Met Receptor Overexpression and Oncogenic Ki-ras Mutation Cooperate to Enhance Tumorigenicity of Colon Cancer Cells in Vivo

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Abstract

We have investigated the influence of Ki-ras oncogene on Met/hepatocyte growth factor (HGF) receptor signaling in human carcinoma cells. The model system used in these studies included the DLD-1 colon cancer cell line with a mutated Ki-ras allele, and the DKO-4 cell line generated from DLD-1, with its mutant Ki-ras allele inactivated by targeted disruption. These cell lines were transduced with cDNAs of either active Met receptor or dominant negative Met receptor. As compared to the DLD-1 cells, constitutive overexpression of Met receptor in this cell line (DLD-1-Met) resulted in increased tumorigenicity in SCID mice. In contrast, overexpression of Met in DKO-4 cells (DKO-4-Met) that have lost oncogenic Ras activity demonstrated suppressed tumorigenicity with respect to the parent DKO-4 cell line. Tumors formed by the DLD-1-Met cells showed increased levels of mitogen-activated protein kinase (MAPK) and lower levels of apoptosis compared to the DKO-4-Met tumors. Overexpression of the dominant negative Met receptor cDNA decreased the Met phosphorylation levels in both DLD-1 and DKO-4 cells, but only suppressed tumorigenicity in the DKO-4 cell line. In vitro, HGF stimulation of DLD-1 cells resulted in a prolonged duration of MAPK activation, while DKO-4 cells exhibited a rapid attenuation of MAPK phosphorylation. The results suggest that Ki-ras mutations and HGF signaling cooperate to enhance tumor growth by increased duration of MAPK activation and decreased apoptosis in human carcinoma cells.

Introduction

Met/hepatocyte growth factor (HGF) receptor is a receptor tyrosine kinase (RTK) that plays an important role in regulating cellular proliferation, motility, morphogenesis, and apoptosis (1). Met receptor levels are found to be elevated in a wide variety of cancer types including lung, colon, and pancreatic cancers (2). Previous reports have indicated that overexpression of the Met receptor enhances tumorigenicity in some cell lines (2), but suppresses tumorigenicity in other cell lines (3). Cross-talk between Met and Fas receptor signaling has delineated one mechanism for this dichotomy of response and provides a rationale for cell death response due to Met overexpression (4). While this cross-talk may provide a mechanism for growth suppression in Met-overexpressing tumors, there remains the question of whether growth promotion in vivo may also be modulated by other Met-interacting signaling molecules.

One of the key effectors of Met receptor signaling is the Ras family of membrane-associated guanine nucleotide exchange proteins (5, 6). Activation of Met by HGF may lead to activation of several pathways downstream of Ras such as the Raf/MEK/mitogen-activated protein kinase (MAPK) (7) and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) (8, 9) pathways. In addition, the Met receptor has a p85 PI3K binding domain that allows for direct activation of the PI3K/PKB pathway in a Ras-independent manner (10). Both the MAPK and the PI3K pathways can contribute to cell survival and proliferation and are thought to play key roles in development of cancers.

The Ras family contains several isoforms including N-ras, H-ras, and Ki-ras. Ki-ras is one of the most frequently mutated genes in a wide variety of human cancers including lung and colorectal cancers (11–13). Oncogenic mutations on Ras constitutively activate downstream signaling cascades in cell lines ectopically expressing the Ras oncogene (14–17). Such activity putatively mimics a perpetual signaling from the upstream RTKs. However, when MAPK activity is measured in cell lines with mutant ras genotype, constitutive elevation of MAPK phosphorylation is not consistently observed (18–21). Furthermore, several reports have shown that the presence of oncogenic Ras mutation does not preclude a significant activation of the MAPK pathway on growth factor stimulation (16, 18). These observations suggest that Ras oncogene may require other interactors to constitutively activate the MAPK signaling cascade in vivo.
Concomitant overexpression of tyrosine kinase receptors including Met receptor is common in human carcinomas that demonstrate high frequency of Ki-ras mutations, including colon, lung, and pancreatic cancers. This led us to hypothesize that mutated Ki-ras oncogene may modulate Met receptor signaling and lead to increased oncogenicity. We have investigated this possibility using colon cancer cell line DLD-1 with an activating Ki-ras mutation (22) as well as its derivative cell line DKO-4 that has had a targeted disruption of the mutant Ki-ras allele but retains the remaining normal Ki-ras allele. The inactivation of the mutant Ki-ras allele in the DLD-1 cell line resulted in both suppressed anchorage-independent growth capacity in soft agar and suppressed s.c. tumor formation in nude mice. We demonstrate here that Met receptor overexpression enhances tumorigenicity only in the cell line with Ki-ras mutation but not in its Ki-ras oncogene-inactivated counterpart, thus identifying Ras oncogene as a key modulator of Met signaling activity in cancer cells.

