MCPIP1 downregulation in clear cell renal cell carcinoma promotes vascularization and metastatic progression

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Abstract

Clear cell renal cell carcinoma (ccRCC) is the most common type of kidney cancer and it forms highly vascularized tumors. The monocyte endoribonuclease MCPIP1 negatively regulates inflammation by degrading mRNA encoding proinflammatory cytokines, such as IL-6, IL-1, and IL-12. MCPIP1 is also a negative regulator of NFκB and AP1 activity and it influences a broad range of miRNA activities. Here we report that MCPIP1 protein levels are decreased during renal cancer progression. In patient-derived tumors, and xenografts established in NOD-SCID or nude mice, low MCPIP1 levels correlated strongly with increased proliferation, tumor outgrowth and vascularity. MCPIP1 activity regulated secretion of VEGF, IL-8, and CXCL12 leading to chemotaxis of microvascular endothelial cells, phosphorylation of VE-cadherin and increased vascular permeability. Mechanistic investigations showed that MCPIP1 regulated ccRCC cell motility, lung metastasis and mesenchymal phenotype by regulating key elements in the EMT signaling axis. Overall, our results illuminate how MCPIP1 serves as a key nodal point in coordinating tumor growth, angiogenesis and metastatic spread in ccRCC.
Introduction

Over the last 20 years, the incidence of renal cancer has increased by 30%, and more than 270,000 of new cases are diagnosed worldwide each year (1). The highest incidences of renal cell carcinoma (RCC) tend to occur in Western Countries (2). Clear cell RCC (ccRCC) is the most common type among renal carcinomas, constituting 80% of all cases (3). Over 30% of patients present with metastasis at the time of diagnosis (4). Despite the use of targeted therapy, the survival time of patients with metastatic ccRCC ranges from three months to five years (4).

Tumor vascularization is a critical step during ccRCC development because it promotes local tumor progression and metastatic spread (5). The tumor production of proangiogenic proteins such as vascular endothelial growth factor (VEGF) promotes the recruitment of endothelial precursor cells from the bone marrow to form new circulatory networks throughout the tumor (6, 7). Angiogenic processes can also be induced by the downregulation of antiangiogenic agents, activation of oncogenes, inactivation of tumor suppressor genes and hypoxia (8). One of the major inducers of angiogenesis during tumor progression is inflammation (9). It has been shown that proinflammatory cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6) or monocyte chemotactic protein 1 (MCP-1) are required for angiogenesis and tumor growth, invasion and metastasis in animal models. Moreover, these factors promote the expression of certain angiogenic factors, e.g., VEGF, whereby they indirectly stimulate the process of tumor vasculature (9, 10).

Monocyte chemotactic protein 1-induced protein 1 (MCPIP1), also known as Regnase-1 encoded by the ZC3H12A gene, is a newly discovered zinc finger protein that is upregulated in macrophages during inflammation in response to certain molecules, such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), and lipopolysaccharide (LPS) (11, 12, 13). MCPIP1 acts mainly as an endonuclease that degrades the mRNA of proinflammatory cytokines, such as
IL-6, IL-1β, IL-12 and IL-2 (13, 14, 15). Additionally, MCPIP1 is a negative regulator of NFκB activity (11, 16, 17) and may suppress miRNA biosynthesis (18). Recently, it has been shown that MCPIP1 is involved in the regulation of viability and proliferation in numerous cancer cell lines (19, 20, 21). Moreover, MCPIP1 overexpression in breast cancer cells induces apoptosis in vitro and reduces tumor growth and metastatic disease in vivo (22). Interestingly, some studies have suggested that MCPIP1 may promote endothelial cell angiogenesis by increasing the secretion of angiogenic factors, such as VEGF and hypoxia-inducible factor 1-alpha (HIF1α) (23, 24). Our recent findings have suggested that MCPIP1 may decrease the level of HIFs in tumor cells, and we showed that MCPIP1 is downregulated in ccRCC samples compared to normal tissue (25). In this study, we demonstrate that decreasing the MCPIP1 protein level correlates with renal cancer progression and better tumor vascularity. We found that the lack of RNase activity of MCPIP1 is responsible for the secretion of proangiogenic factors, the acquisition of the mesenchymal phenotype and the metastatic spread of ccRCC cells. Our findings suggest that ccRCC growth, vascularization and metastasis depend on the level of MCPIP1 and its endonuclease activity.
Materials and Methods

Patient tissue samples

Renal tumor tissue samples were obtained from patients surgically treated for renal cancer in the Centre of Oncology, Maria Sklodowska-Curie Memorial Institute, Cracow Branch, Poland. The study was approved by Institutional Review Board and informed consent was obtained from each patient. All human tissue was collected using protocols approved by the Local Ethics Committee (approval no. 68/KBL/OIL/2011). All samples were subjected to histological evaluation by pathologists, diagnosed according to World Health Organization classification and divided into four groups (I-IV) according to the histologic grading Fuhrman system. In each case, the sample was divided and frozen in liquid nitrogen and stored at -80°C for protein isolation or incubated overnight in RNA-later (Invitrogen) at 4°C and stored in -80°C for RNA isolation. The analysis of the MCPIP1 protein level included 60 samples: 15 samples from each group (I-IV). The microarray analysis included 16 samples.

Microarray assay

Experiment was performed using Affymetrix HuGene ST 2.1 microarrays on 16 RNA samples isolated from ccRCC patient tissues from grades I-IV (8 samples for I+II and 8 samples for III+IV). Total RNA was extracted using Eurx Universal RNA Purification Kit (EURx) according to manufacturer protocol. The quantity of ribosomal RNA and DNA contamination was examined using electrophoresis in 1% denaturing formaldehyde gel. Concentration of total RNA was assessed using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). 100 ng of total RNA were used to synthesis and amplification of ss-cDNA, then samples were fragmented and labeled with biotin. Each step was performed according to Affymetrix GeneChip WT PLUS Reagent Kit Manual. Following fragmentation, 10 µg of cRNA were hybridized for 16 h at 48°C on Affymetrix HuGene2.1 ST Array Strp. Arrays were washed and stained in the Affymetrix Gene Atlas Fluidics Station. Affymetric
HuGene2.1 ST Array Strips were scanned using the GeneAtlas Imaging Station. The data were normalized with Expression Console Software 1.4.1 with RMA algorithm and analyze using Affymetrix Transcriptom Analysis Console (TAC) Software 3.1 with ANOVA one-way between subjects (unpaired). We have followed the Minimum Information About a Microarray Gene Experiment (MIAME) guidelines and deposited raw and processed data in the Gene Expression Omnibus (GEO) repository with the accession number: GSE89563.

