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Loss of connexin43 (Cx43) in Sertoli cells leads to spatio-temporal alterations in occludin expression

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Summary. Within the testis, Sertoli cell (SC) junctional complexes between somatic SC create a basal and apical polarity within the seminiferous epithelium, restrict movement of molecules between cells, and separate the seminiferous epithelium into a basal and adluminal compartment. This barrier consists of membrane integrated proteins known as tight, adherens, and gap junctions, which promote cell-cell contact along the blood-testis-barrier (BTB). Nevertheless, these junctions, which form the basis of the BTB are structures whose function and dynamic regulation is still poorly understood. Thus, in this study, through the use of immunohistochemistry (IHC), semi quantitative western blot (WB) analysis, and real-time-quantitative-PCR (qRT-PCR) we focused on the expression pattern of the main testicular tight junction protein, occludin, in SC. For this, the established transgenic SC specific connexin 43 (Cx43) knockout (SCCx43KO) mouse line was used; both knockout (KO) and wildtype (WT) males of different ages from juvenile to adult were compared. The object was to elucidate a possible role of Cx43 on the expression pattern and regulation of occludin. This conditional KO mouse line lacks the gap junction gene *Gja1* (coding for Cx43) only in SC and reveals impaired spermatogenesis. The qRT-PCR indicates an increase in occludin mRNA in adult KO mice. These results correspond to the occludin protein synthesis of adult mice. Additionally, during puberty, occludin localization at the BTB barrier in KO mice is delayed. Our study demonstrates spatiotemporal alterations in occludin mRNA- and protein-expression, indicating that Cx43 might act as a regulator for BTB formation (and function).

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Introduction

Numerous junctions and proteins are responsible for blood-testis-barrier (BTB) formation, such as tight junctions like *Ocln* (occludin) and *Cldn11* (claudin-11), adhesion proteins like *Cdh2* (N-cadherin), and gap junctions like *Gja1* (connexin43, Cx43) (Furuse et al., 1993, 1998; Martín-Padura et al., 1998; Cyr et al., 1999). How important single members of these junctional complexes are is demonstrated by a study from Saitou and colleagues (2000). Mutations in the *Ocln* gene create male sterility by altering the BTB formation (Saitou et al, 2000; Chung et al., 2001). In a knockout (KO) mouse line of *Cldn11* the male mice are sterile, indicating that claudin-11 is vital for the formation of the BTB (Gow et al., 1999). Similar results were achieved from a KO mouse line of Cx43, the Sertoli cell specific connexin43 knockout (SCCx43KO, further investigated in this paper). The adult male SCCx43KO mice are sterile, yet having a functional BTB (Brehm et al., 2007; Sridharan et al., 2007; Carrette et al., 2010).

In most mammalian species Cx43 is the predominant testicular gap junction protein within the seminiferous epithelium, its immunoreactivity is mainly detected between adjacent Sertoli cell(s) (SC) and between SC and germ cells (GC) (Risley et al., 1992; Steger et al., 1999; Batias et al., 2000; Bravo-Moreno et al., 2001; Brehm et al., 2002, 2007; Tripathi and Tripathi, 2010; Weider et al., 2011a,b). This connexin is additionally known to form an integral component of the SC-SC junctional complexes at the BTB (Risley et al., 1992) and plays a role in the coordination of changes in SC junctional permeability as well as in SC and GC

differentiation (Pelletier, 1995). Furthermore, the synchronization of GC proliferation and differentiation is mediated through the gap junctional (Cx43) network (Decrouy et al., 2004).

Direct evidence for a possible relationship between Cx43, junction dynamics, and spermatogenesis was derived from Carette et al. (2010). This study demonstrated that Cx43 participates in the regulation of adherens and tight junction protein expression by using adult SCCx43KO mice, specific anti-Cx43 siRNA, and gap junction blockers in the SerW3 SC line. Interestingly, using Western blot (WB), immunohistochemistry (IHC), and immunofluorescence (IF), increased protein levels of N-cadherin, beta-catenin, and occludin were observed in adult KO mice compared to their wildtype (WT) littermates (Carette et al., 2010). As no functional alterations of the BTB were detected, it was supposed that Cx43 rather participates in the dynamic process of opening and closing of this barrier and/or permanent BTB closure. In the human testis, an altered expression of *Tjp1* (zonula occludens-1, ZO-1) and ZO-2, and a dysfunction of *Cldn11* have been demonstrated in SC associated with testicular carcinoma *in situ* (Fink et al., 2006, 2009). Comparing 8 day old SCCx43KO and WT males, alterations in the composition of intratubular cells were detectable (Giese et al., 2012): although the SC number was unaltered, the GC population was three times less in the transgenic SCCx43KO mice. In addition, via microarray analysis a total of 658 genes were found to be significantly different in their expression in the SCCx43KO mice. *Ocln* expression was not significantly increased but already showed a higher transcription rate in the KO (Giese et al., 2012).

Little is actually known about the initial and spatiotemporal expression pattern of barrier associated proteins (like occludin) and the dynamic formation of the BTB at the pubertal time period of its formation/assembly. Yet the authors of this study and others propose that Cx43 plays a regulatory role in this process (Pelletier, 1995; Cyr et al., 1999; Segretain et al., 2004; Brehm et al., 2007; Cheng and Mruk, 2012). In 1976, Nagano and Suzuki used occludin as the specific indicator for the initial formation of the BTB at day 16 in pubertal mice. This occludin formation was confirmed by Cyr and others (1999) through the use of IF at post partum (p.p.) day 14 in mice, hence coinciding with the formation of the BTB. It was also determined that occludin expression in the first seven weeks increased dramatically at the base of seminiferous epithelium in mice p.p. (Moroi et al., 1998).

After a collaborative study with Carette and others (2010), numerous questions were still open about tight junctional development at the BTB. Through the use of the SCCx43KO mouse line it was determined that the BTB was functional. This was tested, e.g., via lanthanum nitrate (transmission electron microscopy) which was incapable of penetrating the barrier to enter the lumen. Nevertheless, these adult mice were still sterile and could not reproduce. Yet the loss of Cx43 in SC caused an increase in tight and adherens protein expressions for N-cadherin, beta-catenin, and occludin, as indicated through semi quantitative WB analysis and IF. However, a decrease in the ZO-1 protein expression was revealed (Carette et al., 2010). What effect does a Cx43 KO have on the developmental process of the BTB during puberty and what are its effects on the expression of tight junctional occludin? The importance of a timely correct (spatiotemporal) occludin expression, initial BTB formation, and initiation of normal spermatogenesis are evidently linked with one another.

In this study, through the use of IHC, semi quantitative WB analysis, and real-time-quantitative-PCR (qRT-PCR) we analyzed the expression of the tight junction protein occludin with the presence (WT) or absence (KO) of the gap junction protein Cx43 in the testes of mice from juvenile to adult. For this purpose, the established transgenic mouse line SCCx43KO (Brehm et al., 2007; Sridharan et al., 2007) was used and both KO and WT littermate males were compared.

Materials and methods

Tissue preparation for descriptive methods

Animal experiments were approved by the animal rights committees at the regional commission of Giessen, Germany (decision V54-19c 20/15 c GI 18/1) and the regional commission of Hannover, Germany (decision 33.9-425-05-11A120). Full details of the breeding strategy, PCR genotyping, and confirmation of Cx43 gene loss by *Glb1* (beta-galactosidase) IHC are described elsewhere (Brehm et al., 2007). Briefly, genotyped SCCx43KO$^{-/-}$ (KO) and WT mice from various ages (days 2-23 p.p. and adult mice, >60 days p.p.) were anesthetized with an intraperitoneal injection of a high dose cocktail of ketamine hydrochloride (Medistar, Holzwickede, Germany) and xylazine (Serumwerk Bernburg, Bernburg, Germany). From each mouse, the right testis was fixed in liquid nitrogen (see section: RNA extraction, cDNA synthesis, qRT-PCR, Statistical Analysis; Protein extraction and occludin WB analysis) and the left testis was fixed in Bouin's solution for 24 h and transferred to 70% ethanol. Testes were then dissected and embedded in paraffin wax (see sections: IHC for beta-galactosidase (Confirmation of loss of the floxed Cx43 gene); IHC for Cx43 and occludin) using standard techniques. Five-micrometer sections were stained with hematoxylin-eosin (HE) and evaluated according to the methods described by Russell et al. (1990). For comparative analysis, littermates were chosen. Sections were always processed simultaneously.

