Molecular markers in renal transplant biopsies

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Relatively high mRNA expression of EMT related markers in renal allograft protocol biopsies is associated with favorable outcome

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Abstract

Epithelial to mesenchymal transition (EMT) may determine the balance between the progression of renal allograft dysfunction and regenerative mechanisms in the graft tissue. We investigated whether mRNA levels of EMT-related molecules and histomorphological parameters are of prognostic value in protocol renal allograft biopsies.

Sixty-five patients participating in a prospective trial had protocol biopsies taken 6 and 12 months after transplantation. Quantitative real-time PCR was used to determine mRNA expression of EMT-related markers. The extent of Sirius red staining and the Banff score were determined. Renal function was assessed at 6 and 24 months.

Patients showing an improvement in renal function between 6 and 24 months had significantly higher mRNA expression levels of TGF-β, S100A4, and collagens I and III in their 6-month biopsies compared to patients with deterioration in renal function. Sirius red staining and the Banff score at 6 months were not associated with the course of renal function.

EMT-related molecular markers, but not histomorphological parameters, in protocol renal transplant biopsies taken at 6 months discriminated patient groups with different outcomes. Unexpectedly, increased mRNA expression levels of these markers were associated with improvement of graft function. EMT may be associated with increased tubular repair and connective tissue remodeling, which contribute to stabilization of graft function.

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Introduction

Chronic allograft nephropathy (CAN) remains the main cause of late renal allograft failure [1;2]. CAN, a histological diagnosis that is characterized by the presence of tubulointerstitial fibrosis and tubular atrophy, is accompanied clinically by a progressive decline in renal function. Myofibroblasts in the interstitial compartment are the effector cells in fibrogenesis [3;4]. Epithelial to mesenchymal transition (EMT) of tubular epithelial cells (TECs) is a possible source of myofibroblasts. During EMT, TECs lose epithelial characteristics and acquire a mesenchymal phenotype. In studies of human biopsies that were obtained for clinical indications, TECs expressing mesenchymal markers were observed frequently [5-9].
Iwano *et al.* have shown in a mouse model that EMT is a major source of differentiated myofibroblasts in the interstitium [10]. The origin of myofibroblasts in human renal disease is less clear. An extensive electron microscopic study of human renal biopsies with interstitial fibrosis did not detect fully differentiated myofibroblasts in the tubules,[11] which invalidates the concept that TECs are a major source of ECM-producing myofibroblasts. With respect to outcome, the effect of EMT in kidney grafts is unclear. On one hand, the results from the study by Iwano *et al.* are highly suggestive of a role for EMT in fibrogenesis [10]. On the other hand, local conversion of epithelial cells and activation of resident fibroblasts may be the basis of regenerative mechanisms that limit permanent tissue damage. Hence, the question remains whether EMT assists in restoration of the renal architecture or in the development of fibrosis.
Transforming growth factor-β (TGF-β) is an inducer of EMT,[12;13] but no evidence of a role for TGF-β in the transdifferentiation of TECs into myofibroblasts has been found [13]. TGF-β seems to act as a double-edged sword in renal allograft dysfunction. Relatively high TGF-β expression levels early after transplantation are associated with favorable graft outcome, which suggests beneficial actions of this cytokine [14;15]. In contrast, gene polymorphism studies [16;17] and studies of biopsies with chronic renal allograft rejection suggested a profibrogenic effect of TGF-β [18;19]. *In vitro* studies have shown that TGF-β drives EMT by inducing expression of α-SMA and fibroblast specific protein-1 (which is highly homologous to human S100A4) and by reducing E-cadherin expression in tubular epithelial cells [12;20;21]. In addition, TGF-β induces expression of the transcriptional factor Smad-interacting protein 1 (SIP1) to mediate EMT through direct repression of E-cadherin expression [22-25]. Furthermore, TGF-β exerts its biological effects through the phosphorylation of Smad-2 and -3 [26;27].
Bone morphogenetic protein-7 (BMP-7) acts as an antagonist of TGF-β by inhibiting EMT [28]. In animal models, BMP-7 counteracts TGF-β and reverses chronic renal injury by inducing mesenchymal to epithelial transition (MET) through a Smad-1, -5, and -8-dependent pathway [26;27;29;30]. This finding corroborates the plasticity of tubular epithelial cells to modulate their phenotype [30]. Hepatocyte growth factor (HGF) has also been shown to counteract the actions of TGF-β [31;32]. *In vitro*, HGF counteracts TGF-β by inhibiting tubular EMT and maintaining an epithelial phenotype [33;34].
The identification of EMT-related markers at an early time point after transplantation could reveal novel therapeutic markers to combat chronic

