Transition from Preinvasive Carcinoma *In Situ* to Seminoma Is Accompanied by a Reduction of Connexin 43 Expression in Sertoli Cells and Germ Cells[1]

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Abstract

Carcinoma *in situ* (CIS) represents the preinvasive stage of human germ cell tumors, but the mechanism leading to pubertal proliferation and invasive malignancy remains unknown. Among testicular gap junctional proteins, connexin 43 (Cx43) represents the predominant Cx, and, previously, an inverse correlation between synthesis of Cx43 protein and progression of tumor development was detected. In the present study, using cDNA microarray analysis, *in situ* hybridization, semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) from tissue homogenates, RT-PCR from microdissected tubules with normal spermatogenesis and CIS, and seminoma cells from invasive seminoma, we asked whether reduction of Cx43 protein is accompanied by a change of Cx43 transcripts. We detected a significant downregulation of Cx43 at mRNA level in Sertoli and germ cells starting in seminiferous tubules infiltrated with CIS and resulting in a complete loss in seminoma cells. It was demonstrated that downregulation of Cx43 expression in neoplastic human testis takes place at the transcriptional level and starts in CIS. This reduction of Cx43 expression further suggests that early intratubular derangement in Cx43 gene expression and disruption of intercellular communication between Sertoli cells and/or Sertoli and preinvasive tumor cells may play a role in the progression phase of human seminoma development.

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Introduction

Testicular germ cell tumor is the most common cancer among men aged 17 to 45 years [1]. It comprises a variety of histologic features with two major subtypes, seminoma and nonseminoma [2]. It is known that there are two periods of major changes in gene expression: first at the transition from primordial germ cells to pre-spermatogonia, and later during the pubertal switch from mitotic to meiotic cell division [3]. The transformation of the common precursor, the carcinoma *in situ* (CIS) cell, to atypical germ cells has been suggested to take place in gonocytes at 7 to 10 weeks of gestation [4–6]. It has further been suggested that testicular cancer progression may be correlated with an impaired status of Sertoli cell differentiation [7–11]. In recent years, several genes and chromosomal aberrations have been identified that are considered to be involved in the pathogenesis of testicular germ cell tumorigenesis [12–14], but the molecular mechanism leading to an increased proliferation of CIS cells at puberty followed by progression to invasive malignancy after long latency remains still unknown.

Gap junctions contain channels that connect neighboring cells and constitute the basis for direct intercellular communication [15]. These channels are composed of connexin (Cx) protein subunits. Different subtypes of Cx are expressed in an overlapping spatiotemporal and cell-type-specific pattern [16]. Thus, Cx can be used as specific cellular differentiation markers [17]. In addition, gap junctions play an important role in the maintenance of normal cell growth so that genes for the Cx proteins form a family of tumor suppressor genes [18]. The occurrence of gap junctions in the seminiferous epithelium of different species has been firmly established [19]. Among testicular Cx, Cx43 is the predominant Cx and can be expressed by Sertoli cells, spermatogonia, spermatocytes, peritubular cells, and Leydig cells [19–22]. Testicular gap junctions containing Cx43 seem to be necessary for the regulation of

differentiation of Sertoli cells, the controlled interaction and communication of Sertoli and germ cells, and the coordinated start of differentiation and proliferation of normal germ cells at puberty [19–22]. Cx43 is also present in the endothelial cells of large- and small-caliber blood vessels, where the protein coexists with Cx40 and Cx37 [23,24]. Cx37 is known to be expressed in the vascular endothelium including testicular blood vessels and seems to play a role in vascular development [16,25,26].

Cx43 as a phosphoprotein demonstrates multiple electrophoretic isoforms when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), including a faster migrating form that includes nonphosphorylated (P0 or NP) Cx43 and at least two slower migrating forms, commonly termed P1 and P2. Both P1 and P2 comigrate with P0 following alkaline phosphatase treatment, suggesting that phosphorylation is the primary covalent modification detected in SDS-PAGE analysis [27,28]. This phosphorylation of Cx43 in its life cycle has been implicated in the regulation of a broad variety of Cx processes, such as the trafficking, assembly/disassembly, degradation, as well as the gating of gap junction channels [29,30]. In addition, Cx43 phosphorylation is known to play a role in gap junctional plaque formation and activity [27].

So far, some studies have been published investigating the presence of gap junctions and Cx43 in seminiferous tubules and pathologic human testes. Seminiferous tubules showing spermatogenic arrest at the level of spermatogonia, those with Sertoli-cell-only (SCO) syndrome and infiltrated with CIS cells display an intratubular reduction or loss of Cx43 protein [10,21,22,31]. In a recent study, an aberrant cytoplasmic localization of Cx43 protein was detected in tumor cells of four patients with seminoma and in neoplastic cells originating from the JKT-1 seminoma cell line [32]. Thus, alterations of Cx43 protein synthesis can be correlated to various testicular disorders and male infertility including cancer [10,21,31–35].

To date, there are no reports associating abnormalities in intratubular Cx43 gene expression with spermatogenic impairment in the context of the development of testicular human seminoma from CIS. The present work at mRNA level extends an earlier study on the expression of the tumor suppressor Cx43 in CIS of adult patients [10] that revealed an inverse correlation of Cx43 protein synthesis between Sertoli cells and abnormal preinvasive germ cells. As alterations of Cx protein synthesis in neoplasms are not always paralleled by changes in mRNA expression and because some tumors can express steady-state transcripts without observable immunoreactivity, indicating that they are able to downregulate Cx immunoreactivity independently of mRNA abundance [36,37], we aimed to elucidate whether a reduction of Cx43 protein is accompanied by a downregulation of Cx43 transcripts using cDNA microarray analysis, *in situ* hybridization (ISH), semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) from tissue homogenates, and RT-PCR from microdissected tubules with normal spermatogenesis and CIS, and from seminoma cells from invasive seminoma.

Material and Methods

Testicular Tissue

After obtaining written informed consent, investigations were performed on biopsies from 10 patients (age 28–52 years; mean 32.4 years) with obstructive azoospermia and histologic normal spermatogenesis, 4 patients (age 58–67; mean 62 years) orchiectomized for prostate carcinoma revealing histologic normal spermatogenesis, 20 patients (age 20–45 years; mean 33.8 years) with CIS of the testis and impaired spermatogenesis, and 40 patients (age 25–57 years; mean 39 years) with different stages of seminoma (22 × pT1, 14 × pT2 and 4 × pT3). Patients were not treated with any drugs before surgery.

