Connexin 43 expression in human and mouse testes with impaired spermatogenesis

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Connexin 43 (Cx43) belongs to a family of proteins that form gap junction channels. The aim of this study was to examine the expression of Cx43 in the testis of a patient with Klinefelter's syndrome and of mice with the mosaic mutation and a partial deletion in the long arm of the Y chromosome. These genetic disorders are characterized by the presence of numerous degenerated seminiferous tubules and impaired spermatogenesis. In mouse testes, the expression and presence of Cx43 were detected by means of immunohistochemistry and Western blot analysis, respectively. In testes of Klinefelter's patient only immunoexpression of Cx43 was detected. Regardless of the species Cx43 protein was ubiquitously distributed in testes of reproductively normal males, whereas in those with testicular disorders either a weak intensity of staining or no staining within the seminiferous tubules was observed. Moderate to strong or very strong staining was confined to the interstitial tissue. In an immunoblot analysis of testicular homogenates Cx43 appeared as one major band of approximately 43 kDa. Our study adds three more examples of pathological gonads in which the absence or apparent decrease of Cx43 expression within the seminiferous tubules was found. A positive correlation between severe spermatogenic impairment and loss of Cx43 immunoreactivity observed in this study supports previous data that gap junctions play a crucial role in spermatogenesis. Strong Cx43 expression detected mostly in the interstitial tissue of the Klinefelter's patient may presumably be of importance in sustaining Leydig cell metabolic activity. However, the role of gap junction communication in the control of Leydig cell function seems to be more complex than originally thought.

Key words: Connexin 43, testis, klinefelter syndrome, mutant mice.

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Paper accepted on October 5, 2007

European Journal of Histochemistry
2007; vol. 51 issue 4 (October-December):261-268

Gap junctions are intercellular protein channels that provide a pathway for exchange of ions, second messengers and small molecules. This exchange allows electrical and metabolical coupling of cells as well as coordinated responses of coupled cells to hormones and growth factors (Bruzzone *et al.*, 1996; Saez *et al.*, 2003). Connexins (Cxs) are the principal protein components of gap junctions. Although the presence of several connexin proteins has been reported in various tissues including testes, Cx43 was found to be the most abundantly expressed gap junction protein in testicular cells (Pelletier *et al.*, 1995; Lablack *et al.*, 1998; for review, see Pointis *et al.*, 2005). However, the localization of Cx26 and Cx32 between Sertoli cells and germ cells has been reported by Risley *et al.* (1992). Moreover, *Cx33* and *Cx50* transcripts have been localized to meiotic germ cells (Risley, 2000). Cx mRNAs of *Cx37* and *Cx40* have also been identified in the testis, but their specific cellular localization is unknown.

The history of the discovery of gap junctions in the testis has been reviewed in several papers (Bruzzone *et al.*, 1996; Evans and Martin, 2002; Pointis *et al.*, 2005). Besides the reviews reporting functional features of the channels (Paul, 1995; Simon and Goodenough, 1998) there are others on the role of gap junctions in various mature organs, including endocrine glands (Munari-Silem and Rousset, 1996; for review, see Saez *et al.*, 2003). Recently, the introduction of new techniques and approaches has increased our knowledge of gap junctions (Segretain *et al.*, 2004; Gilleron *et al.*, 2006; Lee *et al.*, 2006).

According to current data, gap junctions are essential in the control of male reproductive functions, especially spermatogenesis (Steger *et al.*, 1999; Batias *et al.*, 1999; Decrouy *et al.*, 2004). A putative role of Cx43 protein in the control of spermatogenesis has also been reported by the group of Minucci in nonmammalian vertebrates (Palmiero *et*