Cell culture

Human clear cell renal cell carcinoma cell line of primary tumor (Caki-2) was obtained from Sigma in 2015. Caki-1 cells were obtained from American Type Culture Collection (ATCC) in 2011. HEK-293 cell line was obtained from ATCC in 2010. Human Microvascular Endothelial Cells (HMEC-1) were obtained in July 2016 from ATCC and Human Umbilical Vein Endothelial Cells (HUVEC) were kindly provided by the Department of Transplantology, Medical College, Krakow, Poland in 2016. The initial vials were expanded and cryopreserved in the laboratory and propagated no longer than fifteen consecutive passages. HUVEC cells were used at passage second or third. All cell lines were routinely validated to be mycoplasma free by PCR every three months.

Caki-2 cells were cultured in McCoy 5A medium (Lonza) supplemented with 10% fetal bovine serum (FBS) (BioWest). Caki-1 was cultured in Eagle's minimal essential medium (EMEM) (Lonza) with 10% FBS. HEK-293 was cultured in DMEM high glucose medium (Lonza) supplemented with 10% FBS. HMEC-1 cell line was cultured in MCDB 131 medium (Lonza) supplemented with 10% FBS, L-Glutamine (2mM), EGF 10 ng/ml, hydrocortizone 1 μg/ml and HUVEC was maintained in EBM-2 medium with supplements for HUVEC (Clonetics). All cell lines were cultured at 37°C in a 5% CO₂ atmosphere. To collect conditioned media from ccRCC cell lines, cells were cultured for 48 h in normoxic (21 %
oxygen) or hypoxic (1 % oxygen) conditions in media supplemented with 0.5 % bovine serum albumin (BSA) (BioShop).

Authentication

All cell lines were authenticated by GenMed, using STR DNA profiling methods within loci of D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA and AMEL. Caki-1, Caki-2 and HUVEC were analyzed in August 2016 and HEK293 cell line was analyzed in April 2017.

Raw and processed data from microarray analysis are deposited in the Gene Expression Omnibus repository: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89563 (accession number: GSE89563).

Stable transduction with viral vectors

To examine the effect of MCPIP1 in ccRCC three different viral vectors were used. To stably inhibit MCPIP1, lentiviral vectors with control (shCtrl) were used (Sigma). Two different shRNAs were tested (sh4 and sh5) and most efficient was chosen for further experiments. For stable overexpression of MCPIP1 doxycycline-dependent TetON system was used (pLIX MCPIP1, pLIX PURO and mutant form pLIX D141N) or retroviral vectors with control (pMX MCPIP1, pMX PURO). Briefly, ccRCC cells were plated at 50 % of confluency. Viral vectors were added at multiplicity of infection (MOI) of 50 with 6 μg/ml polibrene (Millipore). To increase transduction efficiency after 24 h the process was repeated. Cells were incubated with viruses for 24 h, then medium was changed. After additional 24 h, 1 μg/ml of puromycin (InvivoGen) was added to start selection. To induce MCPIP1 overexpression in TetON system, 1 μg/ml of doxycycline (BioShop) was added.

Animal studies

The experiments have been conducted in accordance with an Institutional Animal Care and Use Committee: the Local Ethics Committee of the Collegium Medicum of the Jagiellonian University in Krakow, Poland (approval no 117/2014) and II Local Ethics Committee of
Institute of Pharmacology Polish Academy of Sciences (approval no 20/2017 and 21/2017).
Mice were handled according to the regulations of national and local animal welfare bodies.

Foxn1<sup>nu</sup>/Foxn1<sup>nu</sup> (Envigo or Charles River Laboratory) and NOD-SCID (Charles River Laboratory) 6-weeks old female mice were kept under SPF conditions, with water and food ad libitum. Caki-1 and Caki-2 cells with stable overexpression, mutation (pMX PURO, pMX MCPIP1, pLIX PURO, pLIX D141N, pLIX MCPIP1) or downregulation (shCtrl, shMCPIP1, shCtrl-GFP, shMCPIP1-GFP) of MCPIP1, were injected subcutaneously as a cell suspension (2.5x10<sup>6</sup> cells in PBS-Caki-1, 5x10<sup>6</sup> cells in PBS-Caki-2). Tumor growth was monitored for 6 (shCtrl, shMCPIP1, pLIX PURO, pLIX D141N, pLIX MCPIP1) or 8 (pMX PURO, pMX MCPIP1) weeks. Tumor volume was estimated using caliper measurements, according to the formula: volume=width x depth<sup>2</sup> of tumor or 3D ultrasonography. After tumor and lung excision, RNA isolation and histological analysis were performed.

**3D ultrasonography**

Tumor volume and vascularity in Nude mice were measured every week after i.sc. injections using 3D ultrasonography VEVO 2100 with MS550D transducer (VisualSonics). Mice were anesthetized with 2% isoflurane with air flow 0.5 L/min (Baxter). Vasculature measurements were performed using Power Doppler mode. All results were analyzed in 3D mode using VEVO 2100 Software.

**RNA isolation and quantitative RT-PCR**

Total RNA from cultured cells and tissues was isolated using the Universal RNA Purification Kit (EURx). RNA from total mice lungs were isolated using Fenozoal (A&A Biotechnology). The quantity of ribosomal RNA and DNA contamination was examined using electrophoresis in 1% denaturing formaldehyde gel. Concentration of total RNA was assessed using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using 1 µg of total RNA, oligo(dT) 15 primer (Promega) and M-MLV reverse
transcriptase (Promega). Real-time PCR was carried out using SybrGreen Master Mix (A&A Biotechnology) and Eco Real-Time PCR System (Illumina). For the examination of mice lung metastasis, specific probes for human GAPDH and mouse GAPDH (Life Technologies) were used with Taq PCR Master Mix (EURx). Gene expression was normalized to elongation factor-2 (EF2). mRNA level in each sample was analyzed in duplicates. The relative level of transcripts was quantified by the ΔΔCT method. Sequences of primers (Genomed) and annealing temperatures are listed in Table 1 in Supplementary material.

Western Blot

Cultured cells were washed with ice cold PBS (Lonza), harvested and lysed with Mammalian Protein Extraction Reagent (M-PER) (Thermo Scientific) supplemented with proteinase and phosphatase inhibitors, for 10 min. Lysates were spun for 20 min, 11 000 x g at 4°C. SDS-PAGE electrophoresis was conducted in 10% polyacrylamide gel. After wet transfer onto PVDF membrane (Millipore), membranes were blocked in appropriate buffer (5% BSA or 5% non-fat powdered milk in Tris-buffered saline with 0.1% Tween 20 (Sigma)). Next, membranes were incubated with primary antibodies at 4°C overnight, with gentle agitation. On the following day, membranes were washed three times for 10 min with TBS 0.1% Tween 20 and incubated with secondary antibody for 1h at room temperature with gentle rocking. Chemiluminescence was detected after 5 min incubation with Immobilon Western HRP substrate (Millipore) using ChemiDoc system (BioRad). All antibodies and dilutions are listed in Table 2 in Supplementary material.

