Thrombospondin 2 Functions as an Endogenous Regulator of Angiogenesis and Inflammation in Experimental Glomerulonephritis in Mice

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The role of thrombospondin 2 (TSP2) was investigated in an anti–glomerular basement membrane (GBM) nephritis model that compared TSP2-null mice with wild-type (WT) controls. TSP2-null mice were analyzed for kidney function, renal cortical matrix expansion, influx of inflammatory cells, proliferation, and apoptosis, as well as for capillary rarefaction after induction of anti-GBM disease. Whereas the renal cortex of normal control WT mice did not show any detectable TSP2 staining above background, TSP2 protein expression was clearly upregulated in anti-GBM disease. TSP2 deficiency led to an accelerated and enhanced inflammatory response, as indicated by the influx of CD4+ and CD8a+ cells and monocytes/macrophages. Glomerular fibrin deposition and a matrix-remodeling response were also observed, as indicated by collagens I and IV staining and a proliferative response within the renal interstitium. These changes were accompanied by increased matrix metalloproteinase 2 activity and enhanced α-smooth muscle actin staining in the TSP2-null mice. Neither a compensatory increase in TSP1 nor increased phosphorylation of Smad 2/3, an indicator for TGF-β activity, was observed. The proliferative response of the peritubular endothelium was accelerated and enhanced, leading to a reversal of capillary rarefaction in TSP2-null mice, whereas interstitial cell death was equivalent to that in WT mice. In conclusion, the lack of the matricellular protein TSP2 in mice accelerates and enhances several responses to renal injury and reveals an important role for TSP2 as a major endogenous antiangiogenic and matrix metalloproteinase 2–regulating factor in renal disease.


T he matricellular glycoprotein thrombospondin-2 (TSP2) is involved in cell adhesion, migration, and proliferation and is also a strong inhibitor of angiogenesis (1). Regulation of angiogenesis by TSP2 is due, in part, to modulation of matrix metalloproteinase (MMP) levels by binding and promotion of endocytosis (2). TSP2 is expressed during kidney development (3), but kidney structure and function seem to be unaffected in TSP2-null mice (4). TSP2-null mice display a disordered collagen fibrillogenesis in skin and tendons, a bleeding diathesis, and an increased vessel density in some tissues, supporting its function as an angiogenesis inhibitor in vivo. Despite the predominant role of TSP2 as an endogenous angiogenesis inhibitor in granuloma formation (5), delayed-type hypersensitivity reactions (6), cutaneous wound healing (7), carcinogenesis (8), and rheumatoid arthritis (9), no data are available regarding the role of TSP2 in renal disease. In contrast, TSP1 has been demonstrated to be the major endogenous activator of latent TGF-β in experimental renal glomerulonephritis (10,11), and TGF-β has been shown to be the central cytokine mediating matrix expansion and fibrosis in renal disease. It is interesting that TSP2 theoretically could act as a competitive inhibitor of TSP1-mediated TGF-β activation (12), depending on its spatial and temporal expression during kidney disease.

Considering that accumulation of extracellular matrix, cellular proliferation, influx of inflammatory cells, and capillary injury and repair by angiogenesis are shown to be critical processes during kidney disease, we hypothesized that TSP2, as a matricellular protein, might be an important regulator during glomerulonephritis. In particular, its role as a potential endogenous antiangiogenic molecule is of major interest, because several recent studies pointed to the central importance of the endothelium during inflammatory renal disease (13–19). Whereas complete renal healing after injury in reversible models of renal disease requires successful capillary repair, progressive renal disease is characterized by a progressive loss of the microvasculature (13). Renal capillary repair is mediated in part by an endothelial proliferative response, which depends on the local balance of anti- and proangiogenic factors in the kidney. Infusion of the major angiogenic growth factor, vascular endothelial growth factor (VEGF), selectively stimulated renal capillary repair and thereby slowed disease progression, whereas blockade of VEGF delayed glomerular capillary repair, leading
to increased glomerulosclerosis in experimental glomerulonephritis (15–17).

In contrast to the increasing knowledge of the importance of proangiogenic VEGF in renal disease, very few data are available regarding the function of endogenous antiangiogenic factors. To characterize the biologic role of TSP2 in inflammatory renal disease, normal kidneys as well as kidneys with anti-glomerular basement membrane (anti-GBM) glomerulonephritis in TSP2-null mice, were compared with those in wild-type (WT) mice. The anti-GBM glomerulonephritis model in the mouse is characterized by invasion of inflammatory cells followed by glomerular and interstitial fibrosis and repair (20). Therefore, this model is appropriate to investigate the role of TSP2 in inflammation, matrix accumulation, and cellular proliferation. In addition, TSP2-null and WT mice were compared with respect to capillary rarefaction and endothelial proliferation, regulation of MMP, and TSP1-mediated TGF-β activation.

