Expression of Neutrophil Gelatinase-associated Lipocalin Regulates Epithelial Morphogenesis in Vitro*

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Growth factors such as hepatocyte growth factor (HGF) are highly up-regulated during development and following renal injury and are known to induce marked morphogenic actions in cultured tubular epithelial cells, including scattering, migration, single cell branching morphogenesis, and multicellular branching tubulogenesis. In the present study, we demonstrate that HGF stimulates epithelial cells to express neutrophil gelatinase-associated lipocalin (Ngal), a member of the lipocalin family of secreted proteins that has recently been shown to participate in mesenchymal-epithelial transformation via its ability to augment cellular iron uptake. At concentrations below those found to mediate iron transport, purified Ngal can induce a promigratory and probranching effect that is dependent on ERK activation. The suppression of Ngal expression using short hairpin RNA results in increased cyst formation by tubular cells. However, the simultaneous addition of Ngal and HGF leads to direct association of the two proteins, and results in a partial inhibition of HGF-stimulated activation of c-Met and the downstream MAPK and phosphatidylinositol 3-kinase signaling pathways. This inhibitory effect down-regulates HGF-stimulated single cell migration, and limits branching morphogenesis at both the single cell and multicellular level. These experiments demonstrate that the local expression of Ngal can play a regulatory role in epithelial morphogenesis by promoting the organization of cells into tubular structures while simultaneously negatively modulating the branching effects of HGF.

Following renal tubular injury and during kidney development, secreted growth factors such as hepatocyte growth factor (HGF) are highly expressed and appear to play important roles in stimulating epithelial cell migration, morphogenesis, and tubulogenesis (1, 2). To induce these events, HGF activates signaling pathways involved in actin cytoskeletal rearrangement, cell-matrix attachment, and cell-cell interactions (3–5). In vitro, activation of these pathways stimulates the loss of cell-cell contact (scattering), marked increases in cell migration, and the formation of tubules with multiple branch points. However, in vivo, these responses must be carefully regulated to prevent the formation of abnormal tubular architecture during development and repair.

To identify the factors that epithelial cells utilize for the regulation of HGF-stimulated morphogenesis, we have recently performed a microarray analysis of renal inner medullary collecting duct cells stimulated with HGF (6). One of the messages found to be markedly up-regulated was for neutrophil gelatinase-associated lipocalin (Ngal), a member of the lipocalin family of proteins that is also known as SIP24, 24p3, or lipocalin2. Lipocalins are a diverse family of small secreted proteins that generally bind small hydrophobic ligands, soluble extra cellular macromolecules, and possibly specific cell surface receptors (7–9). Ngal was first isolated as 24p3, an mRNA that was up-regulated in SV-40 infected primary kidney cells (10), and the 24-kDa protein product was later identified and found to be an acute phase reactant secreted by the liver that could also be induced in other organs and a variety of cultured cells by exposure to serum, prostaglandins, LPS, and cytokines such as human fibroblast growth factor and II-1 (11–13).

Despite the multiple stimuli that can induce Ngal expression, at present the functional role of this protein is not well understood. Based on its ability to bind compounds in the extracellular space, one major function appears to be either stabilizing/sequastering these bound molecules or facilitating their transport into the cell. Ngal has been found to associate with and stabilize such proteins as MMP9 in neutrophils (14, 15), whereas it has more recently been shown to bind bacterial siderophores and thereby act as an antimicrobial agent by sequestering iron (16, 17). The iron-binding capacity of Ngal has also been shown to facilitate iron delivery into cells, thus potentially playing an important role in the regulation of iron-sensitive genes that participate in mesenchymal-epithelial transition during development of the proximal parts of the mammalian nephron (18). However, mice lacking Ngal expression are born in normal numbers and have no apparent phenotype when housed in the absence of bacteria (17), suggesting that kidney development is not markedly abnormal, although this has not yet been examined morphologically. The possibility that Ngal may play an important role in the adult kidney has been suggested from the observation that it is highly up-regulated following transient renal ischemia (19) and that an infusion of Ngal can protect against ischemia-mediated tubular injury in the mouse (20).

