Normal and Malignant Prostate Epithelial Cells Differ in Their Response to Hepatocyte Growth Factor/Scatter Factor

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Hepatocyte growth factor/scatter factor (HGF/SF) promotes the proliferation, differentiation, motility, and invasion of epithelial cells by binding to its cell surface receptor, the Met tyrosine kinase. In the prostate, Met is expressed predominantly by prostate epithelial cells (PrEC), whereas HGF/SF is synthesized by prostate stromal cells (PrSC). Met is also expressed in localized and metastatic prostate cancers. Our results show that PrECs in *in vitro* culture maintain expression of Met at a level comparable to DU145 cancer cell expression. HGF/SF secreted by PrSC stimulates tyrosine phosphorylation of the Met receptor. In normal PrEC, HGF/SF causes growth inhibition, sustained phosphorylation of mitogen-activated protein kinase, and increased CK18 expression consistent with cell differentiation. In contrast, HGF/SF significantly stimulates the proliferation of DU145 prostate cancer cells. HGF/SF in the conditioned medium of PrSC specifically induces migration of both normal and malignant prostate epithelial cells through MatriGel-coated Transwell filters. HGF/SF depletion reduces cell migration by approximately 50%. The response of PrEC is specific for HGF/SF since the other growth factors tested do not significantly affect growth or migration of PrECs. These results support the *in vivo* importance of the prostate stroma and specifically of HGF/SF as a unique stromal derived factor in the development and progression of prostate cancer. (Am J Pathol 2001, 159:579–590)

Hepatocyte growth factor/scatter factor (HGF/SF)^{1,2} has been implicated in the communication between stromal and epithelial cells of many different organs. HGF/SF is secreted predominantly by stromal cells and binds to its receptor, Met^{3,4} mostly expressed on epithelial cells. Activation of the Met receptor tyrosine kinase affects a wide range of cellular processes, including growth and differ-

entiation, morphogenesis and invasiveness.^{5–7} The critical function of HGF/SF-Met in early development and during organogenesis is illustrated by the expression of Met in human embryonic tissues and by the identical phenotypes of HGF/SF and Met targeted knockout mice with early placental and hepatic defects.^{8–10}

Gerald Cunha and collaborators^{11,12} demonstrated the reciprocal nature of interactions between the stroma and epithelium in the determination of final organ structure. Several studies have suggested that mechanisms mediating the inductive interactions between the stroma and normal epithelium may also stimulate neoplastic cells during carcinogenesis and metastasis.^{11,13–15} This occurs through inappropriate expression or activation of receptors for stromal derived growth factors in tumor cells.¹⁶ One of these receptors is Met which is aberrantly expressed or activated in a variety of human cancers.^{17–20} Met was originally identified by its oncogenic potential in gene transfer experiments with DNA from transformed osteogenic sarcoma cells using the NIH 3T3 cell focus forming assay.³ Dimerization of the cytoplasmic Met kinase activates its oncogenic function.^{4,21} Constitutive Met activation is achieved through germline or somatic mutations in the Met kinase domain as detected in familial papillary renal cell carcinomas.^{22,23} Transgenic expression of two independent strongly activating Met mutations as well as transgenic expression of *tpo-met* resulted in mammary carcinogenesis.^{24,25} Furthermore, Met can be persistently activated through autocrine stimulation in tumor cells co-expressing Met and HGF/SF.^{26–31} Based on the ability of HGF/SF to cause the dissociation of tumor cells from the primary tumor mass, to stimulate cell motility, and to elicit the activation of proteolytic cascades that degrade the extracellular matrix,^{32–34} the HGF/SF-Met ligand-receptor system is a likely regulator of tumor metastases. As previously demonstrated, autocrine secretion of HGF/SF promotes metastases in a mouse model of tumor metastasis and

Supported by Department of the Army grant DAMA-17-98-1-8592 (to B.S.K.).

Accepted for publication April 19, 2001.

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HGF/SF plays a role in Ras-mediated metastases formation.^{28,30,33,35}

HGF/SF is an important mediator of stromal-epithelial interactions in the normal prostate; however, its specific function on Met-expressing epithelial cells has not been fully defined.³⁶ HGF/SF biological activity in the conditioned medium of mouse prostatic stromal cells, immortalized human myfibroblastic prostate stromal cells (PrSC), and primary PrSC stimulated the scattering, motility, and collagen gel invasion of DU145 prostate cancer cells and the proliferation of mouse prostate epithelial cells (PrEC).³⁷⁻⁴¹ HGF/SF synthesized by bone marrow stromal cells specifically increased the colony size of normal PrEC seeded on bone marrow stroma.⁴⁰ Immunohistochemical Met expression in normal prostatic epithelium is restricted to the basal epithelial cell layer and to a subpopulation of luminal ductal epithelial cells.³⁷ Met has been implicated in prostatic carcinogenesis and metastasis due to its expression in 45 to 84% of locally invasive cancers and its high expression in metastatic prostate cancer cells.^{37,42,43}

The prostatic epithelium is composed of three compartments that are distinguished by their cytokeratin expression profile.⁴⁴ The basal cells express predominantly high molecular weight cytokeratins (CK5 and CK14), the luminal secretory cells express predominantly low molecular weight cytokeratin (CK8 and CK18) and the intermediate cells express a combination of basal and luminal cytokeratins.⁴⁵⁻⁴⁸ There is increasing evidence that basal epithelial cells differentiate into luminal epithelial cells.⁴⁹ The expression level of high and low molecular weight cytokeratins in intermediate cells is heterogeneous, indicating that this compartment contains different cell populations possibly along a linear differentiation pathway. In addition, intermediate cells are likely candidates for the transiently proliferating/amplifying cells in the prostate,^{50,51} whereas secretory luminal cells are non-proliferative. The cytokeratin expression of prostate cancer cells generally corresponds to that of secretory epithelial cells. However, occasional prostate cancers express the basal high molecular weight cytokeratins.^{52,53} Furthermore, tumor cells express growth factor receptors that are normally detected only in basal or intermediate epithelial cells. It is not certain whether the inappropriate expression of growth factor receptors in cancer cells is due to a defect of receptor down-regulation during differentiation or due to re-expression of growth factor receptors as a result of cellular transformation.

