Bone Marrow–Derived Myofibroblasts Contribute to the Renal Interstitial Myofibroblast Population and Produce Procollagen I after Ischemia/Reperfusion in Rats

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Bone marrow–derived cells (BMDC) have been proposed to exert beneficial effects after renal ischemia/reperfusion injury (IRI) by engraftment in the tubular epithelium. However, BMDC can give rise to myofibroblasts and may contribute to fibrosis. BMDC contribution to the renal interstitial myofibroblast population in relation to fibrotic changes after IRI in rats was investigated. A model of unilateral renal IRI (45 min of ischemia) was used in F344 rats that were reconstituted with R26-human placental alkaline phosphatase transgenic BM to quantify BMDC contribution to the renal interstitial myofibroblast population over time. After IRI, transient increases in collagen III transcription and interstitial protein deposition were observed, peaking on days 7 and 28, respectively. Interstitial infiltrates of BMDC and myofibroblasts reached a maximum on day 7 and gradually decreased afterward. Over time, an average of 32% of all interstitial H9251/smooth muscle actin–positive myofibroblasts coexpressed R26-human placental alkaline phosphatase and, therefore, were derived from the BM. BMD myofibroblasts produced procollagen I protein and therefore were functional. The postischemic kidney environment was profibrotic, as demonstrated by increased transcription of TGF-H9252 and decreased transcription of bone morphogenic protein-7. TGF-H9252 protein was present predominantly in interstitial myofibroblasts but not in BMD myofibroblasts. In conclusion, functional BMD myofibroblasts infiltrate in the postischemic renal interstitium and are involved in extracellular matrix production.


Renal tubular ischemia/reperfusion injury (IRI), if not too severe, is, in principle, completely reversible (1), indicating that innate repair mechanisms are activated upon damage. Regeneration and remodeling of the kidney results in recovery of renal function and morphology by tubular epithelial cell replacement (2,3). However, tubulointerstitial remodeling, as a result of an uncontrolled balance between synthesis and degradation of extracellular matrix (ECM) proteins, also can result in tubulointerstitial fibrosis (4,5), which is an important risk factor for progressive renal function loss (6).

Previously, we and others showed that after renal IRI, low numbers of bone marrow–derived cells (BMD) engraft tubuli and differentiate to tubular epithelium (7–10), possibly replacing damaged tubular epithelial cells. These data have been taken to support a therapeutic potential for BMD stem/progenitor cells in renal tubular IRI. However, adverse effects also have been reported in models of lung and liver injury, in which BMD stem/progenitor cells gave rise to myofibroblasts and contributed to fibrosis (11,12).

Renal interstitial myofibroblasts are regarded as major producers of ECM proteins in fibrosis and therefore play a central role in its pathogenesis. Their origin remains the subject of discussion. Myofibroblasts may originate from injured tubular epithelial cells by epithelial-to-mesenchymal transition (13), differentiation of resident fibroblasts (14), or migration of perivascular smooth muscle cells (15). In mouse models of BM transplantation, it was shown that the BM is a source of myofibroblasts for many tissues, including the gut, lung, and kidney (10,16–18). However, functionality of renal BMD myofibroblasts after IRI was not shown.

Here, we investigated the contribution of BMDC to the renal interstitial myofibroblast population. Moreover, we studied the contribution of these cells to ECM production. To this end, we used a rat model of unilateral renal ischemia/reperfusion in F344 rats that were reconstituted with R26-human placental alkaline phosphatase (hPAP) transgenic BM.
Materials and Methods

Animals
Male 6 wk-old F344 rats (Harlan, Horst, The Netherlands) and R26-hPAP rats (F344 background; founders gift of Dr. E. Sandgren, School of Veterinary Medicine, University of Wisconsin–Madison, Madison, WI) that were transgenic for hPAP (19) were placed under conventional housing and diet. Drinking water was supplemented with 1 mg/ml 5-bromo-2-deoxyuridine (Brdu; Sigma, St. Louis, MO) for 3 d before the rats were killed. All animal procedures were approved by the local committee for care and use of laboratory animals and performed according to governmental and international guidelines on animal experimentation.

