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The *met* Proto-Oncogene Receptor and Lumen Formation

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The *met* proto-oncogene product (Met) and its ligand, hepatocyte growth factor/scatter factor (HGF/SF), have been implicated in cell mitogenic response, cell motility, and the promotion of the ordered spatial arrangement of tissue. By means of confocal laser-scanning microscopy, it was shown that Met is expressed in cells bordering lumen-like structures that resemble ducts in the human mammary cell line T47D. In human breast tissue biopsies, Met staining was intense in normal cells bordering mammary ducts but was reduced in adjacent tumor tissue. Met staining in lumen-forming organs colocalizes with staining of antibody to phosphotyrosine, which suggests that the Met receptor and its substrates may be activated in lumen structures or ducts. HGF/SF treatment of human epithelial carcinoma cell lines resulted in the formation of lumen-like structures in vitro. Reduced expression of Met could be related to the extent of tumor cell differentiation.

The human *met* proto-oncogene product (Met), a member of the family of tyrosine kinase growth factor receptors, was identified by means of the activated oncogene *tpr-met* (*1*, *2*). Met is synthesized as a glycosylated 170-kD precursor and cleaved in the external (ligand-binding) domain to yield a mature disulfide-linked α (50-kD), β (140-kD) heterodimer (*1*, *3*, *4*). The Met receptor is expressed in a variety of tissue and cell types, but the highest concentrations are found in epithelial cells (*5*, *6*). Hepatocyte growth factor (HGF) has been shown to be the ligand for the Met receptor. Under physiological conditions, the kinase activity of Met is dependent on the binding of the mature heterodimeric receptor to its ligand (*7*). HGF is a mediator of liver regeneration both in humans and in rodents (*8*) and is a powerful mitogen for hepatocytes and epithelial cells (*9*). Scatter factor (SF) is identical to HGF (*10*, *11*) and was independently shown both to promote epithelial cell motility (scattering) and to cause certain epithelial cell lines to become invasive when assayed in vitro (*11*, *12*). HGF/SF has been shown to be involved in the differentiation that causes Madin Darby canine kidney (MDCK) epithelial cells to morphologically change into branching tubules (*13*).

The expression of Met in cells and tissues of both human and mouse origin was determined. Immunoprecipitation and protein immunoblot analyses showed that Met is expressed in the human breast carcinoma cell line T47D (*14*, *15*). The distribution of Met in T47D cells was examined by immunofluorescence and confocal laser-scanning microscopy (CLSM) with C28 (*16*) or C200 (*17*) rabbit antibodies to a COOH-terminal or an NH_2-terminal peptide of Met, respectively (Figs. 1 through 3). Controls were prepared with C28 antiserum in the presence of competing peptide or without C28 antiserum.

T47D cells in suspension form lumen-like structures resembling mammary ducts (*14*). Analyses of T47D cells in paraffin sections stained with C28 antibody (*16*) showed intense fluorescent staining in cells bordering lumen-like structures (Fig. 1). A marked decrease in intensity was observed when competing C28 peptide was added to the primary antibody or when the primary antibody was omitted (*18*, *19*). Moreover, T47D cells stained with the C200 antibody, directed against the Met extracellular domain, also showed intense fluorescent staining in cells bordering the lumen-like structures (Fig. 1). We observed an 80-fold (± 5-fold) greater fluorescence by CLSM in cells that form the lumen border than in adjacent cells (Fig. 2). The subcellular localization of Met in the T47D cells was also investigated by electron microscopy with indirect immunogold labeling and the C28 Met antibody (*17*). These analyses show that Met was localized to microvilli that protrude into the lumen (Fig. 3).