Despite an understanding of the Met expression pattern in normal and malignant PrECs, the precise function of HGF/SF-Met in the normal prostate and in prostate cancer is not known. To determine whether HGF/SF responsiveness is altered as a result of oncogenic transformation of prostate epithelial cells, we compared the HGF/SF-induced proliferation and migration of normal PrEC and prostate cancer cells. Whereas HGF/SF stimulation of normal primary PrECs resulted in growth inhibition and differentiation, transformed prostate cancer cells proliferated on HGF/SF stimulation. This is the first evidence that a single stromal factor can induce differentiation of primary cultured PrECs and that oncogenic trans-

formation can potentially alter the responsiveness of epithelial cells to stromal-derived growth factors.

Materials and Methods

Antibodies and Proteins

The anti-BrdU monoclonal antibody was purchased from DAKO (Carpinteria, CA), the anti-human HGF/SF monoclonal from Sigma (St. Louis, MO) and the 4G10 anti-phosphotyrosine from Upstate Biotechnology (Lake Placid, NY), hMet (C-28) polyclonal antibodies were obtained from Santa Cruz (Santa Cruz, CA), MAPK and phospho-MAPK antibodies were purchased from New England Biolabs (Beverly, MA). The anti-HGF serum was raised in rabbits immunized with rHGF/SF.³⁴ Growth factor reduced (GFR)-MatriGel was purchased from Becton Dickinson, (Bedford, MA) and polylysine from Sigma and diluted 1:100 in distilled water. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin were obtained from Sigma. Recombinant HGF/SF (rHGF/SF) was purified as described.²⁸

Cell Lines

DU145 cells were obtained from the American Type Culture Collection and cultured in RPMI medium with 10% fetal calf serum (FCS) (Gemini, Calabasas, CA). Madin Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle's medium, 25 mmol/L HEPES pH 7.5, 10% FCS and used in the scatter assay as previously described.³⁴

Tissue Procurement

Prostate tissue was obtained from surgical specimens with Institutional Review Board approval. Briefly, a 0.5 × 0.5 × 0.3 cm piece was collected in transfer medium (50% HAM, 50% F12 supplemented with penicillin and streptomycin) from the posterior prostate, from the side with a negative biopsy result. One- to 8-mm³ pieces were incubated with collagenase (250 U/ml), hyaluronidase (325 U/ml) and 5% FCS for 18 hours, washed on a 100-μm filter (Falcon cell strainer, Becton Dickinson, Franklin Lakes, NJ, catalog no. 2360) and plated in 10-cm dishes in 5 ml of culture medium.

Epithelial Cell Culture

Epithelial cells grew in PrEGM (Clonetics, Baltimore, MD), supplied as a base medium without growth factors (referred to as base medium PrEGM) to be reconstituted with growth factors, or in keratinocyte-SF medium (GIBCO BRL, Rockville, MD). Outgrowth from the tissue pieces was observed after 5 to 6 days. Cells were first passaged after 12 to 14 days by consecutive incubations with phosphate-buffered saline containing/1 mmol/L ethylene diaminetetraacetic acid (EDTA), cell dissociation buffer (GIBCO BRL) and 0.12% trypsin/EDTA. For sub-

Table 1. Immunohistochemical and Reverse Transcription-Polymerase Chain Reaction Expression Profile of Cultured Primary Epithelial and Stromal Cells from the Prostate

Antibody/PCR primers	Company	Dilution	Cultured PrEC	Cultured PrSC	Prostate frozen section control*
K903 (CK5 + CK14) [†]	DAKO	1:200	+	n.d.	PrBEC
CK5 (RT-PCR) [‡]			-/+	-/+	+
Cam 5.2 (CK8 + CK18)	Becton Dickinson	1:1	+	n.d.	PrSEC
CK18	Santa Cruz	1:100	+	n.d.	PrSEC
Androgen receptor	BioGenex	1:1	—	—	PrSEC, PrSC
Vimentin	DAKO	1:200	+/-	+	PrSEC, PrSC
Desmin	DAKO	1:100	n.d.	—	PrSC
SMA	DAKO	1:100	n.d.	+	PrSC

*Cells that stained positive in the frozen sections from the prostate: PrBEC, prostate basal epithelial cells, PrSEC, prostate secretory epithelial cells which comprise most epithelial cells.

[†]Cytokeratin.

[‡]PCR primers for CK5: forward primer: GTC ACC AAC TTG CTG CCA AG; reverse primer: GTT CCT GGT GGA GCA AGA GAA C.

Minute pieces of prostate tissue were placed on coverslips for explant cultures of stromal and epithelial cells. Colonies that formed after 3 weeks were fixed and stained with the antibodies specified. Bound antibodies were detected colorimetrically.

sequent passages, the trypsin/EDTA was reduced to 0.06%. Trypsinized cells were plated at 1×10^4 cells per cm^2 and grown to 80% confluence. Cells were propagated for four passages.

Cells were characterized after outgrowth from tissue pieces that were placed on glass coverslips or after the first passage. Table 1 summarizes the immunohistochemical and reverse transcription-polymerase chain reaction profile of the cultured cells. Most epithelial cells stained positive with the K903 mAb which binds high molecular weight cytokeratins (CK5 and CK14) as well as with Cam 5.2 mAb which binds low molecular weight CK (CK8 and 18) and focally positive for vimentin. The specificity of antibodies for individual cytokeratins was ascertained by negative staining of stromal cells as well as by different reactivities of antibodies of the same isotype. The K903, CK18, androgen receptor and desmin antibodies of the IgG1 isotype and the Cam 5.2, vimentin and SMA antibodies of the IgG2a isotype showed specific staining patterns, consistent with specific binding of individual antibodies to the respective antigenic determinants. Expression of the androgen receptor or Bcl-2 was not detectable by immunohistochemistry. The vimentin positive cells had epithelial and not stromal cell morphology and expressed cytokeratins. Such cells were also observed *in vivo*, at the tips of hyperplastic papillae⁵⁴ and therefore represents an *in vivo* existing cell population and not a culture artifact.