BM Chimeras
In nontransgenic rats, BM was ablated by whole-body irradiation (9 Gy, IBL 637 Cesium-137) and reconstituted with total R26-hPAP BM (1 × 10^6 R26-hPAP cells/recipient, intravenously). Rats were housed in filter-top cages, and drinking water was supplemented with neomycin (0.35% wt/vol) from 1 wk before to 2 wk after irradiation. BM chimism was determined after the rats were killed by enzymatic hPAP staining (see below) on BM cytospots and typically was between 80 and 90%.

Surgical Procedures
Four weeks after BM transplantation, rats were sedated by general isoflurane (2% Forene; Abbot, Hoofddorp, The Netherlands), N_2O (50%), and O_2 (50%) anesthesia. The left renal artery was clamped for 45 min to induce ischemia, followed by reperfusion. Sham-operated control rats underwent the same procedure, except for clamping of the left renal artery.

Kidney Function

Plasma creatinine levels were determined using the enzymatic colorimetric assay CREA plus (Roche, Woerden, The Netherlands), which is a precise and specific quantification method for creatinine (20).

Immunohistochemistry

Immunohistochemistry was performed on 5-μm zinc-fixed, paraffin-embedded sections. Sections were dewaxed, and antigen was retrieved by overnight incubation in 0.1 M Tris/HCl buffer at 80°C (for α-smooth muscle actin [α-SMA] and collagen III staining) or incubation with 0.7 M HCl at room temperature for 30 min and 0.025% (wt/vol) pepsin in 0.35 M HCl at room temperature for 15 min (for Brdu staining). Endogenous AP was heat-inactivated by incubation in substrate buffer (0.1 M Tris/HCl [pH 9.5], 0.1 M NaCl, and 5 mM MgCl_2) at 65°C for 30 min (19) (for α-SMA/hPAP staining). Endogenous peroxidase was blocked with 0.3% H_2O_2 for 30 min, and endogenous biotin was blocked with biotin blocking kit (DAKO, Glostrup, Denmark): Sections were incubated for 1 h with primary antibodies: Rabbit anti-rat collagen III (Biogenesis, Poole, UK), rabbit anti-hPAP (Serotec, Oxford, UK), mouse α-SMA (clone 1A4; DAKO), or anti-BrdU (Sigma-Aldrich), followed by appropriate secondary antibodies for 30 min. Color development was performed with 3,3-diaminobenzidine tetrachloride (α-SMA, collagen III), fuchsin substrate-chromogen system (hPAP; DAKO), or 3-amino-9-ethylcarbazole (Sigma-Aldrich) substrate dissolved in N,N-dimethylformamide (Merck) and 0.5 M acetate buffer (pH 4.9; Brdu).

For enzymatic hPAP staining, endogenous AP was heat-inactivated (see previous paragraph), and sections were incubated in fresh substrate buffer that contained 2% (vol/vol) of the substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche) at room temperature for 5 h.

Sections were counterstained with Mayer’s hemalum (Merck) and mounted in Kaiser’s glycerol gelatin (Merck).

Immunofluorescence staining for confocal microscopy was performed on 5-μm cryostat sections. For double staining of α-SMA and hPAP, the primary antibodies were incubated for 1 h followed by development of α-SMA with tyramide-TRITC (PerkinElmer Life Sciences, Boston, MA) via goat anti-mouse horseradish peroxidase (Southern Biotechnology, Birmingham, AL) and hPAP with FITC-labeled goat anti-rabbit conjugate (Southern Biotechnology). Triple staining of α-SMA and hPAP with goat anti–procollagen I (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-TGF-β (Santa Cruz Biotechnology) required the use different conjugates to prevent cross-reaction. After overnight incubation at 4°C with goat anti–TGF-β and 1 h of incubation with the other primary antibodies, α-SMA was developed with tyramide-TRITC (PerkinElmer Life Sciences) via donkey anti-mouse horseradish peroxidase (Southern Biotechnology), hPAP with Cy5-labeled donkey anti-rabbit conjugate (Jackson ImmunoResearch, Soham, UK), procollagen I with FITC-labeled donkey anti-goat conjugate (Jackson ImmunoResearch), and TGF-β with biotinylated donkey anti-goat (Abcam, Cambridge, UK) with SA-FITC (DAKO). All possible cross-reactions were tested, and none was found. Sections were counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) and mounted with citifluor (Agar Scientific, Stansted, UK).

