- Gow A, Southwood CM, Li JS, Pariali M, Riordan GP, Brodie SE, Danias J, Bronstein JM, Kachar B & Lazzarini RA 1999 CNS myelin and Sertoli cell tight junction strands are absent in *Osp/claudin-11* null mice. *Cell* **99** 649–659. (doi:10.1016/S0092-8674(00)81553-6)
- Guillemot L, Hammar E, Kaister C, Ritz J, Caille D, Jond L, Bauer C, Meda P & Citi S 2004 Disruption of the *cingulin* gene does not prevent tight junction formation but alters gene expression. *Journal of Cell Science* **117** 5245–5256. (doi:10.1242/jcs.01399)
- Hayashi S & McMahon AP 2002 Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Developmental Biology* **244** 305–318. (doi:10.1006/dbio.2002.0597)
- Lim BK, Xiong D, Dorner A, Youn TJ, Yung A, Liu TI, Gu Y, Dalton ND, Wright AT, Evans SM *et al.* 2008 Coxsackievirus and adenovirus receptor (CAR) mediates atrioventricular-node function and connexin 45 localization in the murine heart. *Journal of Clinical Investigation* **118** 2758–2770. (doi:10.1172/JCI34777)
- Meng J, Holdcraft RW, Shima JE, Griswold MD & Braun RE 2005 Androgens regulate the permeability of the blood–testis barrier. *PNAS* **102** 16696–16700. (doi:10.1073/pnas.0506084102)
- Mirza M, Hreinsson J, Strand ML, Hovatta O, Soder O, Philipson L, Pettersson RF & Sollerbrant K 2006 Coxsackievirus and adenovirus receptor (CAR) is expressed in male germ cells and forms a complex with the differentiation factor JAM-C in mouse testis. *Experimental Cell Research* **312** 817–830. (doi:10.1016/j.yexcr.2005.11.030)
- Mirza M, Petersen C, Nordqvist K & Sollerbrant K 2007 Coxsackievirus and adenovirus receptor is up-regulated in migratory germ cells during passage of the blood–testis barrier. *Endocrinology* **148** 5459–5469. (doi:10.1210/en.2007-0359)
- Mirza M, Pang MF, Zaini MA, Haiko P, Tammela T, Alitalo K, Philipson L, Fuxe J & Sollerbrant K 2012 Essential role of the coxsackie- and adenovirus receptor (CAR) in development of the lymphatic system in mice. *PLoS ONE* **7** e37523. (doi:10.1371/journal.pone.0037523)
- Moog-Lutz C, Cave-Riant F, Guibal FC, Breau MA, Di Gioia Y, Couraud PO, Cayre YE, Bourdoulous S & Lutz PG 2003 JAML, a novel protein with characteristics of a junctional adhesion molecule, is induced during differentiation of myeloid leukemia cells. *Blood* **102** 3371–3378. (doi:10.1182/blood-2002-11-3462)
- Mruk DD & Cheng CY 2004 Sertoli–Sertoli and Sertoli–germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocrine Reviews* **25** 747–806. (doi:10.1210/er.2003-0022)
- Nagano T & Suzuki F 1976 The postnatal development of the junctional complexes of the mouse Sertoli cells as revealed by freeze-fracture. *Anatomical Record* **185** 403–417. (doi:10.1002/ar.1091850403)
- Neaves WB 1973 Permeability of Sertoli cell tight junctions to lanthanum after ligation of ductus deferens and ductuli efferentes. *Journal of Cell Biology* **59** 559–572. (doi:10.1083/jcb.59.3.559)
- Pazirandeh A, Sultana T, Mirza M, Rozell B, Hultenby K, Wallis K, Vennstrom B, Davis B, Arner A, Heuchel R *et al.* 2011 Multiple phenotypes in adult mice following inactivation of the coxsackievirus and adenovirus receptor (Car) gene. *PLoS ONE* **6** e20203. (doi:10.1371/journal.pone.0020203)
- Raschperger E, Thyberg J, Pettersson S, Philipson L, Fuxe J & Pettersson RF 2006 The coxsackie- and adenovirus receptor (CAR) is an *in vivo* marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis. *Experimental Cell Research* **312** 1566–1580. (doi:10.1016/j.yexcr.2006.01.025)
- Russell L 1977 Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. *American Journal of Anatomy* **148** 313–328. (doi:10.1002/aja.1001480303)
- Russell LD, Ettlin R, Sinha Hikim A & Clegg E 1990 *Histological and Histopathological evaluation of the Testis*. Clearwater, FL: Cache River Press.
- Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, Noda T & Tsukita S 2000 Complex phenotype of mice lacking occludin, a component of tight junction strands. *Molecular Biology of the Cell* **11** 4131–4142. (doi:10.1091/mbc.11.12.4131)
- Sakaguchi T, Nishimoto M, Miyagi S, Iwama A, Morita Y, Iwamori N, Nakauchi H, Kiyonari H, Muramatsu M & Okuda A 2006 Putative “stemness” gene *jam-B* is not required for maintenance of stem cell state in embryonic, neural, or hematopoietic stem cells. *Molecular and Cellular Biology* **26** 6557–6570. (doi:10.1128/MCB.00729-06)
- Shao M, Ghosh A, Cooke VG, Naik UP & Martin-DeLeon PA 2008 JAM-A is present in mammalian spermatozoa where it is essential for normal motility. *Developmental Biology* **313** 246–255. (doi:10.1016/j.ydbio.2007.10.013)
- Shaw CA, Holland PC, Sinnreich M, Allen C, Sollerbrant K, Karpati G & Nalbantoglu J 2004 Isoform-specific expression of the coxsackie and adenovirus receptor (CAR) in neuromuscular junction and cardiac intercalated discs. *BMC Cell Biology* **5** 42. (doi:10.1186/1471-2121-5-42)
- Sollerbrant K, Raschperger E, Mirza M, Engstrom U, Philipson L, Ljungdahl PO & Pettersson RF 2003 The coxsackievirus and adenovirus receptor (CAR) forms a complex with the PDZ domain-containing protein ligand-of-numb protein-X (LNX). *Journal of Biological Chemistry* **278** 7439–7444. (doi:10.1074/jbc.M205927200)
- Su L, Mruk DD & Cheng CY 2012 Regulation of the blood–testis barrier by coxsackievirus and adenovirus receptor (CAR). *American Journal of Physiology. Cell Physiology* **303** C843–C853. (doi:10.1152/ajpcell.00218.2012)
- Verdino P & Wilson IA 2011 JAML and CAR: two more players in T-cell activation. *Cell Cycle* **10** 1341–1342. (doi:10.4161/cc.10.9.15294)
- Wang CQ & Cheng CY 2007 A seamless trespass: germ cell migration across the seminiferous epithelium during spermatogenesis. *Journal of Cell Biology* **178** 549–556. (doi:10.1083/jcb.200704061)
- Wang CQ, Mruk DD, Lee WM & Cheng CY 2007 Coxsackie and adenovirus receptor (CAR) is a product of Sertoli and germ cells in rat testes which is localized at the Sertoli–Sertoli and Sertoli–germ cell interface. *Experimental Cell Research* **313** 1373–1392. (doi:10.1016/j.yexcr.2007.01.017)
- Willems A, Batlouni SR, Esnal A, Swinnen JV, Saunders PT, Sharpe RM, Franca LR, De Gendt K & Verhoeven G 2010 Selective ablation of the androgen receptor in mouse Sertoli cells affects Sertoli cell maturation, barrier formation and cytoskeletal development. *PLoS ONE* **5** e14168. (doi:10.1371/journal.pone.0014168)
- World Health Organization, DoRHaR 2010 *In WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. Geneva: World Health Organization.
- Young P, Qiu L, Wang D, Zhao S, Gross J & Feng G 2008 Single-neuron labeling with inducible Cre-mediated knockout in transgenic mice. *Nature Neuroscience* **11** 721–728. (doi:10.1038/nn.2118)
- Zen K, Liu Y, McCall IC, Wu T, Lee W, Babbini BA, Nusrat A & Parkos CA 2005 Neutrophil migration across tight junctions is mediated by adhesive interactions between epithelial coxsackie and adenovirus receptor and a junctional adhesion molecule-like protein on neutrophils. *Molecular Biology of the Cell* **16** 2694–2703. (doi:10.1091/mbc.E05-01-0036)

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