RNA extraction, cDNA synthesis, qRT-PCR, statistical Analysis

Total RNA was extracted from testes of six adult mice (n=3 WT and n=3 KO) with TRIzol® reagent (Life Technologies, Karlsruhe, Germany, 10296-010), according to the manufacturer's protocol. First, RNA

was isolated and incubated with 1.5 U/μg RNase-free DNase I (Roche, Mannheim, Germany, 04716728001) for 40 min at 37°C. Then, the strands of cDNA were synthesized via the Superscript II Reverse Transcriptase, according to the manufacturer's protocol (Gibco BRL, Eggenstein, Germany, 18064-014). The resulting cDNA samples were then forwarded to qRT-PCR. The PCR reaction set up (final volume: 20 μl) contained 7.8 μl sterile water, 10 μl iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA, 170-8880), 0.6 μl 10 pmol forward primer, 0.6 μl 10 pmol reverse primer and 1 μl cDNA. Primers (Table 1) were purchased from MWG-Biotech (Ebersberg, Germany). Reactions were run with the CFX96™ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA, 185-5196) under the following cycling conditions: 1 x 95°C 3 min, 40 x [95°C 15 s, 55°C 30 s and 72°C 30 s], and 72°C 7 min. Heat shock protein 90aa1 (*Hsp90aa1*) (Giese et al., 2012) and beta-actin (*Actb*) were used as housekeeping genes. All experiments included controls lacking the reverse transcriptase-enzyme to check for contamination with genomic DNA as well as "no template controls" with sterile water instead of cDNA to check for cross-contamination. Both controls were negative. Due to the non-selective dsDNA binding of the SYBR® Green dye, melting curve analysis and gel electrophoresis were performed to confirm the exclusive amplification of the expected PCR products. Sequencing of the PCR-products was performed by the Qiagen Sequencing Service (Hilden, Germany). Before final calculation of the normalized gene expression was done, the assessment of the exact amplification efficiencies of target and reference genes were carried out using the standard curve method with different dilution steps (undiluted, 1:10, 1:100, 1:1000 and 1:10000). Amplification was carried out as follows: initial template denaturation and activation of the iTaq DNA polymerase (Life Technologies, Karlsruhe, Germany, 10296-010170-8870) for 3 min at 95°C followed by 40 cycles at 95°C for 10 s (denaturation of template) and 60°C for 60 s. The specificity of each PCR reaction was assessed by performing a melting curve analysis.

The data was then calculated in Microsoft Excel 2003 (Microsoft, Redmond, USA) using Pfaffl's formula (Pfaffl, 2001). The delta-delta-C_t values were statistically analyzed via a One-way ANOVA test using the software SPSS version 15.0 (IBM SPSS Statistics, Ehningen, Germany). A p-value of <0.05 was defined as significant with $*p<0.05$ and $**p<0.01$.

Protein extraction and occludin WB analysis

For WB analysis, frozen testicular tissues of forty-two mice were used. Mice ages (in days p.p.): 8 (n=2 mice/genotype), 10 (n=2 mice/genotype), 12 (n=5 mice/genotype), 14 (n=2 mice/genotype), 15 (n=5 mice/genotype), and adult (n=5 mice/genotype) (>60 days p.p.). Protein extraction was carried out using the TRIzol® reagent as recommended by the manufacturers (Life Technologies, Karlsruhe, Germany, 10296-010). Protein concentrations were determined in triplicate via the Bio-Rad DC™ Protein Assay (Life Technologies, Karlsruhe, Germany, 500-0111) according to the manufacturer's protocol. The same amount of protein homogenate (20 μg/30 μl) was loaded per lane. The proteins were fractionated on a 12% polyacrylamide gel at 150 V for 150 min in SDS running buffer (1% SDS, 25 mM Tris, 200 mM glycine). Protein molecular weight was determined via the protein marker PageRuler™ Prestained Protein Ladder 10-170 kDa (Fermentas, St. Leon-Rot, Germany, SM0671). Then, proteins were blotted for 1 h at 1 A/cm^2 (PeqLab, Erlangen, Germany, 52-2020) onto a Protran BA 85 nitrocellulose membrane (Whatman, Dassel, Germany, 10401197), using a blotting buffer (20% methanol, 25 mM Tris, 200 mM glycine, pH 8.3). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline and tween 20 (TBST; pH 7.4) for 45 min at room temperature (RT) and incubated with a polyclonal rabbit anti occludin primary antibody (Invitrogen, Darmstadt, Germany, 71-1500, Table 2) in TBST, over night at 4°C. For the negative control, WB were carried out by omitting the primary antibody. The secondary goat anti-rabbit IgG-HRP antibody (Santa Cruz, Heidelberg, Germany, sc-2004, Table 2) diluted in TBST was applied for 45 min at RT. Then, the membrane was treated with the SuperSignal® West Dura Kit (Thermo Scientific, Schwerte, Germany, 37071) according to the manufacturer's protocol. The membrane was finally photographed on a Bio 1D, Vilber (Lourmat, Eberhardzell, Germany). The occludin protein is detectable at ~65 kDa.

For days 12 p.p., 15 p.p. and adult mice (n=3 mice/genotype and age) a semi quantitative analysis of occludin relative to the housekeeper (loading control) alpha tubulin was performed. To detect the loading control alpha tubulin, the same (occludin) membrane was stripped. First the membrane was washed twice for 5 min in TBS and then an additional two times for 30 min in stripping buffer (0.2 M glycine, 0.05% tween 20,

Table 1. The forward and reverse primer sequences for qRT-PCR.

Gene	Direction	Primer Sequences
Actb	Forward	TTCCTTCTTGGGCATGGAGT
	Reverse	TACAGGTCTTTGCGGATGTC
Cldn11	Forward	CGTCATGGCCACTGGTCTCT
	Reverse	GGCTCTACAAGCCTGCACGTA
Gja1	Forward	ACAGCGGTTGAGTCAGCTTG
	Reverse	GAGAGATGGGGAAGGACTTGT
Hsp90aa1	Forward	AAGAGAGCAAGGCAAAGTTTGAG
	Reverse	TGGTCACAATGCAGCAAGGT
Cdh2	Forward	TGGCAATCAAGTGGAGAACC
	Reverse	ATCCGCATCAATGGCAGTG
Ocln	Forward	ATCCTGTCTATGCTCATTATTGTG
	Reverse	CTGCTCTTGGGTCTGTATATCC
Tjp1	Forward	CCCTACCAACCTCGGCCTT
	Reverse	AACGCTGGAAATAACCTCGTTC

1% SDS pH 2.0). Afterwards, it was washed four times at 5 min in TBST and then blocked in 5% non-fat dry milk dissolved in TBST. The blot was incubated with the monoclonal rabbit anti alpha tubulin primary antibody (New England Biolabs GmbH, Frankfurt am Main, Germany, 2125, Table 2) diluted in TBST overnight at 4°C. The following day the secondary goat anti-rabbit IgG-HRP antibody (Santa Cruz, Heidelberg, Germany, sc-2004, Table 2) diluted in TBST was applied for 45 min at RT. The detection of the ~52 kDa alpha tubulin protein on the membrane occurred as previously described for occludin.

The semi quantitative comparison of occludin with alpha tubulin was analyzed using the BIO-1D, Vilber software (Lourmat, Eberhardzell, Germany). Ages 12, 15 days p.p., and adult littermates were blotted, and each mouse pair was detected three times on separate blots. The statistical analysis was performed using the One-way ANOVA with the software SPSS version 15.0 (IBM SPSS Statistics, Ehningen, Germany). A p-value of <0.05 was defined as significant with $*p<0.05$ and $**p<0.01$.

