Connexin 43 expression in human and mouse testes with impaired spermatogenesis

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Gap junctions are intercellular protein channels that provide a pathway for exchange of ions, second messengers and small molecules. This exchange allows electrical and metabolic coupling of cells as well as coordinated responses of coupled cells to hormones and growth factors (Brizzone 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 (Brizzone 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 (Batlas 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<sup>mos</sup>) 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 ATPase 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 (Stynna 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<sup>mos</sup>) (n=3) and respective controls (n=3), and (2) with a partial deletion in the long arm of the Y chromosome (B10. BR-Y<sup>del</sup>) (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 discontinuous 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% H2O2 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 occurred 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<sup>msm</sup>) and of the the B10. BR-Y<sup>al</sup> (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](image-url)
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).
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 Cxss (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 mouse 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.

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