The Tumor Gene Survivin Is Highly Expressed in Adult Renal Tubular Cells

Implications for a Pathophysiological Role in the Kidney

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The inhibitor of apoptosis protein survivin is of critical importance for regulation of cellular division and survival. Published data point to a restricted function of survivin in embryonic development and cancer; thus survivin has been broadly proposed as an ideal molecular target for specific anti-cancer therapy. In contrast to this paradigm, we report here broad expression of survivin in adult differentiated tissues, as demonstrated at the mRNA and protein levels. Focusing on the kidney, survivin is strongly expressed in proximal tubuli, particularly at the apical membrane, which can be verified in rat, mouse, and human kidneys. In the latter, survivin expression seems to be even stronger in proximal tubuli than in adjacent cancerous tissue. Primary and immortalized human renal tubular cells also showed high levels of survivin protein expression, and RNA interference resulted in a partial G₂/M arrest of the cell cycle and increased rate of apoptosis. In conclusion, survivin may be of importance for renal pathophysiology and pathology. The predominant apical expression of survivin may indicate a further, yet unknown, function. Interventional strategies to inhibit survivin's function in malignancy need to be carefully (re)evaluated for renal side effects, as well as for other possible organ

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The kidney is an organ with a multitude of highly specific tasks, such as maintenance of water and electrolyte homeostasis, blood pressure control, and regulation of erythropoiesis. For most of these, the tubular system is of crucial importance. Renal tubular cells are very sensitive to a large number of clinically relevant stresses, such as hypoxia/ischemia, sepsis, or different toxic agents. The uniform pathomorphological appearance of such lesions is acute tubular necrosis, which leads to acute renal failure and has profound socio-economical impact, as well as high relevance for mortality of the critically ill patients. In addition, acute tubular injury can contribute to the progression of chronic kidney disease.¹

Together with the thick ascending limb of Henle, the proximal tubule is the most sensitive region of the tubular system. Most of the transepithelial transport takes place in the proximal tubule, leading to a very high rate of energy consumption. At the same time the availability of energy substrates is restricted, because tubular cells are not able to perform glycolysis and the peritubular blood supply is easily hampered because of its postcapillary character.^{2,3} Taken together, the proximal tubule is a functionally important but highly susceptible structure. Considering the delicate nature of the proximal tubulus, its extraordinary ability for repair is remarkable, which involves a high rate of proliferation and differentiation processes potentially leading to complete restoration of

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kidney function even after extended tubular necrosis. Therefore, biological mechanisms involved in maintenance of renal function, protection against cellular stresses, and control of repair processes are of great interest.

Apoptosis is an important process for functional maintenance, development, and repair of tubular injury, in which control of apoptosis is a careful balance between pro- and antiapoptotic influences.^{4,5} Interestingly, it has been shown previously that genetic inactivation of apoptosis inhibitors or activators of proliferation in tubular epithelial cells leads to renal cystic disease.⁶

A protein believed to have a bifunctional role in cell division and cell survival, thus influencing both cellular proliferation and apoptosis, respectively, is survivin, the smallest member of the inhibitor of apoptosis gene family (IAP).⁷ The molecule comprises one N-terminal baculovirus IAP repeat (BIR) domain and a long C-terminal α -helix coiled region and forms a stable homodimer in solution.^{8,9} Regulation of survivin spans from transcriptional mechanisms to the post-translational modifications of phosphorylation and ubiquitination, as well as localization to distinct subcellular compartments.^{7,10–12} Survivin has been implicated in binding of the microtubules of the mitotic spindle, centromeres, kinetochores, and intracellular midbodies, enabling coordinated cellular division.^{13,14} Survivin expression is sharply induced in the G₂/M phase, which is primarily governed by transcriptional mechanisms.^{15,16} Likewise, disruption of survivin function leads to cell division defects, with cells becoming polyploid and multinucleated.^{17,18}

In general, the IAP family of proteins are characterized by the presence of one to three BIR domains and their ability to inhibit apoptosis.¹⁹ The latter can be achieved either by direct binding and inhibition of caspases or by interfering with caspase activation. With respect to survivin, these mechanisms have been discussed controversially. Different possibilities have been considered for survivin, such as direct caspase binding, capture of inhibitors of other IAP family members, or maintenance of correct checkpoint mechanisms of the cell cycle, without which cells undergo apoptosis.^{20,21} Nevertheless, disregarding the yet undefined molecular mechanism, a large body of evidence has demonstrated that survivin has indeed a strong potential of antagonizing apoptosis.⁷ Interestingly, considering its bifunctional role, it has recently been shown that the mitotic and anti-apoptotic function of survivin can be separated by mutation of a rev-like or CRM1-dependent nuclear export signal, which could implicate distinct molecular pathways.^{22–24}

The anticipated biological function of survivin is defined by a paradigm of a characteristic oncofetal role. Abundant and ubiquitous expression has been shown in embryonic tissues.²⁵ Likewise, a homozygous knockout for survivin by targeted recombination led to embryonic lethality as early as day 4.5 (postcoitum) of murine development.^{26,27} Already in the initial report of survivin identification, the difference of expression from almost none in adult tissues to pronounced up-regulation in cancerous tissues was described.⁸ Meanwhile, it is firmly established that survivin is a highly expressed

gene in virtually all human solid tumors. Furthermore, numerous studies have demonstrated a convincing correlation between survivin expression in tumors, disease progression, and prognosis.²⁸ Therefore, survivin seems to be a single relevant factor for enhancing tumor aggressiveness.

For many years, there was a prevalent notion that survivin was not or hardly expressed in adult differentiated tissues. Therefore, the possibility of specifically targeting tumor cells by inhibiting survivin was widely proposed. Recently, however, numerous studies have demonstrated survivin expression in physiological tissues and normal cells.^{29,30} Therefore, careful descriptions of survivin expression and function in adult noncancerous tissues are urgently required. This study is the first description of survivin expression in adult rat, mouse, and human kidneys, comparing these data with developing kidneys and renal cell carcinoma. Survivin expression was mainly observed in tubular epithelial cells and podocytes, with strongest expression in proximal tubules. This is of particular interest because both cell types are considered to be quiescent or postmitotic, respectively. Considering the susceptible nature of the proximal tubular apparatus, the high level of survivin expression in these cells may be of importance for renal physiology. Furthermore, the involvement of survivin in renal repair mechanisms could be postulated. Because we have also found expression in other organs, we would like to encourage similar studies in other organs and predict the necessity of a new definition of the function of survivin in pathophysiology and pathology.

Materials and Methods

Unless otherwise stated all chemicals were from Sigma-Aldrich (Taufkirchen, Germany).

Collection of Human Tissues

Tissues were collected from radical tumor-nephrectomies, either snap-frozen in liquid nitrogen (microdissection), fixed in 4% paraformaldehyde (immunohistochemistry), and/or prepared for cell isolation (see below). Written informed consent was obtained from each patient before nephrectomy. Samples of embryonic human kidneys were obtained from a historic collection.³¹ The collection of human tissues for this study was approved by the local ethics committee.

Animal Procedures

Adult male C57BL/6 mice and adult male Sprague-Dawley rats at the age of 3 to 6 months were sacrificed by cervical dislocation and the organs perfusion-fixed with 4% paraformaldehyde (unless stated otherwise), processed, and embedded into paraffin, as described in detail.³² Hypoxic exposure of rats was performed in an INVIVO₂ 400 (Ruskin, Bridgend, UK) at 7% O₂ for mice and 9% O₂ for rats, for 8 hours. For segmental infarction, a branch of the renal artery was ligated before sacrifice,

for the indicated time points. To induce toxic acute renal failure, cisplatin was injected intraperitoneally (8 mg/kg) for the indicated time before sacrifice. Samples of developing kidneys were derived from newborn Sprague-Dawley rats.³¹ All animal procedures were approved by the institutional review board.

Cells and Culture Conditions

For cell culture studies the immortalized cell lines HKC-8 (kindly provided by Dr. L. Racusen, Johns Hopkins University School of Medicine, Baltimore, MD), HK2 (purchased at American Type Culture Collection, Bethesda, MD), and primary human (proximal) tubular cells (hPTs) were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Sigma-Aldrich) in a 1:1 ratio, supplemented with penicillin (50 U/ml) and streptomycin (50 µg/ml). Cells were incubated at 37°C at 5% CO₂ in humidified air. HKC-8 and HK2 were both derived from human proximal tubular cells and immortalized by viral transformation. Their medium additionally contained 10% fetal calf serum (PAA, Cölbe, Germany), insulin (10 µg/ml), and transferrin (5 µg/ml). hPTs were isolated and cultured according to the method of Detrisac and colleagues.³³ In brief, renal cortical tissues were collected from radical tumor-nephrectomy samples at greatest distance from the tumor tissue. The samples were stored and transported from the operating theater in precooled (4°C) Hanks' balanced salt solution. In the sterile cabinet, the renal capsule was removed, and the outer cortex was cut into 1-mm³ pieces. These tissue blocks were cultured in standard plastic dishes with serum-free medium additionally containing epidermal growth factor (10 ng/ml), insulin (10 µg/ml), transferrin (5 µg/ml), hydrocortisone (36 ng/ml), and triiodothyronine (4 pg/ml). On sufficient outgrowth of epithelial cells, trypsin was applied to subculture the cells. Experiments were conducted on passage 3, ~4 to 5 weeks after tissue preparation. For experiments, subconfluent cultures were used. The culture medium was renewed at the beginning of each experiment. Hypoxic exposure was in a Ruskinn INVIVO₂ 400 with 1% oxygen, 5% CO₂, balance nitrogen. Exposure to experimental conditions was for 24 hours.