In the present study, we demonstrate that HGF can increase Ngal secretion and that Ngal can stimulate renal tubular cell...
ERK activation accompanied by a modest increase in cell migration and branching morphogenesis. These effects appear to be physiologically relevant, because knockdown of Ngal expression results in increased cyst formation by tubular cells grown in a three-dimensional matrix. Interestingly, we show that Ngal can bind directly to HGF, thereby diminishing HGF-stimulated c-Met receptor phosphorylation and downstream signaling. These effects are seen within minutes of Ngal addition, at nanogram concentrations of Ngal, and independent of iron-loading, distinguishing them from the effects seen in mesenchymal-epithelial transition. The Ngal-mediated decrease in HGF signaling results in a significant inhibition of HGF-stimulated cell migration and single cell branching morphogenesis, suggesting that up-regulation of Ngal following renal injury is likely to play a modulatory role in tubular cell responses to HGF.

**EXPERIMENTAL PROCEDURES**

**Materials**—The mouse inner medullary collecting duct (mIMCD-3) cells are derived from the mouse inner medullary collecting duct (21) and were a generous gift from D. M. Dubcova (DMEM/F12) with 10% fetal calf serum and used to passage 17 and passage 30. Anti-ERK 1/2, anti-phospho-ERK 1/2, and anti-Akt antibodies were purchased from Cell Signaling Technologies (Beverly, MA), anti-HGFα antibody from SantaCruz Biotechnology, and anti-phosphoMet (Tyr75)-1234 and -1235) antibody from Upstate Biotechnology (phosphorylation of Tyr-1234 and Tyr-1235 has been shown to be critical for the activation of met receptor kinase activity (22)). The anti-Ngal rabbit polyclonal antibody has been described previously (18). Type I collagen was purchased from Upstate Biotechnology (Lake Placid, NY). HGF, Protein A-Sepharose, and tri-reagent were purchased from Sigma. Polyvinylidene difluoride membranes were from Millipore (Billerica, MA). Protein was induced with 0.5 mM isopropyl-1-thio-galactoside at 20 °C. The top compartment was connected, and 1.5 ml of the mixture was pre-equilibrated with 0.45 mM extraction buffer. (Amersham Biosciences) plus added iron, or in membrane was removed by centrifugation, and the supernatant protein was concentrated using the Centricon Filter Unit (Millipore) as per manufacturer's instructions. The protein content was determined using the Bradford assay, and 20 μg of concentrated supernatant protein was used unless otherwise noted. Samples were boiled for 5 min in SDS sample buffer containing β-mercaptoethanol and separated via SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes, blocked with 5% milk, TBST (100 mM Tris, pH 7.5, 0.9% NaCl, 0.1% Tween 20) and hybridized with the Ngal antibody at 1:1000 dilution in a 1% milk, TBST solution. The visualization of proteins was accomplished with horseradish peroxidase-conjugated secondary antibodies and ECL (Amersham Biosciences).

**Stable Knockdown of Ngal Expression by shRNA**—Expression of murine Ngal was stably suppressed using the GeneSuppressor Encoding System (Imgenex). Briefly, 2 primer pairs were synthesized, 1 pair encoding nucleotides 192–213 followed by a 9-base “loop” and the inverted repeat (shRNA1) and the second encoding nucleotides 352–373, again followed by the loop and inverted repeat at 40 ng/ml for up to 8 days with a media change every 3 days unless otherwise noted. Representative structures were photographed at 20× (for the long term assay) or 40× (for the single cell branching assay) using a Nikon microscope with Hoffman modulation. Images were obtained using a SpotRT digital camera and processed using the Photoshop software. 

**HGF/EOP Signaling Experiments**—Mouse mIMCD-3 epithelial cells were lysed in radioimmune precipitation buffer (Amersham Biosciences) plus added iron, or in membrane was removed by centrifugation, and the supernatant protein was concentrated using the Centricon Filter Unit (Millipore) as per manufacturer's instructions. The protein content was determined using the Bradford assay, and 20 μg of concentrated supernatant protein was used unless otherwise noted. Samples were boiled for 5 min in SDS sample buffer containing β-mercaptoethanol and separated via SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes, blocked with 5% milk, TBST (100 mM Tris, pH 7.5, 0.9% NaCl, 0.1% Tween 20) and hybridized with the Ngal antibody at 1:1000 dilution in a 1% milk, TBST solution. The visualization of proteins was accomplished with horseradish peroxidase-conjugated secondary antibodies and ECL (Amersham Biosciences).

