

# 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 pO<sub>2</sub> 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 parenchyma 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, 1994; 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

## SIGNIFICANCE

Much emphasis has been placed in recent years on hypoxia-induced neoangiogenesis, a biological process characterized by migration of endothelial cells toward the tumor mass in order to restore a regular supply of oxygen. The data presented in this study unveil a second, parallel mechanism that employs a symmetric and complementary strategy: it allows single neoplastic cells to escape hypoxia by invading surrounding tissues where oxygen and nutrients are not limited. This phenomenon has important implications in tumor biology. First, it provides evidence for an angiogenesis-independent, hypoxia-induced "invasive switch" that links tumor hypoxia to increased malignancy. Second, it points to the therapeutic necessity of suppressing cell motility when targeting tumor angiogenesis, in order to prevent the potential spread of cancer cells consequent to intratumoral oxygen deprivation.

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; Woolf 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 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 cells 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% O<sub>2</sub>, 3% O<sub>2</sub>, or 21% O<sub>2</sub> plus CoCl<sub>2</sub>, a compound that mimics the effects of hypoxia by inducing Hypoxia Inducible Factor-1 $\alpha$  stabilization (HIF-1 $\alpha$ ; Yuan et al., 2003). After 48 hr, cells were analyzed by immunofluorescence microscopy using anti-Met antibodies. Anti-HIF-1 $\alpha$  antibodies and phalloidin were used as controls.

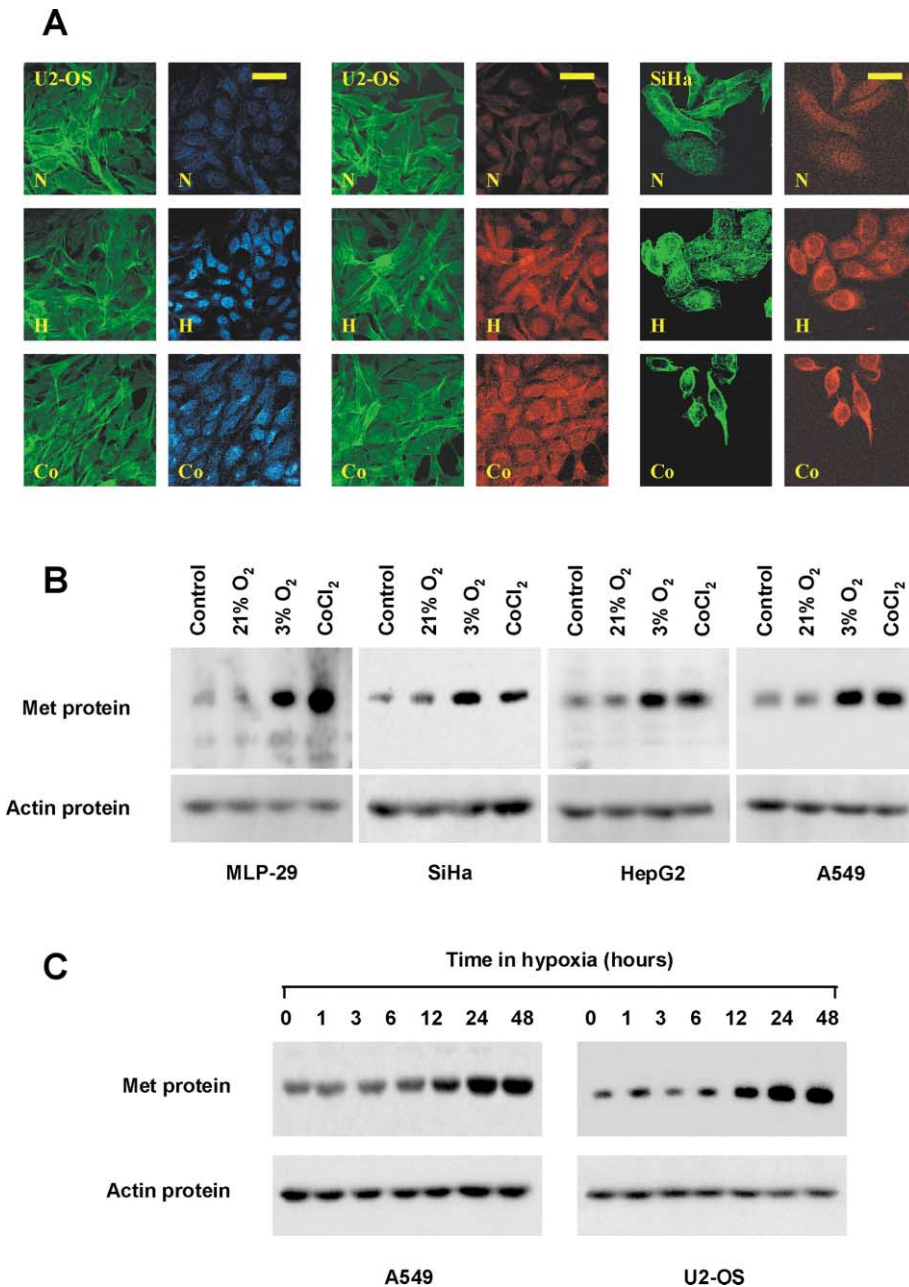
As Figure 1A shows, hypoxia and CoCl<sub>2</sub> treatment markedly increased the levels of Met in all cells analyzed (only the data relative to two cell lines are shown). To quantify the extent of this induction, we performed Western blot analysis of cells subjected to the same conditions and then measured 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 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. As Table 1 shows, hypoxia or CoCl<sub>2</sub> 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 is detectable in normoxic conditions. This difference in regulation conceivably reflects different physiological functions: while VEGF may be dispensable in normoxic conditions, Met plays an important role in controlling cell proliferation, motility, and survival.

### Hypoxia activates the *met* promoter

The promoter region of the human *met* gene contains several putative HIF-1 binding sites (HBSs)—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-1 $\alpha$*  cDNA, and then incubated cells in normoxic conditions. An inactive, mutant form of HIF-1 $\alpha$  (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-1 $\alpha$  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.,



**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 CoCl<sub>2</sub> (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 Aval 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-1 $\alpha$ . Mutagenesis of the AP-1 site

also reduced the ability to respond to hypoxia or HIF-1 $\alpha$ , 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 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