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allograft nephropathy. Studies of human renal biopsies have investigated the presence of EMT only at the protein level [7-9]. However, various studies of human renal biopsies have shown that mRNA expression levels of matrix and matrix-regulating molecules can be of additional diagnostic and prognostic value [14;15;35-37]. In the context of a prospective study comparing area-under-the-concentration-over-time curve systemic exposure of calcineurin inhibitors, protocol biopsies were obtained 6 and 12 months after transplantation. The objective of the present study was to assess the relation between mRNA expression of EMT-related markers in protocol renal transplant biopsies and outcome at 2 years.

Material and Methods

Patient Groups and Study Design

Sixty-five patients, receiving either their first or second graft, were enrolled in a prospective study and received tailored calcineurin inhibitor regimens as described previously [38]. Highly immunized patients with panel reactive antibodies >50% were excluded from this study. Renal function as measured by the Cockcroft formula was assessed 6 months and 24 months after transplantation. In addition, the corrected GFR at 24 months, which is statistically independent of the starting value, was calculated as described previously [39]. Briefly, the corrected GFR at 24 months is defined as the difference between the observed GFR at 24 months and its linear prediction on the basis of the GFR at 6 months. Protocol biopsies were obtained 6 months and 12 months after transplantation and were scored according to the Banff criteria for chronic changes [40]. Protocol biopsies were available from 91% and 88% of the patients enrolled in the study at 6 and 12 months, respectively. Reasons for missing biopsies were increased bleeding risk, secondary refusal by the patient, and lack of specimen for adequate evaluation according to the Banff criteria.

RNA Extraction

Frozen biopsy tissue was available for 52 6-month biopsies. A 2-µm section from each biopsy was analyzed by light microscopy to ascertain the presence of the renal cortex, according to a procedure that was described in a previous study [41]. Ten to fifteen 10-µm sections of renal cortex were cut in a Leica CM3050 S cryostat, collected in an Eppendorf tube, and stored at -70°C until use. RNA was isolated by using RNeasy spin columns (Qiagen, Westburg, The Netherlands) according to the manufacturer's instructions, and 1 µg RNA was converted to cDNA with avian myeloblastosis virus reverse transcriptase (Roche, Mannheim, Germany) in combination with 100 ng oligo dT (Roche) and 500 ng random hexamer primers (Invitrogen, Breda, The Netherlands).

Real-Time PCR

Quantitative real-time polymerase chain reaction (Q-PCR) was performed by using an iCycler™ (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). cDNA samples were diluted 50 times, and 5 ml of the dilution was used for PCR. Primer sequences were designed with Beacon Designer version 4 (PRE-

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MIER Biosoft International, Palo Alto, CA). Primer set sequences were chosen over an exon-intron junction to prevent genomic DNA amplification. Primer sets were evaluated for specificity by cDNA sequencing. Lack of DNA amplification was confirmed by performing a Q-PCR reaction with 10 ng human DNA. mRNA levels were quantified and normalized to the mean mRNA levels of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine guanine phosphoribosyltransferase-1 (HPRT-1). Primer sequences are displayed in Table 1.
To allow the comparison of samples from different Q-PCR plates, a 1:5 dilution range of a reference sample was included in each Q-PCR run.