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incubating serum-starved cells for the indicated time in serum-free antibodies and ECL. A 1:3000 dilution in 5% milk, TBST solution. Visualization of proteins NaCl, 0.1% Tween 20), and hybridized with the appropriate antibody at night. Immunoprecipitates were collected by adding protein-Sepharose IB immunoblotting (18) and washed 3 times with TBST and solution. SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes, blocked with 5% milk, TBST (100 mM Tris, pH 7.5, 0.9% NaCl, 0.1% Tween 20), and hybridized with the appropriate antibody at a 1:3000 dilution in 5% milk, TBST solution. Visualization of proteins was accomplished with horseradish peroxidase-conjugated secondary antibodies and ECL.

**HGF/Ngal Interaction**—Conditioned supernatants were collected by incubating serum-starved cells for the indicated time in serum-free DMEM/P12 followed by centrifugation at 15,000 rpm for 10 min to remove cell debris. The supernatant was then incubated with or without HGF (40 ng/ml) for 1 h, and anti-HGF Ab was added overnight. Immunoprecipitates were collected by adding protein-Sepharose A and washed 3 × with radioimmune precipitation assay buffer, and associated proteins were separated by SDS-PAGE, transferred to Immobilon-P (Millipore) membrane, immunoblotted with anti-Ngal antibody, and visualized with ECL. For purified Ngal association, 1 μg of Ngal was incubated with or without HGF (1 μg) in modified radioimmune precipitation assay buffer (lacking SDS) overnight in the presence of the anti-HGF Ab and collected and analyzed as above. For detecting direct association of HGF and Ngal, purified HGF was isolated by SDS-PAGE, transferred to an Immobilon-P membrane followed by incubation with 1 μg/ml of purified Ngal in 5% milk, TBST overnight at 4 °C. The membrane was washed 3 times with TBST and hybridized with anti-Ngal antibody overnight at 4 °C followed by visualization with ECL. The membrane was stripped with β-mercaptoethanol strip solution (1% β-mercaptoethanol, 2% SDS, 62.5 mM Tris, pH 6.8) followed by incubation with anti-HGF Ab antibody.

**RESULTS AND DISCUSSION**

**Up-regulation of Expression of Ngal Following HGF Stimulation**—Microarray analysis was performed on RNA obtained from serum-starved mIMCD-3 cells stimulated for 24 h with HGF using the NIA15K mouse array. Three separate experiments revealed a 5.0 ± 1.0-fold up-regulation of the message for the secreted lipocalin protein Ngal. To determine whether HGF up-regulated the expression of the Ngal protein, supernatants were collected over 24-h time periods from serum-starved confluent cells after 48 and 72 h of HGF stimulation. Supernatants collected at 48 h revealed a large amount of Ngal even in the absence of HGF treatment (the concentration of Ngal in the supernatant reaches ~100–250 ng/ml by 48 h based on comparison with purified Ngal, data not shown), with a modest increase in the amount of Ngal detected in the supernatants from HGF-treated cells (Fig. 1A). Following 72 h of serum starvation in the absence of HGF there was a substantial decline in basal levels of Ngal secretion, whereas HGF-stimulated cells expressed an ~4-fold greater amount of Ngal at this later time point. These results confirm that Ngal is secreted by IMCD cells in response to serum (as has been shown in multiple other cell types) and demonstrate that HGF can increase both the message expression and the level of secretion of the Ngal protein.

