The transcription factor PAX2 regulates ADAM10 expression in renal cell carcinoma

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ADAM10 is a metalloprotease that plays an important role in the progression and metastasis of various cancers. In the present study, we propose compelling evidence that PAX2 can bind to the promoter of ADAM10 and regulate ADAM10 protein expression in renal cancer cells. We further show that ADAM10 is the major sheddase for the constitutive cleavage of L1-CAM and c-Met, two important proteins involved in the progression of renal cancer. The downregulation of ADAM10 led to a more scattered cell phenotype, which was accompanied by the induction of Slug and the loss of E-cadherin, which is observed during epithelial-to-mesenchymal transition (EMT). In addition, the downregulation of ADAM10 reduced the proliferation but induced the migration of renal cancer cells. Notably, the downregulation of PAX2 led to an increased L1-CAM expression, which was accompanied by a massive metalloprotease-mediated release of soluble L1-CAM. Importantly, soluble L1-CAM induced the proliferation of endothelial cells and the migration of renal cancer cells. Finally, we can demonstrate that the silencing of PAX2 led to an L1-CAM-dependent activation of the PI3K/Akt pathway, one important pathway mediating cancer cell survival. In summary, we identified PAX2 as a regulator of L1-CAM and ADAM10, which play crucial roles in the progression of various cancers including renal cell carcinoma and the downregulation of ADAM10 maybe an earlier step in renal cancer development as it seems to be involved in processes of EMT.

Introduction

In Europe, renal cell carcinoma (RCC) is the seventh most common malignancy among men and 12th among women (1). The incidence of renal cancer is world wide rising, accounting for 209 000 new cases and 102 000 deaths each year worldwide (2). The relative 5 years survival for patients diagnosed with RCC depends strongly on the stage of the diagnosed tumor. While patients diagnosed with localized malignancies have a 5 years survival of 80%, it drops to only 2–18% for patients with distant metastasis (1). Since very few effective therapeutic regimens are available, alternative approaches are urgently needed to prolong patients survival. A new promising approach could be to target the Paired Box Gene 2 (PAX2), which has been identified, as an important factor during renal cancerogenesis (3–8). PAX2 is highly expressed during nephrogenesis, where it is required for tubular branching and differentiation (9,10). Furthermore, re-expression of PAX2 occurs in several human cancers including RCC (11). Importantly, in RCC, different studies have demonstrated that PAX2 is expressed in nearly all histological subtypes (3,5,12,13). Moreover, it was shown that PAX2 is highly expressed in early-stage differentiated tumors, indicating that the re-expression of PAX2 is an important early step during renal carcinoma development. This is underlined by the finding that constitutive PAX2 overexpression in mice led to cyst formation in the kidney (8). Another important function of PAX2 in renal cancer cells is its protection against apoptosis, but the mechanism how PAX2 protects cancer cells from cell death is not well understood (14,15).

Abbreviations: ChIP, chromatin immunoprecipitation; EMT, epithelial-to-mesenchymal transition; EMSA, electrophoretic mobility shift assay; mTOR, mammalian Target of Rapamycin; PAX2, Paired Box Gene 2; PCR, polymerase chain reaction; siRNA, short-interfering RNA; SN, supernatant; TMA, tissue microarray.

Despite the important role of PAX2 during nephrogenesis and renal cancer progression, only few data exist about downstream targets of PAX2 (16–18). Importantly, PAX2 is able to repress the expression of the tumor suppressor gene TP53 (19,20). To better understand the role of PAX2 in renal cancer progression, it is important to identify new transcriptional targets of PAX2.

Beside PAX2, the expression of the metalloprotease ADAM10 has been linked to renal cancer progression. With immunohistochemical analysis of a tissue microarray (TMA) of 104 renal cancer samples, we demonstrated that ADAM10 correlated significantly with tumor stage and its expression was significantly increased in papillary RCC (21). Furthermore, it has been shown that ADAM10 cleaves L1-CAM and c-Met, which both play important roles during renal cancer development (21–24). In this study, we can demonstrate in renal cancer cells that ADAM10 is the major constitutive sheddase of L1-CAM and c-Met, two proteins known to play important roles in the progression of RCC. In addition, we provide compelling evidence that PAX2 can directly regulate ADAM10 expression in renal cancer cells. Furthermore, we found that the downregulation of PAX2 led to an increased ADAM10-independent L1-CAM shedding, which was accompanied by L1-CAM-dependent activation of the PI3K/Akt pathway.

Material and methods

Electrophoretic mobility shift assay analysis
The isolation and preparation of nuclear extracts were performed as described previously (25). A double-stranded oligonucleotide reflecting the specific PAX2-binding site in the ADAM10 promoter was used for electrophoretic mobility shift assay (EMSA) analysis. The oligonucleotide contains 21 bp from –305 to –326 relative to the ADAM10 transcriptional start site (ADAM10 complementary DNA sequence: NM_001110.2): 5’-CCCCCGCTCTACGTGGTGAGG-3’. Additionally, an oligonucleotide with mutations at the specific PAX2-binding site was used to further characterize the retarded complex: 5’-CCCCGGCTGCAGTGTT- GAGG-3’ (bold bases indicate the wild type or mutated PAX2 binding motifs). For the characterization of the retarded complexes, nuclear proteins were preincubated for 30 min with two concentrations of anti-PAX2 antibody.