Stromal Cell Culture

Stromal cells were cultured with MCBC 131 (GIBCO BRL) supplemented with 10% FCS, nonessential amino acids solution (1:100) (GIBCO BRL), insulin (5 $\mu\text{g}/\text{ml}$), transferrin (10 $\mu\text{g}/\text{ml}$), dexamethasone (100 nmol/L), and sodium selenite (5 ng/ml). All supplements were purchased from Sigma. Epithelial cells disappeared after the second passage. Stromal cells were propagated for five passages. The cells expressed vimentin and smooth muscle actin, but did not express desmin or the androgen receptor (Table 1). The presence of epithelial cells in the initial stromal explants served as an intrinsic control for the

antibody specificity. Therefore the cultured cell population represents a subpopulation of myofibroblastic cells. Serum-free stromal cell conditioned medium (PrSC-CM) was obtained from 70 to 80% confluent cultures either by incubation with α -MEM base medium for 48 to 72 hours since this medium by itself did not cause migration of epithelial cells. The PrSC-CM was tested for scatter activity in the MDCK scatter assay before its use in cell migration assays.

Immunohistochemical and Immunofluorescent Staining

Tissues or cells on coverslips were fixed with 50% methanol/50% acetone for 20 minutes at -20°C for staining of cytoplasmic proteins and with 4% paraformaldehyde at room temperature for nuclear staining followed by immediate washing with PBS. 3% bovine serum albumin (BSA) was used for blocking of non-specific antibody binding sites. Antibodies were applied for 45 minutes at concentrations indicated in Table 1 and bound antibodies detected with the Vectastain ABC horseradish peroxidase detection kit (Vector Laboratories, Inc., Burlingame, CA) and 3,3'-diaminobenzidine (DAB) as a substrate. To determine expression of HGF/SF, anti-HGF/SF and preimmune rabbit sera were diluted 1:100 in 3% BSA/PBS. Bound antibody was detected with the Vectastain ABC alkaline phosphatase and the alkaline phosphatase substrate (SK-5100) kit. After staining, dried coverslips were mounted with Cytoseal (Stephens Scientific, Cornwell Corporation, Riverdale, NJ). Images were taken at 200 $\times$ magnification.

For immunofluorescent staining, frozen tissue sections were fixed and incubated with hMet and K903 antibodies (both 1:200 in PBS/3% BSA) for 45 minutes. FITC anti-rabbit (1:500) and Texas Red anti-mouse (1:1000) (both from ICN, Coast Mesa, CA) were used for detection. Immunofluorescent staining was photographed with a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) and an Axiophot 2 camera (Zeiss Gottingen, Germany). Images were taken at 200 $\times$ magnification.

Preparation of Extracellular Matrices

Matrices were prepared as previously described.^{55,56} Briefly, cells were cultured in 6-cm plates, 1 week post confluence. Cell layers were washed twice with PBS (150 mmol/L NaCl, 10 mmol/L NaPO₄, pH 7.5), incubated with 1% Triton X-100 in PBS for 3 minutes followed by a 1-minute incubation with 25 mmol/L ammonium hydroxide to remove nuclei and cytoskeletal elements. After this treatment, the extracellular matrices remained adherent to the tissue culture plate. They were washed three times with sterile PBS, blocked with 3% BSA for 1 hour and incubated with 100 ng of rHGF/SF for 1 hour. After washing, 2×10^6 DU145 cells were adhered for 2 hours. Cells were then lysed in radio immunoprecipitation assay (RIPA) buffer (20 mmol/L Tris-HCl pH 7.5, 1 mmol/L EDTA, 100 mmol/L NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate). The following inhibitors were added: 12.5 μ g/ml aprotinin, 1 μ g/ml leupeptin, 10 μ g/ml pepstatin, 1 mmol/L benzamide, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L β -glycerophosphate, 50 mmol/L sodium fluoride, 1 mm sodium vanadate, 10 μ mol/L sodium molybdate. Met protein was precipitated from 500 μ g of cell lysate and analyzed by Western blotting for tyrosine phosphorylation.

Immunoprecipitation and Western Blotting

DU145 or PrEC were lysed in RIPA buffer. Ten to 30 μ g of whole cell lysate was analyzed by Western blotting for Met expression as previously described.³⁴ Protein concentrations were measured with the BioRad assay. For measurements of tyrosine phosphorylation 300–500 μ g of cell lysate were precipitated with 1 μ g of hMet antibody. Membranes were probed with 4G10 antibody (1:3000) in TBS, 0.05% Tween, 1 mmol/L sodium vanadate, 1 mmol/L sodium molybdate, 1% BSA, 0.5% ovalbumin.

HGF/SF Precipitation

Thirteen ml of cell culture medium were supplemented with 1% Triton X-100 and 10 mmol/L Tris-HCl, pH 7.5, and incubated with 50 μ l of packed heparin-Sepharose or glutathione S-transferase (GST)-Sepharose overnight at 4°C. Beads were washed three times with 1% Triton X-100 in TBS (10 mmol/L Tris-HCl pH 7.5, 100 mmol/L NaCl) and analyzed by SDS-PAGE.

HGF/SF-depleted CM was generated by incubation of 2 ml of PrSC-CM with 15 μ l of Protein-G beads and 10 μ l of hMet mAb (Sigma) or mouse Ig control. The beads were removed by centrifugation. The HGF/SF-depleted medium had no residual scatter activity.