Results

Establishment of Met-Overexpressing and Met Down-Regulated Stable Cell Lines

Using the pBMN-ires-GFP retroviral vector system in the Phoenix packaging cell line, 40% retroviral transduction efficiencies were achieved in the DLD-1 and DKO-4 colon cancer cell lines (data not shown). After fluorescence-activated cell sorting of the GFP-positive cells, high Met-expressing stable cell lines of DLD-1 and DKO-4 were established. These cell lines demonstrated high levels of Met receptor protein, which was constitutively autophosphorylated (Fig. 1). Further stimulation of the cells by HGF did not result in additional increase in autophosphorylation levels of the receptor, indicating that they are in fully activated state. In contrast, stable expression of the dominant negative Met receptor (dn-Met) resulted in significant down-regulation of the endogenous receptor autophosphorylation levels following treatment with HGF.

Met Receptor Overexpression Enhances Tumorigenicity in ras Mutant Cell Lines

Consistent with previous reported results (22), the parental DLD-1 cell line exhibited increased tumorigenicity in SCID mice compared to DKO-4 Ki-ras disrupted progeny (Fig. 2). Constitutive overexpression of the Met receptor in DLD-1 cell line (DLD1-Met) demonstrated enhanced xenograft tumor growth rate as compared to the parental and empty vector transduced control cell lines (Fig. 2). In contrast, down-regulation of the Met receptor activity in DLD-1 cells did not result in a loss of tumorigenicity. This indicates that Ras has a dominant role in the oncogenicity of this cell line, but Met receptor overactivation may enhance its tumorigenic activity. However, either the overexpression or down-regulation of Met receptor activity in the DKO-4 cells without activated ras oncogene resulted in suppressed tumorigenicity, indicating that in the absence of oncogenic Ki-ras signaling, Met receptor does not promote oncogenesis.

Ki-ras Oncogene Potentiates HGF-Met-Induced Anchorage-Independent Growth Activity

Consistent with previously reported data, DLD-1 has higher colony-forming efficiency in soft agar than DKO-4 cells (22). Up- or down-regulation of Met receptor expression did not significantly change the overall soft agar colony-forming efficiency of both the DLD-1 and DKO-4 cells (Fig. 3A). HGF stimulation of the DLD-1 cell lines did not significantly increase their overall colony-forming efficiency, but HGF treatment significantly enhanced the anchorage-independent growth capacity of DKO-4 cells (Fig. 3A). On further examination of the soft agar plates, a noticeable increase in colony size was noted in plates treated with HGF, as compared
to untreated counterparts (Fig. 3B). For DLD-1 cell lines, HGF-induced large colony formation was most noticeably enhanced in dn-Met derivative, indicating that colony number could reach a plateau at a certain level of Met expression in DLD-1. Furthermore, HGF appears capable of inducing the maximum capacity for large colony formation of DLD-1-dn-Met cells with significantly reduced Met receptor activity. In contrast, the absence of Ras mutation limits the maximum capacity of DKO-4 cells to form large colonies, regardless of the Met receptor expression levels (Fig. 3C). The results suggest that the HGF-Met-mediated anchorage-independent growth of these colon cancer cells is markedly sensitized by the Ras oncogene.