Fixation, Embedding, and Histologic Evaluation

Testicular biopsy specimens were cut into two equal pieces. One part was fixed by immersion in Bouin's fixative and embedded in paraffin wax using standard techniques. Sections (5μm) were stained with hematoxylin and eosin and scored, according to Bergmann and Kliesch [38]. The other part was snap-frozen in liquid nitrogen. Diagnosis of CIS and/or seminoma was established by histologic and morphologic examination of the biopsy specimen and by placental alkaline phosphatase (PIAP) immunostaining.

cDNA Microarray Analyses

We compared the gene expression profiles of cells of the 40 seminoma from different patients described above in different stages (pT1, pT2, pT3) with three normal testes using tissue homogenates and cDNA microarrays focusing on the predominant testicular Cx, *Cx43* (probe set ID 32531_at, accession no. NM_000165; corresponding Cx primer sequences can be viewed using probe set IDs under http://www.affymetrix.com/analysis/netaffx/). Additionally, the expression pattern of other Cx was investigated: *Cx31* (probe set ID 41076_at, accession no. AF099730), *Cx31.1* (38903_at, accession no. AF099731), *Cx32* (39598_at, accession no. X04325), *Cx37* (40687_at, accession no. M96789), *Cx47* (39504_at, accession no. AF014643, and 39505_at, accession no. AW007036), and *Cx50* (31778_at, accession no. U34802). Testicular tissues used for microarray analyses were lysed in TRIzol (Life Technologies, Karlsruhe, Germany) and total cellular RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA quality was controlled spectrophotometrically and by gel electrophoresis. Microarray analyses were carried out applying HG-U95Av2 microarrays (Affymetrix, Santa Clara, CA) from 5 μg of total RNA. Fragmentation of cRNA, hybridization, washing, staining, and scanning was performed following the manufacturer's protocols. Expression values were obtained using Affymetrix Microarray Suite Version 5.0. This includes the background correction of the average of the lowest two percentiles of intensities on a 4 × 4 grid on the chip, the introduction of an "ideal mismatch" forced to be lower than the corresponding perfect match, and the use of Tukey's biweight to elicit an expression value out of single probe

intensity pairs. Annotation of the probe sets was taken from the annotation files provided on the Affymetrix home page (version updated in October 2003). Exploratory data analysis, Wilcoxon paired nonparametric tests for difference, and the Mann-Whitney test for the nonparametric independent two-group comparisons were performed with the program SPSS 10 for Windows (SPSS Inc, Chicago, IL) and Microsoft Excel 2000 (Microsoft Corp, Redmond, WA). Differences with $P < .05$ were regarded as statistically significant.

To confirm the *Cx43* (accession no. NM_000165) expression data, we performed RT-PCR with tissue homogenate from 20 patients (5 normal testes and 15 seminoma tissues) using the following primer pairs: 5′-AGG AGT TCA ATC ACT TGG CGT GAC-3′ as forward primer and 5′-GGC TCA GCG CAC CAC TGG TCG CA-3′ as reverse primer. Because most of the frequently used housekeeping genes were differentially expressed in seminoma [39,40], the ubiquitin B (*UBB*) housekeeping gene, which was found to be constantly expressed in seminoma, was chosen for this approach and coamplified as internal standard control. For semiquantitative examination, *UBB* primer pairs (5′-GGC TTT GTT GGG TGA GCT TG-3′ as forward primer and 5′-CTG GGC TCC ACC TCC AGA GT-3′ as reverse primer) were added in the same tube to the Cx43 primers. PCR conditions were as follows: $1 \times 94°C$ for 2 minutes, $30 \times (94°C$ for 1 minute, $63°C$ for 1 minute, $72°C$ for 1 minute), $72°C$ for 4 minutes. The generated PCR products were size-fractionated on a 2% agarose gel and detected by ethidium bromide staining. The degree of Cx43 expression was evaluated densitometrically applying the Scion Image software (Scion Corporation, Frederick, MD) and correlated to the expression of *UBB*. Experiments were repeated at least three times.

UV Laser–Assisted Microdissection, Laser Pressure Catapulting, RNA Extraction, and RT-PCR

Microdissection followed by RT-PCR was performed on biopsies from four patients with histologically normal spermatogenesis, on four patients showing CIS, and on three biopsies from patients with seminoma ($1 \times$ pT1, $1 \times$ pT2, $1 \times$ pT3) using the PALM MicroBeam system (PALM, Bernried, Germany). The system consisted of a nitrogen laser of high beam precision (wavelength 337 nm), which was coupled to an inverted microscope (Axiovert 135; Zeiss, Jena, Germany) using the epifluorescence illumination path. The microscope stage was digitally controlled and moved by a computer mouse. The PALM Membrane Slides were irradiated with an ultraviolet lamp for 30 minutes to achieve better adhesion of the sections. For histologic evaluation, the sections were stained with hematoxylin and dehydrated. Tubules of interest were identified by light microscopy and PIAP immunostaining (CIS tubules) and separated from the adjacent interstitial tissue by laser microdissection. With a laser shoot the chosen seminiferous tubules or cells were catapulted into the cap of a test tube (laser pressure catapulting) filled with mineral oil. The same number of seminiferous tubules ($n = 20$) or the same number of seminoma cell clusters of approximately the same size but from different locations ($n = 20$) were collected per tube. Isolated cell profiles were then dissolved in 350 μl lysis buffer containing β-mercaptoethanol and 5 μl carrier RNA (RNeasy Micro Kit, Qiagen). RNA from microdissected human testicular tissue was extracted using the RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol including the use of carrier RNA and DNase-I digestion. Equal amounts of RNA were used for first-strand cDNA synthesis using Sensiscript II reverse transcriptase, as recommended by the manufacturer (Qiagen). PCR analysis from microdissected seminiferous tubules or seminoma cells was carried out with the following primer pairs (accession no, NM_000165): 5′-CCA TCT CTA ACT CCC ATG CAC AGC-3′ as forward primer and 5′-TGG CAC GAC TGC TGG CTC TGC TT-3′ as reverse primer, resulting in a 137-bp amplicon. PCR conditions were as follows: $1 \times 95°C$ for 3 minutes, $15 \times (95°C$ for 1 minute, $66°C$ for 1 minute, $72°C$ for 2 minutes), $45 \times (95°C$ for 1 minute, $62°C$ for 1 minute, $72°C$ for 2 minutes), $72°C$ for 10 minutes. Ten microliters of cDNA was added to 5 μl $10\times$ PCR buffer (including 15 mM $MgCl_2$; Qiagen), 10 μl $5\times$ Q solution, 1 μl dNTPs (Promega, Mannheim, Germany), 0.5 μl *Taq* polymerase (5 U/μl; Qiagen), 1 μl of each primer (10 μM), and DEPC-H_2O to a final volume of 50 μl. For internal (methodological) control due to low amounts of mRNA and for evaluation of RNA quality, we performed RT-PCR for the housekeeping gene β-actin (*ACTB*) from the same RNA samples that were used for Cx43 amplification. The primer sequences for *ACTB* (accession no. BC013835) were 5′-TTC CTT CCT GGG CAT GGA GT-3′ (forward) and 5′-TAC AGG TCT TTG CGG ATG TC-3′ (reverse) resulting in a 90-bp amplicon. PCR conditions were $1 \times 95°C$ for 2 minutes, $50 \times (94°C$ for 1 minute, $55°C$ for 1 minute, $72°C$ for 1 minute), and $1 \times 72°C$ for 10 minutes. PCR products were separated on a 2% agarose gel, visualized by SYBR Green (Sigma-Aldrich, Munich, Germany), and sequenced by Qiagen. Microdissection using serial sections followed by RT-PCR was repeated at least three times per patient.

