

Sertoli-cell-specific knockout of connexin 43 leads to multiple alterations in testicular gene expression in prepubertal mice

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SUMMARY

A significant decline in human male reproductive function has been reported for the past 20 years but the molecular mechanisms remain poorly understood. However, recent studies showed that the gap junction protein connexin-43 (CX43; also known as GJA1) might be involved. CX43 is the predominant testicular connexin (CX) in most species, including in humans. Alterations of its expression are associated with different forms of spermatogenic disorders and infertility. Men with impaired spermatogenesis often exhibit a reduction or loss of CX43 expression in germ cells (GCs) and Sertoli cells (SCs). Adult male transgenic mice with a conditional knockout (KO) of the *Gja1* gene [referred to here as connexin-43 (*Cx43*)] in SCs (SCCx43KO) show a comparable testicular phenotype to humans and are infertile. To detect possible signaling pathways and molecular mechanisms leading to the testicular phenotype in adult SCCx43KO mice and to their failure to initiate spermatogenesis, the testicular gene expression of 8-day-old SCCx43KO and wild-type (WT) mice was compared. Microarray analysis revealed that 658 genes were significantly regulated in testes of SCCx43KO mice. Of these genes, 135 were upregulated, whereas 523 genes were downregulated. For selected genes the results of the microarray analysis were confirmed using quantitative real-time PCR and immunostaining. The majority of the downregulated genes are GC-specific and are essential for mitotic and meiotic progression of spermatogenesis, including *Stra8*, *Dazl* and members of the DM (*dsx* and *map-3*) gene family. Other altered genes can be associated with transcription, metabolism, cell migration and cytoskeleton organization. Our data show that deletion of *Cx43* in SCs leads to multiple alterations of gene expression in prepubertal mice and primarily affects GCs. The candidate genes could represent helpful markers for investigators exploring human testicular biopsies from patients showing corresponding spermatogenic deficiencies and for studying the molecular mechanisms of human male sterility.

INTRODUCTION

A significant decline in human male reproductive function associated with diseases of the male genital tract (azoospermia, cryptorchidism, testicular cancer) has been reported for the past two decades (Asklund et al., 2004; Povey and Stocks, 2010; Pointis et al., 2011). In addition, recent studies revealed that intercellular junctions, specifically gap junctions, might be structures of interest and be involved in this phenomenon (Steger et al., 1999; Brehm et al., 2002; Defamie et al., 2003; Pointis et al., 2011).

Gap junction channels connect neighboring cells in nearly all tissues; they permit an exchange of metabolites, signaling molecules and ions, and regulate numerous physiological functions such as

cell proliferation, differentiation and apoptosis (Goodenough et al., 1996). Each channel is composed of two hemichannels, called connexons, separately contributed by the two participating cells. The connexons themselves are hexamers formed by self assembly of six connexins (CXs) (Bruzzone et al., 1996). To date, the family of CX genes consists of 20 members in the mouse and 21 members in the human genome (Sohl and Willecke, 2004).

In addition to their classical role, recent studies have reported on the channel-independent functions of gap junctions (Stout et al., 2004). These functions comprise, for example, extrinsic guidance of migrating cells owing to CX-mediated cell adhesion, as well as intracellular processes. Among the connexins, CX43 in particular has been shown to exert effects on migration by interfering with receptor signaling, cytoskeletal remodeling and tubulin dynamics (Kameritsch et al., 2011). Undocked gap junction hemichannels might also exert physiological roles because connexons are involved in: the release of, for example, ATP and NAD⁺, Ca²⁺ wave propagation, cell-volume control and the passage of survival signals within the tissue (Spray et al., 2006; Pointis et al., 2010). CXs might also function as scaffold proteins for the attachment of cytoplasmic partner molecules (Huang et al., 1998) and as signaling molecules (Kardami et al., 2007). CX43 interactions with the cytoskeleton have been suggested by several previous studies. The CX43 C-terminus has been shown to bind TJP1 (Giepmans et al., 2001a), a scaffold protein that facilitates linkage of the membrane with the actin cytoskeleton (Itoh et al., 1997; Hartsock and Nelson, 2008). CX43 can also directly bind to tubulin (Giepmans et al., 2001b). In a very

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recent study, CX43 was shown to modulate cell migration by regulating microtubule dynamics associated with abnormal actin stress fiber organization (Francis et al., 2011).

CX43 is the predominant testicular CX in most species, including humans (Risley et al., 1992; Steger et al., 1999; Batias et al., 2000; Bravo-Moreno et al., 2001; Brehm et al., 2002). In adult men, CX43 protein localization has been investigated in testes both with normal spermatogenesis and associated with different forms of spermatogenic impairment (Steger et al., 1999; Brehm et al., 2002; Defamie et al., 2003; Roger et al., 2004; Brehm et al., 2006; Steiner et al., 2011). As in rodent testes, CX43 immunoreactivity was generally present between Leydig cells (LCs) (Pérez-Armendariz et al., 1994) and, within the seminiferous epithelium, CX43 was found to form an integral component of the Sertoli cell (SC)-SC junctional complexes that represent the anatomical base of the blood-testis barrier (BTB) (Risley et al., 1992). Further components of the BTB, in addition to the CX-based gap junctions, are cadherin (CDH)-based adherens junctions and occludin (OCLN)-based tight junctions (Pointis et al., 2005). There is further evidence that CX43 plays a role in the coordination of changes in SC junctional permeability as well as in SC and germ cell (GC) differentiation (Pelletier, 1995). Furthermore, the synchronization of GC proliferation and differentiation is mediated through the gap junctional network (Decrouy et al., 2004).

Within the normal human (and murine) seminiferous epithelium, CX43 immunoreactivity is localized between SCs and GCs (spermatogonia and primary spermatocytes) and along the SC-SC junctional complexes (Steger et al., 1999; Brehm et al., 2002; Defamie et al., 2003). Some information is available concerning the presence of gap junctions and expression of CX43 in seminiferous tubules of pathologic human testes. By means of freeze fracture, no gap junctions were detected in feminized human testes (Nagano and Suzuki, 1976) and the presence of atypical testicular gap junctions was observed in infertile patients (Bigliardi and Vegni-Talluri, 1977). Gap-junction-like cell-membrane specializations were rare in hypospermatogenic and aspermatogenic testes (Schleiermacher, 1980). In addition, testicular histology of these infertile adult patients frequently reveals spermatogenic defects that are associated with heterogeneous SC populations. For example, tubules showing (1) spermatogenic arrest at the spermatogonial level and those with (2) SC-only (SCO) syndrome are characterized by immature populations of SCs expressing fetal differentiation markers, such as anti-Muellerian-hormone (AMH) and cytokeratin 18 (CK18) (Bergmann and Kliesch, 1994), and exhibit a reduction or even loss of CX43 in GCs and SCs (Steger et al., 1999; Brehm et al., 2002; Defamie et al., 2003). Finally, alterations of CX43 expression have been correlated to the development of human carcinoma in situ (CIS) of the testis and testicular tumors (Brehm et al., 2002; Roger et al., 2004; Brehm et al., 2006).

Taken together, there now exist many studies and review articles (e.g. Pointis and Segretain 2005; Pointis et al., 2005; Sridharan et al., 2007b; Pointis et al., 2010; Gilleron et al., 2011; Weider et al., 2011a; Pointis et al., 2011) that support functional roles of CX43 in the regulation of human spermatogenesis: (1) CX43 forms intercellular channels between SCs and proliferating GCs, indicating that this CX is involved in the physiological maturation and proliferation processes of these cell types; (2) alterations of CX43 expression are correlated with testicular disorders in men;

for example, individuals with CIS and seminoma, the most frequent type of human GC tumors, show a reduction of CX43 expression in SCs and tumor cells. In CIS tubules, the reduction of CX43 expression was shown to be regulated at the transcriptional level and accompanied with a dedifferentiation of SCs (Brehm et al., 2006); (3) moreover, possible mutations in the human *CX43* gene have been discussed because a recent study working with a mutant mouse model of oculodentodigital dysplasia (ODDD) showed impaired spermatogenesis, supporting the possibility of subfertility in ODDD human males (Gregory et al., 2011). However, to date, there is still a lack of information on the molecular mechanisms resulting in the impairment of human male reproductive function through CX disruption.

To further investigate the role of CX43, several knockout (KO) and knock-in (KI) mice have been generated. General KO of the *Cx43* gene leads to cardiac malformation and prenatal death; thus, a functional analysis of spermatogenesis was not possible in this KO mouse line (Reaume et al., 1995). Nevertheless, a severe GC loss in the embryonic tissue has been detected, indicating an indispensable role of CX43-mediated cell coupling for GC development (Juneja et al., 1999). Testes of *Cx43*-null mutant embryos neither allow a normal GC proliferation nor differentiation when grafted under the kidney capsule of adult males (Roscoe et al., 2001). However, the detailed mechanisms that lead to sterility of the male *Cx43* mice in the mentioned studies are still unknown (Pointis et al., 2005).

To study the effects of a deletion of *Cx43* on spermatogenesis, a conditional SC-specific KO of the *Cx43* gene (SCCx43KO) has been generated and revealed that *Cx43* expression in SCs is an absolute requirement for normal testicular development and initiation of spermatogenesis (Brehm et al., 2007; Sridharan et al., 2007a). Compared with the generalized KO (Reaume et al., 1995), SCCx43KO mice are viable, but infertile (Brehm et al., 2007; Sridharan et al., 2007a). Weighing of animals and testes displays no differences in the body weight between SCCx43KO and wild type (WT); however, testes were significantly smaller in the KO males. Furthermore, adult SCCx43KO mice show a significantly reduced number of GCs per tubule, whereas the mean number of SCs per tubule is significantly higher compared with WT (Brehm et al., 2007). Interestingly, histological analysis of adult SCCx43KO mice revealed similar spermatogenic alterations as seen in infertile humans, such as an arrest of spermatogenesis at the level of spermatogonia, or SCO syndrome (Brehm et al., 2007; Sridharan et al., 2007a).

The number of GCs that can be supported by each SC is fixed for each species. This species-specific ratio of GCs to SCs is determined prepubertally by proliferation and apoptosis (Rodriguez et al., 1997; Sharpe et al., 2003). A disruption of cell-cell communication is thought to lead to increased apoptosis and GC loss, demonstrating the significant role of testicular CX to maintain the survival of GCs by regulating intercellular communications between GCs and adjacent supporting cells (Lee et al., 2006b). Dye-coupling studies demonstrated that CX43 participates in the coupling between SCs and between SCs and GCs, with the dye transfer from SCs to GCs being mostly unidirectional (Decrouy et al., 2004). Thus, the observed phenotype in adult SCCx43KO mice might, for instance, be due to: (1) a disrupted direct connection; for example, a disturbed flow of signaling molecules and high-energy metabolites from SCs to

GCs via gap junction channels containing CX43 or (2) channel-independent functions.

To get a deeper insight into the molecular mechanisms and possible signaling pathways leading to the observed histological phenotype and infertility in adult SCCx43KO mice, a genome-wide gene expression profiling of 8-day-old WT and SCCx43KO mice was performed. Mice of that age have been chosen because (1) the morphology of the testis was still comparable between the two genotypes and (2) murine spermatogenesis has been initiated, indicated by the first appearance of type B spermatogonia (Bellvé et al., 1977).

