

Hypoxia response and VEGF-A expression in human proximal tubular epithelial cells in stable and progressive renal disease

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Proteinuria, inflammation, chronic hypoxia, and rarefaction of peritubular capillaries contribute to the progression of renal disease by affecting proximal tubular epithelial cells (PTECs). To study the transcriptional response that separates patients with a stable course from those with a progressive course of disease, we isolated PTECs by laser capture microdissection from cryocut tissue sections of patients with proteinuric glomerulopathies (stable $n = 20$, progressive $n = 11$) with a median clinical follow-up of 26 months. Gene-expression profiling and a systems biology analysis identified activation of intracellular vascular endothelial growth factor (VEGF) signaling and hypoxia response pathways in progressive patients, which was associated with upregulation of hypoxia-inducible-factor (HIF)-1 α and several HIF target genes, such as transferrin, transferrin-receptor, p21, and VEGF-receptor 1, but downregulation of VEGF-A. The inverse expression levels of HIF-1 α and VEGF-A were significantly superior in predicting clinical outcome as compared with proteinuria, renal function, and degree of tubular atrophy and interstitial fibrosis at the time of biopsy. Interactome analysis showed the association of attenuated VEGF-A expression with the downregulation of genes that usually stimulate VEGF-A expression, such as epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and HIF-2 α . *In vitro* experiments confirmed the positive regulatory effect of EGF and IGF-1 on VEGF-A transcription in human proximal tubular cells. Thus, in progressive but not in stable proteinuric kidney disease, human PTECs show an attenuated VEGF-A expression despite an activation of intracellular hypoxia response and VEGF signaling pathways, which might be due to a reduced expression of positive coregulators, such as EGF and IGF-1.

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The increasing prevalence of patients on renal replacement therapy has become a major challenge for healthcare systems.¹ Frequently, end-stage renal disease (ESRD) is the terminal phase of a chronic process, and a better understanding of the pathophysiology could lead to the development of new treatment options for stabilizing renal function, thus reducing the incidence of ESRD. Furthermore, to use both new and already available drugs more efficiently, it is highly desirable to identify patients with an adverse prognosis in the early phases of the disease, as not

all subjects show a relentlessly progressive decline in renal function.

Histologically, the risk for ESRD and the rate of excretory function loss correlates substantially better with the degree of tubular atrophy and interstitial fibrosis (TAIF) than with the type and/or extent of glomerular pathology.² A variety of most likely interacting insults have been shown to trigger tubulointerstitial pathology, such as proteinuria,³ leakage of glomerular filtrate into the periglomerular space,⁴ or tubulointerstitial hypoxia and rarefaction of peritubular

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capillaries.⁵ In this context, proteinuria is especially interesting. Although the magnitude of proteinuria often shows a positive correlation with the loss of glomerular filtration rate (GFR), some nonproteinuric renal diseases also progress to ESRD. In contrast, patients with minimal change disease often develop a nephrotic syndrome even though their renal function remains well preserved.

Proximal tubular epithelial cells (PTECs) may mediate the effects of proteinuria on tubulointerstitial damage by a variety of mechanisms, such as epithelial-to-mesenchymal transition (EMT).⁶ Experimental evidence is also available, showing that proteinuria induces changes in gene expression in PTEC, leading to alterations in the composition of proteins secreted mainly into the basolateral compartment, favoring the recruitment of inflammatory cells into the renal interstitium and contributing to fibrogenic reactions and renal scarring.⁷⁻⁹ On the other hand, it has also been suggested that under certain circumstances tubular expression of antifibrotic cytokines, such as hepatocyte-growth factor and bone morphogenetic protein-7 (BMP-7), protects PTEC from apoptosis¹⁰ and reduces interstitial collagen accumulation.¹¹

It is, therefore, suggestive that the balance between protective and deleterious networks is most likely to determine the final outcome of renal diseases rather than alterations in a single gene or a single pathway. Previous studies trying to unravel the molecular pathophysiology have been limited to using a candidate gene approach.¹²⁻¹⁴ Recently, we have combined a large-scale gene expression profiling with a systems biology approach to study the transcriptional response of isolated PTEC in proteinuric glomerulopathies.⁸ In a study performed by Schmid and coworkers,¹⁵ microarray technology and systems biology tools have also been successfully applied to differentiate the tubulointerstitial transcriptional response in human diabetic nephropathy as compared with nondiabetic samples. However, in this study, no microdissection of specific cells was performed.

In this study, we identified gene expression profiles and regulatory pathways in PTEC obtained by laser capture microdissection from biopsies of patients with proteinuric nephropathies that differentiate subjects with a stable and progressive post-biopsical clinical course.

MATERIALS AND METHODS

Kidney Biopsies

We used 31 kidney biopsies obtained from patients with proteinuric renal diseases during their routine diagnostic workup for which we had sufficient clinical follow-up data (Supplementary Table 1). Microarray-based gene expression profiling was performed in 21 of these samples, real-time PCR experiments were performed in 15, and 5 samples were analyzed by both methods.

