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Connexin 33: A Rodent-Specific Member of the Gap Junction Protein Family?

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ABSTRACT: Gap junctional intercellular communication between Sertoli cells and between Sertoli cells and spermatogonia is considered to play a key role in the regulation of both proliferation and differentiation of germ cells. A member of the gap junction protein family, Connexin 33 (cx33), probably has an inhibitory effect on the formation of gap junctions and so far it is the only cx that has been exclusively found in rat and mouse testes. Thus, this connexin seems to be a special member of the cx family. Using immunohistochemistry, Western blot analysis, polymerase chain reaction, and reverse transcription (RT)-PCR (tissue homogenate and microdissected cells), we studied the possible occurrence of cx33 at the protein, the DNA, and the RNA level in human testis. Whereas immunohistochemistry using the only commercially available anti-cx33 antibody showed similar labeling to the rat within the seminiferous epithelium, we could not find any further evidence for the existence of cx33 using Western blot analysis, PCR, and RT-PCR in human testis. Based on the demonstration of the staining pattern of mitochondria in human germ cells and on preabsorption studies, we could demonstrate anti-cx33 antibody cross-reacting with mitochondrial ferritin, a protein localized in the mitochondria of human testicular spermatids. Therefore, we were not able to abide by the suspicion that cx33 is present in human testis. Additionally, it was not possible to demonstrate cx33 via PCR and immunohistochemistry in the testis of different mammals (dog, cattle, pig, horse, and marmoset monkey) with normal spermatogenesis. These data indicate that cx33 seems to be the first rodent-specific testicular cx.

Key words: Gap junctional intercellular communication, cx33, testis, spermatogenesis.

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Gap junctions contain channels that connect neighboring cells and constitute the basis for direct intercellular communication via exchange of ions, metabolites and second messengers (Kumar and Gilula, 1996). These channels are formed by a family of highly related proteins called connexins (cx) that consist of at least 21 members in human and 20 members in rodents (Sohl and Willecke, 2004). In the testis, there are different ways of interaction and communication between different cell populations. Beside paracrine and endocrine communication, the communication via gap junction channels plays an important role (Jegou, 1993; Steger et al, 1999; Brehm et al, 2002). Gap junctions between Sertoli cells and germ cells and between Sertoli cells only are necessary for the regulation of the terminal differentiation of Sertoli cells at puberty and for the differentiation and proliferation of germ cells. Therefore, they play a key role in the regulation of spermatogenesis (Risley et al, 1992; Jegou, 1993; Griswold, 1995; Juneja et al, 1999; Risley, 2000; Roscoe et al, 2001; Risley et al, 2002). In rodents, the cells of the seminiferous tubule express a complex panel of cx (Risley, 2000). In human testis, only 2 members of the cx family are described at present: cx26 and cx43 (Steger et al, 1999; Brehm et al, 2002). However, many other cx are probably implicated in the complex cell-cell interaction and communication in human testis. One of them could be cx33, which has already been detected in the rodent seminiferous tubule.

For the first time, cx33 was described in 1992 by Haefliger et al (Haefliger et al, 1992). This cx seems to be a member of the gap junction protein family with a couple of specialties. Haefliger et al noticed, by Northern blot analysis, that the expression pattern in the rat was restricted to testis tissue only, in contrast with most other cx-family members, which are expressed in a wide variety of organs and tissues. With the more sensitive method of reverse transcription-polymerase chain reaction (RT-PCR), it has been shown that rat cx33 mRNA seems to be present predominantly but not only in testis (Chung et al, 1999). Additionally, Chang et al (1996) observed that cx33 was able to inhibit the expression of cx37 and re-

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duced the expression of cx43. Therefore, a possible inhibitory role for this cx was suggested. A model is proposed where cx33 limits the capacity of cells to build functional channels by enabling formation of heterotypic channels with other cells, while formation of homotypic channels is not possible. In immunogold electron microscopy and immunofluorescence, rat cx33 was described to be part of intratubular Sertoli cell gap junctions but not of interstitial Leydig cell gap junctions. Most cx33 immunoreactivity occurred as stage-dependent in a linear pattern near the basal Sertoli cell junctional complexes. Occasionally, it has been found on adluminal surfaces with most strongly immunoreactive tubules at stages II–VII and weak reactivity at stages IX–XIV (Tan et al, 1996). In RT-PCR experiments with isolated rat testis cells, cx33 mRNA was detected mainly in Sertoli cells but also in pachytene spermatocytes and round spermatids (Risley, 2000). More recently, Willecke et al (Willecke et al, 2002) compared the cx genes in the mouse genome with those in human and observed that only 1 mouse cx gene (cx33) and 2 human cx genes (cx25 and cx59) seem to have no orthologs in the other genome, suggesting that no further cx gene would be discovered in the remaining nonsequenced portion of the human genome.

This study investigates the existence and expression of cx33 gene in normal human testis as well as in arrests of spermatogenesis and Sertoli cell-only syndrome. Because of its inhibitory effect on other cx, it is possible that an overexpression of cx33 alters communication defects between Sertoli cells and germ cells or Sertoli cells to each other. Healthy testis tissue from other mammals, including the marmoset monkey as a representative of the primates, was likewise investigated for cx33 gene status.

Materials and Methods

Testicular Tissue

A) Fixation and Embedding—Testicular tissue was either fixed by immersion in Bouin fixative and embedded in paraffin using standard techniques or was snap frozen in liquid nitrogen and stored at −80°C. Sections from paraffinized material were stained with hematoxylin/eosin and scored for spermatogenesis.

B) Human Testicular Tissue—Investigations were performed on biopsies from 18 human patients with obstructive azoospermia and on testicular tissue from 18 orchiectomized patients treated for prostate carcinoma, both showing histologically normal spermatogenesis (score 10-8; Bergmann and Kliesch, 1998). Additionally, biopsies of 19 infertile patients showing mixed atrophy were used. In these cases, testicular sections showed tubules with an arrest of spermatogenesis at the level of round spermatids, spermatocytes or spermatogonia, or Sertoli cell-only syndrome (SCO syndrome). Informed consent was obtained from all patients providing biopsies for our studies.

C) Testicular Tissue From Different Mammals—Testicular tissue from different mammals, such as rat, dog, cattle, pig, horse, and marmoset monkey with histologically normal spermatogenesis was obtained from routine castrations. Rat testicular tissue was always used as positive control. The samples derived from patients treated in different veterinary clinics belonging to the Department of Veterinary Medicine at the University of Giessen, Germany. Castration methods were performed specifically for each species. All animals were treated humanely.

