Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene

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Summary

Hypoxia unleashes the invasive and metastatic potential of tumor cells by largely unknown mechanisms. The Met tyrosine kinase, a high affinity receptor for hepatocyte growth factor (HGF), plays a crucial role in controlling invasive growth and is often overexpressed in cancer. Here we show that: (1) hypoxia activates transcription of the met protooncogene, resulting in higher levels of Met; (2) hypoxic areas of tumors overexpress Met; (3) hypoxia amplifies HGF signaling; (4) hypoxia synergizes with HGF in inducing invasion; (5) the proinvasive effects of hypoxia are mimicked by Met overexpression; and (6) inhibition of Met expression prevents hypoxia-induced invasive growth. These data show that hypoxia promotes tumor invasion by sensitizing cells to HGF stimulation, providing a molecular basis to explain Met overexpression in cancer.

Introduction

Most tumors have acquired the ability to develop their own blood vessels as they grow. However, the structure and architecture of the tumor vasculature is highly disorganized compared to normal tissues, resulting in irregular and inefficient oxygen delivery. As a consequence, neoplastic lesions are riddled with regions subjected to acute and chronic hypoxia (Harris, 2002; Höckel and Vaupel, 2001; Vaupel et al., 1989). Although a limiting factor for tumor growth, hypoxia appears to represent a positive stimulus for invasion. In fact, clinical studies have clearly demonstrated that the low pO2 tension within a neoplastic lesion is an independent prognostic indicator of poor outcome and correlates with an increased risk to develop distant metastases, independent of therapeutic treatment (Höckel et al., 1996, 1999; Brizel et al., 1996, 1997; Sundfor et al., 1998).

The higher malignancy of hypoxic tumors has been attributed to the ability of hypoxia to select for cells that are more resistant to apoptosis (Graeber et al., 1996; Yu et al., 2002) and to induce the secretion of angiogenic factors (Shweiki et al., 1992; Forsythe et al., 1996; Harris, 2002). However, the formation of metastases is a biological phenomenon too complex to be explained solely on the basis of increased angiogenesis, and experimental evidence suggests that hypoxia can directly increase tumor cell invasiveness (Young et al., 1988; Young and Hill, 1990; Cairns et al., 2001; Postovit et al., 2002; Rofstad et al., 2002).

In order to become metastatic, a neoplastic cell has to disrupt interactions with surrounding cells, cross the basal membrane or parenchima of origin, to migrate through the extracellular matrix, penetrate a blood or lymph vessel, and subsequently extravasate into a foreign tissue, where it still has to implant, proliferate, and generate its own net of capillaries (Woodhouse et al., 1997; Liotta and Kohn, 2001; Hanahan and Weinberg, 2000; Chambers et al., 2002). In addition, a cancer cell that undertakes the metastatic route must acquire the ability to escape death by “anoikis” (the absence of the home environment), which triggers apoptosis of normal cells when they abandon their histological niche (Frisch and Francis, 1984; Frisch and Screaton, 2001).

As a consequence of this complexity, the metastatic process involves a variety of effector molecules that control cell proliferation, survival, motility, cell-cell contacts, and interactions with the extracellular matrix, and is mastered by specific cytokines that orchestrate the coordinated completion of the program (Liotta and Kohn, 2001). Perhaps the most well characterized molecules among these coordinating cytokines are scatter factors (for a review see Trusolino and Comoglio, 2002).

The prototype of the scatter factor family is hepatocyte growth factor (HGF), also known as scatter factor-1 (Nakamura...
et al., 1986, 1989; Stoker et al., 1987; Gherardi et al., 1989; for a review see Rubin et al., 1993). HGF is a pleiotropic cytokine that plays a major role in organ formation during embryogenesis (Schmidt et al., 1995; Uehara et al., 1995; Woof et al., 1995; Takayama et al., 1996; Andermarcher et al., 1996) and in tissue homeostasis in the adult (Miyazawa et al., 1994; Yanagita et al., 1993; Yang et al., 1995; Matsumoto and Nakamura, 1997). Inappropriate activation of the HGF pathway, as often observed in cancer, leads to a malignant process—known as invasive growth—by which tumor cells weaken tissue constraints, migrate, and invade foreign districts, where they give rise to metastases (Birchmeier et al., 1997; Vande Woude et al., 1997; Comoglio and Trusolino, 2002). Interestingly, the high-affinity HGF receptor, encoded by the met protooncogene, is activated in human cancer either by point mutation (Schmidt et al., 1999; Park et al., 1999; Di Renzo et al., 2000) or—in the large majority of cases—by overexpression (Di Renzo et al., 1992, 1995; Liu et al., 1992; Boix et al., 1994). Since met gene amplification is a rare event, the mechanisms underlying Met protein overexpression in tumors remain obscure.

Here we show that hypoxia induces the expression of the Met receptor both in vitro and in vivo. In cultured cells, the Met protein and mRNA levels increase substantially after exposure to low oxygen tension. In experimental tumors, Met protein levels are highly upregulated in coincidence with hypoxic areas, forming an expression gradient that is inversely proportional to blood vessel proximity. By analyzing the human met promoter, we demonstrate that this induction is transcriptional and is mediated by two Hypoxia Inducible Factor-1 binding sites (Semenza, 2001) and an AP-1 site. We also show that hypoxia-induced Met overexpression results in increased sensitivity to HGF, and that low oxygen tension and HGF synergize in inducing cell motility and invasion. Finally, using a gene transfer approach and RNA interference technology, we provide evidence that expression of Met at the levels achieved by hypoxia is necessary and sufficient to sensitize cells to minimal amounts of HGF and thus to activate the invasive growth program.

All together, these data suggest that the HGF receptor is an important mediator of hypoxia-induced tumor invasiveness, providing a molecular explanation for Met overexpression in cancer.

Results

Hypoxia increases the levels of met mRNA

The effect of hypoxia on the expression of met mRNA was analyzed in the same panel of cells described above by Northern blotting and quantified by radioimaging. Table 1 shows, hypoxia or CoCl₂ induced the levels of met mRNA in all cells tested to an extent consistent with the data obtained by Western blot analysis. Figure 2A shows representative experiments for two cell lines. In time-course experiments (Figure 2B), met mRNA induction preceded by a few hours the increase in Met protein, and followed an induction pattern similar to that observed for the HIF-1-inducible gene vegf. However, a major distinction between vegf and met can be made based on their basal expression. In fact, while both vegf and met expression are induced by hypoxia, only met mRNA can be made based on their basal expression. In fact, while both vegf and met expression are induced by hypoxia, only met mRNA can be made based on their basal expression.