IHC for beta-galactosidase (Confirmation of loss of the floxed Cx43 gene)

Beta-galactosidase IHC was performed on all testicular sections of SCCx43KO and WT mice to confirm the efficiency of the SC specific deletion of the Cx43 gene, as described in Brehm et al. (2007). Briefly, sections were treated, after deparaffinization and rehydration, with 3% H_2O_2 and blocked with 3% bovine serum albumin (BSA) for 20 min each, and incubated with the polyclonal anti-beta-galactosidase antibody (Abcam, Cambridge, United Kingdom, ab616, Table 2) overnight. Sections were then exposed to the EnVision™ +Kits HRP Rabbit DAB+ (Dako, Hamburg, Germany, K4011, Table 2) for 30 min, and visualized using the DAB included in the kit according to the manufacturer's protocol. Finally, the sections were then dehydrated and mounted with Eukitt® (Sigma-Aldrich, Munich, Germany, 03989) according to the manufacturer's protocol.

IHC for Cx43 and occludin

IHC stainings for Cx43 (n=2 mice/genotype and age) and occludin (n=2 mice/genotype and age) were performed on all mice (mice age pairs were all from the same litter) and on consecutive sections, with minor changes as described previously (Brehm et al., 2007). Briefly, sections were microwave treated for 3 times 5 min at 600 W in sodium citrate buffer (pH 6.0), blocked with 3% BSA for 30 min and incubated with the respective primary antibodies: polyclonal anti-rabbit Cx43 (Cell Signaling Technology, Frankfurt am Main, Germany, 3512, Table 2) and polyclonal anti-rabbit occludin (Invitrogen, Darmstadt, Germany, 71-1500, Table 2) overnight at 4°C.

The Cx43 sections were then exposed for 30 min at RT to the biotinylated secondary antibody goat anti-rabbit IgG (BioLogo, Kronshagen, Germany, ZU101, Table 2) diluted in PBS with 1% BSA. Sections were treated with Vectastain Elite ABC Kit Standard (Vector Laboratories, Peterborough, United Kingdom, PK-6100, Table 2) for 30 min at RT and immunoreaction was visualized using Peroxidase Substrate Kit AEC (Biologo, Kronshagen, Germany, AE002) solution as recommended by the manufacturer. Following each incubation, sections were washed thoroughly with 0.1 M Tris-HCl buffer, pH 7.4. Slices were counterstained with hematoxylin for 10 s and rinsed with running water. Finally, sections were mounted with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany, 109242).

The occludin sections were then exposed for 30 min at RT with EnVision™ +Kits HRP Rabbit DAB+ (Dako, Hamburg, Germany, K4011, Table 2). The sections were treated with the Peroxidase Substrate Kit AEC (Biologo, Kronshagen, Germany, AE002,) solution as recommended by the manufacturer. For better visualization of occludin localization, slices were not counterstained. Finally, all sections were mounted with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany, 109242).

A negative control was performed by substitution of the primary antibody with buffer, and all controls were negative. Additionally, an isotype control was performed by substituting the primary antibody with a polyclonal anti-rabbit IgG antibody (Sigma-Aldrich, Munich, Germany, R5506) and the respective primary antibody dilutions. All control sections were negative throughout. IHC for Cx43 and occludin was repeated at least twice per mouse.

Results

qRT-PCR of adult mice

Based on the data from Carette and others (2010) who demonstrated an upregulation of *Ocln* in adult

Table 2. The dilutions of the antibodies for IHC and WB.

Target Protein	Specificity	Mono/Poly	Method	Dilutions 1st AB	Dilutions 2nd AB
Alpha tubulin	Mouse	Monoclonal	WB	1:1000	1:5000
Beta-galactosidase	E. coli	Polyclonal	IHC	1:5000	Ready to use
Cx43	Mouse	Polyclonal	IHC	1:100	1:200
Occludin	Mouse	Polyclonal	IHC	1:300	Ready to use
			WB	1:250	1:5000

SCCx43KO mice compared to age-matched WT littermates, our results indicate alterations in *Cldn11*, *Gja1*, and *Ocln*. qRT-PCR analysis (Fig. 1) revealed a highly significant ($p<0.01$) increase in mRNA-expression of *Cldn11* and *Ocln* in KO mice in comparison to their WT littermates. Nevertheless, the tight junction associated *Tjp1* and the adherens junctional *Cdh2* did not alter in mRNA expression. As was expected, the SCCx43KO mice showed a highly significant ($p<0.01$) decrease in mRNA expression of *Gja1*. This served as an additional control for the successful KO.

Beta-galactosidase IHC (confirmation of KO)

As previously established by Brehm et al. (2007), and as an additional control of the KO, each mouse was tested via beta-galactosidase expression. Age independent, the immunostaining for beta-galactosidase indicates that only SC in SCCx43KO mice expressed this protein in the nucleus (Fig. 2B), while the WT SC showed no such expression (Fig. 2A).

Cx43 IHC

As seen by Bravo-Moreno and others (2001), Cx43 is first located in the cytoplasm of the SC of new born mice, Cx43 expression strengthens at day 7 p.p. and begins initial localization towards the BTB region (basal third of the SC). These results are similar to those of Fig. 3. It is evident that no Cx43 is detectable at any age point in the SC of SCCx43KO mice, whereas the SC of the WT mice show the typical Cx43 expression pattern. At ages 2 and 7 (Fig. 3A,C), Cx43 is present in the cytoplasm of SC in seminiferous cords. Fig. 3: images E and G, ages 8 and 10 show a weakened cytoplasmic Cx43 immunolocalization in comparison to the younger mice, yet some Cx43 signaling is detectable at the BTB area. Images I (Fig. 3), A (Fig. 4), and C (Fig. 4), ages 12, 14, and 15 demonstrate a predominant strengthening of Cx43 immunolocalization towards the basal third of the SC, whose nuclei begin to migrate towards the outer edge of the cord. Fig. 4E,G,I, ages 17, 23, and adult reveal tubule and lumen formation, and the localization of Cx43 has reached its definite BTB position between SC and between SC and GC. Furthermore, Cx43 is

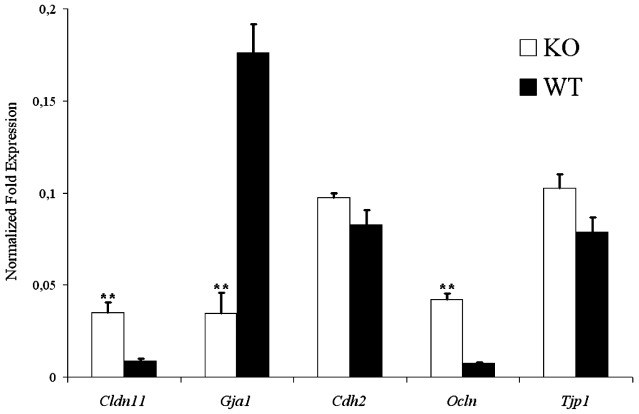

Fig. 1. A graph of the analyzed qRT-PCR between adult WT (n=3) and KO (n=3). Each mouse qRT-PCR was performed in triplicate. The delta-delta-C_t data was calculated using the Pfaffl's formula (Pfaffl, 2001) and was normalized to the housekeeper genes *Actb* and *Hsp90aa1*. The genes *Cldn11* and *Ocln* indicate a highly significantly increased expression in the KO mice in comparison to the WT littermates. As expected, the gene *Gja1* depicts a highly significant decrease in the KO mice in comparison to the WT littermates. No significant changes in the genes *Cdh2* and *Tpj1* could be determined. (**p<0.01).