Immunohistochemistry

A) Cx33: Paraffin Sections and Immunohistochemistry—For cx33 immunostaining, deparaffinized and rehydrated sections from human, dog, pig, horse, marmoset monkey, and rat were boiled for 30 minutes in sodium citrate buffer (pH 6.0), exposed to 20% acetic acid for 15 seconds, blocked with 5% bovine serum albumin (Fluka Chemie AG, Buchs, Switzerland) for 30 minutes, and incubated with the only commercially available polyclonal anti-cx33 primary antibody (1:500; rabbit anti-rat; Biotrend, Köln, Germany) overnight at 4°C. Sections were then exposed to the secondary antibody (1:50; mouse anti-rabbit, IgG; DAKO, Glostrup, Denmark) followed by the third antibody (1:50; rabbit anti-mouse, IgG; DAKO, Glostrup, Denmark) for 30 minutes each. Sections were incubated with a mouse alkaline phosphatase antibody complex (1:100; DAKO, Hamburg, Germany) for 30 minutes. Following each incubation, sections were washed thoroughly with 0.1 M Tris-HCl buffer (pH 7.4; Sigma, Steinheim, Germany). The immunoreactive product was visualized using HistoMark Red (KPL, Gaithersburg, Md). Negative controls were performed by omitting the primary antibody as well as by replacing the primary antibody with rabbit IgG at the same dilution and under similar incubation conditions as the primary antibody. The Leydig cells served as internal negative control.

B) Cx33: Cryostat Sections and Immunofluorescence—In order to demonstrate that the fixation procedure did not change the epitope structure of cx33, we performed immunofluorescence on fresh-frozen tissue of human testis. Cryostat sections from 6 frozen human testis biopsies were attached to microscope slides, fixed with 96% ethanol, blocked with 5% bovine serum albumin for 15 minutes, and incubated with the polyclonal anti-cx33 primary antibody (1:100; rabbit anti-rat; Biotrend, Köln, Germany) for 90 minutes at room temperature. Then sections were exposed to the secondary antibody (1:200; donkey anti-rabbit, fluoresceinisothiocyanate [FITC]-conjugated; Chemicon International, Temecula, Calif) for 30 minutes. Following each incubation, sections were washed with 0.1 M phosphate buffered saline (PBS; pH 7.4; Sigma, Steinheim, Germany). Negative controls were performed by omitting the primary antibody.

C) Cross-Reaction of the cx33 Antibody—In order to clarify if the cx33 antibody cross-reacts with mitochondrial proteins in the human spermatids, 2 assays were performed: first, the mitochondria in human testis tissue were visualized by immunostaining with an anti-cytochrome c oxidase antibody; the signal localization in round spermatids was compared with that obtained for cx33. Second, based on similarities between the cx33 antibody and a protein in human testis, mitochondrial ferritin, the unspecific detection of the latter by our cx33 antibody, was investigated by an immunoabsorbant assay.

C1) Mitochondrial Staining Pattern: Paraffin Sections and Immunohistochemistry—We performed immunohistochemical staining of deparaffinized and rehydrated paraffin sections from human using a mouse monoclonal antibody against cytochrome c oxidase (1:500; mouse anti-human; Molecular Probes, Leiden, Netherlands). The sections were boiled in sodium citrate buffer (pH 6.0) for 30 minutes, exposed to 20% acetic acid for 15 seconds, blocked with bovine serum albumin for 30 minutes, and incubated with the primary antibody described above for 90 minutes at room temperature. Sections were then exposed to the secondary antibody (1:50; rabbit anti-mouse, IgG) for 30 minutes. Afterward, the sections were treated with a mouse alkaline phosphatase antibody complex (1:100; DAKO, Glostrup, Denmark). Following each incubation, sections were washed thoroughly with 0.1 M Tris-HCl buffer, ph 7.4. The immunoreactive product was visualized using HistoMark Red (KPL). Negative controls were performed by omitting the primary antibody.

C2) Mitochondrial Ferritin: Paraffin Sections and Immunohistochemistry—Cx33 antibody was incubated with recombinant human mitochondrial ferritin (kindly provided by Prof Arosio, University of Brescia, Italy) for 60 minutes at room temperature (10 μg mitochondrial ferritin/500 μL diluted antibody [1:500]). After centrifugation, we used the supernatant for immunohistochemical staining of human sections as described above.

Protein Extraction and Western Blot Analysis

Proteins were extracted from unfixed frozen human and rat tissue by using the TRIzol® reagent as recommended by the manufacturer (Life Technologies, Karlsruhe, Germany). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% resolving gels under reducing conditions and blotted onto a Westran® polyvinylidene fluoride transfer membrane (Schleicher and Schuell, Dassel, Germany). The membrane was blocked with 5% nonfat dry milk in 0.1 M phosphate buffered saline (PBS; pH 7.4) and incubated with polyclonal anti-cx33 antibody (1:500; rabbit anti-rat; Biotrend, Köln, Germany) overnight. A biotinylated goat anti-rabbit IgG antibody (1:500; DAKO, Glostrup, Denmark) was used as secondary antibody. Finally, the membrane was treated with Vectastain Elite ABC Kit (Vector, Burlingame, Calif) and developed with True Blue Peroxidase Substrate (KPL). Control Western blots were performed by omitting the primary antibody.

RT-PCR

The RT-PCR assay for detection of cx33 mRNA was first performed on RNA extract from whole human testis tissue. Because we suggested a weak expression of cx33, certain fractions of cells of the seminiferous epithelium, where the cx33 expression was presumptively high, were investigated. The cell isolation was performed by ultraviolet laser-assisted microdissection leading to a higher specificity and sensitivity of the assay.

A) RNA Extraction and RT-PCR From Tissue Homogenate—Total RNA from frozen rat and human testicular tissue was extracted by using the TRIzol® reagent (Life Technologies) as recommended by the manufacturer. Then it was incubated with RNase-free DNase I (10 U/μL; Roche, Mannheim, Germany; 1–3 U/1 μg RNA, 40 minutes, 37°C) to digest genomic DNA. The DNase activity was stopped by incubating the probe at 72°C for 10 minutes. First-strand cDNA synthesis was performed using Superscript II RNase H^- reverse transcriptase (Invitrogen, Karlsruhe, Germany) and Omniscript reverse transcriptase (Qiagen, Hilden, Germany) according to the manufacturer's protocols, showing identical results. Four different primer sequences were used for PCR: 2 primer pairs for cx33 according to literature defined by Chung et al (Chung et al, 1999; 648-base pair [bp] amplicon; 5′ CAG AAA CAT ACA GGA AAG CAT ATC 3pr = sense; 5′ AAT GTT AAT GTC CAG TAC CCT TGC 3′ = antisense) and Risley (Risley, 2000; 878-bp amplicon; 5′ ATG AGT GAT TGG AGT GCC TTA CAC 3′ = sense; 5′ ACA TGG TTC TGT TCT CTT TAC 3′ = antisense) and 2 self-designed pairs of primers: 5′ ATG AGT GAT TGG AGT GCC TTA CAC 3′ = sense, 5′ GGC CAT GTA GGT (C/T)AG CAG CAA (C/T)CT 3′ = antisense (cx33W, 484-bp amplicon) and 5′ GGT GTC AIT GGT GTC TTT TGT C 3′ = sense, 5′ CTA GAA IIT ATG GTG GTG AGA AC 3′ = antisense (cx33E, 165-bp amplicon). These 2 pairs of primers amplify smaller amplicons and, by using wobbles, respectively, Inosin, they are more unspecific for rat and mouse cx33, more specific for the human genome, and were finally chosen to exclude amplification of highly homologous cx43. The sequences were based on the NCBI accession numbers AK040693 and M76534. The synthesized primers were purchased from MWG Biotech (Ebersberg, Germany). For PCR, 1 μL of cDNA was added to 5 μL 10× PCR buffer (10× Buffer; Promega, Mannheim, Germany), 2 μL $MgCl_2$ (25 mmol/L; Promega), 1 μL dNTPs (10 mmol/L; Promega), 0.5 μL polymerase (5 U/μL; Taq DNA polymerase; Promega), 1 μL of each primer (10 μmol/L), and DEPC-H_2O to a final volume of 50 μL. Then different PCR conditions were used: 1) according to Chung et al (1999) and Risley (2000) and 2) the following programs for the primer pair cx33W: 1× 95°C for 3 minutes, 10× 94°C for 40 seconds, 55°C (down to 50°C) for 1 minute, 72°C for 2 minutes, 25× 94°C for 40 seconds, 52°C (down to 47°C) for 1 minute, 72°C for 2 minutes, and 1× 72°C for 10 minutes; and for the primer pair cx33E: 1× 95°C for 3 minutes, 10× 94°C for 40 seconds, 59°C (down to 48°C) for 1 minute, 72°C for 2 minutes, 25× 94°C for 40 seconds, 56°C (down to 45°C) for 1 minute, 72°C for 2 minutes, and 1× 72°C for 10 minutes. PCR products were finally separated on a 2% agarose gel, visualized by ethidium bromide (Sigma, Steinheim, Germany), and the sequences analyzed by Qiagen sequencing service.