The intense Met-specific staining of T47D cells bordering the lumen prompted us to examine Met expression in normal and abnormal human breast tissue. Fifty human breast carcinoma biopsies were examined by CLSM with C28 Met antibody (*17*). Normal and tumor tissue from a representative breast biopsy is shown (Fig. 4). We found intense Met fluorescence in cells that form a normal mammary duct (Fig. 4A). Thus, the intensity of staining was always greater (~80-fold) in the duct-forming cells than in the adjacent nonductal cells (*18*, *20*). The pattern of Met staining in the mammary duct was similar to but higher than that observed in the T47D lumen-like structures (Figs. 1 through 3). By contrast, Met staining in adjacent breast tumor tissue was always reduced, but even in the disrupted architecture of the tumor, Met fluorescence was evident in lumen- or duct-like structures (Fig. 4C).

Met is rapidly phosphorylated on tyrosine residues after HGF/SF activation (*7*), and labeling with a monoclonal antibody to phosphotyrosine (anti–P-Tyr) colocalizes with Met staining in cells activated with the Met ligand HGF/SF (*15*). We co-stained breast tissue samples with anti–P-Tyr and found intense anti–P-Tyr fluorescence that colocalized with Met staining in the cells forming the normal breast duct (Fig. 4B). These results suggest that Met and its substrates may be activated in these cells. However, we cannot exclude that other tyrosine kinase receptors and other substrates are activated. As with the lower Met staining of tumor tissue (Fig. 4C), we also observed reduced amounts of anti–P-Tyr staining in the tumor cells (Fig. 4D); however, even the lower amounts of anti–P-Tyr fluorescence colocalized with Met staining. It has been proposed that HGF may be a tumor suppressor gene (*21*), and this has also been suggested for *met* (*22*); both of these genes are localized on the long arm of chromosome 7. Monosomy 7 or 7q

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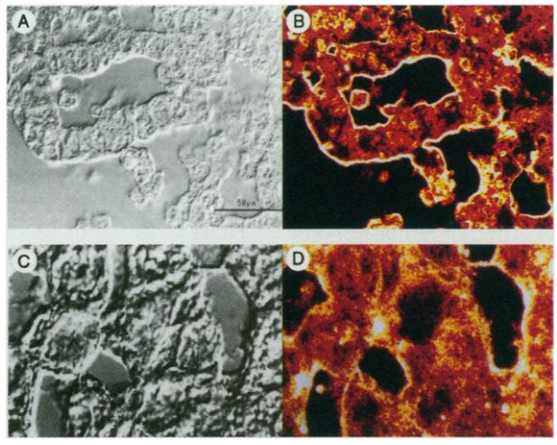

Fig. 1. Lumen-like structures in T47D cells in culture. (**A**) Nomarski image. (**B**) Immunofluorescence of cells stained with C28 antibody to human Met. (**C**) As in (A). (**D**) Immunofluorescence of cells stained with C200 antibody to human Met. Scale bar (50 μm) in (A) indicates relative size for (A) through (D).

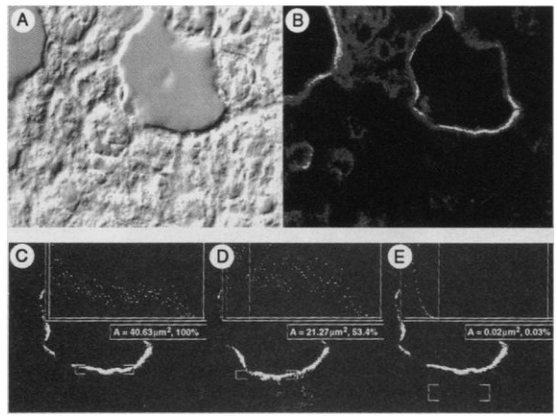

Fig. 2. T47D cells forming a duct-like structure. (**A**) Nomarski image. (**B**) Immunofluorescent staining of the same region with Met C28 antibody. (A) and (B) are ×2.45 magnification of the lower right-hand portion of Fig. 1, A and B, respectively. Overall magnification is ×490. (**C** through **E**) Quantitative determination of the Met-specific immunofluorescence. The histograms show the distribution of fluorescence of the bracketed areas. Distribution of fluorescence in the apical region of the lumen is shown for the right lumen (C), upper left lumen (D), and an adjacent region (E). Brighter staining indicates increased fluorescence. A, area of defined fluorescence intensity.