Hypoxia activates the met promoter

The promoter region of the human met gene contains several putative HIF-1 binding sites (HBS) both in sense and antisense orientation—through which HIF-1 promotes gene transcription in hypoxic conditions (Semenza, 2001; Figure 3A). To study the effect of hypoxia on met transcription, we subcloned progressively shorter fragments of the human met promoter (Gambarotta et al., 1994) into a reporter plasmid upstream to a luciferase gene, thus generating four different promoter constructs (P1, from −2619 to +353; P2, from −295 to +353; P3, from −32 to +353; P4, from +89 to +353). We then transfected the various reporter plasmids into suitable cell lines and incubated cells in normoxic or hypoxic conditions. In a separate set of experiments, we cotransfected the same reporter plasmids with an expression vector containing either no insert or a hif-1α cDNA, and then incubated cells in normoxic conditions. An inactive, mutant form of HIF-1α (Richard et al., 2000) was used as a negative control. Luciferase activity of transfected cells was analyzed to determine promoter activity. As shown in Figure 3B, hypoxia or exogenous wild-type HIF-1α increased transcription from the met promoter by 2–3 fold, while they had no effect on a control reporter plasmid (Basic). Although this extent of induction might seem modest, it should be stressed that the promoters of other genes known to be induced by hypoxia display comparable changes in activity when tested in similar assays (Yamashita et al., 2001; Xu et al., 2000; Maity and Solomon, 2000; Gerber et al., 1997; Levy et al., 1995; Kimura et al., 2000, 1986; as well as in the normal state.

As Figure 1A shows, hypoxia and CoCl₂ treatment markedly increased the levels of Met in all cells analyzed (only the data relative to two cell lines are shown). To quantify the extent of this induction, we performed Western blot analysis of cells subjected to the same conditions and then measured Met signal intensity by chemiluminescence. Anti-actin antibodies were used as controls for protein loading. The results of this analysis are summarized in Table 1. Representative experiments for four cell lines are shown in Figure 1B. To determine the kinetics of Met induction by hypoxia, we also performed time-course experiments in two selected cell lines (A549 and U2-OS; Figure 1C). These experiments revealed that induction of Met begins to appear after approximately 12 hr of continuous exposure to a 3% oxygen environment.

Hypoxia induces the expression of the Met protein

To investigate a possible role of Met in the cellular response to hypoxia, we analyzed the expression of the Met protein in different cell lines cultured in normoxic or hypoxic conditions. To this end, cell lines established from normal tissues (B5/589, human breast epithelium; MLP-29, murine hepatocyte precursors) or tumors (A549, human lung carcinoma; SK-OV-3, human ovarian carcinoma; SiHa, human cervical carcinoma; HepG2, human hepatocarcinoma; U2-OS, human osteosarcoma) were serum-starved to minimize the effect of growth factors on Met expression and then incubated in the presence of 21% O₂, 3% O₂, or 21% O₂ plus CoCl₂, a compound that mimics the effects of hypoxia by inducing Hypoxia Inducible Factor-1α stabilization (HIF-1α; Yuan et al., 2003). After 48 hr, cells were analyzed by immunofluorescence microscopy using anti-Met antibodies. Anti-HIF-1α antibodies and phalloidin were used as controls.
Figure 1. Hypoxia induces the Met protein
A: Immunofluorescence analysis by confocal microscopy. Cells (see text) were incubated in normoxia (N), hypoxia (H), or stimulated with CoCl₂ (Co) for 48 hr, and then stained with anti-HIF-1 antibodies (in blue) or anti-Met antibodies (in red). Counterstaining with phalloidin is shown in green. The bar represents a 50 μm indicator.
B: Western blot analysis. Cells were treated as in A and total protein extracts were analyzed for Met protein expression using anti-Met antibodies. Anti-actin antibodies were used as control of protein loading. Control, untreated cells at time zero.
C: Time-course analysis of Met expression. Cells were incubated in hypoxic conditions for the indicated times and Met expression was determined by Western blot analysis as in B.

2000). By this analysis, the minimal hypoxia-responsive region of the met promoter could be restricted to a 264 bp fragment comprised between a SmaI restriction site (located 89 bp downstream the transcriptional start site) and an AvaI restriction site situated in the first untranslated exon (at position +353). This region (P4) contains an AP-1 site (which has been assigned a central role in controlling met transcription; Seol et al., 2000) and two putative HIF-1 binding sites (HBS-4 and -5). We mutagenized these sites individually and compared the activity of each mutant P4 promoter to the activity of wild-type P4 promoter. As shown in Figure 3C, mutagenesis of HBS-4 or HBS-5 resulted in a significantly reduced transcriptional response to hypoxia or to exogenous HIF-1α. Mutagenesis of the AP-1 site also reduced the ability to respond to hypoxia or HIF-1α, but severely impaired the basal activity of the promoter as well. Mutagenesis of the same sites in the context of a P2 promoter construct also impaired its transcriptional response to hypoxia, while mutagenesis of HBS-2, HBS-3, asHBS-1, or asHBS-2 had no effect (data not shown). Taken together, these data show that hypoxia induces transcription from the met promoter and that this transcriptional activation is mediated by two different functional HBSs and an AP-1 site, all located in the 5′ untranslated region (5′ UTR). This situation is not unique, since the location of a functional HBS in the 5′ UTR of a hypoxia-responsive gene has already been reported (Sánchez-Elsner et al., 2002). Furthermore, cooperation between HBSs and AP-1 sites in the transcriptional
response to hypoxia has also been observed for other genes (Yamashita et al., 2001; Damert et al., 1997; Kimura et al., 2000; Norris and Millhorn, 1995; for a review see Michiels et al., 2001).

