Expression of Hepatocyte Growth Factor and Its Receptor Met in Wilms’ Tumors and Nephrogenic Rests Reflects Their Roles in Kidney Development

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Abstract

Purpose: Hepatocyte growth factor (HGF) and its receptor Met are known to play diverse roles in both organogenesis and cancer. Wilms’ tumor (WT) is a prototype for the link between abrogated development and neoplasia, with dysregulation of growth factor/receptor pathways playing key roles. Despite this, an understanding of the HGF/Met axis in the process is lacking.

Experimental Design: Observing copy number alterations at the loci for these genes in WTs and their precursor lesions nephrogenic rests, we examined protein expression by immunohistochemistry and investigated the effects of HGF on an in vitro model of kidney development.

Results: HGF was preferentially expressed in the blastemal cells of nephrogenic rests but not WTs. Met expression was infrequent and restricted to well-differentiated epithelial cells and stroma in both lesions. In an independent cohort of favorable histology WTs on a tissue microarray, HGF was expressed in 15 of 193 (8%) cases and correlated with a predominance of epithelial cells, whereas Met expression was observed in 25 of 179 (14%) cases and was associated with stromal subtypes. In a mouse mesonephric cell line model, we observed Met expression in culture conditions reflecting both mesenchymal and epithelial differentiation, whereas HGF was up-regulated in association with acquisition of a more epithelial-like phenotype. This could be mimicked by exogenous exposure of mesenchymal-like cells to recombinant HGF.

Conclusions: These data show that the relatively infrequent expression of HGF and Met in WT tumorigenesis reflects their roles in nephrogenesis, particularly the mesenchymal-to-epithelial transition, rather than a dependence on oncogenic signaling pathways.

Kidney development is a complex process, consisting of two distinct embryologic origins, nephrogenic (mesenchymal) and ductogenic (ureteric; ref. 1). On induction by the ureteric bud, the metanephric mesenchyme undergoes a series of morphogenetic events resulting in ingrowth of the ureteric bud into the metanephric blastema, mesenchymal to epithelial transition, and formation of tubules of the mature nephron (2). Wilms’ tumor (WT; nephroblastoma), the most common pediatric kidney cancer, can be considered a failure of this transition. It arises from pluripotent renal precursors that are undergoing excessive proliferation resulting in undifferentiated stromal components, blastemal cells similar to the condensing mesenchyme, and primitive epithelial structures resembling comma- and S-shaped bodies and glomeruli (3). The presence of associated nephrogenic rests (4) that consist of foci of persistent embryonal remnant tissues that failed to mature to normal renal parenchyma further points toward impaired differentiation in early renal development.

A number of genes involved in nephrogenesis, especially in the mesenchymal to epithelial transition, have also been implicated in WT tumorigenesis (2, 3). We have previously identified dysregulation of the insulin-like growth factor II/insulin-like growth factor 1 receptor signaling network to be associated with perilobar nephrogenic rests (5) and WT relapse (6). Other growth factor pathways active in the normal and developing kidney are also likely to play a role, with hepatocyte growth factor (HGF)/Met being excellent candidates in this context (7).

HGF (scatter factor) and its high-affinity tyrosine kinase receptor Met are both widely expressed early in development, and deletion of either gene causes lethal disruptions to embryogenesis (8, 9). During nephrogenesis, Met is preferentially expressed by the epithelium of the ureteric bud and the developing collecting duct, whereas HGF is expressed in mesenchymal cells and is subsequently localized to the distal tubules and collecting ducts, consistent with a role as a paracrine regulator for renal tubulogenesis (10). From the time of

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Induction to the stage of metanephric condensation, HGF is stimulating branching morphogenesis, promoting motility, proliferation, invasion, morphogenesis, and survival via its receptor’s multifunctional docking site, capable of recruiting signal transducers resulting in the activation of the Ras/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signal transduction pathways (11, 12). Neutralizing antibodies against HGF blocked branching morphogenesis by the ureteric bud in organ cultures of kidney rudiments and inhibited the early steps in branching morphogenesis by immortalized ureteric bud cells in three-dimensional organ culture as well as glomerulogenesis and nephrogenesis in vivo (13, 14).

Dysregulation of HGF and Met signaling is a crucial feature of many human malignancies (15). On HGF binding, c-Met autophosphorylation occurs on two tyrosine residues (Y1234 and Y1235) within the activation loop of the kinase domain that regulate enzyme activity. Phosphorylation on two tyrosine residues near the COOH terminus (Y1329 and Y1356) forms a multifunctional docking site that recruits intracellular adapters via Src homology-2 domains and other recognition motifs, leading to downstream signaling. In particular, the direct binding of growth factor receptor binding protein 2 to the c-Met docking site through Y1356 links the receptor to the Ras/mitogen-activated protein kinase pathway, regulating cell cycle progression.