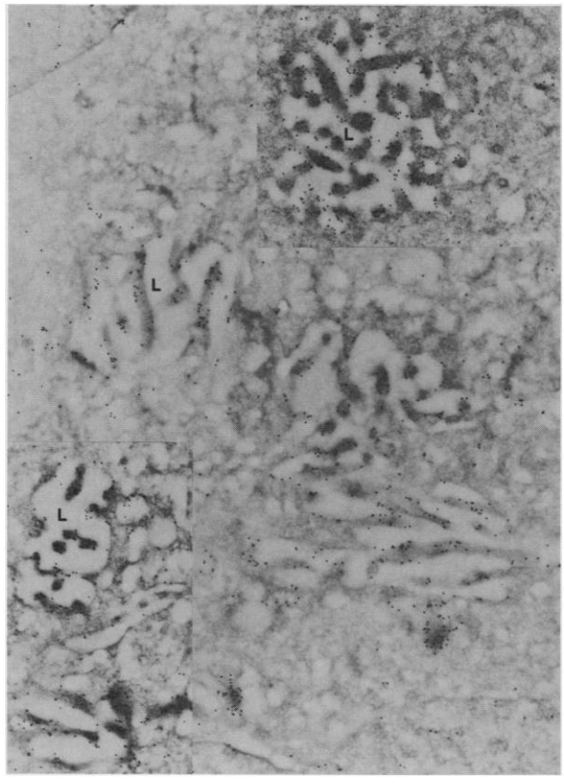

Fig. 3. Subcellular localization of Met in T47D cells. Serial sections (~ 600 nm) through the same lumen-like structure were examined by immunoelectron microscopy with secondary antibody labeled with 10-nm colloidal gold particles (*31*). Met is localized in microvilli that protrude into the lumen (L) and could account for the apical staining observed in cells bordering the lumen in Figs. 1 and 2. The panels represent three sections ~ 4 μm apart. From the bottom, left to right, are the middle, upper, and lower sections. The lumen acts as a point of reference for alignment of the sections (magnification ×15,600).

are often found in certain tumors, and loss of heterozygosity on 7q in breast tumors correlates with significantly shorter metastasis-free survival and overall survival (*22*). This result is consistent with the reduced amounts of Met expression in human breast cancer that we observed in the breast tissue biopsies. This raises the possibility that the reduced organization in the tumor tissue may be related to suppressed Met expression.

Our analyses showed that human Met expression both in vitro and in vivo is concentrated in cells that border lumen-like structures. Because extensive lumen and duct formation occurs in the early development of the digestive tract, we investigated Met expression in this tissue in mouse embryos. These analyses, performed with a COOH-terminal rabbit peptide antibody SP260 (6), showed that in 11-day-old mouse embryos, Met is expressed in the lumen-bordering cells (Fig. 5). As in human samples, intense Met staining was evident on the apical side of the cells forming the lumen of the digestive tract (Fig. 5, A and D) and was 50-fold (± 2-fold) higher in these cells than in the surrounding tissue (*18*, *23*). Serial sections stained in the absence of the primary COOH-terminal mouse Met antibody or in the presence of competing peptide showed much lower amounts of fluorescence (Fig. 5, B and C). Moreover, as in the cells lining the human breast duct, mouse Met staining colocalized with anti–P-Tyr staining in the lining of the embryonic digestive tract (Fig. 5E), as shown in computer-generated overlap analysis (Fig. 5F). Thus, the Met receptor appears to be preferentially expressed in the border cells of the breast duct and in the lumen of the gastrointestinal tract. Met staining in lumen-like structures has been observed in human gastrointestinal lumen and the lumen of biliary ducts and esophagus (*24*). The colocalization of Met staining with anti–P-Tyr immunofluorescence suggests that the Met receptor is activated in the lumen-forming cells of the breast duct and gastrointestinal tract (Figs. 4 and 5).