For each primer pair, control RT-PCR was performed using rat cDNA. Negative controls were performed by omitting reverse transcriptase. For internal control and evaluation of RNA quality, we used RT-PCR for the housekeeping gene β-actin from the same RNA samples that were used for cx33 amplification. The used primer sequences and PCR conditions were those defined by Hess et al (1992).

B) Ultraviolet Laser-Assisted Microdissection and Laser Pressure Catapulting—Microdissection was performed using the PALM® MicroBeam system (PALM, Bernried, Germany), consisting of a nitrogen laser of high-beam precision (wavelength 337 nm), which was coupled to an inverted microscope (Axiovert 135; Zeiss, Jena, Germany) via the epifluorescence illumination path.

The PALM® Membrane Slides (PALM, Benried, Germany)

were irradiated with an ultraviolet lamp for 30 minutes to achieve better adhesion of the sections. For histological evaluation, the sections were stained with hematoxylin and dehydrated.

Sertoli cells and different generations of germ cells of human and rat testicular tissue were identified by light microscopy and separated from the adjacent tissue by laser microdissection. With a laser shoot, the chosen cells were catapulted into the cap of a test tube (laser pressure catapulting) filled with mineral oil. Per tube, either 15 seminiferous tubules containing all germ and Sertoli cells (rat and human) or only round spermatids of 15 seminiferous tubules (according to the immunohistochemical staining of round spermatids in human, see below) were collected and dissolved in 350 μL lysis buffer (RNeasy Micro Kit; Qiagen) with β-mercaptoethanol (Serva, Heidelberg, Germany; 10 μL β-mercaptoethanol/mL lysis buffer) and 5 μL carrier RNA (4 ng/μL; RNeasy Micro Kit, Qiagen). After centrifugation and vortexing, specimens were immediately frozen in liquid nitrogen.

RNA from both human and rat testicular tissue was extracted using the RNeasy Micro Kit (Quiagen) according to the manufacturer's protocol, including the use of carrier RNA and DNase-I digestion. First-strand cDNA synthesis was performed using Sensiscript reverse transcriptase as recommended by the manufacturer (end volume = 20 μL; Qiagen). For PCR from microdissected cells, we used the primer pair named cx33E (165-bp amplicon, described above). Because of the instability of nucleic acids in microdissected cells, the amplicon should not be longer than 200 bp. Ten microliters of cDNA were added to 5 μL 10× PCR-buffer (including 15 mmol/L $MgCl_2$; Qiagen), 10 μL 5× Q-Solution (Qiagen), 1 μL dNTPs (Promega, Mannheim, Germany), 0.5 μL polymerase (5 U/μL; Taq polymerase; Qiagen), 1 μL of each primer (10 μmol/L), and DEPC-H_2O to a final volume of 50 μL. PCR conditions were as follows: 1× 95°C for 3 minutes, 15× 94°C for 40 seconds, 59°C (down to 48°C) for 1 minute, 72°C for 2 minutes, 40× 94°C for 40 seconds, 56°C (down to 45°C) for 1 minute, 72°C for 2 minutes, and 1× 72°C for 10 minutes. For internal control due to low amounts of mRNA, RT-PCR was performed again for β-actin from the same RNA samples used for cx33 amplification. The used primer sequences for β-Actin were 5′ TTC CTT CCT GGG CAT GGA GT 3′ = sense and 5′ TAC AGG TCT TTG CGG ATG TC 3′ = antisense (90-bp amplicon). The primers were purchased from MWG Biotech (Ebersberg, Germany). PCR conditions were 1× 95°C for 2 minutes, 50× 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and 1× 72°C for 10 minutes. PCR products were finally separated on a 2% agarose gel, visualized by SYBR-Green (Sigma-Aldrich, Munich, Germany) and the sequences were analyzed by Qiagen.

DNA Extraction and PCR

For detecting the cx33 gene, DNA from frozen human, rat, dog, cattle, pig, horse, and marmoset monkey normal testicular tissue was extracted by using the QIAamp DNA Mini Kit as recommended by the manufacturer (Qiagen). The extracted DNA was precipitated by mixing with 3 M sodium acetate and 96% ethanol and incubated overnight at −20°C. After centrifugation and washing with 70% ethanol, DNA was dissolved in 10 mM Tris buffer (pH 7.5). Identical PCR conditions and primers were used as described above (RNA extraction and RT-PCR from tissue homogenate). PCR products were finally separated on a 2% agarose gel, visualized by ethidium bromide (Sigma, Steinheim, Germany), and the sequences analyzed (Qiagen). In each case, negative controls were performed by omitting the DNA.

Results

Immunohistochemistry, cx33

In immunohistochemical experiments using the only commercially available cx33 antibody, we found a reproducible signal within the seminiferous epithelium of human and rat testicular tissue showing histologically normal spermatogenesis. In rat testis used as positive control, cx33 immunoreactivity occurred within the adluminal compartment of the seminiferous tubule adjacent to round and elongated spermatids (not stage specific) and within the basal compartment of Sertoli cell cytoplasm (stage specific) as described in the literature (Tan et al, 1996). However, the adluminal signals could have been due to an unspecific staining of the residual bodies (Figure 1A through D).