al., 2003; Izzo *et al.*, 2006). Direct evidence for the involvement of gap junction communication in spermatogenesis has been demonstrated by Juneja *et al.* (1999) and Roscoe *et al.* (2001) using connexin knock-out animals. *Cx43*-deficient mice exhibit a 50% depletion in primordial germ cells in fetal testes. In contrast to Cx43, knock-out mice for other Cxs are viable and no effects on spermatogenesis have been observed. An important role for Cx43 in spermatogenesis has also been evidenced using knock-in mice by Willecke and his group (Plum *et al.*, 2000). They generated two knock-in mouse lines, Cx43KI32 and Cx43KI40, in which the coding region of the *Cx43* gene was replaced by regions of either *Cx32* or *Cx40*, respectively. Both mutant mice are viable, however the males are sterile due to arrest of spermatogenesis at the level of spermatogonia which leads to a Sertoli-cell-only phenotype. A detailed mechanism that leads to the sterility of male mice in the two lines is still unknown. Very recently, the studies on conditional Sertoli cell Cx43 knockouts indicating the importance of this protein in testis and spermatogenesis have been described by Sridharan *et al.* (2007) and Brehm *et al.* (2007). To date, however, there is limited information on gap junctions in pathological testes (Batias *et al.*, 1999; Brehm *et al.*, 2002; Defamie *et al.*, 2003; Roger *et al.*, 2004).

Therefore, in this study we examine the expression of Cx43 protein in testes of a patient with Klinefelter's syndrome, in those of mice with the *mosaic* mutation and a partial deletion in the long arm of the Y chromosome. It should be added that the *mosaic* mutation (*Atp 7a*$^{mo\text{-}ms}$) arose spontaneously in the outbred mouse colony at the Department of Genetics and Evolution, Jagiellonian University in Krakow (Krzanowska, 1966), whereas the Y-del mutation was found during routine chromosomal analysis of B10. BR male mice in The National Institute of Genetics (Mashima, Japan). Since 1987, the Y-del mutants have been bred at the Department of Genetics and Evolution of the Jagiellonian University. Due to phenotypical similarities *mosaic* is classified in the group of X-linked *mottled* mutations in mice. Symptoms of the *mottled* mutations are caused by changes in the *Atp 7a* gene which encodes a protein belonging to the P-type ATP-ase family which is involved in ATP-dependent transport of copper across plasma or intracellular membranes (Gitshier *et al.*, 1998). *Mosaic* is a lethal mutation; affected males usually die on about day 16. Those which survive to reach sexual maturity are valuable research subjects. The *mosaic* mice and Y-del mice have been described in detail in our earlier reports (Styrna *et al.*, 2002; Kotula-Balak *et al.*, 2004a; 2007). They are characterized by small testes, a large proportion of degenerated seminiferous tubules, and, as a consequence, impaired or arrested spermatogenesis.

Materials and Methods

Human and animal materials

Testicular biopsy was obtained from a 31-year-old man with Klinefelter's syndrome, who had been subjected to ICSI-PESA/TESA procedures (ICSI - *intracytoplasmic sperm injection*; PESA - *epididymal sperm aspiration*; TESA - *testicular sperm aspiration*). Human testes of fertile men and of graft donor obtained after a sudden, unexpected death served as controls (n=3).

Mouse testes were obtained from two-month-old males (n=12) divided into two groups: (1) with the *mosaic* mutation (*Atp 7a*$^{mo\text{-}ms}$) (n=3) and respective controls (n=3), and (2) with a partial deletion in the long arm of the Y chromosome (B10. BR-Y^{del}) (n=3) and B10. BR as controls (n=3). Mice were bred in the Department of Genetics and Evolution of the Jagiellonian University (Krakow, Poland). All mice were given a commercial pelleted diet, water *ad libitum*, and maintained under a 12h light-dark cycle. Testicular sections were used for immunohistochemistry, whereas testes homogenates served as a source of protein for Western blot analysis.

Ethics of experimentation

All procedures were performed in accordance with the Polish legal requirements, under the licence given by the National Commission of Bioethics at the Jagiellonian University.