Table 1. **Primer sequences**

Gene	Forward Primer	Reverse Primer
S100A4	TCGGGCAAAGAGGGTGACAAGTTC	CCTGTTGCTGTCCAAGTTGCTCATC
E-cadherin	ACGCATTGCCACATACACTCTC	CTTGTTGTCATTCTGATCGGTTACC
TGF-β	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA
BMP-7	CAGCAGCAGCGACCAGAGG	CACAGTAGTAGGCGGCGTAGC
HGF	TCCAGAGGTACGCTACGAAGTC	CGGTGTGGTGTCTGATGATCC
Sip1	AAATAAGGGAGGGTGGAGTGGAAC	CGGTCTGGATCGTGGCTTCTG
Smad 1	GAGCAGCAGCAGCACCTACC	GGTCTTCAGGAGGCAGGTAAGC
Smad 2	ATTTGCTGCTCTTCTGGCTCAG	ACTTGTTACCGTCTGCCTTCG
Smad 3	GACGACTACAGCCATTCCATCC	CTCCATCTTCACTCAGGTAGCC
Smad 5	GCTTCTGGCTCAATCTGTCAAC	GTGATATTCTGCTCCCCAACCC
Collagen I	CCTCAAGGGCTCCAACGAG	TCAATCACTGTCTTGCCCCA
Collagen III	GAGGATGGTTGCACGAAACA	TGTCATAGGGTGCAATATCTACAATAGG

Histochemistry
Paraffin-embedded tissue was available for 59 6-month biopsies and 57 12-month biopsies. Four-micrometer sections were cut. The extent of interstitial collagen accumulation was visualized in paraffin sections of 6-month and 12-month biopsies with Sirius red (SR) staining, as described previously [42]. Quantification of SR staining by digital image analysis was performed using a Zeiss microscope with a full-color 3CCD camera (Sony DXC 950p, Sony Corporation, Shinagawa-ku, Japan) and KS-400 image analysis software version 4.0 (Zeiss-Kontron, Eching, Germany). An average of 10 microscopic sections from each slide were examined with the 20X objective, analyzing an average area of 63.2 ± 19.5% of the total cortex on each slide.

Statistical Analysis
Statistical analysis was performed with SPSS software version 12.0.1 (SPSS, Inc., Chicago, IL). Log-transformed (^{10}log) mRNA expression levels were used for analysis. Differences between groups were assessed by using the Independent Samples *t* test. Correlations were calculated with Pearson's correlation test. The patient group was analyzed as a whole by using the corrected GFR at 24 months. Subgroup analyses compared patients with an increase in renal function between 6 and 24 months to those with a decrease in renal function between 6 and 24 months. $P < 0.05$ was considered statistically significant.

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Results

Patient Characteristics

According to the course of renal function between 6 and 24 months, the patient cohort was divided into two groups: those with a decrease and those with an increase in renal function. Characteristics of the patient groups are summarized in Table 2.

Table 2. Clinical and histological parameters

Renal Function (6 24 months)	Decrease	Increase
Patients included	33	19
Cockcroft (ml/min)		
6 months	69.4 ± 19.4	63.3 ± 17.1
24 months*	57.8 ± 19.0	70.3 ± 20.9
Donor age (y)	48.1 ± 13.9	42.8 ± 13.0
Recipient age (y)	45.3 ± 13.9	48.3 ± 11.8
Donor gender (m/f)	16/17	10/9
Recipient gender (m/f)	23/10	17/2
Blood pressure (mmHg)		
t0	140/83	144/81
6 months	136/81	137/79
12 months	135/82	138/80
Antihypertensive medication		
6 months	1.59 ± 0.95	2.17 ± 1.04
12 months*	1.76 ± 0.99	2.44 ± 1.10
Cold ischemia time (h)	17.9 ± 14.5	20.5 ± 8.1
Delayed graft function (%)	21	26
Donor source		
Living donor	16	4
Deceased donor	12	12
Donation after cardiac death	5	3
HLA-A, -B, -DR mismatches	2.67 ± 1.81	2.16 ± 1.64
CMV infection (%)	55	58
Subclinical acute rejection (%)		
6 months	18.2	17.6
12 months	16.7	16.7
CAN score ≥ 2 (%)		
6 months	6.1	11.8
12 months	17.2	11.1

[1] Data are means ± SD. CMV, cytomegalovirus; CAN score, Banff score for chronic changes. Subclinical acute rejection is scored according to the Banff criteria. *$P < 0.05$.