Multiple mechanisms of pathway activation including coexpression of HGF and Met, receptor amplification, and point mutations have been described, with overexpression or misexpression often correlating with poor prognosis and, particularly, metastasis (12). In WT, involvement of the HGF/Met pathway has been reported to correlate with increased proliferation rate (16), although this has not been explored further. Little information is presently available on biochemical and biological responses induced by HGF on undifferentiated cells, such as those in nephrogenic rests.

We have noted copy number gains at the HGF and MET loci in WTs and nephrogenic rests and sought to clarify their role in WT biology. Contrary to their role in adult epithelial cancers, we observed that HGF/Met did not confer a worse clinical outcome in WT patients but were instead associated with a cell type–specific expression that reflects their role in renal development and the mesenchymal to epithelial transition.

### Materials and Methods

#### Samples
Archival pathology specimens of perihilar nephrogenic rests and WTs were collected with full Ethical Committee approval and have been described previously (5). Pediatric renal tumor tissue microarrays were constructed containing replicate representative cores (n = 885) from all available cellular components from 274 WTs, 13 clear cell sarcomas of the kidney, 10 mesoblastic nephromas (7 classic and 3 cellular), and 7 rhabdoid tumors of the kidney, and have also been described previously (6, 17–20). Tumors were treated with either immediate nephrectomy or preoperative chemotherapy and delayed surgery, and for the purpose of this analysis, all nonanaplastic WTs are described as favorable histology.

#### Array-based comparative genomic hybridization
Array-based comparative genomic hybridization (array-CGH) was carried out using a 5.8k, 0.9Mb-spaced and/or a 16k, 100kb-spaced BAC array platform as reported previously (5). All raw and processed data have been deposited in Array Express (E-TABM-436).6

#### Immunohistochemistry
Immunohistochemistry was done on 5-μm formalin-fixed paraffin-embedded sections using a rabbit polyclonal antibody either to human HGF (JPI8131, Immuno-Biological Laboratories; directed against the NH2-terminal part of the Human HGF α chain) or Met (JPI8321, Immuno-Biological Laboratories; directed against VDTRAPSWETS) using the Envision horseradish peroxidase system (DAKO) at a dilution of 1:75 for HGF and 1:100 for Met according to the manufacturer’s instructions (21). An additional blocking step of 1% normal goat serum (DAKO) was included for both antibodies. Antigen retrieval for HGF was carried out with 0.1% trypsin (Sigma) in 0.05 mol/L Tris-HCl (pH 7.4) at 37°C; for Met, the slides were boiled for 10 min in 10 mmol/L citrate buffer (pH 6) in the microwave. Positive controls were pleomorphic adenoma (Abcam) for HGF and invasive ductal breast carcinoma (Abcam) for Met. Tumor cell positivity and cellular distribution were assessed independently by three pathologists (N.I.S., G.V., and J.S.R.-F.).

#### Statistical analysis
All statistical tests were done in R 2.6.2.7 Correlations between categorical values were done using the χ2 and Fisher exact tests. Cumulative survival probabilities were calculated using the Kaplan-Meier method, with differences between survival rates analyzed with the log-rank test. Multivariate analysis was carried out using the Cox proportional hazards model.

#### Cell culture
M15 cells derived from mouse mesonephros, a kind gift from Melissa Little (University of Queensland, Brisbane, Australia), were cultured routinely in DMEM (Sigma) supplemented with 10% FCS and 1% l-glutamine (Invitrogen). Cells were grown on sterile 13-mm glass coverslips at cell densities of 30% confluence (subconfluent) and 80% confluence (confluent) in a humidified 5% CO2 atmosphere at 37°C. For the stimulation experiments, cells were serum starved for 24 h and incubated with 100 ng/mL of human recombinant HGF (Merck) for up to 48 h.

#### Immunofluorescence
Cells were grown on glass coverslips in 24-well plates, with ice-cold methanol used to fix cells for up to 5 min before incubation with the following primary antibodies: vimentin S-20 (Santa Cruz Biotechnology; 1/50 dilution), wide-spectrum cytokeratin (Abcam; 1/75 dilution), WT-1 C-19 (Santa Cruz Biotechnology; 1/50 dilution), HGF (Immuno-Biological Laboratories; 1/35 dilution), and Met (Santa Cruz Biotechnology; 1/35 dilution). For the secondary detection, the following antibodies were used in 1/1,000 dilution: Alexa Fluor donkey anti-goat 488, Alexa Fluor donkey anti-

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6 http://www.ebi.ac.uk/arrayexpress/
7 http://www.r-project.org
rabbit 555, and Alexa Fluor donkey anti-rabbit 568 (Invitrogen). PBS with 1% bovine serum albumin, 2% FCS was used for blocking as well as for diluting all the primary and secondary antibodies. Cell nuclei were stained with TOPRO-3. Fluorescence was examined using a laser scanning confocal microscope (Leica).