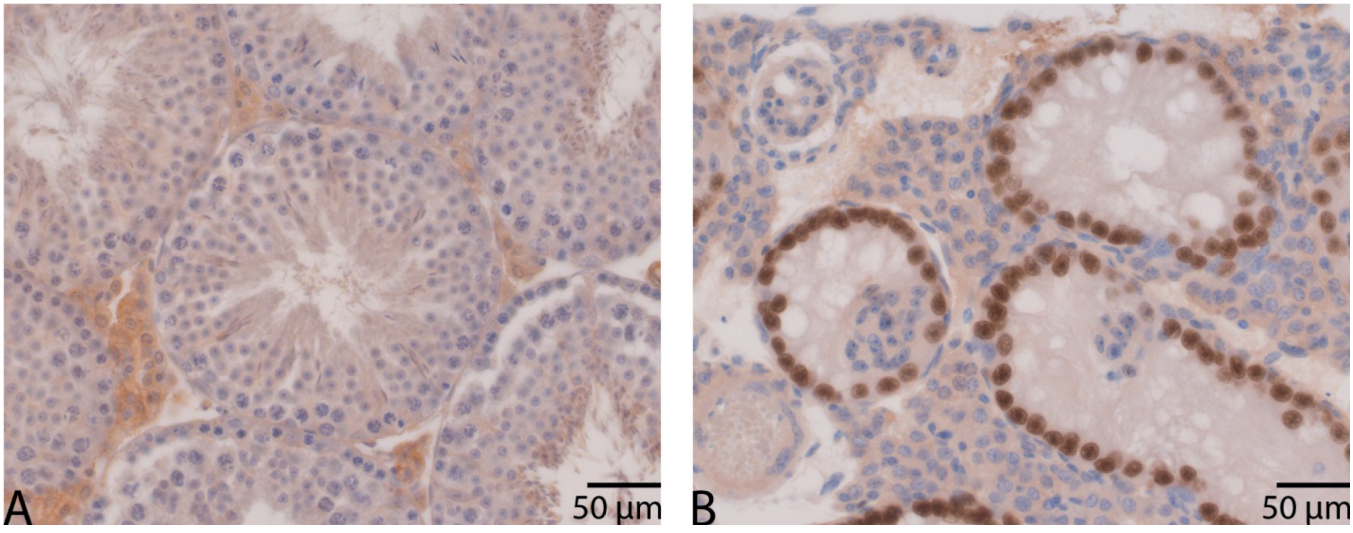

Fig. 2. Beta-galactosidase IHC staining (brown) of adult WT and KO mice tissue sections. The WT **(A)** genotype indicates no nuclear staining of the SC, while the KO **(B)** depicts an evident brown immunostaining restricted to SC nuclei. This serves as an additional confirmation of the successful Cx43 KO.

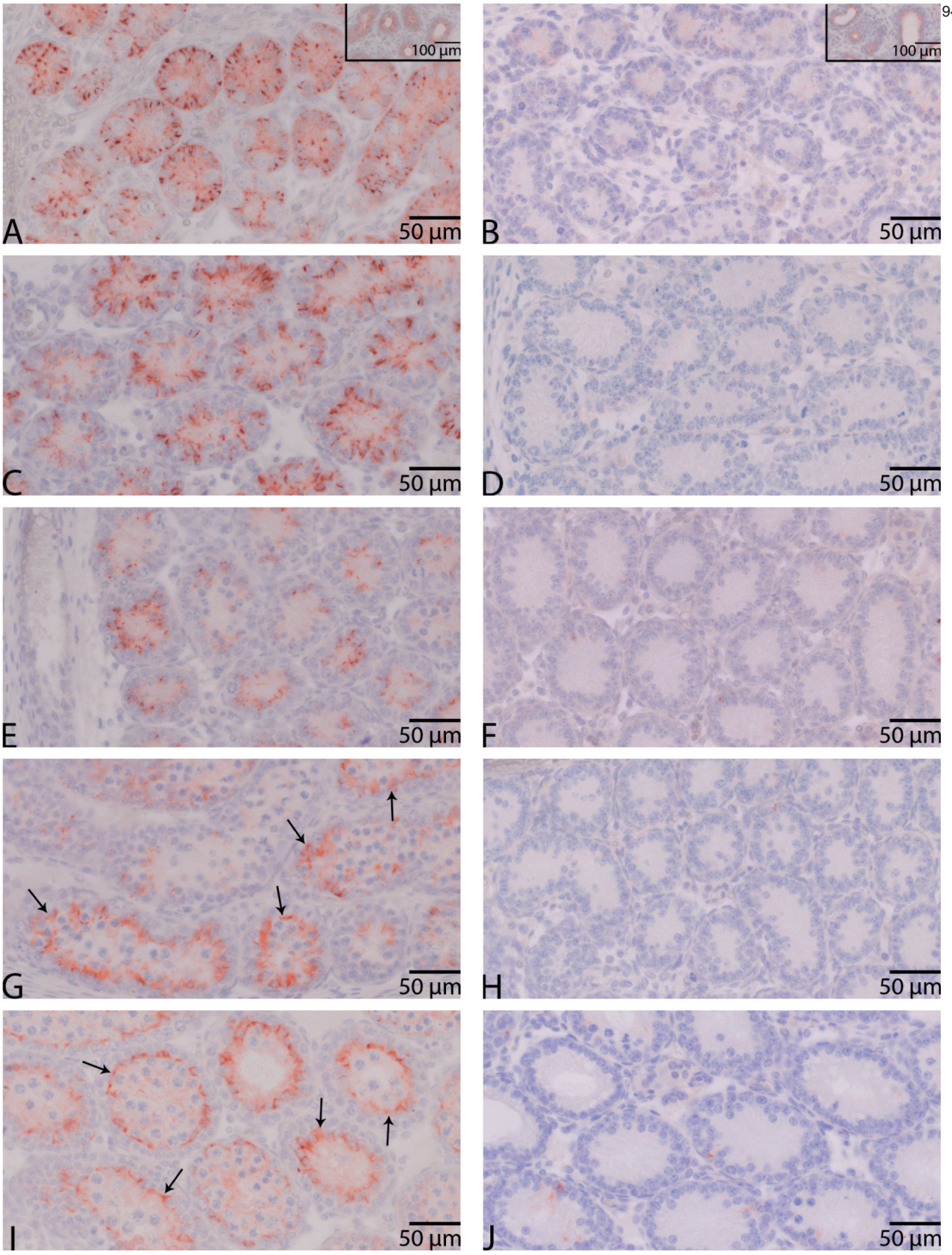

Fig. 3. IHC of testicular Cx43 (red) from WT (**A, C, E, G, I)** and KO (**B, D, F, H, J**,) mice (days 2-12 p.p.). The postnatal development in days: age 2 (**A, B**); age 7 (**C, D**); age 8 (**E, F**); age 10 (**G, H**); age 12 (**I, J**). All sections were counterstained with hematoxylin. The epididymis from the same tissue sections as from images **A** and **B** were used as a positive control for the detection of Cx43 (**inset in images A, B**) and shows the characteristic epididymal Cx43 staining pattern. Within the seminiferous cords/tubules of SCCx43KO mice no Cx43 immunostaining is detectable at all. Images **A** and **C**: In seminiferous cords of WT mice a cytoplasmic immunolocalization for Cx43 in SC is evident. Images **E** and **G**: Seminiferous cords show a weakened cytoplasmic Cx43 immunoreactivity and an initial shift of Cx43 towards the BTB (**G**, arrows). Image **I**: In seminiferous cords/tubules of WT mice Cx43 is now mainly localized at the BTB region (arrows).