**Met is upregulated in hypoxic regions of tumors**

Hypoxia is a common feature of solid tumors, even in highly vascularized lesions. This is due to the disorganized architecture of tumor vessels, which does not guarantee a homogeneous oxygen supply to the tumor mass. As a result, most tumors contain several regions with lower O2 tension, which can be visualized by staining tumor sections with anti-HIF-1α antibodies (Vukovic et al., 2001). To test whether Met is upregulated by hypoxia in vivo, we analyzed Met expression in hypoxic regions of experimental tumors induced in nude mice by subcutaneous injection of human cancer xenografts and in bona fide human tumor samples (Figure 4). In a first approach, tumor sections were double-stained with anti-HIF-1α antibodies and anti-human Met antibodies, and sections were analyzed by confocal microscopy. As expected, HIF-1α signal was only barely detectable in the vast majority of tumor cells, but several “hot spots” of intense HIF-1α staining could be identified throughout the section, both in experimental tumors and in human samples. On the contrary, Met was detectable in all tumor cells, but its levels dramatically increased in coincidence with HIF-1α-positive, hypoxic areas. Figure 4A shows representative microscopic fields in which colocalization of HIF-1α (in red) and Met (in green) is observed (column 1, cervical carcinoma xenograft; column 3, human breast carcinoma). Antibody specificity was determined using epitope-competed anti-Met antibodies (columns 2 and 4). In a second approach, we costained tumor sections with anti-Met antibodies and antibodies against the endothelial marker CD-31. Confocal microscopy analysis revealed that—consistent with a role of oxygen tension in regulating Met expression—cells expressing higher levels of Met are localized in regions distant from blood vessels, whereas cells close to capillaries express very low levels of Met. This is exemplified in Figure 4B (red, CD-31; green, Met) that shows representative microscopic fields (column 1, cervical carcinoma xenograft; column 3, human breast carcinoma). Also in this analysis, anti-Met antibody specificity was demonstrated by epitope competition (columns 2 and 4). We therefore conclude

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**Table 1. Hypoxia and CoCl2 induce met mRNA and Met protein expression in normal and tumor cell lines**

<table>
<thead>
<tr>
<th>Cell line analyzed</th>
<th>Fold induction by hypoxia</th>
<th>Fold induction by CoCl2</th>
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<tbody>
<tr>
<td></td>
<td>met mRNA</td>
<td>Met protein</td>
</tr>
<tr>
<td>BS/589 h. breast epithelium</td>
<td>2.3 ± 0.3</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>A549 h. lung carcinoma</td>
<td>3.3 ± 0.2</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>U2-OS h. osteosarcoma</td>
<td>3.6 ± 0.6</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>SiHa h. cervical carcinoma</td>
<td>3.1 ± 0.3</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>HepG2 h. hepatocarcinoma</td>
<td>2.5 ± 0.2</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>SK-OV-3 h. ovarian carcinoma</td>
<td>2.6 ± 0.3</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>MLP-29 m. hepatocyte precursors</td>
<td>2.7 ± 0.4</td>
<td>3.0 ± 0.7</td>
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The levels of met mRNA and Met protein were determined in the indicated human (h.) or mouse (m.) cell lines as follows: cells were grown to 80% confluence in low serum, serum-starved for at least 24 hours, and then incubated in a 21% O2 environment, in a 3% O2 environment, or in a 21% O2 environment in the presence of 100 μM CoCl2 for an additional 48 hours. The levels of met mRNA were determined by Northern blotting of total RNA using a full-length met cDNA radiolabeled probe, quantified using a phosphoimager apparatus with dedicated software, and normalized to loading controls. The levels of Met protein were determined by Western blotting of total protein extracts using anti-Met antibodies, quantified directly by short-wave chemiluminescence using a dedicated optical scanner and software, and normalized to loading controls. Values (mean ± SD) refer to at least three independent experimental determinations.
that Met is sensitive to oxygen gradients in vivo, and that hypoxic regions of solid tumors overexpress Met.

**Hypoxia sensitizes cells to HGF stimulation**

The data presented above provide evidence that the Met receptor is induced by hypoxia both in vitro and in vivo. To assess whether these changes in protein levels are significant from a biological viewpoint, we analyzed whether a hypoxic environment could affect HGF-induced Met activation and signaling. To this end, the same panel of cells used above was deprived of serum growth factor to minimize background, and then incubated in normoxic or hypoxic conditions for 48 hr. Immediately
Figure 4. Met expression in tumors correlates directly with hypoxia and inversely with proximity to blood vessels

Tumor sections derived from an experimental cervical carcinoma xenograft (columns 1 and 2) or from a bona fide human breast carcinoma (columns 3 and 4) were analyzed by confocal microscopy.

A: Sections were double stained with antibodies against the hypoxic marker HIF-1α (in red) and against the Met receptor (in green). Control of anti-Met antibody specificity was performed by epitope competition (Comp. MET; columns 2 and 4).

B: Sections derived from the same tumors were costained with antibodies against the endothelial marker CD-31 (in red) and against the Met receptor (in green). Anti-Met antibody specificity was determined as in A (columns 2 and 4). The yellow bar represents a 100 μm indicator.
Figure 5. Hypoxia amplifies HGF signaling

A: Met receptor activation analysis in cells preincubated in normoxia or hypoxia. Following stimulation with HGF or no factor, cellular proteins were immunoprecipitated using anti-Met antibodies and analyzed by Western blotting using anti-phosphotyrosine antibodies. Signal intensity was quantified as described in Experimental Procedures. The histogram shows absolute Met tyrosine phosphorylation levels (Met pTyr; a.u., arbitrary units). Values are the mean of three independent experiments.