Materials and Methods

Animal Model and Experimental Design

Animal studies were performed in accordance with the internal animal review board (Regierung von Mittelfranken: 621-2531.31-04/03). Generation of TSP2-null mice was described previously (4). Age- and gender-matched WT and TSP2-null mice on a homogeneous 129/SVJ background were used (4). The mice were fed standard mouse chow (Altromin 1324; Spezialfutterwerke, Lage, Germany) and tap water ad libitum.

Experimental anti-GBM glomerulonephritis was induced in WT and TSP2-null mice by an intraperitoneal injection of a sheep anti-GBM antibody (dosage 0.5 ml/20 g body wt), using a heterologous antibody to whole-rabbit glomeruli as described previously (20). At least seven mice per group were administered an injection on two consecutive days and killed after 3, 7, 14, 21, or 28 d. The day before killing, a 24-h urine collection was performed, and proteinuria and creatinine clearance were determined. On the day of killing, blood was collected via vena cava puncture, and kidneys were perfused with Ringer solution and frozen or fixed in methyl Carnoy’s or paraformaldehyde. In addition, age-matched groups (n = 8) of healthy WT and TSP2-null controls were investigated. For zymography, two healthy and three nephritic WT and TSP2-null mice were killed 14 d after induction of anti-GBM nephritis for generation of renal extracts.

Renal Morphology and Immunohistochemistry

Tissue for light microscopy was fixed in methyl Carnoy’s solution or 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections for indirect immunoperoxidase staining, as described previously (21). Sections were also stained with the periodic acid-Schiff reagent and were counterstained with hematoxylin. In periodic acid-Schiff-stained biopsies, a glomerulosclerosis and tubulointerstitial injury index was determined. Glomerular fibrin was measured using an acid fuchsin orange G staining (22). Snap-frozen tissue was cut into 5-μm sections for detection of CD4+ and CD8a+ cells and complement C3.

To perform immunostaining, we incubated tissue sections with the following primary and secondary antibodies: 19A2, a murine IgG mAb against proliferating cell nuclear antigen (PCNA; Chemicon, Temecula, CA), an indicator of actively proliferating cells; a rat monoclonal IgG2a to mouse CD4 antigen (Caltag Laboratory, Burlingame, CA); a rat monoclonal IgG2a to mouse CD8a antigen (Caltag Laboratory [18]; F4/80, a murine IgG1 mAb to a surface receptor that is present on monocytes, macrophages, and dendritic cells (Caltag Laboratory); MECA-32, a murine IgG1 mAb that is specific for detecting endothelial cells (a gift from R. Hallmann, University of Münster, Münster, Germany); a murine IgG2 mAb to α-smooth muscle actin (α-SMA; Dako, Hamburg, Germany) (21); a rabbit polyclonal antibody to collagen I (Biogenes, New Fields, UK); a biotinylated polyclonal antibody to human collagen IV (Southern Biotechnology Associates, Birmingham, UK); a murine IgG, mAb against TSP1 (Dunn, Labortechnik, Asbach, Germany [21]); a rabbit polyclonal antibody to TSP2 (23); and a rabbit polyclonal antibody to phosphorylated Smad 2/3 (Santa Cruz Biotechnology, Santa Cruz, CA [10]). Glomerular endothelial cells were detected using biotinylated tomato lectin (Sigma, Deisenhofen, Germany) (18). Negative controls for immunostaining included either the omission of the primary antibody or substitution of the primary antibody with equivalent concentrations of an irrelevant murine mAb or preimmune rabbit IgG. For TSP2 staining, paraformaldehyde-fixed tissues were pretreated with 0.025% pronase for 10 min at 37°C. All other immunoperoxidase stainings were done using methyl Carnoy’s-fixed tissues. Binding of the disease-inducing anti-GBM antibody was evaluated using a specific rabbit anti-sheep antibody (Linaris, Wertheim-Bettingen, Germany). After incubation with primary antibodies overnight at 4°C, specific biotinylated secondary antibodies (all from Zyomed, San Francisco, CA) were applied to tissue sections, followed by peroxidase-conjugated Avidin D (Vector Laboratories, Burlingame, CA) and color development with diaminobenzidine, with or without nickel chloride, for nuclear staining.