**Ngal Stimulates Epithelial Cell Migration and Tubulogenesis**—Following renal injury, surviving epithelial cells undergo a process of dedifferentiation into a more mesenchymal phenotype, followed by enhanced cell spreading, migration, and eventual reconstitution of a tubular morphology. Earlier observations that HGF is markedly up-regulated by acute renal injury (2, 25), coupled with more recent data demonstrating that Ngal is also highly expressed in the kidney following renal ischemia (19), led us to investigate the possibility that Ngal is involved in regulating the morphogenic responses of tubular cells. Ngal is of particular interest in renal tubule cell morphogenesis, because it has recently been shown to be capable of binding and stabilizing extracellular proteins such as MMP9 (thereby potentially increasing matrix degradation and subsequent cell migration and tubule formation) (15) as well as facilitating intracellular iron delivery, thus playing a regulatory role in iron-dependent gene transcription during mesenchymal-epithelial transition in kidney development (18). To determine whether Ngal can regulate epithelial cell morphogenesis, the non-iron-loaded protein was purified from BL-21 E. coli lacking siderophore expression as described previously (Fig. 1B) (18). Cell migration was measured in mIMCD-3 cells that had been washed to remove any endogenous Ngal and then stimulated with increasing concentrations of purified Ngal for 4 h. Because the amount of Ngal secreted into the cell supernatant was estimated to be ~100–250 ng/ml at 48 h of culture, we examined doses between 1 and 100 ng/ml. The rate of secretion of Ngal by IMCD cells is sufficiently slow enough to require ~18–24 h of culture before the protein is detected in the supernatant of fully confluent cells, so the endogenously secreted protein is unlikely to significantly affect the net concentration of Ngal in experiments lasting less than 24 h. At doses from 10 to 60 ng/ml, Ngal stimulated epithelial cell migration, with a peak effect at 20–40 ng/ml (Fig. 2A, quantitated in B). Interestingly, concentrations >60 ng/ml resulted in a decrease in cell migration, whereas concentrations ≤1 ng/ml had no effect.

The effects of Ngal on cell migration were reproduced using Ngal purified from siderophore positive XL-1B bacteria (either in the iron-loaded or iron-deficient state), as well as iron-loaded Ngal purified from BL-21 bacteria in the presence of exogenous siderophore and iron (data not shown). This suggests that the migratory effect of Ngal is independent of its iron-transporting capacity. Further support for this idea comes from the observation that the concentration of Ngal mediating the greatest migratory effect (20–60 ng/ml) is significantly below the microgram concentrations needed to demonstrate an iron-dependent transcriptional effect (18).

The observation that both HGF and Ngal were up-regulated following renal injury, and that HGF could directly stimulate Ngal secretion, suggested that cells were likely to be exposed to both factors simultaneously. We therefore tested the effect on cell migration of the addition of both factors. Surprisingly, the addition of Ngal consistently diminished HGF-stimulated cell migration toward the level seen with Ngal alone (Fig. 2C).

Cell migration involves the coordinated regulation of actin cytoskeletal rearrangement with extension of lamellipodia and focal adhesion turnover at the leading edge, which are events similar to those required for in vitro tubule formation. We therefore examined the ability of Ngal to stimulate single cell process formation and multicellular tubule formation in cells
cultured in a three-dimensional matrix. Again, cells were washed to remove endogenous Ngal and then suspended in a type 1 collagen matrix. After 24 h of treatment with purified Ngal, there was a modest but statistically significant increase in single cell process formation (Fig. 3A, quantitated in B). However, similar to the effects seen in migrating cells, the simultaneous addition of Ngal and HGF resulted in a decrease of HGF-stimulated branching morphogenesis toward that seen with Ngal alone.

When mIMCD-3 cells were cultured for 8 days in a Matrigel/collagen mixture, they proliferated and organized into a mixture of multicellular cysts and some tubular structures in the absence of HGF, whereas the predominant type of structure seen following HGF treatment was branching tubules with central lumens (Fig. 3C). In contrast, the culture in the presence of 40 ng/ml Ngal without added HGF led to the formation of multiple elongated structures without detectable central lumens (Fig. 3C, b, occasional cysts were also observed under these conditions), whereas co-culture with Ngal and HGF resulted in a phenotype of elongated multicellular structures with fewer branches than those seen with HGF alone and the absence of lumens in the majority of cases. Cumulatively, the

**Fig. 2.** Ngal stimulates single cell migration. A, a modified Boyden chamber was used to measure cell migration, and representative fields were photographed at 40× magnification. Arrowheads show the membrane pores, arrows demonstrate cells that have migrated through the membrane. B, quantitation of a dose-response curve for Ngal-induced single cell migration. C, quantitation of the migration response to Ngal with or without HGF. This figure is representative of three independent experiments.