Patients were split into two cohorts: 'stable disease (SD)' and 'progressive disease (PD)'. The median follow-up time was 26 months. As shown in Figure 1a and Supplementary

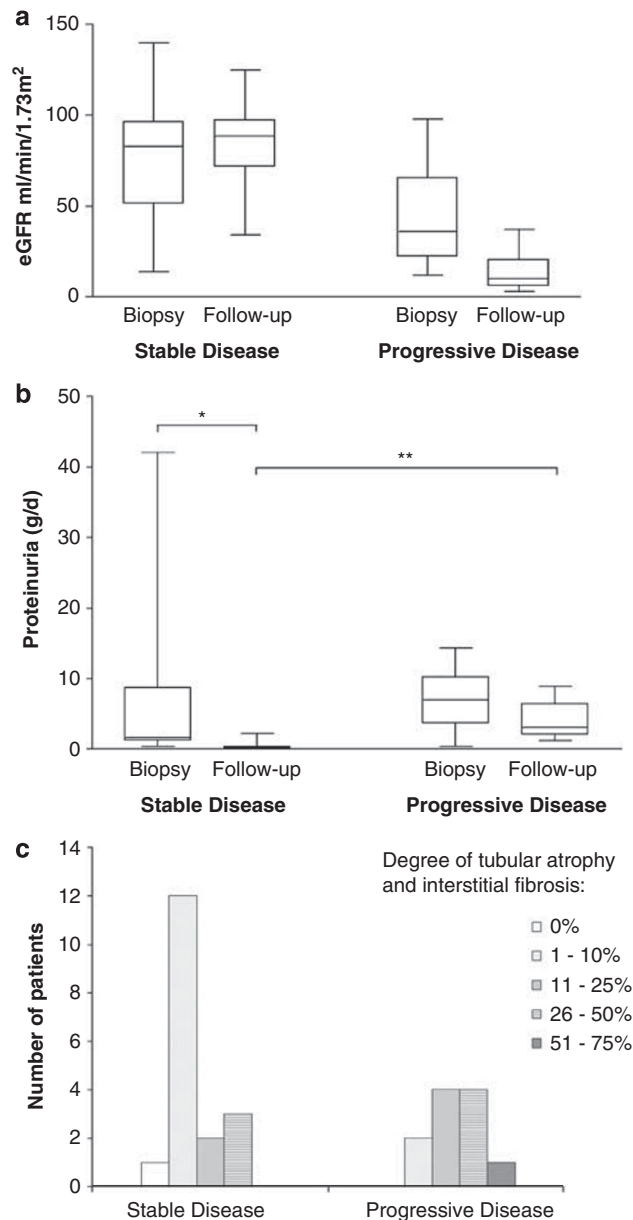


Figure 1 Patient characteristics: Kidney function, proteinuria, degree of TAIF. **(a)** eGFR as indicator of kidney function calculated using the MDRD formula. Progressive patients showed worse renal function at the time of biopsy than stable patients (NS). Renal function declined further over time by more than 30%, or the patients reached ESRD. Median values and the 25th and 75th percentile are shown. **(b)** The degree of proteinuria as calculated using the protein-creatinine ratio is shown. At the time of biopsy, there was a trend toward higher proteinuria in the progressive group ($P = 0.08$). During follow-up, the proteinuria declined significantly in the stable group ($*P < 0.0001$), and the proteinuria was significantly lower in the stable than in the progressive patient cohort ($**P = 0.0005$). Statistics were calculated using two-sided Mann-Whitney U -test with a CI of 95%. **(c)** The distribution of the degree of TAIF in stable and progressive patients is shown. The differences in distribution were not significant following a χ^2 test.

Table 1, progressive patients had an advanced impairment of kidney function at the time of biopsy, which did not improve during follow-up, or they showed a decline of kidney func-

tion of more than 30% (calculated as Δ of the estimated GFR (eGFR), or they reached ESRD. All other patients were defined as stable ($n = 20$). Proteinuria at the time of biopsy was not statistically different between stable and progressive patients, although there was a trend toward higher proteinuria in the progressive group (stable 5.49 ± 2.18 g/day; progressive 7.09 ± 1.28 g/day, $P = 0.08$ two-sided Mann–Whitney U -test). During the course of the disease, the reduction of proteinuria was significantly higher in the stable group (Figure 1b). The number of nephrotic and nephritic patients was not different between the cohorts. TAIF was scored by an independent pathologist following a semiquantitative grading system on haematoxylin and eosin and periodic acid–Schiff- or Pearse-stained sections: 0, no fibrosis; 1, 1–10%; 2, 11–25%; 3, 26–50%; 4, 51–75%; 5, >75%. Although the progressive cohort contained more patients with higher degree of TAIF (Figure 1c), the distribution of the respective grade of TAIF was not statistically significant after a χ^2 test. Therapy with steroids or with ACE-inhibitors or angiotensin receptor blockers did not differ significantly between the cohorts (χ^2 test): 5 of the 17 stable patients (for which medication at the time of biopsy has been documented) and 4 of the 11 progressive patients were on steroid therapy (29 vs 36%, NS). Three of the 17 stable patients and 1 of the 11 progressive patients were treated with ACE-inhibitors (18 vs 9%, NS). This study was approved by the Institutional Review Board of the University of Innsbruck.

Laser Capture Microdissection, RNA Isolation, Quality Control, and RNA Amplification

Processing of cryocut sections, isolation of proximal tubular cells, isolation of total RNA, quality control by RT-PCR, and T-7-based linear amplification of RNA were performed as described on our previous articles.^{8,16} In brief, PTECs were stained for alkaline phosphatase using 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate under RNase-free conditions, and the cells were isolated using the PixCell IIs Laser Capture Microdissection System and CapSure™ LCM Caps (Arcturus, Mountain View, CA, USA). Total RNA was isolated using the Pico Pure™ RNA Isolation Kit (Arcturus, Mountain View, CA, USA). Owing to low RNA amounts, we performed two rounds of linear RNA amplification using the RiboAmp™ RNA Amplification Kit (Arcturus, Mountain View, CA, USA). Amplified Universal Human Reference RNA (Stratagene, La Jolla, CA, USA) served as reference material. The quality of the amplified RNA was assessed by spectrophotometry (A260/280) and with the Agilent Bioanalyzer and RNA6000 LabChip™ Kit (Agilent, Palo Alto, CA, USA). More detailed information on laboratory procedures are freely accessible through our website www.microarray.at.

