Graded activation of the MEK1/MT1-MMP axis determines renal epithelial cell tumor phenotype

Rajeev Mahimkar, Maria Alejandra Alfonso-Jaume\(^2\), Leslie M. Cape, Rajvir Dahiya\(^1\) and David H. Lovett\(^*\)

Department of Medicine and \(^1\)Department of Urology, San Francisco Department of Veterans Affairs Medical Center, University of California, San Francisco, CA 94121, USA and the Northern California Institute for Research and Education, San Francisco CA 94121, USA

\(^2\)Present address: Department of Medicine, W. S. Middleton VAMC, University of Wisconsin School of Medicine, Madison, WI 53705, USA

To whom correspondence should be addressed. Department of Medicine, San Francisco Department of Veterans Affairs Medical Center, University of California, 111J Medical Service, 4150 Clement Street, San Francisco, CA 94121, USA. Tel: +1 415 221 4810, ext. 3568; Fax: +1 415 750 6949; Email: david.lovett@va.gov

Activation of Raf/Ras/mitogen-activated protein kinase (MEK)/mitogen-activated protein kinase signaling and elevated expression of membrane type-1 matrix metalloproteinase (MT1-MMP) are associated with von Hippel–Lindau gene alterations in renal cell carcinoma. We postulated that the degree of MEK activation was related to graded expression of MT1-MMP and the resultant phenotype of renal epithelial tumors. Madin Darby canine kidney epithelial cells transfected with a MEK1 expression plasmid yielded populations with morphologic phenotypes ranging from epithelial, mixed epithelial/mesenchymal to mesenchymal. Clones were analyzed for MEK1 activity, MT1-MMP expression and extensive epithelial–mesenchymal transition. Phenotypes of the MDCK-MEK1 clones were evaluated in vitro with nu/nu mice. Tissue microarray of renal cell cancers was quantitatively assessed for expression of phosphorylated MEK1 and MT1-MMP proteins and correlations drawn to Fuhrman nuclear grade. Graded increases in the MEK signaling module were associated with graded induction of epithelial–mesenchymal transition of the MDCK cells and induction of MT1-MMP transcription and synthesis. Inhibition of MEK1 and MT1-MMP activity reversed the epithelial–mesenchymal transition. Tumors generated by epithelial, mixed epithelial/mesenchymal and mesenchymal MDCK clones demonstrated a gradient of phenotypes extending from well-differentiated, fully encapsulated non-invasive tumors to tumors with an anaplastic morphology, high Fuhrman nuclear score, neuroangio- genesis and invasion. Tumor microarray demonstrated a statistically significant association between the extent of phosphorylated MEK1, MT1-MMP expression and nuclear grade. We conclude that graded increases in the MEK1 signaling module are correlated with M1-MMP expression, renal epithelial cell tumor phenotype, invasive activity and nuclear grade. Phosphorylated MEK1 and MT1-MMP may represent novel, and mechanistic, biomarkers for the assessment of renal cell carcinoma.

Introduction

Recent insights into the molecular pathogenesis of renal cell carcinoma, particularly of the clear cell type, have made major contributions to our current understanding of this common neoplasia and have led to the development of potentially more effective therapies (1,2). Alterations in the von Hippel–Lindau (VHL) gene, through mutation or inactivation of the von Hippel–Lindau (VHL) gene, through mutation or inactivation of the VHL gene, through mutation or inactivation factor-α (HIF-α) results in the transcriptional activation of a number of genes associated with tumorigenesis and neoangiogenesis (3). In addition, VHL inactivation can result in sustained oncogenic epidermal growth factor receptor (EGFR) signaling through the Akt-1 and Ras/mitogen-activated protein kinase kinase (RAF/MEK)/mitogen-activated protein kinase (MAPK) signaling cascades (4,5). MEK (also designated mitogen-activated protein kinase) exists in two isoforms (MEK1/ MEK2) and is a dual-specificity kinase that binds to inactive MAPK. Upon activation of MEK kinase activity via phosphorylation by Raf, MEK phosphorylates MAPK, yielding an active kinase with multiple substrates, including transcription factors.