Light microscopy was performed using a Leica DMLB microscope (Leica Microsystems, Rijswijk, The Netherlands), Leica DC300F camera, and Leica QWin 2.8 software. Fluorescence images were obtained using a Leica TCS SP2 three-channel confocal laser scanning microscope, equipped with lasers that provide 488-, 543-, and 633-nm laser lines. Tissue sections were observed using a 20 × 0.70 NA oil immersion objective lens, stack sections were chosen to obtain a z-resolution, and images were obtained at 1024 × 1024 pixel resolution.

Quantitative Reverse Transcriptase–PCR

Frozen kidneys were homogenized in 4 M guanidinium isothiocyanate (0.7% β-mercaptoethanol). Total RNA was isolated by standard

![Figure 1. Postischemic plasma creatinine levels. Plasma creatinine levels were assessed to determine kidney function. Bars indicate mean levels and SEM of all experimental rats per group. *P < 0.05, **P < 0.005 versus sham.](image-url)
procedures using phenol and chloroform/isoamyl alcohol. After DNase treatment (DNA-free Kit; Ambion, Austin, TX), RNA integrity and absence of DNA contamination were confirmed by gel electrophoresis. Equal amounts (5 μg) of total RNA from quarters of control and experimental kidneys were reverse-transcribed with Moloney murine leukemia virus (M-MuLV) reverse transcriptase in the presence of random hexamers (First-Strand cDNA Synthesis Kit; Fermentas Life Sciences, St. Leon-Rot, Germany). Equal amounts of cDNA (30 ng) were used for all quantitative reverse transcriptase–PCR (qRT-PCR) reactions. For further confirmation that RNA was free from genomic DNA, qRT-PCR also was carried out on total RNA.

For qRT-PCR, we used TaqMan “assay by demand” primer/probe sets for rat β2-microglobulin, collagen III, TGF-β, and bone morphogenetic protein-7 (BMP-7; Applied Biosystems, Foster City, CA; www.appliedbiosystems.com). PCR was performed in triplicate, using an ABI7900HT System (Applied Biosystems), in 384-well microtiter plates in a final volume of 10 μl, 5 μl of which was TaqMan universal PCR Master Mix (Applied Biosystems), 0.5 μl was primer/probe mix, and 4.5 μl was cDNA. Amplifications were performed starting with a 2-min AmpErase UNG activation step at 50°C, followed by a 10-min Amplitaq Gold Enzyme Activation step at 95°C, 45 cycles of denaturation at 95°C for 15 s, and combined primer annealing/extension at 60°C for 1 min. Cycle thresholds (Ct) for the individual reactions were determined.