Protein Extraction and Immunoblot Analysis

Protein extraction of tissues and cells was performed as described.³⁴ In brief, mouse tissues were frozen in liquid nitrogen and stored at -80°C. For cell culture extracts, adherent cells were washed with ice-cold phosphate-buffered saline (PBS) and removed by scraping. Cell pellets and weighed tissues (weight/volume ratio of 1:20) were homogenized into extraction buffer (7 mol/L urea, 10% glycerol, 10 mmol/L Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethyl sulfonyl fluoride with 1 mg/L aprotinin, pepstatin, and leupeptin) using a T8 Ultra-Turrax homogenizer (IKA, Staufen, Germany) for 10 seconds at full speed. Extracts were quantified using the DC protein assay (Bio-Rad, Munich, Germany). For survivin immunoblotting,

proteins were resolved in sodium dodecyl sulfate and 14% polyacrylamide gels and transferred to Immobilon P (Millipore, Bedford, MA) overnight in 10 mmol/L Tris, 100 mmol/L glycine, 10% methanol, and 0.05% sodium dodecyl sulfate. Membranes were blocked with PBS, 5% fat-free dried milk, and 0.1% Tween 20 and probed with either polyclonal antibody, AF886 (1:1000; R&D Systems, Wiesbaden, Germany) or monoclonal antibody, NB500-238 (clone 60.11) (1:1000; Novus Biologicals, Littleton, CO) and horseradish peroxidase-conjugated secondary antibodies. Signals were visualized by chemiluminescence (Pierce, Rockford, IL). Details of all primary and secondary antibodies used are given in Table 1. As a control for correct molecular weight of survivin, we used reticulocyte lysates programmed with full-length human survivin cDNA, with which we performed an *in vitro* transcription and transcription reaction, according to the manufacturer's instructions (TNT Quick Coupled; Promega, Mannheim, Germany).

Survivin Immunohistochemistry

Paraffin-embedded specimens were cut into 4-µm sections, dewaxed, and rehydrated in a series of ethanol washes, and endogenous peroxidase activity was blocked. Slides were coated with 3-aminopropyl-tri-ethoxysilane. Immunohistochemical staining was performed as described in detail.³⁵ A commercial protocol was followed based on a streptavidin-biotin-peroxidase reaction (DAKO, Hamburg, Germany). For antigen retrieval, slides were cooked for 18 minutes in 10 mmol/L citrate buffer (pH 6.0) by using a standardized pressure cooker (Biocare Medical, Walnut Creek, CA). Next, slides were blocked with PBS and 5% fat-free dried milk for 30 minutes at room temperature. Sections were incubated with primary antibodies overnight at 4°C. Thorough rinsing was followed by incubation with biotinylated secondary antibody for 1 hour at room temperature and streptavidin/biotinylated alkaline phosphatase for 30 minutes. Finally, AEC solution (DAKO) was used as chromogen according to the manufacturer's instructions. All incubations were performed in a humidified chamber. Between incubations, specimens were washed three times in Tris-buffered saline (50 mmol/L Tris-HCl and 136 mmol/L NaCl, pH 7.4). Samples were processed in parallel. At equal incubation times, the omission of primary antibody resulted in completely negative signal, at comparable exposure times. Finally, the sections were counterstained with hematoxylin solution according to Mayer (DAKO) and were analyzed with a Leica DMRB microscope (Leica, Bensheim, Germany), partly by using differential interference contrast. Photographs were recorded digitally (Visitron, Puchheim, Germany). The detailed immunohistochemical studies were performed with rabbit anti-survivin antibody (AF886). All principal findings in rat and human tissues were confirmed with a mouse monoclonal antibody (clone 60.11). Specific expression in proximal tubuli was also confirmed by immunohistochemistry with the antibodies mAb clone 32.1 (Novus Biologicals) and pAb NB 500-201 (Novus Biologicals). Details of all primary and secondary antibodies used are given in Table 1.

Table 1. Details of Antibodies Used

Figure	Method	Detected protein	Primary antibody	Concentration/dilution ($\mu\text{g/ml}$)	Secondary antibody (source)	Concentration/dilution ($\mu\text{g/ml}$)
1 (A–F)	IHC	Survivin	pAB AF886 (R&D Systems, Wiesbaden, Germany)	10.0	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (DAKO)	8.0
1 (G–I)	IF	Survivin	pAB AF886 (R&D Systems)	10.0	Red fluorescent dye-labeled anti-rabbit immunoglobulin (Invitrogen)	10.0
2 (A–G)	IHC	Survivin	pAB AF886 (R&D Systems)	10.0	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (DAKO)	8.0
2 (H)	IHC	Survivin	mAB clone 60.11 (Novus Biologicals)	9.0	Polyclonal swine anti-mouse immunoglobulins/ biotinylated (DAKO)	6.0
3 (C–E)	IB	Survivin	pAB AF886 (R&D Systems)	1.0	Polyclonal swine anti-rabbit immunoglobulins/ HRP-conjugated (DAKO)	0.3
4 (A–E)	IHC	Survivin	pAB AF886 (R&D Systems)	10.0	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (DAKO)	8.0
5 (A–F)	IHC	Survivin	pAB AF886 (R&D Systems)	10.0	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (DAKO)	8.0
6 (A)	IB	Survivin	pAB AF886 (R&D Systems)	1.0	Polyclonal swine anti-rabbit immunoglobulins/ HRP-conjugated (DAKO)	0.3
6 (C–F)	IHC	Survivin	pAB AF886 (R&D Systems)	10.0	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (DAKO)	8.0
7 (A)	IF	Survivin	pAB AF886 (R&D Systems)	10.0	Red fluorescent dye-labeled anti-rabbit immunoglobulin (Invitrogen)	10.0
7 (B)	IB	Survivin	pAB AF886 (R&D Systems)	1.0	Polyclonal swine anti-rabbit immunoglobulins/ HRP-conjugated (DAKO)	0.3
S1 (A)	IHC	Synaptopdin	mAB clone G1D4 (Acris, Hiddenhausen, Germany)	10.0	Polyclonal swine anti-mouse immunoglobulins/ biotinylated (DAKO)	6.0
S1 (C)	IHC	NCC	pAB (gift from David Ellison, Portland, OR)	2.0	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (DAKO)	8.0
S1 (E)	IHC	Calbindin	pAB D-28K (Chemicon, Temecula, CA)	0.5	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (DAKO)	8.0
S1 (G and I)	IHC	11 β -HDII	pAB AB1296 (Chemicon)	1.0	Polyclonal rabbit anti-sheep (Vector Laboratories, Peterborough, UK)	6.0
S2 (A)	IHC	Survivin	pAB AF886 (R&D Systems)	10.0	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (DAKO)	8.0
S2 (B)	IHC	Survivin	pAB 500.201 (Novus Biologicals)	10.0	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (DAKO)	8.0
S2 (C)	IHC	Survivin	mAB clone 60.11 (Novus Biologicals)	9.0	Polyclonal swine anti-mouse immunoglobulins/ biotinylated (DAKO)	6.0
S2 (D)	IHC	Survivin	mAB clone 32.1 (Novus Biologicals)	9.0	Polyclonal swine anti-mouse immunoglobulins/ biotinylated (DAKO)	6.0
S3	IHC	PCNA	mAB clone P10 (DAKO)	1.0	Polyclonal swine anti-mouse immunoglobulins/ biotinylated (DAKO)	6.0

Primary and secondary antibodies used for immunohistochemistry, immunofluorescence, and immunoblotting are listed, with inclusion of sources, purpose, and antibody concentrations. IB, immunoblot; IHC, immunohistochemistry; IF, immunofluorescence; NCC, sodium chloride co-transporter; 11 β -HDII, 11 β -hydroxysteroid dehydrogenase type II.

Demonstration of Antibody Specificity by Peptide Preabsorption

Fifty μg of purified full-length recombinant human survivin peptide (R&D Systems) were incubated with 5 μl of primary rabbit anti-survivin antibody (AF886; R&D Systems) in 200 μl of PBS at 37°C for 2 hours. The peptide/antibody solution was centrifuged for 15 minutes at $14,500 \times g$ to pellet any immune complexes. After the supernatant was carefully removed, the pellet was resolved in 500 μl of PBS and 5% fat-free dried milk and used for immunohistochemistry as described above. As control, 5 μl of primary anti-survivin antibody without recombinant human survivin protein, but with 50 μg of bovine serum albumin, were processed in parallel and applied for immunohistochemistry.