Proliferation Assay

Epithelial cells (first or second passage) were deprived of growth factors for 24 to 48 hours. 10^5 cells were plated on growth factor-reduced MatriGel (GFR-MatriGel)-coated glass coverslips in growth factor-free medium. After 12 hours, 20 to 50 ng/ml HGF/SF was added in 1 ml base medium (PrEGM) and after 24 hours, cells were pulsed

with 10 μ l/ml bromodeoxyuridine (BrdU, 3 mg/ml, Sigma) per well for 12 hours (DU145 cells) or 36 hours (PrEC). Coverslips were fixed in 50% methanol:50% acetone, dried, and stained for BrdU incorporation. Images of BrdU stained cells were captured using a Nikon Microphot-SA microscope at 200 $\times$ magnification and a digital video camera (Kontron Elektronik, Pro/Gres 3012) and visualized with Adobe Photoshop software. To determine the percentage BrdU positive cells, approximately 1000 cells on duplicate coverslips were counted. Five separate individuals were analyzed. Statistical analysis was done with Microsoft Excel software.

Migration Assay

DU145 cells or 2×10^5 PrEC were placed in the upper compartment of 10-mm tissue culture inserts (Transwell, Nunc, Naperville, IL) coated with polylysine and GFR-MatriGel (1 mg/ml in RPMI buffer). The same concentrations of growth factors or CM were added to upper and lower compartments. After migration for 8 to 9 hours at 37°C, cells were removed from the upper compartment. Tissue culture inserts were fixed in 3.7% paraformaldehyde and stained with the HEMA 3 stain set (Biochemical Sciences, Inc., Swedesboro, NJ). Dried filters were mounted onto coverslips for quantitative analysis. For each filter, 20 to 28 visual fields at 400 $\times$ magnification were counted.

PrEC Differentiation

PrEC (passage 2 or 3) were grown to 80% confluence and treated with 20 U/ml HGF/SF in base medium (PrEGM, Clonetics) for 4 or 5 days. Cells were lysed in RIPA buffer and 50 μ g of whole cell lysate was analyzed for CK18 expression. For immunohistochemical analysis, cells were grown on coverslips and with 50 U/ml HGF/SF for 2 days.

Results

HGF/SF and Met Expression in Normal Prostate Tissue *in Vivo*

Immunohistochemically analysis of *in vivo* tissue demonstrates that the prostatic stroma largely consists of two cell populations: the desmin and smooth muscle actin (SMA) expressing bundled smooth muscle fibers and the SMA and vimentin positive myofibroblastic cells (Table 1). HGF/SF protein is present diffusely throughout the extracellular matrix and not in the cytoplasm of particular stromal cells (Figure 1, upper panels). HGF/SF protein is not detected in the epithelium. The specificity of the anti-HGF antibody was confirmed by staining with a pre-immune serum from the same rabbit. HGF/SF producing cells cannot be identified by immunohistochemical analysis, but based on the localization of HGF/SF protein in the extracellular matrix it is likely that the synthesized

Figure 1. HGF and Met expression in the prostate. Frozen sections of benign prostate tissue were stained with anti-HGF/SF (**A**) or preimmune serum (**B**) and developed colorimetrically (magnification, $\times 200$). Frozen section stained with anti-Met-FITC (**C**) and anti-high molecular weight cytokeratin (K903) Texas Red antibodies (**D**) (magnification, $\times 200$).

HGF/SF is rapidly secreted and immobilized in the extracellular matrix by heparan sulfate proteoglycans.^{57, 58}

Met is expressed in the epithelium. Consistent with previous reports of immunohistochemically stained prostate sections,^{37, 42} we find highest Met expression and co-localization with high molecular weight cytokeratin in the basal cells of the prostatic ducts and acini. (Figure 1, lower panels). In addition, Met is highly expressed in a few luminal cells of prostatic ducts and uniformly in luminal cells of atrophic glands (data not shown). The overall immunohistochemical staining profile is consistent with Met expression in the basal and intermediate/transiently proliferating cellular compartment of the prostatic epithelium.⁵⁹

HGF/SF Secretion by Cultured Prostate Stromal Cells

We find that the conditioned medium of the primary PrSC reproducibly contains HGF/SF activity as demonstrated by its stimulation of MDCK scattering (Figure 2A). These results are consistent with several reports that demonstrate the secretion of HGF/SF by immortalized PrSC.^{37,39–41} The immunohistochemical profile of the cultured stromal cells shows a myofibroblastic phenotype (Table 1), suggesting that the myofibroblastic subpopulation of prostate stromal cells may be the source of HGF/SF synthesis *in vivo*. The presence of HGF/SF protein in the conditioned medium of PrSC (PrSC-CM) is further demonstrated by the induction of Met tyrosine phosphorylation in DU145 cells (Figure 2B). Further, HGF/SF protein accumulates in the PrSC-CM and can be precipitated with heparin-Sepharose beads and identified by Western

Figure 2. Prostate stromal cells secrete HGF. **A:** Scatter assay. Forty-eight-hour conditioned medium from prostate stromal cells was tested in the MDCK scatter assay. MDCK cells were incubated for 12 hours with preconditioned medium (pre-CM), conditioned medium (CM), or 20 ng of HGF/SF/ml. Cells were photographed at $40\times$ magnification. **B:** Met phosphorylation in DU145 cells by conditioned medium from prostate stromal cells. DU145 cells were incubated for 20 minutes with conditioned (CM) or preconditioned (pre-CM) medium. Cell lysates were precipitated with the Met antiserum and the Western blot was probed with 4G10. **C:** Precipitation of HGF/SF from conditioned medium of prostate stromal cells. Thirteen ml of conditioned or preconditioned medium were incubated with heparin-Sepharose. Conditioned medium was also incubated with GST-Sepharose as a control. Precipitates were analyzed for the presence of HGF/SF on Western blots probed with the anti-HGF/SF monoclonal antibody.

blotting. Thus, a 80-kd band is detected in the heparin-Sepharose precipitates from PrSC-CM with the HGF antiserum but not in precipitates from preconditioned medium or in precipitates with GST-agarose (Figure 2C).