Microarray Hybridization, Analysis, and Data Storage

RNA was labeled and cohybridized to cDNA microarrays as described previously.¹⁶ cDNA microarrays were obtained

from the Stanford Functional Genomics Facility (<http://www.microarray.org/sfgf/>). The arrays contained 41 792 spots, representing 30 325 genes assigned to a UniGene cluster and 11467 ESTs. Arrays were scanned using a GenePix 4000B microarray scanner, and the images were analyzed with the GenePix Pro 4.0 software (Axon Instruments, Union City, CA, USA). All samples were processed in technical duplicates and gene expression values were averaged. Raw data as well as array images were uploaded to the Stanford Microarray Database (SMD) <http://genome-www5.stanford.edu/MicroArray/SMD> and to the Gene Expression Omnibus (GEO) website <http://www.ncbi.nlm.nih.gov/geo/> (GEO series accession number GSE11513). All experiments were performed according to the MIAME (Minimum Information About a Microarray Experiment) guidelines.¹⁷

Statistical and Pathway Analysis

We excluded signals showing intensity values less than 2.5 over background in either channel and focused our analyses on genes with valid data in at least 80% of processed samples, leaving 19 921 cDNA clones in the analysis data set. A two-sample t -test ($P < 0.05$) and a twofold-change criterion were used to identify differentially expressed genes (DEGs) when comparing both patient cohorts. DEGs were functionally annotated using gene ontology terms¹⁸ as provided by the SOURCE tool,¹⁹ the iHOP system (information Hyperlinked Over Proteins²⁰), and PubMed. We furthermore searched for biological pathways enriched/depleted in DEGs using the PANTHER (Protein Analysis Through Evolutionary Relationships) Classification System.²¹

Hypoxia Response and VEGF Signaling Interactome Analysis

We extracted all genes involved in these two pathways as provided by the PANTHER Classification System and extended this list by adding regulators and targets of hypoxia-inducible-factor (HIF) as well as regulatory proteins of vascular endothelial growth factor (VEGF), which were identified in a comprehensive literature search. The complete list of HIF- and VEGF-associated genes is shown in Supplementary Table 1.

Correlation of Transcription Levels of VEGF-A and HIFs with Clinical Parameters and Outcome

The correlations between VEGF-A, HIF-1 α , and HIF-2 α (EPAS1) to the level of proteinuria and the degree of TAIF were calculated. In addition, linear regression models were built with these genes as well as with proteinuria, eGFR, and degree of TAIF as independent variables to predict outcome in the patient cohort at hand.

Real-Time PCR

We used the TaqMan™ PreAmp Master Mix (Ambion, Austin, TX, USA) together with the respective TaqMan

probes (vide infra) for approximately 400-fold amplification of the original RNA as the laser-capture microdissection of proximal tubular cells from frozen sections yields about 1 ng of total RNA per sample. The accuracy of this amplification method was tested, and we did not find any amplification bias (data not shown). In all real-time PCR experiments, we used GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Hs99999905_m1) and PPIA (cyclophilin A; Hs99999904_m1) as endogenous controls. We evaluated the RNA expression values of the following genes: HIF-1 α (Hs00153153_m1), HIF-2 α (EPAS1; Hs01026149_m1), cyclin-dependent kinase inhibitor 1 alpha (CDKN1 α = p21; Hs00355782_m1), and VEGF-A (Hs00173626_m1).

Immunohistochemistry

Frozen tissue samples of four stable and four progressive patients were fixed with cold acetone. For VEGF-A staining, the sections were first incubated with a 1:100 dilution of the affinity-purified rabbit polyclonal VEGF-A antibody (sc-152, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at room temperature. For visualization of VEGF-A expression, a 1:400 dilution of a biotin-conjugated goat anti-rabbit secondary antibody (Jackson Immuno Research Laboratories, Newmarket, UK) and the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) were used. All sections were counterstained with Gill's hematoxylin.

Cell Culture Experiments

Cell culture reagents were obtained from GIBCO (Life Technologies, Lofer, Austria). Human kidney-2 (HK-2) cells were cultured in keratinocyte-serum free medium containing 10% fetal bovine serum, 5 ng/ml recombinant epidermal growth factor, 0.05 mg/ml bovine pituitary extract, 100 U/ml penicillin, and 100 μ g/ml streptomycin.²² The cells were grown at 5% CO₂ at 37°C humidified atmosphere and split in a ratio 1:5 once a week. After growth to subconfluent state, cells were washed once, made quiescent by incubation in serum- and supplement-free medium for 48 h, and then used for the experiments. We performed stimulations with TGF- β 1, epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1) at a concentration of 10 ng/ml in the absence of serum and any other growth supplements. TGF- β 1 was purchased from R&D Systems (Minneapolis, MN, USA), whereas EGF and IGF-1 were obtained from Sigma (St Louis, MO, USA). After 8 and 32 h total RNA was extracted from cultured cells using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) as recommended by the manufacturer.

RESULTS

PTECs in Stable and Progressive Nephropathies Show Differential Gene Expression

On the basis of the kidney function at the time of biopsy and Δ eGFR during a median follow-up of 26 months, patients

were defined as having SD or PD (Figure 1 and Supplementary Table 1). A total of 149 transcripts were differentially expressed between both patient cohorts. A total of 122 of these clones, representing 113 unique genes based on UniGene IDs were upregulated, and 27 cDNA clones were downregulated in PD samples. Among the 113 genes upregulated in the PD cohort, 33 (29%) were involved in regulatory processes, such as signal transduction or regulation of transcription. Other prominent functional categories enriched with DEGs were immune response with 18 (16%) upregulated genes, as well as metabolism (11; 10%) and cell cycle regulation or cell proliferation (5; 4%). Interestingly, in the latter functional class, four of the five genes were associated with inhibition of cell proliferation or induction of cell cycle arrest in response to various stress stimuli (eg, p21). In the immune response group, we found a marked upregulation of interferon-induced transcripts, interferon-inducible proteins, and interferon regulatory factors, as well as an upregulation of members of the complement cascade. Among the 27 genes downregulated in progressive disease, we found decreased expression of BMP-7 and EGF. Microarray-derived gene-expression values of SD and PD samples were confirmed by real-time PCR for five genes, and we did not detect any directional changes in transcription (data not shown).