**Fig. 3.** Ngal regulates HGF-stimulated branching morphogenesis and tubulogenesis. A, mIMCD-3 cells were incubated for 24 h in a collagen matrix in control medium (a); +40 ng/ml Ngal (b); +40 ng/ml HGF (c); or in the presence of HGF + Ngal (d). Representative cells were photographed at 40× magnification. B, quantification of branches/cell as in A. This figure is representative of three different experiments.* $p < 0.01$ versus control; ** $p < 0.001$ versus HGF alone. H+N, Ngal + HGF. C, mIMCD-3 cells were incubated for 8 days in a collagen/Matrigel mixture in control medium (a); +40 ng/ml Ngal (b); +40 ng/ml HGF (c); or in the presence of HGF + Ngal (d). Representative multicellular structures were photographed at 20× magnification.
results of these phenotypic experiments reveal that Ngal has a moderate promorphogenic effect when given alone but acts to moderate the more vigorous migratory and branching effects of HGF.

**Ngal Stimulates Transient ERK Activation**—We have demonstrated previously that cell migration and branching morphogenesis stimulated by HGF and EGF require the activation of the MAPK pathway (5, 26). The ability of Ngal to activate MAPK was therefore examined in cells stimulated with Ngal as compared with HGF. At concentrations that induced cell migration, Ngal addition resulted in a rapid, transient activation of ERK1/2 as judged by immunoblotting with an antibody specific for the phosphorylated form of the proteins (Fig. 4A, quantitated in B). ERK activation was maximal at 10 min (at which point it was 2.5-fold greater than baseline, but was still less than 50% of that seen with HGF) and returned to baseline by 30–60 min. To determine whether Ngal-mediated ERK activation was critical for cell migration, cells were treated with or without the MEK inhibitor U0126 followed by stimulation with Ngal. These experiments revealed that Ngal-mediated cell migration was completely inhibited following treatment with U0126 (Fig. 4C).

The rapid nature of this signaling effect suggests that Ngal may be directly binding to a specific cellular receptor, as has been previously suggested for tear lipocalin (lipocalin-1) (9). However, the lipocalin-1 receptor identified by Wojnar et al. (9) is believed to mediate internalization and degradation of lipocalin, and no direct signaling pathway has been identified downstream of this receptor to our knowledge. In addition, the ability of Ngal to bind to other molecules makes it difficult to exclude the alternative possibility that, despite our purification procedure and the absence of other detectable bands on Coomassie-stained gels, the Ngal has associated with a bacterially produced molecule that is itself mediating the modest ERK activation.

**Ngal Inhibits HGF but Not EGF Signaling**—Our observations that Ngal diminished HGF-stimulated cell migration and tubule formation led us to examine the possibility that Ngal might inhibit HGF-dependent cell signaling. Cells were treated with HGF alone or HGF that was preincubated with Ngal for 10 min prior to addition to the cells. Although HGF alone resulted in a 7-fold increase in ERK activation and Ngal alone again caused a 2.5-fold stimulation, the mixture of Ngal and HGF resulted in a level of ERK activation that was 60% less than that seen with HGF alone (Fig. 5A, quantitated in B). Similarly, stimulation of the phosphatidyl inositol 3-kinase pathway (as judged by an antibody to the phosphorylated/activated form of the downstream kinase Akt) was diminished in cells treated with the HGF-Ngal mixture as compared with HGF alone (Fig. 5C, quantitated in D). Inhibition of the activation of both pathways led us to examine the level of phosphorylation of the c-Met receptor itself. Using a phosphospecific antibody that recognizes the activated receptor, the combination of Ngal and HGF was found to markedly diminish activation of the receptor as compared with treatment with HGF alone (Fig. 5E, quantitated in F). To determine whether these inhibitory effects of Ngal occurred with other morphogenic stimuli, we examined the effect of Ngal on EGF-stimulated cell signaling. In contrast to the results seen when Ngal was mixed with HGF, Ngal failed to inhibit EGF-stimulated ERK activation (Fig. 5G, quantitated in H) or Akt activation (Fig. 5I, quantitated in J). Of note, our cell lysis conditions primarily solubilize cytosolic- and membrane-associated proteins, with poor solubilization of nuclear proteins. The translocation of much of the activated ERK into the nucleus therefore results in a decrease in total ERK in the cell lysates following EGF stimulation (Fig. 5G, lanes 2 and 4) and HGF stimulation (A, lane 2). Consistent with the observed decrease in ERK activation following stimulation with HGF and Ngal, total ERK in the lysate is not significantly diminished in this case (Fig. 5A, lane 4).