Chromatin immunoprecipitation assay
The chromatin immunoprecipitation (ChIP)-IT Express kit (Active Motif, Rixensart, Belgium) was used to perform the ChIP assay as described in the manual. The incubation mixture contained 50 µl sheared chromatin, 100 µl protein G magnetic beads and 3 µg PAX2 antibodies. The primer pairs 5’-CCCGCGTCCTACGTT- GAGGAA-3’ and 5’-CCCTGGGACAGAAACGCGGC-3’ was designed to amplify a 207 bp product of the human ADAM10 promoter which contains the PAX2-binding site sequence (NM_001110.2).

Antibodies
The monoclonal PAX2 antibody that was used for western blot, EMSA and immunohistochemistry was ordered from Epitomics (Burlingame, CA). For ChIP assay, we used a monoclonal PAX2 antibody from Abnova (Heidelberg, Germany). The monoclonal antibodies L1-CAM-11A were a kind gift from Prof Dr Peter Aliev (German Cancer Research Center, Heidelberg, Germany). The monoclonal ADAM10 antibody was ordered from Diaclone (Besancon, France). To detect ADAM17 (H-300) and c-Met (C-12) expression, antibodies from Santa Cruz Biotechnology (Heidelberg, Germany) were used. For western blot analysis, the phospho-S6 (Ser235/236), mammalian Target of Rapamycin (mTOR) (7C10), phospho-mTOR (Ser2448) (D92C), Slug (C19G7), Akt and Phospho-Akt (Ser473)(S87F11) antibodies were obtained form Cell Signaling Technology (Frankfurt am Main, Germany). The β-catenin antibody was obtained from BD Bioscience (Heidelberg, Germany) and the β-actin antibody was obtained from Sigma-Aldrich (Taufkirchen, Germany).

Reagents
The ADAM10- and ADAM17-specific metalloprotease inhibitors, GI254023X (10 µM) and GW280264X (10 µM), were kindly provided by Dr Andreas Ludwig (Aachen, Germany) and are described elsewhere (26). The inhibitors GM6001, TAPI-2 and XXI (Compound E) were obtained from Calbiochem (Darmstadt, Germany). Staurosporine was ordered from Enzo Life Sciences AG (Lausen, Switzerland). The human recombinant L1-CAM (1 µg/ml) protein

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and LY204002 (10 μM) was obtained from Millipore (Schwalbach, Germany) and Rapamycin (20 ng/ml) was ordered from Serva (Heidelberg, Germany).

Cell culture

The renal carcinoma cell line A498 was obtained from Dr K. Joerger (Innsbruck Medical University, Innsbruck, Austria), whereas the renal carcinoma cell line Foehn and the ovarian cancer cell line SKOV3ip were a kind gift from Prof Dr Peter Altevogt (German Cancer Research Center, Heidelberg, Germany). The human endothelial cell line EAhy 926 was provided by Dr Edgell (University of North Carolina, Chapel Hill, NC) and the glioblastoma cell line T98G was provided by Dr Kleber (German Cancer Research Center). The cell line 786-O was provided by Dr Prof Wilhelm Krek (ETH, Zurich, Switzerland).

Western blot analysis

Protein isolation and western blot analysis has been described before (27).

Fluorescence microscopy

Immunofluorescence analysis of tissue sections and cells has been described earlier (28).

Complementary DNA synthesis and PCR analysis

RNA from cultured cells was isolated using the RNA Easy Kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Equal amounts of total cellular RNA (1 μg) were reverse-transcribed with random primer by the use of M-MulV Reverse Transcriptase (Fermentas, Sankt Leon-Rot, Germany). Transcribed complementary DNAs were used for polymerase chain reaction (PCR) with specific primers for ADAM10 (467 bp, 5'-AGAAGATTG GACATACTTTGGAGCTCCA-3' and 5'-GCCATCATATATCCCTTGCAC/AGT-3'), PAX2 (277 bp, 5'-CCCCGGTCTTCCCTCATA-3' and 5'-CCACACACTGGGAGATCT-3'), β-catenin (530 bp, 5'-GTTGCTGCA CATCGAGGATAC-3' and 5'-CGTAGACTGATCAGTACCTG-3'), SNAIL (72 bp, 5'-TTCTCACGGAATACTGCAACAG-3' and 5'-CGTGTGCGTTCGGATGTG-3'), Slug (116 bp, 5'-CCCTGAGATGGCATACTTGAC-3' and 5'-CTTTCCTGCCGGTGAGTCTCA-3') and β-actin (234 bp, 5'-GACCTGCGAG CAAGAGATGG-3' and 5'-AGACTGTGGTGGCGTACAG-3'). PCR products were amplified using Taq DNA polymerase (NatuTec, Frankfurt, Germany) and subjected to electrophoresis through 2% agarose gels and ethidium bromide staining.