Pathway Analysis

We identified nine biological pathways that were significantly enriched in DEGs upregulated in PD, whereas we did not find any significantly enriched biological pathways in SD (Table 1). Among the top ranked pathways in PD samples, we found hypoxia response through HIF activation ('hypoxia response') and VEGF signaling. We focused on these two pathways, as hypoxia and VEGF expression have been described to play a role in human progressive tubulointerstitial fibrosis (reviewed by Schrijvers *et al*²³). To confirm the activation of these pathways, we analyzed the expression of all pathway members with a differential expression between the cohorts of >1.7-fold, as, in our experience, a difference in transcription between two samples >1.7-fold represents a reliable reproducibility. A total of 72 of these pathway members were spotted on the arrays used, and 12 transcripts showed an induction in PD, whereas 4 genes were downregulated (Table 2). Several key mediators of a hypoxia response-like HIF-1 α and HIF-1 β and CREB-binding protein (CREBBP)—a cofactor of the HIF complex—showed an upregulation, supporting the results from pathway analysis. Surprisingly, VEGF-A—a major target gene of HIF—was downregulated in PD samples. This contradiction of the activation of hypoxia response and VEGF signaling pathways on the one hand and downregulation of VEGF-A RNA on the other hand prompted us to evaluate the expression of other HIF targets as well as of HIF and VEGF regulators in our samples.

Hypoxia Response and VEGF Signaling Interactome Analysis

On the basis of a scientific literature screening, we identified 39 HIF-1 α target genes, 18 positive and 7 negative HIF regulators, and 33 positive and 5 negative regulators of VEGF with

Table 1 Significantly enriched pathways in PD samples

Pathways upregulated in PD samples	t-Test
PDGF signaling pathway	0.001
VEGF signaling pathway	0.005
Hypoxia response through HIF activation	0.011
Cytoskeletal regulation by Rho GTPase	0.013
p53 pathway	0.022
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha-mediated pathway	0.027
p53 pathway feedback loops 2	0.034
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha-mediated pathway	0.046
Inflammation mediated by chemokine and cytokine signaling pathway	0.049

Nine significantly enriched biological pathways were identified using PANTHER in the upregulated DEGs in the PD cohort. The *P*-value of a χ^2 test is given. DEG: differentially expressed gene.

Table 2 Up- and downregulated pathway members

Accession number	Gene name	Symbol	Pathway component	Fold change
W88746	CREB-binding protein (Rubinstein–Taybi syndrome)	CREBBP	CREBBP/p300	2.877
AA9765284	PTK2B protein tyrosine kinase 2 beta	PTK2B	FAK	2.539
AA709154	Rho GTPase activating protein 1	ARHGAP1	Rac	2.475
AA935529	Nitric oxide synthase 1 (neuronal)	NOS1	eNOS	2.191
AA419268	Phosphoinositide-3-kinase, regulatory subunit 2 (p85 beta)	PIK3R2	PI3K	2.059
AA058828	Fms-related tyrosine kinase 1	FLT1	VEGFR-2	1.945
W44318	Mitogen-activated protein kinase 1	MAPK1	MEK/Erk	1.913
AA293870	Protein kinase C, epsilon	PRKCE	PKC	1.866
AA485731	Phosphoinositide-3-kinase, regulatory subunit 2 (p85 beta)	PIK3R2	PI3K	1.814
W47003	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	HIF1A	HIF1-a	1.792
AA708437	Phosphoinositide-3-kinase, catalytic, beta polypeptide	PIK3CB	PI3K	1.765
W47196	Aryl hydrocarbon receptor nuclear translocator	ARNT	HIF1-b	1.748
H57180	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	PLCG2	PLC-g	-1.750
AA608530	FK506-binding protein 12-rapamycin-associated protein 1	FRAP1	mTOR	-1.764
AA630298	PTK2 protein tyrosine kinase 2	PTK2	FAK	-1.798
R19956	Vascular endothelial growth factor A	VEGFA	VEGFR-2	-2.516

A total of 16 members of the hypoxia response and VEGF signaling pathways showing fold-change values > 1.7 or < -1.7 were detected. The fold-change values of the respective GenBank cDNA clones along with the Gene Names, the official Gene Symbols, and the pathway component names are provided.

expression values in our data set (Supplementary Table 2). Of the HIF targets, cyclin-dependent kinase inhibitor 1A (CDKN-1A, p21), transferrin (TF), transferrin receptor (TFRC), and the VEGF receptor FLT-1 were upregulated, whereas aldolase A (ALDO-A), carbonic anhydrase 12 (CA-12), and VEGF-A were downregulated. Of the 18 positive regulators of HIF, the SRC oncogene, mitogen-activated protein kinase 1, and a subunit of the phosphoinositide-3-kinase (PIK-3CB) were upregulated, whereas we observed a downregulation of IGF-1 and EGF. There were no changes in expression of the seven negative regulators of HIF. In the group of positive regulators of VEGF, NOS-1, SRC, prostaglandin E receptor 4 (PTGER-4), PDGF-D, HIF-1 α and the transcription factor SP3 were induced in PD samples. On the other hand, positive regulators of VEGF, which showed a downregulation in PD samples included EGF, IGF-1, HIF-2 α (EPAS-1), and PTGER-3. There were no changes in the expression of the five negative regulators of VEGF. We confirmed the upregulation of HIF-1 α and p21, and the downregulation of HIF-2 α and VEGF-A by real-time PCR in the array patient cohort as well as in independent patients (Figure 2).