Results

Chromosomal rearrangements lead to recurrent gain of 7q21 in nephrogenic rests and WTs. We have previously shown the genomic changes associated with the development of WTs from their nonobligate precursor lesions nephrogenic rests by array-CGH (5). In that study, we noted that in two cases, WTs and their associated rests harbored alterations on chromosome 7, which led to a gain of DNA copy number close to the centromere on the long arm. In one case, there was a low-level focal gain in both the tumor and the rest of ~5 Mb at 7q21, whereas in another we observed a complex rearrangement of chromosome 7 involving loss of the short arm, high-level gain of 7q11-q21, and loss of 7q22-qter (Fig. 1). Again, this genomic alteration was seen in both the nephrogenic rest and adjacent WT, although there were additional changes in copy number seen only in the tumor, including concurrent gain of 1q and loss of 16q. In both of these cases, the minimal region spanned the HGF locus at 7q21.11.

HGF and MET are preferentially expressed in nephrogenic rests. With the focal copy number gain of the HGF locus observed in nephrogenic rests, and the known gains of the whole of chromosome 7 (involving both the HGF and MET loci) in WTs and rests, we sought to investigate the expression of the growth factor and its receptor in a series of tumors and precursor lesions by immunohistochemistry using fully optimized, reproducible, and specific antibodies. In total, we assessed 46 nephrogenic rests and 36 WTs from 43 patients. HGF expression was observed in 25 of 46 (54%) of rests, including those with 7q21 gain (above), and was noted in all cellular components including immature blastema, stroma, and well-differentiated tubules. Immunopositivity ranged from focal clusters comprising <10% of the whole lesion to 100% of rest cells (representative images are shown in Fig. 2). By contrast, we observed no positive staining in any of the WT cases in this series.

Met expression was seen in 10 of 46 (22%) nephrogenic rests, restricted to well-differentiated epithelial cells or stromal compartments. Similarly, 5 of 36 (14%) of WTs were also Met positive, again restricted to either well-differentiated tubules or tumorigenic stroma (representative images are shown in Fig. 2). In this series, all blastemal components of rests and tumors were negative. The data are summarized in Table 1.

Expression of HGF and c-Met in WTs is associated with epithelial and stromal cell types and good prognosis. To further probe the possible clinicopathologic association of HGF/Met positivity in a larger, independent WT cohort, we examined expression on a pediatric renal tumor tissue microarray. In contrast to our smaller series of whole sections, we observed HGF expression in 15 of 193 (8%) of favorable histology WTs. This was not, however, a statistically significant difference from the smaller series (P = 0.137, Fisher’s exact test). This was mostly restricted to epithelial and stromal cell types, with only 3 of 101 (3%) cases exhibiting blastemal cell positivity (Supplementary Table S1). There were no associations with age at diagnosis or tumor stage, and although HGF positivity showed a trend toward an association with better outcome in favorable histology WT, regardless of treatment protocol, this failed to reach statistical significance due to the small number of positive cases and events therein (Supplementary Fig. S2). Expression in the epithelial cell components was significantly correlated with the predominance of that cell type in the tumor as a whole (P = 0.0015, Fisher’s exact test). Due to the small number of positive cases, it was not possible to analyze the possible effect of HGF expression on outcome independently of histologic subtype. Both epithelial and stromal-predominant
WTs are recognized to have an excellent outcome and this may account for the generally better outcome, at least in those cases exposed to preoperative chemotherapy (22).

An analogous situation was observed for Met expression, with 25 of 179 (14%) positive cases (Supplementary Table S1). There were few tumors with blastemal cell positivity (3 of 98, 3%); expression was largely restricted to epithelial and stromal compartments, with a significant association with stromal cell type predominance ($P = 0.008$, Fisher’s exact test) and a nonsignificant trend toward better prognosis (Supplementary Fig. S2). Once again, there were no association with age, stage, or treatment protocol. Coexpression with HGF was observed in only a small proportion of tumors (6 of 171, 4%), and although the clinical associations did not reach statistical significance, it is notable that there were no relapses or deaths observed in any case with HGF/Met coexpression (Supplementary Fig. S2). Multivariate analyses for HGF and/or Met expression with respect to relapse-free and overall survival were not statistically significant.