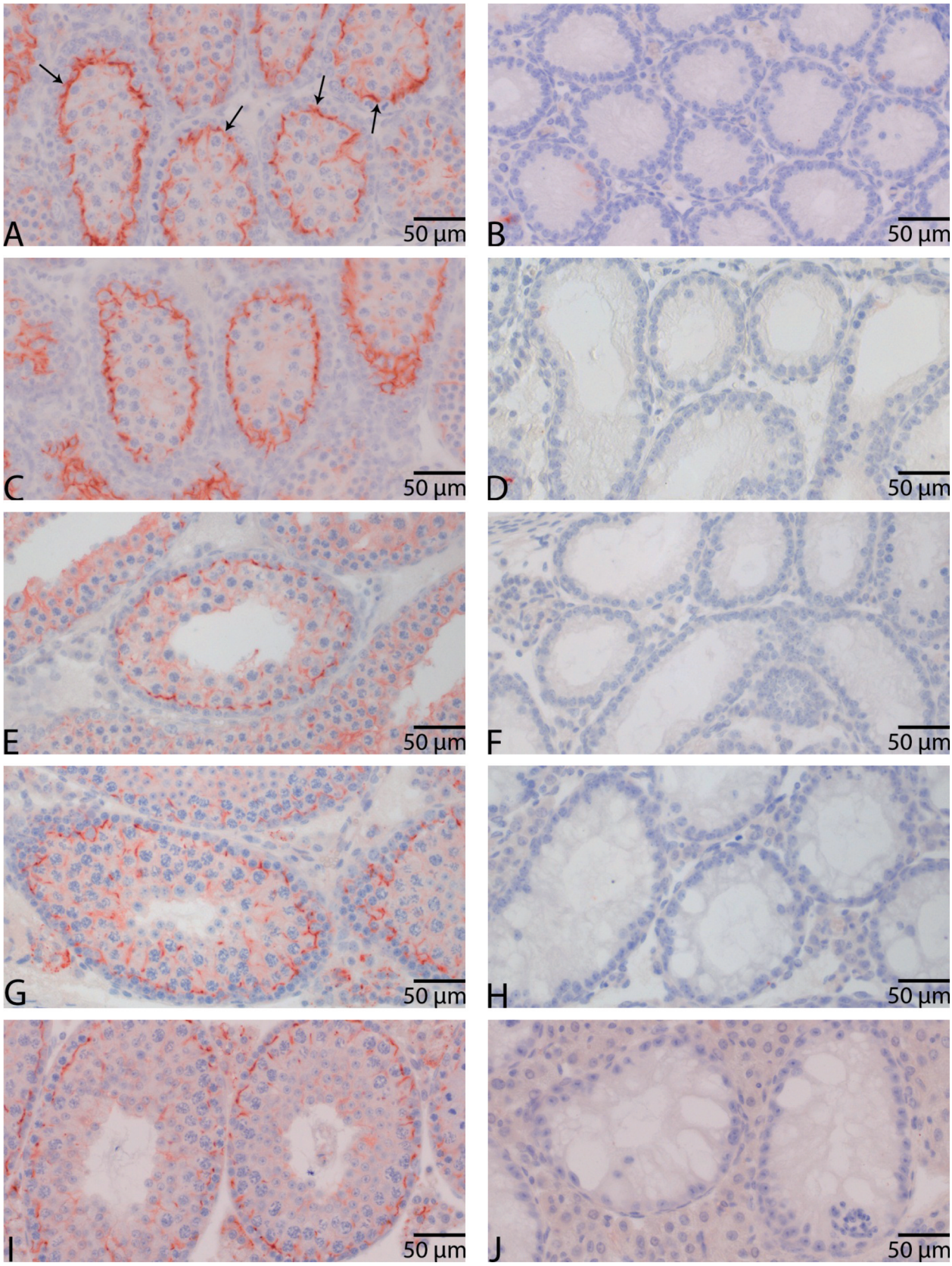

Fig. 4. IHC of testicular Cx43 (red) from WT (**A, C, E, G, I)** and KO **(B, D, F, H, J**) mice (days 14 p.p.-adult). The postnatal development in days: age 14 (**A, B)**; age 15 (**C, D**); age 17 (**E, F**); age 23 (**G, H**); adult (**I, J**). All sections were counterstained with hematoxylin. Within the seminiferous cords/tubules of SCCx43KO mice no Cx43 immunostaining is detectable at all. Images **A** and **C**: In seminiferous cords/tubules of WT mice Cx43 is mainly localized at the BTB region (**A**, arrows). Images **E, G,** and **I**: Typical intratubular Cx43 immunoreactivity. Cx43 is predominantly localized at the basal third of the seminiferous epithelium. In addition, Cx43 can also be seen adluminally in SC-GC contacts.

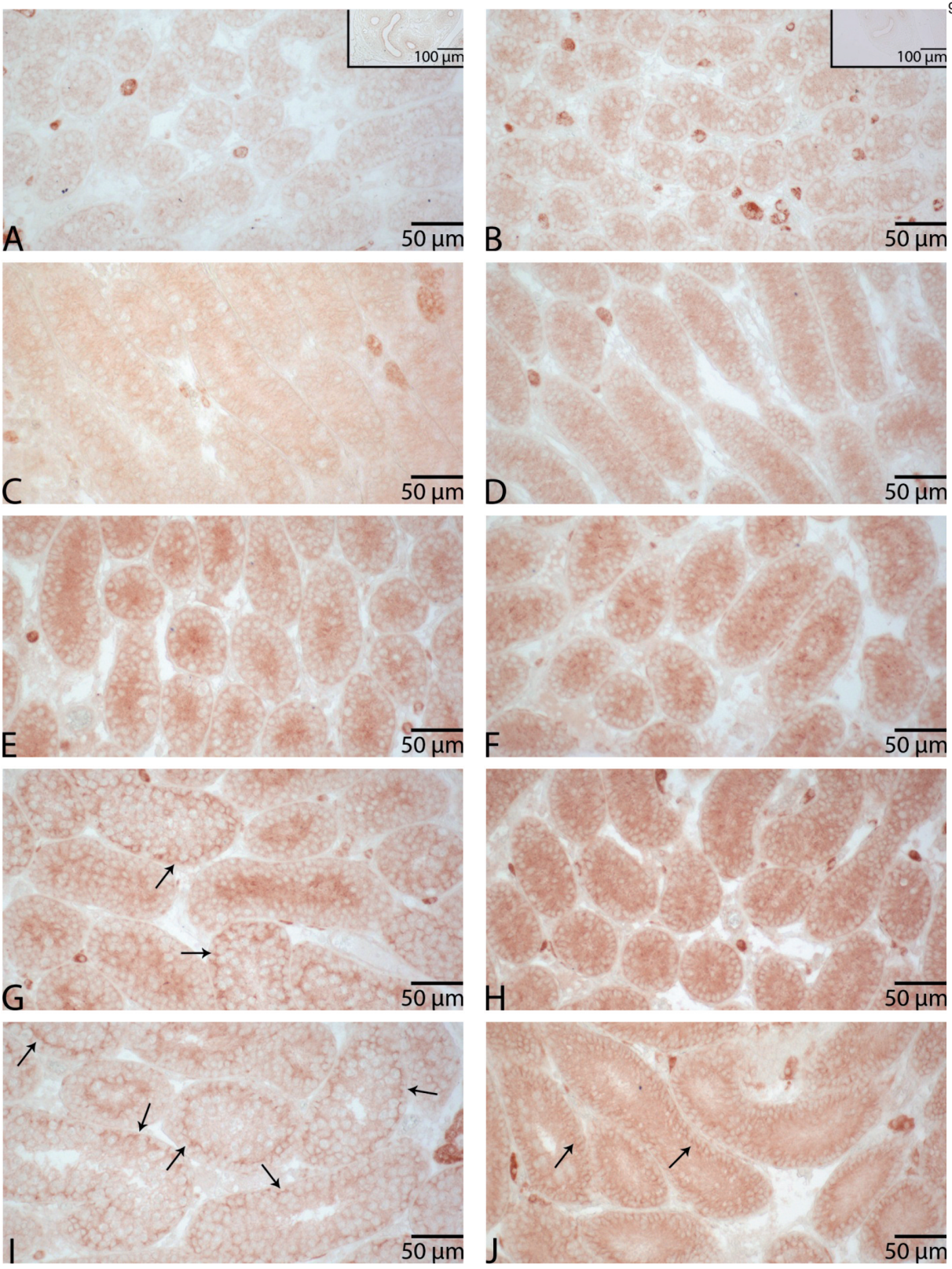

Fig. 5. IHC of testicular occludin (brown) from WT (**A, C, E, G, I**) and KO (**B, D, F, H, J**) mice (days 2-11 p.p.). The postnatal development in days: age 2 (**A, B**); age 7 (**C, D**); age 8 (**E, F**); age 10 (**G, H**); age 11 (**I, J).** The epididymis from the same tissue sections as from images **A** and **B** was used as a positive control for the detection of occludin (**inset in images A, B**) and shows the characteristic epididymal occludin staining pattern. Images **A, B, C, and D**: Seminiferous cords of WT and KO mice show a weak and diffuse cytoplasmic occludin immunoreaction. Images **E and F**: Occludin immunoreactivity seems stronger but is still cytoplasmic and diffuse. Image **G**: An initial shift of occludin to the basal third of SC is visible in seminiferous cords of WT mice (arrows). Image **H**: Seminiferous cords of SCCx43KO mice still exhibit a diffuse and cytoplasmic Cx43 immunolocalization. Image **I**: Initial seminiferous tubule formation. In almost all WT seminiferous cords/tubules occludin is localized at the BTB region (arrows). Image **J**: Initial seminiferous tubule formation. Only single cords/tubules show an occludin immunoreactivity at the BTB region in SCCx43KO mice (arrows).