MTT assay

5 000 cells per well were seeded in 96-well plates. Then 0.5 mg/ml of MTT solution (Sigma) was added for 2 h. Reaction was stopped by adding 5 mM HCl (POCH) in isopropanol (Chempur) and stirred for 30 minutes to dissolve the formazan crystals. Absorbance was measured at 570 nm with the reference wavelength of 500 nm using Tecan Spectra Fluor Plus.
Microplate Reader (Tecan Group Ltd.). Measurements were performed 24, 48, 72 and 96 h after seeding. Three independent experiments were performed in triplicate.

**Cell proliferation**

Cells were cultured on 24-well plates (20,000 cells per well). After 24, 48, 72 and 96 h, cells were trypsinized and counted in Bürker Chamber. Three independent experiments were performed in triplicate. Results are expressed as number of cells per 1 ml.

**Migration assay**

Cells were grown on 24-well plates to full confluence. Right before experiment 10 mM of hydroxyurea (Sigma) was added to media to block cell proliferation. A scratch was generated with a small pipette tip. Then plate was transferred to microscope culture chamber with 37°C, 5% CO₂. Images were acquired every 10 min for 16 h using Leica DMI6000B inverted widefield fluorescence microscope (Leica Microsystems). All images were recorded using 10x dry objective with Leica LAS X image acquisition software and analyzed with Hiro v. 1.0.0.4 software.

**Chemotaxis Assay**

The directional movement of HMEC-1 cells toward conditioned medium was evaluated using modified Boyden’s chamber with 8-μm pore polycarbonate membrane inserts (Transwell; Costar-Corning, Cambridge, MA, USA). Cells were seeded into the upper chamber of an insert at a density of 2 x 10⁴ in 100 μl, in duplicates. The lower chamber was filled with pre-warmed conditioned medium mixed with MCDB131 medium with 0.5 % of BSA (1:1) or with 0.5% BSA DMEM as a negative control. After 24 hours, cells that did not migrate were scraped off with cotton wool from the upper membrane while cells that had transmigrated to the lower side of the membrane were fixed with methanol, stained with Wright solution and counted under inverted microscope with high power field (HPF). At least five fields were counted.
each time and the mean number of cells per HPF was calculated. Three independent experiments in duplicates were performed.

**Branch points assay**

50 μl of growth factor reduced Matrigel (BD Biosciences) was plated in a 96-well plate and incubated at 37°C for 30 min. HMEC-1 cells were detached, counted, and single-cell suspensions at a density of 10 000 cells per well in 200 μl of conditioned media and proper controls were plated on the Matrigel. 4 h after seeding endothelial tube formation was stained with Calcein AM (1 μM). Images were captured using Olympus IX50 inverted microscope (Olympus) with 4x dry objective. Formation of tubule-like structures was analyzed with Angiogenesis Analyzer for ImageJ (Carpentier G., Angiogenesis Analyzer for ImageJ (available online: http://imagej.nih.gov/ij/macros/toolsets/Angiogenesis%20Analyzer.txt).

**Fibrin Beads Assay**

To study angiogenesis fibrin beads test was performed. Shortly, latex beads (Sigma) were incubated with endothelial cells for 6 h (approximately 400 cells per bead) to coat the beads. After 24 h beads were resuspended in 2 mg/ml fibrinogen (Sigma) solution in medium with 0.5% BSA. To generate gel, 0.625 U/ml of thrombin (Sigma) was added, then beads were embedded in 24-well plate. After polymerization, on the top of the gel ccRCC cells were seeded (30 000 cells per well) in medium supplemented with 0.5% BSA. Medium was changed every second day. To evaluate migration and formation tubular structures by endothelial cells, images were taken every day for six days using Nikon Eclipse Ti-S (Nikon) microscope with 10x objective. For migration analysis ImageJ (National Institutes of Health) software was used.

**Enzyme-linked immunosorbent assay (ELISA)**

To evaluate levels of secreted proteins in conditioned media (IL-8, VEGF and IL-6) or in mice plasma (SDF-1) DuoSet ELISA assays were performed (R&D Systems) according to the
manufacturer’s instructions. Absorbance was measured at 450 nm with the reference wavelength of 540 nm using Tecan Spectra Fluor Plus Microplate Reader. Three independent experiments were performed in triplicate.

**Flow cytometry**

Mouse blood were collected at the end of experiment, next erythrocytes were lysed with High-Yield Lyse Fixative-Free Lysing Solution (Life technologies) for 30 min. Lysed blood were diluted 100x in PBS, and 10 ml of each sample was taken for analyze. Measurement was performed using Attune NxT Acousting Focusing Cytometer and analyzed with Attune software v2.4.

**Immunofluorescence staining**

Cells were plated on glass coverslips in 12-well culture plates at density of 100 000 cells per well and grown to full confluence. Then cells were stimulated with conditioned media for 3 h and fixed for 15 min in 4% methanol-free formaldehyde (Thermo Scientific) in PBS at room temperature. Next, slides were permeabilized and blocked in blocking buffer (1% BSA in PBS with 0.3% Triton X-100 (Sigma)) at room temperature for 1 h. Incubation with primary antibody for VE-Cadherin (Abcam) diluted 1:100 in PBS with 1% BSA was performed overnight at 4°C in a humidifying chamber. On the following day secondary antibody AlexaFluor 488 conjugated goat anti-rabbit (1:1000; Thermo Fisher Scientific) was added for 1 h in the dark at room temperature in a humidifying chamber. All samples were mounted with prolong gold antifade reagent with DAPI (Invitrogen) and sealed with nail polish. Images were taken with Leica DMI6000B inverted widefield fluorescence microscope with 100x or 63x oil immersion objective with Leica LAS X image acquisition software.

**Immunohistochemical staining**

Tumor sections were stained with hematoxylin-eosin to visualize tumor morphology. Immunohistochemical evaluation was performed using primary rabbit polyclonal anti-CD31...
antibody (1:50, Abcam) and EnVision Detection Systems Peroxidase/DAB, Rabbit/Mouse (DakoCytomation, Denmark, UK) to visualize tumor vascularization. All images were taken using Nikon Eclipse Ti-S microscope with 10x objective. For analysis ImageJ software was used. Slides for CD31 on frozen sections: tissues were pre-fixed in pure buffered formaldehyde (Chempur), washed in PBS, incubated 12 h in 30% sucrose at 4°C and embedded in OCT (VWR chemicals). 9μm sections were stained immunohistochemically with primary rat anti-mouse CD31 antibody or isotype control (1:150, BD Pharmingen) and subsequently with secondary goat anti-rat AlexaFluor 594 conjugated antibody (1:1000; Thermo Fisher Scientific). Slides were mounted with Dako Fluorescent Mounting Medium (Dako). Images were taken with Leica DMI6000B inverted wiedefield fluorescence microscope with 40x or 63x oil immersion objective with Leica LAS X image acquisition software.

**Statistical analysis**

*In vitro* experiments were conducted in three independent repetitions. The number of animals or patient samples is indicated in the figure legends. All results are shown as the mean±SD. For comparison of two groups, two-tailed unpaired Student’s *t*-test, two-way or one-way ANOVA with post-hoc Bonferroni test were used. The p-values are marked with the asterisks in the charts (* p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001 versus control).