Tissue sections were incubated overnight with CD4 or CD8a primary antibodies, followed by a secondary Cy3-labeled donkey anti-rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA). The autologous antibody response to the anti-GBM antibody was evaluated by specific staining for mouse IgG using a Cy3-labeled goat anti-mouse IgG antibody (Molecular Probes, Leiden, The Netherlands) and for C3 using a FITC-conjugated antibody (Cappel, ICN Biomedicals, Eschwege, Germany).

Immunohistochemical Double Staining

To determine the number of proliferating endothelial cells, we performed double immunostaining for PCNA, a marker of cell proliferation, and MECA-32, an endothelial cell-specific marker, as described previously (21). Briefly, the first primary antibody (MECA-32) was incubated overnight at 4°C, followed sequentially by a biotinylated secondary antibody, peroxidase-conjugated Avidin D, and color development with diaminobenzidine without nickel chloride. After incubation in 3% H2O2/methanol for 20 min, the second primary antibody (proliferating cell nuclear antigen [PCNA]) was applied overnight at 4°C, followed by peroxidase-conjugated rat anti-mouse IgM antibody (Zymed) and diaminobenzidine with nickel chloride as the second color reagent. The controls for these double-staining procedures consisted of either omitting or replacing the secondary antibody or omitting or replacing the primary antibody with an irrelevant mouse mAb. The number of proliferating endothelial cells was evaluated by counting the number of cells that stained for both PCNA (black) and TSP2 (red) on anti-GBM nephritis and were protected using biotinylated tomato lectin (Sigma, Deisenhofen, Germany) (10). Glomerular endothelial cells were detected using biotinylated tomato lectin (Sigma, Deisenhofen, Germany) (18). Negative controls for immunostaining included either the omission of the primary antibody or substitution of the primary antibody with equivalent concentrations of an irrelevant murine mAb or preimmune rabbit IgG. For TSP2 staining, paraformaldehyde-fixed tissues were pretreated with 0.025% pronase for 10 min at 37°C. All other immunoperoxidase stainings were done using methyl Carnoy’s-fixed tissues. Binding of the disease-inducing anti-GBM antibody was evaluated using a specific rabbit anti-sheep antibody (Linaris, Wertheim-Bettingen, Germany). After incubation with primary antibodies overnight at 4°C, specific biotinylated secondary antibodies (all from Zymed, San Francisco, CA) were applied to tissue sections, followed by peroxidase-conjugated Avidin D (Vector Laboratories, Burlingame, CA) and color development with diaminobenzidine, with or without nickel chloride, for nuclear staining.

Quantification of Histochemical and Immunohistochemical Staining

Cortical expression of collagen I and collagen IV and glomerular mouse IgG, sheep IgG, and complement C3 deposition was quantified using computer-assisted image analysis software (MetaVue; Visitron Systems, Munich, Germany) to analyze positively stained areas. At
least 20 fields per section were analyzed at \( \times 200 \) magnification or at least 25 glomeruli at \( \times 400 \) magnification.

TSP2 and TSP1 were graded semiquantitatively using a score from 0 to 3 (0, no positive staining; 1, minor staining including single cells or small focal areas; 2, moderate staining with expanded positively stained areas; 3, prominent staining of large areas). For each biopsy, 20 fields at \( \times 400 \) magnification were evaluated in a blinded manner.

Glomerular capillary loss was determined using a semiquantitative score that ranged from 0 to 4 (0, normal; 1, loss of <25% of glomerular capillaries; 2, loss of 26 to 50% of glomerular capillaries; 3, loss of 51 to 75% of glomerular capillaries; and 4, >75% of glomerular capillaries lost). Peritubular capillary loss was examined through a \( 10 \times 10 \) ocular grid and a \( \times 40 \) objective. The grid covers an area of 0.0625 mm\(^2\) at this magnification. Each square that contained no MECA-32\(^+\) capillary was counted (15). This scoring system thus inversely reflects capillary rarefaction.

Tubulointerstitial injury was defined as inflammatory cell infiltrates, tubular dilation and/or atrophy, or interstitial fibrosis. Tubulointerstitial injury was graded on a scale of 0 to 4 (0, normal; 1, involvement of <25% of the cortex; 2, involvement of 26 to 50% of the cortex; 3, involvement of 51 to 75% of the cortex; and 4, involvement of >75% of the cortex) (24). Fibrin and glomerulosclerosis were graded on a scale of 0 to 4 (0, normal/no staining; 1, <25% of the glomerulus involved; 2, 26 to 50% of the glomerulus involved; 3, 51 to 75% of the glomerulus involved; and 4, >75% of the glomerulus involved). Myofibroblast accumulation, as assessed by \( \alpha\)-SMA staining, was graded semiquantitatively on a scale of 0 to 3 (0, only staining of vascular smooth muscle cells; 1, positive Bowman’s capsules; 2, <25% of the tubulointerstitium positive; and 3, >25% of the tubulointerstitium positive). For \( \alpha\)-SMA evaluation, 12 fields (\( \times 200 \) magnification) were analyzed.

