

Histomorphological traceability of cell signaling events leading to tissue fibrosis and cancer

Ph.D. Thesis

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1 Introduction

One of the most common maladies of the 21st century is chronic kidney disease. Increased life expectancy and illnesses (diabetes, hypertension, high blood lipid concentration) associated with kidney damage may lead to end-stage renal disease and kidney failure.

According to the definition of the United States National Kidney Foundation chronic kidney disease is a "structural and / or functional kidney damage occurring for a minimum of 3 months due to various causes". The definition was also accepted and implemented by the Hungarian nephrology related organizations.

Surveys carried out around the world indicate that 10-16% of world-wide population may suffer from chronic kidney disease.

Due to the steady increase in the number of chronic kidney disease, the development of renal impairment and the detection of the molecular and cellular processes leading to it are of particular importance in order to prevent kidney disease. Unfortunately, there is currently no specific drug available to restore the structure of damaged kidneys to their original condition. Currently, our therapeutic toolbox contains pharmacologically active substances only slowing down the process of fibrosis. These drugs are primarily antihypertensives inhibiting the function of the renin-angiotensin-aldosterone system. Current antihypertensive drugs have wide-spectrum effects and therefore their potential side effects may be significant. A much more accurate understanding of the renal fibrosis process is needed to allow the development of novel drugs enabling precisely targeted therapies.

Our team has examined the intracellular role of certain novel signaling molecules identified in recent years for their potential involvement in the development of renal fibrosis, and in my dissertation summarizes the results of this work.

2 Objectives

Since SCAI is also expressed in the kidneys, and considering the factors leading to renal fibrosis and EMT, as well as the role of SCAI in regulating MRTF dependent transcription, we proposed to investigate its potential protective role in a model of fibrotic EMT and in the regulation of α -SMA expression.

In addition, we aimed to investigate the role of PAI-1 protein in the processes leading to renal fibrosis progression and ECM homeostasis damage. We also proposed to investigate whether the in vitro described role of SCAI could be confirmed and supported by immunohistochemical studies of human tissue samples.

At the beginning of my work, I set the following hypotheses and goals:

1. Changes in SCAI or PAI-1 gene expression during renal fibrosis process can be observed and these changes can be detected in animal and human cells and tissues on mRNA and protein level.
2. I proposed to examine novel signaling mechanisms regulating certain well defined markers of EMT and renal fibrosis, such as α -SMA and PAI-1.
3. I hypothesized that the function of SCAI described during renal fibrosis may correlate with expression patterns in human kidney tumors and formulated the question to what extent the expected reduction in SCAI expression characterizes different tumors.

Based on the hypotheses, I had the following questions:

1. What is the effect of SCAI on EMT in various animal and human cell types and tissues?
2. Can SCAI be detected in human malignant tumors and what is its expression pattern in certain tumors?
3. Is PAI-1 regulation AngII dependent?

3 Methods

3.1 Cell culture

Porcine proximal tubular epithelial cells (LLC-PK₁/AT₁) were obtained from Dr. R. Harris. mIMCD-3 kidney medullary collecting duct cells were purchased from ATCC (ATCC, Manassas, VA, USA).

3.2 TGF- β 1 transgenic mouse

CBA.B6-Alb / TGF- β 1 (cys223,225ser) transgenic mouse strain was a gift from Dr. S.S. Thorgeirsson. The mice were raised in a germ-free environment at the Semmelweis University NET GMO division, 10 hours / 14 hours in a light and dark cycle. Mice received rodent nutrients and had free access to drinking water. All animal experiments were approved by the Animal Welfare Committee of the Semmelweis University (XIV-I-001 / 2146-4 / 2012) and the animal handlings were in line with the National Institute of Health (NIH, USA) Laboratory Animal Recommendations and Rules.

3.3 Transient transfection and luciferase promoter activity measurements

In the experiments involving α -SMA promoter activity, cells grown on 6 well plates were transfected at low or high confluence with FuGene6 (Roche, Mannheim, Germany). Luciferase activity was measured using the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) on a Victor X3 2030 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA). As internal control, Renilla luciferase was used. In the PAI-1 promoter activity assays, LLC-PK₁/AT₁ cells grown in 60 mm dishes at 50% confluence were transfected by the calcium phosphate precipitation method.