Short-interfering RNA transfection

The following short-interfering RNA (siRNA) duplexes (MWG Biotech AG, Ebersberg, Germany) were used for downregulation of the corresponding protein expression: PAX2 siRNA (PAX2 siRNA1), 5'-GAAGUCAAGUGCGAGUCAUUT-3'; L1-CAM siRNA, 5'-AGGGAUUGUUGUCCAC-UUAAAT-3'; ADAM10 siRNA, 5'-AGACAUUGAAGGUAAUAUU-3'; ADAM17 siRNA, 5'-GAGAAGUCGUAGUACUUUGTCT-3'; PAX2 siRNA2, 5'-GCAU CUGCGUGCGACUUAG-3'; PAX2 siRNA3, 5'-GUGAAGUGAGUAGU CUGA-3'. ADAM15 siRNA has been described before (29). As a negative control, unspecific scrambled siRNA duplexes (5'-AGGAUGUGUAA UCGCGUCUGT-3') were used. Twenty-four hours before transfection 0.4 × 10^6 cells were seeded in six-well plates. Transfection of siRNA was carried out using Oligofectamine (Invitrogen, Karlsruhe, Germany) together with 10 nM siRNA duplex per well as described (30).

Immunohistochemical analysis

Immunohistochemical analysis has been described before (21).

Tissue microarray

For immunofluorescence analysis, we used the TMA KD811 from Biomax (Rockville, MD) that consists of 33 different renal cancer cores that have a diameter of 1.5 mm and a thickness of 5 μm. The TMA consists of 12 clear cell renal carcinoma cores, 19 transitional cell carcinoma cores of the renal pelvis and 2 papillary transitional cell carcinoma cores of the renal pelvis. For immunohistochemical analysis, we used the TMA KD1002 that consists of 40 cases of malignant clear cell renal carcinoma and 10 normal kidney tissue cores with a diameter of 1 mm and a thickness of 5 μm.

Cell cycle analysis

Cells were transfected with siRNA as described before. After 96 h, cells were trypsinized, washed in phosphate-buffered saline and fixed with 70% ethanol at −20°C. After centrifugation, cells were incubated in hypotonic solution containing 50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100 and 20 μg/ml RNaseA for 30 min at 37°C. Finally, cells were analyzed using flow cytometry in a linear mode. Each assay was performed in triplicates and repeated at least thrice.

Cell migration assay

The cell migration assay has been described earlier (31).

Shedding assay

After 72 h of siRNA transfection, cells were cultured for 24 h under serum-free conditions. Cell debris was removed by centrifuging and 10% of trichloroacetic acid was added to the supernatant (SN) and incubated for 10 min on ice. The precipitated proteins were centrifuged at 16 000g and washed two times with absolute ethanol. After drying the protein pellet, proteins were solved in sodium dodecyl sulfate sample buffer and analyzed by western blot analysis.

Proliferation assay

The proliferation assay has been described before (27).

Isolation of microvesicles

SNs were collected and centrifuged for 10 min at 1200g and 20 min at 10 000g to remove cellular debris. Microvesicles were collected by centrifugation at 100 000g for 4 h using a Beckman SW60 rotor. Afterward, the isolated microvesicles were resuspended in sodium dodecyl sulfate sample buffer and analyzed by western blot analysis.

Statistical analysis

For the analysis of statistical significance, Student’s t-test was used.

Results

ADAM10 cleaves L1-CAM and c-Met in RCC cells

Members of the ADAM family are mainly responsible for the cleavage of growth factors, adhesion molecules and cell surface receptors (32,33). To determine whether the metalloproteases ADAM10 and ADAM17 are involved in the constitutive cleavage of L1-CAM and c-Met in renal cancer cells, we downregulated with siRNA each metalloprotease. Seventy-two hours after siRNA transfection, we analyzed the lysates and the precipitated SN of A498 cells by western blot analysis. Interestingly, the knockdown of ADAM10 led to an increase of ADAM17 and L1-CAM expression (Figure 1a). Importantly, the release of L1-CAM and c-Met in the SN was strongly inhibited by the downregulation of ADAM10. In addition, the knockdown of ADAM17 was compensated by an upregulation of ADAM10 and led to an induced shedding of L1-CAM and c-Met (Figure 1a; SN). Interestingly, we observed that the downregulation of ADAM10 led to morphological changes of renal cancer cells as shown in Figure 1b by bright field microscopy. Compared with control siRNA (sc-siRNA)-transfected cells, the ADAM10-specific siRNA-transfected cells showed a more scattered and round like phenotype (Figure 1b).

To analyze if this morphological change was due to processes observed in epithelial-to-mesenchymal transition (EMT), we determined the expression of E-cadherin, the E-cadherin repressor Slug and mesenchymal protein α-smooth muscle actin after the knockdown of ADAM10. The downregulation of ADAM10 led to an induction of Slug (Figure 1c, upper right panel) and concomitant to the reduction of the epithelial marker protein E-cadherin (Figure 1c, middle right panel and Figure 1d). In addition, after silencing ADAM10, we observed an upregulation of the mesenchymal protein α-smooth muscle actin (Figure 1c, lower right panel). In contrast, the messenger RNA levels of β-catenin and Snail and the localization of β-catenin were not changed after silencing ADAM10 (supplementary Figure S1A and B is available at Carcinogenesis Online). Furthermore, we can show that ADAM10 siRNA-transfected A498 cells showed a decreased proliferation compared with scrambled siRNA-transfected cells (Figure 1e). Since the downregulation of E-cadherin has been reported to increase cell migration (34) we determined the role of ADAM10 in cell migration. As shown in Figure 1F silencing of ADAM10 significantly increased the migration of renal cancer cells.