In human testis, the cx33 signal occurred differently. We found a signal only in the adluminal compartment in round and elongated spermatids (Figure 2A through C). The staining within round spermatids was noticed as a spotted and not stage-dependent signal in the periphery of the cytoplasm, similar to mitochondrial localization in germ cells (Russell et al, 1990). This indicates that the used antibody may cross-react with a mitochondrial protein. A staining in the basal compartment of the tubules in the cytoplasm of Sertoli cells, as described for rat tissue, was not detected in human. In human testicular tissue showing histologically impaired spermatogenesis (arrest of spermatogenesis at the level of round spermatids, spermatocytes, and spermatogonia, or SCO syndrome), we only could find weak isolated signals in round spermatids in tubules with arrest of spermatogenesis at the level of round spermatids, indicating a specific staining in spermatids. In all other tubules missing round and elongated spermatids, the immunostaining was absent (data not shown). In addition, immunohistochemical experiments using testicular tissue from dog, pig, horse, and marmoset monkey with histologically normal spermatogenesis did not show any specific signal (data not shown).

Immunofluorescence experiments using frozen human testicular tissue with histologically normal spermatogenesis supported the above described findings for paraffin immunohistochemical staining. Here, we also detected a signal in the adluminal compartment of the seminiferous tubule in spermatids (Figure 2D).

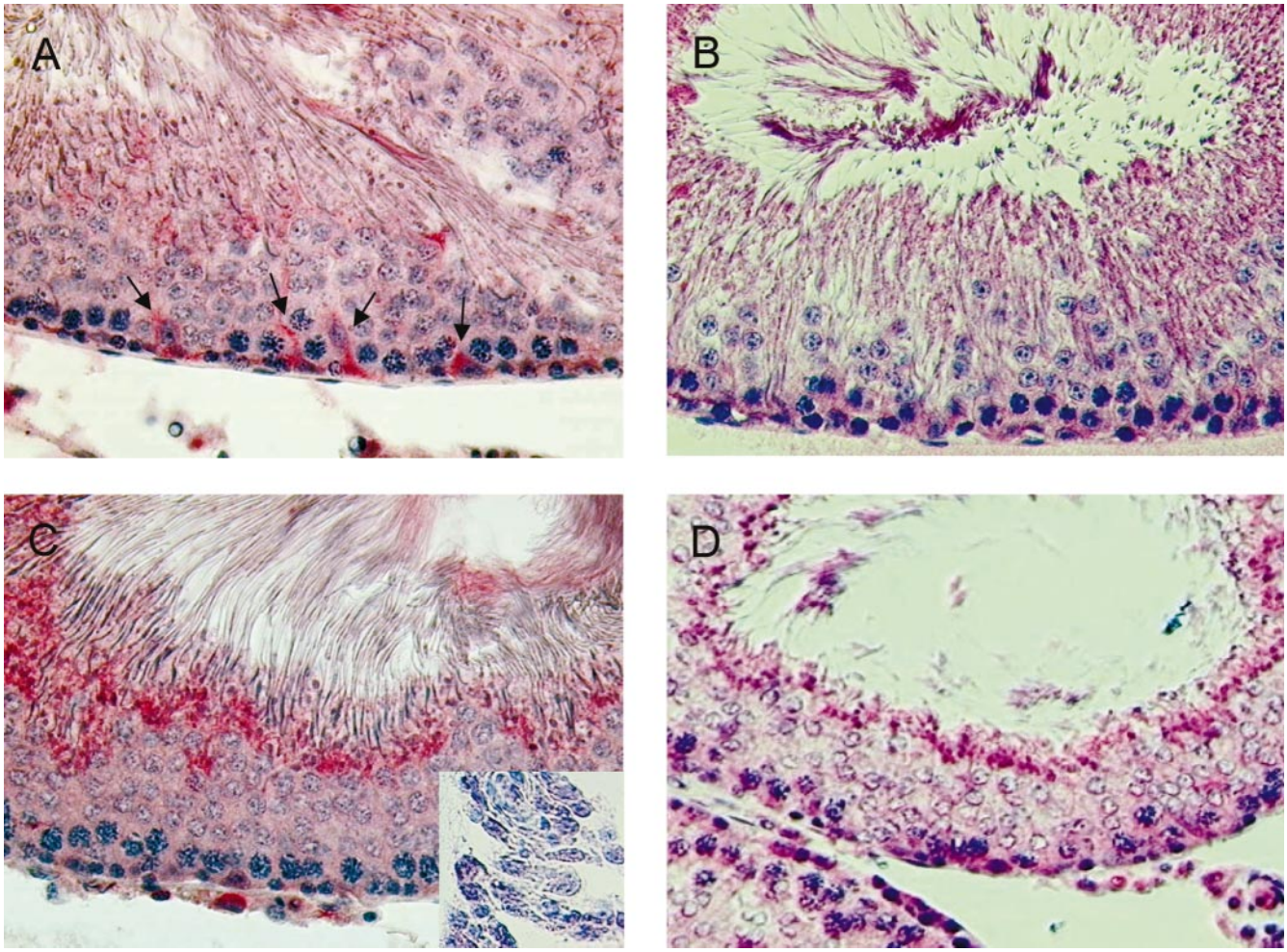

Figure 1. Immunostaining of rat testis with normal spermatogenesis for cx33 (positive control), primary magnification 40×. **(A)** Cx33 is present in the basal compartment in the cytoplasm of Sertoli cells (arrows). **(B)** Negative control using rabbit IgG, no signal can be seen. **(C)** A signal is present in the adluminal compartment adjacent to round and elongated spermatids (arrows); inset: Leydig cells as internal control. **(D)** Negative control using rabbit IgG. A signal is present in the adluminal compartment adjacent to round and elongated spermatids, too.

Immunohistochemistry, Cross-Reaction of the Anti-cx33 Antibody

Immunohistochemical experiments using the antibody against human cytochrome c oxidase for detecting mitochondrial staining pattern in round spermatids in human tissue with normal spermatogenesis exhibited a comparable spotted signal in the periphery of the cytoplasm of round spermatids as described above using the anti-cx33 antibody (Figure 2E).

Using the recombinant human mitochondrial ferritin for blocking the antibody against cx33 followed by immunohistochemical staining of human testicular tissue with normal spermatogenesis showed total disappearance of the described staining pattern of cx33 (Figure 2F and G).

Western Blot Analysis

Western blot experiments for cx33 using whole-testis protein extracts from rat and human revealed a single band between 27 and 33 kD in rat testis tissue. In contrast, no immunoreactive band using human testicular tissue with normal spermatogenesis in Western blot analysis was detectable (Figure 3). No cross-reaction of the anti-cx33 antibody with cx43 was observed.

PCR

For detecting the cx33 gene in human and different mammals, PCR with human, dog, cattle, pig, horse, and marmoset monkey testis tissue and rat testis tissue as positive control showing normal spermatogenesis was performed. The results for each used primer pair were comparable. The rat testis tissue showed the expected cx33 amplicon. This identity was confirmed by sequencing. Although the cattle and dog testis tissues showed also bands in the expected position, these amplicons were not specific by sequencing. All other species, including human, showed no amplicon with the cx33 specific primers (Figure 4A).