**Ki-ras Mutation and Met Overexpression Cooperate to Enhance MAPK Phosphorylation in Mouse Tumor Tissue**

To further characterize the increased growth rate of the DLD-1-Met xenograft tumors, cell lines and tumor tissues were investigated for their levels of phosphorylated MAPK and PKB. These pathways signal downstream from the Met receptor and are both potentially involved in cell proliferation and survival. Tumor tissue and cell lines were studied by immunoblot for their levels of Met and dn-Met receptor, as well as phosphorylated receptor levels (Fig. 4A).

Both the DLD-1 and DKO-4 demonstrated similar basal levels of Met receptor expression, which indicates that Met receptor level was not modulated by Ras mutation in these lines (Fig. 4A, left panel). While both the tumor tissue and cell lines had expression of Met and dn-Met constructs, only cell lines grown in tissue culture showed elevated receptor phosphorylation. Cell lines grown in tissue culture all had similar constitutive levels of MAPK and PKB phosphorylation, regardless of Ki-ras genotype, or stable overexpression of Met or dn-Met (Fig. 4A, left panel). DLD-1 tumors overall exhibited slightly increased MAPK phosphorylation as compared to DKO-4 tumors (Fig. 4A,
FIGURE 4. DLD-1 tumors have elevated MAPK phosphorylation and low rates of apoptosis. A, Western blots for phospho-PKB (pPKB), phospho-MAPK (pMAPK), phospho-Met (pMET Y1234/1235), murine Met (for dn-Met construct), and total Met. Loading control is total MAPK. Left panel shows 18–24 h serum-starved cell lines at 80% confluence; right panel shows tumor tissue extracts. B, Immunohistochemistry for phospho-MAPK in DLD-1, DLD-1-Met, DKO-4, and DKO-4-Met tumor tissue. C, Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay results for DLD-1 and DKO-4 tumor sections. Mean number of positive cells from 10 fields ± SE, counted at ×400 magnification.
right panel), but DLD-1-Met tumors demonstrated further markedly elevated MAPK phosphorylation levels when compared to both the DLD-1 control and all of the DKO-4 tumors, as detected by both immunoblot and by immunohistochemistry (Fig. 4, A and B). It is worth noting that there was no significant difference in the levels of phosphorylated PKB in DLD-1 and DKO-4 cell lines or xenograft tumor tissue, regardless of Ki-ras genotype or Met receptor expression level, indicating a dominant role for MAPK signaling in this system.

**FIGURE 5.** HGF caused a sustained MAPK phosphorylation in cell lines with Ki-ras mutation in contrast to those with wild-type ras genotype. A, Cell lines were grown to approximately 50% confluence, serum deprived for 18–24 h, then stimulated with HGF (10 ng/ml). The total cell protein extracts were immunoblotted by antibodies to phospho-MAPK (shown) and the unphosphorylated form of MAPK to confirm loading (data not shown). B, Quantification of phospho-MAPK signal by densitometry. The changes in phospho-MAPK levels during an 8-h period were presented in relative scale to show the maximum signals (5 or 30 min post-stimulation) as 100%. The data are representative of duplicate or triplicate trials for each cell line. C, Cell lines were treated as in A and then immunoblotted using antibodies specific to phospho-PKB. Membranes were subsequently reprobed with antibody to the unphosphorylated form of PKB. The data presented are representative of triplicate experiments. D, Quantification of phospho-PKB signal by densitometry. The changes in phospho-PKB levels during an 8-h period are presented as above in B. The data are representative of triplicate studies for each cell line. E, Patterns of Raf and MEK activation by HGF. Cells were treated as in A and the levels of phospho-Raf1 and phospho-Mek1/2 during the 8-h time course estimated by Western blot. The data presented are representative of duplicate experiments.
Met and Ki-ras-Mediated Growth Response Is Not Due to a Decreased Rate of Apoptosis

To determine if the Met and Ki-ras-regulated increase in tumor growth was due to a decrease in apoptosis or an increase in cellular proliferation, TUNEL staining was performed on tumor tissue sections. DLD-1-Met tumors did not show decreased rates of apoptosis compared to DLD-1 parent and vector control tumor sections (Fig. 4C). DKO-4 and DKO-4 vector control tumors had elevated rates of apoptosis compared to the DLD-1 tumors (Fig. 4C), consistent with their reduced tumor growth rate in SCID mice (Fig. 2). The DKO-4-Met tumors demonstrate a particularly enhanced rate of apoptosis as compared to DKO-4 controls (Fig. 4C), which was also consistent with tumor growth suppression observed in vivo (Fig. 2).

