Matrix Metalloproteinase 13 (MMP13) and Tissue Inhibitor of Matrix Metalloproteinase 1 (TIMP1), Regulated by the MAPK Pathway, Are Both Necessary for Madin-Darby Canine Kidney Tubulogenesis*

A classic model of tubulogenesis utilizes Madin-Darby canine kidney (MDCK) cells. MDCK cells form monoclonal cysts in three-dimensional collagen and tubulate in response to hepatocyte growth factor, which activates multiple signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway. It was shown previously that MAPK activation is necessary and sufficient to induce the first stage of tubulogenesis, the partial epithelial to mesenchymal transition (p-EMT), whereas matrix metalloproteinases (MMPs) are necessary for the second redifferentiation stage. To identify specific MMP genes, their regulators, tissue inhibitors of matrix metalloproteinases (TIMPs), and the molecular pathways by which they are activated, we used two distinct MAPK inhibitors and a technique we have termed subtraction pathway microarray analysis. Of the 19 MMPs and 3 TIMPs present on the Canine Genome 2.0 Array, MMP13 and TIMP1 were up-regulated 198- and 169-fold, respectively, via the MAPK pathway. This was confirmed by two-dimensional and three-dimensional real time PCR, as well as in MDCK cells inducible for the MAPK gene Ref. Knockdown of MMP13 using short hairpin RNA prevented progression past the initial phase of p-EMT. Knockdown of TIMP1 prevented normal cystogenesis, although the initial phase of p-EMT did occasionally occur. The MMP13 knockdown phenotype is likely because of decreased collagenase activity, whereas the TIMP1 knockdown phenotype appears due to increased apoptosis. These data suggest a model, which may also be important for development of other branched organs, whereby the MAPK pathway controls both MDCK p-EMT and redifferentiation, in part by activating MMP13 and TIMP1.

Epithelial organs such as the kidney, lung, and salivary gland develop from a process termed branching morphogenesis or tubulogenesis (1–7). Although tubulogenesis is incompletely understood, genes involved in this process are conserved in different organs, and indeed throughout evolution (8). Of all mammalian organs, kidney development is probably the best understood (9) and the kidney is particularly well suited for studies of tubulogenesis (10).

Because of the complexity of organogenesis (the human kidney is composed of more than 20 cell types and 1 million nephrons (10, 11)) and the transitory nature of cyst and tubule formation, it is difficult to study these processes in vivo. Therefore, relatively little was known about tubulogenesis prior to the development of the Madin-Darby canine kidney (MDCK) hepatocyte growth factor (HGF) in vitro assay. The MDCK cell line was derived from the kidney tubules of a normal cocker spaniel in 1958 (12, 13) and, for the past several decades, has been one of the most widely used reagents for studying important and fundamental issues in epithelial cell biology (14). When MDCK cells are seeded singly within a three-dimensional collagen matrix, over 10 days they form monoclonal structures that are characterized by a polarized epithelium surrounding a fluid-filled space, apical microvilli, a solitary cilium, and apical tight junctions (15, 16), meeting the most rigorous definition of “cysts” (17). Exposure of preformed MDCK cysts to HGF causes the cysts to develop branching tubules (18) in a process that resembles renal branching morphogenesis in vivo (11).

Detailed studies using MDCK cells grown in a collagen matrix until the cyst stage and induced with HGF showed that tubulogenesis consists of the following two morphologically defined stages: an initiation stage, termed partial epithelial-mesenchymal transition (p-EMT) that occurs in the first 24 h following HGF induction, and subsequent redifferentiation
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that proceeds over the next 48–72 h (5, 7, 10). In morphologic terms, the p-EMT stage involves the formation of actin extensions and chains of cells, which have lost their polarity, extending off the basolateral surface of the cysts, whereas redifferentiation involves the generation of tubular lumens and the re-emergence of epithelial cell polarity (5). HGF (also known as scatter factor) is mitogenic, motogenic, and morphogenic, and binding of HGF to its CMET tyrosine kinase receptor, which is located on the basolateral surface of MDCK cells (19), activates a multitude of signaling pathways, including phosphoinositide 3-kinase, phospholipase C, protein-tyrosine phosphatase 2, cytosolic phospholipase A$_2$, and MAPK/ERK to name a few (as reviewed in Ref. 20).

We and our colleagues previously showed that the MAPK pathway of Raf-MEK-ERK is both necessary and sufficient to initiate p-EMT, whereas matrix metalloproteinases (MMPs) are necessary for the redifferentiation stage of MDCK tubulogenesis (7, 21). MMPs are zinc-containing endopeptidases that are involved in remodeling the extracellular matrix and are crucial for organ development. Tissue inhibitors of matrix metalloproteinases (TIMPs) are endogenous specific inhibitors of MMPs that bind the active site of the MMP catalytic domain. To date, 28 MMPs and 4 TIMPs have been identified in vertebrates (22).