RESULTS

GC number is significantly reduced in 8-day-old SCCx43KO mice

Comparison of testis weight revealed no significant differences between WT and SCCx43KO mice on day 8 post partum (p.p.). However, morphological and histological examination detected obvious differences in the composition of intratubular cells between the two genotypes (Fig. 1A-D). GC number was three times higher in testes of WT mice, whereas the number of SCs (and LCs) was not affected by the deletion of *Cx43* (Figs 1, 2).

Differential gene expression in WT and KO mice

Microarray analysis revealed 658 significantly regulated genes [$P \leq 0.05$ and $-2 \geq \text{fold change (FC)} \geq 2$]. Of these, 135 genes showed higher gene expression values, whereas 523 displayed lower gene expression values in SCCx43KO mice compared with WT mice. Annotation was possible for 475 downregulated and 106 upregulated genes. Table 1 gives an overview of the highest ranked genes and the functional category they belong to. All significantly regulated genes can be found in supplementary material Table S1A,B.

Hierarchical clustering of the 658 significantly regulated genes shows two clusters clearly differing between WT and KO mice (Fig. 3). Within each cluster, the gene expression profiles of the biological replicates of WT1-3 and KO1-3 mice were homogenous, indicating reproducibility of the microarray results. Within the WT group,

two subclusters are present, i.e. WT1 versus WT2/3. This small difference in the expression profile can be explained because WT2 and 3 were from the same litter, whereas WT1 was another litter from the same mouse.

Gene ontology (GO) of the differentially regulated genes revealed clusters of biological categories representing functional annotation groups. The upregulated genes were assigned to three clusters, involved in chemotaxis, cell migration and cytoskeleton organization (Table 2). The downregulated genes could be assigned to 16 clusters, with the majority of these genes being involved in spermatogenesis, cell cycle/meiosis, transcription, sexual differentiation, cell migration, DNA modification and translation (Table 2). The detailed functional annotation clusters can be found in supplementary material Table S2A,B.

Pathway analysis for functions with the differentially regulated genes resulted in spermatogenesis as a top function. The 29 genes within spermatogenesis were mainly downregulated and involved in subfunctions such as meiosis of male GCs or expression of RNA, highlighting the influence of the KO gene *Cx43* on the different aspects of spermatogenesis (Fig. 4).

Additionally, pathway analysis revealed ten networks each containing interactions of nine or more genes from the list of differentially regulated genes (supplementary material Table S3). The top network contained the KO gene *Cx43* and genes that were overall involved in infectious disease, gene expression and antigen presentation. The network is depicted in Fig. 5A, showing the interaction of *Cx43* with these genes. Remarkably, most of the genes in the network involving *Cx43* are downregulated and involved in gametogenesis, gene activation, quantity of gap junctions, cell division, cell differentiation and cell migration.

Further interactions of *Cx43* are indicated in the predefined canonical 'GC-SC junction pathway' (Fig. 5B). It is shown that interaction between SCs and GCs can be supported by protein complexes and/or proteins such as CDHE- or CDHN-CTNNB1 (CDHE/N-CTNNB1), PONSIN-AFADIN-NECTIN2, ITG α 6 β 1, TNFR and TGF β R.

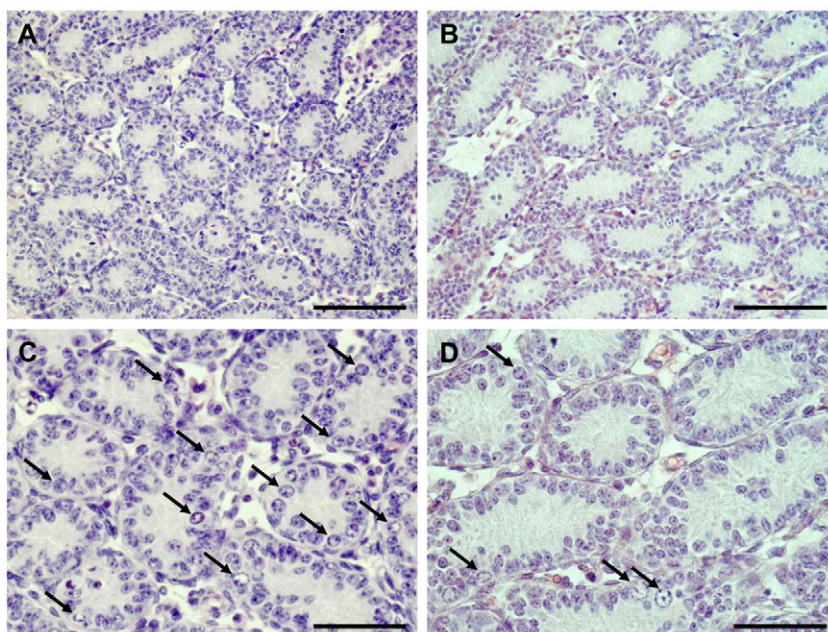


Fig. 1. Representative histology of WT and SCCx43KO mouse testis at day 8 p.p. H&E staining of a WT mouse testis (A,C) shows SC and type A and B spermatogonia (arrows). By contrast, in seminiferous cords of an SCCx43KO mouse (B,D), only single GCs (arrows) are detectable. Scale bars: (A,B) 100 μ m; (C,D) 50 μ m.

Table 1. Overview of the highest ranked up- and downregulated genes

Gene symbol	Entrez ID	Gene name	t-test P-value	RP P-value	FC KO/WT
Upregulation (FC>3.0)					
Cell migration/chemotaxis					
<i>Acta1</i>	11459	actin, alpha 1, skeletal muscle	0.01	0.00	11.99
<i>Pcp4</i>	18546	Purkinje cell protein 4	0.00	0.00	59.79
<i>Ccl17</i>	20295	chemokine (C-C motif) ligand 17	0.00	0.00	3.02
<i>Ccl9</i>	20308	chemokine (C-C motif) ligand 9	0.02	0.00	3.13
<i>S100a9</i>	20202	S100 calcium binding protein A9 (calgranulin B)	0.00	0.00	3.02
<i>Tns1</i>	21961	tensin 1	0.02	0.00	3.11
<i>Mid1</i>	17318	midline 1	0.03	0.00	3.52
Metabolism					
<i>Adh7</i>	11529	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	0.01	0.00	3.62
<i>Adpgk</i>	72141	ADP-dependent glucokinase	0.00	0.00	21.50
Receptors					
<i>Arhgef12</i>	69632	Rho guanine nucleotide exchange factor (GEF) 12	0.01	0.00	6.02
<i>Dkk2</i>	56811	dickkopf homolog 2 (<i>Xenopus laevis</i>)	0.00	0.00	6.01
<i>Esr2</i>	13983	estrogen receptor 2 (beta)	0.04	0.00	3.01
<i>Vmn1r208</i>	171252	vomer nasal 1 receptor 208	0.03	0.00	3.55
Protein transport					
<i>Cadps</i>	27062	Ca ²⁺ -dependent secretion activator	0.01	0.00	3.21
<i>Kpna2</i>	16647	karyopherin (importin) alpha 2	0.00	0.00	7.36
Defense response					
<i>Defb9</i>	246079	defensin beta 9	0.02	0.00	3.66
Downregulation (FC<-5.7)					
Spermatogenesis					
<i>Stra8</i>	20899	stimulated by retinoic acid gene 8	0.03	0.00	-26.70
<i>Sohlh1</i>	227631	spermatogenesis and oogenesis specific basic helix-loop-helix 1	0.00	0.00	-6.98
<i>Dazl</i>	13164	deleted in azoospermia-like	0.04	0.00	-6.04
<i>Rnf17</i>	30054	ring finger protein 17	0.01	0.00	-9.64
Reproductive system development/cell cycle					
<i>Hnf1b</i>	21410	HNF1 homeobox B	0.00	0.00	-6.32
<i>Lhx8</i>	16875	LIM homeobox protein 8	0.01	0.00	-7.42
<i>Sox3</i>	20675	SRY-box containing gene 3	0.00	0.00	-13.30
<i>Tex11</i>	83558	testis expressed gene 11	0.02	0.00	-15.44
<i>Piwi2</i>	57746	piwi-like homolog 2 (<i>Drosophila</i>)	0.03	0.00	-5.89
<i>Taf7l</i>	74469	TAF7-like RNA polymerase II, TATA box binding protein (TBP)-associated factor	0.03	0.00	-6.66
<i>Sycp2</i>	320558	synaptonemal complex protein 2	0.01	0.00	-8.90
<i>Eaf2</i>	106389	ELL associated factor 2	0.00	0.00	-6.85
<i>Hormad1</i>	67981	HORMA domain containing 1	0.02	0.00	-7.68
Regulation of transcription					
<i>Hmx1</i>	15371	H6 homeobox 1	0.04	0.00	-20.53
<i>Otx1</i>	18423	orthodenticle homolog 1 (<i>Drosophila</i>)	0.03	0.00	-9.38
<i>Utf1</i>	22286	undifferentiated embryonic cell transcription factor 1	0.02	0.00	-7.27
<i>Snai3</i>	30927	snail homolog 3 (<i>Drosophila</i>)	0.00	0.00	-6.11
<i>Grhl1</i>	195733	grainyhead-like 1 (<i>Drosophila</i>)	0.01	0.00	-6.83
<i>Pou3f3</i>	18993	POU domain, class 3, transcription factor 3	0.00	0.00	-6.13
<i>Zfy2</i>		zinc finger protein 2, Y linked (<i>Zfy2</i>)	0.00	0.00	-5.84
<i>Dmrtb1</i>	56296	DMRT-like family B with proline-rich C-terminal, 1	0.04	0.00	-68.15
<i>Dmrct2</i>	71241	doublesex and mab-3 related transcription factor like family C2	0.00	0.00	-7.31
Transport					
<i>Crabp1</i>	12903	cellular retinoic acid binding protein I	0.00	0.00	-12.69
<i>Clic6</i>	209195	chloride intracellular channel 6	0.02	0.00	-6.92
Immune response					
<i>Zbp1</i>	58203	Z-DNA binding protein 1	0.00	0.00	-9.70

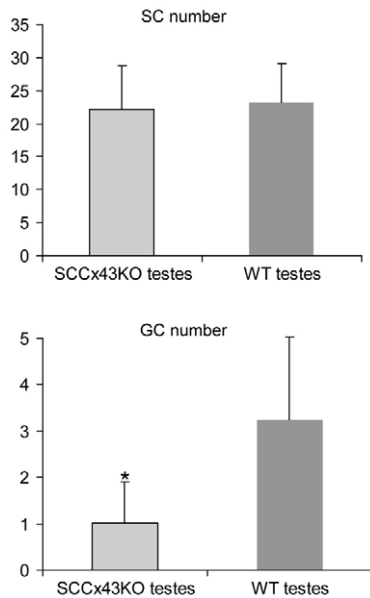


Fig. 2. Comparison of SC and GC numbers of WT and SCCx43KO mouse testis at day 8 p.p. Average number of SCs and GCs per cord, comparing the two genotypes at day 8 p.p. GC number is significantly reduced in KO mice. Error bars represent standard deviation (s.d.). The asterisk indicates the statistical significant difference ($P < 0.05$) between the WT and SCCx43KO mice. Seminiferous cords of WT mice contain 3.24 ± 1.78 GCs and 23.17 ± 5.92 SCs. Cords of KO mice have 1.02 ± 0.89 GCs and 22.17 ± 6.64 SCs.