VEGF-A and HIF-1 α Gene Expression Predict Outcome Better than Degree of Proteinuria, eGFR, and Tubulointerstitial Fibrosis Alone

The expression of HIF-1 α correlated positively with the level of proteinuria (Pearson $r = 0.67$), whereas the expression of VEGF-A was marginally negatively correlated to proteinuria

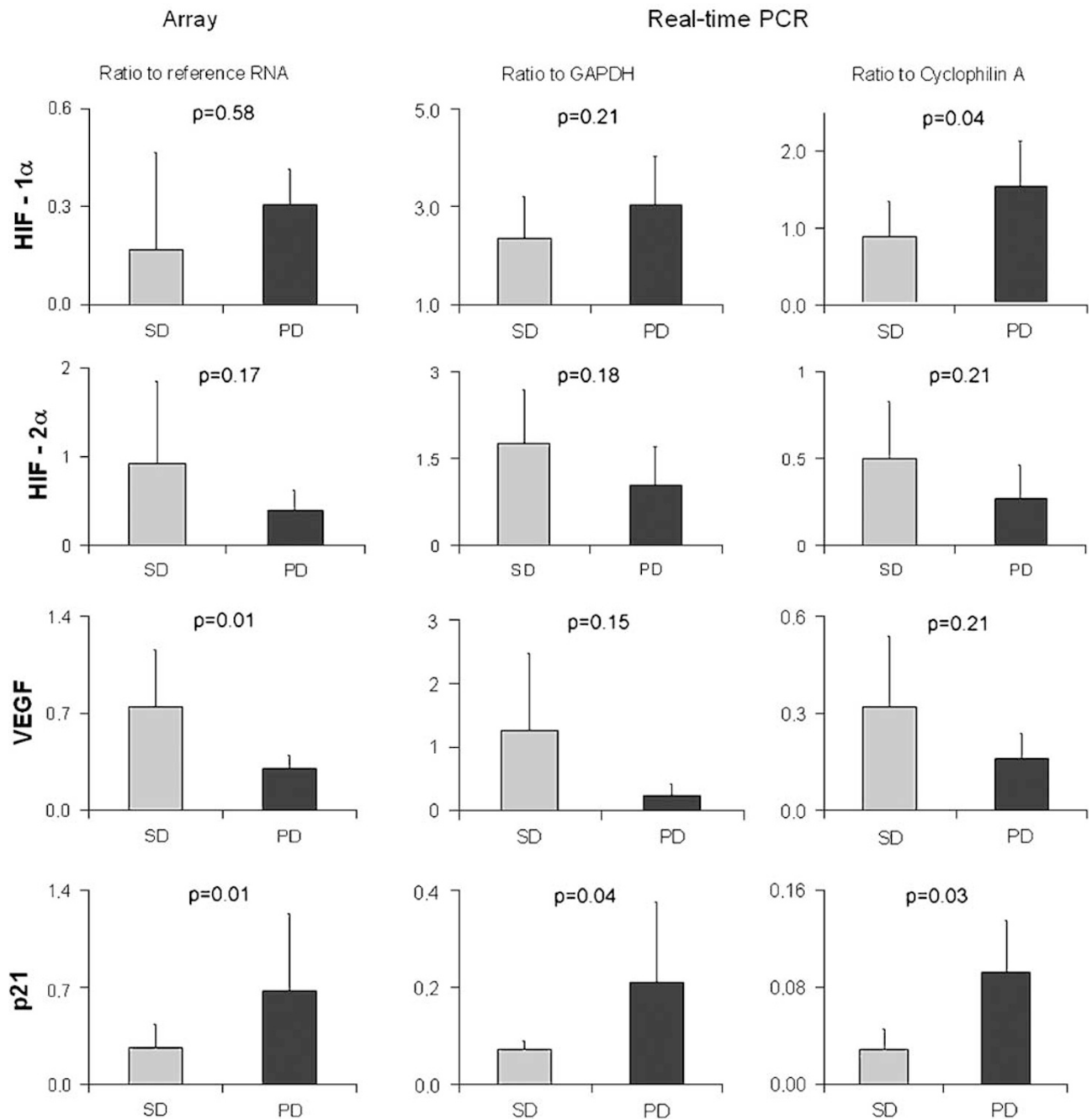


Figure 2 Real-time PCR of selected genes in an expanded patient cohort. We analyzed the RNA expression of HIF-1 α , HIF-2 α , VEGF-A, and p21 in patients with available microarray results as well as in an expanded patient cohort (Table 1). Microarray expression data are shown as ratio to the reference RNA (left column). Real-time PCR expression values are shown as ratios to the respective housekeeper gene (GAPDH and cyclophilin A, right columns). Error bars represent the standard deviation of the mean. SD: stable disease, PD: progressive disease. The *P*-value was calculated using a two-sided *t*-test with a CI of 95%.

(Pearson $r = -0.22$). A negative correlation of VEGF-A to the degree of interstitial fibrosis (Pearson $r = -0.49$) was detected. HIF-2 α also showed a negative correlation of -0.51 to the level of fibrosis. Neither a correlation between HIF-2 α and the level of proteinuria nor a correlation of HIF-1 α to the degree of interstitial fibrosis could be detected.