After establishing a correlation between the expression of Met and lumen formation, we tested whether Met-HGF/SF could induce lumen formation in human epithelial carcinoma cells in vitro. Cells that expressed Met were exposed to varying concentrations of HGF/SF (1 to 100 ng/ml) (*25*). The results with two human colon carcinoma cell lines, SW480 and HT29, are shown in Fig. 6. We observed formation of lumen structures in these cells when they were grown on glass (70% confluent) and treated with HGF/SF. Lumen formation was cell density–dependent and not obvious when cells were grown in plastic tissue

culture flasks or treated with epidermal growth factor instead of HGF/SF under the same conditions (*18*).

There are studies that cite differences in cell growth and morphology when the cells are grown on glass instead of plastic. These differences are presumably due to cell-to-cell and cell-to-substratum interactions (*26*). Lumen formation was also observed in vitro with T47D and MCF7 breast carcinoma cell lines after HGF/SF treatment (*18*). These analyses showed that lumen formation was dependent on the dose of HGF/SF (Fig. 6). Thus, when cells were treated with 1 to 10 ng of HGF/SF per milliliter of medium, we observed lumen-like structures with a uniform size. The regularity in the size may indicate that the number of cells forming the lumen border is controlled and that cell-to-cell interactions are involved in this formation. The *sevenless* gene in *Drosophila*, a tyrosine kinase growth factor receptor that specifies cell fate in ommatidia formation through cell-to-cell interactions (*27*), may possibly serve as a model for Met-HGF/SF interaction. At high concentrations of HGF/SF (100 ng/ml), lumen formation was abrogated (Fig. 6).

The scattering response of HGF/SF establishes that this factor is responsible for cell motility and differentiation (*28*). MDCK cells grown in collagen gels in the presence of HGF/SF form bracing tubules, which suggests that the three-dimensional geometry of cell-to-substrate interactions directs MDCK cells to organize into tubules in response to HGF/SF (*13*). Here, we demonstrate that HGF/SF can induce lumen structures in epithelial cancer cell monolayers in vitro.

Mammary gland epithelia undergo developmental changes during pregnancy to become secretory. In culture, mammary epithelial cells can regain their differentiated phenotype only when appropriate hormonal and substratum conditions are provided (*29*, *30*). Met expression in normal breast duct cells suggests that HGF/SF and Met may be involved in the differentiation of ductal epithelium in the mammary gland. Moreover, rodent mammary gland differentiation can be achieved in vitro when the cells are grown on a suitable basement membrane matrix (*30*). Although this could be caused by many factors, it is possible that the extracellular matrix contains traces of HGF/SF or that