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There were no significant differences between patient groups in donor and recipient age and gender, donor source, cold ischemia time, occurrence of delayed graft function, blood pressure, total HLA mismatches, incidence of CMV infection, and GFR 6 months after transplantation. The patient group that showed an increase in renal function over time used significantly more antihypertensive medication. There was no significant difference between patient groups in the number of patients who showed subclinical rejection in their 6 and 12 months protocol biopsies or in the Banff score for chronic changes assessed at 6 and 12 months.

mRNA Assessments

Q-PCR assays were performed on cDNA of protocol biopsies obtained 6 months after transplantation to assess the expression levels of different EMT-related markers. The mean mRNA levels are summarized in Table 3, and those mRNAs with significantly different expression levels between groups are depicted in Figure 1.

Patients who showed an increase in GFR between 6 and 24 months ($n = 19$) had significantly higher mRNA expression levels of TGF-β (–0.20 ± 0.31 *versus* –0.39 ± 0.26), S100A4 (–0.43 ± 0.69 *versus* –0.75 ± 0.34), collagen I (0.16 ± 0.60 *versus* –0.36 ± 0.49), and collagen III (0.09 ± 0.55 *versus* –0.29 ± 0.50) in their 6-month biopsies compared to patients with a decrease in GFR ($n = 33$) ($P < 0.05$). Patients with higher E-cadherin mRNA expression levels showed a decrease in GFR between 6 and 24 months (–0.44 ± 0.55 *versus* –0.19 ± 0.34) ($P < 0.05$).

Using the Pearson's correlation test to find associations between different outcome variables in a linear fashion, mRNA expression levels and corrected GFR at 24 months were compared. Correlations between log-transformed mRNA

Table 3. Log-transformed mRNA expression levels of EMT-related markers 6 months after transplantation in patients with a decrease or increase in renal function

Gene	Decrease in Cockcroft (n = 33)	Increase in Cockcroft (n = 19)	P
S100A4	-0.75 ± 0.34	-0.43 ± 0.69	0.029*
E-cadherin	-0.19 ± 0.34	-0.44 ± 0.55	0.048*
TGF-β	-0.39 ± 0.26	-0.20 ± 0.31	0.022*
BMP-7	-0.004 ± 0.30	0.13 ± 0.33	0.197
HGF	1.80 ± 0.48	1.74 ± 0.71	0.730
Sip1	-0.22 ± 0.27	-0.10 ± 0.46	0.242
Smad 1	1.80 ± 0.23	1.63 ± 0.67	0.180
Smad 2	2.00 ± 0.20	1.95 ± 0.26	0.376
Smad 3	-0.38 ± 0.54	-0.42 ± 0.48	0.814
Smad 5	2.08 ± 0.18	2.07 ± 0.28	0.937
Collagen I	-0.36 ± 0.49	0.16 ± 0.60	0.001*
Collagen III	-0.29 ± 0.50	0.09 ± 0.55	0.014*