Figure 2. Postischemic renal interstitial collagen III deposition. Expression levels of collagen III were determined by quantitative reverse transcriptase–PCR (RT-PCR) on RNA that was isolated from kidneys at various time points after ischemia/reperfusion injury (IRI) induction (A). Mean ΔΔCt values are shown in combination with SEM of all experimental rats per group. Mean ΔΔCt values indicate gene expression Ct values normalized against expression levels of an internal calibrator (β2-microglobulin) and mRNA expression levels of an external calibrator. Differences in expression levels between experimental and healthy control rats were expressed as fold variance of expression, calculated as 2^ΔΔCt (21). Fold variance of 1 indicates that there are no transcriptional differences compared with healthy control rats, >1 is considered increased transcription, and <1 is considered decreased transcription. Bars indicate mean levels and SEM of all experimental rats per group. Con, contralateral. All sham and contralateral groups showed similar results and therefore are shown as one group. In healthy control kidneys, the mean ΔΔCt was 2.46 ± 0.40. Postischemic collagen III protein deposition was quantified by computerized morphometry (B). Scoring was performed on sham (■), ischemic (□), and contralateral (△) kidneys. All sham groups showed similar results and therefore are shown as one group. *P < 0.05, **P < 0.005 versus sham rats. (C through G) Photomicrographs of collagen III staining are shown of sham kidney (day 1; C); contralateral kidney of an ischemic kidney (day 1; D); and kidneys that were subjected to ischemia and isolated on days 1 (E), 28 (F), and 112 (G) after IRI induction. Magnification, ×200.
using ABI Prism SDS 2.0 data processing software (Applied Biosystems). Ct values >40 were not included in calculations. Ct values were normalized against β2-microglobulin expression levels (21). For correction for interassay variance, Ct values were normalized against an external calibrator that consisted of RNA from healthy and ischemic rat kidneys. Differences in expression levels between experimental and control rats were expressed as fold variance of expression, calculated as $2^{-ΔΔCt}$ (21). Briefly, fold variance of 1 indicates that there are no transcriptional differences compared with healthy control rats, >1 is considered increased transcription, and <1 is considered decreased transcription.

Quantification

Interstitial staining of collagen III, α-SMA, and hPAP was measured by a blinded observer, using computerized morphometry (Leica QWin 2.8 software). Stained areas of 15 to 25 randomly selected fields in cortex and outer medulla were quantified as percentage of total measured area. The quantification was performed at a magnification of ×200 for each rat and time point. Vascular and glomerular expression of collagen III and vascular expression of α-SMA were excluded from measurements. The percentage of hPAP+/α-SMA− cells was determined by counting by two independent investigators, of all α-SMA+, hPAP+, and double-positive cells in 30 microscope fields in cortex and outer medulla per section, for each rat at a magnification of ×200.

Statistical Analyses

Statistical tests were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Differences between controls and experimental groups was determined with one-way ANOVA Dunnett multiple comparison test. P < 0.05 was considered statistically significant.

Results

Postischemic Plasma Creatinine Levels and Renal Interstitial Collagen III Deposition

Plasma creatinine levels were increased significantly until day 28 after IRI. Afterward, plasma creatinine gradually decreased to reach baseline values on days 56 and 112 after IRI (Figure 1).

Presence of collagen III, which is known to be produced excessively during fibrosis, was determined at transcript levels...
and protein level. Collagen III mRNA transcript levels increased progressively, peaking on day 7 and gradually decreasing afterward at later time points (Figure 2A).

Deposition of collagen III on day 1 after IRI (Figure 2, B and E) was comparable to that in kidneys that were subjected to a sham procedure (Figure 2, B and C) and contralateral kidneys (Figure 2, B and D). Renal interstitial deposition of collagen III reached a maximum on day 28 (Figure 2, B and F) and gradually decreased afterward but remained elevated as compared with controls (day 112; Figure 2, B and G). Irradiation, required for BM transplantation, did not elicit renal interstitial fibrosis, as determined by negligible collagen III deposition in contralateral kidneys (Figure 2D).

Postischemic Infiltration of Renal Interstitial BMDC
Renal interstitial BMDC infiltration was readily visible by the presence of hPAP-positive cells. No alkaline phosphatase activity was observed in the postischemic kidney of a nontransgenic F344 rat after heat inactivation of endogenous alkaline phosphatase (Figure 3A). In R26-hPAP BM-transplanted rats, small renal interstitial BMDC infiltrates were observed in kidneys that were subjected to sham operation (Figure 3, B and G), contralateral kidneys (Figure 3, C and G), and kidneys on day 1 after IRI (Figure 3, D and G). The BMDC infiltrate size strongly increased after day 1, reaching a maximum on day 7 after IRI (Figure 3, E and G). The large BMDC infiltrates persisted until day 28 after IRI (Figure 3G) and gradually decreased afterward, manifesting in small interstitial infiltrates on day 112 (Figure 3, F and G). The BMDC infiltrate size in ischemic kidneys, however, remained higher than in contralateral kidneys.