A regression analysis with proteinuria as independent variable and the group assignment of stable and progressive as dependent variable resulted in an adjusted R^2 of 0.234. The adjusted R^2 of the eGFR as independent variable was 0.067. The adjusted R^2 of the degree of TAIF as independent variable was 0.135. A similar analysis with VEGF-A as the independent variable resulted in an adjusted R^2 of 0.556, thus

being a superior predictor of outcome. VEGF-A and HIF-1 α as independent variables together in the model resulted in an even higher R^2 value of 0.677, being significantly superior in comparison with proteinuria, eGFR, or the degree of TAIF alone (P -value: 0.001 following an ANOVA comparing the model with VEGF-A and HIF-1 α with the model with proteinuria).

EGF and IGF-1 Stimulate VEGF-A mRNA Expression in Renal Proximal Tubule Cells *In Vitro*

The findings of the pathway-analysis showed a down-regulation of VEGF-A despite an activation of the hypoxia response and VEGF signaling pathways in the progressive cohort. In the interactome analysis, we did not find any overexpressed negative regulators, but a downregulation of several positive regulators of VEGF-A expression, such as EGF and IGF-1. EGF and IGF-1 have been shown to stimulate VEGF-A mRNA expression in various cell lines.^{24–27} We hypothesized that, also in human proximal tubule cells, EGF and IGF-1 might play a crucial role inducing VEGF-A expression. Thus, we investigated the transcription of VEGF-A mRNA in HK-2 cells in the presence of EGF and IGF-1, respectively. As shown in Figure 3, stimulation of HK-2 cells with EGF led to a time-dependent increase in VEGF-A mRNA expression, which was 1.5-fold (NS) and 1.8-fold ($P < 0.05$) after 8 and 32 h, respectively ($n = 3$). When HK-2 cells were incubated with IGF-1, the increase in VEGF-A transcription was much less pronounced, with no significant change after 8 h, and a 1.2-fold ($P < 0.05$) increase after 32 h, respectively ($n = 3$). Induction of VEGF-A expression by TGF- β 1 served as a positive control.

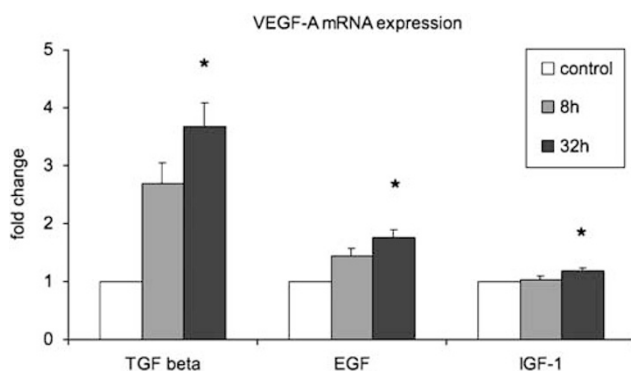


Figure 3 Time-dependent effect of TGF- β 1, EGF, and IGF-1 on VEGF-A expression in HK-2 cells. HK-2 cells were starved for 48 h and were then exposed to 10 ng/ml TGF- β 1, EGF, and IGF-1 for 8 and 32 h in the absence of any additional growth supplement. RNA was extracted using TRI reagent, and real-time PCR was performed using GAPDH as endogenous control. The control samples were arbitrarily set to 1, and the fold change of VEGF-A expression at each time point was calculated using the $\Delta\Delta C_t$ method. The mean of three experiments and the standard deviation is shown. A two-sided pairwise t -test was calculated between each incubation time and the control. * Marks a P -value of < 0.05 .

VEGF-A Protein Expression in Kidney Biopsies

To analyze VEGF-A protein expression, we performed immunohistochemistry in biopsy samples from four SD and four PD samples. VEGF-A was found to be expressed in $36.1 \pm 3.3\%$ of the tubules in stable patients, and in $5.9 \pm 6.7\%$ of the tubules in progressive patients ($P = 0.0286$; Figures 4 and 5). VEGF-A was expressed in all glomeruli regardless of the clinical course of the patients.

DISCUSSION

Even though glomerular damage represents a key initial event in many acute and chronic kidney diseases, the progressive decline of renal function over time is much more closely associated with the development and progression of TAIF.² Glomerular proteinuria might be one of the mediators between these two events because it directly affects the phenotype of PTECs.³ However, several nonproteinuric kidney diseases also show a progressive course of disease; therefore, other mechanisms must also be operative. One of these is probably an alteration of the function of post-glomerular endothelial cells leading to chronic ischemia and loss of peritubular capillaries (reviewed by Rabelink *et al*²⁸). As proteinuric renal diseases usually progress faster, it is tempting to speculate that these processes interact. Hypoxia could potentiate the deleterious effects of proteinuria on PTEC and vice versa glomerular protein leakage might activate PTEC in a manner that negatively affects the neighboring vascular endothelial cells. Surprisingly, however, some proteinuric renal diseases do not progress.

Therefore, in our study, we have focused on transcriptional changes in PTEC obtained from biopsies of patients with stable and progressive proteinuric renal diseases. Although all of the patients had a considerable degree of proteinuria at the time of biopsy, some of the patients also presented with signs of a nephritic renal disease (ie, erythrocyturia and proteinuria in the urinary sediment). However, the frequency of nephritis vs nephrosis did not differ significantly between the stable and progressive cohorts, which was also the case for the therapy with steroids or blockers of the renin-angiotensin system. On the other hand, proteinuria during follow-up was significantly different between the cohorts, with stable subjects showing a 93% mean reduction of proteinuria, and progressive subjects (not on hemodialysis) showing only a 46% mean reduction of proteinuria, respectively. Thus, even though we cannot rule out a potential effect of hematuria on PTEC, we conclude that the dysfunction of PTEC described in our study is primarily due to the effect of protein and yet unknown factors.