When overlaying gene expression data onto the canonical pathway, only the interaction via CDHE/N-CTNNB1 is affected by downregulation of *Cdh1*. The putative connections of CX43 and the canonical pathway are given as orange arrows in Fig. 5B. CX43 can be related to this canonical pathway by interaction with ACTB, TJP1 and with a complex consisting of proto-oncogene products SRC and MTMR2.

The family of CX genes comprises 20 members in the mouse (in the groups *Gja-Gje*). To assess whether other junction genes, especially the members of the gap junction family, were also affected from the KO of *Cx43*, the gene expression of those junction genes available on the microarray was determined (supplementary material Table S4). Beside *Cx43*, none of the remaining junction genes reached statistical ($P \leq 0.05$) or biological ($-2 \geq FC \geq 2$) significance. Nevertheless, *Gja6*, *Gjb3*, *Gjc1*, *Gjd3* and *Tjp3* were biologically downregulated (FC ranging from -1.3 to -1.8), with *Gjb3*, *Gjc1* and *Gjd3* being statistically significant ($P \leq 0.05$). The interaction of the junction genes with *Cx43* is depicted in Fig. 5C.

The majority of genes associated with spermatogenesis are GC-specific

The majority of the differentially expressed genes can be associated with spermatogenesis. The majority of these genes are exclusively expressed in GCs (Table 3).

Validation of the microarray data by quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was applied to validate the microarray data. In total, 19 candidate genes, showing different ranges of values (up, down, unchanged), were selected to be

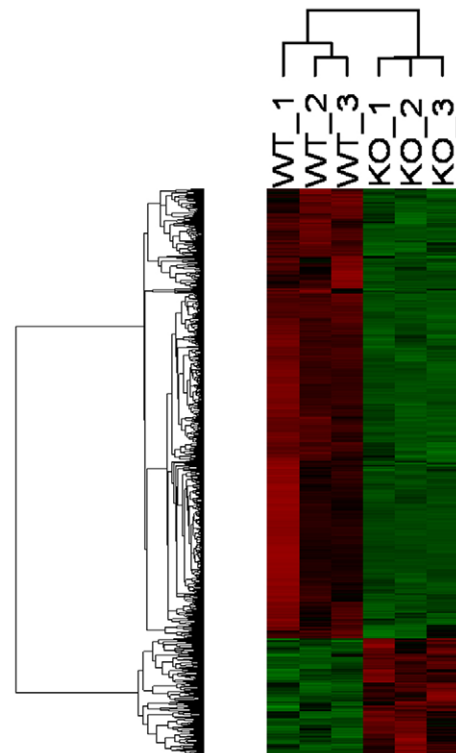


Fig. 3. Hierarchical clustering of 658 significantly regulated genes. Genes are depicted in rows and samples in columns. Green indicates downregulation; red indicates upregulation.

analyzed. Among the chosen genes, some are mainly associated with the BTB formed by SCs, such as *Cdh2*, *Cld11*, *Ocln* and *Tjp1*. Other genes were selected for their FC value or their importance in spermatogenesis. After applying the calculated correction factors, the qRT-PCR data were mostly consistent with the microarray results, displaying a significant correlation between the log ratios of the considered genes in the qRT-PCR and the corresponding log ratios in the microarray analysis with correlation coefficient of 0.86 and significance P -value < 0.01 . Relative mRNA levels of *Sohlh1*, *Ddx25*, *Cdkn2a*, *Stra8*, *Dmrt6*, *Sox3*, *Dazl*, *Cx43*, *Piwil2*, *Tex11* and *Sycp2* were lower in the KO mice compared with WT animals (Fig. 6).

Confirmation of *Cx43* deletion by immunohistochemistry

CX43 immunostaining at day 8 p.p. revealed no immunoreactivity in seminiferous cords of SCCx43KO mice (Fig. 7), whereas, in seminiferous cords of the prepubertal WT males, a typical immature and diffuse adluminal signal was detectable.

Immunohistochemistry confirmed qRT-PCR results at protein level

Selecting two highly downregulated GC-specific genes [*Stra8* (Fig. 8) and *Dazl* (Fig. 9)] and one gene associated with the BTB and with no significant alteration at mRNA level [*Ocln* (Fig. 10)], we applied immunohistochemistry (IHC) to examine their level of protein expression.

In seminiferous cords of the 8-day-old WT mice, many spermatogonia and first preleptotene spermatocytes showed an

Table 2. Functional annotation clustering of up- and downregulated genes

Functional group	Genes	P-value range min.	P-value range max.
Upregulated genes			
Chemotaxis	<i>Robo1, S100a9, Zic1, Apbb1, Ccl17, Ccl6, Themis, Ccl9, Cfi, Ltb</i>	0.002	0.063
Cell migration	<i>Vav3, Tns1, Robo1, Mdga1, S100a9, Esr2, Apbb1</i>	0.005	0.041
Cytoskeleton organization	<i>Acta1, S100a9, Cnn1, Apbb1, Tacc2</i>	0.046	0.186
Downregulated genes			
Spermatogenesis	<i>Adad1, Asz1, Ccnb1ip1, Chrna7, Clgn, D1pas1, Dazl, Ddx25, Dmc1, Dnd1, Figla, Foxl2, Ggt1, Hmga1, Mael, Morc1, Msh4, Ovol1, Piwil2, Rad51c, Rec8, Rnf17, Sohlh1, Sohlh2, Sox3, Spata19, Spata5, Stra8, Sycp2, Sycp3, Taf7l, Tcfl5, Tdrd1, Tex11, Tex15, Tle3, Zbtb16, Zbbp, Rad51c</i>	4.43E-14	2.48E-12
Cell cycle/meiosis	<i>Ccnb1ip1, Cdkn2a, Clgn, Cpeb1, Dmc1, Fanci, Fsd1, Hells, Hormad1, Hormad2, Mael, Msh4, Nek3, Ovol1, Piwil2, Rad51, Rad51c, Rbm38, Rec8, Smc1b, Stra8, Suv39h2, Sycp1, Sycp2, Sycp3, Tdrd1, Tex11, Tex15, Trnp1, Uba3, Uhmk1</i>	2.36E-15	0.004
Transcription	<i>Cbx2, Cdkn2a, Dazl, Eaf2, Ehf, Fgf8, Foxl2, Gabpa, Glis1, Grhl3, Gtpbp4, Hells, Hmga1, Hmx1, Hnf1b, Htatip2, Irf6, Isl1, Krt17, Lin28a, Mael, Nfib, Ovol1, Ovol2, Pax8, Piwil2, Pou2f2, Pou3f3, Rictor, Rnf128, Sall4, Satb1, Shh, Six2, Spink12, Stra8, Tdrd1, Utf1, Zbtb16, Zbtb42</i>	0.001	0.041
Sexual differentiation	<i>Ascl2, Ccnb1ip1, Cdh1, Chrna7, Col4a3bp, Dmc1, Fgf8, Foxf1a, Foxl2, Gabpa, Cx43, Hnf1b, Kif3a, Krt8, Lhx8, Msh4, Ovol2, Sall4, Shh, Six2, Sohlh1, Spint1, Stra8, Sycp2, Tex11, Tex15</i>	0.003	0.049
Cell migration	<i>Asz1, Chrna7, Dab1, Fgf8, Cx43, Isl1, Krt14, Krt17, Lhx2, Lhx6, Mesp1, Ovol2, Pou3f3, Reln, Shh, Taf7l, Tnn</i>	0.004	0.091
DNA modification and translation	<i>Arih1, Asb9, Ate1, Dazl, Dnahc12, Fbxo15, Foxl2, Hells, Herc3, Krt17, Lin28a, Mael, Oas2, Piwil2, Psm8, Psmb9, Rffl, Rictor, Rnf128, Satb1, Siah1b, Stra8, Suv39h2, Tdrd1, Uba3, Uba6, Ube2e3, Uchl1, Usp12, Usp18, Usp37, Usp45, Zyg11b</i>	0.004	0.181

Clusters with at least one significantly overrepresented GO category ($P < 0.05$) have been taken into account. For upregulated genes, three clusters could be determined and for downregulated genes the clusters have been summarized into five representative main groups.

intensive staining for STRA8 (Fig. 8A). By contrast, only single cells of the SCCx43KO mice revealed an immunoreactivity for STRA8 (Fig. 8B). Note the immunonegative spermatogonia in Fig. 8B (arrows). In adult WT mice, a stage-specific immunoreaction for STRA8 can be seen (Fig. 8C), whereas no immunoreaction at all can be detected in seminiferous tubules that show an arrest of spermatogenesis at the level of spermatogonia (arrow) in adult mutants (Fig. 8D).

Regarding the distribution pattern of DAZL, there is also an obvious difference between the two genotypes (Fig. 9A-D). The number of stained cells is drastically reduced in 8-day-old SCCx43KO mice, confirming results obtained by qRT-PCR. Note the immunonegative spermatogonia in Fig. 9B (arrows). In adult mice, a clear immunoreaction for DAZL can be seen in spermatogonia and spermatocytes (Fig. 9C). By contrast, in seminiferous tubules showing an arrest of spermatogenesis at the level of spermatogonia (arrows) in adult SCCx43KO mice, not even spermatogonia show a DAZL immunostaining (Fig. 9D, arrows).

OCLN represents an integral SC membrane protein and is involved in the formation of the BTB. Deletion of *Cx43* in SCs results in no obvious changes in OCLN protein synthesis and localization when comparing 8-day-old WT (Fig. 10A) and mutant (Fig. 10B) animals.

Evaluation of DMRT7 protein synthesis using immunofluorescence

Immunofluorescence (IF) revealed no immunoreactivity for DMRT7 in most seminiferous tubules in 15-day-old KO mice (Fig. 11B), whereas the WT mice showed a specific staining in most

pachytene spermatocytes (Fig. 11A). In adult WT males, a specific staining can be detected in nearly all spermatocytes (Fig. 11C), whereas only paraffin autofluorescence is seen in KO tubules (Fig. 11D). The insert in Fig. 11C shows paraffin autofluorescence in interstitial LCs in negative controls.

DISCUSSION

The prevalence of human couple infertility is extremely high and in many countries affects one in seven couples (Sharpe, 2010). Although the nature and exact proportion of the predominant cause of the problem remains controversial, the World Health Organization (WHO) reports that, in nearly 40% of cases the cause can be attributed to the female, in 20% to the male and in 25% to both; however, in 15% the cause still remains unknown (WHO, 1987). On the basis of these figures, the incidence of human male factor infertility in the general population is approximately 7%, making it more prevalent than type 1 and 2 diabetes mellitus combined, and this proportion is expected to rise. With at least one third of infertile men being diagnosed as having idiopathic infertility associated with impaired spermatogenesis, there is an urgent need to better understand the status of male reproductive health, especially in Europe and industrialized countries (EU Strategic Research Plan, 2010).