Immunohistochemistry

Sections of 5 μm thickness were obtained from formalin-fixed and paraplast-embedded testes. After dewaxing and rehydration, for antigen retrieval, slides were immersed for 2×5 min in 10mM citrate buffer (pH 6.0) and heated in the microwave oven (600 W). The whole procedure has been described in detail elsewhere (Levallet *et al.*, 1998; Hejmej *et al.*, 2005). Briefly, the sections were incubated in the presence of a rabbit polyclonal antibody against Cx43 (dilution, 1:100; Sigma

Chemical Co., St Louis, MO, USA). Next, biotinylated secondary antibody, goat anti-rabbit IgG (dilution, 1: 400; Vector, Burlingame CA, USA) was applied, followed by avidin-biotinylated horseradish peroxidase complex (ABC/HRP; dilution, 1:100; Dako, Glostrup, Denmark). Peroxidase activity was visualized using 3,3′-diaminobenzidine tetrachloride (DAB) as a substrate. Additionally, counterstaining with Mayer's haematoxylin was performed. All the slides were processed simultaneously so that Cx43 staining intensities in pathological gonads versus those of the controls could be compared. In negative-control sections, the primary antibody was omitted, or substituted by irrelevant IgG. At the end, the sections were examined with a Leica DMR microscope (Wetzler, Germany) using Nomarski interference contrast.

Western blot analysis

Samples of freshly dissected testes of the *mosaic* mice and Y-del mice and the respective controls were homogenized in Radio-Immunoprecipitation Assay buffer (RIPA; 0.05 mmol/L Tris-HCl, 0.15 mol/L NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0), and a protease inhibitor cocktail (Sigma). Protein concentration was determined using Bradford reagent (Bio-Rad Protein Assay; Bio-Rad, München, Germany) using serum bovine albumin (BSA) as a standard. Samples were mixed with an equal volume of 2x Laemmli buffer and boiled for 5 min. Then, protein aliquots (50 μg each) were separated on discontinous polyacrylamide gels (4.5% stacking gel and 12.5% separating gel by electrophoresis) under reducing conditions. After electrophoresis, the separated proteins were electrophoretically transferred to nitrocellulose membranes at 100 mA, overnight at 4°C. The membranes were blocked for 1h in TBS containing 1% BSA, and then incubated in the presence of a polyclonal rabbit anti-Cx43 antibody (1:8000; Sigma) for 2h. Next, the membranes were rinsed to remove the unbound antibody and incubated with a goat anti-rabbit IgG (1:1000; Vector) coupled to horseradish peroxidase (Dako) for 1.5h at room temperature. Bands were visualized by DAB (0.5 mg/mL) and 0.3% H_2O_2 dissolved in TBS. In order to check reproducibility of the findings three separate analyses were performed. Homogenates of a rat heart were used as a positive control. As a standard, Prestained SDS-PAGE Standards (Bio-Rad) were used.

Results

Immunohistochemistry

Widespread expression of Cx43 in both human and mouse testes, confirming previous data, was detected in seminiferous tubules of reproductively normal males (Figure 1A, Figure 2A-B), which was not the case in those with various testicular disorders (Figure 1B-D, Figure 2C-F).

In human control testes, staining for Cx43 between neighboring Leydig cells was very strong and linear in pattern, whereas in most of the tubules the signal of moderate intensity occurred in the basal compartment between Sertoli cells and spermatogonia or primary spermatocytes (Figure 1A). In testes of a patient with Klinefelter's syndrome, an absence of Cx43 expression was observed either in hyalinized seminiferous tubules depleted of germ cells (Figure 1B) or in tubules showing Sertoli cell-only-phenotype (Figure 1C). Small sites of Cx43 staining occured basal to spermatogonia (arrowheads) or between the remaining spermatocytes and Sertoli cells (Figure 1D). Human Leydig cells maintained strong to very strong intensity of Cx43 staining. Additionally, massive hyperplasia of Leydig cells was observed (Figure 1B-D). No staining was ever observed in the negative controls (see, insert in Figure 1A). However, the results obtained in the present study should be made with the caution, since there is only a single observation for a Klinefelter's patient.