**Table 1.** Hypoxia and CoCl<sub>2</sub> induce *met* mRNA and Met protein expression in normal and tumor cell lines

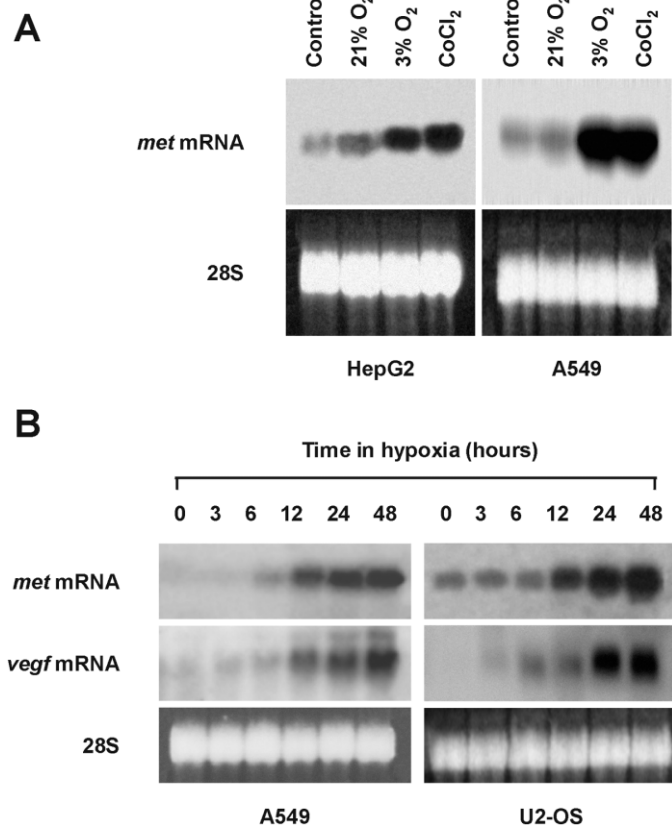
| Cell line analyzed |                          | Fold induction by hypoxia |             | Fold induction by CoCl <sub>2</sub> |             |
|--------------------|--------------------------|---------------------------|-------------|-------------------------------------|-------------|
| Name               | Origin                   | <i>met</i> mRNA           | Met protein | <i>met</i> mRNA                     | Met protein |
| B5/589             | h. breast epithelium     | 2.3 ± 0.3                 | 2.0 ± 0.2   | 2.5 ± 0.7                           | 2.2 ± 0.1   |
| A549               | h. lung carcinoma        | 3.3 ± 0.2                 | 3.1 ± 0.2   | 3.2 ± 0.4                           | 2.5 ± 0.2   |
| U2-OS              | h. osteosarcoma          | 3.5 ± 0.6                 | 3.2 ± 0.9   | 3.0 ± 0.2                           | 2.5 ± 0.2   |
| SiHa               | h. cervical carcinoma    | 3.1 ± 0.3                 | 2.9 ± 0.2   | 2.0 ± 0.4                           | 1.9 ± 0.2   |
| HepG2              | h. hepatocarcinoma       | 2.5 ± 0.2                 | 2.6 ± 0.1   | 2.3 ± 0.6                           | 2.1 ± 0.2   |
| SK-OV-3            | h. ovarian carcinoma     | 2.6 ± 0.3                 | 2.5 ± 0.2   | 2.8 ± 0.2                           | 2.3 ± 0.2   |
| MLP-29             | m. hepatocyte precursors | 2.7 ± 0.4                 | 3.0 ± 0.7   | 3.4 ± 0.3                           | 4.2 ± 0.4   |

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% O<sub>2</sub> environment, in a 3% O<sub>2</sub> environment, or in a 21% O<sub>2</sub> environment in the presence of 100 μM CoCl<sub>2</sub> 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 phosphorimager apparatus with dedicated software, and normalized to loading controls. The levels of Met proteins 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.

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 O<sub>2</sub> 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

**Figure 2.** Hypoxia induces *met* mRNA

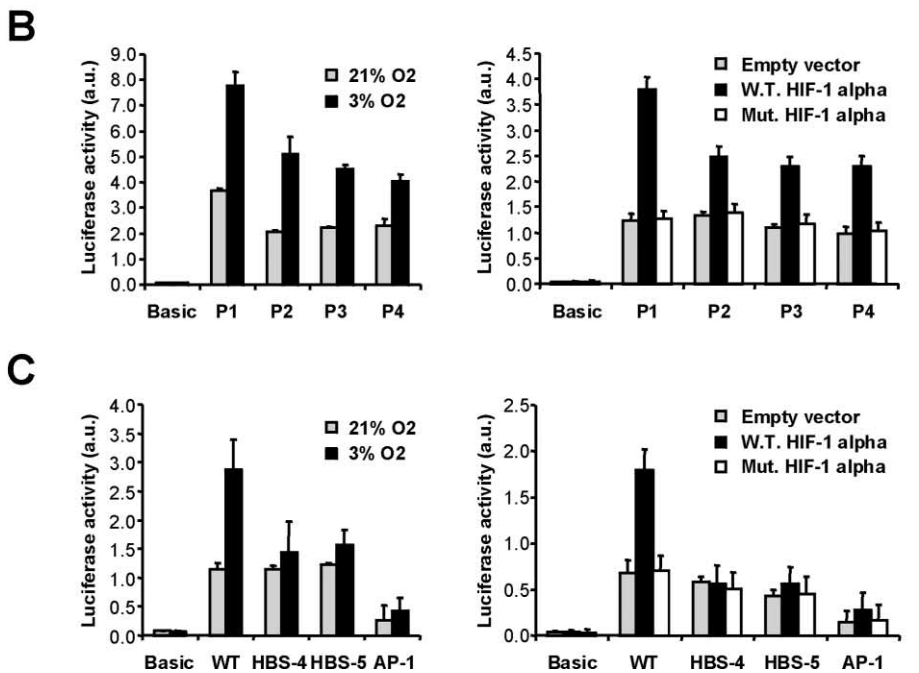
**A:** Northern blot analysis. Cells were subjected to normoxia (21% O<sub>2</sub>), hypoxia (3% O<sub>2</sub>), or CoCl<sub>2</sub> treatment for 48 hr, and the levels of *met* mRNA were determined by Northern blot analysis. Control, untreated cells at time zero. **B:** Time course analysis of *met* mRNA expression. Cells were incubated in hypoxic conditions for the indicated times and *met* mRNA levels were determined by Northern blot analysis. The induction of *vegf* mRNA is shown as a positive control.

**A**

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-411 CCCGGTGGGCGGGGGCGTTAGCGGAGACGTGGGAGAGGCCGAGAGCAAGCTCGGCCCTTCCGGGGTCAG
GGGCCCASmaICCCGCCCGCCCAATCGCCTCTGCACCTCTCCGGTCTCGTTTCGAGCGGGAGGGCCCAAGTC
SmaI
-336 CGAGCGGGTGCAGGAGGGTGCAGCCCTGCATCTGAGCCCGGGTGACACTCGCCTCCCAAGCGCCAGGAGGG
GCTCGCCACGGTCTCCACGCGCGGGACGTAGACTCGGGCCCACTGTGAGCGGAGGGTTCGCGGTCTCTCC
SmaI
-261 GGAGACTCGGTCCCCTTATCTCCGGCTGTGCTAACTTCAGACTGCCTGAGCTGGGGGAGGAGCGCGCAGCCA
CCTCTGAGCCAGGGCGAATAGAGGCCGACACGATTGAAGTGTGACGACTCGACCCCTCTCTCGCGGTCTCGGT
AvaII
-186 GGGCGAGAAAATCTTCCACCTAGAAAGTTTACCTTGTCTGGGGCGGGGCGAGAGCGGGAGGAAACGCGACCCC
CCCCTCTTTTGAAGAGTGGATCTTTCAAAGTGAACAGCACCCCGCCCGTCTCCGCCCTCTTTGCGTGGGG
HBS-2
-111 CGCGGGCCAGGCGCGCGCGGACGGCAGGAAGGGCGGGGCCGATTTCCTCTGGTGTGTCAGTCCCACCT
GCGCCCGTCCGCGCCGCGCTGCGTCTTCCCGCCCGCGTAAAGGAGACCCACCAGGTCAGGGGTGGA
start
-036 CAGCGGTCTCGGAACCCGCGGACTAGGGGACGGACAGCAGCGAGGCGAGACAGACACGTGCTGGGGCGGGCAGG
GTCGCCAGGAGCCTTGGGCGCCTGATCCCTGCCTGTCTGTGCGCTCCGCTGTCTGTGTCACGACCCCGCCGTC
AvaII
+040 CGAGCGCTCAGTCTGGTGCCTGGCGGTGCTCCGGCCCAACGCGCCCGGGCGCGCGGGCGCGCGCCG
GCTCGCGGAGTCAGACCAGCGGACCCACGGAGGCGGGGTTGCGCGGGCCGCGCGCCGCGCGCGCGC
AP-1 HBS-4 SmaI
+115 ATGCCCGCTGAGTCACTGGCAGGGCAGCGCGCGTGGGAAGGGGCGAGGGAGTGCGGCCGCGGGCGGGCGG
TACGGCCGACTCAGTGCAGCTCCGTCGCGCGCACACCTTCCCGCCCTCCACGCCGCGCCCGCCCGCC
HBS-5
+190 GGCCTGGGCTCAGCCCGCCGAGGTGACCCGGAGGCCCTCGCCCGCCGCGCGCCCGAGCGCTTGTGTAGCA
CCGCGACCCGAGTCGGGCGCGCTCCACTGGGCTCCGGGAGCGGGCGCGCCGCGGGGCTCGGAAACACTCGT
+265 GATGCGGAGCCGAGTGGAGGGCGGAGCCAGATCGGGGCGACAGCTGACTTGTGTGAGAGGCGGGGAGGCGC
CTACGCTCGGCTCACCTCCGCGCTCGGTCTACGCCCGCTGTGACTGAACGACTCTCTCCGCCCTCCGCG
HBS-5
+340 GGAGCGCGGTGTGTCC
CCTCGCGCGCACACAGG
AvaII