B: Met signal transduction analysis. Cells incubated in normoxia (N) or hypoxia (H) were stimulated as described in A and then lysed. Protein extracts were immunoprecipitated using anti-Gab-1 antibodies and analyzed by Western blotting using anti-phosphotyrosine antibodies. The same blots were reprobed using anti-Gab-1 antibodies to normalize for the amount of Gab-1 after, cells were stimulated with HGF or no factor as control for affect cell motility in classic model systems used to test HGF activity. The most classic and simple of these is the “scatter” assay (Stoker and Gherardi, 1991), in which cells are induced by HGF (also known as scatter factor) to loosen cell-cell interactions, to degrade extracellular matrix, and to scatter within a few hours. We assayed the effect of hypoxia on HGF-induced cell scattering on MLP-29, HepG2, and U2-OS cells. As for all experiments described so far, cells were serum-starved to reduce any background due to growth factors (including minimal amounts of HGF contained in fetal serum), preincubated in normoxia or hypoxia for 24 hr, and then stimulated with HGF or no factor. After 24 hr, cells were fixed, stained with fluorescinated phalloidin, and analyzed by fluorescence microscopy. As shown in Figure 6A, HGF induced the typical changes in morphology (acquisition of a “fibroblast-like” shape, sprouting of pseudopodia) and a motile response (scattering) in all cell lines analyzed. Consistent with our data on Met activation, hypoxia per se did not significantly affect basal cell morphology, but strongly synergized with HGF in inducing cell scattering. In end-point titration assays performed with progressive 1:2 ligand dilutions, hypoxia amplified HGF-induced cell scattering by at least 2 dilutions (not shown). We next analyzed the effect of hypoxia on HGF-induced cell migration by a Matrigel invasion assay (Medico et al., 1996). This assay measures the ability of cells to migrate through a reconstituted extracellular matrix in response to HGF. Cells (SiHa and U2-OS) were plated onto a layer of Matrigel, serum-starved to minimize any interference by serum growth factors, and then incubated in normoxic or hypoxic conditions, either in the absence or presence of HGF. Again in accordance with our biochemical data, hypoxia did not stimulate basal cell migration, but significantly amplified the

Hypoxia enhances HGF-induced cell motility
Since Met mediates motile cues in both physiologic and pathologic conditions, we set out to investigate whether hypoxia could affect cell motility in classic model systems used to test HGF activity. The most classic and simple of these is the “scatter” assay (Stoker and Gherardi, 1991), in which cells are induced by HGF (also known as scatter factor) to loosen cell-cell interactions, to degrade extracellular matrix, and to scatter within a few hours. We assayed the effect of hypoxia on HGF-induced cell scattering on MLP-29, HepG2, and U2-OS cells. As for all experiments described so far, cells were serum-starved to reduce any background due to growth factors (including minimal amounts of HGF contained in fetal serum), preincubated in normoxia or hypoxia for 24 hr, and then stimulated with HGF or no factor. After 24 hr, cells were fixed, stained with fluorescinated phalloidin, and analyzed by fluorescence microscopy. As shown in Figure 6A, HGF induced the typical changes in morphology (acquisition of a “fibroblast-like” shape, sprouting of pseudopodia) and a motile response (scattering) in all cell lines analyzed. Consistent with our data on Met activation, hypoxia per se did not significantly affect basal cell morphology, but strongly synergized with HGF in inducing cell scattering. In end-point titration assays performed with progressive 1:2 ligand dilutions, hypoxia amplified HGF-induced cell scattering by at least 2 dilutions (not shown). We next analyzed the effect of hypoxia on HGF-induced cell migration by a Matrigel invasion assay (Medico et al., 1996). This assay measures the ability of cells to migrate through a reconstituted extracellular matrix in response to HGF. Cells (SiHa and U2-OS) were plated onto a layer of Matrigel, serum-starved to minimize any interference by serum growth factors, and then incubated in normoxic or hypoxic conditions, either in the absence or presence of HGF. Again in accordance with our biochemical data, hypoxia did not stimulate basal cell migration, but significantly amplified the
Hypoxia and HGF synergize in inducing cell motility and invasion

**A:** Scatter assay. Serum-starved cells were preincubated in normoxia (21% O₂) or hypoxia (3% O₂) for 24 hr and then stimulated with HGF or no factor in the same conditions for additional 24 hr. Following staining with fluoresceinated phalloidin, cells were analyzed by confocal microscopy.

**B:** Matrigel invasion assay. Cells seeded onto a layer of Matrigel were serum-starved and incubated in normoxia or hypoxia. After 24 hr, cells were stimulated with HGF in the same conditions for additional 24 hr. The number of cells migrated through the Matrigel layer was scored by microscopy following staining with crystal violet.

**C:** Collagen invasion assay. The ability of MLP-29 and U2-OS cells to form branched tubular structures in a three-dimensional collagen matrix was tested in normoxia (N) and hypoxia (H), with or without exogenous HGF. These experiments were run in the presence of 10% FBS.

*Figure 6. Hypoxia and HGF synergize in inducing cell motility and invasion.*

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response to HGF in all cells tested (Figure 6B). A similar effect was also achieved by stimulation with CoCl₂ (data not shown).

**Hypoxia and HGF synergize in inducing invasion**

HGF is a proinvasive cytokine *par excellence*. The genetic program activated by HGF leads epithelial cells to abandon their site of origin within a tissue, to migrate through the extracellular matrix, and to invade adjacent tissues (Brinkmann et al., 1995; Birchmeier et al., 1997). The most reliable in vitro assay that dependably measures HGF-induced invasiveness is the collagen invasion assay (Montesano et al., 1991). This assay—also known as the “branching morphogenesis” assay—highlights the potential of cells to invade a tridimensional collagen gel, forming typical branched structures. This invasion process represents the *summa* of the invasive growth phenotype and results from the fine integration of all the pleiotropic effects induced by HGF, including cell proliferation, motility, differentiation, and survival. To study the effect of hypoxia on cell invasion, we performed a collagen invasion assay using different cell lines (U2-OS, MLP-29, SiHa) in normoxic or hypoxic conditions. It should be stressed that—in contrast to the other bioassays described above—collagen invasion assays must be performed in high serum in order to prevent massive cell death. Preformed cell spheroids were embedded into a collagen gel, preincubated in 21% O₂ or 3% O₂ for 24 hr, and then stimulated with HGF or no factor in the same oxygen environment. After 24 hr, cell colonies were analyzed by microscopy and representative spheroids photographed. As shown in Figure 6C, hypoxia dramatically amplified the proinvasive effect of HGF (only the data relative to U2-OS and MLP-29 cells are shown). Stimulation with CoCl₂ also achieved a strong synergistic effect with HGF (data not shown). However, in contrast with the results obtained in the other bioassays performed, low pO₂ also induced branching morphogenesis on its own, as previously observed by RT-PCR analysis in both normoxic and hypoxic conditions (data not shown), the latter phenomenon can be explained either by implying a mechanism totally unrelated to Met, or by hypothesizing that hypoxia-induced Met overexpression sensitizes cells to the minimal amounts of HGF contained in serum. This was tested directly by the following experiments.