We used a systems biology approach to better describe these complex interactions. In patients with progressive renal disease, we found an activation of the intracellular hypoxia response and VEGF signaling pathways. Even though chronic renal failure has been proposed to be associated with hypoxia, the role of hypoxia-inducible transcription factors and the expression of HIF target genes in chronic kidney diseases has

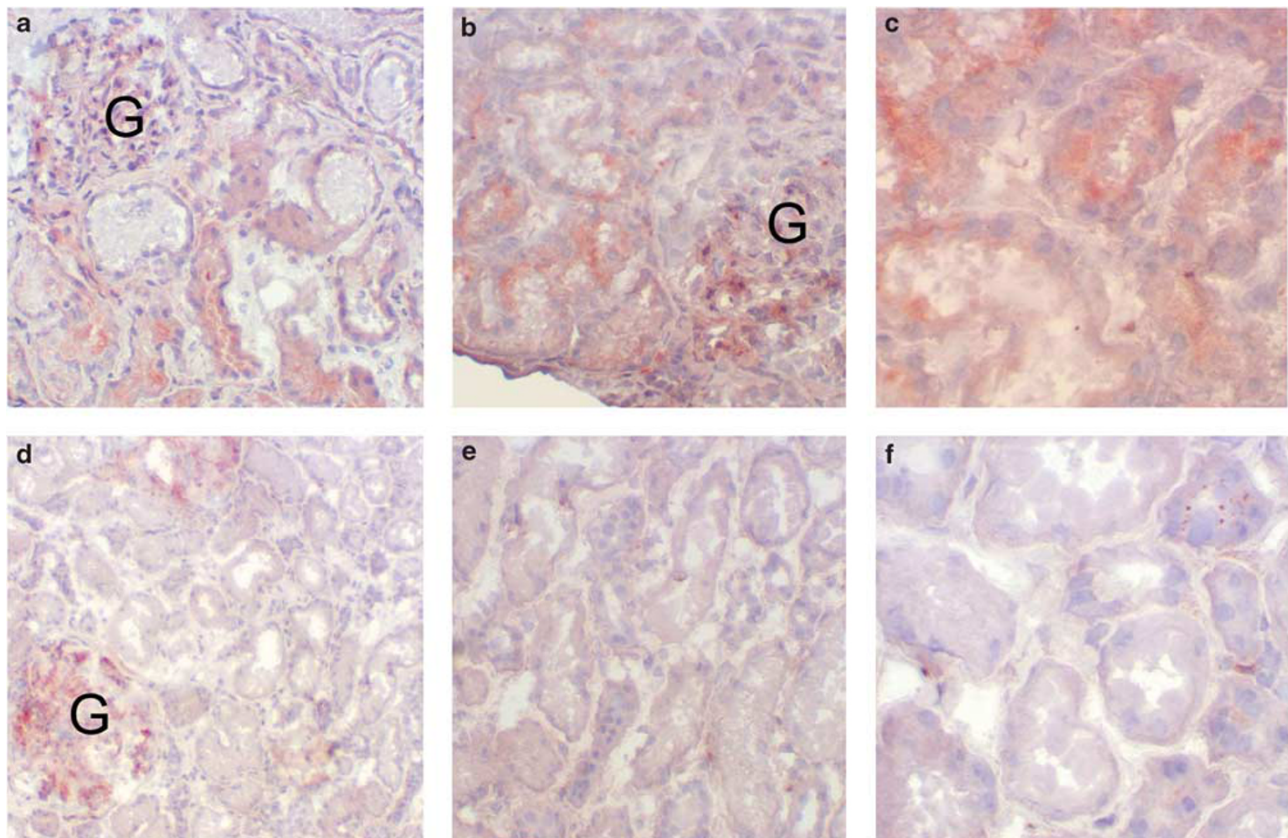


Figure 4 VEGF immunostaining. Immunostaining for VEGF in stable (a–c, $n = 4$) and progressive patients (d–f, $n = 4$): PTEC from stable patients showed strong intracellular VEGF protein expression, whereas VEGF protein expression was strongly diminished in progressive patients, which is in accordance with RNA expression data. Original magnification: (a, d) $\times 100$, (b, e) $\times 200$, (c, f) $\times 400$. (a–f): We detected strong VEGF signals in most glomeruli (G) regardless of the clinical course.

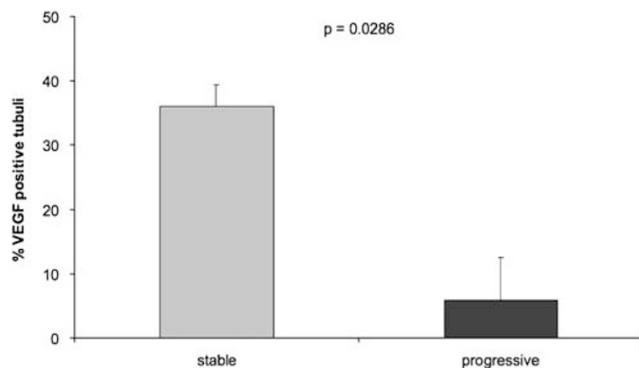


Figure 5 Quantification of tubular VEGF protein expression. VEGF-positive and VEGF-negative tubuli were counted in kidney biopsies from four stable and four progressive patients. The mean percentage of VEGF-positive tubuli \pm standard deviation of the mean is shown. Two-sided Mann–Whitney U-tests showed a significant difference in VEGF protein expression between stable and progressive patients ($P = 0.0286$).