The transcription factor PAX2 binds to the ADAM10 promoter

To identify transcription factors that are involved in the regulation of ADAM10 in renal cancer cells we performed a computational analysis of the ADAM10 promoter and identified a putative PAX2 binding site (−312 bp) in the ADAM10 promoter (Supplementary Figure S2A and S2B are available at Carcinogenesis Online). To determine a direct interaction of PAX2 with the promoter of ADAM10 we performed EMSA. Double-stranded DNA probes representing the specific PAX2 binding site of the ADAM10 promoter were incubated with nuclear
Fig. 1. ADAM10 is the major metalloprotease that cleaves L1-CAM and c-Met under constitutive conditions (a) Western blot analysis of total lysates and trichloroacetic acid-precipitated proteins from SN of A498 cells transfected with specific siRNA against ADAM10 (A10-siRNA), ADAM17 (A17-siRNA) or both proteins (A10/A17-siRNA). As a control, scrambled siRNA (sc-siRNA) was used. Soluble proteins from the SN were investigated with specific antibodies against L1-CAM or c-Met and densitometrically analyzed in a separate diagram. The total lysates were investigated with antibodies against ADAM10, ADAM17 or L1-CAM. To confirm equal protein loading, β-actin antibody was used. The protein amounts of lysates of three independent experiments were densitometrically analyzed and its relative value to β-actin is depicted in a separate graph. P-values were determined of four independent experiments and are considered statistically significant compared with sc-siRNA-transfected cells. (b) A498 cells were transfected with control siRNA or with siRNA against ADAM10. Seventy-two hours after the transfection, phase contrast images were taken of the cells. Scale bar in phase contrast images represents 50 μM. (c) Immunofluorescence analysis of A498 cells transfected with control (sc-siRNA) or ADAM10-siRNA. Images in the upper panel depict ADAM10 (red) and Slug (green) expression, images in the middle panel show ADAM10 (red) and E-cadherin (green) expression and images in the lower panel demonstrate alpha-smooth muscle actin (αSMA, red).
PAX2 can regulate ADAM10 expression

To determine, whether the transcription factor PAX2 can regulate ADAM10 expression in renal cancer cells, we downregulated PAX2 expression. Importantly, the downregulation of PAX2 by siRNA1 led to an almost complete loss of ADAM10 expression (Figure 3a). The transfection of PAX2 siRNA3, which did not lead to a complete loss of PAX2 protein levels, only reduced ADAM10 levels to 40% compared with scrambled siRNA-transfected cells (Figure 3a). The regulation of ADAM10 by PAX2 was confirmed by immunofluorescence and messenger RNA expression analysis (Figure 3b and c). To determine whether ADAM10 could play a role in the induction of apoptosis we downregulated both proteins by siRNA and analyzed the amount of apoptotic cells by cell cycle analysis. As shown in Figure 3e the downregulation of PAX2 led to a strong induction of apoptosis in renal cancer cells, whereas silencing of ADAM10 had no effect on renal cancer cell death.

Silencing of PAX2 led to a strong release of L1-CAM into the SN of renal cancer cells

Based on our previous results we investigated the role of PAX2 in the ADAM10 mediated release of L1-CAM. Surprisingly, we observed...
that the downregulation of PAX2 led to a massive release of soluble L1-CAM (sL1-CAM) into the SN, which could be inhibited by the broad spectrum metalloprotease inhibitors GM6001 and TAPI-2 (Figure 4a). In contrast, the specific ADAM17 inhibitor GW280264X (GW) and ADAM10 inhibitor GI254023X (GI) did not significantly reduce the release of L1-CAM (Figure 4b). In contrast, by using specific siRNA targeting ADAM10, ADAM15 and ADAM17 we could show that ADAM15 and ADAM17 siRNA reduced the L1-CAM shedding ∼50% whereas ADAM10 siRNA had no effect on the release of L1-CAM as expected (Figure 4c). We further analyzed if the inhibition of mTOR by rapamycin has an effect on the release of L1-CAM. Importantly, rapamycin was able to reduce L1-CAM shedding to almost basal levels (Figure 4d).

To determine whether the induced L1-CAM shedding by PAX2 siRNA could be due to released microvesicles, we isolated microvesicles from cell SNs of siRNA-transfected renal cancer cells. As shown in Figure 4e, staurosporine, which induces apoptosis and the release of microvesicles (35), led to a massive release of L1-CAM-containing microvesicles, whereas no induction was detected in the