Ki-ras Mutation Sustains HGF-Induced MAPK Phosphorylation

To further investigate the effect of Ki-ras oncogene on the activation of MAPK by HGF, serum-starved cells were stimulated with HGF over an 8-h time course. For this study, additional cell lines were used. These included DKs-8, another Ras oncogene-inactivated progeny of DLD-1, as well as another colon carcinoma cell line, HCT-116, with its HKh-2 and Hke-3 Ki-ras disrupted clones (22). We assessed the basal Met receptor levels in these cell lines and did not find any significant influence of the receptor level by Ras mutation in this system (data not shown). Furthermore, none of these cell lines expressed HGF at any significant level in an autocrine fashion (data not shown). All cell lines, regardless of their Ki-ras genotype, showed a rapid increase (within 5–30 min) in MAPK phosphorylation in response to HGF, indicating that they are all equally responsive to HGF-induced signaling activation. More importantly, we noted that elevated levels of phosphorylated MAPK were maintained for up to 8 h after HGF stimulation in parental DLD-1 and HCT-116 cell lines (Fig. 5A). In contrast, the phospho-MAPK levels more rapidly and dramatically returned to near-basal levels by 3 h post-stimulation in all cell lines (data not shown). Hence, these cell lines are equally responsive to HGF-induced signaling activation. Moreover, the speed, amplitude, and duration of MAPK phosphorylation were variable and correlated better between cells of similar lineage than with Ki-ras genotype. The densitometry data from three independent studies demonstrated that PKB phosphorylation kinetics were similar for all cell lines (Fig. 5D). Similar to MAPK activation, the levels of phosphorylated PKB had returned to basal levels within 24 h after growth factor stimulation in all cell lines (data not shown). Consistent with in vivo results, PKB signaling by HGF stimulation was not significantly influenced by Ki-ras genotype in these lines.

Ki-ras Modulation of HGF Response Is Regulated at the MAPK Level

Activation of Ras triggers a kinase cascade, which causes phosphorylation of Raf, MEK, and eventually MAPK (5). To investigate at which level in the signaling cascade that ras mutation modulates the sustained MAPK phosphorylation in cell lines stimulated by HGF, we studied the time course of Raf-1 and MEK activation in colon cancer cell lines DLD-1 and DKO-4 following treatment with HGF. The phospho-Raf-1 antibody used for these studies detects phosphorylation at Ser259, which is indicative of suppression in Raf-1 activity. Both the parent DLD-1 and mutant ras knockout DKO-4 lines initially demonstrated relatively high levels of Raf-1 phosphorylation corresponding to suppressed activity. Following stimulation by HGF, the level of phosphorylated Raf-1 decreased during the first 30 min (increased activity), then returned to the normal level by 3 h (Fig. 5D). For both cell lines, MEK phosphorylation (active MEK1/2) also peaked within 5 min after HGF stimulation, but it rapidly attenuated to basal levels by 30 min post-stimulation (Fig. 5D). The effect of ras oncogene on the duration of MAPK activation by HGF signaling appeared regulated at the MAPK level.

Discussion

We have demonstrated that overexpression of the HGF receptor Met in the DLD-1 colon carcinoma cell line, harboring an oncogenic Ki-ras mutation, results in an increase in tumor growth rate in vivo. This increase in tumorigenicity of the DLD-1-Met cell line appeared dependent on an increase in proliferation rather than a decrease in apoptosis rates (Fig. 4C). Further, this increase in tumor growth rate is dependent on the presence of activated Ki-ras, as DKO-4 cells with a disrupted Ki-ras oncogene showed growth suppression in vivo when Met receptor was overexpressed in this line, possibly due to increased apoptosis rate. Overall, the data indicate that Ras oncogene can sensitize colon cancer cells to the growth effect of Met receptor activation.