Survivin Immunofluorescence

Paraffin-embedded kidney sections were processed as above and incubated with a primary rabbit anti-survivin antibody (AF886; R&D Systems). After incubation overnight at 4°C and washing with Tris-buffered saline, tissues were incubated with red fluorescent dye-labeled anti-rabbit IgG (1:100; Invitrogen, Karlsruhe, Germany) at 37°C for 1 hour. Finally, the nuclei were stained with 4,6-diamidino-2-phenylindole (6.5 $\mu\text{g}/\text{ml}$; Invitrogen) for 1 hour, and the stained sections were observed and photographed with a Nikon fluorescence microscope (Düsseldorf, Germany).

Laser-Assisted Microdissection

Frozen tissue of human kidneys was embedded in tissue freezing medium (Leica Instruments, Nussloch, Germany) and cut into 10- μm -thick sections onto PEN-membrane covered slides (PALM MicroLaser Technologies, Bernried, Germany). The sections were fixed with acetone, counterstained with hematoxylin solution according to Mayer, and then microdissected by a PALM MicroLaser System. In general, ~ 50 cortical tubular structures per slide were cut and collected without differentiation into proximal or distal tubuli in PALM adhesive caps. For comparison, ~ 30 glomeruli per slide were collected from the same samples.

RNA Extraction and Real-Time PCR

Survivin mRNA expression was assayed by performing real-time PCR from different mouse tissues and from the microdissected tubuli and glomeruli of human kidneys. RNA extraction from the latter was performed by column purification using the RNeasy micro kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and all RNA transcribed into cDNA, using the Sensiscript RT kit (Qiagen). For whole tissue extraction, mouse kidney tissues were weighed and lysed into RNA-Bee (Tel-Test, Friendswood, TX) under electric homogenization in a weight/volume ratio of 1:10, following the manufacturer's instructions. RNA was dissolved in distilled water and

quantified, and 1 μg of total RNA was subjected to cDNA synthesis (Superscript II; Roche, Mannheim, Germany). For real-time PCR intron-spanning primer sequences for mouse (sense: 5'-ATGCCACCTTCAAGAACTG-3', antisense: 5'-GGCCAAATCAGGCTCGTTCT-3'; product size, 111 bp) and human (sense: 5'-CTTGGCCCAGTGTTCCT-3', antisense: 5'-CCTCCCAAAGTGCTGGTATT-3'; product size, 111 bp) survivin were used. Controls were mouse $\beta 2$ -microglobulin (B2M, sense: 5'-GCTATCCAGAAAACCCCTCA-3', antisense: 5'-CCGTTCTTCAGCATTGGAT-3'; product size, 131 bp), mouse cyclophilin A (sense: 5'-GTCTCCTTCGAGCTGTTTG-3', antisense: 5'-TGGCGTGTAAGTCACCACC-3'; product size, 131 bp), mouse hypoxanthine phosphoribosyl transferase (HPRT, sense: 5'-CGTCGTGATTAGCGATGATG-3', antisense: 5'-GTCCATAATCAGTCCATGAG-3'; product size, 119 bp) and human β -actin (sense: 5'-AGTCCTGTGGCATCCACGAAA-3', antisense: 5'-GTCATACTCCTGCTTGCTGA-3'; product size, 281 bp). All primers were used at a concentration of 300 nmol/L, with 55°C as annealing temperature. A commercial 2 $\times$ SYBR Green PCR Mix (Eurogentec, Seraing, Belgium) was used according to the manufacturer's instructions. PCR was performed with 50 cycles, taking 2 μl of cDNA into the reaction with an end volume of 25 μl . Values for survivin were related to their controls using the $2^{-\Delta\text{ct}}$ calculation method.

Survivin Knockdown by siRNA

For survivin knockdown, short interfering RNA (siRNA) of the sequence sense, 5'-CUGGACAGAGAAAGAGC-CATT-3' and antisense, 5'-UGGCUCUUUCUCUGUCCAGTT-3' were used (as described by Rödel and colleagues³⁶). As a negative control, we used an siRNA targeting green fluorescence protein (GFP) of the sequence sense, 5'-GGUGUGCUGUUUGGAGGUCTT-3' and antisense, 5'-GAACUCCAAACAGCACACCTT-3', at a concentration of 100 nmol/L each. Knockdown was performed as previously described,³⁷ 24 hours before analysis for cell cycle or initiation of apoptosis assays (see below).

Cell Cycle Analysis

Both adherent and detached hPTs were collected by trypsinization and washed three times with PBS for 5 minutes by centrifugation at $125 \times g$. Cells were resuspended in a staining solution containing 1.5 $\mu\text{mol}/\text{L}$ propidium iodide and 25 $\mu\text{g}/\text{ml}$ RNase A and incubated for 30 minutes. The samples were subjected to fluorescence-activated cell sorting analysis on a FACSCalibur (BD Biosciences, Heidelberg, Germany).

Annexin V Binding Assay

Survivin knockdown was performed 24 hours before apoptotic stress, which was performed by exposing the hPTs to cisplatin or *in vitro* ischemia, as detailed above. The exposure of anionic phospholipids was measured by binding of fluorescein isothiocyanate-labeled annexin V

(AxV; Roche) to the latter. The cells were stained for 30 minutes at 4°C with 200 ng of AxV-fluorescein isothiocyanate and 500 ng of propidium iodide in 500 μ l of Ringer solution. The samples were immediately measured using an EPICS flow cytometer (Coulter Inc., Hialeah, FL). Dead cells (apoptotic and necrotic) expose phosphatidylserine and display increased FL1 values. Propidium iodide-negative and -positive cells are referred to as apoptotic and necrotic, respectively.

Caspase 3/7 Activity Assay

Alternatively, stress-induced apoptosis in hPTs was also studied by measuring the activity of the caspases 3 and 7 in a 96-well microplate format, using a commercial kit (Caspase-Glo; Promega). Cells were seeded at 1.5×10^5 per 3.5-cm well, 24 hours before knockdown was performed. Apoptotic stress was induced 24 hours after adding siRNA, which was upheld for further 24 hours. For analysis, cells were incubated for 90 minutes in a luciferase substrate mix, and lumines-

cence activity was measured in a luminometer (Berthold, Bad Wildbad, Germany).

Statistics

All values are presented as means $\pm$ SEM. Student's paired *t*-test was applied to reveal statistical significances. *P* values less than 0.05 were considered significant. Statistical analyses were performed using SPSS Software for Windows (version 13.0; SPSS, Inc., Chicago, IL).

Results

As a first step, we characterized survivin expression and cellular distribution in normal adult renal tissues by immunohistochemistry. We achieved best morphological results for the kidney by perfusion-fixation in rats. Therefore, we performed the most detailed studies in rat tissues, subsequently confirming each major finding in murine and human kidney samples. Immunohistochemistry

Figure 1. Survivin expression in adult rat kidney. Immunohistochemistry for survivin (red staining) from adult rat perfusion-fixed kidneys. **A:** Low-power image from the kidney ranging from cortex to the upper papilla emphasizes the strong cortical expression of survivin with gradual decrease toward the papilla. **B–E:** Cortical sections from the kidney display strong expression of survivin in proximal tubuli. **D:** Weaker glomerular staining most likely originates from podocytes (see also Supplemental Figure 1 at <http://ajp.amjpathol.org>). **E:** Vascular endothelial cells do not show any staining in the healthy kidney. The changeover of outer to inner medulla shows moderate survivin expression in the S3 segments of proximal tubuli (**F**), which is weaker than the cortical S1 and S2 sections but stronger than the faint staining in medullary and papillary collecting ducts (**F** and **G**; see also Supplemental Figure 1 at <http://ajp.amjpathol.org>). Original magnifications: $\times 12.5$ (**A**); $\times 100$ (**B**, **F**, and **H**); $\times 200$ (**G**); and $\times 400$ (**C** and **D**).

revealed striking expression of survivin mainly in cortical tubular structures. A low-power image of the rat kidney displays the cortical character of survivin staining (Figure 1A). The staining intensity gradually weakens toward the medulla, with no overt signal in the papilla, as judged by this low magnification. Higher magnification of the renal cortex clearly reveals the origin of the strong cortical staining, namely the proximal tubuli, which are discernible by thickness of the epithelium and the existence of brush borders (Figure 1, B and C). Interestingly, strongly positive proximal tubuli, were frequently neighbored by negative (or almost negative) tubular segments, where staining on consecutive sections revealed the distal origin (Supplemental Figure 1, see <http://ajp.amjpathol.org>). The corticomedullary border zone displays a distinct separation of strongly positive S2 and moderately positive S3 segments in the outer medulla from almost negative structures in the inner medulla (Figure 1F). In the medulla, merely very weak staining was observed in collecting ducts (Figure 1, G and H), as differentiated by consecutive staining with anti-11- β -hydroxysteroid-dehydrogenase type II (Supplemental Figure 1, I and J; see <http://ajp.amjpathol.org>).