![Fig. 3. PAX2 regulates ADAM10 expression (a) Total cell lysates of A498 cells transfected with specific siRNA against ADAM10 (A10-siRNA), against PAX2 (PAX2 siRNA1/2/3) were prepared and western blot analysis was performed. As a control, scrambled siRNA (sc-siRNA) was used. Membranes were incubated with antibodies against ADAM10 and PAX2. β-Actin was used to determine equal protein loading. (b) ADAM10 expression (red) was analyzed by immunofluorescence analysis of A498 cells transfected with siRNA against ADAM10 (A10-siRNA), PAX2 (PAX2 siRNA) or control siRNA (sc-siRNA). Nuclei of A498 cells were stained with 4',6-diamidino-2-phenylindole (DAPI). Scale bar represents 20 μM. (c) Messenger RNA from A498 cells treated with transfection reagent alone (Mock) or without complementary DNA (neg.ctrl.) were used as controls. Reverse transcription–PCR was performed with ADAM10 and PAX2 specific primers. Specific β-actin reverse transcription–PCR was used as an internal control. (d) Western blot analysis of total cell lysates of the endothelial cell line EAhy, the glioblastoma cell line T98G or the ovarian cancer cell line SKOV3ip, transfected with control siRNA (sc-siRNA) or PAX2 (PAX2 siRNA)-specific siRNA. Membranes were incubated with specific antibodies against ADAM10 and PAX2. β-Actin was used as a loading control. (e) The percentage of apoptotic A498 cells (cells in sub-G1/G0 phase) was measured by cell cycle analysis as described in Materials and Methods and are shown in a graph. P = 0.03 considered statistically significant compared with sc-siRNA-transfected cells. P = 0.14 considered not statistically significant compared with sc-siRNA-transfected cells.](image-url)
Fig. 4. Downregulation of PAX2 induces the release of soluble L1-CAM. (a) Western blot analysis (with L1-CAM-specific antibodies) of soluble L1-CAM (sL1-CAM) levels in trichloroacetic acid-precipitated SNs of A498 cells transfected with specific siRNA against PAX2 (PAX2 siRNA), L1-CAM (L1-siRNA) or control siRNA (sc-siRNA). In addition, the metalloproteinase inhibitors (GM6001 and TAPI-2) were preincubated to inhibit metalloproteinase-mediated cleavage of L1-CAM. Soluble L1-CAM was densitometrically analyzed and depicted in a separate diagram. P-values were determined of three individual experiments and are statistically significant compared with PAX2 siRNA-transfected cells (ctrl). (b) Levels of soluble L1-CAM (sL1-CAM) were determined by western blot analysis.
SNs of sc-sc-siRNA and PAX2 siRNA-transfected renal cancer cells. In Figure 4f, the efficient downregulation of PAX2 is shown by western blot analysis.

To examine if sL1-CAM from the SN of renal cancer cells can induce the proliferation of endothelial cells, we added the SNs of siRNA-transfected cells to endothelial cells (Figure 4g). Importantly, only the SN of the PAX2 silenced A498 cells significantly induced the proliferation of endothelial cells, whereas the SNs of control siRNA (sc-siRNA) or the PAX2 and L1-CAM siRNA (PAX2/L1 siRNA)-transfected cells had no effect on endothelial cell proliferation. Additionally, we added soluble L1-CAM to A498 cells or coated the membrane of a transwell chamber with sL1-CAM and performed migration assays. Increased migration of renal cancer cells was observed when soluble L1-CAM was added directly to A498 cells (Figure 4h). In contrast, no induced migration was seen when soluble L1-CAM was coated on the membranes of the transwell chambers (Figure 4h). These observations indicate that the downregulation of PAX2 activates different metalloproteinases, which increases L1-CAM shedding and subsequently induces endothelial proliferation and cancer migration.

Silencing of PAX2 induces the activation of the PI3K pathway

As rapamycin reduced the induced L1-CAM shedding (Figure 4d), we wanted to determine if the downregulation of PAX2 activates the PI3K pathway. As shown in Figure 5a, the downregulation of PAX2 increased the expression of L1-CAM and the phosphorylation of the protein kinase S6 (Figure 5a). Furthermore, by additional silencing of L1-CAM (PAX2/L1 siRNA), we could show that the activation of S6 kinase was L1-CAM dependent (Figure 5a). In contrast, by using a γ-secretase inhibitor (CE), we could show that the γ-secretase mediated cleavage of L1-CAM is not involved in the phosphorylation of the S6 kinase (Figure 5a).

To confirm if the phosphorylation of S6 is transmitted through the PI3K pathway, we treated PAX2 siRNA-transfected renal cancer cells after the PAX2 knockdown either with the mTOR inhibitor rapamycin or the PI3K inhibitor LY294002 (Rapa, Ly29, Figure 5b). Both inhibitors strongly reduced the phosphorylation of S6, whereas rapamycin strongly reduced the phosphorylation of mTOR and S6 but had no effect on the phosphorylation status of Akt (Figure 5b). Furthermore, the PI3K inhibitor LY294002 reduced the phosphorylation of Akt and S6 but did not reduce the phosphorylation of mTOR (Figure 5b). In summary, we can show that PAX2 downregulation induces an L1-CAM-dependent activation of the PI3K pathway.

To confirm the effects after PAX2 downregulation in other renal cancer cell lines, we analyzed the regulation of ADAM10, the shedding of L1-CAM and phosphorylated S6 in the renal cancer cell lines Foehn and 786-0 (supplementary Figure S3A and Bis available at Carcinogenesis Online).