Impaired spermatogenesis (with e.g. an arrest of spermatogenesis at different stages and/or SCO syndrome) can result from a variety of causes. For example, genetic defects like Klinefelter syndrome and cryptorchidism exhibit a fivefold increased risk for the development of testicular cancer, which represents the most

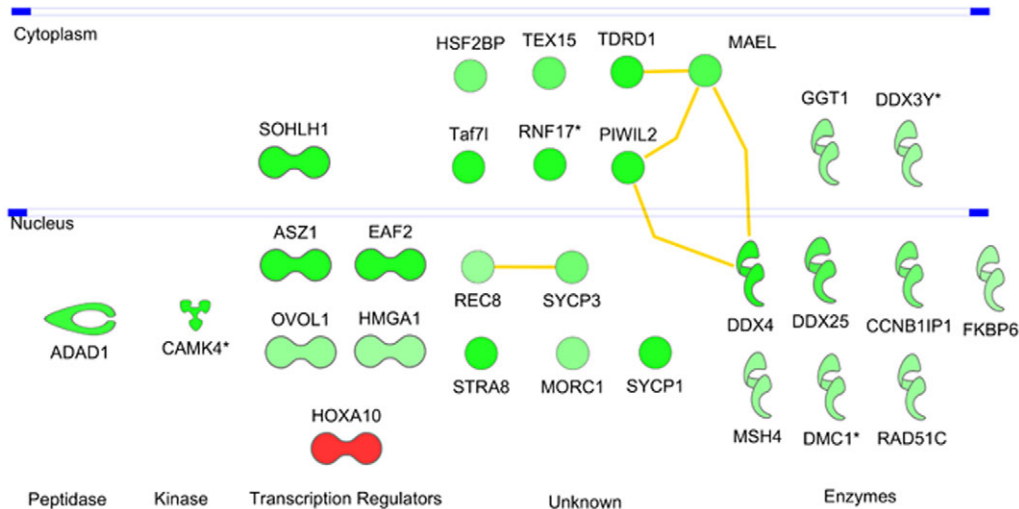


Fig. 4. Interaction network of functions involved in spermatogenesis. Analysis of all regulated genes in IPA revealed spermatogenesis as a top function. Known protein-protein interactions for the involved genes are highlighted by yellow lines. Colors represent the FC in expression of the genes. Red, significant upregulation with $FC > 2$; green, significant downregulation with $FC < -2$. Genes in dark green show higher FC compared with genes in light green (e.g. *Sohlh1* with $FC -7.0$ and *Rec8* with $FC -2.4$).

common cancer among men (Møller and Skakkebaek, 1999; Skakkebaek et al., 2001; Asklund et al., 2004). In approximately 25% of these men, an aberrant spermatogenic appearance is also evident in the 'assumed normal' contralateral testis. In a series of prospective and well-standardized studies over the past 10 years, it has been found that the prevalence of an abnormally low sperm count in young men is as high as 15-20% (Jørgensen et al., 2006; Andersson et al., 2008). Testicular biopsies of these men often reveal mixed atrophy showing seminiferous tubules with qualitatively normal spermatogenesis directly adjacent to tubules with spermatogenic arrest and/or SCO syndrome (Steger et al., 1996; Steger et al., 1998).

Recent studies revealed that the gap junction protein CX43 seems to be involved in different forms of spermatogenic disorders (Steger et al., 1999; Brehm et al., 2002; Defamie et al., 2003; Steiner et al., 2011) and in the pathogenesis of human testicular germ cell tumors (Brehm et al., 2002; Steiner et al., 2011), so a transgenic mouse model (*SCCx43KO*) has been established to investigate cellular and molecular mechanisms that are essential for male GC differentiation and to identify aberrant signaling pathways in 'male factor infertility due to impaired spermatogenesis' (Brehm et al., 2007; Sridharan et al., 2007a).

In the present study, using the microarray technology and after applying the calculated correction factors, several candidate genes possibly involved in the observed reduction of GC number, in preventing the initiation of spermatogenesis and thus in the disturbed progression beyond mitosis of the remaining GCs in adult *SCCx43KO* mice have been detected. These genes (connected with a CX43 deficiency) could also represent helpful markers for investigators exploring the molecular mechanisms of human male sterility. GO analysis revealed chemotaxis, cell migration and cytoskeleton organization as the major functional clusters affected within the upregulated genes. Interestingly, genes associated to the group of cell migration are also highly present within the group of downregulated genes, indicating an

important role for CX43-mediated communication in this functional gene class.

Within the seminiferous epithelium, CX43 is thought to be important for both the SC-SC functional synchronization and the SC-GC crosstalk (Pointis et al., 2010). Here, direct contact-dependent junctional pathways play a pivotal role in the regulation of GC and SC proliferation and differentiation, and the maintenance of the male phenotype (Mruk and Cheng, 2004). In addition to (intermingled) CX43 gap junctions, the other junctions are composed of specialized proteins implicated in cell adhesion, the regulation of paracellular diffusion, the establishment and maintenance of cell polarity, the regulation of cell attachment, and the regulation of actin proteins (Cheng and Mruk, 2002; Derangeron et al., 2009; Cheng et al., 2011). CX43 interactions with the cytoskeleton have been suggested by several previous studies. The C-terminus of CX43 has been shown to bind TJP1 (Giepmans et al., 2001a), facilitating the linkage of the membrane with the actin cytoskeleton (Itoh et al., 1997; Hartsock and Nelson, 2008). CX43 can also directly bind to tubulin (Giepmans et al., 2001b). In a very recent work, CX43 was shown to modulate cell migration by regulating microtubule dynamics associated with abnormal actin stress fiber organization (Francis et al., 2011). In this context, in the present study, *Tuba3* [encoding for TUBA3 ($FC -4.5$)] and different *Krt* genes (*Krt14*, *17*, *24* and *8*; encoding for keratins) were significantly downregulated, implying alterations in cytoskeleton dynamics.

During embryonal development, human SCs contain both CK18 and vimentin; however, only the latter persists in adult testis (Franke et al., 1979) and CK18 expression nearly disappears after the 20th week of gestation. CK18 intermediate filaments are completely lost around birth, thereby signaling the earliest transition of SCs to a mature state. CK18 expression, therefore, indicates a state of undifferentiation (Rogatsch et al., 1996; Franke et al., 2004). Interestingly, co-expression of vimentin together with CK18 occurs in SCs of adult testes under pathological conditions

Table 3. List of differentially expressed GC-specific genes associated with spermatogenesis

Gene	Testicular localization	Publication	KO-model-derived functions in male spermatogenesis	Publication
<i>Ovo1</i>	Primary and secondary spermatocytes	Dai et al., 1998	Regulation of pachytene progression of male GCs	Li et al., 2005
<i>Spag6</i>	Sperm	Sapiro et al., 2002	Maintenance of sperm flagellar motility and structural integrity of mature sperm	Sapiro et al., 2002
<i>Sohlh1</i>	Prespermatogonia, spermatogonia	Ballow et al., 2006	Regulation of spermatogonial differentiation into spermatids	Ballow et al., 2006
<i>Lhx8</i>	GC	Pangas et al., 2006	Possible role in regulation of spermatogonial differentiation into spermatocytes	Pangas et al., 2006; Ballow et al., 2006
<i>Dazl</i>	Spermatogonia type B, spermatocytes	Ruggiu et al., 1997	Progression through meiotic prophase and/or GC differentiation	Saunders et al., 2003
<i>Stra8</i>	Spermatogonia type A and B, preleptotene spermatocytes, early leptotene spermatocytes	Zhou et al., 2008	Regulation of meiotic initiation	Anderson et al., 2008
<i>Hnf1b</i>	GC	Reber and Cereghini, 2001	Homozygous vHNF1 mice die before gastrulation	Reber and Cereghini, 2001
<i>Sox3</i>	Spermatogonia	Raverot et al., 2005	Differentiation of spermatogonia and/or initiation and progression of meiosis	Raverot et al., 2005
<i>Zpbp2</i>	Sperm	Lin et al., 2007	Morphological normal sperm formation and/or fertility	Lin et al., 2007
<i>Taf7l</i>	Spermatogonia, spermatocytes, round spermatids	Cheng et al., 2007	Development of normal sperm count and motility	Cheng et al., 2007
<i>Tex11</i>	GC	Wang et al., 2001	Essential for spermatid development and completion of spermatogenesis	Tsai-Morris et al., 2004; Yang et al., 2008
<i>Sycp2</i>	GC	Wang et al., 2001	Differentiation of zygotene-like spermatocytes into normal pachytene spermatocytes (synaptonemal complex assembly and chromosomal synapsis during male meiosis)	Yang et al., 2006; Yang et al., 2008
<i>Piwil2</i>	GC	Beyret and Lin, 2011	Spermatocytes without PIWI proteins are arrested in the pachytene stage	Beyret and Lin, 2011
<i>Ddx25</i>	GC	Gutti et al., 2008	Post-transcriptional regulator of spermatogenesis (as a component of mRNP particles), controls apoptosis in spermatocytes of adult mice	Gutti et al., 2008

deletion would be expected first to lead to an altered expression pattern related to this cell population. However, our results can be explained by the juxtaposition and dependency of the two cell types, which is interrupted by the deletion of the *Cx43* gene. As shown by dye transfer studies, the coupling between SCs and GCs is unidirectional from SCs to GCs (Decrouy et al., 2004). Together with the observation that the gene expression of mainly GCs is influenced, our results might lead to the assumption that the interruption of poorly characterized mediators of signals from SCs to GCs affect primarily the normal development of early GC populations, preventing the initiation of spermatogenesis. Examples for such GC-specific genes are depicted in Table 3. The physiological relevance of the genes identified by the microarray study is underscored by the finding that at least eight downregulated genes have already been shown to be essential for normal spermatogenesis. Using qRT-PCR, IHC and IF, we were able to verify the microarray results for selected genes of interest, such as *Stra8*, *Dazl* and *Dmrt7*.

Stra8 (FC -26.70) is a vertebrate-specific factor expressed in GCs in response to retinoic acid (RA) (Anderson et al., 2008). STRA8 protein expression at day 8 p.p. is restricted to the spermatogonia and first preleptotene spermatocytes (Zhou et al., 2008). RA is known to be essential for normal spermatogenesis. The actions of RA are mediated through six distinct ligand-dependent

transcription factors, including three RA receptors (RAR α , β and γ) and three retinoid X receptors (Zhou et al., 2008). It is well known that RA stimulates the expression of *Cx43* (Batias et al., 2000; Tanmahasamut and Sidell, 2005; Vine and Bertram, 2005). A recent publication by Chung et al. further demonstrated that a deletion of RAR α leads to an alteration of gap-junction-based cell coupling (Chung et al., 2010). KO studies of the *Stra8* gene revealed its importance for functional spermatogenesis: its deletion leads to infertility of male and female mice. In male KO mice, infertility is caused by a failure in meiotic initiation, blocking the transition into the meiotic prophase (Anderson et al., 2008). The close relation between *Cx43* and *Stra8* is affirmed by their overlapping regulatory mechanisms, their common requirement during GC development and the dependency of *Stra8* expression on *Cx43* expression. Whether this interaction is causally related or the result of altered interactions between GCs and SCs leading to a loss of signaling molecules that are essential for *Stra8* transcription and STRA8 protein synthesis in GCs requires further investigation. However, in a very recent study a close relationship between RA signaling, STRA8 expression and the control of meiotic entry in the human fetal gonad was shown (Childs et al., 2011).