3.4 Immunofluorescent microscopy

Cells were grown on sterile 8-well Nuc Lab-Tek II Chambered Coverglass (Nalge Nunc International, Rochester, NY, USA) and then fixed in Dulbecco Modified PBS (DPBS) containing 4% paraformaldehyde, permeabilized, blocked and incubated with primary antibodies. After thorough washing, samples were incubated with the appropriate

fluorophore-conjugated secondary antibodies. Nuclei were visualized with DAPI (Invitrogen, Carlsbad, CA, USA). Coverslips were mounted with Fluorescence Mounting Medium (Dako, Glostrup, Denmark). Slides were examined using an Olympus FV500-IX confocal laser scanning microscope (Olympus Optical Co. Europe, Hamburg, Germany).

3.5 Immunohistochemistry of human tissue

To test the expression pattern of SCAI protein, formalin fixed, paraffin embedded tissue samples were used, which were selected from the histological archive of 2nd Department of Pathology, Semmelweis University. Ethical permission was obtained from the Ethical Committee of Semmelweis University (TUKEB 5/2011 and IKEB 2017/2011). Tissue samples were fixed with 4% neutral buffered formalin at the start of histological processing for 24 hours. After embedding tissue samples in paraffin, 3-4 μ m thick slices were made. After blocking, SCAI antibody was used as primary antibody followed by Supersensitive Rabbit Link (BG-HK336-9R, Supersensitive Link, Biogenex, Fremont, Ca, USA) and alkaline phosphatase conjugated streptavidin (Biogenex, Fremont, Ca, USA). Development was performed using Dako Liquid Permanent Red (K064011, Dako, Glostrup, Denmark). Nuclei were stained with a Mayer hematoxylin solution (Sigma Aldrich, St. Louis, MO, USA).

Histological sections from the kidney were examined by a Leica DMR HC (Leica Microsystems, Wetzlar, Germany) light microscope. Intestinal histograms from the colon were scanned using a Panoramic P250 scan scanner with 40x lens and Hitachi camera (3DHitech Kft., Budapest, Hungary), and in the Panoramic Viewer program, the NuclearQuant module evaluated the cellular signal intensity after manual selection of tumor-free mucosa and tumor area. A modified Histo-score (H-score, Hirsch score) was calculated on a 4-point scale (0: no signal, 1: weak signal, 2: medium signal, 3: strong signal), resulting in H-scores ranging between 0 and 300.

3.6 qRT-PCR measurements

For the quantitative RT-PCR (RT-qPCR) measurements, total RNA from mIMCD-3 cells was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the

manufacturer's instructions. For studies using TGF- β 1 transgenic mice 100 mg kidney tissue was homogenised, and total RNA was isolated using SV Total RNA Kit (Promega, Madison, WI, USA). Subsequently, in each experiment, a total of 2 μ g of RNA was subjected to reverse transcription with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) using random primers. The average value is expressed as $2^{-\Delta\Delta C_t}$.

Three parallel measurements were performed per experiment and the measurements were repeated two times.

3.7 Gene microarray data analysis

Gene expression data were downloaded from the National Cancer for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database.

The normalized expression values of SCAI mRNA of the sample series were compared.

3.8 Statistical analysis

In the case of promoter experiments, Student t-test and 1-way ANOVA were used for statistical analyzes.

When analyzing the data of breast and colon expression databases Kruskal-Wallis and Dunn's multiple comparison tests were used.

In the TGF- β 1 transgenic mouse model experiments, Mann-Whitney U-test was used.

4 Results

4.1 Role of SCAI during EMT

4.1.1 Expression of SCAI protein in LLC-PK₁/AT₁ cells

In LLC-PK₁/AT₁ cells endogenous SCAI and the GFP-tagged SCAI were primarily observed in the nuclei, cytoplasmic expression was not significant.