Presence of Renal Interstitial Myofibroblasts
Renal interstitial myofibroblasts were detected using α-SMA staining. Interstitial α-SMA–positive myofibroblasts were detected sporadically in sham kidneys (Figure 4, A and G), contralateral kidneys (Figure 4, B and G) and kidneys that were isolated on day 1 after IRI (Figure 4, C and G). After day 1, we observed a strong increase in renal interstitial α-SMA–stained area, with a peak on day 7 (Figure 4, D and G). After day 28 after IRI (Figure 4, E and G), the renal interstitial α-SMA–stained area was decreased gradually, up to day 112 (Figure 4, G).
F and G). Irradiation, required for BM transplantation, did not elicit renal interstitial fibrosis, as determined by the absence of myofibroblasts in contralateral kidneys (Figure 4B).

**Proliferation of Renal Interstitial Cells**

Proliferation during 3 d before termination of the rats was assessed by BrdU incorporation. After IRI, most BrdU-positive nuclei were present in tubular epithelial cells (7) and were found only occasionally in interstitial cells (Figure 5, day 14 after ischemia).

**BMD Myofibroblasts**

After renal ischemia, cells that were double-positive for hPAP (BMDC) and α-SMA (myofibroblasts) were observed, albeit exclusively in the interstitium. From day 7 after IRI on, the number of renal interstitial hPAP-positive BMDC that coexpressed α-SMA increased significantly compared with contralateral kidneys (Figure 6A). Over time, the number of renal interstitial hPAP-positive BMDC that coexpressed α-SMA remained relatively stable, with an average of 4% of all interstitial hPAP-positive BMDC coexpressing α-SMA (Figure 6A).

On day 3 after IRI, the number of renal interstitial α-SMA–positive myofibroblasts that coexpressed hPAP significantly exceeded that in contralateral kidneys (Figure 6B) and further increased until day 14. During the whole observation period, an average of 32% of all renal interstitial α-SMA–positive myofibroblasts coexpressed hPAP (Figure 6B).

To confirm that the observed hPAP/α-SMA–double-positive cells were BMDC that differentiated to myofibroblasts and not overlying cells, we used confocal microscopy of hPAP (Figure 6C) and α-SMA (Figure 6D) immunofluorescence staining. Overlap of signal in both channels (Figure 6E) was present in 10 of 14 consecutive z-planes.

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**Figure 5.** Proliferation of renal interstitial cells. Proliferation was assessed by 5-bromo-2-deoxyuridine (BrdU) incorporation. BrdU-positive nuclei (black, indicated with arrows), here shown on day 14 after IRI, were present mainly in tubular epithelial cells. The insert shows the presence of BrdU-positive nuclei in interstitial cells. Magnification, ×400.

To determine whether BMD myofibroblasts expressed ECM proteins, we studied their ability to produce procollagen I. Using fluorescence staining that combined α-SMA, procollagen I, and hPAP, we confirmed procollagen I production by α-SMA–positive myofibroblasts (Figure 6, F through I, arrowheads). In addition, all observed hPAP/α-SMA–double-positive cells (BMD myofibroblasts) stained positive for procollagen I (Figure 6, F through I, arrows). Overlap of signal in all three channels was present in five of the six consecutive z-planes.

**Transcript Levels of Fibrosis-Regulating Factors TGF-β and BMP-7**

Expression levels of TGF-β and BMP-7 in postischemic kidneys were normalized against β2-microglobulin expression levels and expression levels of an external calibrator and were expressed as ΔΔCT values (Figure 7). After IRI, ΔΔCT values for TGF-β and BMP-7 at most time points were significantly different from ΔΔCT values of healthy control kidneys (Figure 7). Differences in expression levels between experimental and control rats were expressed as fold variance of expression, calculated as 2^−ΔΔCT (21) (Figure 7). TGF-β transcript levels doubled on day 1, peaked on day 7 (2.7-fold increase), and decreased gradually afterward, to return to baseline levels on day 56 after IRI (Figure 7A). BMP-7 transcript levels were 10 times decreased on day 1 after IRI, as compared with control values, and remained decreased at all studied time points (Figure 7B).