In mouse control testes, the Cx43 signal of weak to moderate intensity was present between almost all Leydig cells (Figure 2A-B). Interestingly, the distribution pattern of Cx43 varied among the seminiferous tubules suggesting that Cx43 expression is stage-dependent (Figure 2A-B). A very strong staining was detected either apical to spermatogonia and basal to primary spermatocytes or between Sertoli cells in the region of the blood-testis barrier (Figure 2A, insert). Staining was also localized between Sertoli cells and secondary spermatocytes or round spermatids close to the lumen. In other tubules a very strong staining was restricted to the adluminal compartment where elongated spermatids are in close contact with Sertoli cells (Figure 2A). The immunoreactive signal occurred also basal and apical to spermatogonia, possibly between Sertoli cells and spermatogonia or pri-

mary spermatocytes (Figure 2B, insert). Differential distribution of the immunoreactive signal seems to be specific for Sertoli cells and dependent on the stage of the spermatogenic cycle, as indicated by previous authors (for review, see Pointis *et al.*, 2005).

In testes of the mosaic and Y-del mice, the staining intensity between neighboring mouse Leydig cells that formed numerous clusters was not reduced, being moderate or even strong (Figure 2C-F). Hyperplasia of Leydig cells was observed (Figure 2C-D, 2F) as in a human sample (Figure 1B-D). No staining or decrease in Cx43 expression in seminiferous tubules, likely associated with spermatogenic arrest conditions, was observed (Figure 2C-F) as reported previously for other testicular disorders (for review, see Pointis *et al.*, 2005; Pointis and Segretain, 2005). No staining was ever observed in the negative controls (Figure 2E-F inserts).

Western blot analysis

Immunodetectable Cx43 protein was observed as bands at the 43 kDa region of the SDS gel in testicular homogenates of control and mutant mice (Figure 3). The intensity of the immunoblots was similar in testes of mice with the *mosaic* mutation (*Atp 7a*$^{mo\text{-}ms}$) and of the the B10. BR-Y^{del} (lanes A2 and B2, respectively) when compared to the respective controls (lanes A1 and B1, respectively). Western blot analysis identified a 43 kDa band in rat heart as well (lane C), which was run as a positive control.

Discussion

During the last decade the testis has been thoroughly scrutinized for the presence of gap junction channels with both morphological and functional assays (Perez-Armendariz *et al.*, 1996; Chang *et al.*, 1996; Steger *et al.*, 1999; Risley, 2000).