While performing these experiments, we compared the effects of premixing the Ngal and HGF and then adding the mixture to the cells versus preincubating the cells in Ngal followed by the addition of HGF. We found that premixing the Ngal and HGF had the greatest inhibitory effect on HGF signaling (data not shown), suggesting that Ngal might selectively bind or degrade HGF. To test this possibility, we examined the
ability of HGF and Ngal to associate in solution and by overlay. As previously shown, supernatants from confluent serum-starved cells cultured for 36 h contained large amounts of Ngal (Fig. 6A, lane 4). The addition of HGF to this conditioned supernatant resulted in the co-immunoprecipitation of HGF and Ngal (Fig. 6A, lanes 2 and 3), as did incubation of the purified bacterially expressed Ngal with HGF (Fig. 6B, lane 2).

In no experiments did we detect evidence of degradation of HGF following association with Ngal. Immobilization of purified HGF by SDS-PAGE and transfer to Immobilon-P, followed by overlay with Ngal, confirmed that HGF and Ngal can directly interact (Fig. 6C).

The ability of either bacterially expressed or endogenous Ngal to associate with HGF, coupled with the observation that the purified Ngal can inhibit HGF-stimulated Met receptor activation and signaling, led us to investigate the possibility of HGF-induced cell signaling. A–F, whole cell lysates were obtained from serum-starved mIMCD-3 cells stimulated for 10 min with either 40 ng/ml HGF, 40 ng/ml Ngal, or a mixture of HGF and Ngal (H+N, preincubated for 10 min before stimulation). Lysates were immunoblotted (IB) with antibodies to activated ERK1/2 (A, α-pERK), to activated Akt (C, α-pAkt), or to activated c-Met (E, α-pmet) followed by blotting with an antibody to the appropriate protein to determine equality of loading. Quantitation of five experiments was performed for normalized ERK activation (B), Akt activation (D), and Met activation (F) as described. *, p < 0.01 versus control; **, p < 0.05 versus HGF alone.

G–J, cells were stimulated with either EGF (20 ng/ml), Ngal (40 ng/ml), or EGF + Ngal (E+N) as above. Whole cell lysates from five separate experiments were analyzed for ERK activation (G, quantitated in H) and Akt activation (I, quantitated in J). *, p < 0.01 versus control; ***, p = ns versus HGF alone.
**Fig. 6. Ngal associates with HGF.**