The intensity of staining in glomeruli was clearly below the tubular staining, but survivin appeared to be expressed in podocytes (Figure 1D). Marking podocytes with an anti-synaptopodin antibody in consecutive sections confirmed that at least some of these stained moderately positive for survivin (Supplemental Figure 1, A and B; see <http://ajp.amjpathol.org>). The regional distribution and intensity of survivin staining in rat kidney is summarized in Table 2.

It is important to note that the staining of murine (Figure 2, A and B) and human kidneys (Figure 2, C–H) showed comparable results. In all species, the predominant signal was seen in cortical proximal tubuli, with low-intensity staining in the medulla, primarily by collecting ducts (Figure 2D). A small number of human kidneys were available ($n = 5$), which were not derived from a tumor-affected

Table 2. Regional Survivin Protein Expression in Rat Kidney

Structure	Cell type/region	Staining intensity
Glomeruli	Podocytes	+
	Endothelial cells	–
	Mesangial cells	–
Interstitium	Endothelial cells	–
	Fibroblasts	–
Tubular apparatus	S1	+++
	S2	+++
	S3	++
	Thin limbs	– (+)
	Thick ascending limbs	– (+)
	Distal convoluted tubule	– (+)
	Connecting tubule	– (+)
	Collecting ducts	+

The extent of survivin protein expression in different regions and structures of the kidney is estimated. The average signal intensity for each cell type within the different zones of the kidney is indicated as follows: –, no staining; +, weakly positive; ++, strongly positive; +++, very strong staining. – (+) is indicated when findings were either negative or very weakly positive in independent staining experiments.

Figure 2. Survivin expression in mouse and human adult kidney and tumor. Immunohistochemistry for survivin in perfusion-fixed mouse kidneys or immersion-fixed human kidneys. Sections through the mouse kidney stained for survivin reveals an identical survivin staining pattern to the rat, namely strong expression in the cortical proximal tubules (A and B), with much less expression in distal tubules and glomeruli, which closely resembles the expression in human kidneys (C–H). D: In human kidney, weak expression can also be seen in medullary collecting ducts. The specificity of the main antibody used (AF886) was further evaluated on consecutive sections of human kidney by antibody preabsorption, with (F) or without (E) full-length survivin protein, processing both sections strictly in parallel. E: Preabsorption almost abolished immunoreactivity. To compare survivin expression levels of human kidneys to corresponding renal tumors, patient samples were collected where the border between kidney and tumor is included and analyzed with two independent antibodies (Table 1, see <http://ajp.amjpathol.org>). As expected, ambient cytoplasmic staining was observed in renal cell carcinoma with both antibodies. However, the signal intensity derived from the adjacent renal tissue, which again originates from proximal tubular cells, was clearly higher than in the cancerous tissues. Original magnifications: $\times 40$ (G and H); $\times 100$ (A, D, E, and F); $\times 200$ (C); $\times 400$ (B).

kidney. These showed no qualitative difference for survivin expression from the tumor nephrectomized samples (data not shown).

To ascertain that the pattern of staining was specific, we aimed to confirm these findings with a number of independent antibodies. Altogether, we were able to confirm the results with two polyclonal and two monoclonal antibodies (Supplemental Figure 2, see <http://ajp.amjpathol.org>), where omission of primary antibody gave no signal (data not shown). In addition, we performed peptide preabsorption on human tissue and with the antibody we used primarily (AF886). Consecutive sections, processed strictly in parallel either with (Figure 2F) or without (Figure 2E) the

Figure 3. Validation of renal tubular survivin expression. **A:** Frozen human kidney sections were processed by laser-assisted microdissection, cutting cortical tubular sections and whole glomeruli, which served as a control. The microdissected material from 10 patients was analyzed for mRNA expression of survivin by semiquantitative real-time PCR. **B:** The relative gene expression of four representative patients, in which no or very little expression is seen in glomeruli (G), but substantial expression can be seen in tubular extracts (T). **C:** Primary hPTs of 10 patients were generated and whole cell lysates immunoblotted for survivin. The position of the molecular weight marker (Page-Ruler; Fermentas, St. Leon-Rot, Germany) at 17 kDa is indicated on the right. Extracts from immortalized human proximal tubular cell lines (HKC-8 and HK2) were loaded as control. To verify the expression of survivin in renal tissue, immunoblots were performed from mouse (D) and human (E) kidney lysates. As control for the correct molecular weight, a whole cell extract from the mouse hepatoma cell line Hepa1c4 was loaded next to *in vitro*-transcribed and -translated (IVTT) recombinant survivin protein, derived from the full-length human cDNA. Unprogrammed lysates of the same system (reticulocyte lysate) were loaded to exclude the existence of unspecific bands in the proximity of survivin mobility. Furthermore, lysates from hPTs treated with control siRNA against the green fluorescent protein (GFP) or survivin have been loaded. Total protein loaded was 20 μ g for Hepa1c4, 30 μ g for hPT, 100 μ g for murine kidneys, and 200 μ g for human kidney lysates. **C–E:** The polyclonal rabbit anti-survivin antibody AF886 was used in all immunoblots.

blocking peptide, clearly showed that the tubular signal was markedly quenched by preincubation with full-length survivin protein, therefore further strengthening the specificity of our findings.

Considering the strong expression in cortical proximal tubules we were interested in comparing the expression levels of survivin from kidney to its derived tumor. Therefore, we collected paraffin-embedded samples where the kidney is directly adjacent to the renal cell carcinoma ($n = 10$), which were clear cell in most cases. We found marked expression of survivin in these tumors, with predominant cytoplasmic staining of a somewhat focal character. However, in each case staining intensity of the kidney, either in direct proximity to the tumor or at greatest possible distance, was much stronger than in renal cell carcinoma, which is demonstrated in consecutive sections in Figure 2, G and H. Thus survivin expression in proximal tubuli is not only specific but seems to be also relatively high, even as compared with renal tumor tissue.

To strengthen further the evidence of survivin expression in adult renal tissue, we aimed to verify mRNA and protein expression with techniques other than immunohistochemistry. Renal tubuli from frozen sections of human kidneys ($n = 10$) were prepared for laser-assisted microdissection. Tubular segments were dissected, without the possibility of differentiating proximal from distal tubuli (Figure 3A). As a control, glomeruli were isolated and analyzed in parallel. Semiquantitative real-time PCR from these samples showed very weak, if any, expression in glomeruli, whereas survivin expression was readily detectable in tubuli (Figure 3B). In addition, we established primary cultures from cortical tubular cells of patients who had to undergo radical nephrectomy ($n = 10$). These cells display a typical cobblestone morphology, have a high expression level of E-cadherin and cytokeratin 18, and thus share characteristic epithelial features (data not shown). In whole cell extracts from these cells, survivin was readily detectable by immunoblotting (Fig-

Figure 4. Survivin localization on the apical membrane. Immunohistochemistry for survivin on human (A), mouse (B), and rat (C) kidney sections and rat small bowel (D–F). **A–C:** Survivin in the normal adult kidney profoundly localizes to the apical membrane in all species investigated. This finding is most prominent in perfusion-fixed rat kidneys (C), where for technical reasons tissue preservation is best. **E:** Interestingly, rat intestine epithelial cells, which are similarly involved in transport, also display a predominance of apical staining, although substantial amounts of survivin staining can also be seen in the cytoplasm. **F:** However, in the basal crypts, where no transport takes place but intensive proliferation occurs, survivin can be seen in the cytoplasm without membranous enhancement. **G** and **H:** Immunofluorescence of rat kidney sections using different detection systems than in the conventional immunohistochemistry also confirms the predominant apical staining pattern. 4,6-Diamidino-2-phenylindole staining in the right hand panel marks the nuclei of all cells (I), in the identical position as shown in H. Original magnifications: $\times 40$ (G); $\times 200$ (D); $\times 400$ (A–C, E, F, and H–I).

ure 3C). Furthermore, whole kidney lysates from adult mice (Figure 3D) and from human kidneys (Figure 3E) showed specific signals for survivin by immunoblotting. The correct molecular weight, predicted to be between 16 and 17 kDa, was verified by using human or mouse cell extracts, where appropriate, *in vitro*-transcribed and -translated (IVTT) recombinant survivin protein, or lysates derived from cell cultures with specific knock-down. Obviously, murine and human survivin proteins migrate comparably. The more variable signal intensities for survivin protein in human kidney lysates could be caused by different surgical clamping times and non-identical regions of the kidney. Considering the dominant expression of survivin in the renal cortex, samples derived from the renal medulla would be considered to have less protein.