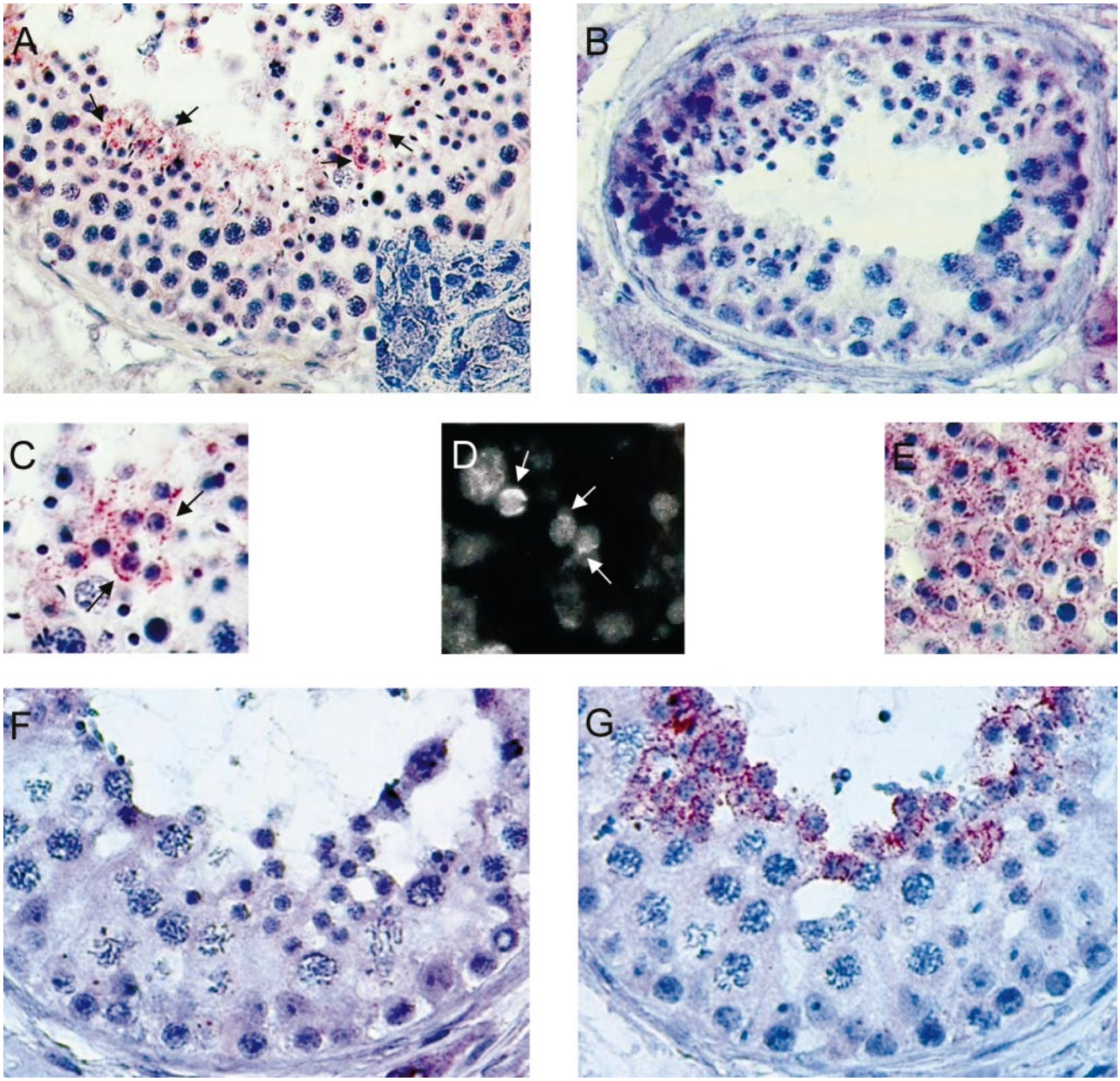

Figure 2. Immunostaining of human testis with normal spermatogenesis for cx33, primary magnification 40×. **(A)** Paraffin section and immunohistochemistry; inset: human Leydig cells as internal control. **(B)** Negative control using rabbit IgG, no signal can be seen. **(C)** Paraffin section and immunohistochemistry, human round spermatids. **(D)** Cryosection and immunofluorescence. In **A**, **C**, and **D**, a spotted signal is found in the adluminal compartment in the circumference of the cytoplasm of round and elongated spermatids (arrows). **(E)** Immunostaining of human round spermatids for cytochrome c oxidase (mitochondrial staining pattern), primary magnification 40×. A comparable spotted signal in the periphery of the cytoplasm as described using the anti-cx33 antibody was found. **(F, G)** Preabsorption studies with recombinant human mitochondrial ferritin using human testis demonstrating normal spermatogenesis, primary magnification 40×. **(F)** Immunostaining using anti-cx33 antibody preincubated with mitochondrial ferritin showing complete disappearance of cx33 immunoreactive signal. **(G)** Immunostaining of the same seminiferous tubule using unblocked anti-cx33 antibody.

RT-PCR (Testis Homogenate and Microdissected Cells)

RT-PCR of human and rat testis homogenate with normal spermatogenesis using 4 different pairs of cx33 primers with varying specificity and different size of amplicons resulted each in 1 clear signal of the expected size in rat used as positive control. The identity of the amplicons was confirmed by sequencing. No specific signal in human was found. The housekeeping gene β-actin, used for internal control and evaluation of RNA quality, showed a single band of the expected size in all human samples (Figure 4B).