**Ethics statement**

The animal experiments were approved by the Local Ethics Committee of the Collegium Medicum of the Jagiellonian University in Krakow, Poland (approval no 117/2014) and II Local Ethics Committee of Institute of Pharmacology Polish Academy of Sciences (approval no 20/2017 and 21/2017). Biopsies of renal tumors were obtained from patients surgically treated for renal cancer in the Centre of Oncology, Maria Sklodowska-Curie Memorial
Institute, Cracow Branch under the supervision of the Local Ethics Committee (approval no. 68/KBL/OIL/2011).

Results

**MCPIP1 decreases during ccRCC progression, and a low MCPIP1 level induces ccRCC cell proliferation in vitro and tumor growth in vivo.**

To assess MCPIP1 expression during ccRCC progression, we analyzed 60 tumor samples. Western blot analysis revealed that MCPIP1 expression varied depending on the tumor grade, with MCPIP1 expression decreasing significantly with tumor progression (Fig. 1A).

Microarray analysis showed a high level of proangiogenic factors in tumor tissue during ccRCC progression (Fig. 1B, C). We observed a high expression of VEGFA, FLT1, CDH5 or EPAS1 transcripts for all grades (I-IV). The expression was slightly decreased in grades III and IV compared with grades I and II (Fig. 1B, C). We also observed that the transcript levels of HGF, MET, SRC, ZEB2, IL-6 and LIF, which are related to tumor invasiveness and metastasis, were increased during tumor progression (Fig. 1B, C), together with a decrease of MCPIP1 protein (Fig. 1A). Transcript of MCPIP1 (ZC3H12A) level in tumor samples was generally low (Fig. 1B, C).

The observation that the MCPIP1 protein level decreases as the tumor grade increases led us to examine the role of MCPIP1 in ccRCC. In the first step, we downregulated MCPIP1 at the mRNA and protein levels using six different siRNAs together with the proper controls (Suppl. Fig. S1A) and two lentiviral vectors expressing shMCPIP1#4 and #5 sequences (Suppl. Fig. S1B). The shMCPIP1#5 lentiviral vector efficiently downregulated MCPIP1 was selected for further experiments and was called shMCPIP1 (Suppl. Fig. S1C). To determine whether MCPIP1 silencing influences ccRCC viability and proliferation, cells were cultured for 96 h, and MTT and proliferation assays were performed. We found that MCPIP1 depletion in ccRCC cells significantly enhanced tumor cell viability and proliferation in both investigated
cell lines, Caki-1 and Caki-2 (Fig. 1C, D; Suppl. Fig. S1D). The largest difference between the control and MCPIP1-deficient cells was observed after 96 h of culture. To examine the growth of human ccRCC in vivo, we established a xenotransplantation model in NOD-SCID (Caki-1) mice. We observed that the inhibition of MCPIP1 affected both tumor growth and weight (Fig. 1E and F, Suppl. Fig. S2A, B, C). We obtained opposite results after the stable overexpression of MCPIP1 using retroviral vectors (Suppl. Fig. S3A, B), where the tumors formed by Caki-1 pMX MCPIP1 were significantly smaller than those in the controls (Suppl. Fig. S3C). The main function of MCPIP1 is the negative regulation of inflammation by acting as an endoribonuclease. In the next step, we checked whether the RNase activity of MCPIP1 is important for tumor growth in vivo (Fig. 1G, H). We expanded our in vivo model with cells overexpressing MCPIP1 (pLIX MCPIP1) and cells carrying a point mutation of D141N that completely abolishes MCPIP1 RNase activity (13) (pLIX D141) (Suppl. Fig. S3A, B). Our findings show that for both investigated xenograft models, Caki-1 and Caki-2 cells overexpressing MCPIP1 formed the smallest, slowly growing tumors, whereas control cells and cells lacking RNase activity produced similar tumors (Fig. 1G, H).

**MCPIP1 regulates tumor vascularization in vivo through its endonuclease activity.**

Staining for CD31 (platelet endothelial cell adhesion molecule, PECAM-1), a vascular marker, showed that more advanced grading (III and IV) was correlated with significantly increased lumen areas of blood vessels compared with those seen in grade I and II tumors (Fig. 2A).

To study the role of MCPIP1 in tumor vascularity development, we analyzed tumors that were formed by cells with MCPIP1 downregulation. CD31 and hematoxylin-eosin staining of tumor sections showed an increase in the number of functional blood vessels with larger lumen areas (Fig. 2B, Suppl. Fig. S4A). Furthermore, the analysis of tumor by 3D
ultrasonography revealed increased vascularization of tumors generated by ccRCC cells with MCPIP1 depletion (Fig. 2C). We also observed more CD31-positive cells during tumor development in tumors formed by MCPIP1-deficient cells (Fig. 2D).

As MCPIP1 acts as an endonuclease, we analyzed the vascularity of tumors generated by MCPIP1 overexpressing and lacking RNase activity (D141N) cells. We observed increased vascularization of tumors formed by ccRCC cells carrying a D141 mutation (Fig. 2E, F). Further analysis of CD31 staining revealed that the lack of RNase activity of MCPIP1 led to an increase in the number of blood vessels with larger diameters in Caki-1-derived tumors (Fig. 2E). Similar results were obtained in Caki-2 tumors using 3D ultrasonography (Fig. 2F).

Low MCPIP1 levels in ccRCC cells promote endothelial cell migration and tubule formation and destabilize endothelial junctions.

In response to stimulation with conditioned media isolated from ccRCC cell lines, we first observed an increased chemotactic response of microvascular endothelial cells migrating toward the media isolated from shMCPIP1 Caki-1 and Caki-2 cells compared with that of cells that were chemoattracted by the media isolated from control Caki cells (Fig. 3A). Moreover, HMEC-1 cells suspended in conditioned media isolated from ccRCC cells with MCPIP1 downregulation formed higher numbers of branch points in the angiogenesis assay (Fig. 3B). To show the direct influence of MCPIP1 silencing in ccRCC cells on the activity of endothelial cells, we performed a co-culture of HMEC-1 cells growing on beads in fibrin gel with ccRCC cells growing on top. We observed that endothelial cells under the influence of tumor cells lacking MCPIP1 showed an increased number of tubular structures (Fig. 3C, left panel) and higher migration rates of endothelial cells (Fig. 3C, right panel).