Taken together, these data demonstrate survivin gene and protein expression in rat, mouse and human kidneys. Survivin protein expression mainly derives from proximal tubular cells, which we have been able to show by im-

munochemistry and real-time PCR from microdissected tubuli and primary tubular cultures. Importantly, the majority of our data stems from normal adult kidneys, which shows very little baseline proliferation or apoptosis.^{4,38} Thus, the question arises what function survivin has in this organ. Interestingly, we have frequently observed survivin expression mainly on the apical membrane in human, murine, and rat kidney (Figure 4, A–C, respectively). This finding was most pronounced, when tissues were optimally perfusion-fixed, which was best achieved in rat, for technical reasons. In paraformaldehyde immersion-fixed kidneys and moderately well-preserved human kidneys, survivin staining was less intense on the apical membrane and localized more strongly to the cytoplasm (data not shown). However, the localization of survivin was also more cytoplasmic in the S3 segments of proximal tubuli and regionally fluctuating in the cortex. Immunofluorescence staining techniques, using separate secondary antibodies and detection systems, confirmed the dominant membranous staining pat-

tern (Figure 4, G–I). Because proximal tubules are highly active in water and solute transport, we studied epithelial cells with similar function in small bowel and hepatic bile duct cells. Judging from immunohistochemistry, survivin was not expressed in the latter (data not shown). However, in small bowel we found strong expression of survivin in enterocytes, as has been reported by others in the basal crypts of colonic mucosa.^{39,40} In such crypts from the small bowel, we detected strong staining of cytoplasmic localization (Figure 4F). The same was true for the differentiated, transporting epithelia of the mucosal villi, although survivin did show an enhancement of signal at the apical membrane (Figure 4, D and E), similar to our findings in the kidney. Thus, it is tempting to speculate that survivin expression is either involved in transport processes or at least localization to the membrane is a feature of transporting epithelia, if expressed.

Considering the restricted expression pattern of survivin in adult kidneys, we were interested in the expression profiles of developing kidneys, where survivin has been reported to be strongly expressed.²⁵ We studied a small series of human embryonic samples (Figure 5, A–C), which have been derived from abortions for medical reasons and have been studied before,³¹ as well as from newborn rats (Figure 5, D–F), in which renal development is incomplete. Survivin was readily detectable in these kidneys, but rather surprisingly only in epithelial structures, as seen before in adult kidneys. Furthermore, the area that showed most growth and least differentiation, the so-called nephrogenic zone with the S-shaped bodies (Figure 5F) in the upper cortex, showed much weaker expression than the more developed tubules,

lying further subcortically. Therefore, cellular expression patterns are comparable in developing and adult kidneys, and strongest expression is not confined to the undifferentiated state of the structures, which may have been expected.

Having established that survivin shows a distinct expression in proximal tubuli of normal kidneys, we next examined the expression characteristics in some relevant pathological models. The proximal tubuli are frequently the site of primary damage in acute renal failure, characterized by the histopathological picture of acute tubular necrosis. For the pathogenesis of acute tubular necrosis, hypoxia and/or ischemia, as well as toxic stresses, are frequently causative. We therefore studied these influences in respect to survivin expression (Figure 6). We have seen a consistent down-regulation of survivin by hypoxia in primary or immortalized human proximal tubular cells (Figure 6A). Interestingly, this effect was equally strong with iron chelation using dipyrityl, which is a chemical inducer of hypoxia-inducible factor-1 (HIF-1), possibly indicating an involvement of this important transcription factor. The repression of survivin was not confined to tubular cells but could also be observed in two renal carcinoma cell lines (data not shown). Ischemia has been reported to up-regulate survivin expression in brain and heart.^{41,42} However, in kidney we did not observe recruitment of further structures displaying survivin expression under hypoxia (Figure 6B) or segmental infarction (Figure 6, C and D). Two subtle differences were noted. First, survivin did seem to localize more toward the cytoplasm, which was more pronounced for the infarct. Second, the level of survivin staining appeared to be

Figure 5. Survivin expression in developing kidney. Immunohistochemistry for survivin in developing human (A–C) and rat (D–F) kidneys. Surprisingly, human embryonic kidneys and newborn rat kidneys showed staining for survivin only in tubular structures, which is similar to adult tissues. Interestingly, the area of most intense growth and where the most undifferentiated structures can be found, the so-called nephrogenic zone in the outer cortex, shows markedly less intense staining than in the more developed central regions. Whereas developing proximal tubular structures show a high level of survivin expression (C, upper structure), developing collecting ducts do not show any staining (C, lower structure). Similarly survivin is not detectable in developing glomeruli (B, E) or interstitial and endothelial cells. Very low level of staining can be seen in the typical S-shaped bodies of rat kidneys in the nephrogenic zone (F, center). Original magnifications: $\times 100$ (A and D); $\times 400$ (B, C, E, and F).

Figure 6. Influence of hypoxia, infarction, and cisplatin on survivin expression. The influence of different stresses on survivin were studied, which have clinical relevance for injury to the proximal tubular cells and can cause the development of acute renal failure. **A:** In cell cultures of primary (hPT) or immortalized (HKC-8) human tubular cells 24 hours of hypoxia (H, 1% O_2) or exposure to the chemical inducer of hypoxia-inducible transcription factor dipyrindyl (DP, 100 μ mol/L) leads to substantial down-regulation of survivin protein, as measured by immunoblotting. **B:** Treating rats with hypoxia (9% O_2 , 8 hours) did not induce a qualitative difference of survivin staining in the kidney as assessed by immunohistochemistry. However, survivin protein in general seemed to be less abundant than under control conditions and more cytoplasmic. **C and D:** Segmental infarction in the rat kidney again did not show a difference in the staining pattern for survivin. The border between the ischemic/necrotic (**left**) and vital (**right**) tissue can be clearly seen in the **middle**. The ratio of apical to cytoplasmic staining of survivin in ischemic tubular cells seems to change in favor of cytoplasmic localization under tissue injury, yet apical staining is still present. Induction of acute renal failure by cisplatin (**E and F**) led to a substantial redistribution of cellular localization of survivin, where the apical orientation is completely lost and the protein is exclusively cytoplasmic.

less strong than under control conditions, although this finding is difficult to quantify. Hence, immunoblotting for survivin of whole organ extracts from hypoxic kidneys did not show a difference in survivin expression (data not shown). A time course experiment of 1, 3, and 7 days after segmental infarction revealed continuously increasing signs of proliferation (Supplemental Figure 3A, see <http://ajp.amjpathol.org>), as judged by proliferating cell nuclear antigen staining. However, in the perinecrotic zone, which is believed to be ischemic, a decrease in tubular survivin staining was noted and was maximal at day 7.

The other pathological model we studied was cisplatin intoxication, which leads to acute renal failure and uremia, with spontaneous clinical and morphological restoration, if the conditions were not too severe.⁴³ Five days after application of cisplatin, clear morphological signs of acute renal failure were seen (Figure 6, E and F), extending into the cortex and proximal tubuli. At this stage, a mean creatinine concentration of 0.59 mg/dl was measured (0.17 mg/dl in untreated controls). Again, the pattern of staining in proximal tubular cells was qualitatively no different in these diseased kidneys than in normal kidneys but seemed somewhat weaker. Immunoblotting of whole organ extracts revealed a moderate decrease of survivin protein at day 7 after injection, but not at any other time point (data not shown). However, the cellular localization of survivin was strikingly different in that it was homogeneously distributed over the cytoplasm with no enhancement of signal on the membrane. A time course experiment of 1, 3, and 7 days after cisplatin injection showed less extensive morphological injury, with most

damage in the S3 segments of the proximal tubuli (Supplemental Figure 1B, see <http://ajp.amjpathol.org>). The rise of creatinine serum levels was moderate with 0.17, 0.20, 0.74, and 0.21 mg/dl at the respective time points (0, 1, 3, and 7 days). In these experimental series no cytoplasmic redistribution of survivin in the proximal tubuli was observed, yet a substantial decrease of survivin staining was noted in the S3 segments, which was already present at day 1.

The prominent functions of survivin are cell cycle regulation and prevention of apoptosis, in which the protein is active in the cytoplasm or nucleus. Interestingly, a recent study has described localization of a survivin splice variant at membrane ruffles in endothelial cells,⁴⁴ where the function is yet to be determined. Recruitment of survivin from the membrane into the cytoplasm may indicate a functional shift, which could be involved in tissue protection and repair.

Survivin has been extensively studied in cell cycle and apoptosis assays in tumor cells, with little available data in primary cells and none in primary renal cells. Survivin immunolabeling of hPTs cultured on glass slides revealed an interesting difference in survivin localization with the majority of cells showing primarily cytoplasmic staining and a minority of cells (~20% of cells) with dominant nuclear staining, which may be caused by cells nearing mitotic division (Figure 7A, top). Rarely were we able to detect immunofluorescent labeling to structures that most likely represent the mitotic spindle apparatus (Figure 7A, bottom). Of note, the signal intensity in these cells was much higher than in neighboring, nondividing cells, probably reflecting the previously described mi-

Figure 7. Subcellular localization and influence of survivin (knockdown) on cell cycle of primary tubular cells. **A:** Immunofluorescence for survivin on primary hPTs cultured on glass slides (**left**). **A: Right:** 4,6-diamidino-2-phenylindole-staining of the identical positions. The **top** clearly shows the difference in subcellular localization from predominant cytoplasmic in the majority of the population to predominant nuclear in individual cells. The latter may indicate the timely proximity to mitosis. The **bottom** shows survivin localizing to the mitotic spindle apparatus of a dividing cell. Furthermore, protein expression is much higher in this dividing cell, than in the surrounding cells. Exposure time of **top left** 1/4 second, of **bottom left** 1/16 second. **B:** RNA interference was performed on hPTs, either for GFP as a control or for survivin, which led to a pronounced decrease of protein levels, as measured by immunoblotting. **C and D:** Twenty-four hours after survivin knockdown in hPTs, measurement of DNA content by using the fluorescence-activated cell sorting-propidium iodide staining method revealed a G₂/M arrest, indicating failure of cells to exit mitosis. The difference of G₂/M fractions, as well as the reduction in G₁ phase, between survivin and GFP knockdown was statistically significant (*). **C:** The mean values of three independent experiments from different patients, where the error bars are SEM. **D:** The original results of one representative experiment.

otic up-regulation. These data implicate that survivin is also responsible for cell division processes in primary tubular cells.