Dazl (FC -6.04), also established as a GC-specific gene, can be detected in the cytoplasm of type B spermatogonia and less abundantly in pachytene, preleptotene and zygotene spermatocytes

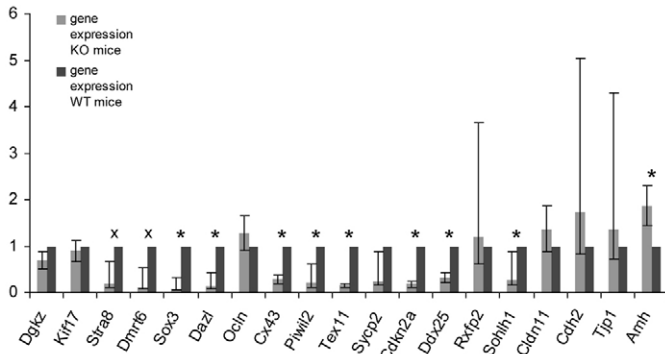


Fig. 6. Relative gene expression of WT mice versus SCCx43KO mice after applying the calculated correction factors. Bars represent mean \pm s.d. mRNA levels of three independent qRT-PCR experiments. Calculations were realized determining *Actb* and *Hsp90ab1* as housekeeping genes and applying the $\Delta\Delta C(t)$ method. The genes *Sox3*, *Dazl*, *Cx43*, *Pih1l2*, *Tex11*, *Ddx25*, *Cdkn2a* and *Sohlh1* were significantly downregulated (* $P < 0.05$), whereas the downregulation of *Stra8* and *Dmrt6* was close to significant (x). The only significant upregulation was detected for *Amh*.

(Ruggiu et al., 1997). Like *Stra8*, KO studies revealed a pivotal role for this gene in spermatogenesis. Interestingly, the nature of the *Dazl* KO is very similar to the observed one in SCCx43KO mice. In both murine lines the males are infertile and exhibit a reduced number of GCs, and the remaining GCs show an arrest of spermatogenesis, precluding progression through meiotic prophase (Saunders et al., 2003). A comparison of significantly regulated genes in 5- and 7-day-old testes of WT and *Dazl* KO mice with results from the present study showed that just two genes were significantly regulated in both experiments, namely *Ovol1* and *Nfx2* (Maratou et al., 2004). These results could lead to the conclusion that there might be a synergistic action of *Dazl* and *Cx43*, rather than a shared pathway leading to the meiotic failure observed in the two mutant mouse lines. Moreover, *Dazl* has been demonstrated recently to have a significant contribution in the differentiation of embryonic stem cells into pre- and post-meiotic GCs (Kerr and Cheng, 2010), and humans with spermatogenic failures showed significantly lower levels of *Dazl* transcripts compared with men with normal spermatogenesis (Lin et al., 2001). Furthermore, these data provide evidence for an important role of *Nxf2* and *Ovol1* for GC development beyond mitosis.

The DM family of genes encode a widely conserved transcription factor family; these proteins are involved in sexual differentiation

at least along three phyla (Murphy et al., 2007). Mammals express at least seven DM domain genes (*Dmrt1-7*) (Kim et al., 2003). *Dmrt1* is known to be essential for sexual differentiation in mammals (Raymond et al., 2001), whereas little information is available for *Dmrt6* [also known as *Dmrtb1* (FC -68.15)]. Kim et al. detected a very high expression of *Dmrt2*, 5, 6 and 7 in adult testis (Kim et al., 2007). Because it is known that *Dmrt7* [also known as *Dmrtc2* (FC -7.31)] is essential for male meiosis and spermatogenesis, we explored its protein expression comparing WT and SCCx43KO mice. Because DMRT protein is restricted to pachytene spermatocytes (and sperm), 15-day-old mice were used for this experiment. In these SCCx43KO mice, only single spermatocytes were present and showed a weak signal for DMRT7 protein, suggesting that the reduction of gene expression of DM family members observed in 8-day-old KO males proceeds and is finally abrogated in older KO mice, particularly because *Dmrt* mRNA has also been detected in spermatogonia (Kawamata et al., 2007). This hypothesis was confirmed by DMRT7 IF in adult KO mice. Based on the fact that at least two members of the DM-domain family (*Dmrt6* and *Dmrt7*) show an altered expression pattern, it is very probable that this gene family displays a contribution to the disturbed spermatogenesis and GC loss observed in adult SCCx43KO mice. Furthermore, *Dmrt1* has been linked with human testicular germ cell tumor development (Looijenga et al., 2006) and susceptibility (Kanetsky et al., 2011).

Beyond the genes confirmed by qRT-PCR and IHC or IF, the expression of several more genes, for example *Sohlh1* (FC -6.98), *Sox3* (FC -13.30), *Sycp2* (FC -8.90) and *Pih1l2* (FC -5.89), was significantly altered in our study and might also be interesting new candidate genes for comparative human investigations. *Sohlh1* and *Sox3* single KO mice show an arrest of spermatogenesis at the level of spermatogonia and, comparable to the present mouse model, their deletion leads to a disruption of GC differentiation beyond early developmental stages and to infertility (Raverot et al., 2005; Ballou et al., 2006). By contrast, the deletion of *Sycp2* and *Pih1l2* leads to a spermatogenic arrest from zygote to early pachytene stages (Kuramochi-Miyagawa et al., 2004; Yang et al., 2006). The Piwi family genes play essential roles in stem cell self-renewal and gametogenesis (Lee et al., 2006a). Thus, it might be possible that *Cx43* acts as a molecular upstream promoter of *Pih1l2* that is in turn known to be able to modulate the expression of stem-cell-specific genes, including *Stra8* or *Cx45*. These genes require further examination to elucidate their possible role in the phenotype of adult SCCx43KO mice and their connection to CX43.

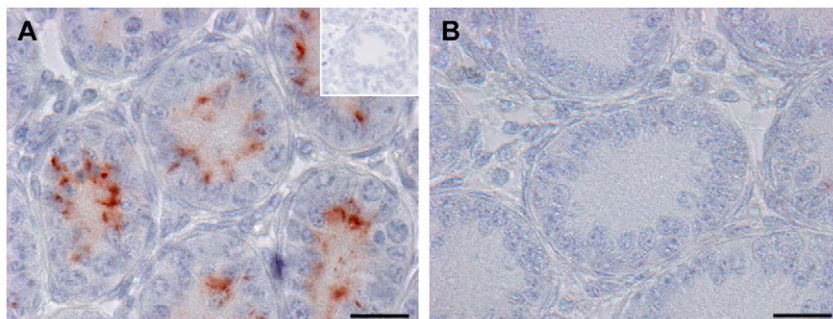


Fig. 7. Confirmation of *Cx43* deletion by IHC. In seminiferous cords of WT mice (A) a typical prepupal diffuse adluminal staining pattern for CX43 can be seen. By contrast, cords of SCCx43KO mice show no CX43 immunoreactivity at all (B). Insert in A: negative control. Scale bars: 20 μ m.

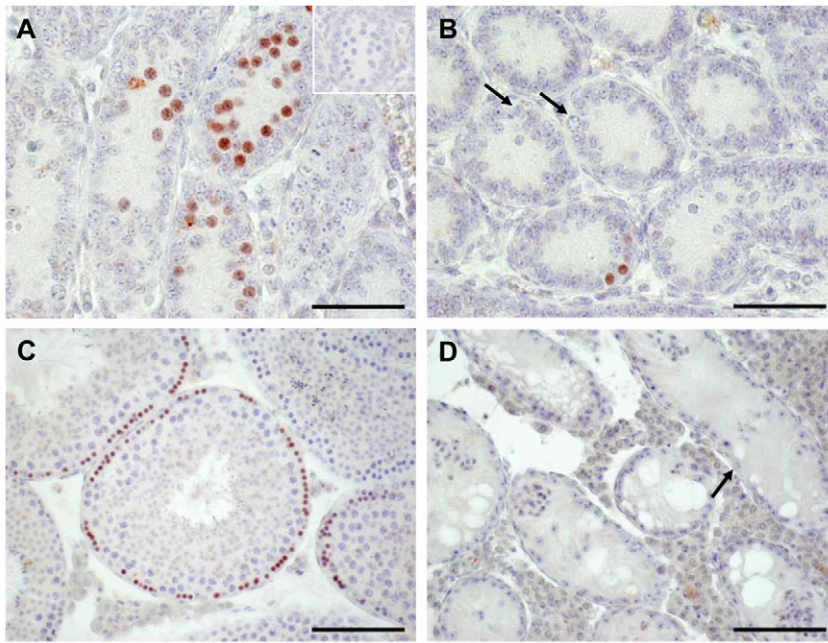


Fig. 8. IHC confirmed the findings obtained by microarray analysis and qRT-PCR for *Stra8* (GCs). The occurrence of STRA8 protein revealed obvious differences between WT (A,C) and SCCx43KO (B,D) mice. In seminiferous cords of the 8-day-old WT mice, many spermatogonia and first preleptotene spermatocytes show an intensive staining for STRA8 (A). By contrast, only single cells of the SCCx43KO mice revealed an immunoreactivity for STRA8 (B). Note the immunonegative spermatogonia in B (arrows). In adult WT mice, a stage-specific immunoreaction for STRA8 can be seen (C), whereas no immunoreaction at all can be detected in seminiferous tubules of adult mutants showing an arrest of spermatogenesis at the level of spermatogonia (arrow, D). Insert in A: representative negative control for STRA8 experiments. Scale bars: (A,B) 50 μ m; (C,D) 100 μ m.

CX43 gap junctions between SCs are further known to form an intercellular communication network within the seminiferous epithelium corresponding to an initial 'syncytium-like organization' that allows the tubular coordination of SC metabolism and, indirectly, via metabolic and signaling coupling, the synchronization of GC proliferation and differentiation. At present, it cannot totally be excluded that the failure to initiate spermatogenesis and the observed GC deficiency in adult SCCx43KO mice reflects an altered state of SC maturation and a sign for functional immaturity of the SC, as proposed by Sridharan et al. (Sridharan et al., 2007a). Thus, impaired GC development as early as day 8 p.p. might represent underlying abnormalities in SCs, because there exists a reciprocal

regulation of SC and GC differentiation, and functional SCs are a prerequisite for normal spermatogenesis (Sharpe et al., 2003; Griswold, 1995). Also, seminiferous tubules of adult humans with different forms of spermatogenic impairment house immature SC populations (Steger et al., 1996). Thus, a disturbed maturation of nursing SCs might consequently result in an asynchronous and altered differentiation of SC-dependent GCs, leading to alterations of candidate gene expression in GCs of SCCx43KO mice (and infertile men).