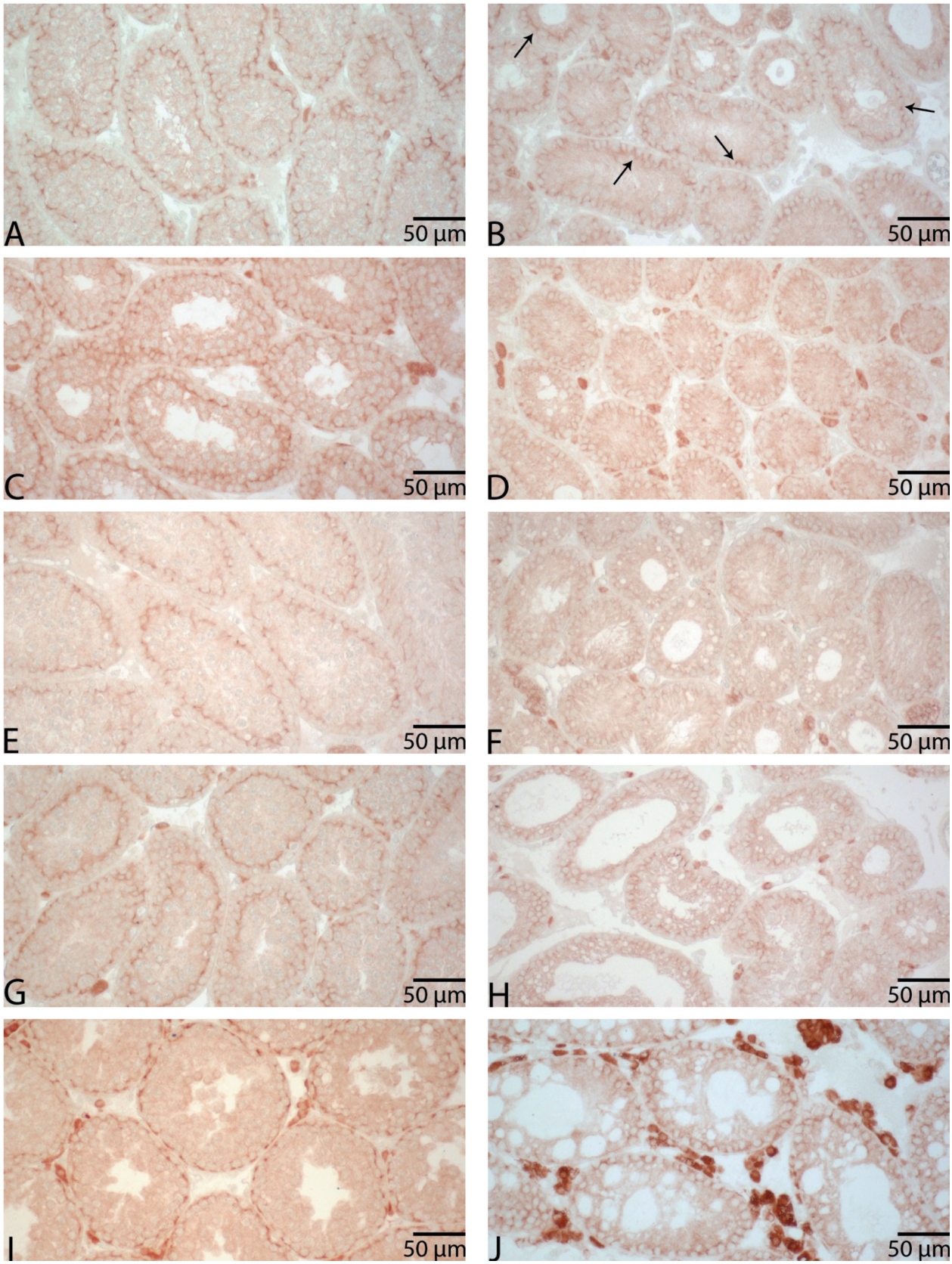

Fig. 6. IHC of testicular occludin (brown) from WT (**A, C, E, G, I)** and KO (**B, D, F, H, J**) mice (days 12-23 p.p.). The postnatal development in days: age 12 (**A, B**); age 13 (**C, D**); age 14 (**E, F**); age 15 (**G, H**); age 23 (**I, J**). Image **A:** All seminiferous tubules show an evident occludin localization to the BTB. Image **B**: Increased number of tubules showing occludin immunoreactivity at the BTB region compared to Fig. 5 image **J** (arrows). Images **C-J**: Seminiferous tubules with the typical wavy like occludin immunoreactivity in WT and KO mice.

occasionally found adluminally in SC, as here the gap junction formation between SC and GC (e.g. spermatocytes) additionally takes place.

Occludin IHC

Cyr and others (1999) describe the localization of occludin to the cytoplasm of the cells at days 5 and 7 p.p., while at day 14 and 23 the protein had localized to the BTB. Similar results were achieved in this study as seen in Figs. 5-7. Between days 2 and 8 (Fig. 5A-F) there appears to be no specific (functional) localization of this tight junction protein in the seminiferous cords of either the KO or WT. At age 10 in some of the cords of the WT mice (Fig. 5G) an initial shift of occludin localization to the BTB can be seen, while in the KO (Fig. 5H), occludin distribution still remains diffuse. One day later, the majority of seminiferous cords/tubules in WT mice displays a distinct wavy like band of occludin immunostaining at the BTB region (Fig. 5I), whereas only single cords in the KO mice show this occludin distribution pattern (Fig. 5J). Here it is evident that the KO occludin expression is delayed by at least 1 day (from day 10 to 11) in comparison to the WT littermates. At age 11 (Fig. 5I,J), the seminiferous cords begin developing into tubules with the formation of a lumen. In the KO mice, initiation of spermatogenesis is drastically altered. After day 12, the WT (Fig. 6A,C,E,G,I, Fig. 7A) and the KO (Figs. 6B,D,F,H,J, 7B) mice exhibit a comparable tubular morphology and occludin can be mainly localized to the BTB, yet the seminiferous tubules of the KO mice tend to be smaller in diameter.

Occludin WB

Fig. 8 image A depicts a representative qualitative occludin WB from WT and KO mice (n=2 mice/genotype and age). Here various juvenile ages (day 8, 10, 12, 14, and 15 p.p.), around the time of BTB formation, are analyzed with a focus on the pubertal time range, as well as an adult comparison. A specific band for occludin can be detected in all samples at ~65 kDa. At day 8 and 10 there appear to be no obvious differences between occludin protein expression. However starting at the ages of 12, 14, and 15 the WB tends to indicate a stronger expression in the KO mice. In adult KO mice, a much stronger band for occludin can be seen. To further analyze this visual difference, semi quantitative WB (Fig. 8B,D) were performed for ages 12, 15 days p.p., and adults (n=3 mice/genotype and age). Each mouse was blotted three times and the expression of occludin was quantified according to the housekeeper gene (loading control) alpha tubulin (Fig. 8C,E). It can be seen that occludin is only significantly upregulated in adult KO males (Fig. 8C) whereas the juvenile ages do not show significant differences (Fig. 8E).