The functional role of survivin was then studied by using specific siRNA, which led to a significant knockdown in hPTs (Figure 7B). Analysis of cell cycle distribution by propidium iodide staining and fluorescence-activated cell sorting 24 hours after transfection of siRNA revealed that there was indeed an influence of survivin on cell cycle regulation of these primary tubular cells (Figure 7, C and D). As has been reported previously in tumor cells, interfering with survivin's function leads to an en-

hancement of the fraction of cells within G₂/M phase, indicating a failure to exit mitosis.

In addition to cell cycle regulation, we assayed for influences of survivin on apoptosis by annexin V binding and caspase 3/7 activity (Figure 8). Confirming previous reports, which have described apoptotic effects of tumor cells but not primary cells by interfering with survivin function,⁴⁵ we did not observe an increased rate of apoptosis in primary tubular cells by survivin knockdown. Next, we tested whether cellular stresses in combination with survivin knockdown revealed a difference. Exposure to 50 μ mol/L cisplatin increased the cellular fraction of apoptotic cells approximately fourfold (Figure 8A, middle) and caspase 3/7 activity by almost 10-fold (Figure 8B, middle). Incubation of hPTs to an atmosphere of 1% oxygen was not sufficient to induce apoptosis (data not shown), whereas the addition of glucose deprivation (termed *in vitro* ischemia) caused a 10-fold increase of caspase 3/7 activity but only a minor increase of annexin V binding. The combination of ischemia with siRNA against survivin led to a doubling of the apoptotic cell fraction, whereas no relevant increase was observed with caspase activity (Figure 8, A and B; right). On the other hand, simultaneous treatment of hPTs with cisplatin and survivin knockdown further enhanced the rate of apoptosis, as measured by both techniques. (Figure 8, A and B; middle). Thus, these data provide evidence that survivin has a role for survival and cell-cycle regulation in renal tubular cells, at least under cell culture conditions.

Considering the discrepancy of our findings with the paradigm of specific oncofetal expression and function, we finally determined survivin expression in adult organs other than the kidney. We assayed the mRNA expression in numerous organ lysates of C57BL/6 mice by real-time PCR (Supplemental Figure 4A, see <http://ajp.amjpathol.org>). All organs studied (brain, heart, liver, lung, and kidney) showed significant expression levels for survivin, in which signal intensity for all tissue extracts rose above threshold before reaching 35 cycles for each organ. Normalizing the expression levels to the kidney (set to 1.0), the relative gene expression (arbitrary units) was 0.56-fold for brain, 3.51-fold for heart, 3.55-fold for lung, and 4.29-fold for liver, with cyclophilin A as control gene. Using B2M and HPRT as controls, these figures were partly different (data not shown), most likely reflecting differing expression levels of these genes in the assayed organs. Therefore, by merely using real-time PCR in this way, we cannot accurately define the expression levels between organs. Immunoblotting for survivin protein of lysates from frozen tissues of the same mice displayed distinct signals at the predicted length of 16 to 17 kDa with two independent antibodies, with an apparently higher sensitivity of the monoclonal antibody (mAb 60.11, Supplemental Figure 4B, see <http://ajp.amjpathol.org>). Of the tissues investigated, the lung showed the strongest signal, whereas survivin was almost undetectable in brain. Hence, by using sufficiently sensitive techniques, we were able to detect survivin expression in various adult tissues on mRNA and, more importantly, protein level. A fascinating task of future studies will be the clarification of survivin's function in these organs, including the kidney.

Figure 8. Influence of survivin (knockdown) on apoptosis of primary tubular cells. The influence of RNA interference against survivin on apoptosis of hPT was measured by annexin V binding (**A**) and caspase 3/7 activity (**B**). GFP siRNA was used as a control. The error bars are SEM, in which each experiment has been performed on three independent patient samples. In resting cultures, survivin knockdown did not show any effect on apoptosis, with any assay. However, differences were seen when the cells were stressed for 24 hours, with either cisplatin (**middle**) or *in vitro* ischemia (**right**). The latter is a combination of glucose withdrawal and hypoxia of 1% O₂. Both models lead to a substantial increase of caspase 3/7 activity, when compared with untreated cells (**B**), whereas only cisplatin exposure leads to a marked increase of annexin V binding (**A, middle**). A clear increase in the apoptotic fraction of cells was seen by survivin knockdown under both cell stresses (**A**, statistically significant for cisplatin and for *in vitro* ischemia, *). Using the caspase 3/7 activity assay, the survivin knockdown achieved a further increase only under cisplatin (**B**, *).

Discussion

Cellular studies have shown that survivin, the smallest member of the IAP protein family, has a bifunctional role for cellular division and survival decisions. As a consequence, a paradigm of an oncofetal expression pattern and function of survivin has emerged. As such, survivin is broadly believed to play a restricted role in embryonic development and tumor biology. However, throughout the last few years survivin has been reported to be expressed in a number of adult cells and tissues with relatively high proliferation rates, such as hematopoietic cells,^{46,47} T lymphocytes and thymus,^{15,48,49} gastric mucosa⁵⁰ and colonic crypts,^{15,39,40} testis,^{15,51,52} endometrium,⁵³ placenta,⁵⁴ and hair follicle.⁵⁵ Because survivin is critical for mitotic cell division and highly up-regulated in mitosis (Figure 7A),^{15,16} expression in these cells and tissues may simply display the anticipated high proliferation rate. In the current study, we describe pronounced expression of survivin in the proximal tubular apparatus, as well as weak expression in glomerular podocytes of the kidney. In contrast to embryonic and cancer cells, the tubular cells are believed to be quiescent and podocytes postmitotic. In general, proliferation rates within the normal adult kidney are minimal, whatever cell population studied, including tubular cells, and were found to be less than 1%.³⁸ However, our data show homogeneous expression of survivin throughout the tubular structures (Figures 1, 2, and 4), which cannot therefore be explained by mitotic activity.

Next to regulation of cellular division, survivin's second known role is inhibition of apoptosis. However, in the

normal adult kidney, very little apoptosis can be found unless the kidney is in a diseased state, such as in acute tubular necrosis, ischemia, or intoxication.^{4,5} In these settings, apoptosis can be both important for pathogenesis and critical for repair mechanisms. Importantly, in this study we describe strong survivin expression in the healthy tubular apparatus. It is therefore tempting to speculate that survivin contributes to a constitutive anti-apoptotic activity for the integrity and function of the kidney. Interestingly, previous studies provided evidence that genetic inactivation of the apoptosis inhibitors Bcl-2 and AP-2β led to the development of cystic degeneration in the kidney^{56,57} thus implicating that indeed continuous antiapoptotic activity in the kidney is required. In this context it is of particular interest that strongly active pro-apoptotic proteins such as the Fas ligand have been found to be constitutively expressed in the normal proximal tubular apparatus.⁵⁸ Further to this anticipated physiological function for maintenance of renal integrity, a role of survivin for cellular survival in renal injury could be hypothesized. Notably, a number of pathological models in different organs have demonstrated expression, regulation, and implicated function of survivin in adult tissues. In particular, survivin was shown to be up-regulated in brain trauma,⁵⁹ ischemia,⁴² and liver regeneration and liver cell survival⁶⁰ and down-regulated in gastric mucosa by indomethacin.⁶¹ For the kidney no data yet exist concerning survivin, neither for pathology nor pathophysiology. However, considering the metabolic burden of the renal tubular apparatus and the marginal perfusion rates, potent protective mechanisms are likely to be of impor-

tance. Intriguingly, survivin has been reported to interact physically with the heat shock protein 90 (HSP90),⁶² which is a molecular chaperone with a central role in the cellular stress response. In models of ischemic acute renal failure, HSP90 has been shown to be induced and have effects on cellular integrity.^{63,64} Furthermore, repair processes such as those after acute tubular necrosis require a large amount of proliferation. In its function as mitotic regulator, survivin could therefore play a role in renal repair. It is of interest that none of the studied renopathological models—hypoxia, segmental infarction, or cisplatin intoxication—led to a significant qualitative difference of survivin expression (Figure 6). Thus, the healthy, embryonic (Figure 5), and severely diseased kidney all show a predominance of survivin expression in the proximal tubuli, with no or much less expression in other renal cell populations.