In addition to the favorable histology WTs, there was also 1 of 8 (12.5%) anaplastic WT cases with high levels of both HGF and Met. No Met expression was observed in any clear cell sarcomas of the kidney, mesoblastic nephroma, or rhabdoid tumors of the kidney. We did, however, observe HGF expression in 2 of 12 clear cell sarcomas of the kidney and 1 of 4 rhabdoid tumors of the kidney.

Table 1. Summary of HGF and MET expression in nephrogenic rests and WT whole sections

<table>
<thead>
<tr>
<th></th>
<th>HGF, n (%)</th>
<th>MET, n (%)</th>
<th>HGF/MET coexpression, n (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Strong</td>
<td>Weak</td>
<td>Negative</td>
</tr>
<tr>
<td>Nephrogenic rest</td>
<td>12/46 (26)</td>
<td>13/46 (28)</td>
<td>21/46 (46)</td>
</tr>
<tr>
<td>WT</td>
<td>0/36 (0)</td>
<td>0/36 (0)</td>
<td>36/36 (100)</td>
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Fig. 2. Expression of HGF and Met in whole sections of nephrogenic rests and WTs. Photomicrographs showing typical patterns of HGF and Met expression by immunohistochemistry in distinct nephrogenic rests and WTs from different patient samples. First row, a perilobular nephrogenic rest (PLNR) with strong, diffuse HGF staining in blastemal cells. Second row, a WT showing no immunoreactivity. Third row, an intralobular nephrogenic rest (ILNR) with strong Met expression in the stromal compartment. Fourth row, a WT with strong stromal immunoreactivity. Low power, original magnification ×200; high power, original magnification ×400.
Themouse mesonephric M15 cells as a model for mesenchymal to epithelial transition during nephrogenesis. M15 cells derived from mouse mesonephros have been widely used to model WT-1 function during nephrogenesis due to the constitutive expression of the protein (data not shown). We sought to investigate the roles of HGF and Met in these cells by exploiting their reported ability (23) to show a mesenchymal to epithelial transition when grown at low and high confluence in vitro. Sparsely grown cells at subconfluence showed a distinct mesenchymal appearance, with spindle-shaped cytoplasm and strong vimentin expression. By contrast, when grown at high confluence, the cells grew as packed sheets with a more cuboidal epithelial-like morphology and expressed cytokeratin (Fig. 3). Met was consistently expressed at the cell membrane in

Fig. 3. Mouse mesonephric M15 cells mimicking the mesenchymal to epithelial transition during nephrogenesis. M15 cells grown at different cell densities showing an epithelial morphology (phase-contrast microscopy) and cytokeratin expression (red immunofluorescence) at confluence and a mesenchymal phenotype (spindle-shaped morphology and vimentin expression (green)) when grown in subconfluent conditions. Nuclei were counterstained with TOPRO-3.

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Fig. 4. Expression of HGF and Met in M15 cells with epithelial and mesenchymal phenotypes. M15 cells grown at confluence and subconfluence stained by immunofluorescence for HGF (red, cytoplasm) and Met (green, cell membrane). Consistent staining is observed for Met, whereas an up-regulation of HGF is seen in confluent conditions. Nuclei were counterstained with TOPRO-3. Merge indicates an integrated image of all three channels.
M15 cells grown under both conditions. HGF, by contrast, although expressed in the cytoplasm at all cell densities, showed a clear up-regulation when grown at high confluence (Fig. 4). This leads to increased Met signaling via mitogen-activated protein kinase, possibly via autocrine/paracrine activation, with elevated levels of phosphorylated extracellular signal–regulated kinase 1/2 (data not shown).

Exogenous exposure to HGF in subconfluent M15 cells drives a more epithelial phenotype. We hypothesized that M15 cells grown at subconfluence retained a mesenchymal phenotype, which converted to an epithelial one when grown at higher density, and that HGF/Met may play a role in this transition. Addition of recombinant HGF to M15 cultures produced little change to confluent cells, which were seen to already express high levels of the growth factor. Subconfluent cultures, however, showed a clear up-regulation of cytokeratin in the cytoplasm, in addition to the perinuclear expression noted in the absence of HGF, after 1-hour exposure to the growth factor (Fig. 5). Mesenchymal cells are known to express keratins in perinuclear aggregates before the development of a filamentous network throughout the cytoplasm and subsequent attachment to the cell membrane to form the cytoskeletal network of an epithelial cell (24, 25). By 24 hours, the cells, continually cultured in low-density conditions, showed extensive cytoskeletal filaments reminiscent of epithelial cells and strong membranous staining, which were absent from the unstimulated cells (Fig. 5). These cytokeratin positive cells retained their spindle-shaped morphology and expression of vimentin, even up to 48 hours of exposure to HGF (data not shown).