Production of Digoxigenin-Labeled cRNA Probes for ISH

Digoxigenin (DIG)-labeled cRNA probes were generated as described previously [41]. Briefly, the 137-bp PCR product of the human Cx43 gene was subcloned in pGEM-T (Promega). Plasmids were transformed in the XL1-Blue *E. coli* strain (Stratagene, Heidelberg, Germany) and extracted by column purification according to the manufacturers instruction (Qiagen). After sequencing, vectors containing the Cx43 insert were digested with *Nco*I and *Not*I (New England Biolabs, Frankfurt, Germany) for the production of sense cRNA (*Nco*I) and antisense cRNA (*Not*I), respectively. Subsequently, *in vitro* transcription was performed using $10\times$ RNA-DIG Labeling Mix (Boehringer Mannheim, Mannheim, Germany) and RNA polymerases T7 and SP6 (Promega).

In Situ *Hybridization*

In situ hybridization was performed on biopsies of 10 patients with normal spermatogenesis, on biopsies of 10 patients with CIS, and on 20 biopsies of patients with seminoma ($10 \times$ pT1, $6 \times$ pT2, $4 \times$ pT3) with minor changes

as described previously [41,42]. Briefly, deparaffinized tissue sections were incubated in active DEPC water for 2 × 12 minutes at 40°C, postfixed in 4% paraformaldehyde for 10 minutes, exposed to 20% acetic acid, and prehybridized in 20% glycerol for 30 minutes. Sections were then incubated with the DIG-labeled sense or antisense cRNA probes. Both cRNAs were used at a dilution of 1:25 in hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, 2× standard saline citrate (SSC), 1× Denhardt's solution, 10 μg/ml salmon sperm DNA (Sigma, Deisenhofen, Germany), and 10 μg/ml yeast t-RNA (Sigma). Hybridization was performed overnight at 40°C in a humidified chamber containing 50% formamide in 2× SSC after posthybridization washes. Subsequently, sections were incubated with the anti-DIG Fab antibody conjugated to alkaline phosphatase (Boehringer) overnight at 4°C. Staining was visualized by developing sections with nitroblue-tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (KPL, Gaithersburg, MD) in a humidified chamber protected from light. Finally, sections were rehydrated for 5 minutes in deionized water and then dehydrated through successive baths of ethanol and xylol, and mounted in Eukitt resin (Merck, Darmstadt, Germany). For each test, negative controls were performed using DIG-labeled cRNA sense probes. Mouse testes were used as positive control. ISH was repeated at least twice.

Protein Extraction and Western Blot Analysis

For Western blot analysis, testicular biopsies from eight men were snap frozen in liquid nitrogen and stored at −80°C until further processing. Histologic evaluation of cryosections of these biopsies before Western blot experiments revealed normal spermatogenesis in four cases and seminoma in four cases (2 × pT2, 2 × pT3). Protein extraction was carried out using TRIzol reagent as recommended by the manufacturer (Life Technologies). Cx43 represents a phosphoprotein and was therefore incubated (10 U/μl, 60 minutes, 37°C) with calf intestinal alkaline phosphatase (CIP, New England Biolabs) to elucidate whether variations in electrophoretic mobility may reflect different phosphorylation states of this protein. Briefly, same amounts of protein per lane were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% resolving gels under reducing conditions using the Mini-PROTEAN system (BioRad, Munich, Germany). Proteins were then blotted onto a Westran polyvinylidene fluoride transfer membrane (Schleicher & Schuell, Dassel, Germany) and blocked with 5% nonfat dry milk dissolved in 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 0.1% Tween for 1 hour under gentle agitation before treatment with the polyclonal anti-Cx43 antibody overnight (1:1000; Zytomed, Berlin, Germany). A biotinylated goat anti−rabbit antibody (1:1000; DAKO, Hamburg, Germany) was used as secondary antibody. The primary and secondary antibodies were dissolved 1:1000 in PBS buffer. Finally, the membrane was treated with Vectastain Elite ABC Kit (Vector, Burlingame, CA) and developed with True Blue Peroxidase Substrate (KPL). Control Western blots were carried out by omitting the primary antibody. Western blot experiments were repeated three times.

Immunohistochemistry (Single and Double Immunostaining)

Single immunohistochemical staining for Cx43 was performed on consecutive sections of the same patients used for ISH, as described previously [10]. Briefly, sections were microwave treated for 30 minutes at 1000 W in sodium citrate buffer (pH 6.0), blocked with 3% BSA for 30 minutes and incubated with the polyclonal anti-Cx43 primary antibody (1:500; Zytomed) overnight at 4°C. Sections were then exposed to the secondary antibody (1:50, mouse anti−rabbit IgG, DAKO) followed by the third antibody (1:50, rabbit anti−mouse IgG, DAKO) and, finally, mouse alkaline phosphatase anti−alkaline phosphatase (APAAP) antibody complex (1:100, DAKO) for 30 minutes each. The immunoreaction was visualized using HistoMark Red (KPL) resulting in a red color on positive cells.

Double immunostaining for Cx43 and the inhibin-α subunit for the simultaneous detection of Leydig cells in seminoma patients was performed as follows: Cx43 and inhibin-α subunit epitopes were exposed by microwaving three times for 5 minutes at 1000 W in sodium citrate buffer (pH 6.0) and then were allowed to stand for an additional 20 minutes. After blocking with 3% BSA for 30 minutes, rabbit polyclonal anti-Cx43 primary antibody (Zytomed) was used after diluting 1:500, and tissue sections were incubated overnight at 4°C, followed by the secondary antibody (1:50; mouse anti−rabbit IgG; DAKO), by the third antibody (1:50; rabbit anti−mouse IgG; DAKO), and, finally, by the mouse APAAP antibody complex (1:100, DAKO) for 30 minutes each. The immunoreaction was visualized using NBT/BCIP (KPL) to produce a blue/pink color on positive cells. Then, the sections were incubated with 3% H_2O_2, with 3% BSA for 30 minutes each, and with the monoclonal primary anti−inhibin-α antibody (1:50, Serotec, Oxford, UK) overnight at 4°C. On the next day, sections were incubated with the biotinylated secondary antibody (1:50; goat anti−mouse IgG, DAKO) and by incubation using the ABC Kit (Vector) for 30 minutes each. Immunoreactivity was visualized using diaminobenzidine (Biologo, Kronshagen, Germany) to produce a brown color on positive cells. Sections were finally slightly counterstained with instant hematoxylin (Shandon, Pittsburgh, PA). For each immunoreaction, control incubations were performed by substituting buffer for the primary antibody. Experiments were repeated three times.