VHL inactivation in renal cell carcinoma also induces sustained phosphorylation and activation of the tyrosine kinase activity of the MET protein, which further contributes to tonic activation of the MAPK signaling cascade (6). Sustained activation of the MEK1 signaling module disrupts epithelial polarity in Madin Darby canine kidney (MDCK) renal epithelial cells and induces expression of membrane Type-1 matrix metalloproteinase (MT1-MMP, also denoted MMP-14), suggesting that tonic activation of this signaling module contributes to the morphologic and phenotypic changes seen in renal cell carcinoma (7). In support of this, constitutive activation of the MEK/MAPK signaling pathway has been documented been in a variety of human tumors (8). Within the context of renal cell carcinoma, MAPK activation was observed in the majority of tumors and the extent of MAPK activity correlated with tumor grade (9).

Local invasion and distal metastasis are important predictors of clinical outcomes in renal cell carcinoma, and increased expression of several matrix metalloproteinases (MMPs), including MMP-2, MMP-9 and MT1-MMP has been reported in clinical renal cell carcinoma tissues (10,11). Increased expression of MMP-2 and MMP-9 correlates with poor prognostic features in renal cell carcinoma, including tumor grade and vascular invasion (12,13). The ability of tumor cells to successfully invade three-dimensional extracellular matrices is critically dependent upon the activity of the membrane-associated MMP class, particularly MT1-MMP (14–16). Petrella et al. (17,18) have recently identified MT1-MMP as a transcriptional target of HIF-2α and determined that the invasive activity of VHL+/− renal cell carcinoma cells in vitro was dependent upon MT1-MMP activity. In addition to transcriptional activation of MT1-MMP by HIF-2α, collagen-induced MT1-MMP synthesis in cultured endothelial cells is dependent upon the activity of the MEK1/MAPK signaling cascade (19). Furthermore, MT1-MMP exerts a positive feedback stimulatory effect on the MEK1/MAPK axis through transactivation of the EGFR (20), thereby stimulating cellular migration.

Given the above observations, we postulated that graded expression of active MEK1 would result in a co-ordinated graded induction of MT1-MMP synthesis that would progressively affect the invasive activity of tumor cells. To approach this issue, we generated clonal populations of MDCK renal epithelial cells expressing increasing amounts of constitutively active MEK1 protein. Graded expression of MEK1 correlated with progressive epithelial–mesenchymal transition, MT1-MMP expression and invasive activity in vitro and in vivo. These observations were validated by tissue microarray analysis of a panel of human renal cell carcinoma tissues, which demonstrated a significant association of tumor grade with levels of phosphorylated MEK1 protein and MT1-MMP expression.

Materials and methods

Cell culture
The renal epithelial MDCK cell line, the VHL+/− renal cell carcinoma cell line Caki-1 and the VHL−/− renal cell carcinoma cell line 786-O were obtained from ATCC (Manassas, VA) and cultured according the supplier’s instructions.

Abbreviations: DAB, diaminobenzidene; EGFR, epidermal growth factor receptor; HIF, hypoxia-inducible factor; MAPK, mitogen-activated protein kinase; MDCK, Madin Darby canine kidney; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 matrix metalloproteinase; PBS, phosphate-buffered saline; VHL, von Hippel–Lindau.
Generation of stable MEK1-MDCK transfectants

MDCK cells were cotransfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA) with pTK-hygro (50 ng; Clontech, Mountain View, CA) and pUSEamp-MEK-HA (500 ng, Millipore, Billerica, MA). pUSEamp-MEK-HA encodes a constitutively active MEK1 with substitution of aspartic acid for serines at 218 and 222 and an HA epitope tag. Control cells were transfected with pTK-hygro alone. Forty-eight hours after transfection, cells were selected with hygromycin (250 μg/ml). Six discrete clones with distinct morphologic features on phase contrast microscopy ranging from epithelial to fully mesenchymal were derived by single cell cloning. These clones were characterized as detailed in the Results section. The clonal populations were expanded through five passages, harvested, aliquoted and frozen for use in subsequent experiments.