4.1.2 SCAI inhibits TGF- β 1-induced α -SMA promoter activity

The cotransfection of SCAI inhibited TGF- β 1 induced α -SMA promoter activity (relative promoter activity: 4.96 ± 0.33 vs 2.31 ± 0.13 , $p < 0.05$).

4.1.3 SCAI protein inhibits cell-cell contact disassembly-induced α -SMA promoter activity

In LLC-PK₁/AT₁ cells, GFP-SCAI overexpression reduced the α -SMA promoter activity increase induced by cell-cell contact disruption by about 25% (relative promoter activity: 8.57 ± 0.31 vs. 6.18 ± 0.35), suggesting that, in addition to the partial inhibitory effect of SCAI, other signal pathways not affected by SCAI may play a role in intracellular signaling pathways triggered by calcium withdrawal. The presence of GFP-SCAI Δ nt protein did not interfere with α -SMA promoter activity (relative promoter activity: 8.57 ± 0.31 vs. 10.02 ± 0.41)

4.1.4 SCAI inhibits TGF- β 1 induced α -SMA protein expression in LLC-PK₁/AT₁ cells

TGF- β 1 treatment lead to α -SMA expression in approximately 20-22% of LLC-PK₁/AT₁ cells. In contrast, only ~2% of GFP-SCAI expressing cells displayed α -SMA expression upon TGF- β 1 treatment.

4.1.5 TGF- β 1 treatment reduces SCAI mRNA content of mIMCD-3 cells

GAPDH-normalized SCAI mRNA expression significantly decreased in mIMCD-3 mouse kidney medullary collecting duct cells following 12 hours of TGF- β 1 treatment (0.92 ± 0.05 vs 0.58 ± 0.02 $p < 0.01$).

4.1.6 SCAI mRNA expression decreased in kidneys of TGF- β 1 transgenic mice

In the kidney of 14 days old TGF- β 1 transgenic mice, the amount of SCAI mRNA was significantly reduced compared to normal 14 days old mouse kidney tissue.

4.2 Characterization of SCAI expression patterns in healthy tissue and malignant tumors

4.2.1 SCAI protein can be detected in healthy human kidney tissue of different ages, and is reduced in fibrotic kidney

In healthy kidney tissue, SCAI protein was detected in the fetal kidney during the 20th week of pregnancy in the nuclei of glomerular and tubular epithelial cells. In a kidney tissue obtained from a young child, intensive nuclear SCAI can also be detected in glomerular cells, in mesangial cells and podocytes. Primary tubular epithelial cells in the adult kidney have nuclear SCAI marking. In the blood vessels significant expression was detected in endothelial cells, while smooth muscle cells were less stained for SCAI. In contrast, SCAI expression is significantly reduced in the remaining tubular cells of fibrotic kidneys.

4.2.2 SCAI protein can be detected in certain malignant tumors of the kidney

In Fuhrman grade III differentiated clear cell kidney tumor cells no SCAI expression was detected. Much to our surprise, blastema dominant Wilms tumors showed significant SCAI marking (high risk Wilms tumor).

4.2.3 SCAI mRNA expression decreased in breast malignant tumors, but is increased in colon tumors

Based on data obtained from gene expression databases, we found that SCAI expression decreases in breast malignant tumors, but increases in colon cancer malignancies. In colorectal cancer, SCAI expression is not affected by tumor stage or metastatic status. In our immunohistochemical studies we have shown that the nuclear expression of SCAI in the healthy mucous membrane is weak, and the H-score was between 0 and 4.7. In the colorectal malignant tumors the expression of the SCAI protein was more pronounced with H-scores between 4.64 and 67.61.

4.3 AngII dependent regulation of PAI-1 in the context of ECM homeostasis

AngIV treatment did not modify PAI-1 promoter activity. AngII, on the other hand, dose dependently increased PAI-1 promoter activity at different concentrations (10^{-10} M/L - 10^{-6} M/L), an effect inhibited in the presence of a specific inhibitor (candesartan) of AT₁R.