Met Expression and Phosphorylation in Cultured Prostate Epithelial Cells

Met is expressed in cultured PrEC but not in cultured PrSC (Figure 3A). Whereas the initial explant culture contains cells with different levels of Met expression, after the first passage, PrECs uniformly express Met (data not shown). In addition, most cultured cells uniformly express high molecular weight (CK5 and CK14) and low molecular weight (CK8 and CK18) cytokeratins as demonstrated by reactivity with K903 and Cam 5.2 antibodies. (Table 1). CK18 is predominantly expressed in the center of epithelial cell clusters and in stellate appearing cells that grow on top of the epithelial monolayer. Overall, the majority of cultured cells express both high and low molecular weight cytokeratins and therefore correspond to the intermediate/transiently proliferating epithelial cell population.^{50,51}

A similar amount of Met protein is expressed in PrECs and DU145 prostate cancer cells that are grown in 10% FCS (Figure 3A, lanes 1 and 3). Both the 170-kd precursor and the 140-kd mature form of Met are detected. In contrast, Met protein is not detectable in PrSC (Figure 3A, lane 2). The 140-kd Met receptor in cultured PrEC was phosphorylated on tyrosines by HGF/SF in the PrSC-CM (Figure 3B). Preconditioned medium containing 10% FCS did not cause tyrosine phosphorylation of Met (Figure

Figure 3. Met expression and phosphorylation in cultured PrEC. **A:** Met expression. Twenty μ g of cell lysate from PrEC, PrSC, or DU145 cells was analyzed for Met expression. DU145 were cultured in 10% or 5% fetal calf serum as indicated. **B:** Phosphorylation of Met by conditioned medium from prostate stromal cells. Epithelial cells were incubated with 20 ng of HGF/SF, conditioned (CM), or preconditioned (preCM) for 20 minutes. Met was precipitated from 300 μ g of cell lysate and analyzed for tyrosine phosphorylation with 4G10. **C:** Met expression and phosphorylation after HGF/SF stimulation. PrEC or DU145 prostate cancer cells were incubated with 20 ng/ml HGF/SF for the indicated time periods and Met expression was analyzed by Western blotting of 30 μ g of whole cell lysate (**first row**). Met was precipitated from 300 μ g of cell lysate and analyzed for tyrosine phosphorylation with 4G10 (**second row**). The blot was reprobed with anti-Met to show the entire amount of precipitated Met protein (**third row**).

3B). Furthermore, the phosphorylated Met in the PrECs bound to *gsl-Grb2* and to a fusion protein of the *Src* homology 2 domain of the regulatory subunit of PI-3 kinase (p85/PI-3K) (data not shown). These results demonstrate that Met in PrEC can be activated by HGF/SF in the PrSC-CM.

After binding to HGF/SF, Met receptor expression declines through degradation of Met by ubiquitinylation.⁶⁰ To evaluate a possible difference in the down-regulation of Met in normal and transformed prostate epithelial cells, confluent cultures of PrEC and DU145 cells were serum-starved and incubated with 20 U of rHGF/SF/ml. Whereas in the DU145 cells, the total amount of Met protein expression rapidly diminished, only a small decrease occurred in normal PrEC during the 24 hours of HGF/SF stimulation (Figure 3C). Although the kinetics of Met down-regulation were different between PrEC and DU145 cancer cells, the kinetics of Met phosphorylation were similar, with a peak at 1 hour after HGF/SF stimulation (Figure 3C). After 24 hours of HGF/SF stimulation, no phosphorylated Met was detected in PrECs despite persistent Met protein expression.

HGF/SF Inhibits Cell Proliferation of Normal PrEC

HGF/SF stimulates proliferation in certain cell types while inhibiting proliferation in others (reviewed in Ref. 7). To determine whether HGF/SF stimulates or inhibits the proliferation of normal PrEC, we examined the mitogenic effects of various growth factors on PrEC cells from a single individual. Adhesion of normal PrEC cells to GFR-

Figure 4. HGF/SF inhibits the proliferation of prostate epithelial cells. **A:** Stimulation of epithelial cells with various growth factors. 1×10^3 epithelial cells from one individual were plated on GFR-MatriGel-coated coverslips. 12 hours later they were stimulated with 20 ng of rHGF/SF, 100 ng of EGF, 100 ng of bFGF, and 50 ng of insulin per ml. BrdU was added 12 hours later for 36 hours. The incorporation of BrdU was detected by immunohistochemistry as described in Materials and Methods. Between 800 and 1000 cells were counted on duplicate coverslips for each condition. The % proliferation indicates the number of positive cells per 100 cells counted. **B:** HGF/SF treatment of normal PrECs. Cells from four different individuals (including three separate primary cultures from individual 4, labeled PrEC 4 a, b, c) were plated on GFR-MatriGel-coated coverslips. Twenty ng of HGF/SF was added 12 hours later. Proliferation was determined by BrdU incorporation as described in Materials and Methods.

MatriGel stimulates cell proliferation compared to polylysine adhesion (data not shown). HGF/SF added 12 hours after plating cells on GFR-MatriGel markedly inhibited the proliferation of normal PrEC, when compared to untreated PrEC ($P = 8 \times 10^{-7}$) (Figure 4A). In contrast, insulin stimulated cell proliferation ($P = 0.003$), while EGF treatment was slightly, but not significantly inhibitory ($P = 0.036$) and bFGF treatment had no effect on cell proliferation. To confirm the growth inhibitory effects of HGF/SF, cells from four additional individuals were analyzed (Figure 4B). In all four separate PrEC isolates, exposure to HGF/SF reproducibly inhibited the BrdU incorporation with a mean decrease of 52.12%. The inhibition of cell proliferation in experiments with cells derived from the same prostate (Figure 4B, PrEC4 a, b, c) was between 20% and 59%. Cell proliferation was also inhibited in cells that were cultured in growth factor-depleted medium but not replated on GFR-MatriGel (data not shown).