Quantitation of MEK1 activity in transfectants

Near-confluent cell layers from the respective MEK1 clones and control were washed twice in cold phosphate-buffered saline (PBS), harvested by scraping and spun at 400 g for 10 min. MEK1 activity was determined using a MEK1 immunoprecipitation kinase assay kit (Millipore) according to the manufacturer’s instructions, using 100 μg protein per sample. In this assay immunoprecipitated MEK1 is used to phosphorylate recombinant inactive MAPK1. The phosphorylated MAPK1 is separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Invitrogen), blotted onto polyvinylidene difluoride (GE Healthcare, Piscataway, NJ) and blocked overnight at 4°C in StartBlock (Thermo Fisher Scientific). For detection of MEK1 activity the blots were incubated sequentially with rabbit anti-MEK1 antibody (1 μg/ml; Millipore) and horseradish peroxidase-coupled goat anti-rabbit F(ab')2 (20 ng/ml; Invitrogen) in StartBlock (Thermo Fisher Scientific). Detection of MEK1-MMP, the blots were incubated sequentially with rabbit anti-MEK1-MMP antibody (1 μg/ml; Millipore) and horseradish peroxidase-coupled goat anti-rabbit F(ab')2 (20 ng/ml; Invitrogen) in StartBlock. Vimentin was detected using rabbit anti-vimentin (1 μg/ml, Clone 36; Transduction Laboratories, Lexington, KY) followed by fluorescein isothiocyanate-conjugated rat anti-IgG2a (5 μg/ml; Invitrogen) in 1% goat serum/PBS at 25°C for 2 h. For detection of the MEK1-HA epitope tag, slips were incubated with murine monoclonal anti-HA IgG (5 μg/ml in 1% goat serum/PBS at 25°C for 2 h, followed by streptavidin–rhodamine conjugate as detailed above.

Western blots

Total cellular extracts were generated by lysis of cells using T-Per (Thermo Fisher Scientific, Rockford, IL) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Total cell extracts (20 μg/sample) were resolved by reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Invitrogen), blotted onto polyvinylidene difluoride (GE Healthcare, Piscataway, NJ) and blocked overnight at 4°C in StartBlock (Thermo Fisher Scientific). For detection of MT1-MMP, the blots were incubated sequentially with rabbit anti-MT1-MMP antibody (1 μg/ml; Millipore) and horseradish peroxidase-coupled goat anti-rabbit F(ab')2 (20 ng/ml; Invitrogen) in StartBlock. Vimentin was detected using rabbit anti-vimentin (1 μg/ml; Abcam) and horseradish peroxidase-coupled goat anti-rabbit F(ab')2 (20 ng/ml; Invitrogen). E-cadherin was detected using anti-E-cadherin IgG2a (1 μg/ml, Clone 36; Transduction Laboratories) and goat anti-mouse F(ab')2 (20 ng/ml; Invitrogen). Peroxidase activity was detected by chemiluminescence with the ECL Plus detection system (GE Healthcare).

The protein content of the analyzed cellular extracts was individually confirmed prior to performance of the western blots to assure equal protein loading of the respective lanes. In addition, blots were probed with rabbit anti-GAPDH (1 μg/ml; Abcam), followed by development at detailed above.

Transient transfection with MT1-MMP luciferase reporter constructs

Subconfluent cultures of cells were washed and transfected with Lipofectamine2000 (Invitrogen) using 0.4 μg of plasmid DNA from the control
Control and MEK1-transformed MDCK cells were grown to subconfluency, with a value of 100% assigned to the control epithelial clone A (gray columns). MT1-MMP transcriptional activity was assessed as detailed in Materials and Methods using the murine MT1-MMP promoter driving a reporter luciferase cassette (black columns; Data are expressed as mean ± SD of quadruplicate determinations for each clone). Panel II: Western blot analysis of MDCK clone cell extracts for MT1-MMP. The dominant detected MT1-MMP band has the apparent molecular mass of the proenzyme form (62 kDa). Panel III: Quantitative MT1-MMP enzymatic assay of MDCK cellular extracts as detailed in Materials and Methods. Results are displayed as ng active MT1-MMP protein/100 μg cellular extract (mean ± 1 SD). Panel IV: Invasive activity of the respective control and MEK1-MDCK clones was performed as detailed in Materials and Methods using a collagen-based cell invasion kit (Data are expressed as mean ± SD of quadruplicate determinations; *P < 0.05 by t-test).