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that Met is sensitive to oxygen gradients in vivo, and that hypoxic regions of solid tumors overexpress Met.

#### Hypoxia sensitizes cells to HGF stimulation

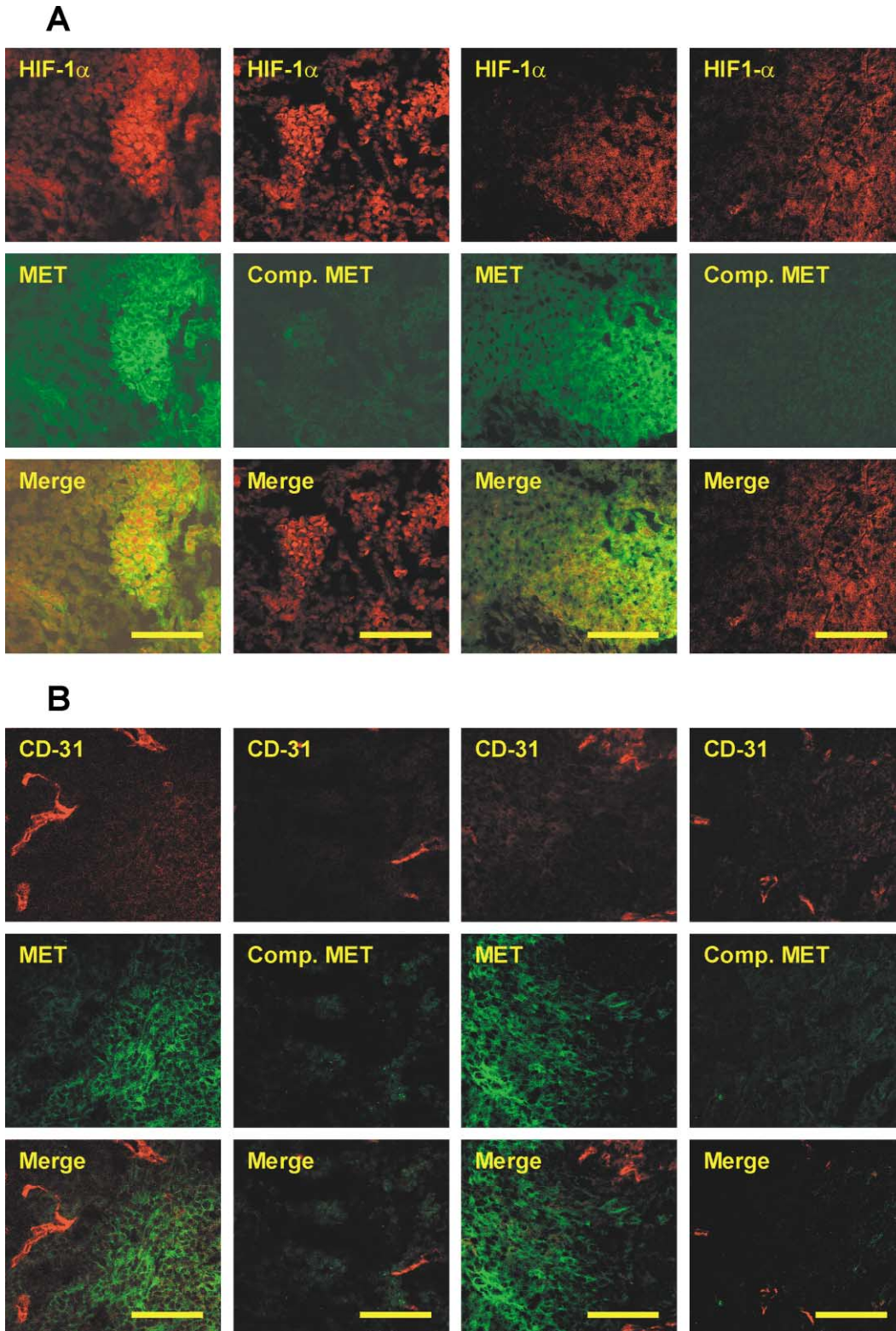
The data presented above provide evidence that the Met receptor is induced by hypoxia both in vitro and in vivo. To assess

**Figure 3.** Hypoxia induces *met* transcription

**A:** Nucleotide sequence of the human *met* promoter. Nucleotide +1 corresponds to the start site according to Gambarotta et al., 1994. Putative HIF-1 binding sites in sense orientation (HBSs) or in antisense orientation (asHBSs) are shown in red (the core consensus sequence "CGTG" is underlined). An AP-1 site is indicated in green. Restriction sites used for subcloning (SmaI, AvaII) are underlined in black.

**B:** Analysis of promoter (luciferase) activity using U2-OS cells (overlapping results were obtained with MLP-29 cells; not shown). Left histogram: cells were transfected with the various promoter constructs (P1-4) and then incubated in normoxic (21% O<sub>2</sub>) or hypoxic (3% O<sub>2</sub>) conditions. Right histogram: cells were cotransfected with P1-4 and an expression vector containing either no insert or a *hif-1α* cDNA, and then incubated in normoxic conditions. Luciferase activity was normalized for transfection efficiency using a *Renilla* reporter plasmid. WT HIF-1α, wild-type HIF-1α; Mut. HIF-1α, inactive HIF-1α mutant; Basic, promoterless reporter construct; a.u., arbitrary units.

**C:** Site-directed mutagenesis analysis. Cells were transfected as in **B** and the activity of wild-type (WT) P4 construct was compared with the activity of mutagenized P4 constructs in which putative transcriptional elements (HBS-4, HBS-5, AP-1) had been destroyed by site-directed mutagenesis.