Discussion

As it is likely that gap junctions provide a cross-talk between SC and/or SC and GC to coordinate the function and localization of multiple junctions at the complex BTB (Cheng et al., 2010; Mok et al., 2011), the objectives of the present study were to investigate the effects of a SC specific deletion of Cx43 on occludin mRNA levels, occludin protein synthesis, and immunolocalization of occludin protein in the testis during the time period of BTB formation.

Also, it has further been reported that Cx43 and occludin can co-localize within intercellular junctional complexes, with this co-localization being related to the binding of both proteins to cytosolic tight junctional scaffolding proteins such as ZO-1 (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998; Cyr et al., 1999; Kojima et al., 1999; Pelletier, 2011).

In the present study, using qRT-PCR, an alteration of gene expression profiles for *Ocln, Cldn11,* and *Gja1* between adult WT and SCCx43KO mice was discovered. In particular, mRNA of tight junction integral membrane proteins occludin and claudin-11 were significantly upregulated in the KO mice, while Cx43 mRNA was downregulated due to the transgenic

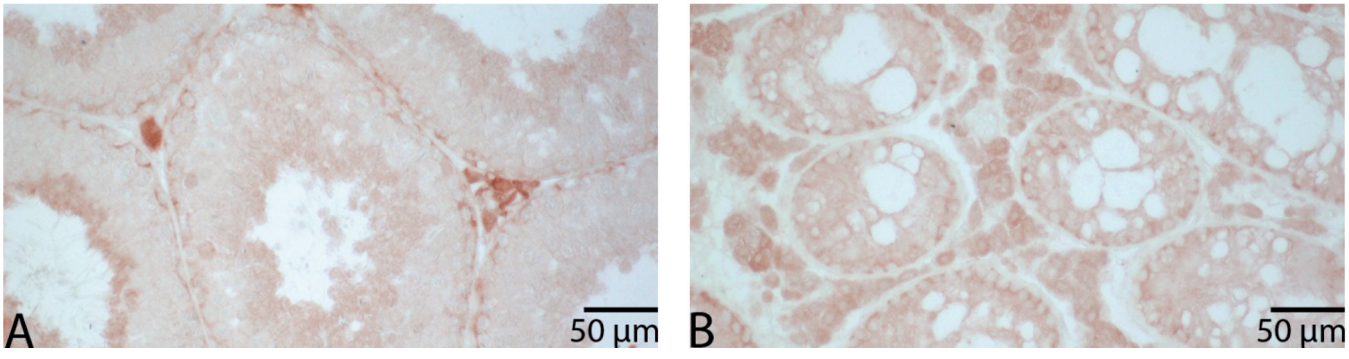

Fig. 7. IHC of testicular occludin (brown) from adult WT **(A)** and KO **(B)** mice. Images **A** and **B**: Characteristic occludin distribution pattern in adult mice.

Cre/loxP-system. In this context, claudin-11 mRNA has been found to be upregulated in human seminiferous tubules with testicular carcinoma *in situ*, while Cx43 was transcriptionally downregulated (Brehm et al., 2002, 2006; Fink et al., 2009). The present study further revealed that the specific deletion of Cx43 in SC did not result in significant changes of *Cdh2* (N-Cadherin) and *Tjp1* (ZO-1) mRNA levels in adult mice.

The comparative immunohistochemical time study for Cx43 and occludin focused on the peripubertal distribution pattern of these two proteins. As expected, Cx43 was only expressed in the WT mice and not in the seminiferous epithelium of the KO animals. Similar to the results from Bravo-Moreno et al. (2001), Cx43 protein was expressed throughout the cytoplasm at days 2 and 7 p.p., and not until days 8 and 10 does Cx43 begin to localize towards the basal third of the SC. BTB localization of Cx43 strengthens at ages 12, 14, and 15, which coincides with the initiation of a functional BTB assembly, tubular lumen formation, and SC polarization (Jégou, 1993). First, small intratubular cell clusters could be seen in KO mice starting from day 12 p.p. in agreement with results from Weider et al. (2011b). By days 17 and 23, Cx43 in the seminiferous tubules forms

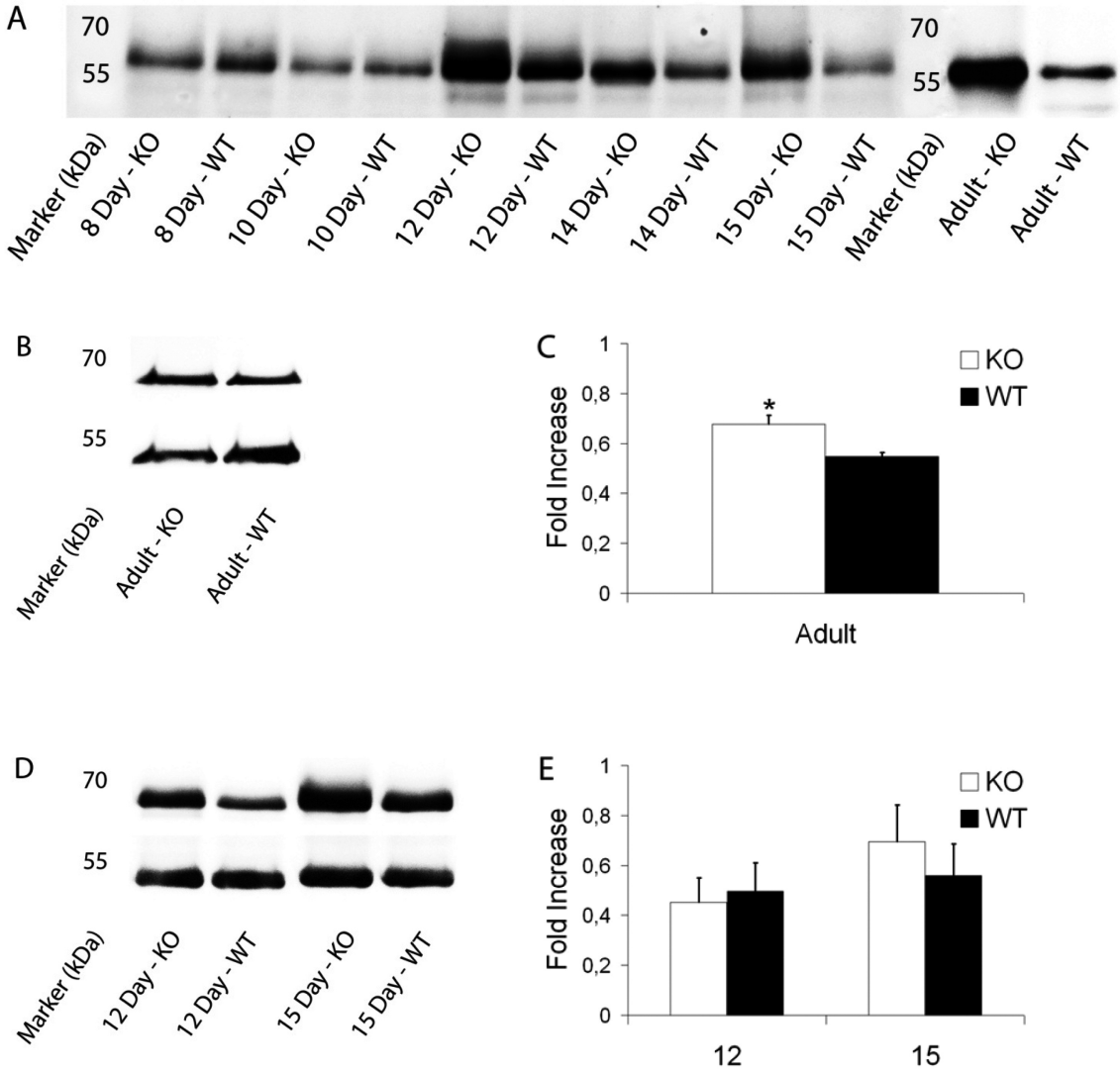

Fig. 8. A. Representative qualitative WB depicting occludin (~65 kDa) from days 8, 10, 12, 14, and 15 p.p. SCCx43KO and WT mice (n=2 mice/genotype and age). **B.** Representative semi quantitative WB showing occludin and alpha tubulin (~52 kDa) from adult KO and WT mice (n=3 mice/genotype and age). **C.** Statistical analysis from WB with adult mice using alpha tubulin as a loading control. Significance between KO and WT was determined (*p<0.05). **D.** Representative semi quantitative WB showing occludin and alpha tubulin from postnatal days 12 and 15 KO and WT mice (n=3 mice/genotype and age). **E.** Statistical analysis from WB of 12 and 15 day old mice using alpha tubulin as a loading control.