Here we used subtraction pathway microarray analysis to identify specific candidate MMP “tubulogenes,” their TIMP regulators, and the molecular pathways by which they were activated. The resulting candidate tubulogenes genes were then knocked down in MDCK cells using short hairpin RNA (shRNA), and the phenotype and mechanism of action were studied during HGF-induced tubulogenesis.

MATERIALS AND METHODS

MDCK Culture, HGF/4-HT Treatment, and RNA Isolation—Low passage type II MDCK cells were obtained from K. Mostov (University of California, San Francisco) and used between passages 3 and 10 as described previously (23, 24). These cells were originally cloned by Daniel Louvard at the European Molecular Biology Laboratory (EMBL) and came to Keith Mostov via Karl Matlin. Cells were cultured in modified Eagle’s minimum essential medium containing Earle’s balanced salt solution and glutamine supplemented with 5% fetal calf serum, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin. MDCK cells were seeded at confluency on 24-mm Transwell filter units coated with collagen (Costar, Cambridge, MA). Pore size on all filters was 0.4 $\mu$m. Cell monolayers were used for experiments after 6–7 days of culture with daily changes in medium. Recombinant HGF at 100 ng/ml was added to the basolateral compartment of MDCK cell monolayers following a 1-h pretreatment either with or without U0126 (UO) (Promega) or PD09859 (PD) (Sigma), both inhibitors of MEK, at 10 and 50 $\mu$m, respectively.

MDCK shRNA-expressing cell lines and controls were plated out in a three-dimensional type I collagen matrix as described previously (21) and allowed to mature into hollow cystic structures over the course of 2 weeks. Cysts were then stimulated either in the presence or absence of hepatocyte growth factor at a concentration of either 100 ng/ml for 24 h or a concentration of 30 ng/ml for 4 days, and the HGF-containing media were changed daily. MDCK cell cysts subjected to HGF at 100 ng/ml for 24 h have been shown to induce p-EMT, the initial stage of tubulogenesis, in virtually 100% of MDCK cell cysts (6, 21); however, the same concentration applied to MDCK cells for 4 days was observed to result in cell scatter. Therefore, a lower concentration of HGF (30 ng/ml) was chosen, which, when added for 4 days, was observed to result in mature tubule formation in some cysts. Recombinant human HGF was very generously provided by the late R. Schwall (Genentech, South San Francisco).

Type II MDCK cells containing inducible Raf fused to the estrogen receptor (25) were grown on two-dimensional Transwell filters as described above. In these cells, the kinase activity of Raf was induced using an estrogen analog, 4-hydroxytamoxifen (4-HT) (Sigma), at a concentration of 1 $\mu$m as described previously (25, 26).

Total RNA was obtained from MDCK cell monolayers using the RNeasy Mini Protocol (Qiagen) for the isolation of total RNA from animal cells. For the microarray studies, RNA samples were visualized by agarose RNA electrophoresis. RNA concentration and purity were measured by determining the 260 nm/280 nm ratio. All ratios were greater than 1.8.

Microarray Analysis—For our experiments, all protocols were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. All conditions were performed in quadruplicate except where otherwise stated. Fifteen $\mu$g of total RNA, collected from MDCK cells grown for 6 days on Transwell filters and exposed to 0 or 24 h of HGF, with and without U0126 or PD09859, were converted to first-strand cDNA. Second-strand cDNA synthesis was followed by in vitro transcription for linear amplification of each transcript and incorporation of biotinylated CTP and UTP. The cRNA products were fragmented to 200 nucleotides or less, heated at 99 °C for 5 min, and hybridized for 16 h at 45 °C to the GeneChip® Canine Genome 2.0 Array (Affymetrix Inc), containing 42,860 Canis familiaris probe sets for >20,000 predicted genes.

The GeneChip® Canine Genome 2.0 Array chips were then washed at low and high stringency and stained with streptavidin-phycocerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycocerythrin stain. A confocal scanner was used to collect fluorescence signal at 3 nm resolution after excitation at 570 nm. The average signal from two sequential scans was calculated for each microarray feature. A complete microarray expression data set has been submitted to the National Center for Biotechnology Information’s (NCBI) Gene Expression Omnibus (GEO) data base (GEO Submission GSE9435, NCBI tracking system 15360129).

Affymetrix GeneChip Operating System (GCOS, version 1.4) was used to quantitate the expression levels for the genes represented on the GeneChip® Canine Genome 2.0 Array; default values provided by Affymetrix were applied to all analysis parameters. Border pixels were removed, and the average intensity of pixels within the 75th percentile was computed for each probe. The average of the lowest 2% of probe intensities occurring in each of 16 microarray sectors was set as background and subtracted from all features in

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that sector. Probe pairs were scored positive or negative for detection of a specific gene sequence by comparing signals from the perfect match and mismatch probe features. The number of probe pairs meeting the default discrimination threshold ($\tau = 0.015$) was used to assign a call of absent (A), present (P), or marginal (M) for each assayed gene, and a $p$ value was calculated to reflect confidence in the detection determination. One of the samples, HGF + UO, was observed to have been degraded during processing and was therefore excluded from subsequent analyses.