**Postischemic Renal Presence of TGF-β Protein**

To address TGF-β expression at protein level and determine which cells were responsible for the increased TGF-β mRNA expression in postischemic kidneys, we used immunofluorescence staining that combined TGF-β with α-SMA and hPAP. In healthy kidneys TGF-β protein was present mainly in proximal tubular cells but also in arterial structures and sporadically in interstitial cells (data not shown). After ischemic damage, renal TGF-β protein was observed occasionally in proximal tubular epithelial cells (Figure 8, A through D). However, using triple staining of TGF-β, α-SMA, and hPAP, we could identify myofibroblasts (TGF-β– and α-SMA–double-positive yellow cells) as main producers of TGF-β in postischemic kidneys (Figure 8, E through H). We did not observe, however, TGF-β expression in BMD myofibroblasts (TGF-β, α-SMA, and hPAP triple-positive; Figure 8).

**Discussion**

Previously, we showed the ability of BMDC to engraft renal tubuli and differentiate to tubular epithelial cells in a rat model of IRI (7). Our study investigated the contribution of BMD myofibroblasts to the renal interstitial myofibroblast population and, for the first time, their functional capacity to produce ECM proteins after IRI. We show that the contribution of BMDC to the renal myofibroblast population amounted to >30%. Moreover, BMD myofibroblasts produced procollagen I, suggesting an active role of these cells in the production of ECM proteins in postischemic remodeling. Resident renal myofibroblasts produced TGF-β, thereby possibly contributing to
Figure 6. BMD myofibroblasts. Coexpression of BMDC and myofibroblast markers was assessed by immunohistochemical hPAP/α-SMA double staining on postischemic kidneys. (A and B) Coexpression of hPAP and α-SMA was quantified in relation to the renal interstitial population of BMDC (A) and to the renal interstitial myofibroblast population (B). Bars indicate mean levels and SEM of all experimental rats per group. All sham groups showed similar results and therefore are shown as one group. *P < 0.05, **P < 0.005 versus sham kidneys. (C through E) BMD myofibroblasts were detected by immunofluorescence staining for hPAP (C; green) in combination with α-SMA (D; red) on postischemic kidney tissue (shown here day 7 after ischemia). The overlay shows FITC, TRITC, and 4′,6-diamidino-2-phenylindole (DAPI) channels, and the x/z and y/z planes on the right and underneath the merged picture demonstrate true co-localization of the markers (E). Arrows indicate co-localization. (F through I) Production of procollagen I by BMD myofibroblasts was assessed by immunofluorescence staining for procollagen I (F; green) in combination with α-SMA (G; red) and hPAP (H; white) on postischemic kidney tissue (shown here day 7 after ischemia). The overlay (I) shows FITC, TRITC, Cy5, and DAPI channels, and the x/z and y/z planes on the right and underneath the merged picture demonstrate true co-localization of the three markers. Arrowheads indicate an example of a procollagen-positive myofibroblast (α-SMA/procollagen I–double-positive). Arrow indicates an example of a procollagen I–positive BMD myofibroblast (α-SMA/hPAP/procollagen I–triple-positive).
findings of BMD, intrarenal mechanisms of myofibroblast formation. However, perivascular smooth muscle cells (15) have been identified as differentiation of resident fibroblasts (14), and migration of epithelial-to-mesenchymal transition (13,26), differentiation choices is of pathophysiologic relevance. In this study, we investigated the contribution of BMDC to the renal interstitial myofibroblast population and found it to amount to >30%. This is three times as high as in a study in unilateral ureteral obstruction (UUO) in mice (18). This difference may reflect differences between our respective models. Possibly, the method of Y-chromosome in situ hybridization used by Roufosse et al. (18) for detection of BMD myofibroblasts can lead to an underestimation by division of the nucleus in sections and loss of the Y chromosome for detection.