Results

cDNA-Microarray Analyses

Microarray analysis identified several up- and down-regulated genes including Cx43 in three normal testicular specimens and 40 seminoma of different stages (pT1, $n = 22$; pT2, $n = 14$; pT3, $n = 4$). The gene expression profiles of 4 samples of tumor stage pT1 were compared with those of 3 normal tissue samples resulting in 12 single comparisons representative for the 43 samples investigated. The expression of 1490 probe sets was determined as differentially expressed in at least 80% of the comparisons with a P value <.001 and >.999 for increased and decreased

changes, respectively. Thereof, the expression of 635 probe sets was increased and of 855 decreased. For *Cx43*, two probe sets were present on the gene array: 2018_at and 32531_at. The first showed very weak signal intensities and was given as absent, whereas the probes of 32531_at were positive in 84% of the samples. All 3 normal testicular specimen expressed Cx43 and 32 of 40 seminoma were positive for this gene. The expression of Cx43 was significantly 9.1-fold downregulated in seminoma tissues (Figure 1*A*). *Cx31* and *Cx47* were absent in all samples investigated. *Cx32* was expressed only in samples with unimpaired spermatogenesis and *Cx50* was unregulated in 81% of 43 samples. *Cx31.1* was expressed in 87% of 43 samples and 2.1-fold significantly increased in seminoma compared with normal testicular specimen. *Cx37* was expressed in 27 of 40 seminoma tissues with a significant 1.9-fold increase compared with normal testicular tissues. The down- and upregulated *Cx* genes in seminoma versus normal testicular tissue obtained by microarray analysis are summarized in Table 1.

To confirm the reduced expression of Cx43, semiquantitative RT-PCR using *UBB* as housekeeping gene were carried out by investigating 5 samples from normal testicular specimen and 15 seminoma (Figure 1, *B* and *C*). The 2.8-fold downregulation of *Cx43* expression in seminoma was significant (Figure 1*D*). However, the upregulation of Cx31.1 could not be confirmed by RT-PCR (data not shown).

RT-PCR following Laser Microdissection of Isolated Seminiferous Tubules and Seminoma Cells

To evaluate Cx43 mRNA expression, microdissection of whole seminiferous tubules and seminoma cells was performed. Same numbers of seminiferous tubules with normal spermatogenesis, with CIS cells and residual spermatogenesis, and tubules showing only CIS and Sertoli cells (CIS-only tubules), and seminoma cells from different stages were excised from testicular tissue. Subsequent RT-PCR analysis of these tubules and/or cell profiles revealed transcripts of Cx43 in tubules showing normal spermatogenesis and in tubules containing both CIS cells and residual spermatogenesis. Tubules resembling only CIS and Sertoli cells were found either positive (in very few samples) or mostly negative. Seminoma cells isolated from various sections exhibiting different seminomatous stages (pT1–pT3) showed no signal for Cx43 mRNA at all (Figure 2).
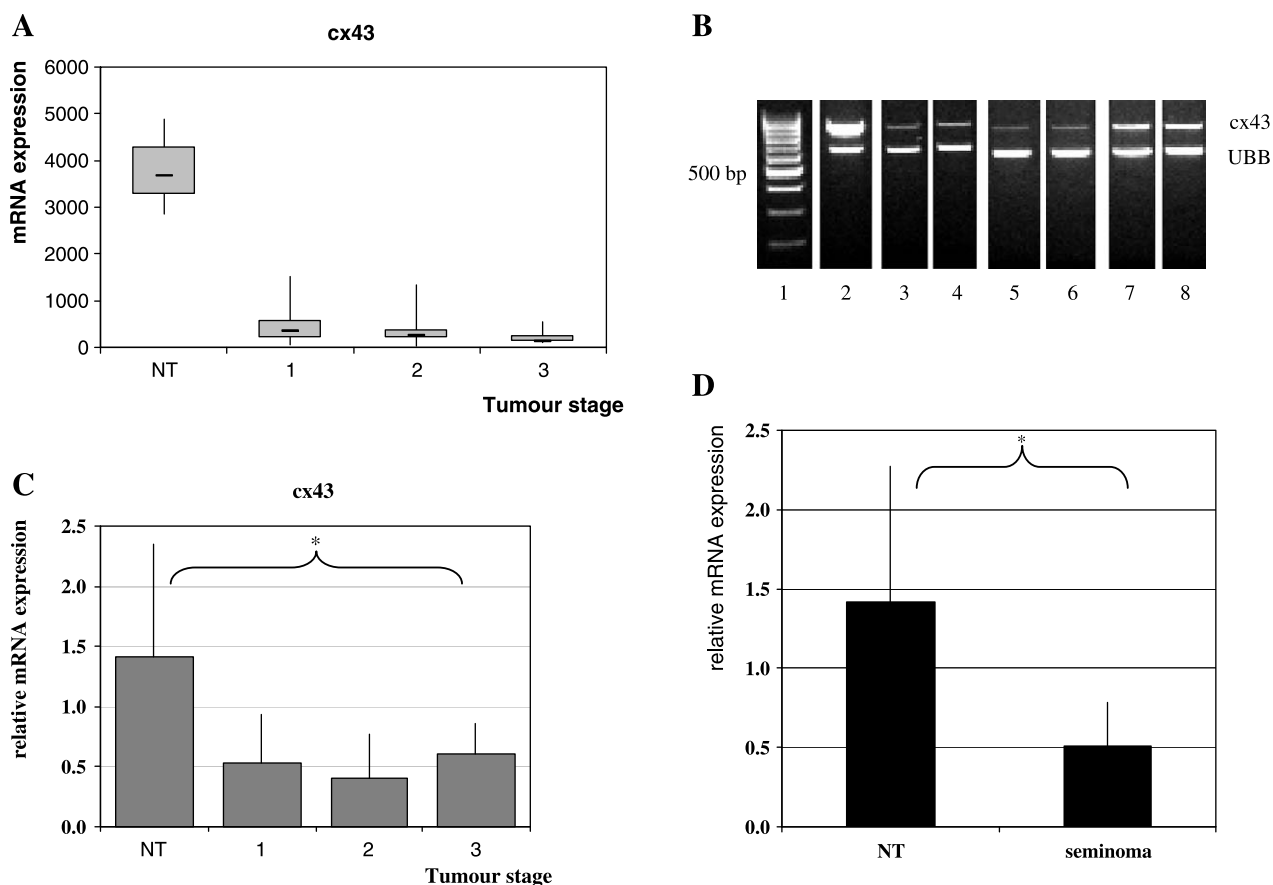
Figure 1. *(A) cDNA microarray analysis revealed a 9.1-fold downregulation of Cx43-expression in seminoma (tumor stages pT1–pT3). (B) Representative semiquantitative RT-PCR with UBB as housekeeping gene revealed a strong band for Cx43 at 827 bp in a man with normal spermatogenesis (lane 2) and a 2.8-fold decrease of Cx43 expression in patients with seminoma stages pT1 (lanes 3 and 4), pT2 (lanes 5 and 6), and pT3 (lanes 7 and 8). Lane 1: 100-bp DNA ladder. (C) Relative Cx43 mRNA expression compared with UBB and related to tumor stages pT1 to pT3. Here, the representative results from three independent PCR reactions of 5 normal testis and 15 seminoma specimens (pT1: n = 8, pT2: n = 4, pT3: n = 3) were analyzed. (D) Relative Cx43 mRNA expression in normal testis and seminoma compared with UBB. NT, normal testis; pT1-3: tumor stages 1 to 3. *P = .016.*