Affymetrix cell files, containing probe intensities, were imported into GeneSpring (version 7.3, Agilent Technologies) where GC-robust multiarray average was applied to calculate probe set signal levels. The GCOS-generated present/absent/marginal determinations for each probe set on each GeneChip® Canine Genome 2.0 Array chip were imported as well. To assess global inter-sample relationships, we exported the data for all genes that were present in at least 3 of the 15 samples and performed hierarchical clustering using GeneSpring.

We were interested in identifying differentially regulated genes in three basic comparisons, HGF alone versus control (standard medium), HGF + UO versus HGF alone, and HGF + PD versus HGF alone. For each comparison, all genes on the array were first filtered to retain those that were present in all replicates of at least one of the conditions. The filtered lists were then exported to Microsoft Excel, where Significance Analysis of Microarrays (version 2.21, Stanford University) was applied in a two-class unpaired mode, with 500 permutations to identify differentially regulated genes with a $\geq 2$-fold change and a false discovery rate of $<0.01%$. These three gene lists were each then subdivided to include a group of genes that were up-regulated and a group of genes that were down-regulated, yielding six lists as follows: HGF > control, HGF < control, HGF + PD > HGF, HGF + PD < HGF, HGF + UO > HGF, and HGF + UO < HGF.

Real Time PCR—Fifteen µg of total RNA, collected from MDCK cells grown for 6 days on Transwell filters and exposed to 0 or 24 h of HGF ± UO126 or PD09859, were converted to first-strand cDNA. cDNA and the TaqMan primer/probe system, individualized for each mRNA, were used in conjunction with the 7700 PRISM sequence detection instrument (both Applied Biosystems) as described in the Applied Biosystems technical manual. When the reaction product amplification exceeded the threshold value, the corresponding cycle number was termed $C_P$. Fold change between conditions was calculated through an exponential function of the observed difference in $C_T$ as described previously (27). The values were normalized to a control mRNA, the 18S ribosome, and all real time PCR studies were performed at least three times in triplicate.

Western Blotting—Western blot analysis was performed as described previously (28). For caspase 3 analysis, filters were probed with rabbit anti-cleaved caspase 3 at a concentration of 1:1000 (Asp-175; Cell Signal Technology, Danvers, MA). This antibody recognizes active caspase 3 and is a marker for apoptosis (29). Filters were then probed with horseradish peroxidase-labeled donkey anti-rabbit antibody at 1:5000 dilution. Filters were developed using SuperSignal West Femto luminescent detection kit (Pierce) and visualized on Kodak X-Omat film (Eastman Kodak Co.).

Creation of shRNA Vectors—The lentiviral vector psicoR (30) was used to generate all shRNA-containing vectors in this paper. The software program Psicoligomaker was used to select 19-mer shRNA sequences against both canine MMP13 and TIMP1 genes. The sequence selected for MMP13 shRNA knockdown was 5'-GACTCATCTGAGTGAATTCAAGAGATTCACCTCAAGATGCTTTTTTTT-3' and MMP13shRev (5’-TCGAGAAAAGACACTCTTGGAGTTGAATCTCTGAGATTTTCAGATCAGTTCAATTTCTCAATTCAGATG-3’) and TIMP1shRev (5’-TGTTCAATCCCATCCCCGTAA-3’ (located in exon 6), and the primers used to generate this sequence were MMP13shFwd (5’-TGTTCAATCCCATCCCCGTAA-3’ and TIMP1shRev (5’-TCGAGAAAAGACACTCTTGGAGTTGAATCTCTGAGATTTTCAGATCAGTTCAATTTCTCAATTCAGATG-3’). The sequence of the scrambled shRNA used as a control was 5’-GTCAAGTCTCACCCTGGTGCT-3’ and the primers used to generate this sequence were scrFwd1 (5’-TGTCAAGTCTCACCCTGGTGCT-3’) and scrRev1 (5’-TCGAGAAAAGACACTCTTGGAGTTGAATCTCTGAGATTTTCAGATCAGTTCAATTTCTCAATTCAGATG-3’). Annealed, phosphorylated oligonucleotides were then ligated into HpaI/Xhol-cut psicoR vector, and clones with the appropriate size insert were selected for sequence analysis. One clone of each plasmid with the correct sequence was chosen and DNA prepared using a maxi-prep DNA kit (Qiagen).