Measurement of MT1-MMP enzyme activity

Control and the respective MEK1 MDCK clones were grown to subconfluency, washed with 4°C PBS, and extracted with 50 mM Tris/ClHCl, 0.5 mM CaCl2, 1 mM ZnCl2, 0.01% (vol/vol) Nonident P-40 and 0.25% (vol/vol) Triton X-100. Cell suspensions were incubated for 10 min at 4°C followed by centrifugation at 2500g for 10 min at 4°C and stored at −80°C until use. MT1-MMP activity was measured on 20 μg cellular protein samples with the Sensolyte 520 MMP-14 assay (AnaSpec, Fremont, CA) according to the manufacturer’s instructions. This assay uses a quenched FRET peptide substrate and was normalized using a standard curve generated with activated recombinant MT1-MMP protein (AnaSpec). Assays were performed in quadruplicate and data are expressed as the mean ± SD.

MT1-MMP inhibition studies

Control and MEK1-transformed MDCK cells with a fully mesenchymal phenotype (clone F, see Figure 1) were cultured in OptiMEM (Invitrogen). To inhibit MT1-MMP activity, cells were incubated for increasing time periods (0–96 h) with a murine monoclonal IgG1, directed against the catalytic domain of the enzyme (10 μg/ml, clone LEM-2/15.6; Millipore) or with control murine IgG1, at the same concentration. Monoclonal clone LEM-2/15.6 targets the amino acid sequence 218–233 within the catalytic domain of MT1-MMP and has been demonstrated to inhibit MT1-MMP activity (21). Cells were stained for expression of E-cadherin and vimentin as detailed above and western blots for E-cadherin and vimentin were performed as detailed above.

Invasion assay

The invasive activity of control and MEK1-MDCK clones was quantitatively determined using the Chemicon 96-well Collagen-Based Cell Invasion Assay Kit. Quadruplicate wells were loaded with 1 × 10⁵ cells and the number of cells traversing the membranes determined at 48 h using CyQuant CR dye solution. The invasion assays were repeated three times.

In vivo subcutaneous tumor assay

Subconfluent cultures of three discrete MEK1 clones (clones B, D and F, see Figure 1) were washed in PBS, harvested by centrifugation and suspended in a 1:1 mixture of medium/Matrigel (BD Biosciences, San Jose, CA). Cell suspensions (1 × 10⁶) from the transfection controls and the respective MEK1 clones were injected subcutaneously into the flanks of groups of six athymic nu/nu female mice (Jackson Laboratory, Bar Harbor, ME). The mice were killed at 4 weeks and excised tumors were fixed in buffered formalin, paraffin embedded and stained with hematoxylin/eosin. For MT1-MMP immunohistochemistry, the blocks were rehydrated, endogenous peroxide blocked and antigen retrieval performed with Sigma protease, 2 mg/ml for 10 min at 37°C. Following avidin/biotin block (Vector Laboratories), the sections were incubated for 90 min with a 1:10 dilution of biotinylated ARK; DakoCytomation, Carpinteria, CA) primary anti-MT1-MMP murine

**Fig. 2.** Quantitative analyses of MEK1 activity, MT1-MMP transcription rates, protein synthesis, MT1-MMP enzymatic activity and invasion. Panel I: MEK1 enzymatic activity was measured in the respective MDCK clones, with a value of 100% assigned to the control epithelial clone A (gray columns). MT1-MMP transcriptional activity was assessed as detailed in Materials and Methods using the murine MT1-MMP promoter driving a reporter luciferase cassette (black columns; Data are expressed as mean ± SD of quadruplicate determinations for each clone). Panel II: Western blot analysis of MDCK clone cell extracts for MT1-MMP. The dominant detected MT1-MMP band has the apparent molecular mass of the proenzyme form (62 kDa). Panel III: Quantitative MT1-MMP enzymatic assay of MDCK cellular extracts as detailed in Materials and Methods. Results are displayed as ng active MT1-MMP protein/100 μg cellular extract (mean ± 1 SD). Panel IV: Invasive activity of the respective control and MEK1-MDCK clones was performed as detailed in Materials and Methods using a collagen-based cell invasion kit (Data are expressed as mean ± SD of quadruplicate determinations; *P < 0.05 by t-test).
monoclonal antibody (clone 113-5B7; Research Diagnostics, Flanders, NJ). Washed slides were incubated with streptavidin/ horseradish peroxidase followed by development with diaminobenzidine (DAB) according to the manufacturer’s instructions. The counterstain was hematoxylin.