**Discussion**

In many adult epithelial and some pediatric cancers, an activated Met pathway is associated with increased invasiveness, metastasis, and poor clinical outcome and is an excellent candidate for novel targeted therapies (15). Despite its discovery in an oncogenic context, the physiologic role of HGF signaling through Met is in diverse developmental processes during embryogenesis. In WT s, a prototype malignancy of differentiation failure, expression of HGF/Met seems to reflect these developmental roles.

During nephrogenesis, HGF and Met form a paracrine loop, with the growth factor expressed largely by the mesenchyme and the receptor preferentially in the ureteric bud epithelium. HGF not only stimulates branching morphogenesis but is also a potent inducer capable of morphogenic, motogenic (“scatter”), and mitogenic effects on kidney development (26). Addition-
ally, Met may be coexpressed with HGF in at least a part of the mesenchymal tubulogenic cell population, where it may either act in the rescue from apoptosis or else have a part in the conversion process (1).

As a result of abrogated nephrogenesis, residual embryonal cells may persist in the mature kidney as nephrogenic rests, molecular genetic precursor lesions of WTs (4). We observed a preferential retention of HGF expression in more than half of our series of nephrogenic rests, whereas such expression was rarely noted in WTs. In particular, HGF expression in the immature blastemal cells was widespread in rests and almost entirely absent from tumors, suggesting that loss of expression from these primitive embryologic cells may be associated with the acquisition of malignancy.

Where HGF expression was noted in WTs, it was significantly associated with an enrichment of epithelial cells, consisting of both chemo-naive "epithelial predominant" and pretreated "epithelial type" tumors. The role of HGF signaling via Met has been purported to play a key role in the mesenchymal to epithelial transition during nephrogenesis (27). We have used the mouse mesonephros M15 model, which, although not truly reflecting all of the complex processes of human renal development in vivo, forms a useful system in which to study the epithelization process of the mesonephric mesenchyme. In this context, we were able to show that HGF participates in the up-regulation of epithelial cytokeratin filaments in cells with an otherwise mesenchymal phenotype. Thus, it is possible that WTs with a preponderance of malignant epithelial cells are driven by excessive HGF/Met signaling. It is notable that the WT49 (anaplastic, metastatic) WT cell line, known to contain an intact HGF/Met pathway (28),8 showed extensive vimentin and cytokeratin expression and a more consistent epithelioid morphology on continued passages.

In the normal kidney, Met expression is limited to epithelial cells in the proximal convoluted tubule, loop of Henle, and the collecting duct, with the glomeruli, distal convoluted tubule, and stroma consistently negative (29). We observed Met expression in the most well-differentiated tubular epithelial cells of both nephrogenic rests and WTs, but we also noted a strong immunoreactivity in stromal cells of both lesions, with a significant association with the predominance of stromal cells in the tumors regardless of treatment protocol.

HGF and Met are known to play a significant role during the development of skeletal muscle, such that signaling through this cascade controls the migration of myogenic precursor cells in the embryo (30). Expression of Met in tumorigenic stromal cells, many with rhabdomyoblastic differentiation, raises the possibility of its role in determining myogenic cell fate during abnormal kidney development and WT tumorigenesis. Possible functional links to WT-1, mutated forms of which are strongly correlated with stromal histology, remain to be elucidated.

Our data differ from those of the only previous publication of HGF/Met expression in a small series of WTs, which reported extensive positivity and a correlation with increased cell proliferation (16). Our study differs from this earlier report not only in terms of a considerably larger number of cases examined but crucially in the antibodies and immunohistochemistry protocols used, selected after extensive and careful optimization from a variety of sources. We further saw no association with an increased proliferation index by Ki67 staining or with any other markers previously analyzed in our sample cohorts (6, 17–20).

Postchemotherapy abundance of epithelial or stromal cells is reported to predict for better clinical outcome (22), and it is likely that the trends we observed were a reflection of this histologic type, although the numbers of positive tumors in our cohort were too small to allow for robust analyses within treatment groups. Our observations of HGF and Met-positive WTs showing a trend toward better prognosis may be counterintuitive when considered in the context of oncogenic signaling; however, it is likely a reflection of their specific roles in renal cell differentiation and may yet play an important role in diverse aspects of WT tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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