Table 1. Expression Data (mRNA) for Cx-Encoding Genes Present on the Microarray in 43 Tissue Samples regarding Different Tumor Stages.

Gene		Probe Set ID	NCBI ID	P-call (%)	NT		pT1		pT2		pT3	
					Mean	SD	Mean	SD	Mean	SD	Mean	SD
Downregulated connexins												
GJA1	Cx43	32531_at	X52947	81	3804	1028	476	357	405	394	224	210
GJA8	Cx50	31778_at	U34802	5	591	200	87	70	101	92	95	39
GJB1	Cx32	39598_at	X04325	7	678	95	338	110	272	122	414	244
GJA7	Cx45	33041_at	U03493	65	429	67	406	262	352	96	547	478
Upregulated connexins												
GJB5	Cx31.1	38903_at	AF099731	91	157	37	301	116	335	96	379	160
GJA4	Cx37	40687_at	M96789	63	189	49	354	102	377	90	305	52

In Situ *Hybridization*

In seminiferous tubules showing normal spermatogenesis, Cx43 mRNA was detected around the nuclei of Sertoli cells (*transparent arrows*), spermatogonia (*transparent arrowhead*) and spermatocytes (*black arrow*, Figure 3*A*). No signal was detectable in the cytoplasm of round spermatids (Figure 3*A*, *white arrow*). Seminiferous tubules from mouse testis with normal spermatogenesis used as positive control revealed similar intratubular location for Cx43 mRNA (Figure 3*A*, *inset*).

The expression pattern of Cx43 mRNA in CIS-only tubules (containing both Sertoli cells and CIS cells) was heterogeneous. In very few tubules, a hybridization signal was mainly present in the cytoplasm of Sertoli cells (Sertoli cell nuclei: *transparent arrows*; CIS cells are marked with a uniform line; Figure 3*B*), whereas in most CIS-only tubules the signal in Sertoli cells or tumor cells was either markedly reduced (Figure 3*C*) or absent (Figure 3*D*). Note a strong signal in the cytoplasm of peritubular cells (Figure 3*C*, *white arrowheads*) and endothelial cells of small blood vessels (Figure 3*D*, *curved black arrow*).

In patients revealing pure seminoma, only small cell clusters (Figure 3*E*), single cells (Figure 3*F*, *curved transparent arrows*), and/or endothelial cells (Figure 3*F*, *inset b*) were found to express Cx43 mRNA, whereas seminoma cells showed no hybridization signal for Cx43. Control incubations with Cx43 cRNA sense probes were in all cases negative (Figure 3*B*, *inset*, and *F*, *inset a*; scale bars = 50 μm).

Immunohistochemistry

In seminiferous tubules showing normal spermatogenesis, a steady Cx43 staining was detected at the level of the blood-testis barrier (BTB) between Sertoli cells, between Sertoli cells and spermatogonia, and between Sertoli cells and primary spermatocytes (Figure 4*A*). In addition, a strong signal was visible between interstitial Leydig cells (Figure 4*A*, *black arrow*).

The expression pattern of Cx43 protein in CIS-only tubules was found to be heterogeneous, corresponding to the location of Cx43 mRNA. In very few tubules, a disorganized immunoreactive signal was present at the level of the BTB (CIS cells: *tranparent arrows*, Figure 4*B*). However, in the majority of these tubules no immunostaining for Cx43 was detectable (Figure 4*C*), whereas immunoreactive signals between adjacent interstitial Leydig cells persisted (Figure 4, *B* and *C*, *black arrows*).

In seminomatous tissue, only between cells belonging to single cell clusters within connective tissue (Figure 4*D*, *black arrow*), and between endothelial cells of small blood vessels (Figure 4*E*, *arrowheads*) immunostaining for Cx43 could be detected independent of staging. Double immunostaining for Cx43 (blue/pink) and inhibin-α (brown) clearly colocalized both proteins in the same cell type and identified these cells as Leydig cells (Figure 4*D*). Seminoma cells showed no immunoreaction for inhibin-α (Figure 4*D*) or for Cx43 (Figure 4, *D* and *E*; scale bars = 50 μm).

Western Blot Analysis

Normal testicular tissue revealed three immunoreactive bands between 41 and 45 kDa. Incubation with CIP changed the electrophoretic profiles of Cx43. Observed bands at 45 kDa (Cx43-P2) and 43 kDa (Cx43-P1) were lost after CIP treatment resulting in one strong band at 41 kDa (Cx43-P0). These results indicate that these two bands represent double- and single-phosphorylated Cx43 isoforms.