Figure 5. Stimulation of DU145 prostate cancer cells by HGF/SF. **A:** Stimulation of DU145 cell migration. DU145 cells migration through polycarbonate filters coated with MatriGel in the presence and absence of HGF/SF. 6 high powered fields (HPF; 400 $\times$) were counted. Mean of three separate experiments with error bar indicating the variation of the mean. **B:** Stimulation of DU145 cell proliferation. Cells were plated in serum-free medium on GFR-MatriGel-coated coverslips. 12 hours later, cells were stimulated with 20 ng/ml HGF/SF and pulsed with BrdU. BrdU incorporation was determined by immunohistochemical staining. Approximately 800 cells were counted per coverslip. Mean of three separate experiments with error bar indicating the variation of the mean. **C:** Phosphorylation of Met. DU145 prostate cancer cells were incubated with 20 ng of HGF/SF, 100 ng of EGF, 100 ng of bFGF, or 50 ng of insulin for 15 minutes. Met was precipitated and analyzed for tyrosine phosphorylation. The blot was reprobed with the Met antibody to show equal amount of Met protein in all lanes. **D:** Binding of HGF/SF to extracellular matrix. Plates coated with epithelial or stromal extracellular matrix were blocked and incubated with 100 ng of HGF/SF as indicated. After washing, DU145 cells were adhered for 2 hours and lysed in RIPA buffer. Met was precipitated from 500 μ l of DU145 cell lysate and analyzed for tyrosine phosphorylation.

Stimulation of DU145 Cells with HGF/SF

We show that HGF/SF stimulates proliferation and migration of DU145 prostate cancer cells and causes specific phosphorylation of the Met receptor (Figure 5 A–C), which is consistent with previous reports.^{41,37} HGF/SF stimulates the migration of DU145 cells through GFR-MatriGel-coated Transwell filters. Whereas few DU145 cells migrate in serum-free medium on MatriGel-coated Transwell filters, the addition of HGF/SF greatly enhances cell migration (Figure 5A). HGF/SF only induced cell migration across filters coated with GFR-MatriGel but not polylysine (data not shown).

Since previous studies with DU145 cells demonstrated either no effect of HGF/SF⁴¹ or an increased³⁷ or decreased⁶¹ cellular proliferation, we evaluated the stimulation of DU145 cells in our experimental system. In our system, HGF/SF stimulates proliferation of DU145 cells that were deprived of exogenous growth factors and replated on GFR-MatriGel. Under these conditions, the baseline proliferation was $14\% \pm 3.5\%$ as shown by BrdU incorporation. Stimulation with HGF/SF produces a 100% increase of BrdU positive cells, clearly demonstrating that HGF/SF stimulates the proliferation of DU145 cells in the absence of exogenously added growth factors (Figure 5B). The increase in cell proliferation was greater at a higher plating density (data not shown). The Met expres-

sion of PC-3 or TSU-Pr1 prostate cancer cell lines show neither an increase nor decrease of cell proliferation on HGF/SF treatment under the same experimental conditions (data not shown).

To determine the specificity of Met receptor phosphorylation, DU145 prostate cancer cells were treated with various growth factors or adhered to extracellular matrix from PrEC or PrSC. Following treatment with HGF/SF, EGF, bFGF, or insulin, Met was only tyrosine phosphorylated on incubation with HGF/SF (Figure 5C). Cell adhesion alone did not induce tyrosine phosphorylation of the Met receptor (Figure 5D). However, tyrosine phosphorylation of Met occurred when the matrix from PrEC or PrSC was treated with HGF/SF. The upper band in Figure 5D in the HGF/SF containing lanes reacted with the Met antibody on reprobing of the blot and therefore represents the phosphorylated Met receptor. Taken together, our results show that HGF/SF stimulates migration and proliferation of DU145 prostate cancer cells. Further, HGF/SF immobilized by the matrix of PrEC or PrSC induces phosphorylation of Met. However, it remains to be determined whether the migratory response of DU145 cells to HGF/SF stimulation is a result of oncogenic transformation or an inherent property of PrEC.

Activation of MAPK and c-Jun Kinase (Junk)

HGF/SF stimulation led to rapid phosphorylation of MAPK in normal PrECs and DU145 prostate cancer cells (Figure 6A). Phosphorylation of MAPK reached a maximum within 10 minutes of HGF/SF stimulation. However, the duration of MAPK phosphorylation differed significantly between normal PrEC and DU145 prostate cancer cells. Whereas in the normal PrEC, MAPK phosphorylation was sustained, in the DU145 prostate cancer cells MAPK phosphorylation was transient. This was not due to a decrease in total MAPK protein. In normal PrECs, MAPK phosphorylation persisted even in the absence of phosphorylated Met receptor (Figure 6B). JunK was phosphorylated on HGF/SF stimulation of PrECs as well as DU145 prostate cancer cells. In both cells the phosphorylation of p46/JunK was transient with a peak at 10 minutes and a decline that was faster than the decline of Met phosphorylation. Phosphorylation of the p54 isoform of JunK was only detectable in DU145 cells (Figure 6C). While MAPK phosphorylation kinetics are different between normal PrECs and DU145 tumor cells, JunK phosphorylation kinetics are similar.

HGF/SF Stimulates the Migration of PrEC

Similar to the response of DU145 cells (Figure 5A), HGF/SF also induced migration of normal PrEC through MatriGel-coated polycarbonate filters. Compared to insulin, EGF and bFGF which did not significantly stimulate cell migration, HGF/SF robustly stimulated migration of normal PrECs (Figure 7A). To determine whether HGF/SF could also stimulate PrEC migration under more physiologically relevant conditions, serum-free PrSC-CM which contains HGF/SF was analyzed for its ability to stimulate