PCNA\(^+\) nuclei were counted separately in glomerular, interstitial, and tubular cells. F4/80\(^+\) cells were also analyzed in 20 fields (\( \times 400 \) magnification). To count CD4\(^+\) and CD8a\(^+\) cells, we analyzed 12 fields each (\( \times 400 \) magnification).

**Apoptosis Assay**

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL) assay, as described previously (25). Briefly, 4% paraformaldehyde–fixed sections were deparaffinized and rehydrated, followed by an antigen-retrieval step that comprised boiling in 0.01 M sodium citrate buffer for 2 min, treatment with proteinase K (Boehringer Mannheim, Mannheim, Germany), and an incubation period with 300 enzyme units of terminal deoxynucleotidyl transferase and Bio-14-dATP (Life Technologies-BRL, Grand Island, NY). Biotinylated ATP was detected by the ABC staining method (Vector). DNAse-treated slides were used as positive controls.

Cells were regarded as TUNEL-positive when their nuclei were stained black and displayed a typical apoptotic morphology with chromatin condensation. The number of apoptotic cells was counted in 20 to 30 areas of each biopsy, using \( \times 400 \) magnification.

**Zymography**

For zymography, proteins from the cortex of kidneys were homogenized in 1% (wt/vol) SDS that contained 100 mM Tris (pH 6.8), using an Ultra Turrax for extraction (Janke & Kunkel GmbH, Staufen, Germany). After sedimentation of insoluble particles by centrifugation at 14,000 \( \times g \) for 10 min, equal aliquots of 20 µg of protein were separated by 10% SDS-PAGE with 0.06% (wt/vol) gelatin in the resolving gel. SDS was removed by agitation of gels for 120 min in 1% (wt/vol) Triton X 100 in 100 mM Tris (pH 7.5), followed by three subsequent washings for 15 min in 100 mM Tris (pH 7.5). After incubation in the same buffer for 18 h at 37°C, gels were stained with 0.1% (wt/vol) Coomassie Brilliant Blue R250 in methanol:acetic acid:H\(_2\)O (40:10:50) and agitated in the same solution until the stacking gel was destained.

**Miscellaneous Measurements**

Urinary protein was measured by the BioRad protein assay, whereas SDS-containing protein samples were measured using the BioRad DC assay with BSA as a standard following the manufacturer’s instructions (BioRad Laboratories, Munich, Germany). Urinary creatinine and urea were measured using an autoanalyzer (Beckman Instruments, Brea, CA).

**Statistical Analyses**

All values are expressed as mean \( \pm \) SEM. Statistical significance (defined as \( P < 0.05 \)) was evaluated using the \( t \) test.

**Results**

**TSP2 is Upregulated Predominantly in the Tubulointerstitium during anti-GBM Disease**

In healthy TSP2-null mice, no renal histologic abnormalities were seen by light microscopy when compared with WT mice (data not shown). Whereas the renal cortex of normal control mice did not show any detectable TSP2 staining above background (Figure 1A), TSP2 protein expression was sometimes variable but clearly upregulated in anti-GBM disease starting at approximately day 7, peaking on day 21, and remaining at a relatively high level up to day 28 (Figure 1E). TSP2-expressing cells were restricted predominantly to spindle-shaped cells within the tubulointerstitium (Figure 1B), whereas early in disease, a more focal periglomerular cell–associated pattern (Figure 1C) and later in disease a more ubiquitous cell- and matrix-associated distribution (Figure 1D) was observed. Some glomerular crescents also stained positively for TSP2 but at a lower intensity compared with the tubulointerstitial expression. TSP2-null mice were used as a negative control and showed minimal background staining (data not shown). A consistent but relatively weak staining, obviously not related to TSP2, could be seen in all middle- and large-size vessels of the renal cortex of WT and TSP2-null mice using this antibody (data not shown).

A compensatory increase in TSP1 protein was not found in either healthy or anti-GBM nephritic TSP2-null mice, compared with WT mice (Figure 2C). TSP1 was detected predominantly in cells of Bowman’s capsule, in the periglomerular tubulointerstitium, and sometimes within the glomerulus (Figure 2A and B). In addition, the lack of TSP-2 did not influence TGF-β activation, as assessed indirectly by measurement of phosphorylated Smad 2/3, which function as signal transduction proteins for TGF-β (Figure 2D).