In seminoma, only a weak immunoreactive band at 45 kDa (Cx43-P2) could be demonstrated. In addition, this weak band was lost after CIP treatment resulting in the Cx43-P0 isoform (Figure 4*F*, *arrow*).
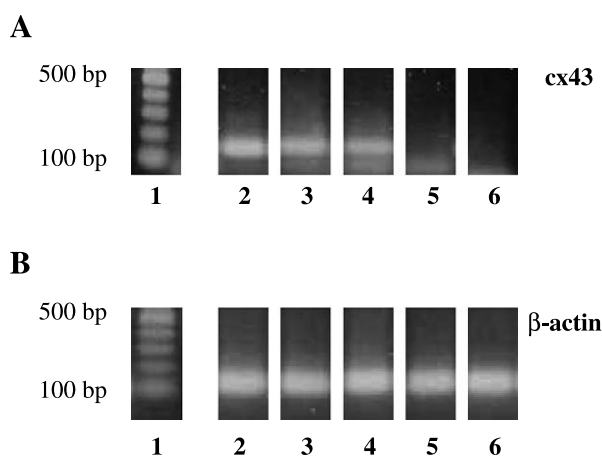
Figure 2. *Representative RT-PCR from microdissected tubules and seminoma cells using primer pairs for Cx43 (A) and β-actin (B). Seminiferous tubules with normal spermatogenesis (lane 2) and seminiferous tubules with both residual spermatogenesis and CIS (lane 3) revealed a 137-bp PCR product for Cx43. Tubules resembling only CIS and Sertoli cells (CIS-only tubules) were found positive or in most cases negative (lanes 4 and 5). Seminoma cells isolated from different seminomatous stages (pT1–pT3) showed no signal for Cx43 mRNA (lane 6). Lane 1: 100-bp DNA ladder.*
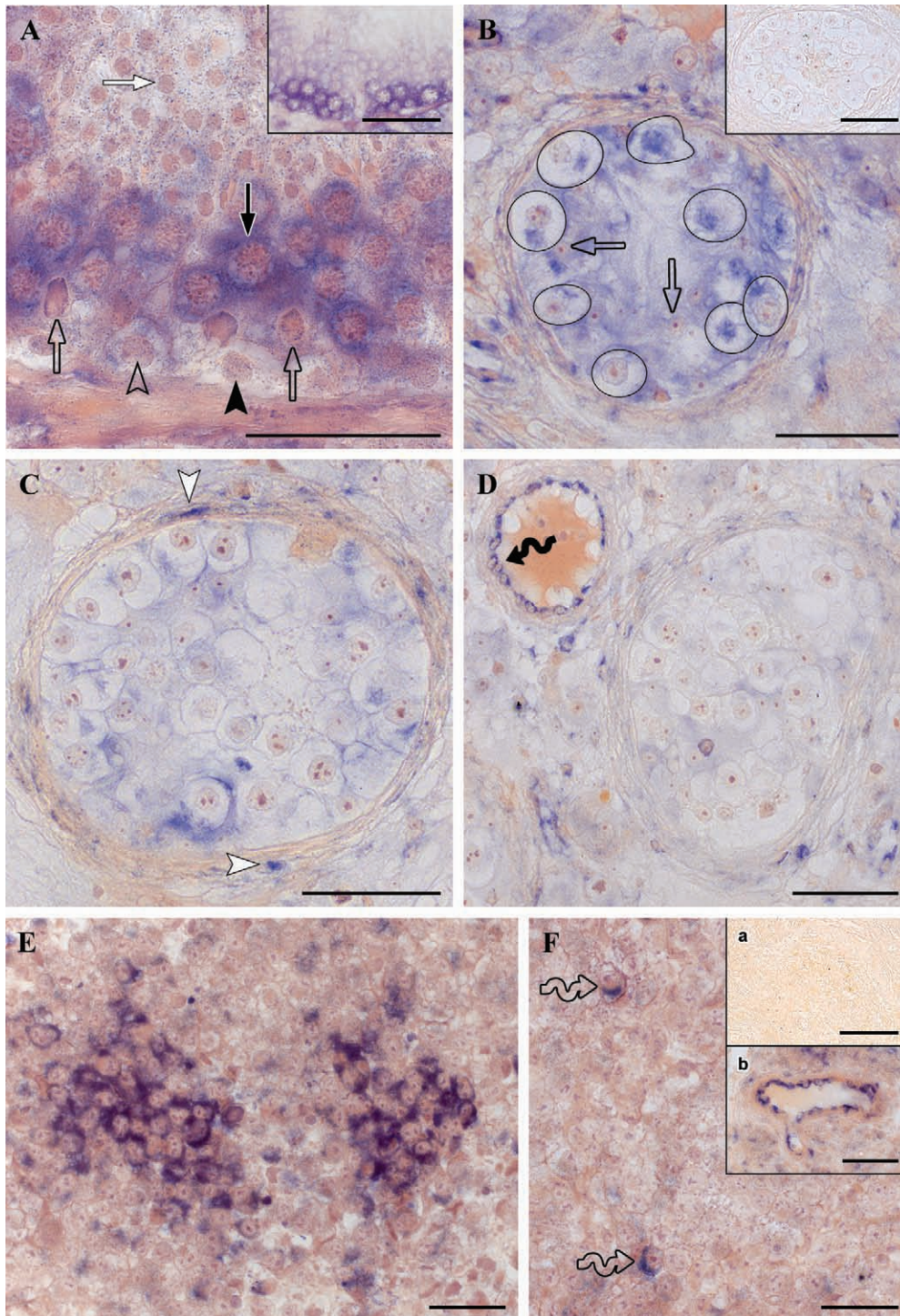
Figure 3. (A) In normal spermatogenesis, ISH revealed a strong signal for Cx43 mRNA around the nuclei of Sertoli cells (transparent arrows), spermatogonia (transparent arrowhead), and primary spermatocytes (black arrow). Note an immunonegative spermatogonia (black arrowhead) and immunonegative round spermatids (white arrow). Seminiferous tubules from mouse testis with normal spermatogenesis used as positive control showed an identical expression pattern (inset). CIS-only tubules displayed a heterogeneous expression pattern of Cx43 mRNA. In very few tubules, a hybridization signal was mainly present in the cytoplasm of Sertoli cells (CIS cells are marked with a uniform line; Sertoli cell nuclei: transparent arrows, B). However, in most tubules the signal in Sertoli cells was either markedly reduced or completely absent (C and D). A strong signal was detectable in the cytoplasm of peritubular cells (white arrowheads, C) and in endothelial cells of small blood vessels (curved black arrow, D). In seminoma, only single cell clusters (E), single cells (curved transparent arrows, F), and/or endothelial cells (F, inset b) were found to express Cx43 mRNA, whereas seminoma cells showed no hybridization signal for Cx43. Control incubations with Cx43 cRNA sense probes were in all cases completely negative (B, inset, and F, inset a). Scale bars = 50 μm.