**Met overexpression is sufficient to induce branching morphogenesis**

The ability of renal epithelial cells to form branched structures under hypoxic conditions in the absence of exogenous HGF has been proposed to depend upon increased levels of HIF-1α, as overexpression of exogenous HIF-1α in the same cells led to a similar phenotype in normoxic conditions (Maranchie et al., 2002). This suggests that a yet unidentified transcriptional target of HIF-1 is involved in the invasive growth process. To investigate on a possible direct cause-effect relationship between Met induction by hypoxia and increased invasion, we overexpressed exogenous Met at levels comparable to those achieved by endogenous Met under hypoxic conditions, and tested whether

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**Figure 7.** Met upregulation mediates hypoxia-induced branching morphogenesis

**A:** Overexpression of exogenous Met is sufficient to induce branching morphogenesis in normoxic conditions. Western blot: Met expression in wild-type U2-OS cells in normoxia (21% O₂) or hypoxia (3% O₂) is compared to Met expression in U2-OS cells transfected with the indicated plasmid constructs in normoxic conditions. WB, Western blot. Histogram: stable transfectants analyzed by Western blot were subjected to a branching morphogenesis assay in normoxic conditions (N; all groups) or in hypoxic conditions (H; empty vector only). The percentage of branched colonies (sprouted spheroids) was scored by microscopy. Values (mean ± sd) refer to three experiments performed in quadruplicate. Empty V, empty vector; WT Met, wild-type Met; β-Met, β-chain Met; K1128A Met, kinase-inactive Met.

**B:** Inhibition of Met expression by RNA interference prevents hypoxia-induced branching morphogenesis. Western blot: Met knock-down. U2-OS cells were transfected with no oligo, double strand met oligoribonucleotides (MET1 + MET3) or mutant double strand met oligoribonucleotides (MET1* + MET3*), incubated in normoxic or hypoxic conditions, and then analyzed by Western blotting as indicated. Histogram: transfectants analyzed by Western blot were subjected to a branching morphogenesis assay in normoxic (N) or hypoxic (H) conditions, either in the presence or absence of HGF. Branched colonies were scored as in A. Values (mean ± sd) refer to three experiments performed in triplicate.
this was sufficient to induce branching morphogenesis in normoxic conditions. To this end, we employed an expression vector with a weak promoter (Michieli et al., 1999). Stable U2-OS transfectants were obtained (see Figure 7A, Western blot panel) that expressed exogenous wild-type Met (WT Met), a kinase-inactive Met (K1128A Met), or an engineered Met—consisting of the β-chain only—deprived of the functional domain responsible for interaction with HGF (β-Met; Michieli et al., 1999). The latter form of Met is properly exposed at the cell surface, but is not activated by HGF. However, β-Met responds biologically to ligand-mimetic antibodies directed against the extracellular portion of the Met β-chain and is indistinguishable from wild-type Met in kinase assays, thus demonstrating that deletion of the HGF-interacting domain does not alter the overall functionality of the receptor (Michieli et al., 1999). Cells transfected with empty vector (Empty V) were used as control. Transfected cells were subjected to a branching morphogenesis assay in normoxic conditions (all groups) or in hypoxic conditions (empty vector only). The percentage of “sprouted” spheroids was calculated as described in Experimental Procedures. As shown in Figure 7A (histogram), overexpression of exogenous wild-type Met in normoxic conditions (WT Met N) closely reproduced the proinvasive effect of hypoxia in control cells (Empty VH). This effect depends on Met kinase activity because the kinase-inactive form of Met did not induce branching morphogenesis (K1128A Met N). Interestingly, impairment of the ability of Met to interact with HGF completely abrogated the proinvasive effect consequent to receptor overexpression (β-Met N). It can therefore be concluded (1) that Met overexpression is sufficient to induce branching morphogenesis in the presence of serum, and (2) that interaction with HGF (contained in serum) is indispensable to achieve this proinvasive effect. With regard to this, we determined that HGF can be affinity-purified from fetal bovine serum using the extracellular portion of human Met, and that hypoxia dramatically amplifies the ability of serum to phosphorylate Met in receptor activation experiments similar to those described in Figure 5 (data not shown).

**Inhibition of Met expression prevents hypoxia-induced invasive growth**

To further strengthen the idea that Met induction by hypoxia is responsible for the observed invasive phenotype, we knocked down Met expression in U2-OS cells or MLP-29 cells by RNA interference (Hannon, 2002). To this end, cells were transfected with two 19-base pairs, double-stranded oligoribonucleotides derived from two distinct regions of *met* cDNA in which the nucleotide sequence is completely identical in the mouse and human species. Cells transfected with point-mutated *met* oligos or no oligos were used as controls. As revealed by Western blot analysis using anti-Met antibodies (Figure 7B, Western blot panel), transfection of wild-type *met* oligos (MET1-MET3) efficiently reduced Met expression in both normoxia and hypoxia, while mutated *met* oligos (MET1*-MET3*) had no effect (only the data relative to U2-OS are shown). Anti-actin antibodies were used as control of protein loading. Transfected cells were subjected to a branching morphogenesis assay in normoxic or hypoxic conditions, either in the presence or absence of exogenous HGF. The percentage of sprouted spheroids was quantified as above. As shown in Figure 7B (histogram), inhibition of Met expression by RNA interference completely prevented hypoxia-induced branching morphogenesis, while mutated *met* oligos had no effect. RNA interference also abolished the response to exogenous HGF in both normoxic and hypoxic conditions, demonstrating that Met expression is efficiently inhibited from a biologic viewpoint. We thus conclude that Met expression is necessary in order to observe the proinvasive effect of hypoxia, at least in the branching morphogenesis assay and in the cell systems analyzed. Taken together, the data presented here suggest that hypoxia sensitizes cells to HGF by increasing Met levels, thus activating an invasive growth program that leads to cell migration and extracellular matrix invasion.

**Discussion**

The data presented in this study demonstrate that Met is induced under hypoxic conditions both in vitro and in vivo. They also show that Met upregulation by hypoxia results in increased sensitivity to HGF stimulation, and that HGF and hypoxia synergize in inducing invasive growth.