**A**, conditioned media were obtained by incubating serum-starved mIMCD-3 cells in DMEM/F12 for 0, 24, or 36 h (36 h supernatant (Sup.) is loaded in lane 4 to demonstrate secretion of Ngal by the cells). HGF was then added to the conditioned media, and associated proteins were immunoprecipitated (IP) overnight with α-HGF and separated by SDS-PAGE and immunoblotted (IB) with anti-Ngal. B, incubation of purified HGF with or without purified Ngal followed by immunoprecipitation of HGF (lanes 1 and 2) resulted in co-immunoprecipitation of HGF and Ngal (lane 2). The HGF antibody incubated with Ngal in the absence of HGF failed to bring down Ngal (lane 3). Lane 4 is loaded with 100 ng of purified Ngal. C, purified HGF was isolated on SDS-PAGE and transferred to Immobilon-P prior to overlay with purified Ngal and immunoblotting with α-Ngal (lane 2) followed by strip and reprobe with α-HGF (lane 1). D, conditioned media (CM) were prepared by incubation of mIMCD-3 cells in serum-free DMEM/F12 for 48 h. Fresh, serum-starved mIMCD-3 cells were washed and stimulated for 10 min with HGF diluted to a final concentration of 40 ng/ml in either conditioned media or control media, followed by cell lysis and immunoblotting for activated c-Met (α-pmet) and total Met (α-met). E, fully confluent parental mIMCD-3 cells (C), cells expressing both shRNA1 and shRNA2 (1+2), and cells expressing the scrambled shRNA (Sc), were cultured for 48 h in serum-free medium, 100 µl of the supernatant was separated by SDS-PAGE, and Ngal expression was detected by immunoblotting. Cell lysates from each cell type were immunoblotted with α-ERK to demonstrate equal numbers of cultured cells. WCL, whole cell lysates. F, wild-type mIMCD-3 cells were suspended in a collagen matrix overlaid with either fresh DMEM/F12 media (FM), conditioned media obtained from fully confluent mIMCD-3 cells (CM+Ngal), or conditioned media obtained from fully confluent mIMCD-3 cells expressing shRNA1 and shRNA2 (CM-Ngal) with or without HGF. Branches/cell were quantitated at 24 h. n = 3 wells counted, this experiment is representative of six separate experiments. G, wild-type mIMCD3 cells, cells stably expressing shRNA1 and shRNA2 (Ngal knockdown), and cells stably expressing the scrambled shRNA (scrambled) were cultured in a Matrigel/collagen matrix in the presence of HGF without media change. Representative fields were photographed on day 6 at 40×. Arrows demonstrate cysts in the Ngal knockdown cells.
that Ngal secreted by epithelial cells can act in an autocrine fashion to regulate HGF signaling. To test this, conditioned media containing Ngal were obtained by culturing serum-starved mIMCD-3 cells for 48 h in DMEM-F12 followed by centrifugation to remove cell debris. HGF (40 ng/ml) was then added to either the conditioned media (thus allowing HGF to bind to the Ngal in the conditioned media) or control DMEM/F12 prior to stimulation of mIMCD-3 cells for 10 min. Immunoblotting of cell lysates revealed an ~50% decrease in c-Met activation by the HGF added in conditioned media as compared with that added in the control media (Fig. 6D). To determine whether this inhibition of HGF signaling resulted in an alteration in the phenotypic response to HGF, mIMCD-3 cells suspended in type I collagen were stimulated with HGF suspended in either fresh or conditioned media, and cell branching was quantitated at 24 h. Similar to the results with purified Ngal (Fig. 3B), conditioned media significantly decreased HGF-stimulated cell branching (Fig. 6F). However, because conditioned media is likely to contain multiple secreted factors in addition to Ngal, we developed cell lines stably expressing two shRNA constructs (shRNA1 and shRNA2) to selectively knock down Ngal expression in the conditioned media (Fig. 6E). Conditioned media obtained from these cells failed to inhibit HGF-stimulated cell branching (Fig. 6F).

To examine the importance of endogenous Ngal secretion on the long term phenotype of tubule formation, wild-type mIMCD-3 cells were compared with cells in which Ngal expression had been stably knocked down using shRNA1 and 2 and to cells expressing the same vector containing a scrambled shRNA. The cells were cultured for 6 days in 2% serum with HGF without a medium change to allow Ngal accumulation in the supernatants of the parental and scrambled cell lines (confirmed by Western blot, data not shown). Interestingly, although wild-type and scrambled shRNA control cells formed elongated cords/tubules under these conditions, the Ngal knockdown cells formed a mixture of tubular structures and cysts (Fig. 6G). The quantification of individual structures revealed that ~65% of the multicellular structures formed by the cells lacking Ngal secretion were cysts, whereas greater than 90% of structures were branching tubules or cell cords in wild-type cells and cells expressing the scrambled shRNA. Thus, it appears that Ngal serves to limit HGF-stimulated morphogenic signaling but is itself required for efficient tubulogenesis.

In conclusion, Ngal is secreted by renal tubular cells in response to serum or HGF and can bind HGF in solution. This association of Ngal and HGF appears to be independent of the iron-binding capacity of Ngal and can decrease the ability of HGF to bind to the c-Met receptor and activate downstream signaling, resulting in a decrease in HGF-stimulated cell migration and branching. In addition, Ngal itself appears to play a stimulatory role in the organization of epithelial cells into elongated tubular structures. The observation that Ngal null mice survive after birth suggests that renal development is not grossly abnormal in these animals, although a careful examination of the kidneys for cyst formation or branching defects has not yet been performed. However, prior work demonstrating that Ngal is highly up-regulated following renal injury suggests that the local expression of Ngal at the site of tubular injury may serve to enhance reparative tubule formation in the adult kidney while also muting HGF-stimulated epithelial morphogenesis, possibly preventing dysregulated branching during tubule repair.

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