Vascular permeability and angiogenesis are regulated by VE-cadherin, a vascular endothelial adhesion molecule, specifically and exclusively expressed by endothelial cells and important
in the maintenance of vascular integrity (26). Tyrosine phosphorylation of VE-cadherin is associated with weak junctions and impaired barrier function (27). To determine whether the silencing of MCPIP1 in ccRCC cells influences the level, localization and phosphorylation of VE-cadherin in endothelial cells, we stimulated two types of endothelial cells, HUVECs (Fig. 3D) and HMEC-1s (Suppl. Fig. S4B), with conditioned media isolated from ccRCC cells. Western blot analysis showed that endothelial cells exhibited VE-cadherin phosphorylation in response to the media (Fig. 3D, Suppl. Fig. S4B). The phosphorylation level was very strong after stimulation with media from Caki-1 shMCPIP1 cells (Fig. 3D, Suppl. Fig. S4B). We observed a similar pattern after stimulation with media isolated from Caki-2 cells with MCPIP1 downregulation (Fig. 3D, Suppl. Fig. S4B). The phosphorylation of VE-cadherin in endothelial cells after stimulation with medium from ccRCC overexpressing MCPIP1 (pMX MCPIP1) was significantly impaired (Fig. 3D, Suppl. Fig. S4B).

Because VE-cadherin is a component of endothelial cell-to-cell adherens junctions and because loosening cell-cell contacts is the first step in inducing angiogenesis, we evaluated the influence of ccRCC conditioned media on endothelial cell organization, vascular integrity and cellular localization using VE-cadherin immunofluorescent staining (Fig. 3E). We observed that media isolated from ccRCC with silenced MCPIP1 blocked the adhesive function of VE-cadherin, disrupted the monolayer integrity of HUVECs and increased endothelial cell dissociation (Fig. 3E). By contrast, the influence of the media isolated from ccRCC with MCPIP1 overexpression (pMX MCPIP1) and control cells (pMX PURO) was similar and did not change the cell organization effectively or in a visible manner (Fig. 3E).

**Silencing of MCPIP1 in ccRCC cells increases the secretion of proangiogenic factors due to the lack of RNase activity.**
Among angiogenic stimuli, the best characterized are VEGF (6, 7, 26), IL-6 (28) and a member of the CXC chemokine family, IL-8 (29). In the next step of our study, we examined whether a change in the level of MCPIP1 protein in ccRCC cells affects the secretion of IL-8, VEGF and IL-6 (Fig. 4A, B, C). Using real-time RT-PCR, we observed that MCPIP1 downregulation increased the transcript level of IL-8, VEGF and IL-6 both under normoxia and hypoxia conditions (Fig. 4A, B, C). Next, conditioned media from ccRCC cells were evaluated using enzyme-linked immunosorbent assay (ELISA), and the analysis revealed that low MCPIP1 levels significantly promoted the secretion of IL-8, VEGF and IL-6 under hypoxic conditions (Fig. 4A, B, C).

The RNase activity of MCPIP1 was crucial for maintaining the low level of proangiogenic factors in ccRCC cells because a point mutation of D141 significantly increased the level of IL-8, VEGF and IL-6 transcripts (Fig. 4D) and protein (Fig. 4E) under hypoxia conditions compared with the control and MCPIP1-overexpressing cells.

**MCPIP1 regulates the level of SDF-1 due to its RNase activity and influence on c/EBPβ and increases the metastatic ability in vivo.**

It has been previously shown that VEGF, together with stromal cell-derived factor (SDF)-1, synergistically induces neoangiogenesis in tumors (30). Moreover, it has been demonstrated that SDF-1, together with its receptor, CXCR4, promotes VEGF expression (31). We noticed that the downregulation of MCPIP1 in ccRCC cells significantly increased SDF-1 and CXCR4 expression in both investigated cell lines (Fig. 5A). Moreover, we observed a significant increase in SDF-1 expression in tumors isolated from nude mice injected with ccRCC cells with silenced MCPIP1 (Fig. 5B, left panel). Furthermore, analysis of blood serum from mice injected with MCPIP1-deficient cells showed higher levels of secreted human SDF-1 (Fig. 5B, right panel).
Interestingly, we observed decreased SDF-1 expression in ccRCC cells with MCPIP1 overexpression, whereas control and D141N cells had a similar level of SDF-1 (Fig. 5C). We found that the mRNA level of SDF-1 and CXCR4 was significantly decreased after MCPIP1 upregulation in both the Caki-1 and Caki-2 cell lines (Suppl. Fig. S5A, B). MCPIP1 was shown to degrade the transcript of CCAAT/enhancer-binding protein beta (c/EBPβ) (32), a transcription factor regulating the transcription of SDF-1 (33). Our findings show that the downregulation of MCPIP1 led to a significant increase in c/EBPβ and that the RNase activity of MCPIP1 regulates its expression in ccRCC cells (Fig. 5D).

SDF-1, together with its receptor CXCR4, is responsible for the invasive phenotype and metastatic behavior of many tumor types (34). Our studies have shown that silencing MCPIP1 increased the metastatic ability in vivo (Fig. 5E). We observed that nude mice injected i.sc. with Caki-1 shMCPIP1 had considerably more lung metastases than did mice injected with control cells both during tumor development and after 6 weeks of tumor growth (Fig. 5E). Stable upregulation of MCPIP1 resulted in a decrease in the number of tumor cells metastasizing to the lungs (Fig. 5F, Suppl. Fig. S5C). Furthermore, we observed an increase in the number of circulating shMCPIP1-GFP cells in the mouse blood (Fig. 5G, Suppl. Fig. S5D-E). These findings were in concordance with lung metastasis (Fig. 5E).

**MCPIP1 downregulation affects the metastatic progression of ccRCC cells according to increased motile activity and an enhanced mesenchymal phenotype.**

The process of metastasis requires the acquisition of the ability to perform active migration, and we noticed that motile activity was strongly increased in ccRCC shMCPIP1 cells compared with that in control cells (Fig. 6A). Analysis of the individual tracks of both cell types revealed that the motile activity of MCPIP1-deficient cells was higher than that of
control cells (Fig. 6B). A 16-h time-lapse recording revealed that parameters for distance and speed were significantly higher for shMCPIP1 cells (Fig. 6C).

Because MCPIP1 downregulation in ccRCC led to increased migration, we assumed that MCPIP1 silencing at least partially modified the cell phenotype into a more aggressive form. To expand on these observations, we evaluated potential changes in the level of various epithelial to mesenchymal transition (EMT) markers and the c-Met receptor known to regulate cell motility (35). Western blot analysis confirmed by densitometry revealed that MCPIP1 silencing suppressed E-cadherin and upregulated β-catenin and vimentin. Moreover, c-Met and Src protein levels were increased. We also observed the increased phosphorylation of c-Met receptors in shMCPIP1 cells (Fig. 6D, Suppl. Fig. S6). Interestingly, similar results were obtained in the normal human renal cell line HEK293 after the downregulation of MCPIP1 (Fig. 6D). Further analysis of E-cadherin repressors revealed that MCPIP1 downregulation induced the expression of the transcription factors Snail and ZEB2 (Fig. 6E).