The Met receptor has been shown to be overexpressed in a large number of human neoplastic lesions, but the molecular mechanisms underlying this upregulation have not been clarified so far. Importantly—as stated above—higher levels of Met are due to increased gene expression rather than gene amplification in the vast majority of cases. Since hypoxia is a common feature of most solid tumors, our data provide a molecular explanation for these observations and suggest that hypoxia is an important factor determining the levels of Met in cancer. Interestingly, Met has been shown to be overexpressed in renal cell carcinomas bearing genetic alterations in the vhl tumor suppressor gene (Oh et al., 2002), and the VHL protein has been found to inhibit HGF-induced renal cell invasion (Koochekpour et al., 1999; Maranchie et al., 2002). Given the pivotal role of the VHL protein in targeting HIF-1α to proteolytic degradation in normoxic conditions (Maxwell et al., 1999), our finding that HIF-1 contributes to met gene expression provides a detailed molecular mechanism underlying the above biological observations.

Our Met activation analysis revealed that a 3-fold increase in protein levels—which might appear a modest change in biochemical terms—results in a dramatic amplification of signal transduction downstream of Met. This can be explained by the following considerations. Firstly, activation of the Met kinase is autocatalytic and intermolecular (Naldini et al., 1991). Upon ligand stimulation, receptor oligomerization occurs, and trans-phosphorylation between sister protomers results in kinase activation. Activated receptors in turn phosphorylate other protomers, thus promoting an enzymatic chain reaction. Intuitively, such a process—an autocatalytic enzymatic reaction—is characterized by a nonlinear relation between receptor concentration and reaction velocity. Secondly, the Met receptor has recently been shown to interact with other receptor species that amplify its signal (Orian-Rousseau et al., 2002; Trusolino et al., 2001), enhance its clustering (Giordano et al., 2002), or trans-phosphorylate the receptor (Follenzi et al., 2000). Thanks to these physiological amplification systems, a modest increase in Met levels may elicit more important downstream effects following HGF stimulation. In the long term—as observed for instance in hypoxic areas of tumors—a positive feedback loop may contribute to sustain and amplify Met overexpression, since the Met pathway induces both the met gene itself (Boccaccio et al., 1994) and HIF-1 activity (Tacchini et al., 2001).
The observation that hypoxia sensitizes cells to HGF stimulation has important potential implications in cancer biology. In fact, HGF is ubiquitously present at high concentrations in plasma and in the extracellular matrix of tissues, where it accumulates—due to its affinity for proteoglycans (Lyon et al., 1994)—under the form of inactive precursor (pro-HGF). Pro-HGF is converted into active HGF in tissues by urokinase (uPA), a protease of the plasminogen cascade (Naldini et al., 1992). Since virtually all tumors—and invasive ones in particular—overexpress urokinase (Aguirre Ghiso et al., 1999), it is reasonable to predict that active HGF is not limiting in neoplastic lesions. Therefore, hypoxia-induced sensitization to HGF may importantly contribute to increase the invasive behavior of a tumor.

Given the pivotal role that the Met receptor plays in tumor invasion and metastasis, the results obtained in this work are particularly relevant to explain why hypoxia increases the malignancy of neoplastic lesions. Until now, great emphasis has been placed on hypoxia-induced neoangiogenesis, mediated chiefly by secretion of VEGF. This molecular phenomenon has actually become a paradigm of the cellular response to hypoxia, and has been assigned a major role in determining a malignant conversion (the angiogenic switch) of the cancer lesion. Indeed, the secretion of angiogenic factors can be interpreted as a concrete attempt of the tumor mass to restore a normal oxygenation rate to cancer cells.

Our data provide evidence for a second type of cellular reaction to oxygen deprivation—symmetric and complementary to the angiogenic response from a strategic viewpoint—that leads to an invasive switch of the tumor mass. In fact, the results presented here show that cells subjected to hypoxia activate a motility program and start invading the extracellular matrix, resembling the typical features of the invasive growth phenotype. Our gene transfer experiments and RNA interference analysis also prove that this increased invasiveness is due to higher levels of Met. It could thus be suggested that increased motility and invasion represent the manifestation of a cellular plan aimed at escaping the hostile hypoxic environment, attempting to colonize the adjacent tissue(s) where oxygen and nutrients are not limited. This mechanism could also play an important role during embryo development or tissue regeneration, where oxygen gradients represent signals for morphogenetic invasive processes, particularly for the formation of branched, tubular organs.

The notion that hypoxia activates motile and invasive cues in tumor cells has important therapeutic implications. Following the initial observations that natural antiangiogenic polypeptides could function as potent tumor suppressors in mice (O’Reilly et al., 1994), several therapeutic strategies have been attempted to contain tumor growth by suppressing neoangiogenesis. This approach has achieved successful results in many experimental systems, and some antiangiogenic compounds are currently being tested in clinical trials (Folkman, 1999). However, as our knowledge on tumor angiogenesis increases, it has become clear that this strategy has the important drawback of inducing tumor hypoxia (Blagosklonny, 2001). This issue has recently been shown to be crucial for the therapeutic outcome, since it allows for selection of more aggressive tumor cells (Yu et al., 2002). Our results raise further concerns on the efficacy of antiangiogenic therapy per se, because they suggest that reduced tumor vascularization—while inhibiting tumor growth—would promote the spread of neoplastic cells toward a more oxygenated environment.

On the other hand, our data suggest that effective tumor treatment could be achieved by combining antiangiogenic therapy with antinvasive drugs, such as Met inhibitors (Boccaccio et al., 1998; Bardelli et al., 1999; Morotti et al., 2002) or HGF antagonists (Date et al., 1998; Michieli et al., 1999). This would perhaps achieve efficient tumor “suffocation” while preventing the escape of cancer cells from hypoxic areas.

**Experimental procedures**

**Cell culture**

The following cell lines were purchased from ATCC (Rockville, MD): HepG2, SiHa, A549, SK-OV-3, and U2-OS. MLP-29 cells were obtained as described (Medico et al., 1996). B5/S89 cells were a gift of Dr. Jacalyn H. Pierre (NCI Bethesda, MD). Cells were maintained in DMEM (HepG2, SiHa, SK-OV-3), Iscove’s DMEM (U2-OS), or RPMI (A549, B5/S89), all supplemented with 10% FBS (Sigma, St. Louis, MO). B5/S89 were grown in the presence of 10 ng/ml human recombinant EGF (Sigma). A 3% O2 environment was obtained using a Heraeus BB 6220 oxygen electrode incubator (Heraeus, Hanau, Germany). CoCl2 (Merck, Darmstadt, Germany) was used at a concentration of 100 μM.