RT-PCR using microdissected cells showed comparable

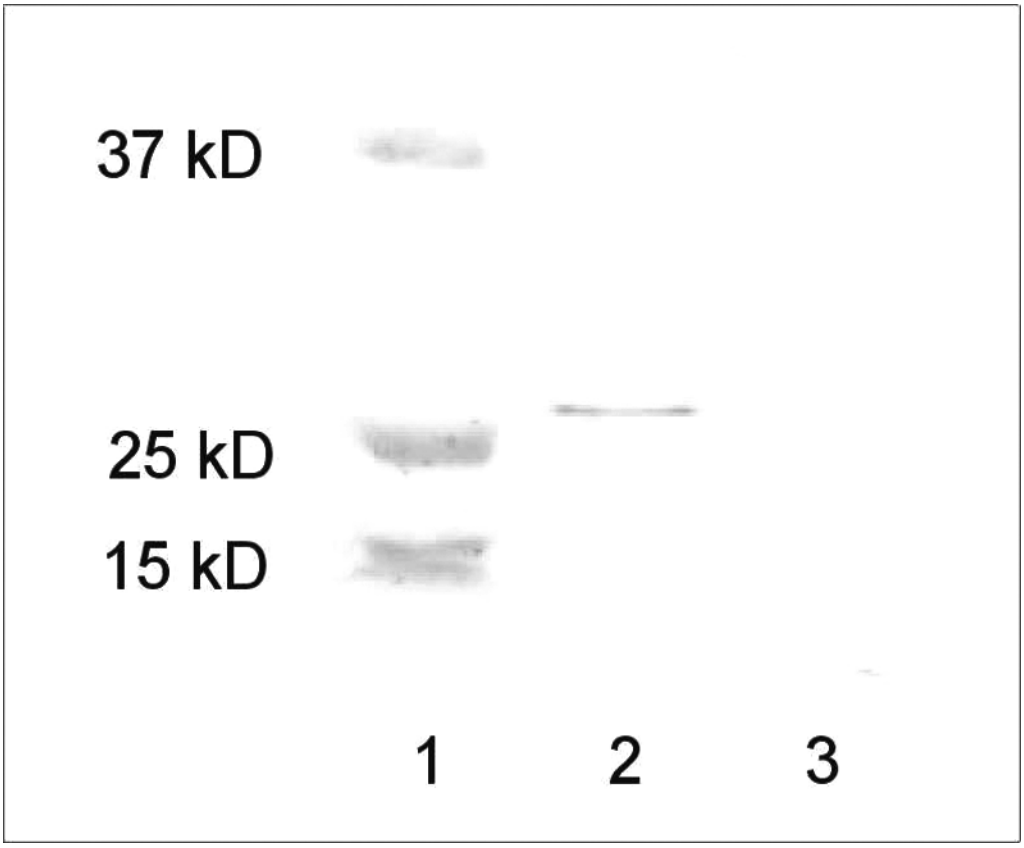

Figure 3. Western blot analysis of rat and human testis homogenates with normal spermatogenesis using anti-cx33 antibody. Lane 1, ladder; lane 2, rat testis (positive control); lane 3, human testis. A single immunoreactive band could be observed in rat testicular tissue, while no immunoreactivity was detected in human testicular tissue.

results. Rat samples containing RNA from whole seminiferous tubules resulted in a cx33-specific band confirmed by sequencing. No specific or only unspecific amplicons could be detected in human testis RNA extracts. The identity of the amplicons was confirmed by sequencing. Additional experiments using only dissected round human spermatids did not show any signal in human as well. The housekeeping gene β-actin used for internal control due to a low amount of mRNA in microdissection showed a single band of the expected size in all human samples (Figure 4C and D).

Discussion

Most cx genes being described in rodents have orthologs in the human genome, showing high sequence identity. For cx33 (mouse) and cx59 and cx25 (both human), orthologs in the other genome have not been found until now (Willecke et al, 2002). Because of the high testicular cx33 expression in rodents and its unique inhibitory effect on the formation of gap junctions probably resulting in an important function due to the regulation of spermatogenesis, our investigations were focused on the possible occurrence of the cx33 gene, protein, and mRNA in human testis with both normal and impaired spermatogenesis and additionally in the testis of different mammals on the DNA and protein level.

Immunohistochemistry of normal human testis with a commercially available antibody against cx33 revealed signals situated in round and elongated spermatids. Therefore, a possible hypothesis for the signal in the adluminal compartment of the rat seminiferous epithelium therefore could have been the necessity of cx33 to modulate gap junctional communication if elongating spermatids in the adluminal compartment change their contact pattern to Sertoli cells by the differentiation of tubulo-bulbar complexes before spermiation. However, a comparison between the immunohistochemical staining pattern of human and rat testis tissue questioned the specificity of the signals in human. The localization of the immunoreactive signal in the rat testis demonstrated the same results as described with a different anti-cx33 antibody (Tan et al, 1996). The signals were present both in the basal and adluminal compartment. However, the latter were not stage specific and due to an unspecific reaction of the