been less well studied.^{5,29} In this study, we were able to show in PD samples elevated RNA levels not only for HIF-1 α and HIF-1 β but also for CREB binding protein, which represents

a transcriptional coactivator of HIF. Binding of HIF to the hypoxia responsive element usually results in the expression of a variety of target genes, including erythropoietin, phosphoglycerate kinase (PGK), and also VEGF-A. Their activation may protect the cells from hypoxia, a mechanism that in PTEC has been proposed to be nephroprotective.³⁰ However, HIF signaling *per se* is also associated with stimulation of collagen synthesis, increased expression of tissue-inhibitor of metalloproteinase-1, decreased expression of matrix-metalloproteinase-2,³¹ activation of plasminogen activator inhibitor-1,³² activation of connective tissue growth factor,³³ TGF- β 1 and PDGF.⁵ Recently, Higgins *et al*³⁴ showed that hypoxia enhanced EMT of primary tubule cells through HIF-1 α *in vitro*, and deletion of HIF-1 α in tubule cells in a murine model of chronic kidney disease inhibited the development of tubulointerstitial fibrosis. HIF-1 α is also increasingly expressed in the tubulointerstitial compartment of patients with progressed diabetic nephropathy.³⁴ Hypoxia and the upregulation of HIF-1 α also inhibits cell growth by upregulation of the cyclin-dependent kinase inhibitors p21 and p27,³⁵ findings that were consolidated by our experiments. In summary, activation of the HIF signaling pathway has the

capacity to both promote and inhibit the progression of TAIF.

It is interesting to note that in our study the RNA and protein expression of VEGF-A was suppressed in patients with progressive renal failure, despite activation of the HIF transcription factors. In line with this finding are results by Tanaka *et al*,³⁶ which also described a decreased VEGF protein expression despite HIF activation in two rat models of chronic renal failure. Recent publications also suggest a decline of VEGF-A expression in progressive diabetic nephropathy.³⁷ In a more general context, these findings are of significant importance because the expression of VEGF-A in the tubulointerstitium is most likely to play a major role in neo-angiogenesis of peritubular capillaries, with reduced VEGF-A levels being responsible for peritubular capillary rarefaction, tubular atrophy, loss of functional nephrons, and impaired recruitment of endothelial progenitor cells.²³ The significance of these hypotheses is further supported by our results in which attenuated expression of VEGF-A and increased expression of HIF-1 α together predicted outcome of chronic renal disease to a higher extent than well-established parameters, such as proteinuria, eGFR, or the degree of TAIF at the time of biopsy.

To elucidate the association between activation of the hypoxia response pathway and the suppressed VEGF-A expression, we performed a systems biology analysis to obtain a functional interaction network of HIF/VEGF pathway members and their regulators. In the progressive cohort, we did not find any differential regulation of negative regulators of VEGF-A expression. However, several positive regulators of VEGF-A transcription, such as EGF, IGF-1, PTGER-3, and HIF-2 α were significantly downregulated. Our *in vitro* experiments confirmed the positive regulatory effect of EGF and IGF-1 on VEGF-A RNA expression in human PTECs, although the effect was more pronounced with EGF than with IGF-1. As EGF and IGF-1 also represent positive regulators of HIF-1 α activation, it is tempting to speculate that in progressive chronic kidney disease, the activation of hypoxia response and VEGF signaling pathways depends on hypoxia, but the induction of VEGF-A transcription requires the presence of coregulatory factors.

HIF-2 α —also named endothelial PAS domain protein 1 (EPAS-1)—is a member of basic-helix-loop-helix/PAS domain containing transcription factor family showing a high sequence homology to HIF-1.³⁸ In the kidney, HIF-1 α and HIF-2 α have been shown to be expressed in a cell-type-specific manner (for review, see Eckardt *et al*²⁹), with HIF-1 α mostly being expressed in glomeruli and in tubule cells, and HIF-2 α being detected in podocytes, in endothelial cells of the peritubular capillaries and in interstitial cells. Both transcription factors are activated under hypoxia, but they differ in their transactivation domains, which implies that they may activate different target genes and/or require distinct transcriptional cofactors.³⁹ In a hu-

man fetal kidney cell line, Xia *et al*⁴⁰ showed that endogenous VEGF-A is upregulated by HIF-2 α overexpression, and downregulated when HIF-2 α transcription is inhibited. Interestingly, in this study, the transcriptional regulation of VEGF-A was independent of HIF-1 α transcription. These results and our findings support the hypothesis that HIF-2 α overexpression might represent an additional key regulator of tubular VEGF-A expression in progressive chronic kidney disease.

Besides differential activation of hypoxia-related pathways, we also found a reduced expression of BMP-7 in PTEC in the PD cohort. As it has been shown that BMP-7 has the potential to act as an antifibrotic protein in the kidney,¹³ a reduced expression of BMP-7 as observed in our PD cohort may represent one detrimental mechanism finally leading to increased tubulointerstitial fibrosis.

In conclusion, our results indicate an activation of hypoxia response and VEGF signaling pathways in PTEC in progressive, but not in stable, kidney disease. Specifically, the upregulation of HIF-1 α and p21 is in line with the idea of a hypoxia-mediated cell cycle arrest. Despite the hypoxic stimulus, the tubular expression of VEGF-A was strongly attenuated in progressive patients, which in the long run may have a detrimental effect on peritubular endothelial cell survival. The RNA expression of HIF-1 α and VEGF-A has a higher predictive value for clinical outcome than proteinuria, eGFR, and degree of TAIF alone, although these results have to be confirmed in further studies including higher patient numbers. Finally, positive inducers of VEGF-A expression, such as IGF-1, EGF, PTGER-3, and/or HIF-2 α rather than HIF-1 α alone, may play a decisive role in the transcriptional regulation of VEGF-A in chronic kidney disease.

Online supplementary material

Supplemental material is available at Laboratory Investigation's website. Additionally, the raw data summary, tables, webtables, figures and additional information can be found on our website <http://www.microarray.at>. Raw data from each of the microarray experiments is accessible through the Gene Expression Omnibus (GEO) website <http://www.ncbi.nlm.nih.gov/geo/> (GEO series accession number GSE11513).

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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