**Biological assays**

For the scatter assay, MLP-29, U2-OS, and HepG2 cells were seeded (1 × 104 cells/well) on glass coverslips in 24-well plates in medium supplemented with 2% FBS. After adhesion, cells were serum-starved and incubated in normoxic or hypoxic conditions for 24 hr. Cells were then stimulated with 15 ng/ml HGF (R&D Systems, Minneapolis, MN) and incubated in the indicated conditions for an additional 24 hr. Cells were fixed, stained with fluorescein-labeled phallolidin (Sigma), analyzed by confocal microscopy, and photographed. Invasion assays were performed in Transwell chambers (Corning Costar, Cambridge, MA). The upper side of the filters was coated with Matrigel (Collaborative Research, Waltham, MA) at a concentration of 1.15 μg/cm2. Cells were seeded (1 × 105 cells/well) onto the layer of Matrigel using 2% FBS-containing medium, serum-starved after 5 hr, preincubated for 24 hr in normoxia or hypoxia, and then stimulated with 30 ng/ml of recombinant HGF for 24 hr. At the end of the treatment, cells on the upper side of the filters were mechanically removed, and those migrated onto the lower side were fixed with 11% glutaraldehyde, stained with crystal violet, and counted. Collagen invasion assays were performed using preformed spheroids as described (Meyer et al., 1999). Briefly, spheroids were generated by incubating cells overnight (700 cells/well) in nonadherent 96-well plates (Greiner, Frickenhausen, Germany) in the presence of 0.24 g/ml methylcellulose (Sigma). Spheroids were embedded into a collagen matrix containing 1.3 mg/ml type I collagen from rat tail (BD Biosciences, Bedford, MA) and 10% FBS using 96-well plates (40 spheroids/well). Embedded spheroids were preincubated in normoxia or hypoxia for 24 hr, and then stimulated with 30 ng/ml HGF or no factor for 24 hr in the same oxygen environment. The percentage of sprouted spheroids was scored by microscopy by analyzing all spheroids contained in each well.

**In vitro immunofluorescence**

For analysis of Met expression, cells were plated on collagen-coated glass coverslips, serum-starved, and then incubated in the indicated conditions (normoxia, hypoxia, or normoxia plus 100 μM CoCl2). After 48 hr, cells were fixed (3% paraformaldehyde, 4% sucrose), permeabilized (0.2% Triton X-100), washed (0.2% BSA in PBS), and blocked (2% BSA). Human cells (HepG2, SiHa, A549, SK-OV-3, U2-OS, B5/S89) were incubated with a mixture of two anti-human Met monoclonal antibodies (DO-24 and DN-30; Prat et al., 1998) or an anti-HIF-1α monoclonal antibody (a gift of Prof. Antonio Bargellesi, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). MLP-29 cells were incubated with anti-mouse Met rabbit polyclonal antibodies (SP260; Santa Cruz, Santa Cruz, CA). Incubation with the appropriate Alexa Fluor 546-tagged secondary antibody (Molecular Probes, Eugene, OR) was performed together with FITC-conjugated phallolidin (Sigma). Cells were then mounted in Mowiol 4-88 (Hoechst, Strasbourg, France). Slides were analyzed with a Laser Radiance 2100 confocal microscope (Biorad, Hemel Hempstead, UK) using fixed parameters in the laser settings.
Western blot analysis
For Met expression analysis, cells were solubilized in boiling Laemmli buffer, sonicated, and cleared by centrifugation. Total protein concentration was determined using a BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL) and equal amounts (40 µg) of proteins were resolved by SDS-PAGE on an 8% gel under reducing conditions. Separated proteins were transferred onto a Hybond nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden) and analyzed by Western blotting using anti-human Met (C-12; Santa Cruz) or anti-mouse Met (SP260; Santa Cruz) antibodies. After incubation with the appropriate secondary antibodies, nitrocellulose-bound antibodies were detected by short-wave chemiluminescence using an ECL-plus kit (Amersham). Quantification of ECL signal was performed using a Phosphorlmager apparatus and dedicated Image Quant software (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA). Anti-actin goat polyclonal antibodies used as control of protein loading were purchased from Santa Cruz (C-11).

Gene probes and Northern blot analysis
Total RNA was isolated from hypoxic and normoxic cells with RNAwiz (Ambion, Austin, TX), resolved on a 0.8% agarose-formaldehyde gel (15 µg each sample), and transferred to Hybond-N+ nylon membrane (Amersham). The VEGF165 probe (Gene Bank # M32977) was obtained by RT-PCR using with rabbit polyclonal anti-human Met antibodies (C-12, Santa Cruz) and VEGF165 cDNA (wild-type and mutant). Total RNA was isolated from hypoxic and normoxic cells with RNAwiz (Amersham). HepG2-derived total RNA as template and the following oligonucleotides as primers: 5’- GAGCGACGGATCCGGTCGGGCCTCCGAAACCATGAACTTTCTG 3’; 5’- CCAGCGGCGTGGGCGTCGTTCGAGATCTGAGTGACG 3’.

The Met probe was obtained from the pCEV-Met plasmid (Michieli et al., 1999). Probes were labeled by random priming (Megaprime, Amersham) using a 5-fold molar excess of a purified human recombinant Met extracellular domain produced by baculovirus (a gift of Dr. S. Cavassa, University of Torino). For CD-31-Met localization studies, sections were double-stained with rabbit polyclonal anti-human Met antibodies (C-12, Santa Cruz) and either rat monoclonal anti-mouse CD-31 antibodies (MEC-13.3, Pharmingen, San Diego, CA) or mouse monoclonal anti-human CD-31 antibodies (UC70A, Dako A/S, Glostrup, Denmark). Goat anti-rabbit Alexa Fluor 488 and either goat anti-rat Alexa Fluor 488 or goat anti-mouse Alexa Fluor 546 (both from Molecular Probes) were used as secondary antibodies. Epitope competition of the rabbit anti-human Met antibody was performed using the specific blocking peptide (sc-10 P, Santa Cruz) according to the manufacturer’s instructions. Slides were mounted with Mowiol reagent (Hoechst) and analyzed by confocal microscopy using a Laser Radiance 2100 microscope (BioRad).