Multiple genes that are essential for BTB formation, such as *Ocln*, *Tjp1* or *Cld11*, were subjected to qRT-PCR analysis (Saitou et al., 2000). In agreement with the microarray data, examination of their

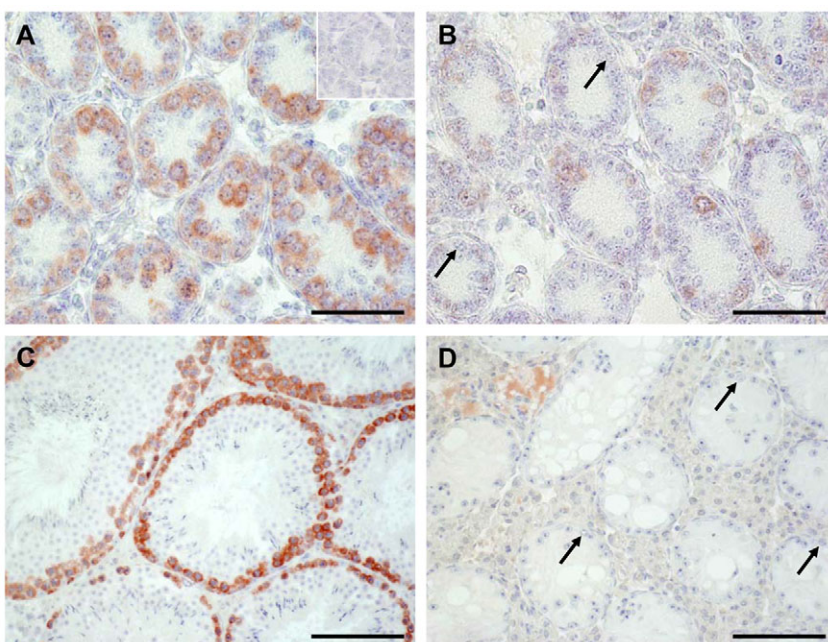


Fig. 9. IHC confirmed also the findings obtained by microarray analysis and qRT-PCR for *Dazl* (GCs). The occurrence of GC-specific DAZL protein revealed obvious differences between WT (A,C) and SCCx43KO (B,D) mice. The number of stained cells is drastically reduced in 8-day-old SCCx43KO mice (B), confirming results obtained by qRT-PCR. Note immunonegative spermatogonia in B (arrows). In adult mice, a clear immunoreaction for DAZL can be seen in spermatogonia and spermatocytes (C). By contrast, in adult SCCx43KO mice not even spermatogonia (arrows) show a DAZL immunostaining (D). Insert in A: representative negative control for DAZL experiments. Scale bars: (A,B) 50 μ m; (C,D) 100 μ m.

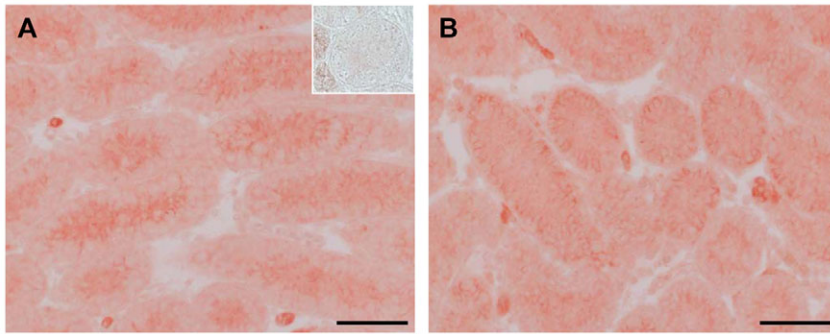


Fig. 10. IHC (without counterstaining) also approved the findings obtained by microarray analysis and qRT-PCR for *Ocln* (SCs). No differences for OCLN are detectable comparing WT (A) and SCCx43KO (B) mice. Insert in A: representative negative control for OCLN experiments. Note faint unspecific binding in LCs. Scale bars: 50 μ m.

mRNA expression on day 8 p.p. revealed no significant differences between WT and SCCx43KO mice, although most genes associated with this barrier showed a higher transcription rate in KO mice. Using IHC, localization of OCLN protein seemed to be similar in WT and SCCx43KO mice, implying no functional alterations.

Direct evidence for a possible relationship between CX43, junction dynamics and spermatogenesis derives from Carette et al. (Carette et al., 2010). By using SCCx43KO mice, specific anti-Cx43 siRNA and gap junction blockers in the SerW3 SC line, this study demonstrated that CX43-based gap junctions participate in the regulation of adherens and tight junction protein expression. Interestingly, increased protein levels of CDHN, β -CTNBN1 and OCLN, but decreased TJP1 levels, were observed in adult KO mice compared with their WT littermates (Carette et al., 2010). Because no significant alterations in *Tjp1* and *Ocln* gene expression profiles have been detected in the present study at day 8 p.p., the observed alterations in TJP1 and OCLN levels in adult KO mice seem to occur after postnatal day 8, together with a functional BTB formation but possibly associated with (1) an impairment in the dynamic process of opening and closing of this barrier and/or (2) permanent BTB closure as suggested by the authors. The close relationship between gap and adherens junctions has been

demonstrated in studies investigating CDHE, and it was shown that CDHE (*Cdh1*; FC -2.8 in the present study) was able to influence the intracellular trafficking and function of both CX26 and CX43 (Hernandez-Blazquez et al., 2001). In the human testis, an altered expression of TJP1 and TJP2, and a dysfunction of claudin-11, have been demonstrated in SCs associated with CIS (Fink et al., 2006; Fink et al., 2009).

Because it has been further demonstrated that CX43 immunolocalization shifts from the luminal to the basal region of the seminiferous epithelium concomitant with BTB formation (Bravo-Moreno et al., 2001), it may be speculated that CX43 has a functional role in the compartmental reorganization of the seminiferous epithelium during puberty. Thus, it is possible that loss of CX43 in SCs results in an altered organization of the seminiferous epithelium leading to (1) morphological, structural and/or metabolic disorganization, (2) altered synchronization of SC-GC metabolism and (3) altered maturation during the first waves of spermatogenesis. This again could result in impaired spermatogenesis in adult SCCx43KO mice.

In contrast to adult SCCx43KO mice, in which the number of SCs per tubule cross-section is significantly higher in comparison to WT mice (Brehm et al., 2007; Sridharan et al., 2007a), no

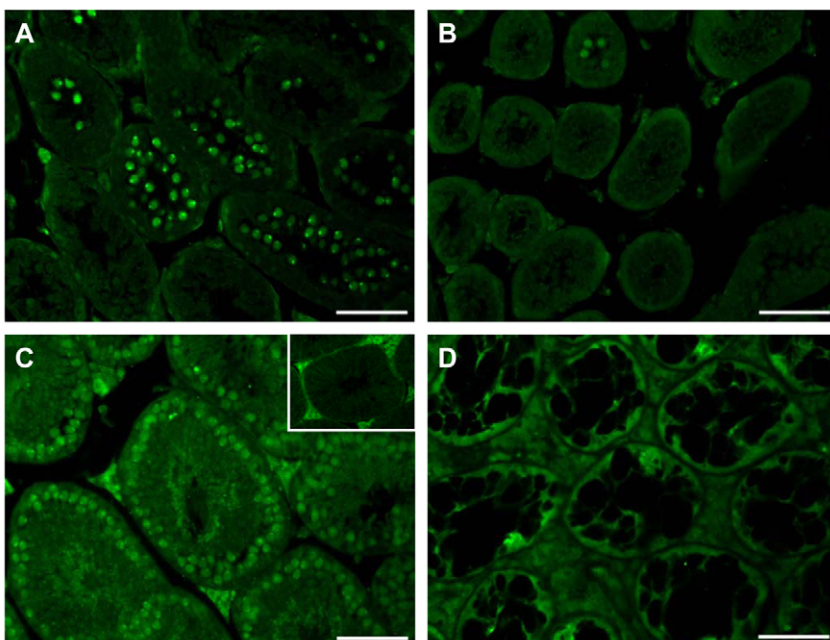


Fig. 11. IF for DMRT7. WT (A,C) and SCCx43KO (B,D) on day 15 p.p. (A,B) and in adulthood (C,D) were compared. Results showed a higher number of DMRT7 immunopositive cells in pubertal WT testis compared with SCCx43KO mice (A,B). In adult WT males, a specific staining can be detected in nearly all spermatocytes (C), whereas only paraffin autofluorescence is seen in KO tubules (D). Insert in C: note paraffin autofluorescence in interstitial LCs in negative controls. Scale bars: 50 μ m.

significant difference concerning the SC number between 8-day-old KO and WT mice can be found. In rodents, SC proliferation takes place during the fetal period until 3 weeks p.p., with a maximum at around 2 days before birth (Orth, 1982). With puberty the mitotic activity stops, SCs mature and the final number of SCs is established. Owing to the Cre mice used in this study (*Amh-Cre*; Cre-expression under the control of the *Amh* promoter), possible alterations do mainly occur in our SCCx43KO mice during the time period with the highest mitotic activity and proliferation of SCs. This fact supports the assumption of a remaining proliferation potential (capacity) of SCs in adult SCCx43KO mice, as suggested by Sridharan et al. (Sridharan et al., 2007b).

By contrast, the GC number was significantly decreased as early as day 8 p.p. in SCCx43KO mice, similar to results seen in adult KO mice. These results are supported by other published data obtained by grafting the testes of *Cx43*-deficient mice under the kidney capsule of WT mice, showing that the GC population fails to expand postnatally in the absence of CX43 (Roscoe et al., 2001). In the last decade, different *Cx43* KO mice have been generated and the importance of *Cx43* to male gametogenesis has been shown: e.g. there is a 50% depletion of primordial GCs in fetal male mice lacking the *Cx43* gene (Juneja et al., 1999) and a significant reduction of GCs in adult SCCx43KO mice (Brehm et al., 2007; Sridharan et al., 2007a) (and in the present study). It was further demonstrated that primordial GCs are already gap-junction intercellular-communication-competent cells and that GC deficiency in *Cx43* KO embryos might arise from an increased apoptosis by abnormal p53 activation (Francis and Lo, 2006). A recent in vitro study confirms the involvement of CX43 gap junctions in the regulation of GC number by controlling spermatogonia survival rather than proliferation (Gilleron et al., 2009). To answer the question of whether the number of GCs is already altered before postnatal day 8 and whether the migration of GCs is altered, new breedings to obtain younger KO mice have been performed. Preliminary results comparing KO and WT mice at day 2 p.p. showed that already at this very early postnatal age the number of GCs per tubule cross-section is drastically reduced in KO mice compared with WT. To investigate even earlier (fetal) developmental ages, for the investigation of primordial GC migration, a study design similar to that of Francis and Lo (Francis and Lo, 2006) and new breeding strategies are planned. These data imply an important role for *Cx43* in SCs on maintaining the number of GCs by regulating CX43 gap junctions. Whether CX43 from SCs is involved in GC growth by controlling primordial GC and/or spermatogonia survival rather than their proliferation, as shown by Gilleron et al. (Gilleron et al., 2009), remains to be elucidated for SCCx43KO mutants in future studies. In this context, it was shown that a low spermatogenic efficiency in infertile men showing an arrest of spermatogenesis at the level of spermatogonia was not only due to postmeiotic events, but also to a decrease in the mitotic activity of spermatogonia (Steger et al., 1998). And, finally, it was demonstrated that many infertile individuals face, besides meiotic defects, the problem of reduced numbers of spermatogonia and spermatogonial stem cells (Hentrich et al., 2011).