A prerequisite for a role of BMD myofibroblasts in renal remodeling is the ability of these cells to produce and deposit ECM proteins in the environment in which BMD myofibroblasts can become functionally active in producing ECM proteins.

Renal remodeling of the ECM after injury initially proceeds as a wound-healing process but can develop into pathologic renal interstitial fibrosis (22). In renal remodeling myofibroblasts, α-SMA–expressing cells with features of both fibroblasts and smooth muscle cells (23) play a dual role. First, in the early phase after injury, myofibroblasts participate in wound healing by producing ECM components (e.g., collagens I and III, tenascin, fibronectin) (24). Second, persisting inflammation after renal injury can lead to persistent myofibroblast activation (25) and thus to excessive ECM production. In conjunction with reduced ECM degradation, this can result in renal interstitial fibrosis, an important risk factor for progressive renal damage (6). In our IRI model, collagen III mRNA levels and protein deposition increased significantly, peaking on days 7 and 28, respectively, but gradually decreased afterward. Together with decreasing BMDC infiltrates and improving kidney function, this is likely to reflect the process of wound healing. However, at the latest time point of the study (day 112), renal interstitial collagen III deposition and BMDC infiltrates still were higher than in control kidneys, which possibly reflect a transition phase between wound healing and fibrosis. Because our model did not result in fibrosis, we determined whether BMD myofibroblasts could contribute to ECM deposition during renal remodeling, rather than to fibrosis.

Although the central role of myofibroblasts in renal remodeling is established, the origin of these cells still is under investigation. Epithelial-to-mesenchymal transition (13,26), differentiation of resident fibroblasts (14), and migration of perivascular smooth muscle cells (15) have been identified as intrarenal mechanisms of myofibroblast formation. However, findings of BMD, α-SMA–positive cells in skin (17,27), lung (27), small intestine (16), colon (16), stomach (17), liver (11), and kidney (10,17,18) provide evidence for an additional source of myofibroblasts.

In this study, we investigated the contribution of BMDC to the renal interstitial myofibroblast population and found it to amount to >30%. This is three times as high as in a study in unilateral ureteral obstruction (UUO) in mice (18). This difference may reflect differences between our respective models. Possibly, the method of Y-chromosome in situ hybridization used by Roufosse et al. (18) for detection of BMD myofibroblasts can lead to an underestimation by division of the nucleus in sections and loss of the Y chromosome for detection.

BMDC, in line with their plasticity, can differentiate into different cell types, thereby potentially exerting different roles in renal remodeling after IRI. The regulation of these differentiation choices is of pathophysiologic relevance. In this study, we observed an altered balance between BMP-7 and TGF-β, two major regulators of renal interstitial fibrosis (28). Whereas TGF-β is a well-established inducer of fibrosis by stimulating differentiation to and proliferation of myofibroblasts (29,30), BMP-7 directly counteracts TGF-β–induced ECM production and promotes epithelial differentiation (31,32). The transcriptional increase of TGF-β and decrease of BMP-7 may provide the environmental clues for preferential differentiation of BMDC toward myofibroblasts and subsequent production of ECM proteins. In the postischemic kidney, TGF-β was observed in proximal tubular epithelial cells and arterial structures but not in BMD myofibroblasts. Resident renal myofibroblasts seemed to be the major producers of TGF-β, suggesting that these cells may contribute to the postischemic renal environment in which BMDC differentiate to myofibroblasts.

A prerequisite for a role of BMD myofibroblasts in renal remodeling is the ability of these cells to produce and deposit...
ECM proteins. We addressed the functionality of renal interstitial BMD myofibroblasts by showing production of procollagen I after IRI. This finding is in agreement with data by Iwano et al. (26) in UUO-induced renal fibrosis, in which renal BMD fibroblasts produced collagen I and heat-shock protein 47, a chaperone molecule for collagen I production. By contrast, Roufosse et al. (18) found no collagen I synthesis in interstitial BMDC in a mouse model of UUO-induced renal fibrosis. The reason for these discrepancies is unclear but might result from differences between the models. UUO is a relatively straightforward model of obstruction-induced fibrosis, whereas our ischemia/reperfusion model leads to wound healing rather than fibrosis. Alternatively, differences in transgenic BM transplantation models (BMDC expressing green fluorescence protein under the promoter of the fibroblast-specific protein-1, luciferase under the promoter of collagen I gene, or hPAP) or specific type of BMDC (BMD myofibroblasts, fibroblasts, or all interstitial BMDC) may be involved.