Promoter analysis
The various met promoter constructs (P-4 to P-1) in the pGL-2 vector (Promega, Madison, WI) have been described previously (Gambardella et al., 1996). Putative HBSIs were mutated in their core region GGTG by a recombinant-PCR based approach (as described in Michieli et al., 1999) as follows: HBS-4, TCTC; HBS-5, TTTT. The AP-1 consensus sequence GGTGAGTC was mutated into TTTAGTC. Accuracy of the mutagenesis procedure was verified by direct sequencing of mutant plasmids. Analysis of promoter activity was performed in U2-OS or MLE-2 cells (105 cells/100 mm plate) transfected using Lipofectin reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For hypoxia-induced transcription, cells were transfected with 10 µg of the appropriate promoter construct and 0.1 µg of a TK-Renilla reporter plasmid (Promega). Following transfection, cells were incubated for 24 hr in normoxic conditions and then exposed to normoxia or hypoxia for 48 hr. For HIF-1-induced transcription, cells were transfected with 3 µg of the appropriate promoter construct, 7 µg of a pCDNA-3 expression vector (Invitrogen) containing either no insert or a human hif-1α cDNA (a gift of Prof. J. Pouyssegur, University of Nice, France), and 0.1 µg of the TK-Renilla reporter plasmid. The inactive mutant form of hif-1α was generated as described (Richard et al., 2000). Following transfection, cells were incubated for 24 hr in normoxic conditions. To measure luciferase activity, cells were processed using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions, and 10 µl of cell lysate was used to determine reporter enzyme activity using a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany). Each experimental point was performed in triplicate and luciferase activity was normalized on Renilla activity to standardize transfection efficiency.

Tumor analysis
All experiments with mice were performed according to international ethical guidelines (EEC Council Directive 86/609, NIH Guide for the Care and Use of Laboratory Animals, NIH Publication # 85-23, 1985). Mice were housed in a specific pathogen-free facility, granted by the University of Torino Ethical Board and by the Italian Ministry of Health. Human samples were obtained following written informed consent by the patient prior to surgery. SiHa human cervical carcinoma cells were inoculated subcutaneously (2 × 106 cells/animal) in 200 µl of PBS into the left posterior flank of three seven-week-old nude/nu female mice on Swiss CD-1 background (Charles River Laboratories, Calco, Italy). After tumors had reached a size ranging between 0.6 and 1.0 cm in diameter, mice were sacrificed and tumors were excised, embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and immediately frozen in liquid nitrogen. Human breast carcinoma samples were obtained from the IRCC Pathology Division and immediately frozen after surgery as described above. For immunofluorescence analysis, 8 µm thick frozen sections were fixed with 4% paraformaldehyde and blocked with 5% goat serum (Vector Laboratories, Burlingame, CA) in PBS containing 1% BSA and 0.3% Triton. For HIF-1α-Met localization studies, sections were double-stained with rabbit polyclonal anti-HIF-1α antibodies (H-206, Santa Cruz) and a mixture of two monoclonal anti-human Met antibodies (DO-24 and DN-30, Pratt et al., 1998). Goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 546 (both from Molecular Probes) were used as secondary antibodies. Epitope competition of the rabbit anti-human Met antibody was performed using the specific blocking peptide (sc-10 P, Santa Cruz) according to the manufacturer’s instructions. Slides were mounted with Mowiol reagent (Hoechst) and analyzed by confocal microscopy using a Laser Radiance 2100 microscope (BioRad).

Met activation and signal transduction analysis
For Met phosphorylation analysis, cells brought to 90% confluency in 2% FBS were serum-starved for 24 hr and then subjected to normoxia or hypoxia for additional 48 hr in the same culturing conditions. Stimulation with HGF (R&D, 30 ng/ml) was performed for 10 min at 37°C. Cells were lysed in RIPA buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1% deoxycholate, 0.5% SDS) containing 1 mM Na3VO4, and a cocktail of protease inhibitors (pepsatin, leupeptin, aprotinin, soybean trypsin inhibitor, PMSF). Cell extracts were sonicated and cleared by centrifugation. Total protein concentration was determined using a BCA Protein Assay Reagent (Pierce) following dilution of SDS concentration to 0.1%, equal amounts of proteins were immunoprecipitated using either monoclonal anti-human Met antibodies (DO-13, Ruco et al., 1996) or polyclonal anti-mouse Met antibodies (SP260, Santa Cruz). Immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting as described above using anti-phosphotyrosine antibodies (Ubl, Lake Placid, NY). The same blots were reprobed using anti-human Met (C-12, Santa Cruz) or anti-mouse Met (SP260, Santa Cruz) antibodies. For Gab-1 activation analysis, cells were subjected to the same treatments as above and then lysed in EB buffer (100 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100) containing 1 mM Na3VO4, and the above-described cocktail of protease inhibitors. Cell extracts were cleared by centrifugation and total protein concentration was determined as above. Equal amounts of proteins were immunoprecipitated using polyclonal anti-Gab-1 antibodies (Ubl), resolved by SDS-PAGE and analyzed by Western blotting using anti-phosphotyrosine antibodies. The same blots were reprobed with anti-Gab-1 antibodies or anti-Met antibodies. Quantification of ECL signal was performed as described above.

Gene transfer and Met knockdown
U2-OS cells (105 cells/100 mm plate) were transfected using Lipofectin reagent as described above with 10 µg of the pCEV expression vector containing either no insert or the appropriate human met cDNA (wild-type met and β-met-lacZ, K1228 met, Crepaldi et al., 1994). Following selection with the appropriate selective agent, stable transfectants were pooled and analyzed for Met expression by Western blot of total cell
extracts. Inhibition of Met expression was achieved by RNA interference using a 1:1 mixture of the following double-stranded oligoribonucleotides:

MET1 5’ AGCUACAGGUCUGACACUU 3’
MET3 5’ GCUAAGGGAGGGCAUU 3’

In order to verify the specificity of the knockdown effect, each oligoribonucleotide was point-mutated (*) to generate a pair of matched control oligos (mutated bases are underlined):

MET1 5’ AGCUACAGGUCUGACACUL 3’
MET3 5’ GCUAAGGGAGGGCAUL 3’

Oligoribonucleotides were produced using the Silencer siRNA Construction Kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Transfections (200 nM total oligoribonucleotide mixture) were performed using Oligofectamine reagent (Invitrogen) according to manufacturer’s instructions. Cells were incubated for 72 hr prior to Met levels determination or biological testing.

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