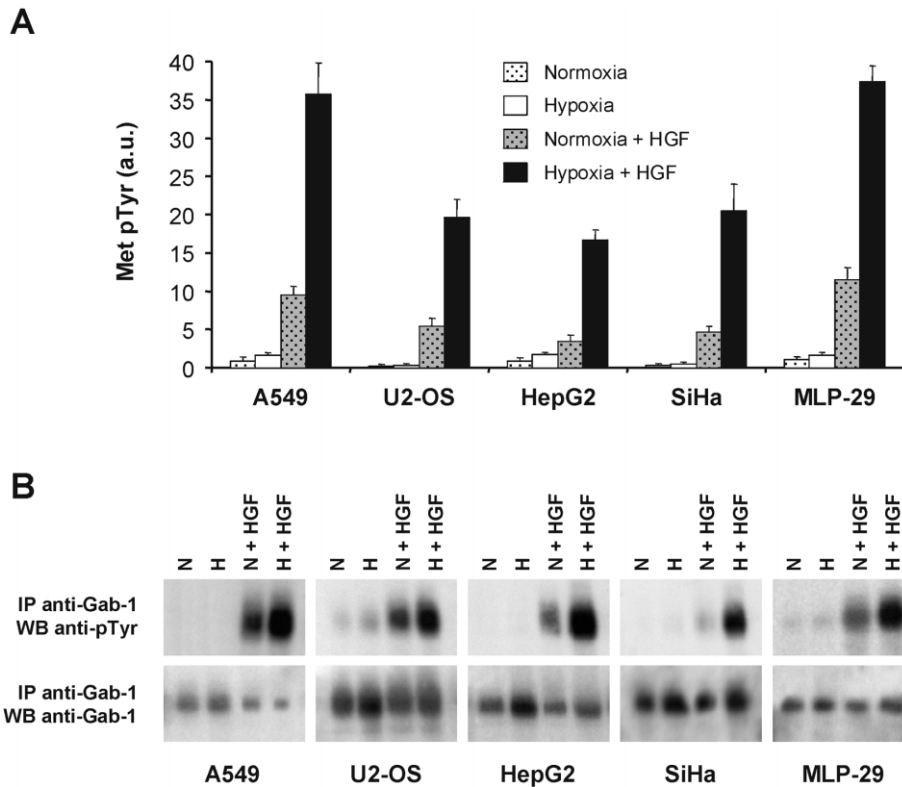


**Figure 4.** Met expression in tumors correlates directly with hypoxia and inversely with proximity to blood vessels

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-1 $\alpha$  (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  $\mu$ m 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.

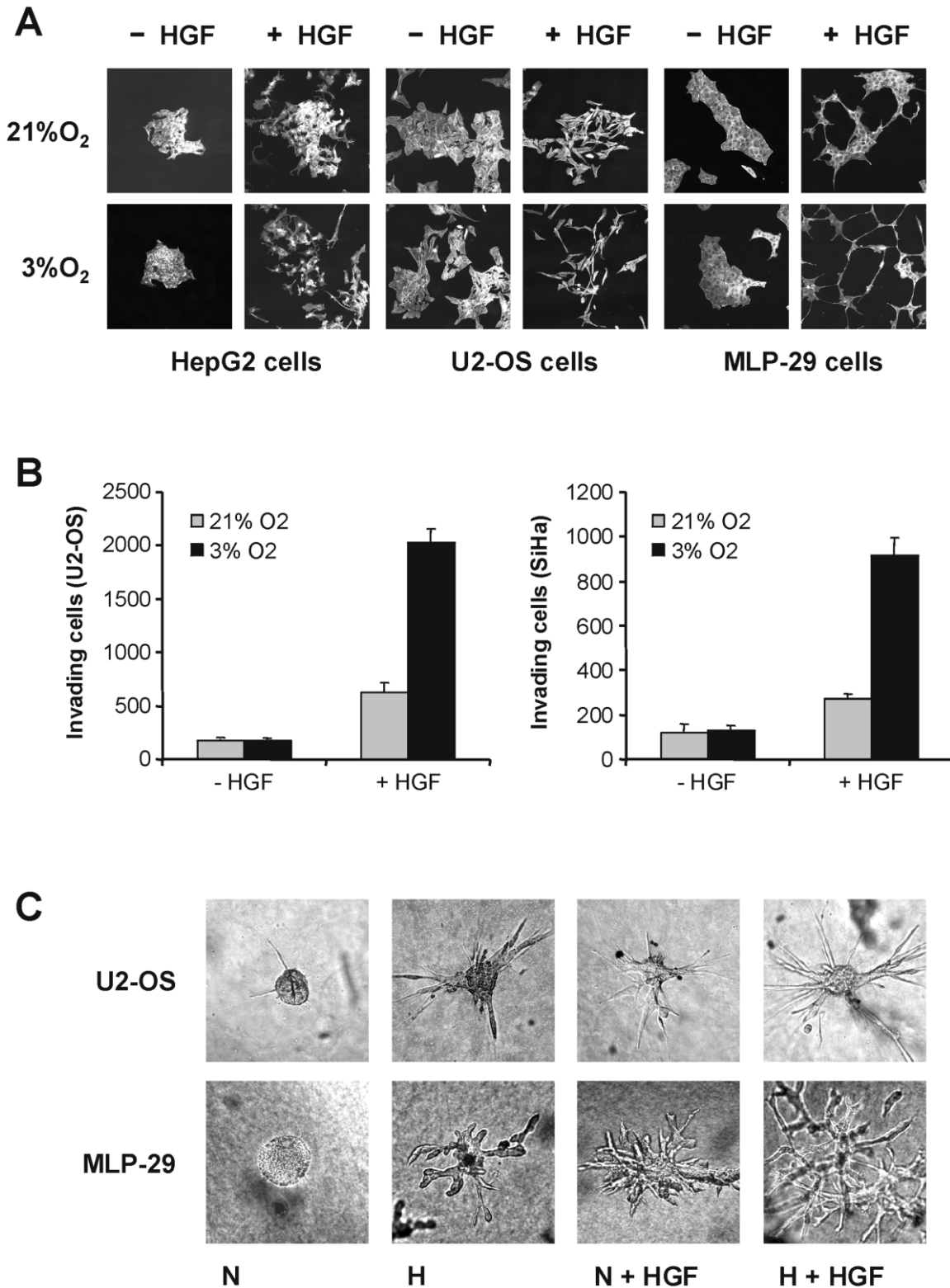
**B:** Met signal transduction analysis. Cells incubated in normoxia (N) or hypoxia (H) were stimulated as described in **A** and then lysed. Protein extracts were immunoprecipitated using anti-Gab-1 antibodies and analyzed by Western blotting using anti-phosphotyrosine antibodies. The same blots were reprobated 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 10 min, and then lysed. Cellular proteins were immunoprecipitated using anti-Met antibodies and analyzed by Western blot using anti-phosphotyrosine antibodies. Strikingly, while hypoxia had little effect on Met basal tyrosine phosphorylation, it dramatically amplified the response to HGF in all cells analyzed (Figure 5A). As a second read-out for Met activity, we also analyzed the activation of one of its key signal transducers, the multiadaptor protein Gab-1 (Weidner et al., 1996). In experiments analogous to those described above, cellular proteins were immunoprecipitated using anti-Gab-1 antibodies and then analyzed by Western blot using anti-phosphotyrosine antibodies. The same blots were subsequently reprobated with anti-Gab-1 antibodies to assess Gab-1 levels and with anti-Met antibodies to determine the association between Gab-1 and the Met receptor. Remarkably, hypoxia amplified several fold (3–9 fold depending on cell line) the extent of Gab-1 phosphorylation in response to HGF (Figure 5B), and dramatically increased the amount of Met associated with Gab-1 (not shown). Again, the basal levels of Gab-1 phosphorylation and association with Met were not significantly changed by hypoxia itself. The slight changes in Gab-1 levels observed in these experiments are probably due to altered immunoprecipitation efficiency (consequent to changes in epitope presentation or protein sequestration by other signal transducers). In fact, direct Western blot analysis of the same protein extracts did not reveal any significant change in Gab-1 levels (not shown). We conclude that hypoxia sensitizes cells to HGF stimulation and amplifies HGF signaling.