Further, we investigated the potential role of certain signaling molecules during AngII-induced PAI-1 promoter activation. In several experimental steps, we found that Smad7, ERK and JNK signaling molecules did not participate in the activation of PAI-1 promoter. At the same time, genistein, a wide spectrum tyrosine kinase inhibitor, significantly inhibited AngII-induced PAI-1 promoter activity (3.82 ± 0.55 vs 1.55 ± 0.52 , $p < 0.05$) but the specific PKC inhibitor (bisindolyl maleimide) was ineffective.

5 Conclusions

Based on the experiments presented above we concluded the following:

1. Concerning the role of SCAI during EMT, the following conclusions have been made in various animal and human cell types and tissues:

a. In LLC-PK₁/AT₁ cells endogenous SCAI and the GFP-tagged SCAI were primarily observed in the nuclei, cytoplasmic expression was not significant.

b. SCAI inhibited TGF- β 1-induced α -SMA promoter activity in LLC-PK₁/AT₁ cells. Consistently, the presence of N-terminally truncated SCAI did not have an inhibitory effect on TGF- β 1-induced α -SMA promoter activity.

c. SCAI partially inhibited cell-cell contact disruption-induced α -SMA promoter activity.

d. SCAI expression significantly inhibited TGF- β 1-induced α -SMA protein expression in LLC-PK₁/AT₁ cells.

e. TGF- β 1 treatment resulted in a significant decrease in SCAI mRNA levels in mIMCD-3 mouse kidney medullary collecting duct cells.

f. In the TGF- β 1 transgenic mouse model, excessive production of TGF- β 1 resulted a significant reduction of SCAI mRNA levels in the kidney when compared to normal murine kidney tissue.

g. SCAI protein was detected in the fetal kidney in the nuclei of glomerular and tubular epithelial cells. At younger age intensive nuclear SCAI is also detected in the glomeruli in mesangial cells and podocytes. In the adult kidney, tubular epithelial cells and endothelial cells express nuclear SCAI, whereas smooth muscle cells of the vessels are negative. In fibrotic kidneys interstitial SCAI was not detectable, while the exhibited tubular cells showed reduced nuclear SCAI staining. SCAI staining was not detected in sclerotic glomeruli.

2. The following conclusions can be drawn from the study of SCAI expression patterns in tumors:

a. There was no evidence of SCAI expression in adult clear cell kidney tumor cells, but childhood Wilms tumor cells are high expressors of SCAI.

- b. As evidenced by data obtained from gene expression databases, SCAI mRNA levels significantly decreased in ductal in situ and invasive breast cancer.
- c. Database gene expression data evidenced that the amount of SCAI mRNA in T1-T4 stage malignant colon tissues was significantly elevated compared to normal colon tissue.
- d. As evidenced by data obtained from gene expression database, SCAI mRNA expression levels did not significantly differ between the primary colon cancer and their corresponding metastatic tissue.
- e. In our immunohistochemical studies SCAI protein was detectable in colon cancer malignant cells, primarily in the nucleus, and colon cancer cells expressed higher amounts of SCAI protein compared to tumor-free colon tissue.

3. The role of the PAI-1 protein in ECM homeostasis is as follows:

- a. In LLC-PK₁/AT₁ cells AngIV did not activate the PAI-1 promoter.
- b. In LLC-PK₁/AT₁ cells, PAI-1 promoter activity was increased in a dose-dependent manner by AngII and this promoter-activating effect was achieved through AT₁R.
- c. The overexpression of Smad7 protein did not interfere with AngII-induced PAI-1 promoter activity in LLC-PK₁/AT₁ cells, therefore the effect of AngII is not Smad signaling dependent, and there is no evidence of AngII-TGF- β 1 crosstalk in this signaling pathway.
- d. Inhibition of MEK and JNK in LLC-PK₁/AT₁ cells had no effect on the AngII-induced activation of PAI-1 promoter.
- e. In LLC-PK₁/AT₁ cells, AngII-induced PAI-1 promoter activity is tyrosine kinase dependent, but PKC is not involved in this process.

6 Bibliography of the candidate's publications

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