**Heterologous and Autologous Antibody Responses Are Similar in WT and TSP2-Null Mice**

The induction of nephritis, as assessed by binding of sheep anti-GBM antibody on day 3, was equal in TSP2-null and WT mice (Figure 3A). In addition, the autologous antibody response, as indicated by deposition of mouse IgG (Figure 3B), and deposition of complement, as assessed by glomerular C3...
TSP2-Null Mice Demonstrate an Accelerated Response to Renal Injury

The influx of inflammatory cells, myofibroblast accumulation, matrix deposition, and cellular proliferation, which are characteristic responses to injury in anti-GBM disease, were investigated in both WT and TSP2-null mice at all time points, as follows.

Inflammation. In the cortex of WT mice, an influx of T cells, as assessed by staining for CD4 and CD8a, started on day 7 during anti-GBM disease, peaked on day 21, and remained at this high level on day 28 (Figure 3, D and E). In contrast, the influx of CD4⁺ and CD8a⁺ cells in TSP2-null mice was transiently increased on days 7 and 14 (1.6- to 2.4-fold higher than found in WT mice) but decreased later, reaching 2.2- to 3.5-fold reduced numbers on day 28 (Figure 3, D and E). Glomerular infiltration with CD4⁺ T cells was similar in both groups (Figure 3G). In healthy controls, the degree of monocyte/macrophage infiltration was equal in TSP2-null and WT mice (Figure 3F). After induction of anti-GBM disease, the influx of monocytes/macrophages was increased 1.6-fold on day 14 in TSP2-null mice (Figure 3F). In contrast to the high number of T cells found on day 28 in WT mice (Figure 3, D and E), the number of monocytes/macrophages declined in both groups (Figure 3F).

Myofibroblast Accumulation and Matrix Deposition. Because MMP-2 is involved in epithelial-to-mesenchymal transi-
tion (EMT) and because its levels are regulated by TSP2, we investigated MMP-2 activity by zymography in the renal cortex of healthy and nephritic kidneys. Extracts from healthy renal cortex showed equivalent MMP-2 activity in WT and TSP2-null mice. In contrast, whereas extracts from nephritic renal cortex, collected 14 d after disease induction, did not show a significant increase in WT mice, a three-fold increase in MMP-2 activity was observed in TSP2-null mice (Figure 4, A and B). To examine further the potential link of MMP-2 activity and myofibroblast accumulation/differentiation, we stained for α-SMA. De novo expression of tubulointerstitial α-SMA started on day 7 and peaked on days 21 and 28 in WT mice (Figure 4C). In contrast, TSP2-null mice expressed transiently more α-SMA in the tubulointerstitium on days 7 and 14 (more than two-fold increased) but showed significantly lower levels on day 28, compared with WT mice (Figure 4C).

Cortical matrix and basement membrane deposition, as indicated by collagen I (Figure 4D) and collagen IV (Figure 4E) staining, respectively, peaked on day 21 in the anti-GBM model.
in parallel with the influx of inflammatory cells. No differences in matrix deposition were seen in healthy mice of both groups (Figure 4).

Deposition of collagens I and IV was accelerated in TSP2-null mice, showing peak expression of both proteins 7 d earlier than in WT mice on day 14 (Figure 4, C and D). Compared with WT mice, collagen I deposition in TSP2-null mice was significantly higher on day 14, whereas collagen IV accumulation was increased on day 7.

**Cellular Proliferation.** Basal cortical proliferation in healthy TSP2-null and WT mice was comparable (Figure 5, A, C, and E). In WT mice, tubular cell proliferation was only slightly enhanced on day 7 and reached a maximum on day 21 (three-fold enhanced compared with healthy mice; Figure 5A). In contrast, tubular cell proliferation was significantly increased in TSP2-null mice, peaking on day 7 and reaching values five times higher than those found in WT mice. On day 14, tubular proliferation was still significantly higher compared with WT mice and declined to nearly healthy control values on day 21 (Figure 5A). Interstitial cell proliferation was also enhanced and accelerated in TSP2-null mice, reaching high values on day 7 (Figure 5C). These levels peaked on day 14 (three-fold increased compared with WT) and declined on day 21, when interstitial cell proliferation in WT mice was found to be maximal. Glomerular cell proliferation was also significantly increased in TSP2-null mice on day 14 (Figure 5E).