#### 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



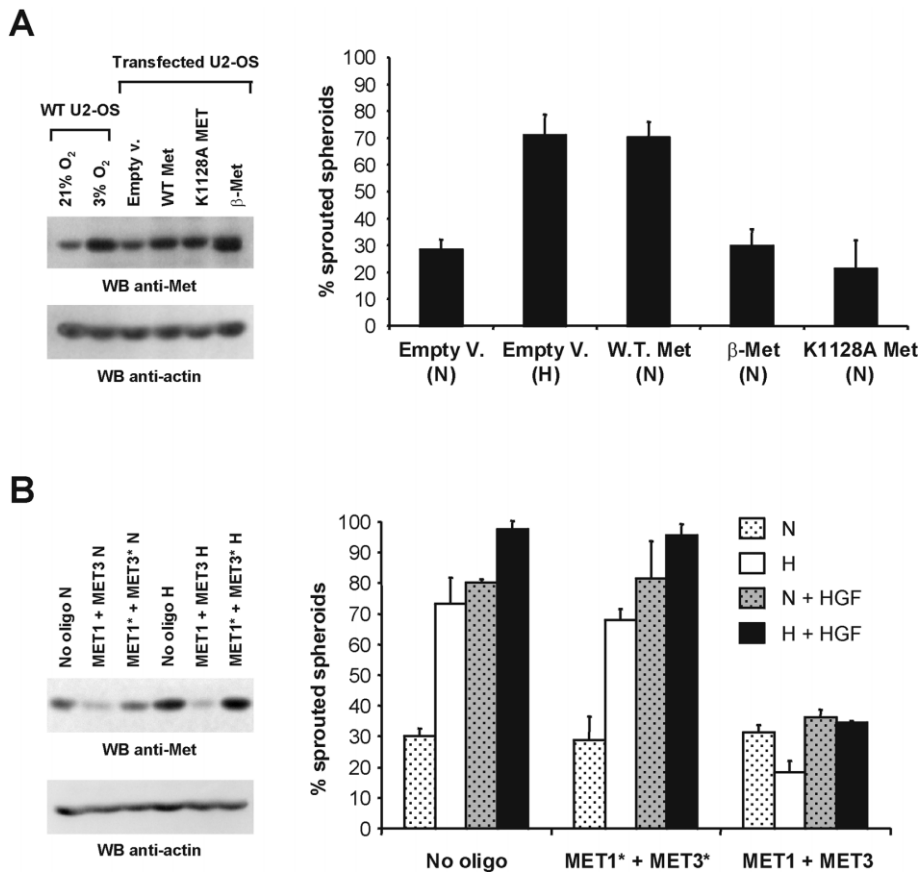
**Figure 6.** Hypoxia and HGF synergize in inducing cell motility and invasion

**A:** Scatter assay. Serum-starved cells were preincubated in normoxia (21% O<sub>2</sub>) or hypoxia (3% O<sub>2</sub>) 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 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% O<sub>2</sub>) or hypoxia (3% O<sub>2</sub>) 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.

response to HGF in all cells tested (Figure 6B). A similar effect was also achieved by stimulation with CoCl<sub>2</sub> (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% O<sub>2</sub> or 3% O<sub>2</sub> 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 dra-

matically amplified the proinvasive effect of HGF (only the data relative to U2-OS and MLP-29 cells are shown). Stimulation with CoCl<sub>2</sub> also achieved a strong synergistic effect with HGF (data not shown). However, in contrast with the results obtained in the other bioassays performed, low pO<sub>2</sub> also induced branching morphogenesis on its own, as previously observed in renal cells (Maranchie et al., 2002). Since the cell lines used in our experiments proved to be negative for HGF expression 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

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  $\beta$ -chain only—deprived of the functional domain responsible for interaction with HGF ( $\beta$ -Met; Michieli et al., 1999). The latter form of Met is properly exposed at the cell surface, but is not activated by HGF. However,  $\beta$ -Met responds biologically to ligand-mimetic antibodies directed against the extracellular portion of the Met  $\beta$ -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 ( $\beta$ -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 oligo 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 mu-

tated *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; Marchie et al., 2002). Given the pivotal role of the VHL protein in targeting HIF-1 $\alpha$  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 antiinvasive 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). B5/589 cells were a gift of Dr. Jacalyn H. Pierce (NCI Bethesda, MD). Cells were maintained in DMEM (HepG2, SiHa, SK-OV-3), Iscove's DMEM (U2-OS), or RPMI (A549, B5/589), all supplemented with 10% FBS (Sigma, St. Louis, MO). B5/589 were grown in the presence of 10 ng/ml human recombinant EGF (Sigma). A 3% O<sub>2</sub> environment was obtained using a Heraeus BB 6220 oxygen electrode incubator (Heraeus, Hanau, Germany). CoCl<sub>2</sub> (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 ( $1 \times 10^4$  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 fluoresceinated phalloidin (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 115 μg/cm<sup>2</sup>. Cells were seeded ( $1 \times 10^5$  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 CoCl<sub>2</sub>). 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, B5/589) 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 phalloidin (Sigma). Cells were then mounted in Mowiol 4-88 (Hoechst, Strasbourg, France). Slides were analyzed with a Laser Riance 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 (80 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 STORM 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 HepG2-derived total RNA as template and the following oligonucleotides as primers:

5' GACGACGGATCCGGTTCGGGCTCCGAAACCATGAACCTTTCTG 3'  
5' CGGCGGGTCGACCCGCCTCGGCTTGTCACATCTGCAAGTACG 3'

The Met probe was obtained from the pCEV-Met plasmid (Michieli et al., 1999). Probes were labeled by random priming (Megaprime, Amersham) using [ $\alpha$ -<sup>32</sup>P] dCTP. Hybridization was carried out at 42°C for 16 hr in the presence of 50% formamide. Nylon membranes were washed twice at room temperature with 2× SSC-0.1% SDS and twice at 45°C with 0.1× SSC-0.1% SDS, and autoradiographed. Image acquisition and analysis were performed using a STORM apparatus and dedicated Image Quant software (Molecular Dynamics). Ribosomal 28S RNA signal was used as loading control.