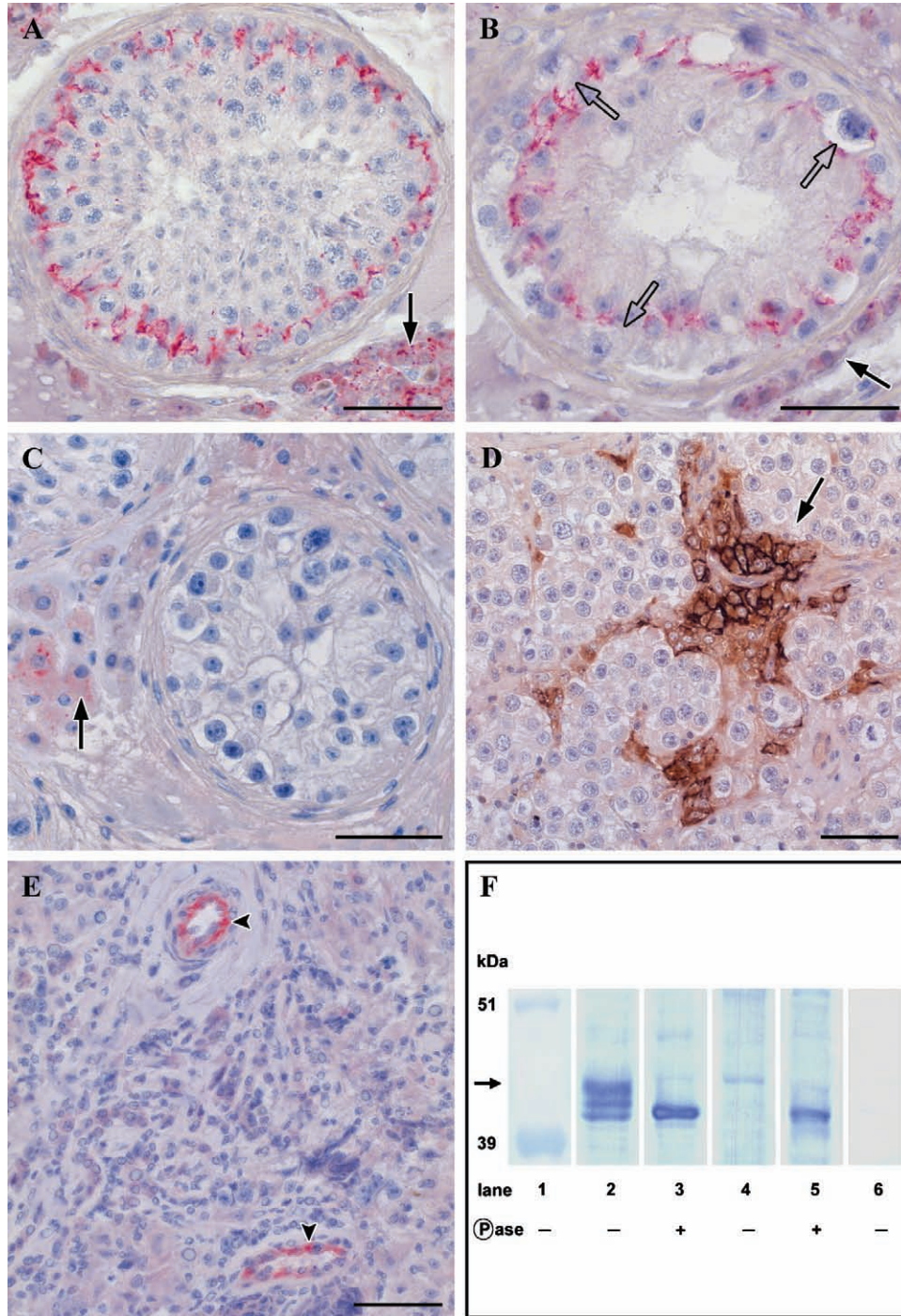
Figure 4. *In seminiferous tubules showing normal spermatogenesis, a strong immunoreactivity at the level of the blood-testis barrier, apical to spermatogonia, basal to primary spermatocytes, along the Sertoli cell junctional complexes and between interstitial Leydig cells (black arrow, A) was observed. The expression pattern of Cx43 protein in CIS-only tubules was found to be heterogeneous. In very few tubules, a disorganized immunoreactive signal was present at the level of the blood-testis barrier (CIS cells: transparent arrows, B). Most of these tubules were found completely immunonegative for Cx43 protein (C). Leydig cells displayed a strong and persistent Cx43 immunoreactivity (black arrows, B and C). In seminoma, only between cells of single cell clusters within connective tissue (black arrow, D) and between endothelial cells of small blood vessels (arrowheads, E) an immunostaining for Cx43 could be detected independent of staging. Double immunostaining for Cx43 and inhibin-α clearly colocalized both proteins to the same cell type and identified these cells as Leydig cells (arrow, D). Cx43 protein (blue/pink) was imunolocalized to the plasma membranes of Leydig cells, whereas inhibin-α immunostaining (brown) was detectable in the cytoplasm of the same cell(s) (D). The tumor cells showed no immunoreaction for Cx43 at all (D and E). Scale bars = 50 μm. Representative Western blot analysis (F) from normal testicular tissue revealed three immunoreactive bands between 41 and 45 kDa (lane 2). Incubation with CIP changed the electrophoretic profiles of Cx43. Observed bands at 45 (Cx43-P2) and 43 kDa (Cx43-P1) were lost after CIP treatment, resulting in one strong band at 41 kDa (Cx43-P0, lane 3) indicating that these two former bands represent double- and single phosphorylated Cx43 isoforms. In seminoma, only a weak immunoreactive band at 45 kDa (Cx43-P2) could be demonstrated (lane 4). In addition, this band was lost after CIP treatment, resulting in the Cx43-P0 isoform (lane 5). In negative controls, no immunoreactive bands have been observed (lane 6). Lane 1: protein marker.*