Using immunohistochemistry we demonstrated

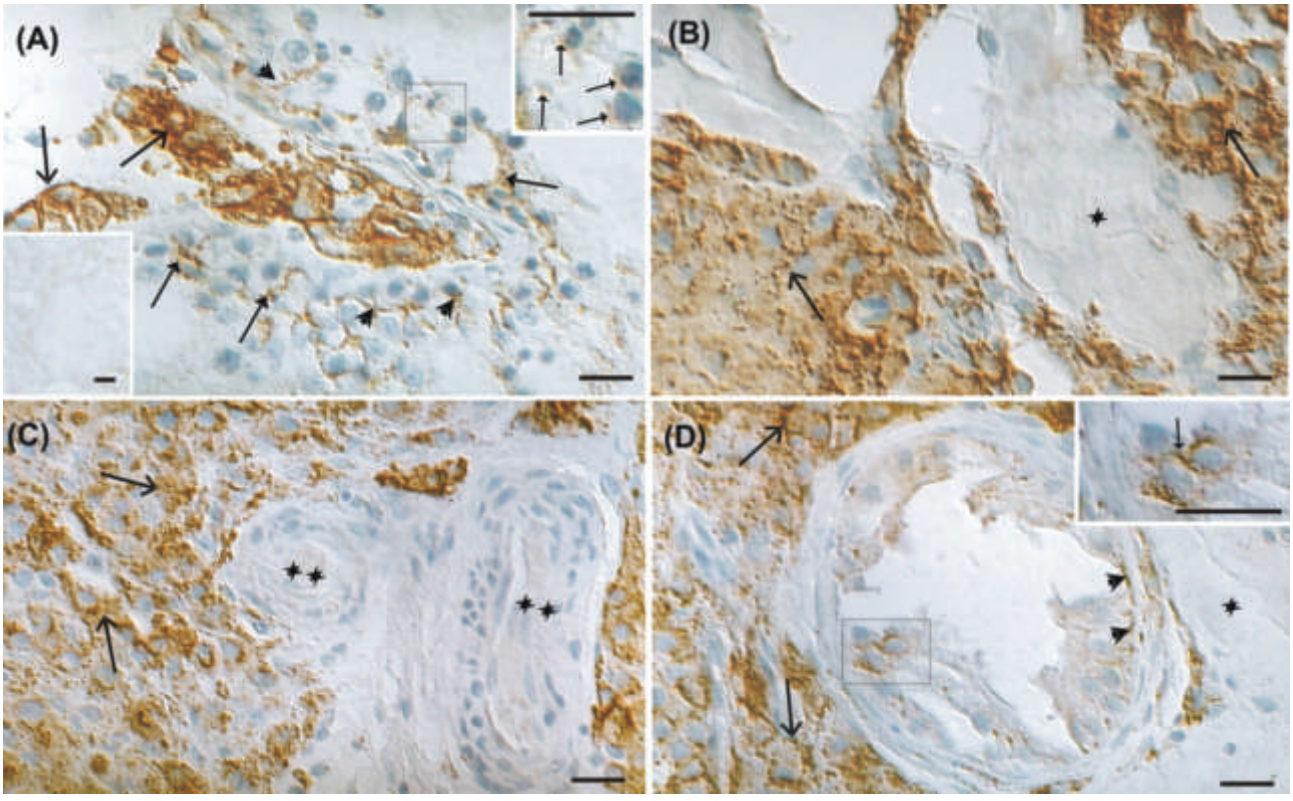

Figure 1. Immunohistochemical staining for Cx43 in human control testes (A) and those of a patient with Klinefelter's syndrome (B-D). Scale bars represent 20 μm. In most of the tubules of human control testes (A), the Cx43 signal occurs in the basal compartment between Sertoli cells (arrows, see an insert), between Sertoli cells and spermatogonia or primary spermatocytes (arrowheads). Very strong staining intensity is seen in Leydig cells (open arrowheads). In testes of a patient with Klinefelter's syndrome (B) no immunostaining for Cx43 is observed in hyalinized seminiferous tubules depleted of germ cells (asterisk) and in tubules showing Sertoli cell-only-phenotype (double asterisk) (C), whereas small sites of Cx43 staining occur basal to spermatogonia (arrowheads) or between the remaining spermatocytes and Sertoli cells (see, an insert in D). In the interstitial tissue the staining intensity is strong to very strong (open arrowheads). Additionally, hypertrophy and hyperplasia of Leydig cells is seen (B-D). No positive staining is observed when the primary antibody is omitted (see, an insert in A).

the expression pattern of Cx43 in testes of a patient and of mice either with *mosaic* mutation or with a partial deletion of Y chromosome versus those of reproductively normal males. Additionally, by Western blot analysis we confirmed the presence of the Cx43 protein in mouse testes. In the former, Cx43 appears as punctuate structures localized to cell-cell contacts between adjacent cells as reported previously in humans (Steger *et al.*, 1999) and rodents (Risley, 2000; Batias *et al.*, 2000). Thus, the presence of gap junctions between Sertoli cells and spermatogonial/spermatid stages supports earlier studies indicating a role for these structures in Sertoli cell-germ cell communication (Steger *et al.*,