**Cell Death/Apoptosis.** The TUNEL staining technique was used for the evaluation of dead/apoptotic cells within the tubular (Figure 5B), interstitial (Figure 5D), and glomerular (Figure 5F) compartments of the kidney. In WT and TSP2-null diseased mice, whereas the number of interstitial and glomerular TUNEL-positive cells peaked on day 14, the number of tubular TUNEL-positive cells was only slightly elevated up to day 21 but peaked markedly on day 28, suggesting secondary damage possibly via interstitial signals. No significant difference in the number of TUNEL-positive cells was found at any time in any compartment in TSP2-null versus WT mice (Figure 5, B, D, and F).

**TSP2-Null Mice Demonstrate Active Capillary Repair**

Because TSP2 is known to be a potent angiogenesis inhibitor, we also compared the degree of cortical and glomerular capillary repair/loss after injury in both groups of mice. Glomerular fibrin deposition, as a marker for endothelial injury, was investigated using acid fuchsin orange G staining (Figure 6, A and B). In TSP2-null mice, glomerular fibrin deposition was transiently enhanced in response to renal injury, showing more than a two-fold higher fibrin deposition on days 7 and 14 (Figure 6A). A capillary rarefaction index, reflecting the loss of renal capillaries, was used as described in the Materials and Methods section. The intact peritubular capillary network of a healthy WT mouse with a low rarefaction score (Figure 6C, brown staining) and a WT GBM-nephritic cortex (day 28) with the loss of capillaries (Figure 6D) are shown as representative examples. In WT mice, the cortical capillary rarefaction index increased continuously from approximately 8% on day 0 to 30% on days 21 and 28 (Figure 6E), indicating a substantial progressive loss of peritubular capillaries during anti-GBM disease. In TSP2-null mice, the cortical capillary rarefaction index also increased similarly to WT mice, up to day 14. After day 14, a major difference was seen between the two groups: Whereas WT mice still showed a progressive loss of peritubular capillaries, in TSP2-null mice, the capillary rarefaction index decreased to 15% on day 28 (Figure 6E), indicating active capillary repair. In WT mice, the course of glomerular capillary loss was similar to the time course of cortical capillary rarefaction (Figure 6F). In contrast to the time course of capillary rarefaction within the cortex, TSP2-null mice (Figure 6E) showed a significantly higher glomerular capillary rarefaction index on day 14, compared with WT mice (Figure 6F). However, in contrast to
WT mice, the glomerular capillary rarefaction index of TSP2-null mice improved during later time points and was almost significantly decreased on day 28 ($P = 0.06$; Figure 6F), indicating active glomerular, together with peritubular, capillary repair.

### Active Peritubular Capillary Repair Is Due to Increased Endothelial Cell Proliferation But Not Decreased Cell Death

The number of proliferating peritubular endothelial cells was determined using a specific double-staining technique (MECA-32/PCNA; Figure 6G). During anti-GBM disease, WT, compared with control mice, demonstrated a several-fold increase in the number of proliferating endothelial cells, starting on day 7, peaking on day 14, and decreasing thereafter (Figure 6H). Endothelial cell proliferation in TSP2-null mice showed the same time course but was elevated (up to six times) on days 7 and 14, compared with WT mice (Figure 6H).

Because even double staining with the TUNEL assay and an endothelial cell marker did not clearly indicate the endothelial origin of apoptotic cells, or, alternatively, the apoptotic cells could have been taken up by endothelial cells, we evaluated interstitial versus tubular TUNEL-positive renal cells, as described previously. No significant difference in the number of TUNEL-positive interstitial cells was found at any time point in TSP2-null mice versus WT mice (Figure 5D).

### Sclerosis and Functional Data

Glomerulosclerosis increased to a maximum on day 21 after induction of anti-GBM nephritis in WT mice and decreased on day 28. In TSP2-null mice, glomerulosclerosis was 2.7 times higher on day 3, reaching a maximum on day 14, and was significantly decreased on day 21 (Figure 7A), indicating an accelerated progression and repair of disease. The tubulointerstitial sclerosis index, reflecting inflammatory cell infiltrates, tubular dilation, and/or atrophy or interstitial fibrosis was significantly increased in TSP2-null versus WT mice on day 3 but not different at other time points of anti-GBM disease (Figure 7B).

In both WT and TSP2-null mice, proteinuria was increased at all time points during anti-GBM disease (Figure 7C), compared with normal healthy mice (day 0). Proteinuria in TSP2-null compared with WT mice showed a tendency to higher values on day 7 ($P = 0.06$) and was significantly increased on day 14 ($P = 0.001$; Figure 7C), at a time, when influx with inflammatory cells, matrix accumulation, myofibroblast accumulation, and endothelial proliferation were also clearly increased in TSP2-null versus WT mice. In contrast, on day 28, at a time of improved capillary rarefaction and for the first time during anti-GBM disease, proteinuria in TSP2-null mice showed a tendency to lower levels compared with WT mice (Figure 7C). Assessment of renal function by creatinine clearance or serum urea (Figure 7, D and E) did not demonstrate any significant difference between the two groups of mice but mimicked changes or tendencies that were similar to those in other parameters, such as proteinuria, sclerosis, and inflammation.