**Tissue microarray staining for MT1-MMP and phospho-MEK1**
Sequential sections of tissue microarray of human renal cancers (Imgenex, San Diego, CA) were stained for MT1-MMP and phospho-MEK1. MT1-MMP was stained as detailed above with the exception that the counterstain was methyl green. To detect phosphorylated MEK1, the avidin/biotin-blocked tissue arrays were stained with a rabbit monoclonal anti-phospho-MEK1 antibody (20 μg/ml; Epitomics, Burlingame, CA) for 90 min at room temperature, followed by incubation with biotinylated goat anti-rabbit F(ab')2 (5 μg/ml; Invitrogen) and developed with DAB/hydrogen peroxide using standard methodology. Tumor nuclear grade was scored by an independent pathologist.

**Quantitative chromogen detection was performed**
The RGB image of an entire stained tissue section for each sample was captured at high resolution (1200 d.p.i.) and digitized under identical conditions. Each digitized image histogram was normalized using the ‘Image Adjust Auto tool’ of Adobe PhotoShop (version 7.0.1). In Adobe PhotoShop, the DAB reaction product was selected using the ‘Magic Wand’ and ‘Select-Similar’ tools, and pixel intensity for the DAB reaction product was quantified using the ‘Edit-Selection-Select All’ and ‘Analyze-Measure’ tools within ImageJ (version 1.38x; National Institutes of Health). Pixel intensity for MT1-MMP or phospho-MEK1, for each tissue section, was graphically represented along with its corresponding Fuhrman nuclear grade. Spearman correlation coefficients were calculated to examine the association of tumor grade with phosphorylated MEK1 and MT1-MMP protein intensities. The non-parametric Kruskal–Wallis test was used to test for differences in phospho-MEK1 and MT1-MMP across the four tumor grades.

**Results and discussion**
Stable transfection of the renal epithelial cell line MDCK with a constitutively active MEK1 construct resulted in a broad range of morphologic phenotypes. Observation of individual clonal populations by phase contrast microscopy indicated that the stable transfectants exhibited morphologic features extending from a conserved epithelial phenotype to a fully transitioned phenotype characterized by an extended and migratory fibroblastic morphology (Figure 1, panel I). Notably, a number of clones were observed with intermediate features common to both epithelial cells and fibroblasts when observed by phase contrast microscopy. For the purposes of this study, we isolated a panel of clonal populations with morphologic features extending from typical epithelial (clone B) through intermediate phenotypes (clones C and D) to fully fibroblastic phenotypes (clones E and F). E-cadherin staining and organization were used as qualitative markers of the epithelial phenotype, whereas staining and organization of vimentin was used as a marker for the degree of epithelial–mesenchymal transition. Control MDCK cells (Figure 1, panel II, A) and the epithelial clone (B) showed maintenance of cell surface staining characteristic of intact E-cadherin junctional complexes. The intermediate clones showed progressive dissolution of the junctional complexes with increased cytoplasmic localization of E-cadherin staining (C and D). E-cadherin distribution was primarily cytoplasmic in the fully fibroblastic clones (E and F).

Staining for vimentin revealed an inverted pattern of expression as the MDCK cells transitioned from the epithelial to mesenchymal phenotypes. Vimentin staining in the controls and the epithelial cell clones (Figure 1, panel III, A and B) was limited to a delicate filamentous pattern in a subcortical distribution. Vimentin staining progressively increased in intensity as the cells transitioned to the mesenchymal phenotype, with a diffuse cytoplasmic distribution (C and D). In the fully mesenchymal phenotypes, vimentin staining was intense and organized in dense filamentous structures with a predominantly perinuclear concentration (E and F). Vimentin exists as a 54 kDa non-phosphorylated form and as a 57 kDa phosphorylated form (22). The 57 kDa phosphorylated form regulates intermediate filament assembly and cellular migration (22,23). Western blots of the respective clones demonstrated a graded increase in both forms of vimentin as a function of the extent of epithelial–mesenchymal transformation (Figure 1, panel IV).