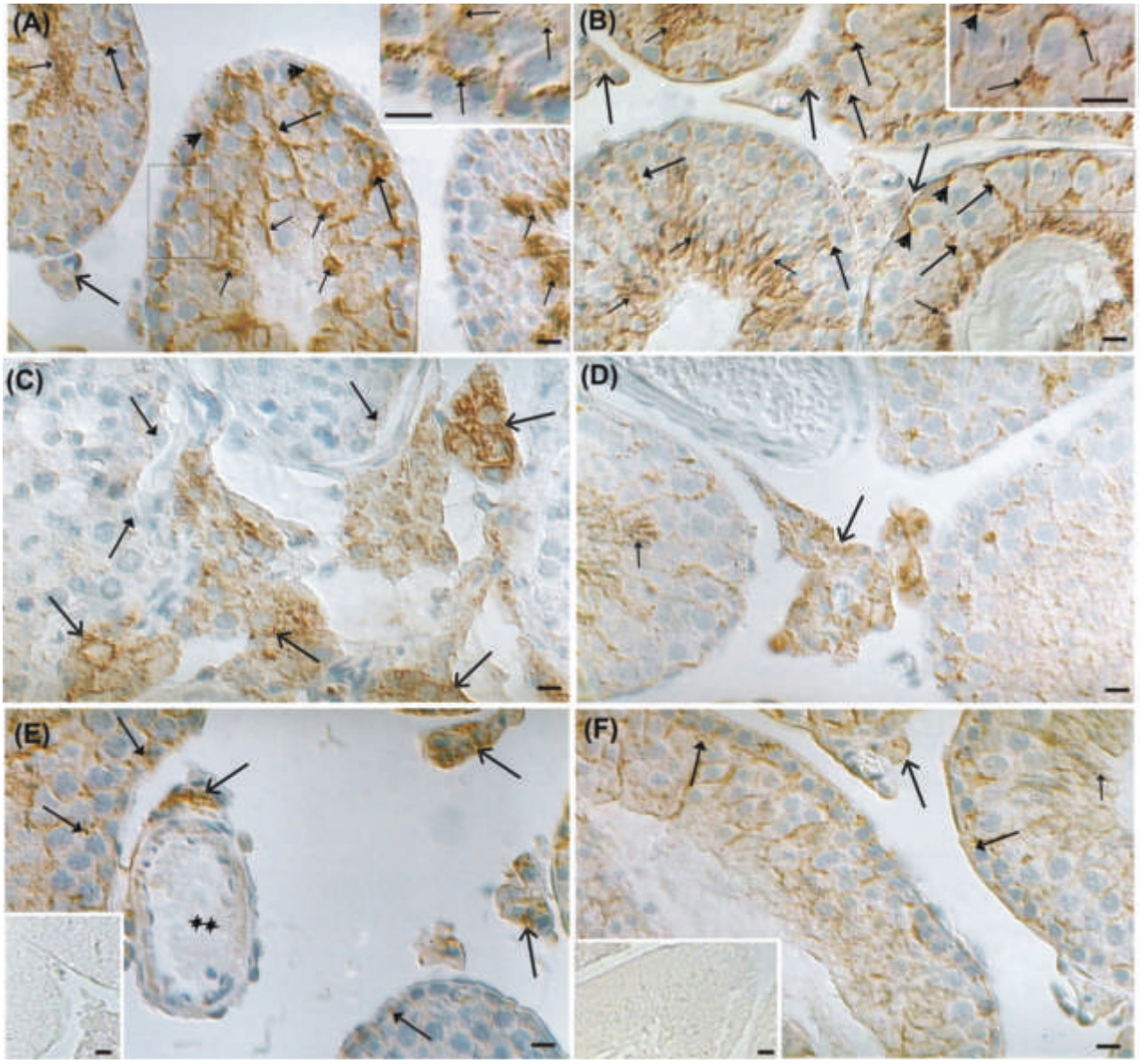

Figure 2. Immunohistochemical staining for Cx43 in respective control mouse testes [for the mosaic (A), for Y-del (B)] and those with the mosaic mutation (C, E) and a partial deletion in the long arm of the Y chromosome (D, F). Scale bars represent 20 μm. In control mouse testes (A, B) very strong staining occurs apical to spermatogonia and basal to primary spermatocytes (arrowheads) between Sertoli cells in the region of the blood-testis barrier (arrows) (see, an insert in A), and between secondary spermatocytes and round spermatids close to the lumen (short arrows). On the other hand, strong staining is confined to either the adluminal compartment (B, left tubule), or the immunoreactive signal occurs basal and apical to spermatogonia (arrowheads) possibly between Sertoli cells and spermatogonia (B, right tubule) or primary spermatocytes (arrows) (see, an insert in B). The staining is also localized between Sertoli cells and elongated spermatids (short arrows). Moderate staining intensity is seen in small groups of Leydig cells (open arrowheads). In testes of both mutants (C-F) a clear reduction of the Cx43 signal is observed within all seminiferous tubules (short arrows), whereas the intensity of the staining between neighboring Leydig cells that form clusters is moderate to strong (open arrows). In testes of the mosaic mice (E) no staining is observed in the seminiferous tubule showing Sertoli cell-only-phenotype (double asterisk). No positive staining is observed when the primary antibody is omitted (see, inserts in E and F).

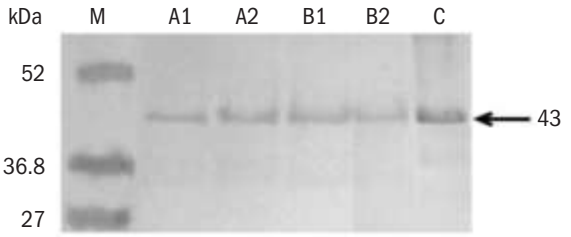