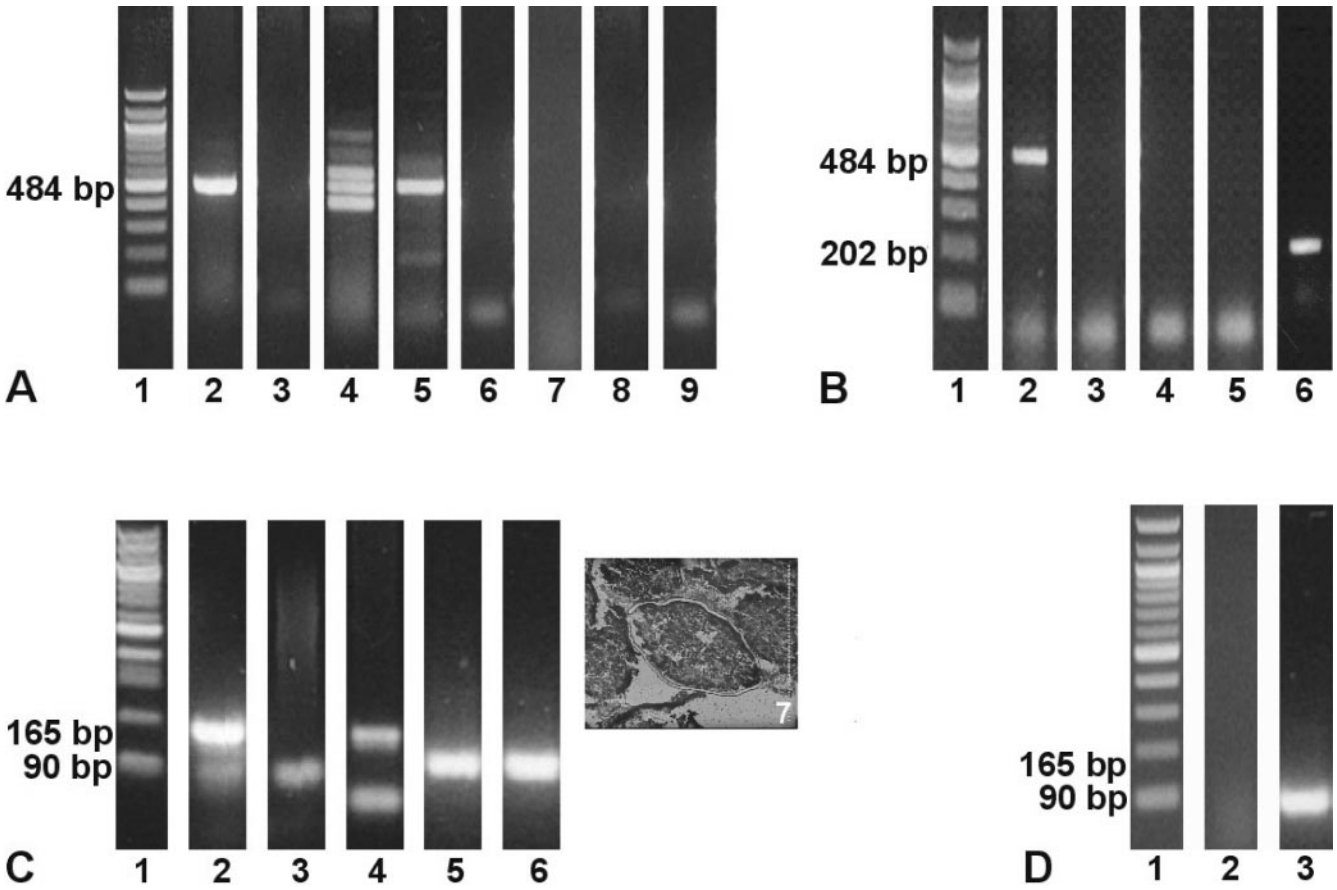

Figure 4. PCR and RT-PCR (tissue homogenate and microdissected cells). **(A)** PCR of testis homogenates of rat, human, dog, cattle, pig, marmoset monkey, and horse with normal spermatogenesis using the primer pair cx33W resulting in a 484-base pair (bp) amplicon. Lane 1, ladder; lane 2, rat (positive control); lane 3, human; lane 4, dog; lane 5, cattle; lane 6, pig; lane 7, horse; lane 8, marmoset monkey; lane 9, negative control. In rat, a specific signal for cx33 of the expected size was observed, while in human, pig, horse, and marmoset monkey, no signal, and in canine and bovine only unspecific (sequencing) signals could be detected. **(B)** RT-PCR of testis homogenates of rat and human with normal spermatogenesis using the primer pair cx33W resulting in a 484-bp amplicon and β-actin for internal control resulting in a 202-bp amplicon. Lane 1, ladder; lane 2, cx33 rat testis (positive control); lane 3, cx33 rat testis (negative control); lane 4, cx33 human testis; lane 5, cx33 human (negative control); lane 6, β-actin human belong to lane 4 as internal control. A specific signal for rat cx33 and for human β-actin of expected size can be seen. In human, no signal for cx33 was visible. **(C)** RT-PCR of microdissected Sertoli cells and germ cells of rat and human testis with normal spermatogenesis using the primer pair cx33E resulting in a 165-bp amplicon and β-Actin for internal control resulting in a 90-bp amplicon. Lane 1, ladder; lane 2, rat (positive control); lanes 3 and 4, human; lanes 5 and 6, β-actin human belonging to lanes 3 and 4, respectively; 7, dissected Sertoli and germ cells. A specific signal for cx33 of the expected size was detectable in rat, but either no signal or only an unspecific signal (sequencing) was found in human. **(D)** RT-PCR of microdissected human round spermatids using the primer pair cx33E resulting in a 165-bp amplicon and β-actin as internal control resulting in a 90-bp amplicon. Lane 1, ladder; lane 2, human; lane 3, β-actin human (internal control) belonging to lane 2. No signal for cx33 was detected in human round spermatids, while β-actin signal confirmed the mRNA integrity.

antibody with residual bodies. Because the signals situated in the cytoplasm of rat Sertoli cells were absent in human, a direct comparison of these 2 stainings was not possible. However, the spotted signal in the adluminal compartment detected in human testis tissue was comparable with the location of mitochondria in spermatids (Russell et al, 1990). We approved this hypothesis by demonstrating the localization of mitochondria in human germ cells by cytochrome c oxidase immunostaining. By comparing the sequence of the only commercially available peptide antibody against 17 amino acids of the carboxy-terminal of cx33 (confidential information of the manufacturer, therefore not specified) with human proteins in testis, we noticed that a protein named mitochondrial ferritin, which has been described in literature to be located in the mitochondria of round and elongated spermatids (Drysdale et al, 2002) as a possible candidate for cross-reaction containing 6 identical amino acids. We were able to confirm this assumption by successful preabsorbtion studies with recombinant human mitochondrial ferritin leading to a complete disappearance of cx33 immunohistochemical staining. The results indicate that the used anti-cx33 antibody cross reacts in human testis with mitochondrial ferritin. Therefore, we were not able to confirm the suspicion that cx33 protein exists in human testis with normal spermatogenesis.

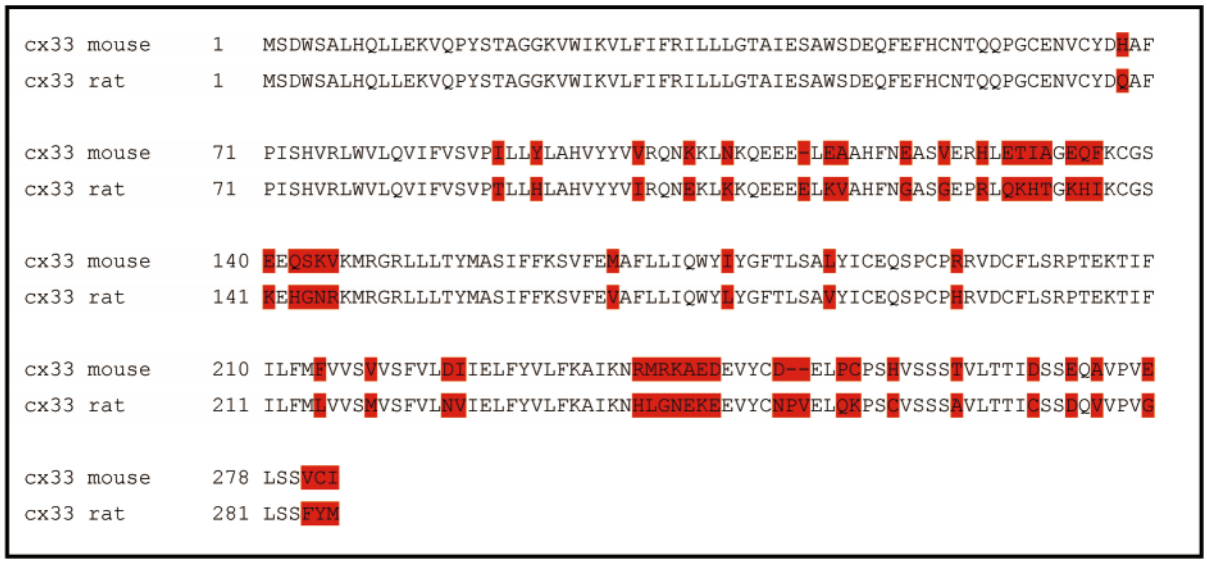

Figure 5. Sequence alignment of the mouse and rat cx33 protein. Various differences (red) can already be seen within these 2 species.

Although cx33 expression in human testis could not be demonstrated in healthy tissue, we decided to investigate its expression in human testicular tissue with impaired spermatogenesis. One demanding example is cx26, of which expression is very weak to almost undetectable in normal spermatogenesis but obviously stronger in arrest of spermatogenesis at the level of spermatogonia (Brehm et al, 2002). However, the expected immunohistochemical staining of Sertoli cells remained negative in all patients investigated.

Additionally, our attempts to verify the immunohistochemical findings in Western blot analysis failed as well as the detection of any specific signal for cx33 in human. Human mitochondrial ferritin could not be detected by Western blot analysis using the commercially available anti-cx33 antibody. A possible explanation is the capacity of the antibody to cross-react only with a tissue-preserved epitope of mitochondrial ferritin, which probably changes its steric features in Western blot analysis.