The level of measured MEK1 activity, as determined by the rates of phosphorylation of recombinant MAPK1 protein, showed a direct relationship with the extent of epithelial–mesenchymal
transformation (Figure 2, Panel I). Control MDCK cells were assigned a relative MEK1 activity of 100%. Cells with an intermediate phenotype (clones C and D) showed relative MEK1 levels of 160 ± 14% and 210 ± 23%, respectively (P < 0.05 as compared with controls). MDCK clones with fully transformed mesenchymal phenotypes expressed relative MEK1 activities of 260 ± 22% and 280 ± 18%, respectively (P < 0.05 as compared with controls). Thus, relatively small, but sustained and graded increases in MEK1 activity are sufficient to induce graded degrees of epithelial–mesenchymal transformation.

There was a similar relationship between the degree of epithelial–mesenchymal transformation and the levels of MT1-MMP transcription (Panel I) and protein synthesis (Panel II). Transcription rates for...
MT1-MMP as assessed with a luciferase reporter construct driven by the MT1-MMP promoter increased nearly 4-fold in the most transformed clones while the relative levels of MT1-MMP protein also increased by ~4-fold. The dominant 62 kDa MT1-MMP band detected on the western blots conforms with the pro- or inactive form of MT1-MMP. The levels of enzymatically active MT1-MMP are highly regulated by a complex process of proenzyme secretion, membrane complex formation, catalytic activation, internalization, degradation or recycling (24–26). We therefore directly quantified the amounts of active MT1-MMP enzyme present in respective MDCK clones as summarized in Figure 2, panel III, the epithelial clones (A and B) and intermediate clone (D) had similar levels of MT1-MMP enzyme activity (~1.0 ng/100 μg cellular protein). The fully mesenchymal MDCK clone F had a higher MT1-MMP activity level of ~1.7 ng/100 μg cellular protein. Thus, while MT1-MMP proenzyme protein progressively increases as a function of MEK1 activity, MT1-MMP enzymatic activity does not and is only elevated in the fully invasive MDCK clone.

Acquisition of an invasive phenotype is an important determinant of tumor behavior and ultimately of prognosis in renal cell carcinoma. Although the prior figures describe a gradient of mesenchymal morphology, kinase activity and MT1-MMP transcription/translation across the MDCK/MEK clonal populations, this was not observed with a quantitative in vitro invasion assay. As summarized in Figure 2, panel IV, clones with intermediate (or mixed epithelial/mesenchymal) phenotypes (C and D) had the same levels of invasive activity observed in the fully epithelial cell clones (A and B). The fully mesenchymal clone F demonstrated a nearly 4-fold increase in invasive activity as compared with the other clones. Thus, acquisition of an invasive phenotype is a feature of MEK1/MT1-MMP-dependent epithelial-mesenchymal transformation seen only in those cells expressing the highest levels of MEK1 and higher levels of active MT1-MMP.

Both MEK1 and MT1-MMP enzymatic activity were required for the maintenance of MDCK epithelial–mesenchymal transformation. The fully mesenchymal MDCK clone F, at the 10 passage, was incubated for 48 h in the presence or absence of the selective MEK1 inhibitor, PD98059 (30 μM) and examined by immunofluorescence staining for the MEK1 protein and with Nomarski optics to define cellular morphology. As shown in Figure 3, panel I, A, MDCK cells from clone F demonstrate an extended migratory morphology with prominent MEK1 protein staining. Inhibition of MEK1 activity reverts the cellular morphology from mesenchymal to fully epithelial (B). We also quantified the effects of selective MEK1 inhibition by PD98059 on MT1-MMP transcription rates. Incubation for 48 h with PD98059 decreased MT1-MMP relative transcriptional activity to 56 ± 24 relative luciferase units as compared with the control value of 245 ± 58 relative luciferase units (P < 0.05). As detailed in Figure 3, panel II, incubation of the fully mesenchymal clone F with a monoclonal antibody directed against the catalytic active site of the MT1-MMP protein induced a reversion to a fully epithelial cell phenotype, as demonstrated by morphology and immunohistochemical staining for E-cadherin and vimentin. Western blot analyses of MT1-MMP antibody-treated cells (panel III) show a progressive decrease in vimentin staining over a 96 h period while E-cadherin increased significantly at the same time point. Incubation with a control monoclonal IgG had no effect on vimentin or E-cadherin expression levels. Thus, sustained expression of both active MEK1 and active MT1-MMP are required for the maintenance of the mesenchymal phenotype. These findings confirm an absolute requirement for the integrity of the MEK1/MT1-MMP axis in maintaining MDCK epithelial–mesenchymal transformation.