Figure 3. Western-blot analysis of Cx43 in testes of control males and of those with impaired spermatogenesis. Control mice (lane A1) and the mosaic mice (lane A2), control B10. BR mice (lane B1) and Y-del mice (lane B2). Lane M contains prestained protein standards with molecular weights in kDa. Rat heart (lane C) used as the positive control.

1999; Bravo-Moreno *et al.*, 2001; Defamie *et al.*, 2003; for review, see Pointis *et al.*, 2005). It should be stressed, however, that the presence of other Cxs (Cx26, Cx31, and Cx33) is especially important for the survival of germ cells and, as a consequence, for the maintenance of spermatogenesis in human and/or rodent testes (Lee *et al.*, 2006). It is of interest that the maintenance of spermatogenesis dependent on the presence of Cx43 protein has been shown in the frog testis, indicating the importance of gap junction communication not only in mammals but also in nonmammalian vertebrates (Palmiero *et al.*, 2003; Izzo *et al.*, 2006).

Examination of testicular sections with pathological disorders revealed a significant reduction or lack of Cx43 expression in the seminiferous tubules. These results are consistent with the experiments of Batias *et al.* (2000) who were the first to show a reduction of Cx43 signal in mice with retinoid X receptorβ deficiency (RXRβ-/-). Interestingly, the reduction of Cx43 immunoreactivity in testes of the *mosaic* mice and Y-del mice was not confirmed by Western blotting experiments. In mutant mouse testes, the Cx43 protein bands were of similar intensity compared with that of the respective controls. We hypothesize that intensity of the Cx43 protein evidenced in the pathological gonads by Western blots might be from the numerous clusters of Leydig cells that showed moderate to strong immunostaining.