Selective and dominant tubular expression of survivin may indicate a particular role in these cells. A finding that could confirm this impression is the predominant membranous expression pattern in the proximal tubuli (Figure 4). The apical orientation of cellular localization and the dominant function of these cells may implicate a role of survivin in transport processes or physiology. The similar findings in enterocytes of the small bowel (but not the basal crypts) could support this assumption. Interestingly, a recent report has provided first evidence for membranous localization of a survivin splice variant in endothelial cells.⁴⁴ By localizing survivin at the membrane ruffles in the spreading edge of these cells, the authors suggested a role for spreading and migration of endothelial cells. The role of survivin at the membrane of the healthy renal tubular cells remains to be determined. It is, however, of interest that pathological models such as cisplatin intoxication (Figure 6) led to a complete dissociation from the membrane into the cytoplasm, where the anticipated effect of cellular protection by antiapoptosis would take place. Thus, recruitment of survivin from the membrane in cellular stress could be postulated.

Considering the intensive research on expression and function of survivin in cancerous tissues, the inability of describing renal expression by other groups remains elusive. We have taken care to validate these findings with different methodological approaches and materials. However, the sensitivity of techniques may play a role, as well as the spectrum of expressed and detected splice variants. To our knowledge, the antibodies tested in our study have not been evaluated for the detection of specific splice variants, as compared with the full-length protein. Interestingly, one of the first reports in the field also mentioned immunodetection of survivin in several adult murine tissues, although embryonic and proliferating tissues were reported to be more prominent.¹⁵ Another report excluded kidney tissue as negative control for immunohistochemistry studies because of unexpected high expression levels in the tubular apparatus,⁶⁵ again using a different antibody than included in our study.

Survivin has been implicated to be of relevance not only in tumor growth and aggressiveness but also in

tumorigenesis.⁶⁶ As such, it is of interest that the proximal tubular apparatus is believed to be the origin of the frequent and aggressive clear cell renal carcinoma.⁶⁷ Whether the constitutively high levels of survivin could contribute to renal tumorigenesis remains speculative to date, yet the hypothesis of survivin deregulation as a contributor for epithelial oncogenesis has been expressed recently.⁵² Although renal clear cell carcinoma has not been studied as extensively as other tumors, a number of reports have shown strong expression of survivin and a convincing correlation to clinical outcome.^{68,69}

In summary, we demonstrate for the first time that the antiapoptotic protein survivin is highly expressed in adult renal epithelial cells. Functional analyses in primary tubular cells indicate that survivin exerts the classic functions of cell cycle regulation and survival control. However, the predominant apical expression of survivin may indicate an additional, yet unknown, function. Importantly, interventional strategies to inhibit survivin function in malignancy need to be carefully (re)evaluated for renal side effects. Interfering with survivin function may lead to serious side effects, either alone or possibly in combination with other cytotoxic agents, such as cisplatin, which itself can cause acute tubular necrosis of the proximal tubular apparatus. Learning more about survivin's function in health and disease will be an important task for future studies.

Note Added in Proof

After this article was accepted for publication, Kindt et al⁷⁰ reported on the role of survivin in toxin-induced acute renal failure.

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References

1. Gill N, Nally JV Jr, Fatica RA: Renal failure secondary to acute tubular necrosis: epidemiology, diagnosis, and management. *Chest* 2005, 128:2847–2863
2. Thadhani R, Pascual M, Bonventre JV: Acute renal failure. *N Engl J Med* 1996, 334:1448–1460
3. Schrier RW, Wang W, Poole B, Mitra A: Acute renal failure: definitions, diagnosis, pathogenesis, and therapy. *J Clin Invest* 2004, 114:5–14
4. Davis MA, Ryan DH: Apoptosis in the kidney. *Toxicol Pathol* 1998, 26:810–825
5. Ortiz A, Justo P, Sanz A, Lorz C, Egido J: Targeting apoptosis in acute tubular injury. *Biochem Pharmacol* 2003, 66:1589–1594
6. Wilson PD: Polycystic kidney disease. *N Engl J Med* 2004, 350:151–164
7. Altieri DC: The case for survivin as a regulator of microtubule dynamics and cell-death decisions. *Curr Opin Cell Biol* 2006, 18:609–615
8. Ambrosini G, Adida C, Altieri DC: A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997, 3:917–921
9. Verdecia MA, Huang H, Dutil E, Kaiser DA, Hunter T, Noel JP: Struc-

- ture of the human anti-apoptotic protein survivin reveals a dimeric arrangement. *Nat Struct Biol* 2000, 7:602–608
10. Zangemeister-Wittke U, Simon HU: An IAP in action: the multiple roles of survivin in differentiation, immunity and malignancy. *Cell Cycle* 2004, 3:1121–1123
11. Reed JC, Bischoff JR: BIRing chromosomes through cell division—and survivin' the experience. *Cell* 2000, 102:545–548
12. Wheatley SP, McNeish IA: Survivin: a protein with dual roles in mitosis and apoptosis. *Int Rev Cytol* 2005, 247:35–88
13. Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC: Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 1998, 396:580–584
14. Skoufias DA, Mollinari C, Lacroix FB, Margolis RL: Human survivin is a kinetochore-associated passenger protein. *J Cell Biol* 2000, 151:1575–1582
15. Kobayashi K, Hatano M, Otaki M, Ogasawara T, Tokuhisa T: Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. *Proc Natl Acad Sci USA* 1999, 96:1457–1462
16. Li F, Altieri DC: Transcriptional analysis of human survivin gene expression. *Biochem J* 1999, 344:305–311
17. Li F, Ackermann EJ, Bennett CF, Rothermel AL, Plescia J, Tognin S, Villa A, Marchisio PC, Altieri DC: Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat Cell Biol* 1999, 1:461–466
18. Chen J, Wu W, Tahir SK, Kroeger PE, Rosenberg SH, Cowser LM, Bennett F, Krajewski S, Krajewska M, Welsh K, Reed JC, Ng SC: Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-independent growth. *Neoplasia* 2000, 2:235–241
19. Schimmer AD: Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. *Cancer Res* 2004, 64:7183–7190
20. Johnson ME, Howerth EW: Survivin: a bifunctional inhibitor of apoptosis protein. *Vet Pathol* 2004, 41:599–607
21. Okada H, Mak TW: Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat Rev Cancer* 2004, 4:592–603
22. Colnaghi R, Connell CM, Barrett RM, Wheatley SP: Separating the anti-apoptotic and mitotic roles of survivin. *J Biol Chem* 2006, 281:33450–33456
23. Knauer SK, Kramer OH, Knosel T, Engels K, Rodel F, Kovacs AF, Dietmaier W, Klein-Hitpass L, Habtemichael N, Schweitzer A, Brieger J, Rodel C, Mann W, Petersen I, Heinzel T, Stauber RH: Nuclear export is essential for the tumor-promoting activity of survivin. *FASEB J* 2007, 21:207–216
24. Knauer SK, Bier C, Habtemichael N, Stauber RH: The survivin-Crm1 interaction is essential for chromosomal passenger complex localization and function. *EMBO Rep* 2006, 7:1259–1265
25. Adida C, Crotty PL, McGrath J, Berrebi D, Diebold J, Altieri DC: Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. *Am J Pathol* 1998, 152:43–49
26. Uren AG, Wong L, Pakusch M, Fowler KJ, Burrows FJ, Vaux DL, Choo KH: Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype. *Curr Biol* 2000, 10:1319–1328
27. Conway EM, Pollefeyt S, Steiner-Mosonyi M, Luo W, Devriese A, Lupu F, Bono F, Leducq N, Dol F, Schaeffer P, Collen D, Herbert JM: Deficiency of survivin in transgenic mice exacerbates Fas-induced apoptosis via mitochondrial pathways. *Gastroenterology* 2002, 123:619–631
28. Altieri DC: Validating survivin as a cancer therapeutic target. *Nat Rev Cancer* 2003, 3:46–54
29. Li F, Brattain MG: Role of the survivin gene in pathophysiology. *Am J Pathol* 2006, 169:1–11
30. Fukuda S, Pelus LM: Survivin, a cancer target with an emerging role in normal adult tissues. *Mol Cancer Ther* 2006, 5:1087–1098
31. Bernhardt WM, Schmitt R, Rosenberger C, Munchenhausen PM, Grone HJ, Frei U, Warnecke C, Bachmann S, Wiesener MS, Willam C, Eckardt KU: Expression of hypoxia-inducible transcription factors in developing human and rat kidneys. *Kidney Int* 2006, 69:114–122
32. Rosenberger C, Mandriota S, Jurgensen JS, Wiesener MS, Horstrup JH, Frei U, Ratcliffe PJ, Maxwell PH, Bachmann S, Eckardt KU: Expression of hypoxia-inducible factor-1 α and -2 α in hypoxic and ischemic rat kidneys. *J Am Soc Nephrol* 2002, 13:1721–1732
33. Detrisac CJ, Sens MA, Garvin AJ, Spicer SS, Sens DA: Tissue culture of human kidney epithelial cells of proximal tubule origin. *Kidney Int* 1984, 25:383–390
34. Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU: Widespread hypoxia-inducible expression of HIF-2 α in distinct cell populations of different organs. *FASEB J* 2003, 17:271–273
35. Rödel F, Hoffmann J, Grabenbauer GG, Papadopoulos T, Weiss C, Gunther K, Schick C, Sauer R, Rodel C: High survivin expression is associated with reduced apoptosis in rectal cancer and may predict disease-free survival after preoperative radiochemotherapy and surgical resection. *Strahlenther Onkol* 2002, 178:426–435
36. Rödel F, Hoffmann J, Distel L, Herrmann M, Noisternig T, Papadopoulos T, Sauer R, Rodel C: Survivin as a radioresistance factor, and prognostic and therapeutic target for radiotherapy in rectal cancer. *Cancer Res* 2005, 65:4881–4887
37. Warnecke C, Zaborowska Z, Kurreck J, Erdmann VA, Frei U, Wiesener M, Eckardt KU: Differentiating the functional role of hypoxia-inducible factor (HIF)-1 α and HIF-2 α (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2 α target gene in Hep3B and Kelly cells. *FASEB J* 2004, 18:1462–1464
38. Nadasdy T, Laszik Z, Blick KE, Johnson LD, Silva FG: Proliferative activity of intrinsic cell populations in the normal human kidney. *J Am Soc Nephrol* 1994, 4:2032–2039
39. Zhang T, Otevrel T, Gao Z, Ehrlich SM, Fields JZ, Boman BM: Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. *Cancer Res* 2001, 61:8664–8667
40. Gianani R, Jarboe E, Orlicky D, Frost M, Bobak J, Lehner R, Shroyer KR: Expression of survivin in normal, hyperplastic, and neoplastic colonic mucosa. *Hum Pathol* 2001, 32:119–125
41. Santini D, Abbate A, Scarpa S, Vasaturo F, Biondi-Zoccai GG, Bussani R, De Giorgio F, Bassan F, Camilot D, Di Marino MP, Feroce F, Baldi F, Silvestri F, Crea F, Baldi A: Surviving acute myocardial infarction: survivin expression in viable cardiomyocytes after infarction. *J Clin Pathol* 2004, 57:1321–1324
42. Conway EM, Zwerts F, Van Eygen V, DeVriese A, Nagai N, Luo W, Collen D: Survivin-dependent angiogenesis in ischemic brain: molecular mechanisms of hypoxia-induced up-regulation. *Am J Pathol* 2003, 163:935–946
43. Heyman SN, Lieberthal W, Rogiers P, Bonventre JV: Animal models of acute tubular necrosis. *Curr Opin Crit Care* 2002, 8:526–534
44. Caldas H, Fangusaro JR, Boue DR, Holloway MP, Altura RA: Dissecting the role of endothelial survivin- Δ Ex3 in angiogenesis. *Blood* 2007, 109:1479–1489
45. Mesri M, Wall NR, Li J, Kim RW, Altieri DC: Cancer gene therapy using a survivin mutant adenovirus. *J Clin Invest* 2001, 108:981–990
46. Fukuda S, Pelus LM: Regulation of the inhibitor-of-apoptosis family member survivin in normal cord blood and bone marrow CD34⁺ cells by hematopoietic growth factors: implication of survivin expression in normal hematopoiesis. *Blood* 2001, 98:2091–2100
47. Gurbuxani S, Xu Y, Keerthivasan G, Wickrema A, Crispino JD: Differential requirements for survivin in hematopoietic cell development. *Proc Natl Acad Sci USA* 2005, 102:11480–11485
48. Kobayashi Y, Yukiue H, Sasaki H, Fukai I, Yokoyama T, Kiriyaama M, Yamakawa Y, Maeda M, Fujii Y: Developmentally regulated expression of survivin in the human thymus. *Hum Immunol* 2002, 63:101–107
49. Okada H, Bakal C, Shahinian A, Elia A, Wakeham A, Suh WK, Duncan GS, Ciofani M, Rottapel R, Zuniga-Pflucker JC, Mak TW: Survivin loss in thymocytes triggers p53-mediated growth arrest and p53-independent cell death. *J Exp Med* 2004, 199:399–410
50. Chiou SK, Moon WS, Jones MK, Tarnawski AS: Survivin expression in the stomach: implications for mucosal integrity and protection. *Biochem Biophys Res Commun* 2003, 305:374–379
51. Weikert S, Schrader M, Krause H, Schulze W, Müller M, Miller K: The inhibitor of apoptosis (IAP) survivin is expressed in human testicular germ cell tumors and normal testes. *Cancer Lett* 2005, 223:331–337
52. Spaulding B, Pan D, Ghadersohi A, Nielsen G, Jensen S, Gellert F, Ling X, Zhang M, Black A, Li F: Characterization of the 12C4 survivin monoclonal antibody and insight into the expression of survivin in human adult tissues. *Histopathology* 2006, 49:622–633
53. Konno R, Yamakawa H, Utsunomiya H, Ito K, Sato S, Yajima A:

- Expression of survivin and Bcl-2 in the normal human endometrium. *Mol Hum Reprod* 2000, 6:529–534
54. Ka H, Hunt JS: Temporal and spatial patterns of expression of inhibitors of apoptosis in human placentas. *Am J Pathol* 2003, 163:413–422
55. Botchkareva NV, Kahn M, Ahluwalia G, Shander D: Survivin in the human hair follicle. *J Invest Dermatol* 2007, 127:479–482
56. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ: Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* 1993, 75:229–240
57. Moser M, Pscherer A, Roth C, Becker J, Mucher G, Zerres K, Dixkens C, Weis J, Guay-Woodford L, Buettner R, Fassler R: Enhanced apoptotic cell death of renal epithelial cells in mice lacking transcription factor AP-2 β . *Genes Dev* 1997, 11:1938–1948
58. Lorz C, Ortiz A, Justo P, Gonzalez-Cuadrado S, Duque N, Gomez-Guerrero C, Egido J: Proapoptotic Fas ligand is expressed by normal kidney tubular epithelium and injured glomeruli. *J Am Soc Nephrol* 2000, 11:1266–1277
59. Johnson EA, Svetlov SI, Pike BR, Tolentino PJ, Shaw G, Wang KK, Hayes RL, Pineda JA: Cell-specific upregulation of survivin after experimental traumatic brain injury in rats. *J Neurotrauma* 2004, 21:1183–1195
60. Deguchi M, Shiraki K, Inoue H, Okano H, Ito T, Yamanaka T, Sugimoto K, Sakai T, Ohmori S, Murata K, Furusaka A, Hisatomi H, Nakano T: Expression of survivin during liver regeneration. *Biochem Biophys Res Commun* 2002, 297:59–64
61. Chiou SK, Tanigawa T, Akahoshi T, Abdelkarim B, Jones MK, Tarnawski AS: Survivin: a novel target for indomethacin-induced gastric injury. *Gastroenterology* 2005, 128:63–73
62. Fortugno P, Beltrami E, Plescia J, Fontana J, Pradhan D, Marchisio PC, Sessa WC, Altieri DC: Regulation of survivin function by Hsp90. *Proc Natl Acad Sci USA* 2003, 100:13791–13796
63. Morita K, Wakui H, Komatsuda A, Ohtani H, Miura AB, Itoh H, Tashima Y: Induction of heat-shock proteins HSP73 and HSP90 in rat kidneys after ischemia. *Ren Fail* 1995, 17:405–419
64. Bidmon B, Endemann M, Muller T, Arbeiter K, Herkner K, Aufricht C: HSP-25 and HSP-90 stabilize Na,K-ATPase in cytoskeletal fractions of ischemic rat renal cortex. *Kidney Int* 2002, 62:1620–1627
65. Koch CA, Vortmeyer AO, Diallo R, Poremba C, Giordano TJ, Sanders D, Bornstein SR, Chrousos GP, Pacak K: Survivin: a novel neuroendocrine marker for pheochromocytoma. *Eur J Endocrinol* 2002, 146:381–388
66. Li F: Role of survivin and its splice variants in tumorigenesis. *Br J Cancer* 2005, 92:212–216
67. Bander NH, Finstad CL, Cordon-Cardo C, Ramsawak RD, Vaughan ED Jr, Whitmore WF Jr, Oettgen HF, Melamed MR, Old LJ: Analysis of a mouse monoclonal antibody that reacts with a specific region of the human proximal tubule and subsets renal cell carcinomas. *Cancer Res* 1989, 49:6774–6780
68. Mahotka C, Krieg T, Krieg A, Wenzel M, Suschek CV, Heydthausen M, Gabbert HE, Gerharz CD: Distinct in vivo expression patterns of survivin splice variants in renal cell carcinomas. *Int J Cancer* 2002, 100:30–36
69. Parker AS, Kosari F, Lohse CM, Houston Thompson R, Kwon ED, Murphy L, Riehle DL, Blute ML, Leibovich BC, Vasmatazis G, Cheville JC: High expression levels of survivin protein independently predict a poor outcome for patients who undergo surgery for clear cell renal cell carcinoma. *Cancer* 2006, 107:37–45
70. Kindt N, Menzebach A, Van de Wouwer M, Betz I, De Vriesse A, Conway EM: Protective role of the inhibitor of apoptosis protein, survivin, in toxin-induced renal failure. *FASEB J* 2007 [Epub ahead of print]