**MCPIP1 overexpression impairs the malignant phenotype of ccRCC cells.**

Subsequent studies have shown that MCPIP1 overexpression in ccRCC cells caused a significant decrease in motile activity (Fig. 7A, B, C). Activation of MCPIP1 expression resulted in a decrease in the distance and speed of ccRCC cells (Fig. 7A, B). Moreover, when we evaluated the levels of EMT markers, we observed a strong increase in E-cadherin and a decrease in vimentin and β-catenin. Furthermore, the levels of the activated and total form of the c-Met receptor, as well as those of Src kinase, were significantly reduced (Fig. 7D). The analysis of the influence of MCPIP1 overexpression and activation on Snail and ZEB2 transcription factors revealed that a high level of MCPIP1 significantly reduced the expression of both factors (Fig. 7E). The lack of RNase activity in pLIX D141N cells did not
change the expression of both transcription factors compared with that in control cells (Fig. 7E), indicating the direct influence of the MCPIP1 protein.

Discussion

In this study, we revealed for the first time that MCPIP1 plays an important role in the regulation of angiogenesis and metastasis of ccRCC. We present evidence that the abolished RNase activity of MCPIP1 is responsible for the secretion of proangiogenic factors and, consequently, the better vascularization of emerging tumors. Furthermore, we show that a low MCPIP1 level is crucial for the acquisition of the mesenchymal phenotype and that the downregulation of MCPIP1 is associated with the progression of ccRCC.

MCPIP1 has been shown to suppress chronic inflammation by promoting the mRNA decay of proinflammatory cytokines (13, 14), as well as regulating NFκB and AP1 activity (11, 16, 17). There is growing evidence that MCPIP1 plays a role during the process of tumorigenesis. It has been already demonstrated that MCPIP1 regulates the viability and proliferation of neuroblastoma (20), HeLa, HepG2 (19), ccRCC (25) breast cancer cells (21). Previous findings have demonstrated that MCPIP1 degrades the mRNA of antiapoptotic gene transcripts in breast cancer cells, leading to apoptosis and tumor regression (22). Moreover, the same study showed that MCPIP1 expression in breast cancer samples was reduced compared with that in healthy tissue, and low MCPIP1 expression was associated with tumor progression and poor survival (22). Our group has already demonstrated that ccRCC tumors have a lower level of MCPIP1 than normal renal tissues, and MCPIP1 overexpression decreases viability and proliferation of ccRCC cells (25). These findings led us to evaluate the level of MCPIP1 during tumor progression and the influence of MCPIP1 downregulation on ccRCC.
The present study showed that the MCPIP1 protein level was decreased together with the increased grading of the tumor; however, the MCPIP1 (ZC3H12A) transcript level is generally low in tumor patient samples, regardless of the tumor grade. In our models, low MCPIP1 levels significantly increased the cell proliferation, tumor growth and tumor weight of ccRCC. By contrast, MCPIP1 overexpression decreases the proliferation \textit{in vitro} and reduced tumor growth \textit{in vivo} as it was demonstrated for breast cancer (22). Interestingly, the analysis of tumors generated by cells carrying a point mutation of D141 (D141N) that completely abolishes MCPIP1 RNase activity (13) demonstrated that the loss of endoribonuclease activity of MCPIP1 significantly increased tumor growth in both investigated models. This observation suggests that, at least in this model, the RNase activity of MCPIP1 is crucial in the process of tumor growth.

ccRCC is a highly vascularized cancer, and dysregulation of angiogenesis is implicated in its development. It has been already presented that high-grade ccRCC had a higher endothelial cell proliferation fraction, larger vessel area and higher VEGF protein expression (36). Consistent with these findings, the analysis of ccRCC patient-derived tumors revealed that low- and high-grade tumors differ in their blood vessel structures. Grade III and IV tumors were characterized by an increased area of vessels expressing CD31 antigen.

In our study, for the first time, we found that MCPIP1 downregulation in ccRCC led to an increased tumor vasculature. Moreover, our findings demonstrated that tumors formed by ccRCC lacking RNase activity have better vascularization than control and MCPIP1-overexpressing tumors. This observation proves that MCPIP1 might regulate the process of ccRCC vascularization via its RNase activity.

The blood vessel formation process depends on endothelial cells forming blood vessels, tumor cells and secreted proangiogenic factors. One of the initial responses of endothelial cells to induce angiogenesis is endothelial cell migration to form capillary tube networks (7).
It has been shown that MCPIP1 may promote angiogenesis by increasing the secretion of angiogenic factors, such as VEGF and HIF1α, in human umbilical vein endothelial cells (23, 24). It has also been demonstrated that MCPIP1 enhances the angiogenic potential of murine mesenchymal stem cells (37). The authors showed that MCPIP1 overexpressing MSCs expressed increased levels of proteins involved in angiogenesis and induction of differentiation (37).

In the present study, we provide evidence that MCPIP1 downregulation in ccRCC cells induces the secretion of proangiogenic factors by tumor cells, including VEGF, a key regulator of physiological angiogenesis (6), IL-8, an inducer of endothelial cell proliferation, survival and capillary tube organization (38) and IL-6, a major tumor-promoting cytokine and stimulator of angiogenesis (28). In addition, our results show that low MCPIP1 levels in ccRCC cells influenced the biology of endothelial cells by promoting its migration, capillary network formation and phosphorylation of VE-cadherin, which was required for the control of vascular integrity (27). Our study demonstrated that the secretion of proangiogenic factors is controlled by the RNase activity of MCPIP1, and MCPIP1 overexpression decreases the level of proangiogenic factors. It is in concordance with previous findings showing that MCPIP1 regulates the level of IL-6 and IL-8 transcripts by binding to stem-loop structures present in 3’UTRs (13, 39). VEGF expression is induced by HIF (Hypoxia Inducible Factors), and we have previously demonstrated that MCPIP1 overexpression reduces the level of HIFs and transcripts for VEGF (25). However, it was suggested that HIF1-independent mechanisms regulating VEGF expression may also exist. IL-8 can rescue the proangiogenic phenotype in HIF1α-deficient cancer cells (40).

It has been reported that the overexpression of VEGF stimulates angiogenesis by upregulating SDF-1 and, thereby, recruits CXCR4-positive proangiogenic myeloid cells (41). SDF-1 is a chemotactic chemokine that promotes the recruitment of endothelial progenitor cells from the
bone marrow (42). It has also been documented that SDF-1 together with its receptor, CXCR4, promotes VEGF expression (43) and that VEGF together with SDF-1 synergistically induces neoangiogenesis in tumors (30). Our results revealed that MCPIP1 silencing promotes SDF-1 expression in vitro and in vivo and RNase activity of MCPIP1 is responsible for the level of SDF-1 transcript (Fig. 5). Moreover, MCPIP1 degrades CCAAT/enhancer-binding protein beta (c/EBPβ) (32), a transcription factor regulating the transcription of SDF-1 (33). These observations not only demonstrate the impact of MCPIP1 on tumor development, such as promoting angiogenesis but also highlight the role of MCPIP1 downregulation in potentiating SDF-1-CXCR4 signaling. The CXCR4/SDF-1 axis can coordinate the metastasis of various of cancers (34), and because of the increased expression of CXCR4 in MCPIP1-deficient cells, we examined the influence of MCPIP1 downregulation on the metastatic potential of ccRCC.