Next determined whether L1-CAM shedding or the PI3K activation is involved in the induction of apoptosis after PAX2 silencing. Therefore, we incubated renal cancer cells after PAX2 siRNA transfection with the broad spectrum metalloprotease inhibitor GM6001 or the mTOR inhibitor rapamycin. Interestingly, GM6001 and rapamycin reduced significantly the number of apoptotic cells (Figure 5c). To analyze whether soluble L1-CAM is involved in PAX2-mediated apoptosis, we added recombinant L1-CAM protein to the PAX2-silenced cells. Soluble L1-CAM was not able to increase apoptosis in PAX2-downregulated cells; in contrast, soluble L1-CAM significantly reduced the apoptosis of PAX2-silenced renal cancer cells (Figure 1d).

ADAM10 and PAX2 are coexpressed in RCC samples

To analyze whether PAX2 and ADAM10 are coexpressed in renal cancer tissue, we analyzed the expression of PAX2 and ADAM10 by immunofluorescence analysis on a TMA, containing biopsies with different types of renal cancer. Representative staining of samples that express both proteins (PAX2 and ADAM10, Figure 6a), ADAM10 alone (Figure 6b) or PAX2 alone (Figure 6c) are shown. The expression pattern of ADAM10 and PAX2 are summarized in Figure 6d and the samples that coexpress PAX2 and ADAM10 are summarized in Table 1. Interestingly, all 12 clear cell carcinoma samples coexpressed ADAM10 and PAX2, whereas in transitional cell carcinoma from the renal pelvis, only 12 of 19 samples coexpressed ADAM10 and PAX2. Coexpression was also observed in one of two papillary transitional carcinoma samples originating from the renal pelvis.

To analyze in more detail the coexpression in clear cell renal cancer tissue, we analyzed the expression of PAX2 and ADAM10 by immunohistochemistry on a TMA, containing predominantly biopsies of clear cell renal cancer patients. The expression pattern of ADAM10 and PAX2 in clear cell renal cancer samples is summarized in Figure 6e: 10% of clear cell renal cancer patients expressed neither PAX2 nor ADAM10, 13% of the patients expressed PAX2 alone, 6% of the patients expressed ADAM10 alone but the majority (71%) of the patients with clear cell renal cancer coexpressed ADAM10 and PAX2.

In addition, we analyzed the number of patients which coexpressed L1-CAM and ADAM10 (42% of clear cell renal cancer patients, Figure 6f) and the number of patients which coexpressed L1-CAM and PAX2 (52%, Figure 6g).

Discussion

RCC is the most common type of kidney cancer, which is characterized as highly resistant to current therapies (36). Despite the rapid progress in the understanding of RCC, the exact molecular mechanisms that lead to the progression of RCC are still not clear and new strategies to cure RCC are urgently needed.

In this context, ADAMs are an attractive family of proteins that have been shown to be upregulated in several cancers (37). Recently, we could demonstrate by analyzing a TMA of RCC patients that ADAM10 expression correlated significantly with tumor stage and was significantly increased in papillary RCC (21). It has been shown in different cancers types that ADAM10 can cleave L1-CAM, whereas the protease of trichloroacetic acid-precipitated SNs from A498 cells transfected with PAX2 siRNA or control siRNA (sc-siRNA). In addition, cells were treated as a control with dimethyl sulfoxide or with 10 μM ADAM10- and ADAM17-specific inhibitors GI254023X (GI) or GW282064X (GW). Soluble L1-CAM was densitometrically analyzed and is shown in a separate diagram. (c) Levels of soluble L1-CAM (sL1-CAM) were determined by western blot analysis of TCA-precipitated SNs from A498 cells transfected with PAX2 siRNA (PAX2 siRNA) and control siRNA (sc-siRNA) alone or the combination of PAX2 siRNA and ADAM10 siRNA (PAX2/A10-siRNA), Pax2 and ADAM15 siRNA (PAX2/A15-siRNA), Pax2 and ADAM17 (PAX2/A17-siRNA). Soluble L1-CAM was densitometrically quantified and is depicted in a separate graph. (d) Levels of soluble L1-CAM (sL1-CAM) were determined by western blot analysis of trichloroacetic acid-precipitated SNs from A498 cells transfected with PAX2 siRNA or control siRNA (sc-siRNA) with or without the addition of 20 ng/ml rapamycin (Rapa). Soluble L1-CAM was densitometrically quantified and depicted in a separate graph. P-value was determined of three individual experiments and are statistically significant compared with PAX2 siRNA-treated cells. (e) L1-CAM expression in microvesicles isolated from A498 cells was determined by western blot analysis of A498 cells transfected with specific siRNA against PAX2 or control siRNA. Staurosporine (1 μM) was used as a positive control. (f) PAX2 expression was investigated in total cell lysates of A498 cells transfected with specific PAX2 siRNA or control siRNA by western blot analysis. To confirm equal protein loading, β-actin was used. (g) EAhCy cells were stimulated with the SN of A498 cells that were transfected with PAX2 (PAX2 (PAX2 siRNA) or L1-CAM- and PAX2 (PAX2 siRNA)-specific siRNAs and afterward, the anchorage-dependent cell growth of EAhCy cells was determined using an MTT, 3-[4,5-dimethyl-2-y]-2,5-diphenyltetrazolium bromide assay. P = 0.012 considered statistically significant compared with scrambled siRNA-transfected cells. P = 0.007 considered statistically significant to L1-CAM and PAX2 siRNA-transfected cells. P = 0.318 considered statistically not significant compared with L1-CAM and PAX2 siRNA-transfected cells. (h) Soluble L1-CAM was added directly into the transwell chamber (+sL1-CAM) or coated on the membrane of the transwell chamber (sL1-coated) and migration assay was performed with A498 cells as described under Materials and Methods. P = 0.04 considered statistically significant compared with untreated control cells (ctrl). P = 0.14 considered statistically not significant compared with control cells.
that cleaves L1-CAM in RCC has not been described so far. In this study, we could demonstrate for the first time that ADAM10 is the major metallopeptase that constitutively cleaves L1-CAM and c-Met in RCC cell lines. Importantly, L1-CAM overexpression has been demonstrated in many tumor types including RCC, where it occurs in about half of all clear cell types and one-third of all papillary types. Moreover, its expression was linked to metastasis and poor prognosis in RCC patients (22,38). Furthermore, coexpression and the cleavage of L1-CAM by ADAM10 have also been described in melanoma and in colon cancer (27,39,40).