To confirm the specificity of the shRNA knockdown, a second shRNA was created using the pPRIME system, which was generously supplied by the Elledge laboratory (31). The shRNA sequence was designed by pasting the TIMP1 canine mRNA sequence (GenBank™ accession number AY534616) into the program found at the RNAi Central website. The 22-mer shRNA sequence beginning at position 89, TGGAGAGCTCTTGCGGATATTC, was cloned into the p199 vector and then into a lentiviral delivery system for infection into MDCK T23 cells, followed by FACS sorting as described above.

Creation of MDCK shRNA Cell Lines—To prepare lentivirus-containing media, early passage HEK293T cells (ATCC) were transfected according to the calcium phosphate precipitation method with 12 µg of the shRNA-containing vector (either psicoR-MMP13sh, psicoR-TIMP1sh, psicoR-scr, or psicoR vector alone) along with 6 µg each of the packaging vectors pVSVG, pMDL, and pREV. After 48 h, 45 ml of lentivirus-containing media were harvested and concentrated in an ultracentrifuge at 16,000 × g at 4 °C for 2.5 h. The viral pellet was resuspended in phosphate-buffered saline and used to infect MDCK strain II or MDCK T23 cells plated at a concentration of 5 × 10^5 at a multiplicity of infection of 10–20. Efficiency of infection was determined by assessing GFP fluorescence using FACS analysis (FACSCalibur, BD Biosciences).

Cre Rescue—MDCK cells expressing MMP13 and TIMP1 knockdown shRNA were grown to 60% confluency and exposed to standard minimum essential medium containing 10 µl of

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Identification of Genes Regulated by HGF as Determined by Microarray Analysis—HGF-induced tubulogenesis in MDCK cells is a complex process requiring the coordination of diverse cellular processes such as proliferation, cytoskeletal rearrangement, motility, differentiation, and extracellular matrix secretion (32). Given that we and our colleagues previously showed, using broad inhibitors of the entire MMP family, that MMPs are necessary for the redifferentiation stage of tubulogenesis (7), we set out here to identify specific candidate MMP and TIMP genes regulated by HGF and their pathway of activation. Because of the large number of MMPs and TIMPs described to date (22), we decided to use nonbiased DNA microarray analysis as our initial screen. The recently released Canine Genome 2.0 Array (Affymetrix) contains 19 MMPs and 3 TIMPs (Fig. 1A), and importantly, all the MMPs and TIMPs that have been reported to be expressed in the kidney are found on the Canine Genome 2.0 Array (Fig. 1B). Our screen was accomplished by first determining the total set of genes regulated by HGF and then, because the MAPK/ERK pathway has been shown to play a central role in HGF-induced tubulogenesis, subtracting out the subset of genes that were still regulated by HGF in the presence of either of two

FIGURE 1. MMPs and TIMPs on the Canine Genome 2.0 Array. A, 19 MMPs and 3 TIMPs are represented on the Canine Genome 2.0 Array (Affymetrix). B, all of the MMPs and TIMPs reported to be expressed in the kidney in a recent review article (22) and, additionally, by our review of the literature (56, 57), are represented on the Canine Genome 2.0 Array.
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distinct inhibitors of the MAPK/ERK pathway, UO and PD, both of which act by preventing phosphorylation of MEK.

For these experiments, MDCK type II cells were grown on a Transwell filter coated with collagen for 6 days and treated according to one of four conditions as follows: exposure to recombinant HGF, to HGF + UO, to HGF + PD, or to standard medium (control). For each condition, performed in quadruplicate, the MDCK cells were exposed to the indicated treatment for a 24-h period, which corresponds to the completion of the p-EMT stage and the beginning of the redifferentiation stage of tubulogenesis. Therefore, all RNA necessary to initiate the redifferentiation stage of tubulogenesis should be present. RNA was harvested from the MDCK cells treated under each condition and then converted to cDNA, which was used to query the *C. familiaris* genes on the GeneChip® Canine Genome 2.0 Array. The details of how we performed the analysis can be found under “Materials and Methods.” We initially evaluated the list of genes that were differentially regulated by hierarchically clustering all genes that were present in at least 3 of the 15 individual samples (one sample in the HGF + UO set was lost during processing).

The resulting dendrogram shown at the top of Fig. 2A indicates that the replicates are very similar for each condition (exposure to recombinant HGF, to HGF + UO, to HGF + PD, or to standard medium) and, furthermore, that gene expression in the HGF + UO condition is similar to gene expression in the HGF + PD condition. The color in Fig. 2A depicts ratios of the expression of a gene in a single sample to the median of expression of that same gene across all the samples.

We defined differentially regulated as representing at least a 2-fold change and used a stringent false discovery rate of <0.01%. The list of genes that were differentially regulated by 24 h of exposure to HGF compared with control medium numbered 3,202 (1,651 genes were up-regulated and 1,551 genes were down-regulated), out of 18,439 total genes present.