*P < 0.05

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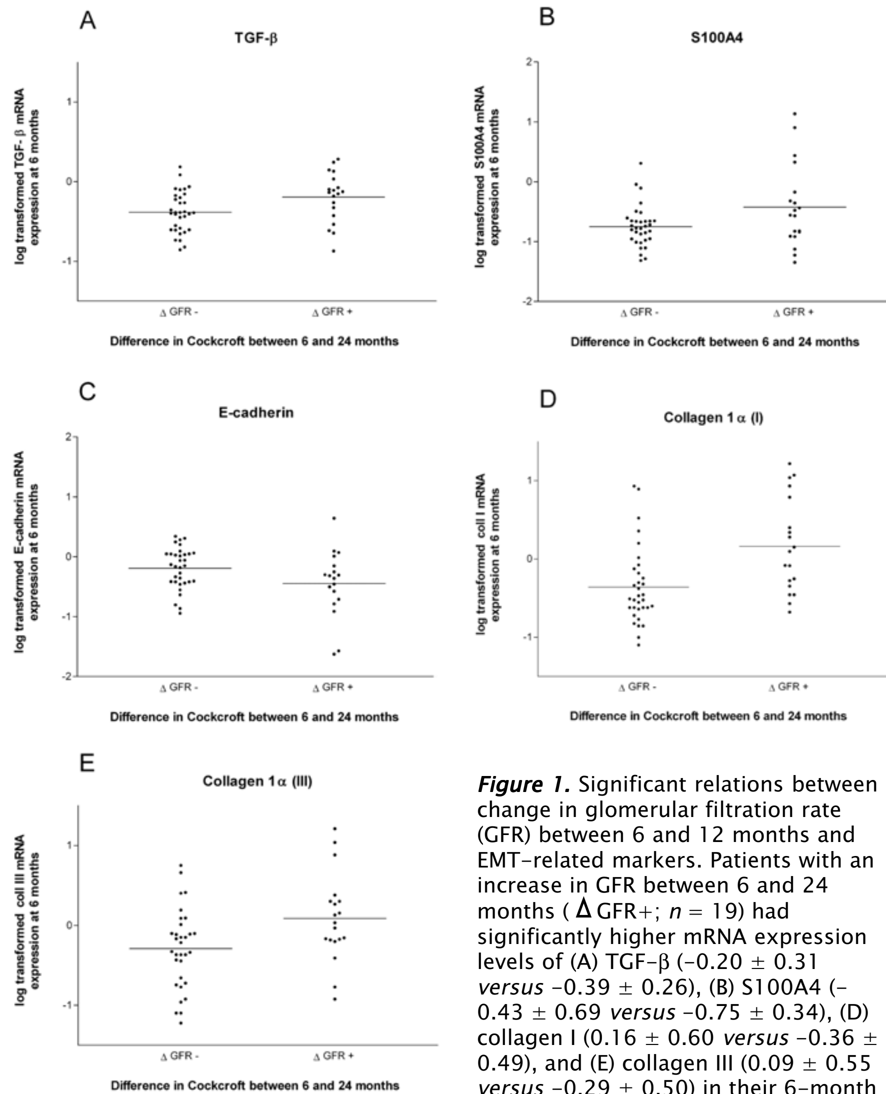

Figure 1. Significant relations between change in glomerular filtration rate (GFR) between 6 and 12 months and EMT-related markers. Patients with an increase in GFR between 6 and 24 months (ΔGFR+; n = 19) had significantly higher mRNA expression levels of (A) TGF-β (–0.20 ± 0.31 *versus* –0.39 ± 0.26), (B) S100A4 (–0.43 ± 0.69 *versus* –0.75 ± 0.34), (D) collagen I (0.16 ± 0.60 *versus* –0.36 ± 0.49), and (E) collagen III (0.09 ± 0.55 *versus* –0.29 ± 0.50) in their 6-month biopsies compared to patients with a decrease in GFR (ΔGFR–; n = 33). (C) Patients with higher E-cadherin mRNA expression levels showed a decrease in GFR between 6 and 24 months (–0.44 ± 0.55 *versus* –0.19 ± 0.34) (P < 0.05).

levels and corrected GFR at 24 months are depicted in Table 4. Six months after transplantation, mRNA levels of TGF-β, S100A4, and collagen I correlated significantly and positively with the corrected GFR at 24 months (r = 0.287, r = 0.274, and r = 0.332, respectively; P < 0.05).

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Table 4. Associations between mRNA levels of EMT-related markers in 6-month protocol biopsies and the corrected glomerular filtration rate (GFR) at 24 months

	Corrected GFR at 24 months	P
S100A4	0.274	0.049*
E-cadherin	-0.116	0.419
TGF-β	0.287	0.039*
BMP-7	-0.037	0.797
HGF	-0.070	0.628
Sip1	0.102	0.470
Smad 1	-0.150	0.289
Smad 2	0.077	0.594
Smad 3	0.038	0.797
Smad 5	0.121	0.393
Collagen I	0.332	0.016*
Collagen III	0.269	0.056