We observed that the silencing of MCPI1 increased the number of circulating shMCPI1 cells in mouse blood and increased lung metastases, whereas the enhanced MCPI1 expression in ccRCC cells reduced tumor growth and metastasis in immunodeficient mice (Fig. 1, Fig. 5, Suppl. Fig. S3). Our observation revealed a significantly increased motile activity of ccRCC cells, decreased E-cadherin and increased level of vimentin and β-catenin. The reduction of E-cadherin correlated with the expression of the Snail and ZEB-2 transcription factors.

Snail and ZEB2 were shown to repress E-cadherin expression and promote invasive properties (44, 45). Overexpression of MCPI1 decreased the migratory potential and increased E-cadherin levels together with decreased vimentin, β-catenin, Snail and ZEB-2 expression (Fig. 7), indicating the role of MCPI1 in acquiring the mesenchymal phenotype of ccRCC cells. It was previously shown that IL-1, IL-6 and IL-8 induce tumor cell migration, invasion and epithelial-mesenchymal transition and MCPI1 regulating half time of IL-6 and
IL-1, IL-8 transcripts, controlling the level of transcription factors (NFκB, C/EBPβ) and signaling proteins (JNK, Akt) may influence on the appearance of EMT signs (13, 16, 17, 39). We previously found that E-cadherin expression and the invasive properties of cervical carcinoma depend on the c-Met receptor (46). In this study, we observed an increased level of c-Met and strong phosphorylation of the receptor together with the elevated level of kinase Src after MCPIP1 downregulation (Fig. 6). MCPIP1 overexpression reduced the level of c-Met, its endogenous phosphorylation and decreased the level of kinase Src in ccRCC (Fig. 7). It has been previously shown that the c-Met gene is a direct target of NFκB, and MET participates in NFκB-mediated cell survival (47). We suspect that the MCPIP1 regulation of NFκB can indirectly influence the level and function of the c-Met receptor.

In conclusion, we provide evidence that the endoribonuclease activity of MCPIP1 regulates tumor angiogenesis and the metastatic cascade and may contribute to ccRCC progression.

Our data highlight the importance of MCPIP1 as a novel tumor suppressor and enable us to postulate that potential MCPIP1 inducers can be used therapeutically for ccRCC in the future.

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References


Figure Legends

Figure 1. Influence of MCPIP1 on tumor progression, cell viability and proliferation.

(A) Analysis of MCPIP1 levels in tumor tissue specimens from patients. Representative western blot of 12 samples with GAPDH as the loading control (left panel). Quantification of the protein level of 60 tumor samples, divided into four groups according to tumor grade (I-IV) (right panel). Red samples were taken for further microarray analysis. (B) Hierarchical clustering of 11 genes that are significantly associated with ccRCC tumor progression and angiogenesis. Each row represents an individual tissue sample. The scale represents gene expression levels from 2.03 to 10.98 in log2 scale. On the left, quantification of the signal from microarray. n (I + II) =8, n (III + IV)= 8. Statistics was performed using One-way ANOVA between subjects (unpaired).

(C, D) Effect of MCPIP1 downregulation on cell viability (MTT assay) and proliferation in Caki-1 (C) and Caki-2 (D) cell lines after 96 h of culture. (E) Effect of MCPIP1 depletion in the Caki-1 cell line on tumor growth in vivo. Left panel, caliper measurements of tumor volume during 6 weeks; right panel, weight of tumors. (F) Representative images of tumor sections after hematoxylin/eosin staining. Tumors were collected 6 weeks after subcutaneous injection of cells. Animal studies involved 30 NOD-SCID mice: Caki-1shCtrl, N=14; Caki-1shMCPIP1, N=16. p-values were estimated using two-tailed unpaired Student’s t-test. (G, H) Effect of MCPIP1 overexpression and mutation in Caki cell lines on tumor growth in vivo. (G) Left panel, caliper measurements of the Caki-1 tumor volume during 6 weeks; right panel, weight of tumors. The results are presented as means±SEM. Animal studies involved 15 NOD-SCID mice: Caki-1pLIXPURO, N=5; Caki-1pLIXD141N, N=5; Caki-1pLIXMCPIP1, N=5. (H) Left panel, caliper measurements of the Caki-2 tumor volume during 6 weeks; right panel, weight of tumors. The results are presented as means±SEM. Animal studies involved 9 nude mice: Caki-2pLIXPURO, N=3; Caki-2 pLIXD141N, N=3; Caki-2pLIXMCPIP1, N=3.
p-values were estimated using two-tailed unpaired Student’s t-test. *p<0.05, **p<0.01 MCPIP1 versus control. #p<0.05, ##p<0.01 MCPIP1 versus D141N

**Figure 2. Influence of MCPIP1 on tumor vascularization.**

(A) CD31 immunohistochemical staining of patient tumor sections. Representative images (left panel) of sections from each grade. Right graph, quantification of functional vessel area with a visible lumen. n (I + II) =3, n (III + IV)= 7. (B) CD31 immunohistochemical staining of tumor sections. Representative images (left panel). Quantification of functional vessels with a visible lumen (middle graph), quantification of lumen area (right graph). Tumors were collected 6 weeks after subcutaneous injection. The study involved 12 NOD-SCID mice: those injected with Caki-1 shCtrl, N=6; those injected with Caki-1shMCPIP1, N=6. (C) Tumor vascularity measured by 3D ultrasonography for 5 weeks (left panel) and USG images of tumors. The red color represents blood vessels (right panel). The fourth measurement was removed due to technical problems. The study involved 15 nude mice: Caki-1shCtrl, N=5; and Caki-1shMCPIP1 N=10. (D) CD31 immunofluorescent staining of OCT tumor sections. Representative images during tumor development 21, 28, 35 and 41 days after i.sc. injections. The study involved 8 NOD-SCID mice: Caki-1shCtrl, N=4; and Caki-1shMCPIP1 N=4. (E) CD31 immunohistochemical staining of tumor sections after overexpression or mutation of MCPIP1. Representative images (left panel). Quantification of lumen area for vessels with a lumen diameter ≥ 200 μm (right graph). Tumors were collected 6 weeks after subcutaneous injection. The study involved 15 NOD-SCID mice: injected with Caki-1pLIX PURO, N=5; Caki-1pLIX D141N, N=5; Caki-1pLIX MCPIP1, N=5. (E) Tumor vascularity measured by 3D ultrasonography for 5 weeks (left panel) and USG images of tumors. The red color represents blood vessels (right panel). The study involved 9 nude mice: Caki-2 pLIX PURO,
N=3; Caki-2pLIX D141N, N=3; Caki-2pLIX MCPIP1, N=3. The results are presented as the mean±SEM. p-values were estimated using two-tailed unpaired Student’s t-test.