Identification of Genes Regulated by HGF via the MAPK/ERK Pathway—Because the MAPK/ERK pathway has been shown to be a major pathway in HGF-induced tubulogenesis, we also wanted to determine what percentage of genes differentially expressed during HGF-induced tubulogenesis are controlled via this pathway. Using the list of genes that were differentially regulated by HGF, we identified those genes that were specifically regulated by HGF through the MAPK/ERK pathway using a process that we have termed subtraction pathway microarray analysis. A Venn Diagram approach was used to generate a list of genes that were up-regulated in MDCK cells by HGF via the MAPK/ERK pathway. This was done by identifying the genes that were up-regulated by HGF alone compared with the control medium and that were down-regulated in both the HGF + PD and HGF + UO conditions compared with HGF alone (Fig. 2B). Conversely, a list of genes that were down-regulated in MDCK cells by HGF via the MAPK/ERK pathway was generated by identifying the genes that were down-regulated by HGF alone compared with the control medium and that were up-regulated in both the HGF + PD and HGF + UO conditions compared with HGF alone (Fig. 2B). Using this strategy, we found that of the 1,651 genes up-regulated by HGF, 1,066 (~65%) were up-regulated specifically via the MAPK/ERK pathway. Of the 1,551 genes down-regulated by HGF, 1,039 (~67%) were down-regulated specifically via the MAPK/ERK pathway. The lists of known genes that were differentially regulated greater than 3-fold, compared with control, by HGF via the MAPK pathway are shown in supplemental Figs. 1 and 2.

**Enrichment for MAPK/ERK Pathway Genes Is Confirmed for Our Screen**—To confirm our enrichment of MAPK/ERK pathway genes, we used DAVID annotation tools. As these tools do not yet cover the canine genome, we first found probe sets from the human GeneChip HG-U133 Plus 2.0 (Affymetrix) that were orthologous to the 2,105 differentially expressed MAPK/ERK genes. We then uploaded this list of probe sets to DAVID and used the functional annotation tool to map the list against KEGG pathways. Our list was significantly enriched (p = 0.00242) for known MAPK/ERK genes.

**MMP and TIMP Genes Are Regulated by HGF via the MAPK/ERK Pathway**—The spatial and temporal expression of MMPs and TIMPs in the kidney is complex and not completely characterized. As noted, all of the MMPs and TIMPs that have been reported to be expressed in the kidney (Fig. 1B) are included on the Canine Genome 2.0 Array (22). Of the MMPs and TIMPs surveyed, we found MMP13 and TIMP1 to be up-regulated 198- and 169-fold, respectively, by HGF via the MAPK pathway (Fig. 3). Of the 1,651 genes significantly up-regulated by HGF, MMP13 and TIMP1 had the fourth and ninth highest fold changes, respectively. TIMP2 and TIMP3 were also slightly up-regulated via the MAPK pathway (Fig. 3).

**Confirmation of the Microarray Results**—To confirm the microarray results, we used real time (quantitative) PCR and three different assays. Real time PCR was performed in two-dimensional filter grown cells (Fig. 4A), in Raf-induced two-dimensional cultured cells (Fig. 4B), and in three-dimensional collagen-cultured cells (Fig. 4C). Raf induction involved use of an MDCK cell line in which MAPK pathway activation is con-

![](https://example.com/image3.png)

**FIGURE 3. MMP and TIMP genes differentially regulated by HGF via the MAPK pathway.** Of the 19 MMPs and 3 TIMPs represented on the Canine Genome 2.0 Microarray, only MMP13, TIMP1, TIMP2, and TIMP3 were significantly regulated by HGF (see “Materials and Methods” for a detailed description of the analysis). All of these genes were regulated via the MAPK/ERK pathway.
Additionally regulated (25). These cells (MDCK Raf:ER) stably express an inducible form of Raf-1 kinase, because of transfection with a plasmid construct containing a fusion of the Raf-1 kinase domain with the estrogen receptor ligand binding domain (33). Binding of the estrogen analog 4-hydroxytamoxifen (4-HT) to the estrogen receptor moiety activates the Raf-1 domain, which then leads to phosphorylation and activation of downstream MEK and ERK (7, 25).

Using real time PCR in two-dimensional filter-grown cells, in Raf-induced two-dimensional cultured cells, and in three-dimensional collagen-cultured cells, we confirmed that MMP13 and TIMP1 activation were dramatically increased by HGF via the MAPK pathway (Fig. 4). TIMP2 and TIMP3 were slightly increased by HGF via the MAPK pathway by microarray and real time PCR analysis of two-dimensional filter-grown MDCK cells, but not by real time PCR analysis after induction of Raf in two-dimensional culture nor by real time PCR analysis of MDCK cells grown in three-dimensional collagen (supplemental Fig. 3). No other MMPs were significantly regulated by HGF.