Interaction between Ras oncogene and HGF-Met signaling pathway in oncogenesis has been reported previously. Webb et al. (23) showed that transformation of NIH3T3 and C127 cells by Ras oncogene resulted in Met receptor overexpression and increased tumorigenicity and metastatic potential. In vivo,
this is also accompanied by increased HGF expression. These results corroborate our finding that the co-activation of ras oncogene and Met receptor activity can enhance malignancy in cancer cells.

In a subsequent study, Furge et al. (24) reported that downregulation of the Met receptor signaling activity by expression of the same dominant negative Met cDNA as used in our current study, resulted in suppression of tumorigenicity and metastatic potential in a cell line transformed with H-ras G12V. Our study shows no tumor growth suppression in DLD-1-dn-Met cell lines, indicating that Met suppression is insufficient to subvert endogenous Ki-ras activation. These results indicate that there are potentially differential responses between endogenously mutated and ectopically expressed Ras oncogene in cells. Alternatively, there may be an isoform-specific interaction between Ki-ras and Met in a colon cancer model. This finding has particular relevance to the design of novel cancer therapeutics which target down-regulation of Met receptor signaling.

While there is a good correlation between the higher tumorigenicity and anchorage-independent growth capacity of DLD-1 cells compared to its Ki-ras oncogene-inactivated progeny DKO-4 cells, the in vivo effect of Met overexpression in enhancing tumorigenicity of DLD-1 cells was not reflected in the results of soft agar assay. This may be due to the semiquantitative nature of the soft agar assay and its limit to detect enhanced proliferation in cells with high basal soft agar colony forming efficiency. Nevertheless, the data provide some insights into the interaction of Met receptor signaling and Ki-ras oncogene. The Ki-ras oncogene appears to sensitize these colon cancer cells to HGF-Met-induced proliferation even at the low Met receptor activation level present in the dn-Met-expressing cell line. Although the dn-Met construct significantly decreased the HGF-induced Met auto-phosphorylation in DLD-1-dn-Met cells, a low level of activation was still observed in response to HGF, and this was sufficient to maximally enhance the soft agar colony forming efficiency in these cells (Fig. 3C). However, the increase in colony-forming ability seen in DLD-1-dn-Met in response to HGF was not seen in vivo, where the DLD-1-dn-Met cell line appears to grow at a similar rate as the control (Fig. 2). In contrast, the highest level of HGF-induced Met receptor activation in DKO-4 cells failed to abrogate the loss of anchorage-independent growth capacity due to inactivation of the Ki-ras oncogene. The result is consistent with the in vivo results showing that Met overexpression stimulates tumorigenicity in DLD-1 cells with oncogenic ras mutation, but not in the DKO-4 cells that have lost its activated Ras oncogene activity. Overall, the data suggest that Ras oncogene activation is dominant over Met receptor activation in oncogenesis, and Ras does not subvert but actually synergizes with Met in stimulating proliferation in vivo.

The observed increased phospho-MAPK but not the phospho-PKB levels in DLD-1-Met tumors compared to the parent DLD-1 provides a mechanistic insight into the interaction between increased Met signaling and Ras oncogene. On the basis of this finding, we positied that Ki-ras oncogene might influence the sensitivity of tumor cells to HGF-induced Met receptor by altering the nature of signaling responses. Consistent with this hypothesis, we have shown that HGF-Met-induced MAPK activation was prolonged in tumor cells with activated ras oncogene but not in cells with wild-type Ras. This effect was demonstrated in several isogenic colon cancer lines, and was also consistently observed in a panel of lung cancer cell lines (data not shown). The effect appears HGF and MAPK pathway specific, because the same effect was not demonstrated following EGF stimulation, or with PKB activation following HGF or EGF treatment (data not shown).

Temporal differences in cellular signaling may have significant phenotypic manifestations. The duration of MAPK signaling response affects differentiation in PC12 cells exposed to either EGF or NGF (25) (reviewed in Ref. 26). In Madin-Darby canine kidney (MDCK) cells, morphogenesis is also promoted by HGF but not EGF, and is linked to the sustained activation of MAPK by HGF (27). These morphological changes could be due to changes in gene expression, caused by alterations in activity of transcription factors such as Fos (28).