Figure 6. Phosphorylation of MAPK and JunK. **A:** MAPK phosphorylation. Cells were stimulated with 20 ng of HGF/SF/ml for the indicated time points. Thirty μ g of cell lysate from prostate epithelial cells (PrEC) or DU145 cells (DU145) was analyzed for MAPK phosphorylation using the phospho-MAPK antibody. The gel was reprobed with anti-MAPK. **B:** Comparison of Met and MAPK phosphorylation kinetics. Densitometric measurements of pMet bands from Figure 3B and pMAPK bands are plotted. The time scale on the x axis is the same as indicated in A. **C:** JunK phosphorylation. Cells were stimulated with 20 ng of HGF/SF/ml for the indicated time points. One hundred μ g of cell lysate from prostate epithelial cells (PrEC) or DU145 cells (DU145) was analyzed for JunK phosphorylation using the phospho-JunK antibody. A parallel gel with the same amount of cell lysate was probed with anti-JunK.

the migration of PrEC. Serum-free PrSC-CM stimulated the migration of PrEC (Figure 7B). Depletion of HGF/SF with an HGF/SF monoclonal antibody but not with control mouse IgG reduced cell migration by 50%. The residual migration stimulating activity was likely due to stromal factors other than HGF/SF, since the HGF/SF depleted medium did not stimulate scattering of MDCK cells. These results demonstrate that HGF/SF is an important stromal factor for the migration of normal and malignant prostate epithelial cells.

Stimulation of CK18 Expression by HGF/SF

CK18 expression is induced during epithelial differentiation in the prostate.⁴⁴ Based on the inhibition of cell proliferation and the sustained activation of MAPK in PrEC, the induction of cellular differentiation by HGF/SF was ascertained. Using CK18 as a differentiation marker for PrECs, the differentiation of HGF/SF-treated PrEC cultures was examined. HGF/SF increased the overall expression of CK18 by three- to fourfold (Figure 8A). Immunohistochemical analysis revealed that HGF/SF stimulation increased the number of highly CK18 positive stellate cells rather than causing a uniform increase in CK18 expression in all PrECs (Figure 8B). Thus, HGF/SF can induce differentiation of a subset of cultured PrECs.

Figure 7. Stimulation of PrEC migration by HGF/SF. **A:** Prostate epithelial cells migrate on HGF stimulation. Cells were placed in the upper chamber of a MatriGel-coated Transwell. Equal concentrations of growth factors (EGF, 1 μ g/ml; bFGF, 100 ng/ml; insulin, 500 ng/ml) were added to the upper and lower compartment of the Transwell chambers. After 7 hours, non-migrating cells were removed from the upper chamber with a cotton swab. The filter was then stained with Diffquick. Eight 200 $\times$ fields were counted per filter. The graph shows the average of two experiments with cells from different individuals. **B:** Migration of prostate epithelial cells in stromal cell-conditioned medium. Serum-free CM from prostate stromal cells was diluted 1:1 with epithelial medium in the absence of growth factors and used in the migration assay. Stromal conditioned medium was depleted of HGF/SF by incubation with 20 μ l anti-HGF/SF monoclonal antibody and protein-G beads. Normal mouse IgG was used as the control.

Discussion

Our study shows that malignant transformation of prostatic epithelial cells modifies their response to a stromal factor which can ultimately lead to tumor growth, invasion, and metastasis. How does malignant transformation depend on factors secreted by the stroma? Our data comparing normal and malignant prostate epithelium demonstrate that at least one stromal-derived factor, HGF/SF, stimulates proliferation of transformed DU145 prostate cancer cells while it stimulates differentiation of normal prostate epithelial cells. HGF/SF actually inhibits the proliferation of normal PrEC, induces increased ex-

Figure 8. Cytokeratin 18 expression. **A:** HGF/SF stimulates CK18 expression. PrEC were incubated with base medium (BM), 20 ng/ml HGF/SF in base medium (HGF/SF), or growth medium (KGM). Fifty μ g of cell lysate was analyzed for CK18 expression by Western blot. **B:** Immunohistochemical analysis of HGF/SF-treated PrEC. Cells were incubated for 2 days with base medium (BM) or 50 ng/ml HGF/SF. Cells were stained for CK18 expression by immunohistochemistry. Magnification, $\times 200$.

pression of CK18 and causes sustained phosphorylation of MAPK. In contrast, HGF/SF causes transient MAPK phosphorylation in DU145 cells and induces mitogenic stimulation. Although HGF/SF had opposite effects on proliferation of normal PrEC and DU145 cells, it induced cell migration in both cell types. Furthermore, HGF/SF is the major motility factor in the conditioned medium of cultured prostate stromal cells, responsible for approximately 50% of the migration-inducing activity. While our study focuses on changes in the epithelial response to stromal factors, others have demonstrated alterations in tumor-associated stromal cells causing aberrant stromal-epithelial interactions during oncogenesis.^{62–66} It will be interesting to determine whether the “tumor stroma” produces increased HGF/SF activity compared to normal stroma.

HGF/SF synthesis *in vivo* occurred only in the prostatic stroma, and our *in vitro* culture system implicates myofibroblastic stromal cells as a source for HGF/SF synthesis. Interestingly, myofibroblastic cells comprise the predominant stromal cell type in the papillae of hyperplastic acini where epithelial differentiate into tall secretory cells (data not shown). Since the activity of secreted HGF/SF is regulated through proteolytic cleavage our immunohisto-

chemical studies cannot assess HGF/SF bioactivity in the prostatic stroma.

In the DU145 cells Met expression declined by approximately 80% after serum starvation; however, Met levels in normal PrEC are not significantly reduced after prolonged growth factor deprivation (Figure 3C). This difference in the regulation of Met expression is not due to the secretion of HGF/SF by DU145 cells since we did not find HGF/SF protein in the CM of prostate cancer cell lines. This is in contrast to reports from other transformed epithelial cells that express HGF/SF.^{67,19} Accordingly, under regular culture conditions, Met is not phosphorylated in DU145 cells, and therefore the down-regulation of Met protein in serum-starved DU145 cells (Figure 3C) is likely due to a lack of growth factors. This HGF/SF-independent regulation of Met expression likely occurs through transcriptional activation via an AP-1 response element in the Met promoter.^{35,60,68,69} In contrast, the HGF/SF dependent regulation of Met protein expression, on stimulation with exogenously added HGF/SF, is likely regulated by Met degradation via the ubiquitinylation pathway.⁶⁰ It is presently not known whether the amount of expressed Met receptor determines the responsiveness of benign or malignant prostate epithelial cells to HGF/SF stimulation *in vivo*.