*P < 0.05

Histochemistry

As depicted in Figure 2A, there was no correlation between the extent of Sirius red staining at 6 months and the corrected GFR at 24 months (r = 0.200, P = 0.139). Furthermore, no correlation of the difference in Sirius red staining between 6 and 12 months with the corrected GFR at 24 months was found (r = -0.217, P = 0.115; Figure 2B). The extent of Sirius red staining at 6 months did not differ between patients showing an increase and patients showing a decrease in renal function (13.29 ± 3.79% *versus* 13.47 ± 3.97%; Figure 2C). At 6 months, the Banff score for chronic changes was not different between patients showing an increase and those showing a decrease in renal function over time (Figure 2D). To investigate the role of EMT in the accumulation of extracellular matrix molecules, EMT-related mRNA expression levels were correlated with the extent of Sirius red staining at 6 and 12 months and with the difference in Sirius red staining between 6 and 12 months. None of the 12 EMT-related markers investigated showed a significant correlation with the extent of Sirius red staining (data not shown).

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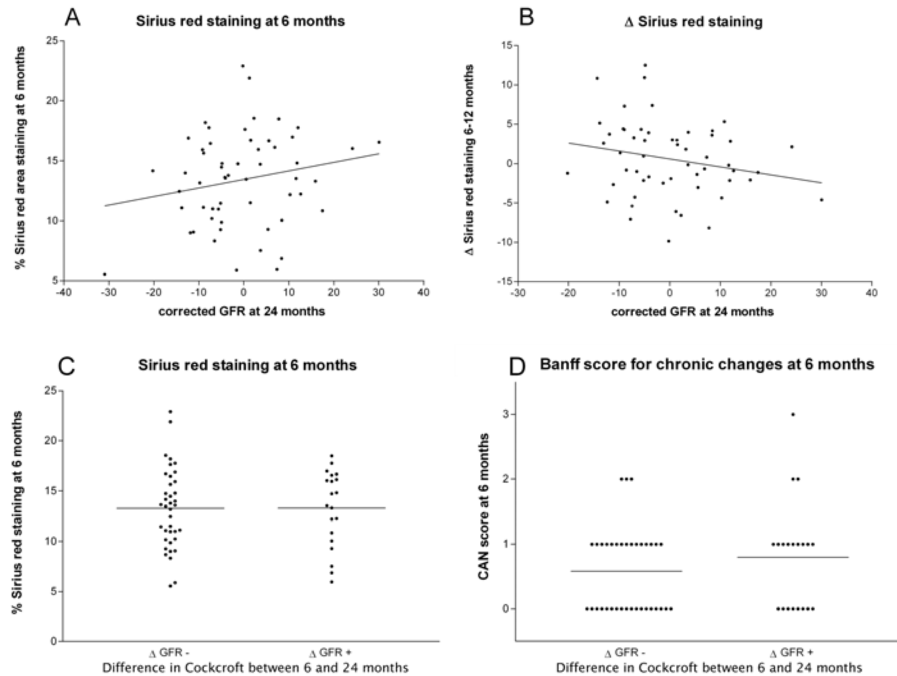

Figure 2. Relations between interstitial collagen deposition and the Banff score for chronic changes. (A) No significant relations between Sirius red staining at 6 months and the corrected glomerular filtration rate (GFR) at 24 months were found ($r = 0.200$, $P > 0.1$). (B) No significant relations between the difference in Sirius red staining between 6 and 12 months and the corrected GFR at 24 months were found ($r = -0.217$, $P > 0.1$). (C) No significant difference in the xtent of Sirius red staining at 6 months was found between patients with an increase (ΔGFR+) and those with a decrease (ΔGFR−) in renal function (13.29 ± 3.79 % *versus* 13.47 ± 3.97 %, $P > 0.1$). (D) No significant difference in the Banff score for chronic changes (CAN) was found between patients with an increase and those with a decrease in renal function.

Discussion

We investigated the relevance of mRNA levels of EMT-related molecules in protocol renal transplant biopsies with regard to graft outcome. Through the identification of markers that are of prognostic value, novel therapeutic targets can be assessed, and renal function can be monitored more closely. Strikingly, we found that markers involved in the process of EMT were elevated in patients who showed an improvement in renal function over time. Clinical and morphological parameters did not discriminate the two patient groups with different outcomes.