Discussion

During normal spermatogenesis, there is a close correlation between Cx43 protein synthesis and Cx43 mRNA expression, as regulation of Cx43 gene expression is tightly controlled at the transcriptional level as can be seen by its stage-dependent expression pattern [43]. Cx43 mRNA was detectable mainly in the cytoplasm of Sertoli cells, in primary spermatocytes and spermatogonia. This corresponds to the location of Cx43 protein between Sertoli cells and between Sertoli cells and germ cells indicating that these normal germ cell populations may be capable of synthesizing Cx43 protein and assembling gap junctions with Sertoli cells. These data are in accordance with previous studies showing a similar Cx43 mRNA expression pattern in mouse and human testis [31,43] and were confirmed by RT-PCR and RT-PCR from microdissected seminiferous tubules applied in this study.

For the first time, the Cx43 transcript expression pattern was demonstrated at different stages of human testicular germ cell neoplasia. The expression of Cx43 mRNA in CIS-only tubules containing both CIS and Sertoli cells was found to be variable. The hybridization signal was, in most tubules, either markedly reduced or totally absent. In very few tubules, however, a signal was mainly present in the cytoplasm of Sertoli cells corresponding with our present and previous immunohistochemical data [10]. This discrepancy of signal intensity in Sertoli cells between CIS-only tubules may be an indication of 1) the known existing partial phenotypic difference of CIS [3], 2) Sertoli cells exhibiting an altered state of maturation compared to Sertoli cells in other CIS-only tubules, or 3) a more progressive stage of tumor development toward seminoma. Specific bands for Cx43 using RT-PCR with testis homogenates from patients showing CIS or CIS-only (data not shown) and seminoma revealed that Cx43 mRNA was still expressed in neoplastic tissue, due to permanent expression of Cx43 in Leydig cells, endothelial cells and/or peritubular cells. In patients showing seminoma, endothelial cells of small blood vessels and isolated clusters of Leydig cells were found to be positive for both Cx43 mRNA and protein. Cx43 and inhibin-α double immunostaining clearly identified these cell clusters as residual Leydig cell clusters, in addition to endothelial cells, as the main source of nonneoplastic cells in seminoma expressing Cx43. Inhibin-α was chosen as this subunit is known to be expressed in human Leydig cells [44]. Furthermore, immunohistochemical stainings revealed no immunoreactive signals for Cx43 between seminoma cells, suggesting that these cells are unable to synthesize Cx43 protein and unable to communicate through Cx43 gap junction channels, and Western blot analyses performed with seminomatous tissue showed only a weak immunoreactive band for Cx43 at 45 kDa. This weak band was identified by CIP treatment as the Cx43-P2 isoform. Phosphorylation of Cx43 is known to play a role in its life cycle, as observations showed that this Cx becomes phosphorylated before its arrival at the plasma membrane and is associated with the accretion of connexons into gap junction plaques [45]. Although Cx43 in nonjunctional plasma membrane regions is not necessarily phosphorylated, Cx43 located in gap junctions has been reported to be primarily

phosphorylated to the P2 form [45]. The role of Cx43-P2 in seminoma remains to be elucidated, but this Cx43 isoform probably originates from membranous Cx43 in residual Leydig cells, as can be seen in our present immunohistochemical stainings. However, the observed loss of Cx43-P0 and Cx43-P1 in Leydig cells in seminoma may indicate 1) an altered or disrupted renewal of these two Cx43 isoforms in seminoma, 2) a Leydig cell–specific property because it has been shown that different tissues (cells) exhibit their own characteristic fingerprint of Cx43 phosphorylation [46], or 3) an aberrant (compensational) hyperphosphorylated state of Cx43 in these cells, as it is known that neoplastic progression also influences the function of the remaining Leydig cells [47].

Interestingly, in CIS tubules a more disorganized staining pattern of Cx43 protein was visible that changed to a complete loss of Cx43 protein in most CIS-only tubules. The phenomenon of an aberrant localization of Cx proteins is well known [32,36,48,49] and may in these few CIS tubules be due to a disturbed intracellular trafficking of Cx43 protein or to the loss of "normal" Sertoli cells as regulators for organized synthesis and assembly of Cx43 to the plasma membrane.

Microdissection of seminiferous epithelium followed by RT-PCR analysis showed, in accordance with foregoing results, that tubules resembling only CIS and Sertoli cells were found in most cases negative, and that seminoma cells isolated from various sections exhibiting different seminomatous stages (pT1–pT3) showed no signal for Cx43 mRNA, indicating that loss of Cx43 protein is, in most cases, accompanied by a loss of Cx43 mRNA. Similar results were found in seminiferous tubules with SCO syndrome; a transcriptional regulation was evident, as there was no synthesis of Cx43 protein and expression of Cx43 mRNA [31]. Data from our cDNA microarray analysis showed a 9.1-fold decrease of Cx43 mRNA expression in patients with different stages of seminoma compared with patients revealing normal spermatogenesis, as confirmed by semiquantitative RT-PCR exhibiting a 2.8-fold decrease of Cx43 mRNA. The varying Cx43 transcript reduction using the two methods is probably caused by the different methodological approaches, i.e., primers/probes and the normalization to the housekeeping gene. However, both data indicate that the tumor suppressor gene Cx43 is significantly downregulated with progression to seminoma and that this downregulation seems to represent a process starting in CIS tubules. Development of seminoma is known to go along with an increase in the number of blood vessels, microvascular density, vascular endothelial growth factor, and thymidine phosphorylase expression [50,51]. Thus, upregulation of Cx37 expression in the present study might be explained by enhanced angiogenesis and formation of new blood vessels associated with tumor progression.

The etiological role of disorders in gap junctional intercellular communication (GJIC) in carcinogenesis was already formulated by Loewenstein and Kanno [52], indicating that tumor cells usually have a low capacity to communicate with each other. The lack of GJIC in cancer cells was extended to cancer cell-normal cell communication, and it has been suggested that this selective communicational isolation of tumor cells from surrounding normal cells helps to escape

from signals keeping proliferation in normal tissues under negative control [53].

Consequently, one possible cause for the still unknown invasive potential of CIS cells could be their growing independence of adjacent Sertoli cells based on their loss of GJIC through gap junctions that contain Cx43. This is in line with studies in other organs showing that impaired expression of Cx43 has been implicated to correlate with neoplastic transformation and increased invasiveness [54,55]. Recently it has been shown that regulation of cell proliferation is mediated not by the channel but by an interaction of the C terminus of Cx43 with an intracellular signaling cascade [56]. Furthermore, the growth regulator protein CCN3 (NOV, nephroblastoma overexpressed) seems to mediate the growth suppression by binding to the C terminus of Cx43 in glioma as well as choriocarcinoma cell lines [57,58]. Thus, further investigation is needed to discriminate between intercellular and intracellular signaling for growth regulation properties of the Cx.

Consistent with our present and previous study [10], Wilgenbus et al. [59] failed to identify Cx43 in human testicular tumors by immunofluorescence. In contrast to our results, Roger et al. [32] found an aberrant intracytoplasmic Cx43 immunostaining in seminoma cells of four patients with seminoma and in the seminoma cell line JKT-1. These results may be due to the low number of patients investigated and by the fact that the authors did not consider the expression of Cx43 in Leydig cells or endothelial cells in seminomatous tissue.

Finally, it is known that normal germ cells exert reciprocal effects on Sertoli cells and that normal spermatogenic progression is required for maintenance of Sertoli cells in their fully differentiated state [60,61]. Interestingly, in human testicular cancer it has been proposed that cancer progression may be correlated with an impaired function and altered differentiation of somatic Sertoli cells [7–11]. Thus, it may be speculated that reduction of Cx43 in Sertoli cells leads to an altered state of Sertoli cell maturation and in turn to enhanced germ cell proliferation, as Sertoli cells are no longer able to transmit signals to adjacent CIS cells keeping their proliferation under negative control.

In conclusion, our data show that progression from CIS to seminoma is associated with an intratubular reduction or even loss of Cx43 gene expression and Cx43 protein synthesis mainly in Sertoli cells, indicating that regulation of their Cx43 expression takes place at transcriptional level. These changes may further be implicated in the proliferation of CIS cells in the progression phase of human seminoma development. Finally, loss of growth-suppressing signals from Sertoli cells through Cx43 gap junction channels to adjacent CIS cells may lead to uncontrolled growth of CIS cells and their increased invasive potential, which, in turn, results in an altered state of Sertoli cell maturation.

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