In addition to L1-CAM, ectodomain shedding has also been described for c-Met, the hepatocyte growth factor receptor tyrosine kinase that is a prominent regulator of cancer cell invasiveness and an activator of the PI3K/Akt pathway (41,42). In hereditary papillary RCC and clear cell RCC, c-Met is often activated (43,44). Recently, it has been shown in hepatocellular carcinoma, that anti-c-Met antibody induced ADAM10-dependent c-Met cleavage and thereby impaired the invasive growth of the tumors (23). The authors suggested that c-Met cannot be cleaved in the absence of ADAM10 and therefore more hepatocyte growth factor can bind to the receptor, which subsequently leads to an increased invasiveness of cancer cells (23). In contrast to these data, our results demonstrate that no stimulation for c-Met shedding was necessary and ADAM10 represents a major constitutive sheddase of c-Met and L1-CAM.

Moreover, we could show that the inhibition of ADAM10 by siRNA leads to a decreased proliferation and a more scattered phenotype of renal cancer cells. It has been described that c-Met and L1-CAM can activate the β-catenin pathway, which is important for the EMT (45,46). Similar to our observation, during EMT, cells become more scattered and round shaped. We did not observe any changes in β-catenin expression or localization after the ADAM10 knockdown. However, after ADAM10 downregulation, E-cadherin, which is important for the formation of the epithelial adherens junctions (47,48) was downregulated. A recent study has shown that the disruption of E-cadherin cell–cell contact is sufficient for a scattered phenotype, whereas the complete loss of E-cadherin is necessary for the induction of EMT (34). In this context, we found that the expression of the E-cadherin repressor slug and the mesenchymal protein alpha smooth muscle actin, which are upregulated during EMT were indeed induced after the ADAM10 knockdown. In contrast to this, SNAIL and β-catenin expression was not changed (supplementary Figure S1A and B is available at Carcinogenesis Online). In agreement with other studies, which show that the loss of E-cadherin leads to increased cell migration, we found that the downregulation of ADAM10 induces cell migration. Future experiments in rodents have to be performed to determine if changes in ADAM10 expression are involved in a more aggressive renal tumor growth in vivo.
Interestingly, downregulation of ADAM17 led to an already described compensation by ADAM10 that was accompanied by an increased L1-CAM and c-Met shedding (49).

Fig. 6. PAX2 and ADAM10 are coexpressed in clear cell renal cancer samples. (a) Representative immunofluorescence image of a clear cell renal cancer tissue sample, which shows coexpression of PAX2 and ADAM10. Renal cancer tissue was stained with specific antibodies for PAX2 (red) and ADAM10 (green). The cells were stained with 4',6-diamidin-2-phenylindol (DAPI) to visualize nuclei (blue). Scale bar represents 20 µM. (b) Representative immunofluorescence image of a papillary transitional carcinoma tissue sample, which shows no PAX2 (red) but ADAM10 (green) expression. (c) Bar plot demonstrating the relative distribution of clear cell, transitional, papillary and granular RCC samples on a TMA that were negative (−; white), positive (+; gray) or strongly positive (++; black) for ADAM10 or PAX2 expression. (d) Bar plot demonstrating the relative distribution of clear cell RCC samples on a TMA that do not express ADAM10 and PAX2 (A10−PAX2−; white), which do not express ADAM10 but express PAX2 (A10−PAX2+; gray), which express ADAM10 but do not express PAX2 (A10+PAX2−; dark gray) or samples that coexpress ADAM10 and PAX2 (A10+PAX2+; black). Expression levels were determined by immunohistochemistry analysis. (f) Bar plot demonstrating the relative distribution of clear cell RCC tissue samples on a TMA that do not express ADAM10 and L1-CAM (A10−L1−; white), which do not express ADAM10 but express L1-CAM (A10−L1+; gray), which express ADAM10 but do not express L1-CAM (A10+L1−; dark gray) or tissue samples that coexpress ADAM10 and L1-CAM (A10+L1+; black). Expression levels were determined by immunohistochemistry analysis.
to identify factors that directly regulate ADAM10 expression in cancer. By analyzing the promoter region of ADAM10, we identified at position \(-313\) till \(-321\) bindings sites for the PAX2 transcription factor (52). Interestingly, recently Prinzen et al. (53) identified a USF-binding site that was overlapping with the new putative PAX2-binding site. Importantly, site-directed mutagenesis of this conserved USF-binding site demonstrated the central role of this sequence in ADAM10 transcription. In previous studies, it has been shown that PAX2 is expressed in nearly all clear cell renal cancers and cell lines (5,7,13). Recently, it has been suggested that the upregulation of PAX2 is an early and essential step during renal carcinogenesis (6,54,55).