The mechanism that results in differential effect of ras mutation on the duration of HGF-induced MAPK activation remains to be determined. One possible explanation for this change, which appears to occur at the level of MAPK, is that HGF induces greater levels of expression or activity of MAPK-specific phosphatases in cells with wild-type genotype as compared to those with mutant Ki-ras genotype. The MAPK phosphatase family consists of 15 members reported to date (29). Among these, we have found that MKP1 and MKP2 are not involved in the cell lines we studied (data not shown).

Collectively, our data provide evidence that HGF and Ki-ras cooperate to modulate MAPK signaling duration and that this temporal difference in signaling results in increased tumor growth rate. Most carcinoma cells express many autocrine- or paracrine-activated tyrosine kinase receptors with overlapping signaling cascades. The differential modulation of tyrosine kinase receptor activity by Ras oncogene may provide a mechanism for predicting the effect of each receptor and ligand on the growth, differentiation, metastatic potential, and therapeutic response of tumor cells.

Materials and Methods

Reagents

Phospho-PKB (Ser473), PKB, phospho-p44/p42 MAPK (Thr202/Tyr204), p44/p42 MAPK, phospho-Met (Tyr1234/Tyr1235), phospho-Raf1(Ser259), and phospho-MEK1/2(ser217/221) antibodies were purchased from Cell Signaling (Beverly, MA). Antibodies against human Met (C28) and murine Met (B3) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant HGF was purchased from R&D (Minneapolis, MN). Phoenix 293T Amphi, DLD-1, and HCT-116 cell lines are available from the American Type Culture Collection (Manassas, VA).

Cell Lines and Culture Conditions

Phoenix 293T Amphi, DLD-1, DKO-4, DKS8, HCT-116, HKe-3, and HKh-2 colon epithelial cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc., Grand Island, NY). The DLD-1...
and HCT-116 colon carcinoma cell lines are heterozygous for G13D Ki-ras mutation. The DKO-4/DKs-8 and Hke-3/HKh-2 lines are derived from DLD-1 and HCT-116, respectively, following disruption of their mutant Ki-ras allele by somatic recombination technique (22).

Met Expression Constructs
The pBMN-Met construct was created by cloning the XhoI cut, full-length 4.6-kb Met cDNA, into the XhoI site of the pBMN-IRE-GFP retroviral vector. The pBMN-IRE-GFP plasmid is part of the Phoenix retroviral packaging system provided by Nolan et al. (http://www.stanford.edu/group/nolan/plasmid_maps/pmaps.html). The dominant negative Met (dn-Met) cDNA was a gift from Dr. Kenneth Lipson (Sugen Inc.), and has three mutations (K1110A, Y1349F, and Y1356F) eliminating phosphorylation at the kinase domain and multifunctional docking site of the receptor. In addition, the 21-amino acid COOH terminus of the human Met cDNA has been replaced by 12 amino acids of murine Met cDNA, allowing detection of this construct in human cells by immunoblotting for murine Met (24). This dn-Met cDNA was directionally cloned into the EcoRI and NotI sites of pBMN-IRE-GFP. The EcoRI site was filled and destroyed during cloning.

Establishment of Stable Met- and dn-Met-Overexpressing Cell Lines
To generate retroviruses, the Phoenix 293T Amphotropic retroviral packaging line was transfected with pBMN, pBMN-Met, and pBMN-dn-Met constructs using LipofectAMINE PLUS transfection reagent (Invitrogen, Burlington, ON, Canada). At 48 and 72 h post-transfection, retrovirus-containing media were harvested and cell debris was removed by centrifugation at 1500 rpm for 15 min. DLD-1 and DKO-4 cell lines were transduced with pBMN, pBMN-Met, and pBMN-dn-Met viral supernatants in the presence of 8 

mg/ml polybrene (Sigma Chemical Co., St. Louis, MO), three times at 48, 56, and 72 h after subculture. At 72 h after the first transduction, the cells were trypsinized into single cell suspension and sorted for high Green Fluorescent Protein (GFP) expression by fluorescence-activated cell sorting. Sorting attained a 90–95% pure population of GFP-expressing cells. Cell lines were monitored by flow cytometry for GFP expression for six to eight passages. No loss of GFP expression occurred in eight passages.