Based on an earlier report by Batias *et al.* (1999), it is clear that a complete population of germ cells is a prerequisite for control level of Cx43 expression. In males, as shown in the study of Roscoe *et al.* (2001), the germ cell population of the testis fails to expand postnatally in the absence of Cx43. Our previous studies revealed that some morphological alterations in mutant mouse testes are likely associated with an increase in conversion of androgens to estrogens in the testis (Kotula-Balak *et al.*, 2004a; 2007). It is possible therefore that hormonal imbalance could lead, altogether with other factors to the impairment of intercellular communication observed in the mutants. Consistent with these findings exogenous 17β-estradiol propionate has been found to reduce the gap junction communication in primary cultures of rat Sertoli cells (Herve *et al.*, 1996). Recently, environmental, anti-fertility (gossypol) and estrogen-like (nonylphenol) agents have also been reported as responsible for impairment of Cx43 gap junctions between Sertoli cells (Fiorini *et al.*, 2004). In our study, the Cx43 immunoreactive signal was found to be present in tubules with partially altered spermatogenesis as shown in normal tubules of mutant mice as well as in a few seminiferous tubules of the patient with Klinefelter's syndrome. However, the staining was undetectable in other tubules that, being mostly hyalinized and depleted of germ cells, frequently showed a "Sertoli-only" cell pattern. A close relationship between Cx43 expression and the severity of spermatogenesis impairment has been published earlier (Steger *et al.*, 1999; Bravo-Moreno *et al.*, 2001; Pointis and Segretain, 2005). Such a relationship has also been demonstrated by Batias *et al.* (1999) in jun-d-null mice in which Sertoli-cell-only tubules were negative for the Cx43. It is interesting to note that the importance of Cx43 gap junctions for spermatogenesis has been indicated by severe depletion of germ cells in prenatal male mice lacking the Cx43 gene (Juneja *et al.*, 1999) or by infertility of homozygous mutants of two knock-in mouse lines in which no differentiated cells indicative of intermediate stages of spermatogenesis were found (Plum *et al.*, 2000). Early morphological studies also showed perturbation of gap junctions in pathological testes. No gap junctions were detected in feminized human testis by means of freeze-fracture (Nagano *et al.*, 1976). Moreover, the presence of atypical testicular gap junctions has been observed in an infertile patient (Bigliardi and Vegni-Talluri, 1977) and in those with azoospermia (Defamie *et al.*, 2003). Furthermore, in human testes with carcinoma in situ and seminoma there were neither immunoreactive signals nor altered trafficking of Cx43 (Brehm *et al.*, 2002; Roger *et al.*, 2004).

According to our observations of pathological

human testes in which moderate to very strong Cx43 expression was detected in the membrane appositions between neighboring Leydig cells, it seems likely that intercellular communication across Cx43 gap junctions could play a role in sustaining of Leydig cell metabolic activity. This assumption has been made based on our previous report (Kotula-Balak *et al.*, 2004b). In the Klinefelter's patient the testosterone concentration of 6.2 ng/mL is regarded to be in the middle range of the normal values for normal men (2.4-8.2 ng/mL), the estradiol level of 18.6 pg/mL is elevated, indicating an increase in androgen metabolism in Leydig cells, however the bioavailable testosterone concentration of 0.34 ng/mL is below the reference range (>0.7 ng/mL). The involvement of Cx43 in the control of Leydig cell function has already been proposed by Bravo-Moreno *et al.* (2001) who demonstrated that Cx43 expression in Leydig cells is regulated in an age- and function-dependent manner. Also You *et al.* (2000) reported that the regulation of testosterone secretion may involve Cx43 communication between adjacent Leydig cells. In contrast, the results of Roscoe *et al.* (2001) and Kahiri *et al.* (2006) indicate that the steroidogenic function of mouse Leydig cells is not compromised by the absence of Cx43. Thus, it seems likely that the role of Cx43 in the control of Leydig cell function is more complex than originally thought.

Collectively, our study adds three more examples of pathological gonads where an absence or a sharp decrease in Cx43 expression was detected within the tubules confirming previous data from other groups that gap junctions play a crucial role in spermatogenesis. Additionally, strong staining for Cx43 localized especially to human interstitial cells may indicate the importance of gap junctions to sustain Leydig cell metabolic activity.

In the future, it will be of interest to investigate whether there are other proteins interacting with connexins in the mammalian testis. It is also likely that genetic studies may uncover additional diseases associated with other connexins.

Acknowledgements

The authors kindly acknowledge a generous donation of mice and human testicular biopsy by Professors Józefa Styrna from the Department of Genetics and Evolution of the Jagiellonian University (Krakow), and Leszek Bablok from the Depertment of Andrology, Institute of Obstetrics and Gynecology, Medical Academy (Warsaw), respectively.

Funding

This work was supported by a grant BW/IZ/ZEHT/12A/2007.

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