We assessed the tumor-forming properties of the MDCK-MEK clones in nu/nu mice. For these studies, mice received a subcutaneous flank injection of MDCK clones with an epithelial phenotype (clone B), an intermediate phenotype (clone D) and the fully mesenchymal invasive clone F. Representative sections are shown in Figure 4, panel I. Tumors derived from the epithelial phenotype MDCK clone (panels A–C) were characterized as well-differentiated adenocarcinoma with developed tubulocystic structures and abundant intraluminal mucin. Tumors derived from clone F, the intermediate phenotype and higher levels of active MEK1/MT1-MMP, were more invasive, displaying areas with a dense stroma and surrounded by a very dense fibrous capsule. There was no evidence for local invasion or neoangiogenesis. The Fuhrman nuclear grade score for these tumors was 1.2 ± 0.2 (n = 50 scored nuclei).

Tumors derived from the MDCK clone with an intermediate mesenchymal phenotype (clone D) remained encased in a relatively dense capsule but were considerably more cellular with a Fuhrman nuclear grade score of 1.8 ± 0.5 (panels D–F, n = 50 scored nuclei). In contrast, tumors derived from the fully mesenchymal MDCK clone F were highly cellular, lacked capsule formation and demonstrated local invasion into the surrounding soft tissues and muscle (panels G–H). The cellular morphology was anaplastic in nature with a Fuhrman nuclear grade score of 3.6 ± 0.3 (n = 50 scored nuclei).

Immunohistochemical staining of the tumors for MT1-MMP expression was performed (Figure 4, panel II). There was little to no detectable MT1-MMP cellular expression in the tumors derived from the epithelial MDCK clones (B), whereas cells lining cystic structures were noted to express MT1-MMP in the tumors derived from the intermediate MDCK clone (C). There was intense cellular staining in the tumors derived from the fully mesenchymal MDCK clones, with prominent staining of columns of cells invading muscle and adipose tissue (D).

We next examined the relationship between VHL status, EGFR and MET signaling with rates of MT1-MMP transcription and synthesis using VHL+/+ Caki-1 clear cell carcinoma cells and VHL−/− 786-0 clear cell carcinoma cells. These studies are summarized in Figure 5. MT1-MMP transcription rates were approximately six times greater in the VHL−/− 786-O cells as compared with the VHL+/+ Caki-1 cells (Figure 5, Panel I).
The effects of two signaling inhibitors on MT1-MMP transcription rates were also assessed. In the concentration used (10 nM), K252a is a potent and selective inhibitor of the receptor tyrosine kinase activity of c-MET (27). The compound 4557w [4-(4-benzyloxyanilino)-6,7-dimethoxyquinazoline] is a potent and selective inhibitor of EGFR tyrosine kinase activity (28). MT1-MMP transcription rates in both cell types were significantly reduced by either the EGFR or MET chemical inhibitors, indicating that basal transcription of MT1-MMP is primarily mediated by a constitutively active receptor tyrosine kinase-coupled Ras/Raf/MEK/MAPK signaling cascade.

Similar findings were observed in terms of MT1-MMP protein synthesis (Figure 5, Panel II). VHL+/+ Caki-1 cells synthesized approximately one-fifth the amount of MT1-MMP as compared with the VHL−/− 786-0 cells. MT1-MMP protein synthesis rates in both cell types were significantly reduced by either the EGFR or MET chemical inhibitors, indicating that basal transcription of MT1-MMP is primarily mediated by a constitutively active receptor tyrosine kinase-coupled Ras/Raf/MEK/MAPK signaling cascade.