Figure 3. Effect of the MCPIP1 level in ccRCC cells on the endothelial cell lines HMEC-1 and HUVEC.

(A) HMEC-1 chemotaxis to conditioned media from Caki-1 (left graph) and Caki-2 (right graph) with MCPIP1 downregulation. The results are represented as the percent of control. (B) Quantification of the branch points developed by HMEC-1s cultured in Matrigel with conditioned media from Caki-1shCtrl or Caki-1shMCPIP1 cells (left graph). Representative images of branch points after staining with Calcein AM (right panel). (C) Effect of the coculture of Caki-1 cells (shCtrl or shMCPIP1) with the HMEC-1 cell line. The left panel represents the percent of tubular structures at all beads. Images are of representative beads; the arrows indicate tubular structures. On the right panel, quantification of the mean area of migrated HMEC-1s per bead with representative images of migration. (D) Western blot analysis of total and phosphorylated VE-cadherin with α-tubulin as the loading control in the HUVEC cell line after stimulation with conditioned media from Caki-1 and Caki-2 cell lines with MCPIP1 up- or downregulation. (E) Immunofluorescent staining for VE-cadherin. Representative merged images of HUVECs stimulated with conditioned media for 3 h from Caki-1 and Caki-2 cell lines with MCPIP1 up- or downregulation (DAPI for nuclei; VE-cadherin antibody labeled with fluorescent dye AlexaFluor 488). The arrows indicate disrupted monolayer integrity.

Figure 4. Level of proangiogenic factors in ccRCC cell lines.

Caki-1 (left graphs) and Caki-2 (right graphs) cells with MCPIP1 downregulation after 48 h of culture under normoxic or hypoxic conditions. IL8 (A), VEGF (B) and IL-6 (C) mRNA
expression estimated by real-time PCR. The mRNA level of CakishCtrl cells under normoxia was set to 1. The levels of secreted IL-8, VEGF and IL-6 in conditioned media were obtained using ELISA. Caki-1 cells with MCPIP1 overexpression or D141N mutation after 48 h of culture under normoxic or hypoxic conditions. (D) mRNA expression estimated by real-time PCR for IL8, VEGF and IL6. The mRNA level for CakipLIX PURO cells under normoxia was set to 1. The levels of secreted IL-8, VEGF and IL-6 in conditioned media were obtained using ELISA. The results are presented as the mean±SD of N=3. p-values were estimated using two-way ANOVA with post-hoc Bonferroni test.

Figure 5. MCPIP1 affects the SDF-1-CXCR4 axis and metastasis of ccRCC cells.

(A) mRNA expression of SDF-1 and CXCR4 in Caki-1 (left panels) and Caki-2 (right panels) cells with MCPIP1 depletion. (B) In vivo quantification of SDF-1 level in nude mice injected subcutaneously with Caki-1 with MCPIP1 downregulation. Left panel, mRNA expression of human SDF-1 in tumors. Right panel, level of secreted SDF-1 into blood serum. The studies involved 15 nude mice: those injected with Caki-1shCtrl, N=5; those injected with Caki-1shMCPIP1, N=10. (C) Quantification of SDF-1 mRNA expression in Caki-1 cells with overexpression or mutation of MCPIP1. (D) mRNA expression and protein level of c/EBPβ in Caki-1 cell lines with downregulation of MCPIP1 (left panels), or overexpression and mutation of MCPIP1 (right panels). (E) mRNA analysis of lung metastasis using real-time PCR in NOD-SCID mice during 6 weeks of tumor development (left graph) or nude mice after 6 weeks (right graph) injected subcutaneously with Caki-1 with MCPIP1 downregulation. The study involved 8 NOD-SCID mice: those injected with Caki-1shCtrl-GFP, N=4; Caki-1shMCPIP1-GFP, N=4 and 10 nude mice: Caki-1shCtrl N=4; Caki-1shMCPIP1, N=6. (F) mRNA analysis of lung metastasis using real-time PCR in NOD-SCID mice injected subcutaneously with Caki-1 with MCPIP1 overexpression. Tumors were
collected 6 weeks after subcutaneous injection. The study involved 10 NOD-SCID mice: Caki-1pLIX PURO, N=5; Caki-1pLIX MCPIP1, N=5. (G) Flow cytometer analysis of circulating GFP-positive cells in mouse lysed blood during tumor development after 28, 35 and 41 days after i.sc. The study involved 8 NOD-SCID mice: those injected with Caki-1 shCtrl-GFP, N=4; those injected with Caki-1shMCPIP1-GFP, N=4. p-values were estimated using two-tailed unpaired Student’s t-test.

Figure 6. Influence of MCPIP1 downregulation on cell motility and EMT markers.
(A) Motile activity of Caki-1 (left panels) and Caki-2 (right panels) cells after MCPIP1 silencing. Upper panel, representative images after 0 h and 8 h. (B) Representative migratory paths during a 16-h time-lapse recording experiment, N=18 cells for each cell line (C) Quantification of the speed and distance of ccRCC cells during the 16-h experiment. The results are presented as the mean±SD of three independent experiments, with the number of analyzed cells for each cell line, N=100. (D) Analysis of EMT markers in ccRCC cells and the normal renal cell line HEK293 after MCPIP1 downregulation. Left panel, representative western blot with α-tubulin as a loading control for the Caki-1 cell line; middle panel, Caki-2; right panel, HEK293. (E) mRNA level of transcription factors Snail and ZEB2 in Caki-1 and Caki-2 cells, quantified with real-time PCR. The results are presented as the mean±SD of three independent experiments. p-values were estimated using two-tailed unpaired Student’s t-test.

Figure 7. Effect of MCPIP1 upregulation on cell motility and EMT markers.
Motile activity of Caki-1 cells with MCPIP1 upregulation. (A) Quantification of the distance and (B) speed of ccRCC cells during the 16-h experiment. The results are presented as the mean±SD of three independent experiments with the number of analyzed cells of each cell line N=100. (C) Representative images after 0 h and 8 h (upper panel) representative
migratory paths during a 16-h time-lapse recording experiment, N=18 cells for each cell line (lower panel). (D) Analysis of EMT markers in ccRCC cells after MCPIP1 upregulation. Left panel, representative western blot with α-tubulin as the loading control. Right panel, densitometric quantification. Caki-1pLIX PURO was set to 1, and the results are presented as the means±SD of two experiments. (E) mRNA level of the transcription factors Snail and ZEB2 in the Caki-1 cell line, quantified with real-time PCR. p-values were estimated using the two-tailed unpaired Student’s t-test.
Fig. 3

A. HMEC-1 chemotaxis to Caki-1 CM

B. Branch points

C. Beads assay

D. Conditioned media from Caki cell lines

E. Caki-1 CM

Caki-2 CM
Fig. 5
MCPIP1 downregulation in clear cell renal cell carcinoma promotes vascularization and metastatic progression

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