### Promoter analysis

The various *met* promoter constructs (P1-4) in the pGL-2 vector (Promega, Madison, WI) have been described previously (Gambiarotta et al., 1996). Putative HBSs were mutated in their core region CGTG 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 GCTGAGTCACT was mutated into TTATAGTCACT. Accuracy of the mutagenesis procedure was verified by direct sequencing of mutant plasmids. Analysis of promoter activity was performed in U2-OS or MLP-29 cells (10<sup>6</sup> 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 72 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 Wilbad, 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). Authorization was 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 × 10<sup>6</sup> cells/animal) in 200 µl of PBS into the left posterior flank of three seven-week-old immunodeficient *nu/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, Prat 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 was performed by preincubating the anti-Met antibody mixture with 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 (JC70A, Dako A/S, Glostrup, Denmark). Goat anti-rabbit Alexa Fluor 488 and either goat anti-rat Alexa Fluor 546 or goat anti-mouse Alexa Fluor 546 (all 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 Na<sub>3</sub>VO<sub>4</sub> and a cocktail of protease inhibitors (pepstatin, 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 kit (Pierce). Following dilution of SDS concentration to 0.1%, equal amounts of proteins were immunoprecipitated using either monoclonal anti-human Met antibodies (DQ-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 (UBI, Lake Placid, NY). The same blots were reprobated 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 Na<sub>3</sub>VO<sub>4</sub> 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 (UBI), resolved by SDS-PAGE and analyzed by Western blotting using anti-phosphotyrosine antibodies. The same blots were reprobated 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 (10<sup>6</sup> 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  $\beta$ -*met*, Michieli et al., 1999; K1228A *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' ACUCUAGAUGCUCAGACUU 3'

MET3 5' GUCAUAGGAAGAGGGCAUU 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' AGUCUAGAUGCUCACACUU 3'

MET3\* 5' CUCAUAGGAAGACCCCAUU 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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#### References

- Aguirre Ghiso, J.A., Alonso, D.F., Farias, E.F., Gomez, D.E., and de Kier Joffe, E.B. (1999). Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. *Eur. J. Biochem.* 263, 295–304.
- Andermarcher, E., Surani, M., and Gherardi, E. (1996). Co-expression of the HGF/SF and c-met genes during early mouse embryogenesis precedes reciprocal expression in adjacent tissues during organogenesis. *Dev. Genet.* 18, 254–266.
- Bardelli, A., Longati, P., Williams, T.A., Benvenuti, S., and Comoglio, P.M. (1999). A peptide representing the carboxyl-terminal tail of the met receptor inhibits kinase activity and invasive growth. *J. Biol. Chem.* 274, 29274–29281.
- Birchmeier, W., Brinkmann, V., Niemann, C., Meiners, S., DiCesare, S., Naudorf, H., and Sachs, M. (1997). Role of HGF/SF and c-Met in morphogenesis and metastasis of epithelial cells. *Ciba Found. Symp.* 212, 230–240.
- Blagosklonny, M.V. (2001). Hypoxia-inducible factor: Achilles' heel of antiangiogenic cancer therapy. *Int. J. Oncol.* 19, 257–262.
- Boccaccio, C., Gaudino, G., Gambarotta, G., Galimi, F., and Comoglio, P.M. (1994). Hepatocyte growth factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF. *J. Biol. Chem.* 269, 12846–12851.
- Boccaccio, C., Andò, M., Tamagnone, L., Bardelli, A., Michieli, P., Battistini, C., and Comoglio, P.M. (1998). Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. *Nature* 391, 285–288.
- Boix, L., Rosa, J.L., Ventura, F., Castells, A., Bruix, J., Rodes, J., and Bartrons, R. (1994). c-met mRNA overexpression in human hepatocellular carcinoma. *Hepatology* 19, 88–91.
- Brinkmann, V., Foroutan, H., Sachs, M., Weidner, K.M., and Birchmeier, W. (1995). Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. *J. Cell Biol.* 131, 1573–1586.
- Brizel, D.M., Scully, S.P., Harrelson, J.M., Layfield, L.J., Bean, J.M., Prosnitz, L.R., and Dewhirst, M.W. (1996). Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. *Cancer Res.* 56, 941–943.
- Brizel, D.M., Sibley, G.S., Prosnitz, L.R., Scher, R.L., and Dewhirst, M.W. (1997). Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. *Int. J. Radiat. Oncol. Biol. Phys.* 38, 285–289.
- Cairns, R.A., Kalliomaki, T., and Hill, R.P. (2001). Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. *Cancer Res.* 61, 8903–8908.
- Chambers, A.F., Groom, A.C., and MacDonald, I.C. (2002). Dissemination and growth of cancer cells in metastatic sites. *Nature Rev. Cancer* 2, 563–572.
- Comoglio, P.M., and Trusolino, L. (2002). Invasive growth: from development to metastasis. *J. Clin. Invest.* 109, 857–862.
- Crepaldi, T., Prat, M., Giordano, S., Medico, E., and Comoglio, P.M. (1994). Generation of a truncated hepatocyte growth factor receptor in the endoplasmic reticulum. *J. Biol. Chem.* 269, 1750–1755.
- Damert, A., Ikeda, E., and Risau, W. (1997). Activator-protein-1 binding potentiates the hypoxia-inducible factor-1-mediated hypoxia-induced transcriptional activation of vascular-endothelial growth factor expression in C6 glioma cells. *Biochem. J.* 327, 419–423.
- Date, K., Matsumoto, K., Kuba, K., Shimura, H., Tanaka, M., and Nakamura, T. (1998). Inhibition of tumor growth and invasion by a four-kringle antagonist (HGF/NK4) for hepatocyte growth factor. *Oncogene* 17, 3045–3054.
- Di Renzo, M.F., Olivero, M., Ferro, S., Prat, M., Bongarzone, I., Pilotti, S., Belfiore, A., Costantino, A., Vigneri, R., Pierotti, M.A., and Comoglio, P.M. (1992). Overexpression of the c-MET/HGF receptor gene in human thyroid carcinomas. *Oncogene* 7, 2549–2553.
- Di Renzo, M.F., Olivero, M., Giacomini, A., Porte, H., Chastre, E., Mirossay, L., Nordlinger, B., Bretti, S., Bottardi, S., Giordano, S., et al. (1995). Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. *Clin. Cancer Res.* 1, 147–154.
- Di Renzo, M.F., Olivero, M., Martone, T., Maffe, A., Maggiora, P., Stefani, A.D., Valente, G., Giordano, S., Cortesina, G., and Comoglio, P.M. (2000). Somatic mutations of the MET oncogene are selected during metastatic spread of human HNSC carcinomas. *Oncogene* 19, 1547–1555.
- Folkman, J. (1999). Angiogenesis research: from laboratory to clinic. *Forum (Genova)* 9 (Suppl. 3), 59–62.
- Follenzi, A., Bakovic, S., Gual, P., Stella, M.C., Longati, P., and Comoglio, P.M. (2000). Cross-talk between the proto-oncogenes Met and Ron. *Oncogene* 19, 3041–3049.
- Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., and Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.* 16, 4604–4613.
- Frisch, S.M., and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* 124, 619–626.
- Frisch, S.M., and Screaton, R.A. (2001). Anoikis mechanisms. *Curr. Opin. Cell Biol.* 13, 555–562.
- Gambarotta, G., Pisto, S., Giordano, S., Comoglio, P.M., and Santoro, C. (1994). Structure and inducible regulation of the human MET promoter. *J. Biol. Chem.* 269, 12852–12857.
- Gambarotta, G., Boccaccio, C., Giordano, S., Andò, M., Stella, M.C., and Comoglio, P.M. (1996). Ets up-regulates MET transcription. *Oncogene* 13, 1911–1917.
- Gerber, H.-P., Condorelli, F., Park, J., and Ferrara, N. (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. *J. Biol. Chem.* 272, 23659–23667.
- Gherardi, E., Gray, J., Stoker, M., Perryman, M., and Furlong, R. (1989). Purification of scatter factor, a fibroblast-derived basic protein that modulates epithelial interactions and movement. *Proc. Natl. Acad. Sci. USA* 86, 5844–5848.