### Discussion

Whereas several studies in cutaneous wound healing, granuloma formation, carcinogenesis, and rheumatoid arthritis have demonstrated that TSP2 is a potent factor in matrix re-
The first major finding of this study is that TSP2 is normally upregulated in acute anti-GBM glomerulonephritis and that its absence in TSP2-null mice accelerates several healing responses in the injured kidney, such as inflammation, myofibroblast accumulation, cellular proliferation, fibrin deposition, and matrix accumulation but not apoptosis, as discussed next. At early time points in WT diseased mice, immunohistochemical analysis of TSP2 protein revealed its presence de novo predominantly within the tubulointerstitium, apparently associated mainly with fibroblast-like cells. At later time points, deposition of TSP2 in the extracellular matrix, in particular in fibrotic areas within the tubulointerstitium, was also documented. This spatiotemporal deposition of TSP2 fits very well with the changed time course of disease in TSP2-null mice and is consistent with changes that were seen in wound healing and granuloma formation (5,7).

TSP2 deficiency led to an accelerated and increased influx of T cells and monocytes/macrophages within the renal cortex that could, by itself, alter the time course of disease. This altered inflammatory response in TSP2-null mice was not due to differences in induction of anti-GBM nephritis, because neither heterologous nor autologous responses were different in the two groups. Such increased inflammation was also reported in delayed-type hypersensitivity reactions that were elicited in the skin of TSP2-null mice (6). The acceleration and increase of inflammation in TSP2-null mice may be due to enhanced microvascular leakage (6) and altered regulation of proinflammatory mediators (9). In rheumatoid arthritis, TSP2 suppressed the production of the proinflammatory mediators IFN-γ and TNF-α and induced the depletion of tissue-residing T cells (9). Although no data are available regarding the mechanism by which TSP2 regulates these mediators, the direct or indirect modulation of the expression or function of chemokines would be consistent with its characteristic features as a matricellular protein (1,26).

In parallel with the inflammatory response, TSP2 deficiency also led to an accelerated and enhanced proliferative response in both the tubular and the interstitial compartments of the diseased kidney, whereas glomerular cell proliferation was only transiently enhanced on day 14. Although the complete mechanism of the alteration of cellular proliferation by TSP2 is unknown, work in vitro has demonstrated that TSP2 can inhibit cellular proliferation by inhibition of cell-cycle progression in endothelial cells (27). Although studies in vitro have revealed effects of TSP2 on cell death (1,27) our data in vivo do not show any increases in cell death by TUNEL staining, in the presence or absence of TSP2.

In agreement with studies of cutaneous wound healing and sponge granuloma formation (5,7), TSP2 deficiency also led to an accelerated matrix deposition and remodeling, as assessed by staining for collagens I and IV. Accelerated matrix deposition as a result of compensatory upregulation of TSP1 was excluded in this study. As shown by Schulz-Cherry et al. (12), TSP1 can activate TGF-β by simultaneous binding to the latency-associated protein and TGF-β. TSP2 shares the sequence that can bind to the latency-associated protein but lacks the second required binding site (12). Therefore, TSP2 might act as an
endogenous competitive inhibitor of TSP1-mediated TGF-β activation. However, the level of phosphorylated Smad 2/3, an indicator of active TGF-β, did not differ between nephritic WT and TSP2-null mice. Unchanged levels of activated TGF-β were also found in TSP2-null mice during the formation of sponge granulomas (5).

The second major finding of our study is that TSP2 deficiency can reverse an already established glomerular and peritubular capillary loss in renal disease. The importance of the maintenance of the renal microvasculature after injury is becoming increasingly recognized, as shown by several recent studies (13–19) in which a central role of the potent proangiogenic factor VEGF has also been established (15–17). In this study, we demonstrate that TSP2 functions as a major endogenous negative regulator of renal capillary repair. Studies, mainly by Kang et al. (13), have provided evidence for the new concept that the loss of peritubular capillaries by induction of chronic ischemia may not just be associated with but are potentially the cause of renal disease progression. Several groups have demonstrated that despite an early proliferative response of the peritubular capillary endothelium in renal disease models such as the aging kidney, the remnant kidney, and chronic cyclosporine A nephropathy, this response is not sustained and the disease process leads to progressive capillary loss (28–31). Whereas VEGF infusion in rats with remnant kidneys was able to prevent progressive capillary loss by induction of an enhanced endothelial proliferative response (32), we demonstrate in this study of the anti-GBM model that the lack of the endogenous angiogenesis inhibitor TSP2 restores the loss of peritubular capillaries.