In addition to previous studies that showed tubular BMDC engraftment and epithelial differentiation after IRI (7–10), our study supports the contribution of BMDC to the renal myofibroblast population. Remarkably, the contribution of BMDC to the myofibroblast population largely exceeds their contribution to the tubular epithelial population. This is in accordance with recent studies that showed that postischemic repair of tubular epithelium occurs only marginally by differentiation of BMDC but mainly by proliferation of resident epithelial cells (10,33).

The different BMDC phenotypes in the kidney after IRI point toward different roles in the postischemic process, as also supported by differences in the time courses in which the different BMDC phenotypes are present. We previously showed that tubular epithelial BMDC engraftment peaked on day 14, whereas the peak in tubular epithelial proliferation was observed 1 wk earlier (7). Our study showed that already on day 3 after IRI, 27% of all renal interstitial myofibroblasts were derived from the BM. Taken together, these data suggest that postischemic replacement of damaged tubular epithelial cells already was accomplished by proliferation of resident epithelial cells after the first week. Therefore, the BMDC, possibly influenced by upregulation of TGF-β in the postischemic renal environment, may differentiate preferably to myofibroblasts rather than to tubular epithelial cells.

It is not clear what the ontogeny of BMD myofibroblasts and BMD tubular epithelial cells is and whether BMD myofibroblasts and BMD tubular epithelial cells are derived from the same source. It has been proposed that myofibroblasts differentiate from a circulating precursor cell type (the circulating fibrocyte [34]). The fibrocyte is a distinct mesenchymal cell type that arises in ex vivo cultures of peripheral blood and has the capacity to differentiate in vitro into α-SMA–positive myofibroblasts (34,35). Although fibrocytes are recruited to the damage site after injury (34,35), it is improbable that they are the only source of renal interstitial myofibroblasts, because the large numbers of BMD myofibroblasts in our study contrast with the low numbers of circulating fibrocytes (0.1 to 0.5% of human blood cells) reported previously (34,35). Moreover, the sporadic presence of interstitial cell proliferation argues against local amplification of the myofibroblast population. An alternative source of renal BM myofibroblasts may be monocytes, because these cells can transdifferentiate (36–38) as well as synthesize and secrete fibrosis-promoting growth factors and cytokines (39).

Data on tubular engraftment of BMDC (7–10,40), as well as promising results after infusion of BMDC (6,41,42), have provided support for the therapeutic potential of BMDC in tubular

Figure 8. Postischemic renal presence of TGF-β protein. TGF-β protein expression in postischemic kidneys (day 3 [A through D] and day 7 [E through H] after ischemia) was determined by immunofluorescence staining that combined TGF-β (A and E; green) with α-SMA (B and F; red) and hPAP (C and G; white). Overlays show FITC, TRITC, Cy5, and DAPI channels and the x/z and y/z planes on the right and underneath the merged pictures demonstrate co-localization of TGF-β and α-SMA (D and H). Magnification of the indicated parts are shown on the far right of E through H. *Examples of TGF-β tubular structures. Arrowheads indicate infiltrated BMDC (hPAP+). Arrows indicate TGF-β+ myofibroblasts (α-SMA/TGF-β+).
epithelial recovery after acute renal failure. However, in line with data from others (10,17,18), our results show that BMDC can differentiate to renal interstitial myofibroblasts as well. By producing ECM proteins, these cells can have a beneficial role in wound healing. However, in case of persistent inflammation and a disturbed balance between ECM production and degradation, these cells can play an adverse role by promoting processes that ultimately lead to fibrosis. Therefore, for therapeutic application of BMDC, caution is called for.

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Disclosures
None.

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