- Giordano, S., Corso, S., Conrotto, P., Artigiani, S., Gilestro, G., Barberis, D., Tamagnone, L., and Comoglio, P.M. (2002). The semaphorin 4D receptor controls invasive growth by coupling with Met. *Nat. Cell Biol.* 4, 720–724.
- Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W., and Giaccia, A.J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 379, 88–91.
- Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* 100, 57–70.
- Hannon, G.J. (2002). RNA interference. *Nature* 418, 244–251.
- Harris, A.L. (2002). Hypoxia—a key regulatory factor in tumor growth. *Nature Rev. Cancer* 2, 38–47.
- Höckel, M., and Vaupel, P. (2001). Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J. Natl. Cancer Inst.* 93, 266–276.
- Höckel, M., Schlenger, K., Aral, B., Mitze, M., Schaffer, U., and Vaupel, P. (1996). Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res.* 56, 4509–4515.
- Höckel, M., Schlenger, K., Höckel, S., and Vaupel, P. (1999). Hypoxic cervical cancers with low apoptotic index are highly aggressive. *Cancer Res.* 59, 4525–4528.
- Kimura, H., Weisz, A., Kurashima, Y., Hashimoto, K., Ogura, T., D'Acquisto, F., Addeo, R., Makuuchi, M., and Esumi, H. (2000). Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. *Blood* 95, 189–197.
- Koochekpour, S., Jeffers, M., Wang, P.H., Gong, C., Taylor, G.A., Roessler, L.M., Stearman, R., Vasselli, J.R., Stetler-Stevenson, W.G., Kaelin, W.G., Jr., et al. (1999). The von Hippel-Lindau tumor suppressor gene inhibits hepatocyte growth factor/scatter factor-induced invasion and branching morphogenesis in renal carcinoma cells. *Mol. Cell. Biol.* 19, 5902–5912.
- Levy, A.P., Levy, N.S., Wegner, S., and Goldberg, M.A. (1995). Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J. Biol. Chem.* 270, 13333–13340.
- Liotta, L.A., and Kohn, E.C. (2001). The microenvironment of the tumour-host interface. *Nature* 411, 375–379.
- Liu, C., Park, M., and Tsao, M.S. (1992). Overexpression of c-met proto-oncogene but not epidermal growth factor receptor or c-erbB-2 in primary human colorectal carcinomas. *Oncogene* 7, 181–185.
- Lyon, M., Deakin, J.A., Mizuno, K., Nakamura, T., and Gallagher, J.T. (1994). Interaction of hepatocyte growth factor with heparan-sulfate. Elucidation of the major heparan sulfate structural determinants. *J. Biol. Chem.* 269, 11216–11223.
- Maity, A., and Solomon, D. (2000). Both increased stability and transcription contribute to the induction of the urokinase plasminogen activator receptor (uPAR) message by hypoxia. *Exp. Cell Res.* 255, 250–257.
- Maranchie, J.K., Vasselli, J.R., Riss, J., Bonifacio, J.S., Linehan, W.M., and Klausner, R.D. (2002). The contribution of VHL substrate binding and HIF1- $\alpha$  to the phenotype of VHL loss in renal carcinoma. *Cancer Cell* 1, 247–255.
- Matsumoto, K., and Nakamura, T. (1997). HGF: its organotrophic role and therapeutic potential. *Ciba Found. Symp.* 212, 198–211.
- Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399, 271–275.
- Medico, E., Mongiovi, A.M., Huff, J., Jelinek, M.A., Follenzi, A., Gaudino, G., Parsons, J.T., and Comoglio, P.M. (1996). The tyrosine kinase receptors Ron and Sea control “scattering” and morphogenesis of liver progenitor cells in vitro. *Mol. Biol. Cell* 7, 495–504.
- Meyer, M., Clauss, M., Lepple-Wienhues, A., Waltenberger, J., Augustin, H.G., Ziche, M., Lanz, C., Buttner, M., Rziha, H.J., and Dehio, C. (1999). A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Fit-1) receptor tyrosine kinases. *EMBO J.* 18, 363–374.
- Michieli, P., Basilico, C., Pennacchietti, S., Maffe, A., Tamagnone, L., Giordano, S., Bardelli, A., and Comoglio, P.M. (1999). Mutant Met-mediated transformation is ligand-dependent and can be inhibited by HGF antagonists. *Oncogene* 18, 5221–5231.
- Michiels, C., Minet, E., Michel, G., Mottet, D., Piret, J.P., and Raes, M. (2001). HIF-1 and AP-1 cooperate to increase gene expression in hypoxia: role of MAP kinases. *IUBMB Life* 52, 49–53.
- Miyazawa, K., Shimomura, T., Naka, D., and Kitamura, N. (1994). Proteolytic activation of hepatocyte growth factor in response to tissue injury. *J. Biol. Chem.* 269, 8966–8970.
- Montesano, R., Matsumoto, K., Nakamura, T., and Orci, L. (1991). Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 67, 901–908.
- Morotti, A., Mila, S., Accornero, P., Tagliabue, E., and Ponzetto, C. (2002). K252a inhibits the oncogenic properties of Met, the HGF receptor. *Oncogene* 21, 4885–4893.
- Nakamura, T., Teramoto, H., and Ichihara, A. (1986). Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. *Proc. Natl. Acad. Sci. USA* 83, 6489–6493.
- Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989). Molecular cloning and expression of human hepatocyte growth factor. *Nature* 342, 440–443.
- Naldini, L., Vigna, E., Ferracini, R., Longati, P., Gandino, L., Prat, M., and Comoglio, P.M. (1991). The tyrosine kinase encoded by the MET proto-oncogene is activated by autophosphorylation. *Mol. Cell. Biol.* 11, 1793–1803.
- Naldini, L., Tamagnone, L., Vigna, E., Sachs, M., Hartmann, G., Birchmeier, W., Daikuhara, Y., Tsubouchi, H., Blasi, F., and Comoglio, P.M. (1992). Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. *EMBO J.* 11, 4825–4833.
- Norris, M.L., and Millhorn, D.E. (1995). Hypoxia-induced protein binding to O<sub>2</sub>-responsive sequences on the tyrosine hydroxylase gene. *J. Biol. Chem.* 270, 23774–23779.
- O'Reilly, M.S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R.A., Moses, M., Lane, W.S., Cao, Y., Sage, E.H., and Folkman, J. (1994). Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79, 315–328.
- Oh, R.R., Park, J.Y., Lee, J.H., Shin, M.S., Kim, H.S., Lee, S.K., Kim, Y.S., Lee, S.H., Lee, S.N., Yang, Y.M., et al. (2002). Expression of HGF/SF and Met protein is associated with genetic alterations of VHL gene in primary renal cell carcinomas. *APMIS* 110, 229–238.
- Orian-Rousseau, V., Chen, L., Sleeman, J.P., Herrlich, P., and Ponta, H. (2002). CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev.* 16, 3074–3086.
- Park, W.S., Dong, S.M., Kim, S.Y., Na, E.Y., Shin, M.S., Pi, J.H., Kim, B.J., Bae, J.H., Hong, Y.K., Lee, K.S., et al. (1999). Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. *Cancer Res.* 59, 307–310.
- Postovit, L.M., Adams, M.A., Lash, G.E., Heaton, J.P., and Graham, C.H. (2002). Oxygen-mediated regulation of tumor cell invasiveness. Involvement of a nitric oxide signaling pathway. *J. Biol. Chem.* 277, 35730–35737.
- Prat, M., Crepaldi, T., Pennacchietti, S., Bussolino, F., and Comoglio, P.M. (1998). Agonistic monoclonal antibodies against the Met receptor dissect the biological responses to HGF. *J. Cell Sci.* 111, 237–247.
- Richard, D.E., Berra, E., and Pouyssegur, J. (2000). Nonhypoxic pathway mediates the induction of Hypoxia-Inducible Factor 1 $\alpha$  in vascular smooth muscle cells. *J. Biol. Chem.* 275, 26765–26771.
- Rofstad, E.K., Rasmussen, H., Galappathi, K., Mathiesen, B., Nilsen, K., and Graff, B.A. (2002). Hypoxia promotes lymph node metastasis in human melanoma xenografts by up-regulating the urokinase-type plasminogen activator receptor. *Cancer Res.* 62, 1847–1853.
- Rubin, J.S., Bottaro, D.P., and Aaronson, S.A. (1993). Hepatocyte growth