Tissue microarrays of controls and 49 specimens of renal cell carcinoma (42 clear cell type, 2 papillary type, 2 collecting duct type and 3 granular type) were stained for phosphorylated MEK1 and MT1-MMP. Digitized images of each specimen were then quantitatively assessed for the levels of phosphorylated MEK1 and MT1-MMP staining using the ImageJ software package and correlated with the corresponding Fuhrman nuclear grade. Representative sections from the tissue arrays are shown in Figure 6 and show a progressive increase in staining for phosphorylated MEK1 (A–C) and MT1-MMP (D–F) as a function of increasing Fuhrman nuclear grade. Figure 6 summarizes the quantitative assessment of phosphorylated MEK1 and MT1-MMP expression. Spearman correlation coefficients were calculated to examine the association of tumor grade with phosphorylated MEK1 and MT1-MMP. Tumor grade is significantly associated with both phosphorylated MEK1 (r = 0.44, P = 0.002) and MT1-MMP (r = 0.56, P < 0.0001, respectively, Spearman correlation coefficients).

Montesano et al. (7) first reported a relationship between high level MEK1 expression, elevated MT1-MMP synthesis and acquisition of an invasive phenotype in three-dimensional culture. In the current study, we have attempted to build on this initial observation and to provide a mechanistic linkage between graded activation of the MAPK/MT1–MMP axis and renal cell carcinoma phenotypic features directly associated with clinical outcomes. The levels of relative MEK1 activity in the MDCK clonal populations were in the same range as reported for human renal cell carcinoma samples (29), indicating that
the observed phenotypes are unlikely to be the result of gross MEK1 overexpression. The functional linkage between both components of the MEK/MT1–MMP axis for the determination of the final cellular phenotype is underscored by the observation that expression of MT1-MMP alone in MDCK cells generates tumor cells that maintain a well-differentiated, fully epithelial non-invasive phenotype (30).

Sustained activation of the MEK signaling module undoubtedly alters the expression of numerous genes in addition to MT1-MMP. It is intriguing, however, that inhibition of MT1-MMP enzymatic activity with an antibody directed against the catalytic site is sufficient to revert fibroblastic, fully mesenchymal MDCK cells to a differentiated epithelial phenotype. Thus, both sustained MEK1 activity and MT1-MMP enzymatic activity are required for the development of the fully mesenchymal phenotype, but this phenotype cannot be maintained in the absence of MT1-MMP enzymatic activity.

The relationship between MEK1 and MT1-MMP is bidirectional. A recent study by Sounni et al. (31) demonstrated that binding of TIMP2 to cell surface MT1-MMP-stimulated cellular migration via activation of MEK1/2 phosphorylation, a process that occurs independently of MT1-MMP proteolytic activity (32). Thus, elevated expression of the MT1-MMP proenzyme is sufficient, via MEK1/2 phosphorylation, to induce proliferation and migration, whereas expression of the active enzyme is required for cellular invasion of extracellular matrices. Distinct roles of the catalytic and hemopexin domains of MT1-MMP have been defined in the epithelial–mesenchymal transformation of prostate cancer cells (33,34). This underscores the conclusion that MT1-MMP effects on cellular behavior are multilayered and involve both proteolytic and non-proteolytic activities that are intricately linked to activation of the MEK1/ERK signaling cascade.

MT1-MMP plays a critical role in the ability of tumor cells to invade three-dimensional extracellular matrices (14–16). In addition, MT1-MMP has been shown to induce aneuploidy and chromosomal instability in model epithelial cells systems (35–37). This process may provide an explanation for the association of higher tumor nuclear grade and anaplastic morphology with higher levels of MT1-MMP expression observed in this study.

Induction of MT1-MMP transcription by MEK1 signaling provides at least a partial mechanistic explanation for the efficacy of protein kinase inhibitors for the treatment of renal cell carcinoma (29). Furthermore, MT1-MMP protein synthesis is regulated by the mammalian target of rapamycin (38,39) and the positive treatment results observed in some patients with renal cell carcinoma treated with the mammalian target of rapamycin inhibitor everolimus may be, at least in part, a result of inhibition of MT1-MMP synthesis.

There is considerable interest in the identification and validation of biomarkers for renal cell carcinoma that are either associated with tumor behavior or response to treatment. The level of insulin-like growth factor-1 receptor expression correlates with Fuhrman nuclear grade and the membrane-associated metalloproteinase ADAM has been associated with renal cell cancer progression (40,41). Our current findings suggest that expression of phosphorylated MEK1 and MT1-MMP may also provide new biomarkers that are mechanistically linked and represent potential treatment targets.

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