In our investigations on the DNA level, we initially did not focus on a special chromosome. But it is well known that cx33 is mapped to the X chromosome of rodents (Haefliger et al, 1992; Schwarz et al, 1994) and the map localization of the cx genes in mouse seems to be consistent with their map localization in human (Haefliger et al, 1992; Sohl and Willecke, 2004). Therefore, we focused in the X chromosome of the human genome. For this purpose, we used different data bases and data programs, splitting the rat cx33 gene in smaller segments, bearing in mind that parts of the X chromosome in human have not been sequenced yet. Because all investigations on the DNA level, including PCR experiments and additionally at RNA level using RT-PCR for both tissue homogenate and microdissected cells did not show any specific signal in human, we extended our search for the cx33 gene and protein to different mammals, keeping evolutionary aspects in mind. Because specific results could not been detected on the DNA or protein levels, we conclude that cx33 seems to be a rodent-specific member of the gap junction protein family. Our data indicate that cx33 is not implicated in the impairment of cell-cell communication associated with spermatogenic defects in human testis.

The complete sequencing of the murine genome allows us, for the first time, to make good comparisons of the most closely related available genome sequences, those of mouse and human. If we compare the genome of these species, the overall impression is one of similarity because 80% of the genes have 1-to-1 corresponding counterparts in the other's genome. In contrast, differences among genomes have received less attention. It was noticeable that the differences cluster in 3 regions: in genes that are relevant for immunity, in genes for host defence, and also in reproductive-typical genes due to the high selective pressure (Emes et al, 2003). Many reproductive genes are found among the 10% most divergent genes. This widespread phenomenon might have important consequences, such as the establishment of barriers to fertilization that might lead to speciation (Swanson and Vacquier, 2002). In conclusion, testis-specific cx33 could be 1 more member of the rapidly evolving genes in the reproductive system presenting important functions for rodent spermatogenesis but not in other mammals, including human. This hypothesis is sustained by the results of a DNA sequence comparison showing a very high level of divergence in male reproductive proteins between closely related species, for example, mouse and rat (Sutton and Wilkinson, 1997; Wyckoff et al, 2000). Compar-

ing the cx33 protein sequence of rat and mouse, various differences are also visible (Figure 5). The sequences were based on the NCBI accession numbers BAC30669 and P28233. The physiological role of cx33 in spermatogenesis remains an open question. The results of this study raise the question of whether cx33 really plays an important role in spermatogenesis. Maybe further investigations using transgenic mice will give hints for the precise role of cx33 in rodent spermatogenesis. Additionally, further investigations will clarify if there is another cx in testis responsible for the inhibitory function of cx33 or which other mechanisms or molecules may lead to inhibition of gap junctional conductance.

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References

Bergmann M, Kliesch S. Hodenbiopsie. In: Krause W, Weidner W, eds. *Andrologie: Krankheiten der männlichen Geschlechtsorgane.* Stuttgart, Germany: Enke Verlag; 1998:66–71.

Brehm R, Marks A, Rey R, Kliesch S, Bergmann M, Steger K. Altered expression of connexins 26 and 43 in Sertoli cells in seminiferous tubules infiltrated with carcinoma-in-situ or seminoma. *J Pathol.* 2002;197:647–653.

Chang M, Werner R, Dahl G. A role for an inhibitory connexin in testis? *Dev Biol.* 1996;175:50–56.

Chung SS, Lee WM, Cheng CY. Study on the formation of specialized inter-Sertoli cell junctions in vitro. *J Cell Physiol.* 1999;181:258–272.

Drysdale J, Arosio P, Invernizzi R, Cazzola M, Volz A, Corsi B, Biasiotto G, Levi S. Mitochondrial ferritin: a new player in iron metabolism. *Blood Cells Mol Dis.* 2002;29:376–383.

Emes RD, Goodstadt L, Winter EE, Ponting CP. Comparison of the genomes of human and mouse lays the foundation of genome zoology. *Hum Mol Genet.* 2003;12:701–709.

Griswold MD. Interactions between germ cells and Sertoli cells in the testis. *Biol Reprod.* 1995;52:211–216.

Haefliger JA, Bruzzone R, Jenkins NA, Gilbert DJ, Copeland NG, Paul DL. Four novel members of the connexin family of gap junction proteins. Molecular cloning, expression, and chromosome mapping. *J Biol Chem.* 1992;267:2057–2064.

Hess JF, Borkowski JA, Young GS, Strader CD, Ransom RW. Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. *Biochem Biophys Res Commun.* 1992;184:260–268.

Jegou B. The Sertoli-germ cell communication network in mammals. *Int Rev Cytol.* 1993;147:25–96.

Juneja SC, Barr KJ, Enders GC, Kidder GM. Defects in germ line and gonads of mice lacking connexin43. *Biol Reprod.* 1999;60:1263–1270.

Kumar NM, Gilula NB. The gap junction communication channel. *Cell.* 1996;84:381–388.

Risley MS. Connexin gene expression in seminiferous tubules of the Sprague-Dawley rat. *Biol Reprod.* 2000;62:748–754.

Risley MS, Tan IP, Farrell J. Gap junctions with varied permeability properties establish cell-type specific communication pathways in the rat seminiferous epithelium. *Biol Reprod.* 2002;67:945–952.

Risley MS, Tan IP, Roy C, Saez JC. Cell-, age- and stage-dependent distribution of connexin43 gap junctions in testes. *J Cell Sci.* 1992; 103:81–96.

Roscoe WA, Barr KJ, Mhawi AA, Pomerantz DK, Kiddler GM. Failure of spermatogenesis in mice lacking connexin43. *Biol Reprod.* 2001; 65:829–838.

Russell D, Ettlin RA, Hikim AP, Clegg ED. Staging for laboratory species. In: Russell D, Ettlin RA, Hikim AP, Clegg ED, eds. *Histological and Histopathological Evaluation of the Testis.* Clearwater, FL: Cache River Press; 1990:62–194.

Schwarz HJ, Chang YS, Lalley PA, Willecke K. Chromosomal assignments of mouse genes for connexin 50 and connexin 33 by somatic cell hybridization. *Somat Cell Mol Genet.* 1994;20:243–247.

Sohl G, Willecke K. Gap junctions and the connexin protein family. *Cardiovasc Res.* 2004;62:228–232.

Steger K, Tetens F, Bergmann M. Expression of connexin 43 in human testis. *Histochem Cell Biol.* 1999;112:215–220.

Sutton KA, Wilkinson MF. Rapid evolution of a homeodomain: evidence for positive selection. *J Mol Evol.* 1997;45:579–588.

Swanson WJ, Vacquier VD. The rapid evolution of reproductive proteins. *Nat Rev Genet.* 2002;3:137–144.

Tan IP, Roy C, Saez JC, Saez CG, Paul DL, Risley MS. Regulated assembly of connexin33 and connexin43 into rat Sertoli cell gap junctions. *Biol Reprod.* 1996;54:1300–1310.

Willecke K, Eiberger J, Degen J, Eckardt D, Romualdi A, Guldenagel M, Deutsch U, Sohl G. Structural and functional diversity of connexin genes in the mouse and human genome. *Biol Chem.* 2002;383:725–737.

Wyckoff GJ, Wang W, Wu CI. Rapid evolution of male reproductive genes in the descent of man. *Nature.* 2000;403:304–309.