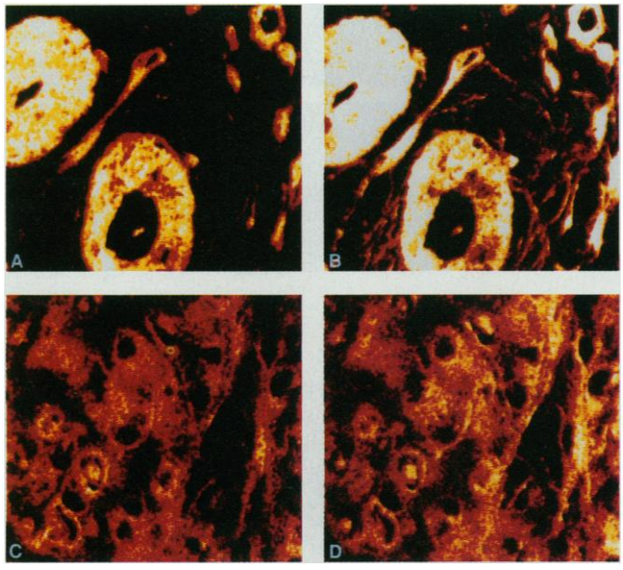

Fig. 4. Expression of Met in breast tissue biopsies. A paraffin-embedded section of a biopsy from breast adenocarcinoma was subjected to indirect immunofluorescence staining with C28 antibody or monoclonal antibody 4G10. The cells and immunofluorescence were analyzed by CLSM. Immunofluorescence analysis of normal mammary duct (**A** and **B**) and adjacent tumor tissue (**C** and **D**) was performed with C28 (A and C) or anti–P-Tyr (B and D). Magnification, ×250.

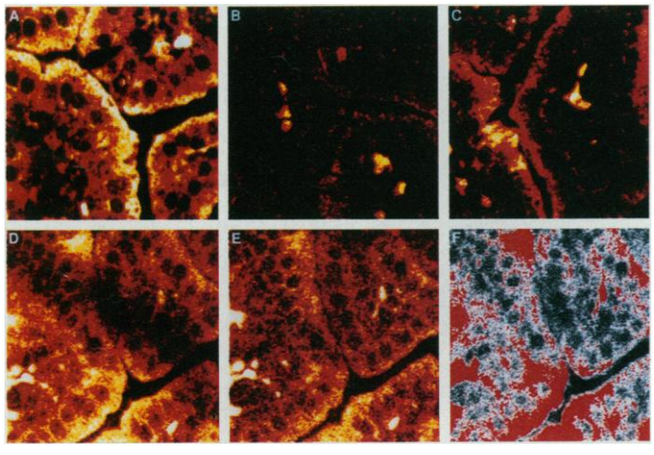

Fig. 5. Expression of Met in the mouse embryonal digestive tract. Eleven-day-old BALB/c mouse embryos were fixed, embedded in paraffin, and subjected to immunofluorescence staining with SP260 and 4G10 antibodies. (**A**) Apical staining of the lumen of the digestive tract with SP260. (**B**) Staining in the absence of the primary SP260 antibody. (**C**) Staining in the presence of SP260 competing peptide. (**D**) As in (A). (**E**) Immunofluorescence with 4G10. (**F**) Analysis of the overlap between the Met and the anti–P-Tyr staining by a confocal image processing program (red indicates increased overlap). Magnification ×220 (A through F).

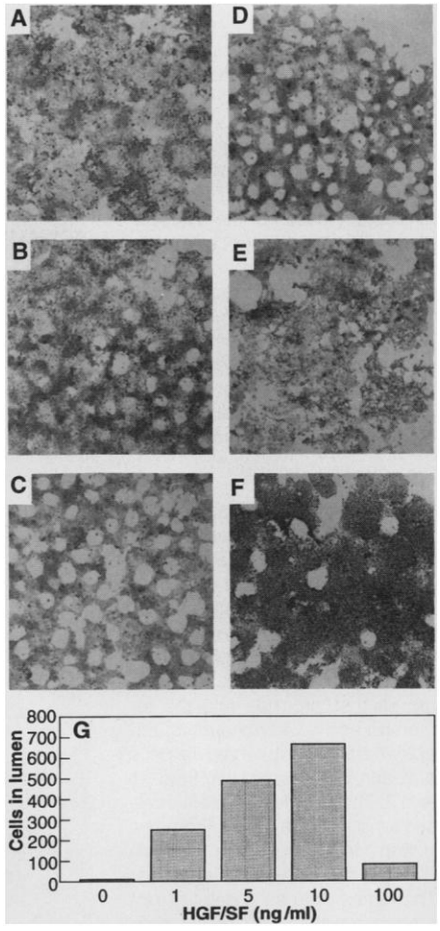

Fig. 6. HGF/SF induces lumen formation in SW480 and HT29 human colon carcinoma cell lines. The cells were treated with the specified concentration of HGF/SF when they were ~70% confluent. SW480 cells grown for 18 hours in complete medium without HGF/SF (**A**) or with HGF/SF at 1 ng/ml (**B**), 5 ng/ml (**C**), 10 ng/ml (**D**), or 100 ng/ml (**E**). (**F**) HT29 cells grown for 18 hours in complete medium with HGF/SF (10 ng/ml). Magnification ×164. (**G**) The influence of HGF/SF concentration on lumen formation. The number of cells forming lumen structures was calculated for each HGF/SF concentration.