Our data reveal that (1) there are considerable differences in the gene expression patterns of SCCx43KO and WT mice at day 8 p.p. (and these differences have a negative impact on protein synthesis in young and adult KO mice), (2) SC-specific deletion of *Cx43* seems

to effect primarily GCs and (3) expression of important GC-specific genes seems to be *Cx43* associated and/or regulated. Because several of the altered genes have been shown to be essential for normal (mitotic and meiotic) progression of GC development and initiation of spermatogenesis, it was additionally demonstrated that corresponding proteins are altered in adult mutants. Thus, these genes and their proteins could be involved in the developmental and histological disorder observed in adult SCCx43KO mice (and in similar human spermatogenic defects).

In conclusion, observed changes in gene expression in the mutant mice suggest a role for SC-GC crosstalk via CX43 gap junctions. However, in addition to its classical role in direct intercellular communication, observed alterations in KO gene expression might also be due to (1) a loss of CX43 hemichannel function in SCs and consequently within the seminiferous epithelium, (2) a loss of direct or indirect interaction with other junctional proteins and/or (3) a loss of interaction or binding of CX43 in SCs to corresponding cytoskeleton partners. Thus, loss of CX43 hemichannels in SCs, which might act as a paracrine conduit to spread factors that modulate the fate of SCs themselves, and GCs can lead to their maturational arrest.

Changes in candidate gene expression patterns could have also been caused by (4) a loss of direct *Cx43* effects on gene transcription, because it was demonstrated in a study working with fetal *Cx43*-null mutants that the loss of the *Cx43* gene is able to exert profound effects on the expression pattern of other testicular CX genes. In fetal testes of *Cx43* KO mice, only four CX mRNAs were detectable (*Cx26*, *Cx37*, *Cx40* and *Cx45*), compared with normal fetal WT testes exhibiting eight CX members (Juneja, 2003). In the present study, a 'close to significant' downregulation of *Gja6* (coding for CX33) was detected, confirming its close association with CX43 (Fiorini et al., 2004). It is finally also possible that (5) a disturbed maturation of nursing SCs might consequently result in alterations in: differentiation of SC-dependent GCs, candidate gene expression in GCs of SCCx43KO mice and the observed GC numbers.

A time-course study comparing SCCx43KO and WT mice from day 6, 10, 12 and 14 p.p. would be useful in confirming the role of these candidate genes and could lead to the identification of altered pathways before and during the first waves of spermatogenesis. Moreover, current studies are comparing histological phenotypes of impaired spermatogenesis and significantly altered candidate genes in SCCx43KO mice with corresponding deficiencies in men by using human testicular biopsies. In addition, these biopsies will be investigated for possible mutations in the *Cx43* gene in SCs or GCs using UV-laser-assisted cell picking (microdissection approach).

It is for all these reasons that the established transgenic SCCx43KO mouse model provides an interesting tool to study and understand the causes of impaired spermatogenesis because it is directly relevant to different corresponding cases of human subfertility and sterility.

METHODS

Mice

Breeding strategy, genotyping and confirmation of *Cx43* gene loss by β -galactosidase IHC of WT and homozygous SCCx43KO mice were carried out as described previously (Brehm et al., 2007). For

all experiments, 8-day-old mice were used ($n=3$ per genotype), except for IF where 15-day-old mice were utilized ($n=3$ per genotype), because DMRT7 protein is known to be first detectable from day 13.5 onwards. Additionally, for STRA8 and DAZL IHC and for DMRT7 IF, adult WT and SCCx43KO mice ($n=3$ per genotype) were investigated. Three KO mice and two WT mice were littermates, one WT mouse was from another litter but from the same parents. Genotyping showed that the used KO animals were all 'homozygously floxed *Cx43* and *Cre* positive' mice, whereas the WT mice (littermates) were '*Cx43* homoflox but *Cre* negative'. Within the WT group (microarray), two subclusters were present, i.e. WT1 versus WT2/3. This small difference in the expression profile can be explained because WT2 and 3 were from the same litter whereas WT1 was from another litter but from the same mouse (parents). Animal experiments were approved by the animal rights committee at the regional commission of Giessen, Germany (decision V54-19c 20/15 c GI 18/1) and the ethics commission at the University of Giessen, Germany (decision 56/05).

Tissue retrieval and treatment

Mice were anesthetized and sacrificed by intraperitoneal injection of an overdose cocktail of ketamine hydrochloride (Medistar, Holzwickede, Germany) and xylazine (Serumwerk Bernburg, Bernburg, Germany). The body and testis weights were defined. Both testes were removed immediately. One testis was snap-frozen in liquid nitrogen and then stored at -80°C until RNA extraction, the other was placed in Bouin's solution overnight for IHC and hematoxylin and eosin staining (H&E), followed by paraffin embedding. For IF the second testis was placed in formalin for 2 hours before paraffin embedding.

Histochemical techniques

For histological evaluation and IHC, 5- μm slices from all KO and WT mice were sectioned. Sections 1, 10, 20, 30, 40 and 50 were stained with H&E according to standard techniques; sections 2, 11, 21, 31, 41 and 51 were used for β -galactosidase IHC. With the latter sections and method, the mean number of SCs (nuclei) per tubule cross-section was determined, avoiding in this way that single spermatogonia mimic and displace SCs, resulting in reproducible SC numbers per tubule cross-section. Then, the number of SCs and GCs per tubule cross-section was counted for at least 25 tubules per section and the average number was calculated to compare the real cell composition of WT and SCCx43KO mice. This quantification method was published previously (Brehm et al., 2007; Weider et al., 2011b). Quantitative microscopic analysis was performed using a Leica DM LB microscope (Leica, Wetzlar, Germany) at a magnification of $400\times$. Statistical evaluation was done using BMDP Statistical Software Package (Statistical Solutions Ltd, Cork, Ireland).

On the basis of this data, a correction factor of $1/(\text{OGC number})$ was calculated for GC-specific genes and a factor of $1/(\text{OGC} + \text{OSC number})$ for genes expressed in GCs and SCs. This implied a factor of approximately $1/3$ for WT and $1/1$ for SCCx43KO mice for genes exclusively expressed in GCs and a factor of ca. $1/26$ for WT and $1/24$ for SCCx43KO mice for genes expressed in both GCs and SCs. The obtained factor was used to correct the data of the microarray analysis by the number of GCs for genes expressed only in GCs or in both cell compartments.

RNA extraction

To carry out microarray analysis, one testis of three mice of each genotype was used. Total RNA was prepared using RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Microarray analysis

Gene expression profiling

RNA was subjected to transcription and hybridization as follows: target preparation was done using the MessageAmp II Kit (Ambion, Applied Biosystems) following the original protocol. Briefly: $1\ \mu\text{g}$ total RNA was used in cDNA synthesis reaction with a poly-A binding primer containing the T7-polymerase promoter. Resulting cDNA was transcribed into cRNA in one round amplification in the presence of 11-Bio-UTP. Double-stranded cDNA and biotin-labeled cRNA were purified using the mini columns included in the kit. The eluted cRNA was quantified with the NanoDrop (NanoDrop Technologies, Rockland, DE) and a quality profile with the Agilent 2100 bioanalyzer (Agilent Technologies) was made. Portions of $20\ \mu\text{g}$ cRNA were subjected to fragmentation in the presence of Mg^{2+} . Subsequently, $10\ \mu\text{g}$ fragmented cRNA (target) was loaded onto CodeLink Mouse Whole Genome Microarray glass slides containing 36,227 probe sets (Applied Microarrays, Tempe, AZ) and hybridized for 18 hours in a Minitron shaker incubator (Infors AG, Bottmingen, Germany) at $37^{\circ}\text{C}/300\ \text{rpm}$ ($n=2$ arrays per sample). Washing and dyeing with Cy-5-coupled streptavidin was done according to the original protocol for CodeLink arrays (Applied Microarrays, Tempe, AZ) and the arrays were scanned using a GenePix 4000 B scanner and GenePix Pro 4.0 Software (Axon Instruments, Arlington, TX).

Spot signals of CodeLink bioarrays were quantified using CodeLink System Software consisting of Batch Submission (V2.2.27) and Expression Analysis (V2.2.25) (GE Healthcare) as outlined in the user's manual. CodeLink Expression Software 1.21 generated background corrected raw as well as median centered intra-slide normalized data. The intra-slide normalized data were used for further analysis. The software automatically calculated thresholds for intra-slide normalized intensities for each array and flagged genes as TRUE when the gene intensity was higher than the threshold or FALSE when the intensity was lower than the threshold. The present call of a microarray was given as the ratio of genes flagged as TRUE/total number of genes on microarray. Microarrays subjected to data analysis showed a mean present call of 83%, indicating a high number of genes above threshold, i.e. being flagged as TRUE. Furthermore, the software flagged each gene value as GOOD, EMPTY, POOR, NEG or MSR, defining different quality measures as outlined in the user's manual. Only gene values flagged as GOOD or EMPTY were used in the following analysis workflow:

(1) Definition of groups: Group 1: WT mice [testes of three WT mice at day 8 p.p. (three biological replicates), each on two microarrays (two technical replicates)]. Group 2: SCCx43KO mice [testes of three KO mice at day 8 p.p. (three biological replicates), each on two microarrays (two technical replicates)]. A total of 12 microarrays (six per group) were subjected to analyses.

(2) Removal of genes with a high number of missing values or of values being flagged as FALSE: genes with missing values $\geq 50\%$ of all arrays in a group were excluded from the dataset. Genes that

were flagged as FALSE in >50% of arrays in each group were also excluded from the dataset. A total of 26,044 probe sets remained after quality control.

(3) Gene expression values deviating more than three times from the group median were classified as outliers and have been removed.

(4) Imputation of remaining missing values: remaining missing values were imputed using sequential K-nearest neighbor (SKNN) imputation (Kim et al., 2004) with $k=5$.

(5) Normalization of imputed dataset: imputed dataset was normalized using quantile normalization in R (Bolstad et al., 2003) and logged to base 2.

(6) Array outlier detection: dissimilarity matrices of the normalized dataset were generated in AVADIS-Pride (Gwadry et al., 2005) to determine outlier arrays within the dataset. No outlier arrays were identified in the dataset.

(7) Correction for differing GC numbers: to exclude that the differentially gene expression was due to the differing numbers of GCs in the two genotypes, microarray data were normalized with the correcting factor obtained by the statistical evaluation of the morphometrical data. There was nearly no difference in the FC of gene expression either with or without adjustment for cell numbers.

(8) Statistical analysis of microarrays: for each gene, the mean value of all technical replicates of a mouse and the FC thereof was calculated in MAYDAY (Battke et al., 2010). To identify differentially regulated genes between WT and SCCx43KO mice, the dataset was subjected to a two-class rank statistics [Rank products (RP)] as described below (Breitling et al., 2004; Breitling and Herzyk, 2005). Additionally, the Student's t -test was applied. For each gene, a significance level of P -value < 0.05 was determined for both tests combined with an FC of at least twofold. A differentially regulated gene was considered to be significant when the P -value in the t -test was less than 0.05, the P -value in RP was less than 0.05 and the FC was equal to/higher than 2 or equal to/less than 2.

(9) Annotation of genes: significantly regulated genes were annotated using the web-based annotation tools SOURCE (Diehn et al., 2003) and the Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resources 2008) (Dennis et al., 2003) as described in the manual.

(10) Hierarchical cluster analysis: hierarchical cluster analysis of the significantly genes was performed in dCHIP using Euclidean distance and centroid linkage.