MMP13 and TIMP1 Inhibition Using shRNA—Given the hundreds-fold induction of MMP13 and TIMP1 by HGF and a lack of induction of TIMP2 and TIMP3 in MDCK Raf:ER cells and in MDCK cells grown in three-dimensional collagen, we focused our efforts on MMP13 and TIMP1. To examine the functional effects of MMP13 and TIMP1 activation during HGF-induced tubulogenesis in MDCK cells, we performed gene knockdown using an shRNA lentiviral vector system. Briefly, 19-mer shRNA sequences against either the canine MMP13 or TIMP1 genes were cloned into the psicoR vector (30), followed by lentiviral infection of MDCK cells with the resulting constructs. Two different control cell lines were simultaneously generated, one containing the psicoR plasmid without any shRNA insert (vector alone) and one containing a scrambled shRNA insert. Additional features of this system include the presence of a GFP reporter gene, whose expression is driven by a promoter distinct from that which drives shRNA expression, as well as loxp sites that flank both the shRNA and GFP genes, allowing for reversible and detectable removal of the plasmid from the MDCK genome.

Using this approach, we were able to obtain a mixed population of MDCK cells containing between 60 and 70% GFP-positive.
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MMP13 shRNA

| Treatment            | Control | MMP13 shRNA |
|----------------------|---------|-------------|
| None (14 Days Growth In Collagen) | ![](image1) | ![](image2) |
| HGF X 24 Hours       | ![](image3) | ![](image4) |
| HGF X 96 Hours       | ![](image5) | ![](image6) |

**FIGURE 5.** shRNA knockdown of MMP13 resulted in an inability to progress beyond the initial stage of p-EMT following HGF induction. A, MDCK cells were infected with lentivirus expressing the shRNA construct for MMP13 as described under “Materials and Methods.” The shRNA construct also encodes GFP, which is transcribed off a separate promoter. Following infection, the MDCK cells with high levels of GFP fluorescence were sorted as single cells using FACs. These cells were allowed to grow to confluence and were then split, and real time PCR was performed. Several clonal MMP13 cell lines are shown with knockdown levels normalized to the scrambled shRNA control (Scr). B, cells demonstrating MMP13 knockdown expressed endogenous GFP and grew in collagen to the cyst stage over 2 weeks in similar fashion to controls (scrambled shRNA or empty pcDNA vector). C, following induction with HGF, cells demonstrating MMP13 knockdown could not progress past the initial stage of p-EMT and showed only actin extensions. A higher magnification view shows chains of cells in the control cell cysts and only actin extensions in the MMP13 knockdown cells. D, following HGF induction for 96 h, control cell cysts showed fully developed tubules with lumens, whereas MMP13 knockdown cysts did not progress past p-EMT. Hoechst stain is a nuclear stain that is blue. Phalloidin-rhodamine is an actin stain and is red. GFP is part of the shRNA construct and is green. The white bar represents 20 μm.

Reference:

**MMP13 and TIMP1 Inhibition Leads to Defects in Cystogenesis and Tubulogenesis**—The clonal cell lines with the highest degree of MMP13 and TIMP1 knockdown (Fig. 5A and Fig. 6A) were then grown in three-dimensional collagen as described previously (21). Briefly, the cells were allowed to grow for 2 weeks to reach the cyst stage and then induced with HGF for 4 days to examine the effect of MMP13 and TIMP1 knockdown on cystogenesis and tubulogenesis. MMP13 knockdown cells formed normal appearing cysts (Fig. 5B) and nearly all (24/25 random cysts in a typical collagen well) initiated p-EMT following 24 h of stimulation by HGF; however, the cysts...
were unable to progress past the actin extension stage of p-EMT (Fig. 5C). Although control MDCK cysts were observed to demonstrate mature tubular lumen formation after 4 days of HGF stimulation (6/25 random cysts in a typical collagen well), MMP13 knockdown cysts exhibited an inability to progress past the first stage of p-EMT with no tubules containing true lumens present (Fig. 5D).

Although virtually all of the GFP-expressing cysts in a varied population of MMP13 knockdown cells displayed an inability to progress past the actin extension stage of p-EMT, most of the TIMP1 knockdown cysts in a varied population displayed normal tubulogenesis in response to HGF. Clonal cell lines were therefore isolated (Fig. 6A), and those cells with very high degrees of TIMP1 knockdown (TIMP1–10 and TIMP1–17 cells) demonstrated a dramatic decrease in “cyst” formation (only one to four cysts present in a typical collagen well). The few multicellular structures that did develop had a very abnormal appearance characterized by a lack of lumen formation. Following induction with HGF, actin extensions were occasionally seen (Fig. 6B, arrow), meaning that these structures could initiate tubulogenesis; however, no chains of cells, cords, or tubules were seen (Fig. 6B).