More than a decade ago, Strutz *et al.* described a novel marker, fibroblast specific protein-1 (FSP-1) [43]. Mouse FSP-1, a member of the S100 calcium-binding family, is highly homologous to human S100A4 and is used as a marker to demonstrate the presence of EMT in tubular epithelial cells. Until now, studies using human renal biopsies have addressed the presence of EMT only at the protein level [5,7,9,44]. Most of these studies have shown that tubular epithelial cells can undergo EMT and thereby possibly contribute to the development of kidney fibrosis. The number of tubular epithelial cells undergoing EMT was correlated with the progression of renal disease and functional loss [7;9;44]. In contrast, we found TGF-β and S100A4 to be upregulated and E-cadherin to be downregulated in 6-month protocol biopsies in the group of patients who showed an improvement in renal function over time. Furthermore, both collagen I and collagen III mRNA were upregulated in the group of patients who showed improved renal function, whereas the deposition of interstitial collagens did not predict renal function at a later time point. This finding may suggest that EMT-related mesenchymal markers play a role in connective-tissue remodeling but do not ultimately lead to excessive accumulation of ECM molecules, which results in a progressive decrease in renal function. Moreover, our results show no significant correlation between mRNA expression of the investigated EMT-related markers and the deposition of interstitial collagens. This finding strengthens our assumption that increased mRNA levels of mesenchymal markers do not necessarily contribute to the development of interstitial fibrosis but might be involved in a mechanism in which the kidney attempts to repair itself in response to injury.

Wound healing upon tissue injury involves inflammation, granulation tissue formation, and remodeling [45;46]. During inflammation, increased cytokine release leads to an increased influx of inflammatory cells and to activation and proliferation of fibroblasts. Fibroblasts and endothelial cells then induce ECM deposition and angiogenesis. Thereafter, the acquired ECM is reorganized by the degradation of collagens. During the course of collagen remodeling, collagen synthesis and catabolism still continue at a low rate. Matrix metalloproteinases, which are proteolytic enzymes that are responsible for collagen degradation, are not only secreted by macrophages, epidermal cells, and endothelial cells, but also by fibroblasts [45]. Detailed knowledge about these steps in the reconditioning of the ECM, particularly that of collagen remodeling, might be of great value in strategies to improve allograft function within the first year of transplantation. The increase in mRNA expression levels of collagens I and III in the group showing an improvement in renal function overtime may

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suggest that collagen remodeling is important in the maintenance of graft function.
The role of EMT in the human kidney is still unclear. The current results are in contrast to those found in earlier studies in which EMT was investigated at the protein level [7-9]. In those studies, EMT features in renal biopsies of allografts with chronic allograft nephropathy and of different renal diseases were associated with an increase in serum creatinine [7;9]. This discrepancy could be attributed to several factors. First, our study was performed on protocol biopsies from groups of patients who had relatively stable graft function. The present results might not be comparable to those from studies in which patients already had an advanced state of disease. The induction of expression of EMT-related markers might suggest that the kidney is attempting to repair injured tubules by restoring an embryonic environment [47]. Second, the protocol biopsies in the current study were obtained at 6 months. Graft function stabilizes within the first year after transplantation, which emphasizes the possibility of connective tissue remodeling through the process of EMT at this relatively early time point. Our study is the first to investigate the role of EMT-related marker expression at the mRNA level in protocol renal allograft biopsies obtained at 6 months. We found that upregulated mRNA expression levels of TGF-β, S100A4, and interstitial collagens correlated with improved renal function at 24 months after renal transplantation. This upregulation of mRNA does not lead to increased deposition of interstitial collagens or to a decrease in renal function over time. These results suggest a possible role for EMT in the process of tubular repair and connective tissue remodeling in the initial stage following renal transplantation. Long-term follow-up might strengthen this assumption, and it would be interesting to relate the current results to outcomes at later time points.

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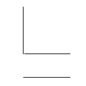

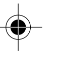

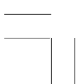
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