(11) Enriched functional categories: enriched functional categories within the differentially regulated genes were determined using DAVID (Dennis et al., 2003) version 2.0. Based on statistical methods, significance levels were calculated that describe the overrepresentation of functional categories by a list of genes – the differentially regulated genes in our case. If a GO category or biological process is significantly overrepresented, which means that a relatively high number of genes assigned to the respective function is present in the selection, this will result in a low P -value. In functional annotation clustering, GO categories are grouped according to the genes from the list of regulated genes that have been assigned to them. An enrichment score is calculated for each cluster, based on the proportion of significantly overrepresented biological processes. Clusters with an enrichment score above 1.2 have been taken into account. Those clusters represented at least

one significantly enriched biological process (i.e. with a P -value of less than 0.05).

(12) Pathway analysis: a relationship between genes and their connection to existing regulatory pathways was examined using Ingenuity Pathway Analysis (IPA), a web-based entry tool developed by Ingenuity Systems, Inc. (<http://www.ingenuity.com>).

Complete data are available at the Gene Expression Omnibus (GEO) database under the accession number GSE23431. This study adhered to the MIAME standards (Brazma et al., 2001).

cDNA synthesis and qRT-PCR

Changes in mRNA levels of altered genes identified by the CodeLink Mouse Whole Genome Bioarray were confirmed using qRT-PCR. Three biological replicates per genotype were processed. DNase treatment was performed according to manufacturer's instructions using the RNase-Free DNase Set (Qiagen, Hilden, Germany). cDNA was synthesized from 15 μ l total RNA (adjusted to 200 nmol/30 μ l) using MultiScribe Reverse Transcriptase (Applied Biosystems Inc., Darmstadt, Germany). Purification of cDNA was realized by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Specific primer pairs were created using Beacon Designer Software (Bio-Rad, München, Germany). Before use in the main experiment, primers were first tested in normal PCR to

Table 4. Primer sequences used in qRT-PCR experiments

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Actb</i>	TTCCTTCTGGGCATGGAGT	TACAGGTCCTTGGCGATGTC
<i>Hsp90ab1</i>	AAGAGAGCAAGGCAAAGTTTGAG	TGGTACAATGCAGCAAGGT
<i>Dgkz</i>	CCTGGAGGTCATCGGCTTCCAC	TCTCGGCACTGCGTCAATCG
<i>Kif17</i>	ACTCCTCCCGTCCCACTCC	GCCAGGCTACCAGGTTGAGC
<i>Stra8</i>	CGCCTTCGCAGACCTACC	GCTCGCTCCCTGGCTGTC
<i>Sox3</i>	GCCCTGGAGAACCCCAAGATG	ACCGCACGCAGTCGCTTG
<i>Tex11</i>	CATCTCTGTGCGTCTTACGTTGC	ATTCCGCTTGCTTCTATGC
<i>Sycp2</i>	GGCTGACCCATCGTGGACAG	ACACCCGCAGACACTTTAGGC
<i>Dmrt6</i>	CGACTACGGGCATCCTCTGAG	GACGCAGGCAGGGCACTG
<i>Sohlh1</i>	CAGGAGGCGGATCTCGTTGAG	ATGCTGTGGCAAGCTGGAG
<i>Rxfp2</i>	TCCGTGGGCGTCTTTGACATC	GCAGCAGCACCAGACCTC
<i>Tjp1</i>	CCCTACCAACTCGGCCTT	AACGCTGGAAATAACCTCGTTC
<i>Ocln</i>	ATCCTGTCTATGCTCATTATTGTG	CTGCTCTGGGTCTGTATATCC
<i>Cldn11</i>	CGTCATGGCCACTGGTCTCT	GGCTCTACAAGCCTGCACGTA
<i>Cdh2</i>	TGGCAATCAAGTGGAGAACC	ATCCGCATCAATGGCAGTG
<i>Cdkn2a</i>	TGGTGAAGTTCTGTGCGATCCC	GGTGGCGCCCTCTTCTCAAG
<i>Dazl</i>	GGAGGCCAGCACTCAGTCTTC	AGCCCTTGACACACCAGTTC
<i>Cx43</i>	ACAGCGGTTGAGTCAGCTTG	GAGAGATGGGAAGGACTTGT
<i>Amh</i>	CCAACGACTCCCGCAGCTC	CTTCCCGCCCATGCCACTC
<i>Ddx25</i>	CTGCCAGACAGTCGAAATGC	TCGCTGCTCCACTGTGAGTTC
<i>Piwil2</i>	CAGAAGACTCCAGCCACCAC	TCAAGACCCATGCCACGGAAC

Actb, actin beta; *Hsp90ab1*, heat shock protein 90 kDa alpha, class B member 1; *Dgkz*, diacylglycerol kinase zeta; *Kif17*, kinesin family member 17; *Stra8*, stimulated by retinoic acid gene 8; *Sox3*, SRY-box containing gene 3; *Tex11*, testis expressed gene 11; *Sycp2*, synaptonemal complex protein 2; *Dmrt6*, DMRT-like family B with proline-rich C-terminal, 1; *Sohlh1*, spermatogenesis and oogenesis specific basic helix-loop-helix 1; *Rxfp2*, relaxin/insulin-like family peptide receptor 2; *Tjp1*, tight junction protein 1; *Ocln*, occludin; *Cldn11*, claudin 11; *Cdh2*, cadherin 2; *Cdkn2a*, cyclin-dependent kinase inhibitor 2A; *Dazl*, deleted in azoospermia-like; *Cx43*, gap junction protein, alpha 1; *Amh*, anti-Muellerian hormone; *Ddx25*, DEAD (Asp-Glu-Ala-Asp) box polypeptide 25; *Piwil2*, piwi-like homolog 2 (*Drosophila*).

TRANSLATIONAL IMPACT

Clinical issue

Although a significant decline in human male reproductive function has been reported for the past 20 years, the molecular mechanisms responsible remain poorly understood. Recent studies indicate that the gap junction protein connexin-43 (CX43) – the predominant testicular connexin in most species, including humans – might be involved. For example, many individuals with impaired spermatogenesis exhibit a reduction or loss of CX43 expression in germ cells and Sertoli cells (SCs; part of the seminiferous tubule). It has been shown that adult male transgenic mice with a conditional knockout of the *Gja1* gene (also referred to as *Cx43*; encoding CX43) in SCs (SCCx43KO) show a testicular phenotype similar to such individuals and are infertile.

Results

Here, the authors sought to identify possible signaling pathways and molecular mechanisms underlying the testicular phenotype and failure to initiate spermatogenesis in SCCx43KO mice. They used microarray analysis to compare testicular gene expression of 8-day-old SCCx43KO and wild-type (WT) mice. This analysis revealed that 658 genes were significantly regulated in testes of SCCx43KO mice. Of these genes, 135 were upregulated and 523 genes were downregulated. Most downregulated genes, including *Stra8*, *Dazl* and members of the DM (*dsx* and *map-3*) gene family, are germ-cell-specific and essential for mitotic and meiotic progression of spermatogenesis. Other genes altered are associated with transcription, metabolism, cell migration and cytoskeletal organization. These data show that deletion of *Cx43* in SCs leads to changes in the expression of multiple genes in prepubertal mice, and affects primarily germ cells.

Implications and future directions

These data indicate that, among the different transgenic mouse models that are used to elucidate the multiple mechanisms underlying human male infertility, SCCx43KO mice provide a unique model for identifying candidate genes in germ cells. The candidate genes identified in this study could represent useful markers in investigations of human testicular biopsies from patients with spermatogenic deficiencies, and for studying the molecular mechanisms of human male infertility.

confirm that only one specific band could be produced. Afterwards, a qRT-PCR with a dilution series was performed to detect the efficiency of the qRT-PCR reaction and to prove that only one product can be detected within the melting curve. A representative number of PCR products were sequenced additionally. Only primers passing the multiple testing without interceptions finally were used. qRT-PCR was performed using IQ SYBR Green Supermix and CFX96 Real-Time System (Bio-Rad, München, Germany). Per sample, 1 μ l cDNA was used for amplification. Specific forward and reverse primers (Table 4) were added to the Mastermix. Cycling conditions were 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 1 minute. After 95°C for 10 seconds melt curve was produced with 65°C to 95°C and an increment of 0.5°C every 5 seconds. *Actb* and *Hsp90ab1* were used as housekeeping genes. All experiments included negative controls lacking cDNA and were carried out as triplicates. Based on the $\Delta\Delta C(t)$ method, evaluation of the qRT-PCR was realized using the CFX Manager Software (Bio-Rad, Germany).

IHC and IF

In order to confirm whether the alterations at RNA level are reflected in protein synthesis, IHC and IF was applied. Slides were dewaxed, rehydrated and microwaved in sodium citrate buffer, pH

6.0. Tissue sections were treated with 3% H₂O₂, blocked with 5% bovine serum albumin to prevent nonspecific binding, followed by incubation with primary antibody at 4°C overnight. Primary antibodies were: (1) rabbit anti-STRA8 (stimulated by retinoic acid gene 8; 1:250; kindly provided by Michael Griswold, Pullman, Washington, DC), (2) rabbit anti-CX43 (1:100; New England Biolabs GmbH, Frankfurt, Germany), (3) rabbit anti-OCN (1:500; Invitrogen, Karlsruhe, Germany) and (4) mouse anti-DAZL antiserum (deleted in azoospermia-like Dazla; 1:250; kindly provided by Howard Cooke, Edinburgh, UK). The slides were covered with a corresponding (compatible) biotinylated secondary antibody (goat anti-rabbit, goat anti-mouse or rabbit anti-goat, from DAKO, Hamburg, Germany) at room temperature (RT) for 1 hour, followed by the application of peroxidase-conjugated streptavidine (VECTASTAIN Elite ABC Standard Kit; Peroxidase, Biologo, Kronshagen, Germany) for 1 hour at RT. Peroxidase activity was visualized by the Peroxidase Substrat Kit AEC (Biologo, Kronshagen, Germany). For IF, formalin-fixed testes were used. After removal of the paraffin wax, rehydration and cooking in sodium citrate buffer with pH 6.0, slices were blocked with 5% goat serum for 1 hour and incubated overnight with the primary rabbit anti-DMRT7 antibody (1:200; kindly provided by David Zarkower, Minneapolis, MN). Exposure of the slices to a biotinylated secondary antibody (goat anti-rabbit; DAKO, Hamburg, Germany) at RT for 1 hour was followed by a 1 hour incubation with NeutrAvidin, DyLight 488 Conjugated (Fisher Scientific GmbH, Schwerte, Germany) and covering with Aqua Polymouth (Polysciences, Inc., Warrington, PA). Negative controls for both IHC and IF were performed by omitting the primary antibody.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

S.G., H.H., T.C., M.B. and R.B. were involved in conception and design of the experiments and co-wrote the manuscript; S.G. and H.H. analyzed the data; S.G. performed most of the experiments, e.g. breeding of SCCx43KO mice and corresponding littermates, H&E stainings, RNA extraction, cDNA synthesis, qRT-PCR and IHC or IF; S.T. performed the microarray analysis; M.M. and K.F. performed statistical analysis and determined the used correction factors; F.G. provided the AMH-Cre mice for the breeding experiments; K.W. prepared figures, tables and supplemental data for publication; all authors read and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at <http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.008649/-/DC1>

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