Our data suggest that the basis for this active capillary repair reaction after day 14 in the TSP2-null mouse is an early substantial increase in the number of proliferating peritubular endothelial cells compared with the response in WT mice. In contrast to endothelial cell proliferation, TSP2 deficiency did not affect endothelial cell death within the tubulointerstitium. It is important to note that enhanced endothelial cell proliferation in TSP2-null mice represents an early change in these mice, suggesting that this change may influence the time course of the disease, as well as other responses such as influx of monocytes/macrophages and matrix deposition. However, the maximal suppression of peritubular endothelial cell proliferation by TSP2 in WT mice, which occurs between days 7 and 14, does not entirely correspond to the peak expression of interstitial TSP2 (days 14 to 21). This seeming discrepancy can be explained by the likelihood that the lower levels of TSP2 in the initial 7- to 14-d period in WT mice seem to be sufficient to inhibit the critical expansion of endothelial cells that are necessary to repair the injury in this renal disease model. Furthermore, TSP2, as a classical matricellular protein, may provide diverse and independent actions on inflammation, proliferation, and matrix remodeling, depending on its environment, the availability of certain cell receptors, and the competition by other proteins (e.g., TSP1). These considerations may also explain why the usual association of an improved capillary bed, as shown in TSP2-null mice on day 28, is not clearly translated into improvement of the tubulointerstitial injury index, as expected by other studies (13). An alternative explanation is that this association of capillary rarefaction and fibrosis is true only for progressive injury models, whereas our anti-GBM model does not seem to progress further from days 21 to 28, as assessed by parameters such as capillary rarefaction, sclerosis index, matrix, etc. Therefore, additional studies in truly chronic progressive renal disease models such as the remnant kidney model or the cyclosporine A nephropathy model should be helpful in clarifying these important questions.

Many of the effects that were observed in TSP2-null mice may be explained by a modulation of the availability of MMP-2. In this study, MMP-2 activity in kidney extracts from TSP2-null mice were higher than in WT mice, confirming findings of other in vitro and in vivo studies (5). MMP-2 can modulate angiogenesis by degradation of basement membranes, mobilization of growth factors, or release of cryptic fragments of matrix molecules (33). Enhanced MMP-2 activity in TSP2-null mice may promote an EMT, as indicated by early increases in cortical myofibroblast accumulation and α-SMA. In a recent publication, overexpression of MMP-2 in renal tubular cells promoted renal EMT and fibrosis (34) in mice. These MMP-2 transgenic mice developed tubular atrophy, renal injury, and inflammation at an age of 8 mo (34). MMP-2–induced fibrosis may potentially be mediated by TGF-β, because in vitro studies showed that MMP-2 can activate latent TGF-β (35). Nevertheless, as discussed before, in our study, no differences in TGF-β activation, as demonstrated by phosphorylation of Smad 2/3, were detected in either TSP2-null or WT mice.

In this study, the lack of TSP2 seems to be beneficial for the regeneration process after renal injury as a result of improved endothelial cell proliferation and capillary repair. However, the lack of TSP2 during the initial 7- to 14-d period caused more inflammation, matrix accumulation, and cellular proliferation and was accompanied by a tendency toward impaired renal function. All of these findings indicate the potential for a lack of TSP2 to cause severe adverse effects under conditions in which improved capillary repair may not compensate for the initial injury. In this regard, it is interesting that TSP2 expression is correlated positively with heart failure in a model of rodent cardiac hypertrophy (36). Conversely, angiotensin II–induced cardiac hypertrophy resulted in cardiac rupture in 70% of TSP2-null mice but in none of the WT controls, suggesting that TSP2 functions as a crucial regulator of the integrity of the cardiac matrix (36).

Conclusion
The lack of the matricellular protein TSP2 in mice accelerates and enhances several early phenotypic responses to renal injury in anti-GBM disease. Potentially, these responses may be mediated by the induction of myofibroblast differentiation and accumulation by MMP-2. Furthermore, TSP2 deficiency not only prevents but also can actually reverse the established glomerular and peritubular capillary loss that occurs in this renal disease model. This improvement is achieved by a stimulation of the proliferative response of the peritubular endothelium. Whether these promising results in TSP2-null mice can
be extended to the treatment of renal diseases will require further studies.

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Disclosures
None.

References
26. Bornstein P, Agah A, Kyriakides TR: The role of throm-