Anchorage-Independent Growth Assay
Petri plates (60 mm, Fisher Scientific, Nepean, ON, Canada) were pre-layered with 2 ml 0.5% Bacto agar (Life Technologies) in DMEM containing 10% FBS. Cells were then plated on top in 2 ml of 0.3% agar–DMEM with 10% FBS. All assays were carried out using three replicate plates at a seeding density of 500 cells/plate. Following overnight incubation in 5% CO2 incubator, 1 ml DMEM supplemented with 10%FBS was added to maintain hydration. For HGF-treated plates, 50 ng HGF was added once on the day following plating. Plates were hydrated once per week with 1 ml medium. After 2 weeks of growth, colonies were stained with an aqueous solution containing 0.5 mg/ml NADH with 0.5 mg/ml nitro blue tetrazolium salt. Following overnight incubation at 37°C, colonies were counted using a dissecting microscope. Small colonies were defined as 100–400 μm in diameter, and large colonies were defined as greater than 400 μm in diameter.

Tumorigenicity Assay
Cells (2 × 106) were injected subcutaneously on the lower abdomen of 5-week-old male SCID mice (n = 4/cell line). Mice were examined every 2 days and tumor length and width was measured using calipers. Tumor volume was calculated using the following formula: (length × width2)/2. At 22 days, mice were euthanized by CO2 asphyxiation, and tumors were excised. Portions of tumors were snap-frozen and stored in liquid nitrogen, or fixed in 10% buffered formalin for routine histopathological processing.

Histology and Immunohistochemistry
Paraffin-embedded tumor tissues were sectioned and immunostained for pMAPK and pPKB using antibodies specific to phospho-MAPK (1:50 dilution) and phospho-PKB (1:50 dilution), using the peroxidase anti-peroxidase method. Immunoreactivity was revealed with aminoethylcarbazole, as described previously (30). TUNEL staining was performed as previously described (31). Apoptotic index was scored by counting the number of positive-staining cells per 10 high power fields (×400 magnification) for each tumor sample.

Growth Factor Stimulation and Cell Lysis
Subconfluent (30–50%) cultures were washed twice with Hanks’ balanced salt solution and incubated in serum-free or supplement-free medium overnight. Two hours before the experiment, cells were washed with Hanks’ balanced salt solution, and the medium replaced with fresh serum-free medium. The experiment was initiated by adding HGF (10 ng/ml) to the media, and total cellular protein was isolated 5 min, 30 min, 3 h, and 8 h later. To prepare protein extracts, stimulated and unstimulated cells were washed twice with cold PBS and lysed for 15 min in ice-cold lysis buffer [50 mM HEPES (pH 8.0), 10% glycerol, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 100 mM NaF, 10 mM Na2PO4, supplemented with 5 μg/ml leupeptin, 5 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate]. Protein was extracted from snap-frozen tumor tissue by homogenizing tissue fragments in ice-cold lysis buffer for 30 s. All lysates were centrifuged at 14000 rpm for 10 min, and the supernatant stored at –70°C before subsequent analyses.

Immunoblotting
Lysates containing 10–35 μg of protein were resolved in a 10% SDS-PAGE and transferred onto polyvinylidene difluoride or nitrocellulose membrane. Equal protein loading was confirmed by staining the gel with amido black dye, and/or by immunoblotting for total PKB and MAPK. Membranes were blocked with Tris-buffered saline (TBS) containing 1% BSA and 0.1% Tween-20 and 5% nonfat milk. The membrane was incubated with primary
antibody respectively for 1 h at room temperature or overnight at 4°C in blocking solution. Membranes were washed three times for 5 min each in TBS containing 0.1% Tween 20, and incubated with HRP-linked anti-rabbit or anti-mouse IgG for 1 h at room temperature. Proteins were visualized using enhanced chemiluminescence (ECL) reagents (Roche-Boehringer Mannheim, Dorval, Quebec, Canada). Signals were quantified by scanning the X-ray films and integrating selected band volumes using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

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References
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