By EMSA, we observed the formation of two complexes, whereas only complex 2 was inhibited by the incubation with PAX2 antibodies, demonstrating the specificity of complex 2. The increase of complex 1 could be explained by conformational change after antibody binding that increases the affinity of PAX2 toward the DNA sequence. Additionally, we confirmed the direct binding of PAX2 to the ADAM10 promoter by ChIP. Furthermore, we present by immunofluorescence western blot and reverse transcription-PCR analysis that PAX2 can regulate ADAM10 expression. Importantly, the downregulation of PAX2 in endothelial cells, glioblastoma cells and ovarian carcinoma cells led to a nearly complete loss of ADAM10 expression, indicating the importance of this finding for other cell types.

From our previous results, we assumed that downregulation of PAX2 would lead to reduced L1-CAM cleavage. Surprisingly, we found increased levels of soluble L1-CAM in the SN of the PAX2 downregulated renal cancer cells. By using different metalloproteinase inhibitors, we could demonstrate that L1-CAM cleavage induced by PAX2 silencing is independent of ADAM10 and partly depends on the activity of ADAM15 and ADAM17. Moreover, by using the mTOR inhibitor, Rapamycin, we could inhibit the induced L1-CAM shedding, which indicates that the activation of the PI3K pathway leads to the activation of metalloproteases that subsequently cleave L1-CAM. In addition, our findings are in agreement with results obtained in ovarian cancer cells, in which apoptotic stimuli induced L1-CAM cleavage by multiple metalloproteases other than ADAM10, whereas under constitutive conditions, L1-CAM cleavage occurred mainly by ADAM10 (35).

Several studies have demonstrated an important role of soluble L1-CAM (sL1-CAM) in the migration, proliferation, angiogenesis and chemoresistance of cancer cells (27,56,57). Furthermore, sL1-CAM stimulated cell migration, chemoresistance and trigger extracellular signal-regulated kinase phosphorylation by binding to integrins (35,58).

In renal cancer cells, we could demonstrate that soluble L1-CAM induced the migration of renal cancer cells, which is an important step during renal cancer development. Importantly, we further provide evidence that soluble L1-CAM can induce the proliferation of endothelial cells and therefore may play an important role in angiogenic processes of RCC. In this cell line, it has been shown that sL1-CAM is a proangiogenic factor (56). RCC is a highly vascular tumor, therefore, it is very important to identify factors that stimulate angiogenesis and may represent new therapeutic targets in the treatment of RCC.

In our study, we could demonstrate that the downregulation of PAX2 led to an increase of the L1-CAM protein that was accompanied by an increased phosphorylation of the ribosomal protein S6 (P-S6). Interestingly, this phosphorylation was inhibited by the additional downregulation of L1-CAM. Additionally, treatment with \(\gamma\)-secretase inhibitors had no effect, whereas PI3K and mTOR inhibitors strongly inhibited the S6 phosphorylation, indicating that \(\gamma\)-mediated cleavage of L1-CAM is not involved in the activation of the PI3K pathway. In addition, we can show that inhibition of mTOR and the inhibition of metalloproteinas in renal cancer cells reduces the number of apoptotic cells after the PAX2 knockdown. The L1-CAM mediated induction of apoptosis after PAX2 knockdown is not mediated through soluble L1-CAM, as soluble L1-CAM reduced the apoptosis of PAX2-silenced renal cancer cells. These data fit to our finding that L1-CAM containing SNs induce the proliferation of endothelial cells, demonstrating that soluble L1-CAM has tumor-promoting function in renal cancer as it induces proliferation and cell survival.

In a TMA with samples of a small cohort of renal cancer patients, we found that PAX2 and ADAM10 were coexpressed in clear cell RCC (in 12 of 12 samples). In addition, in TMA with a larger cohort of clear cell RCC patients, we found in 71% of all RCC samples coexpression of PAX2 and ADAM10, whereas only 6% of the renal cancer patients expressed ADAM10 in the absence of PAX2. This data assume that PAX2 is also involved in the regulation of ADAM10 in RCC patients.

In summary, we present compelling evidence that PAX2 can regulate L1-CAM and ADAM10, which play important roles in various types of cancers, and therefore PAX2 represents an important therapeutic target in RCC patients.

### Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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### Conflict of Interest Statement

None declared.

### References


### Table I. Coexpression of ADAM10 and PAX2

<table>
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<tr>
<th>Renal cancer type</th>
<th>Coexpression (%)</th>
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</thead>
<tbody>
<tr>
<td>Clear cell (n = 12)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>Transitional (n = 19)</td>
<td>12 (63)</td>
</tr>
<tr>
<td>Papillary (n = 2)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Overall (n = 33)</td>
<td>25 (76)</td>
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ADAM10 expression in RCC