To rule out the possibility of a nonspecific effect of the MMP13 and TIMP1 knockdown shRNA, we generated additional knockdown cell lines using the pPRIME system (31) with different shRNA sequences and obtained similar results (data not shown). Finally, the phenotype of MMP13 and TIMP1 shRNA knockdown cell lines could be completely reversed by treatment of these cells with adenovirus expressing Cre recombinase, resulting in excision from the genome of both the GFP reporter gene and the specific shRNA sequence that is flanked by loxP sites (supplemental Fig. 4).

TIMP1 Knockdown Results in an Increase in Apoptosis—TIMP1 has been reported to have anti-apoptotic function that is independent of its MMP inhibitory activity (34). We therefore investigated apoptosis as a cause for the lack of cyst formation seen in the TIMP knockdown cells using antibodies against active caspase 3, a well described marker for apoptosis (29). We found that MDCK cells with the highest levels of TIMP1 knockdown expressed active caspase 3 by both immunofluorescence (Fig. 7A) and Western blot (Fig. 7B).

It has also been reported that cells that lack contact with the basement membrane are rendered susceptible to anoikis, a form of apoptosis (35, 36). We had noticed that TIMP1 knockdown cells adhered less well to the 10-cm culture dishes and could even be detached upon changing of the culture medium. To investigate this further, we performed a cell adhesion assay (37). This involved growing 100,000 MDCK cells on 2 mg/ml collagen (a concentration identical to that used for cyst growth in three-dimensional collagen) for 1 h, performing a series of washes, staining the attached cells with crystal violet, and fixing the cells with formalin. Attachment was quantified by color intensity. TIMP1–10 and TIMP1–17 cells (those with the greatest degree of TIMP1 knockdown) had decreased attachment to collagen compared with the control and other cell lines (Fig. 7C).

DISCUSSION

We report several principal findings here with broad relevance toward understanding the mechanisms of HGF-induced tubulogenesis in MDCK cells and, quite likely, branching morphogenesis in general. First, we identified a large group of genes that were specifically regulated by HGF via the MAPK/ERK pathway following binding of HGF to its CMET tyrosine kinase receptor. We found that 3,202 (17%) of the 18,349 genes represented on the GeneChip® Canine Genome 2.0 Array were differentially regulated by HGF. This is not totally surprising given that HGF is mitogenic, motogenic, and morphogenic in general. First, we identified a large group of genes that were specifically regulated by HGF via the MAPK/ERK pathway following binding of HGF to its CMET tyrosine kinase receptor. We found that 3,202 (17%) of the 18,349 genes represented on the GeneChip® Canine Genome 2.0 Array were differentially regulated by HGF. This is not totally surprising given that HGF is mitogenic, motogenic, and morphogenic in general. First, we identified a large group of genes that were specifically regulated by HGF via the MAPK/ERK pathway following binding of HGF to its CMET tyrosine kinase receptor. We found that 3,202 (17%) of the 18,349 genes represented on the GeneChip® Canine Genome 2.0 Array were differentially regulated by HGF. This is not totally surprising given that HGF is mitogenic, motogenic, and morphogenic in general.
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A  