- factor/scatter factor and its receptor, the c-met proto-oncogene product. *Biochim. Biophys. Acta* 1155, 357–371.
- Ruco, L.P., Ranalli, T., Marzullo, A., Bianco, P., Prat, M., Comoglio, P.M., and Baroni, C.D. (1996). Expression of Met protein in thyroid tumours. *J. Pathol.* 180, 266–270.
- Sánchez-Elsner, T., Botella, L.M., Velasco, B., Langa, C., and Bernabeu, C. (2002). Endoglin expression is regulated by transcriptional cooperation between the hypoxia and transforming growth factor-beta pathways. *J. Biol. Chem.* 277, 43799–43808.
- Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. (1995). Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* 373, 699–702.
- Schmidt, L., Junker, K., Nakaigawa, N., Kinjerski, T., Weirich, G., Miller, M., Lubensky, I., Neumann, H.P., Brauch, H., Decker, J., et al. (1999). Novel mutations of the MET proto-oncogene in papillary renal carcinomas. *Oncogene* 18, 2343–2350.
- Semenza, G.L. (2001). HIF-1, O<sub>2</sub>, and the 3 PHDs: how animal cells signal hypoxia to the nucleus. *Cell* 107, 1–3.
- Seol, D.W., Chen, Q., and Zarnegar, R. (2000). Transcriptional activation of the hepatocyte growth factor receptor (c-met) gene by its ligand (hepatocyte growth factor) is mediated through AP-1. *Oncogene* 19, 1132–1137.
- Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359, 843–845.
- Stoker, M., and Gherardi, E. (1991). Regulation of cell movement: the motogenic cytokines. *Biochim. Biophys. Acta* 1072, 81–102.
- Stoker, N., Gherardi, E., Perryman, M., and Grey, J. (1987). Scatter factor is a fibroblast-derived modulator of epithelial cell motility. *Nature* 327, 239–242.
- Sundfor, K., Lyng, H., and Rofstad, E.K. (1998). Tumour hypoxia and vascular density as predictors of metastasis in squamous cell carcinoma of the uterine cervix. *Br. J. Cancer* 78, 822–827.
- Tacchini, L., Dansi, P., Matteucci, E., and Desiderio, M.A. (2001). Hepatocyte growth factor signalling stimulates hypoxia inducible factor-1 (HIF-1) activity in HepG2 hepatoma cells. *Carcinogenesis* 22, 1363–1371.
- Takayama, H., La Rochelle, W.J., Anver, M., Bockman, D.E., and Merlino, G. (1996). Scatter factor/hepatocyte growth factor as a regulator of skeletal muscle and neural crest development. *Proc. Natl. Acad. Sci. USA* 93, 5866–5871.
- Trusolino, L., and Comoglio, P.M. (2002). Scatter-factor and semaphorin receptors: cell signalling for invasive growth. *Nature Rev. Cancer* 4, 289–300.
- Trusolino, L., Bertotti, A., and Comoglio, P.M. (2001). A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth. *Cell* 107, 643–654.
- Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T., and Kitamura, N. (1995). Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature* 373, 702–705.
- Vande Woude, G.F., Jeffers, M., Cortner, J., Alvord, G., Tsarfaty, I., and Resau, J. (1997). Met-HGF/SF: tumorigenesis, invasion and metastasis. *Ciba Found. Symp.* 212, 119–130.
- Vaupel, P., Kallinowski, F., and Okunieff, P. (1989). Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res.* 49, 6449–6465.
- Vukovic, V., Haugland, H.K., Nicklee, T., Morrison, A.J., and Hedley, D.W. (2001). Hypoxia-inducible factor-1alpha is an intrinsic marker for hypoxia in cervical cancer xenografts. *Cancer Res.* 61, 7394–7398.
- Weidner, K.M., Di Cesare, S., Sachs, M., Brinkmann, V., Behrens, J., and Birchmeier, W. (1996). Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature* 384, 173–176.
- Woodhouse, E.C., Chuaqui, R.F., and Liotta, L.A. (1997). General mechanisms of metastasis. *Cancer* 80, 1529–1537.
- Woolf, A.S., Kolatsi-Joannou, M., Hardman, P., Andermarcher, E., Moorby, C., Fine, L.G., Jat, P.S., Noble, M.D., and Gherardi, E. (1995). Roles of hepatocyte growth factor/scatter factor and the met receptor in the early development of the metanephros. *J. Cell Biol.* 128, 171–184.
- Xu, Q., Ji, Y.S., and Schmedtje, J.F., Jr. (2000). Sp1 increases expression of cyclooxygenase-2 in hypoxic vascular endothelium. *J. Biol. Chem.* 275, 24583–24589.
- Yamashita, K., Discher, D.J., Hu, J., Bishopric, N.H., and Webster, K.A. (2001). Molecular regulation of the endothelin-1 gene by hypoxia. *J. Biol. Chem.* 276, 12645–12653.
- Yanagita, K., Matsumoto, K., Sekiguchi, K., Ishibashi, H., Niho, Y., and Nakamura, T. (1993). Hepatocyte growth factor may act as a pulmotrophic factor on lung regeneration after acute lung injury. *J. Biol. Chem.* 268, 21212–21217.
- Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, K.M., Birchmeier, C., and Birchmeier, W. (1995). Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *J. Cell Biol.* 131, 215–226.
- Young, S.D., and Hill, R.P. (1990). Effects of reoxygenation of cells from hypoxic regions of solid tumors: anticancer drug sensitivity and metastatic potential. *J. Natl. Cancer Inst.* 82, 338–339.
- Young, S.D., Marshall, R.S., and Hill, R.P. (1988). Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. *Proc. Natl. Acad. Sci. USA* 85, 9533–9537.
- Yu, J.L., Rak, J.W., Coomber, B.L., Hicklin, D.J., and Kerbel, R.S. (2002). Effect of p53 status on tumor response to antiangiogenic therapy. *Science* 295, 1526–1528.
- Yuan, Y., Hilliard, G., Ferguson, T., and Millhorn, D.E. (2003). Cobalt inhibits the interaction between hypoxia inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia inducible factor-alpha. *J. Biol. Chem.*, in press.