a wavy-like localization at the BTB region, and in adult mice a stage-specific expression of Cx43 is detectable as already demonstrated in previous studies (Risley et al., 1992; Batias et al., 1999). Looking at the distribution pattern of occludin within the seminiferous epithelium and similar to the findings of Moroi et al. (1998) and Cyr et al. (1999) in WT mice, one notices first a diffuse cytoplasmic expression in SC of WT and KO cords at days 2 and 7 p.p.. At day 8 p.p., a more intense occludin immunoreaction is visible throughout the cytoplasm of SC in both WT and KO. Thus, expression of occludin at day 2, 7, and 8 p.p. seems not to be obviously affected by the absence of Cx43. In WT cords of 10 day old mice and concomitant with initial barrier formation, occludin localization begins to shift towards the BTB region. However, SC in 10 day old KO mice still exhibit a similar diffuse cytoplasmic expression pattern as at day 8. Yet by day 11 p.p., SC in the KO cords show a move of occludin immunostaining towards the BTB in single cords, which is delayed by at least 1 day in comparison to the respective WT littermates. At day 11 almost all of the WT cords show the typical basal localization of occludin. This localization is then comparable to that of day 12 in the KO mice. At around day 12, initial lumen formation begins in both WT and KO mice. Interestingly, Kluin and others (1984) determined that SC proliferation terminates at the age of 12, thus the localization of occludin (and Cx43) to the BTB region could be a possible signaling factor for SC to become post mitotic. The localization of occludin, at day 12 and 13 coincides with the development and formation of the BTB as stated by Nagano and Suzuki (1976), Hellani et al. (2000), Cheng and Mruk (2002), and Hosoi et al. (2002). Cyr et al. (1999) stated slightly different results. In their study using IF, they described that the discontinuous expression of occludin within SC ceased at the age of 14 p.p. (Cyr et al., 1999). These differences might be explained by the fact that these authors' time study jumps from day 7 to 14, while the present study analyzed the postnatal days 7, 8 and 10-15. Interestingly, St-Pierre and co-workers (2003) showed that an altered localization of Cx43 protein during puberty resulted in a delay of the "normal basal occludin shift" in rat SC which was accompanied by an extended cytoplasmic distribution of occludin.

Initial qualitative WB results indicated an increase of occludin protein at postnatal ages 12, 14 and 15, and in adult KO mice. To further investigate these results testicular proteins from ages 12 and 15, and from adult mice were compared using semi quantitative WB with respect to alpha tubulin as a loading control. These additional WB revealed that occludin protein was only significantly upregulated in adult KO mice. Similar WB data was obtained using a rat SC line (subjected to Cx43-siRNA) and adult SCCx43KO mice demonstrating an increase of occludin when Cx43 was lost/absent (Carette et al., 2010).

Comparing occludin protein from the juvenile mice, no significant difference could be detected. However, the KO mice exhibited a greater increase of occludin protein from day 12 to day 15 than their WT littermates. This might be an interesting result as data from a microarray study (Shima et al., 2004) and data obtained from the "Mammalian Reproductive Genetics Database" (mrgd.org) demonstrated that normally a dramatic decrease of Ocln expression occurs in WT mice after day 10 p.p.. Thus it could be possible that SC in SCCx43KO mice are not able to regulate (decrease) their testicular occludin expression. This misregulation may then result in the significant difference of occludin protein found when comparing adult mutants with their WT littermates (Carette et al., 2010; this study).

Our IHC and WB data are supported by a previous study (Li et al., 2010). There it has been demonstrated that Cx43 is crucial for tight junction re-assembly at the BTB during its normal cyclic restructuring (Li et al., 2010), so loss of Cx43 in SC might lead to an accumulation of occludin protein in the cytoplasm of SC and/or the delay in its specific shift to the basal compartment of the seminiferous epithelium. Li and co-workers also showed in vitro that in SC with a Cx43 knockdown fewer junction proteins were found at the cell-cell interface, illustrating a disruption in the kinetics of junction re-assembly. They finally concluded that this specific gap junction protein is necessary to maintain the "dynamic" aspect of the BTB but not its "static" function (Li et al., 2010).

Within the life cycle of a somatic SC, puberty represents the most important time period as this cell type develops from an immature into a mature, morphologic and functional adult SC supporting spermatogenesis (Sharpe et al., 2003). In this context, the observed spatio-temporal alteration of occludin expression in KO mice might be interpreted as a sign for an impaired SC maturation process and/or an abnormal intermediate SC phenotype as supposed by previous studies (Sridharan et al., 2007; Weider et al., 2011b). As it is well known that (1) functional SC are a prerequisite for normal spermatogenesis and that (2) there exists a reciprocal regulation of SC and GC maturation (Sharpe et al., 2003; Griswold, 1995), it is possible that the failure to initiate spermatogenesis in SCCx43KO mice around days 8-12 could have been caused by this altered (and asynchronous to GC) state of SC maturation. It was further speculated that GC (preleptotene and leptotene spermatocytes) represent the source of the stimulus that regulates tight junction (occludin) dynamics (Cheng and Mruk, 2002). Consequently, it is possible that the loss of these GC populations in SCCx43KO males could also have led to the observed spatio-temporal alteration and misregulation of occludin expression and to the altered state of SC maturation.

In addition, results from the present and a previous study showed that Cx43 (and occludin) localization is modulated during the prepubertal period (Bravo-Moreno et al., 2001). A change in the Cx43 spatial distribution of SC to their basal domain seems to be correlated in time with the differentiation from a nonpolarized to a

polarized epithelium (Vergouwen et al., 1991; Bravo-Moreno et al., 2001). As these changes occur in parallel with (1) the maturation and first migration of GC within the SC, with (2) functional BTB formation and with (3) the first wave of spermatogenesis the possibility is raised that loss of Cx43 and consequently loss of direct transfer of molecules via Cx43 gap junctions from SC to GC at this developmental time period might have significantly contributed to the observed impaired spermatogenesis in adult KO males.

The KO of Cx43 in SC leads to male infertility but a yet functional BTB in adult SCCx43KO mice (Brehm et al., 2007; Sridharan et al., 2007; Carette et al., 2010; Giese et al., 2012). Comparable to findings by Handel and Eppig (1979) and Weider et al. (2011b), a tubular lumen formation was present from around day 12 p.p. onwards in both normal and mutant mice. Because lumen formation is known to require the presence of functional tight junctions and the resulting vectorial transport across the epithelium towards the lumen (St-Pierre et al., 2003) it may be supposed that the BTB is also functional in the peripubertal SCCx43KO mice. This supposition has to be confirmed in future experiments.

In summary, the results of the present study show for the first time that there is (1) an alteration in the spatio-temporal expression pattern of occludin in SCCx43KO mice in the peripubertal time period, (2) a delayed shift of occludin immunoreaction to the BTB region in SCCx43KO mice between day 10-12 p.p. and (3) a significant increase of occludin and claudin-11 mRNA expression in adult mutant mice. Thus, our data suggest that Cx43 might represent an important regulator for BTB formation (and function).

In hopes of discovering the molecular mechanisms by which Cx43 in SC participates in the complex regulation of BTB assembly and formation, it will be necessary to perform additional experiments to identify transcriptional targets and signaling pathways of Cx43. For example, in a future study it is planned to perform pathway focused microarray gene expression profiles of e.g. day 10-15 old WT and SCCx43KO mice to investigate and compare the transcriptional consequences from the loss of Cx43 in SC. This might help to provide further information regarding alterations of BTB formation as SC junctional proteins attract more and more attention, and come into focus as being an important cause of altered spermatogenesis and impaired male fertility.

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