Met is constitutively activated in those cells.

The coordination of differentiation and proliferation is an essential feature in the successful development or replacement of tissues, but it is not clear how the decline of proliferation is related to the onset of differentiation. The expression of Met in lumen border cells in vivo and in vitro and the colocalization of Met and anti–P-Tyr immunofluorescence suggest that the Met receptor may be involved in differentiation toward lumen formation. Spatial organization might be dictated by the restricted distribution of the receptor (Fig. 3) (*4*). It is possible that the motogenic (scattering) activity of HGF/SF (*28*) is a fundamental part of this formation.

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31. Fixed cells were embedded in L.R. gold resin (Electron Microscopy Sciences) at −25°C, sectioned with a Nova Ultratome (LKB, Uppsala, Sweden), and picked up with Formvar-coated 200-mesh gold grids (Fort Washington, PA). The grids were washed three times in PBS for 10 min and incubated in 1% bovine serum albumin (BSA) in PBS for 2 hours and in C28 antibody (diluted 1:50 in 1% BSA) at room temperature for 1 hour. Controls were incubated either in the presence of C28 competing peptide or in the absence of the primary antibody. The grids were washed again in PBS, incubated in RPMI 1640 medium for 20 min in 1% BSA, and reacted with goat antibody (to rabbit immunoglobulin) conjugated to gold (10-nm diameter; 1:10 diluted in 1% BSA; Amersham) at room temperature for 1 hour. The grids were finally washed in PBS and distilled water and stained with uranyl acetate and lead citrate. The sections were observed and photographed with an EM 410 electron microscope (Philips Eimdhoven, the Netherlands).

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Regulation of Protein Serine-Threonine Phosphatase Type-2A by Tyrosine Phosphorylation

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Extracellular signals that promote cell growth activate cascades of protein kinases. The kinases are dephosphorylated and deactivated by a single type-2A protein phosphatase. The catalytic subunit of type-2A protein phosphatase was phosphorylated by tyrosine-specific protein kinases. Phosphorylation was enhanced in the presence of the phosphatase inhibitor okadaic acid, consistent with an autodephosphorylation reaction. More than 90% of the activity of phosphatase 2A was lost when thioadenosine triphosphate was used to produce a thiophosphorylated protein resistant to autodephosphorylation. Phosphorylation in vitro occurred exclusively on Tyr^{307}. Phosphorylation was catalyzed by $p60^{v\text{-}src}$ $p56^{lck}$, epidermal growth factor receptors, and insulin receptors. Transient deactivation of phosphatase 2A might enhance transmission of cellular signals through kinase cascades within cells.

Protein phosphatase type-2A (phosphatase 2A) is one of the two predominant enzymes that hydrolyze phosphoserine and phosphothreonine residues in proteins in all eukaryotic cells (*1*). The catalytic subunit of phosphatase 2A is highly conserved among eukaryotic species. There is more than 70% identity in the amino acid sequences of phosphatase 2A from humans and the budding yeast *Saccharomyces cerevisiae*. The 309-residue protein is found in several species as two isoforms, which differ at eight positions (*2*). Phosphatase 2A is thought to exist in animal cells primarily as a heterotrimer (*3*), composed of three subunits, with A = 60 kD, B = 55 kD, and C (catalytic) = 36 kD. However, the AC heterodimer is specifically complexed with

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