A significant difference between normal PrEC and DU145 prostate cancer cells is the duration of MAPK phosphorylation after HGF/SF stimulation (Figure 6A). Since the phosphorylation kinetics of Met and MAPK are similar in normal and transformed cells (Figure 6B), it is likely that MAPK dephosphorylation is slower in the normal PrEC. Besides the correlation of sustained MAPK phosphorylation and cell differentiation in several other tissue culture systems,⁷⁰ HGF/SF induced prolonged MAPK phosphorylation has been associated with the secretion of matrix metalloproteinase-9 (MMP-9) in primary cultures of human keratinocytes.⁷¹ We are currently testing the secretion of MMP-9 from HGF/SF stimulated primary PrEC since this could represent an important mechanism during HGF/SF-mediated tumor invasion and angiogenesis in the prostate.

In vitro culture of PrECs only recapitulates a selective subpopulation of *in vivo* PrEC. An understanding of the relationship between the cultured cells and the *in vivo* prostate epithelium is essential to interpret our findings. Characterization of our PrEC cultures showed that they are comprised of intermediate PrEC^{49,51} that express high and low molecular weight cytokeratins. After the first passage, most cells stained with the basal cell marker, K903 (anti-CK5, CK14), and the luminal cell marker, Cam 5.2 (anti-CK8, CK18). Individual isolates of epithelial cells from different prostatectomy specimens varied in the level of differentiation as shown by differences in CK5 mRNA expression and CK18 immunohistochemical positivity. As previously described,⁴⁴ strongly CK18 positive cells are positioned on top of the epithelial monolayer and are considered to be the most differentiated epithelial population that is obtained in *in vitro* culture. However these cells do not express secretory epithelial markers such as the androgen receptor or prostate specific antigen. It is therefore possible that these cells represent a

more differentiated population of intermediate PrECs and not true secretory prostate epithelial cells. Many more cells showed signs of HGF/SF stimulation such as extension of processes and cell-cell dissociation without an increase in CK18 expression. Furthermore, prolonged HGF/SF exposure did not cause a further increase in the number of differentiated cells, suggesting that HGF/SF induces differentiation in a subpopulation of PrECs. Extrapolation of our *in vitro* results to the *in vivo* observation of high Met expression in atrophic prostate epithelium could be interpreted as an attempt of atrophic luminal cells to differentiate into secretory epithelium.

The point at which cells alter their response to HGF/SF during oncogenic transformation is not known. We have used the DU145 prostate cancer cell line as a model for prostate cancer. This cell line has been derived from a brain metastasis and represents an androgen-insensitive, advanced metastatic prostate cancer.⁷² The proliferation of PC-3 and TSU-Pr1 cells was not effected by HGF/SF under conditions where the proliferation of DU145 cells was increased. It is possible that modifications of signal transduction pathways in PC-3 and TSU-Pr1 cells prevents the transduction of a proliferative signal from the Met receptor, or that Met receptor pathways are constitutively activated by autocrine growth factors. While proliferation of DU145 cells could be reduced to 15% by removal of exogenous growth factors, the proliferation of PC-3 and TSU-Pr1 cells remained at 30 to 35%. A recent report showed HGF/SF stimulated growth and invasion of PC-3 cells in a three dimensional co-culture system and in nude mice,⁷³ suggesting that HGF/SF-mediated cell proliferation depends on the experimental conditions. This might also be the case with DU145 cells, since a recent study demonstrated a growth inhibitory effect of HGF/SF in DU145 cells in the presence of serum.⁶¹ The proliferative response to HGF/SF stimulation of tumor systems derived from other organs is variable.⁷ Among breast cancer cell lines, growth inhibitory⁶⁹ as well as growth stimulatory⁷⁴ effects have been observed. An attempt to analyze HGF/SF stimulation of less aggressive, androgen dependent or responsive prostate cancer cell models failed due to the lack of detectable Met receptor expression. LNCaP, CWR22, LuCaP, or LaPC-4 did not express detectable levels of Met protein. The point at which HGF/SF responsiveness is altered during prostate oncogenesis remains to be determined.

Based on the pro-metastatic effects of HGF/SF and the high expression of Met in prostate cancer metastasis, it is likely that HGF/SF-Met contributes to the metastatic process. The *in vitro* migration of DU145 cells in response to HGF/SF stimulation (Figures 5) predicts that HGF/SF might mediate the motility of cancer cells, their dissociation from the primary tumor and their extravasation into the circulation. Since only approximately 50% of the migration-inducing activity was removed by HGF/SF depletion from the conditioned medium of PrSC, other factors are secreted by PrSC that stimulate epithelial migration. Recently, an activity that stimulates migration of normal and cancerous prostate epithelial cells has been isolated from bone extracts and identified as a low glycosylated fragment of osteonectin.⁷⁵ Interestingly, osteonectin is

expressed in the prostate stroma and could therefore stimulate the motility of epithelial cells in the conditioned medium of PrSC.^{74,76} To analyze the role of Met in prostate cancer progression and the development of tumor metastases, we are currently exploring the relationship of Met expression and prostate-specific antigen recurrence in a cohort of prostate cancer patients who underwent radical prostatectomies for moderately differentiated prostate cancer.

The *in vivo* expression pattern of HGF/SF and Met and the *in vitro* functional analysis highlight the importance of HGF/SF-Met in the development and metastasis of prostate carcinoma and encourage the development of HGF/SF-Met targeted anti-neoplastic and anti-metastatic therapies.

Acknowledgments

We thank Dr. Mukta Webber for suggestions related to the culture of primary prostate epithelial cells. We also thank Dr. Selina Chen-Kiang and members of her laboratory for frequent discussions during the course of this project and Dr. William Muller for comments and suggestions on the manuscript.

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