GFP  Caspase 3  Merge  

Scr  
TIMP1-10  
TIMP1-17  
MMP13-9  

B  

Kd  
30  18  Active Caspase 3  

C  

Color Intensity (nmolatinol units)  
Cell Type  

Scr  TIMP1-10  TIMP1-17  

FIGURE 7. shRNA knockdown of TIMP1 resulted in increased apoptosis and decreased cell adhesion to collagen. A, as early as 5 days of growth in three-dimensional collagen, MDCK cells with high degrees of TIMP1 knockdown showed markedly increased apoptosis as seen by active caspase 3 immunofluorescence staining. Following fixation and staining of the collagen gels, images were obtained using the exact same optical settings (×40 lens and 20-ms exposure, on an Olympus IX70 microscope using IPLab imaging software). Green immunofluorescence is GFP from the shRNA construct; red immunofluorescence is active/cleaved caspase 3 (rabbit anti-caspase 3 primary antibody, followed by Cy3-conjugated donkey anti-rabbit secondary), with the third panel being the merged images. In the TIMP1–17 images, two additional nascent cysts, also with high levels of caspase 3 immunofluorescence staining, are present in different planes of focus. The white bar represents 25 μm. B, MDCK cells with high levels of TIMP1 knockdown (TIMP1–10 and TIMP1–17), control cells with scrambled shRNA (Scr), and control cells with empty vector (psicoR) were grown to confluence in 10-cm tissue culture dishes. Equal amounts of protein, as determined by BCA assay, were loaded in each well, and Western blot using antibody against active/cleaved caspase 3 was performed. Higher levels of active/cleaved caspase 3 are seen in the TIMP1 knockdown compared with the control cells. C, MDCK TIMP1 knockdown cells, MMP13 knockdown cells, and control cells with scrambled shRNA (Scr) were grown to confluence in 10-cm tissue culture dishes. Microtiter plates were coated with collagen at 2 mg/ml (the same concentration used for the tubulogenesis assay). 100,000 cells were added in triplicate to the 96-well plates and incubated for 1 h at 37 °C. Nonadherent cells were removed by washing the wells. Cells were then fixed with 3.7% formaldehyde, stained with 1% crystal violet, and solubilized in 20% acetic acid, and the optical density was measured and quantified with NIH Image J software. Cells with the highest degree of TIMP1 knockdown (TIMP1–10 and TIMP1–17) showed decreased adhesion to collagen. *, p < 0.05; **, p < 0.01. This assay was repeated three times, and a typical analysis is shown.
has been reported that TIMP1 has anti-apoptotic function mediated by phosphatidylinositol 3-kinase and focal adhesion kinase that is independent of its MMP inhibitory activity (34, 39). Consistent with reports showing TIMP1 having an anti-apoptotic function, we found that apoptosis was increased in TIMP1 knockdown cells, as determined by expression of the apoptotic marker active caspase 3 (29). TIMP1 knockdown cells also had decreased adherence to collagen, rendering them susceptible to anoikis, a form of apoptosis (35, 36, 39). During cystogenesis, apoptosis is carefully regulated with centrally located cells undergoing programmed cell death characterized by the expression of active caspase 3 (40). Increased apoptosis and subsequent disruption of this regulated process could be consistent with a phenotype of small aggregations of cells lacking lumens. We propose that the increased apoptosis in MDCK cells with high degrees of TIMP1 knockdown accounts for the abnormal cystogenesis and tubulogenesis phenotypes observed in the three-dimensional collagen growth assay.

Branching morphogenesis of tubular epithelium is a common and important feature of vertebrate organogenesis; examples include collecting ducts of the kidney, the airways of the lung, and milk ducts of the mammary gland (4, 6, 41). The MAPK/ERK pathway has been shown to be important in branching morphogenesis in different organs and, indeed, in very different organisms. For example, the MAPK/ERK pathway is essential for branching of the Drosophila tracheal system following binding of the fibroblast growth factor-like molecule, Branchless, to its receptor tyrosine kinase Breathless (42). Importantly, it has been shown that active ERK regulates branching morphogenesis in the developing kidney and that PD09859 reversibly inhibits branching in a dose-dependent manner (41). Finally, we showed in renal MDCK cells grown in a collagen matrix and stimulated with HGF that the MAPK/ERK pathway is necessary and sufficient for the initial p-EMT stage of tubulogenesis (7, 21).

Regarding the specific biological relevance of MMP13 and TIMP1 to kidney development in vivo, both MMP13 and TIMP1 have been localized to the kidney (22). From review of the literature, although MMP13 expression has not been studied in the developing kidney, TIMP1 expression was detected in the developing mouse kidney at embryonic day 11.5 (E11.5), which is very soon after the initiation of metanephric kidney development at E11. Expression peaked at E16.5 and then decreased and persisted in the adult kidney at lower levels (43). It should be noted that neither MMP13 (44–47) nor TIMP1 (48–51) knock-out mice displayed a relevant kidney phenotype; however, in all the 19 MMP and TIMP single knockouts generated to date, surprisingly subtle phenotypes were described, with all knock-out lines surviving until birth (52). Possible explanations why MMPs and TIMPs do not seem to be essential for development, as determined by single knock-out experiments, include redundancy, compensation, and adaptive development. Indeed, MMPs have many overlapping substrates in vitro, which could indicate genetic redundancy in vivo (52, 53). In fact, redundancy has been demonstrated for Mmp13 mutant mice. Mmp13−/− mice displayed a mild skeletal defect that resolved by 12 weeks, whereas Mmp13−/−, Mmp9−/− double knock-out mice had severe skeletal defects that resulted in drastically shortened bones (46). Compensation has also been shown in the MMP family (52, 54, 55).

It may well be that other MMPs and TIMPs are involved in branching morphogenesis in vivo, possibly depending on the individual organ and the composition of the matrix. However, by using a reductionist approach, we have identified MMP13 and TIMP1 as being necessary for branching morphogenesis in MDCK cells grown to the cyst stage in a three-dimensional collagen matrix and exposed to HGF, suggesting a key role for these molecules in kidney development that may have been otherwise masked by redundancy in in vivo models.

In conclusion, although a number of different intracellular signaling cassettes have been implicated in HGF-induced tubulogenesis in MDCK cells (20), our data strongly suggest that the MAPK/ERK pathway is the predominant pathway in MDCK cells for both the p-EMT and redifferentiation stages of tubulogenesis, and that MMP13 and TIMP1 are necessary for redifferentiation in this model (Fig. 8). It is likely that other branched organs similarly use the MAPK/ERK pathway to activate MMPs and TIMPs during tubulogenesis.

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