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ORIGINAL ARTICLE

Pro-metastatic signaling by *c-Met* through RAC-1 and reactive oxygen species (ROS)

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Overexpression of the c-Met/hepatocyte growth factor receptor(HGF-R) proto-oncogene and abnormal generation of intracellular oxygen species (reactive oxygen species (ROS)) have been linked, by independent lines of evidence, to cell transformation and to malignant growth. By comparing two subpopulations of the B16 mouse melanoma (B16-F0 and B16-F10) endowed with different lung metastasis capacities (low and high, respectively) we found that both the expression/phosphorylation of c-Met and the steady-state levels of ROS positively correlated with metastatic growth. shRNA-mediated downregulation of c-Met in F10 cells led to a parallel decrease in the generation of oxygen species and in metastatic capacity, suggesting that oxidants may mediate the pro-metastatic activity of the HGF receptor. c-Met activation by a ligand elicits the formation of oxidant species through the oxidase-coupled small GTPase Rac-1, a relevant downstream target of the HGF-R. Moreover, cell treatment with the catalytic ROS scavengers EUK-134 and EUK-189 attenuates Met signaling to ERKs and inhibits the anchorage-independent growth of F10 cells, consistent with a critical role for oxygen species in HGF signaling and in aggressive cell behavior. Finally, genetic manipulation of the Rac-ROS cascade at different levels demonstrated its crucial role in the pro-metastatic activity of c-Met *in vivo*. Thus, we have outlined a novel cascade triggered by c-Met and mediated by ROS, linked to metastasis and potentially targetable by new antimetastatic, redox-based therapies.
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Introduction

Molecular changes underlying the gain of metastatic capacity by malignant cells are still incompletely understood, and their identification represents an important avenue to the development of new, more effective antimetastatic therapies.

Since the seminal studies of Fidler and Kripke (1977), demonstrating the pre-existence of clones endowed with different intrinsic metastatic capacities within a heterogeneous malignant population, the mouse melanoma B16 model has been extensively used to investigate the molecular circuitry involved in triggering the metastasic switch. Recently, this experimental model has been successfully exploited for the implementation and validation of a genome-wide screen aimed at the identification/isolation of metastasis-associated genes (Clark *et al*., 2000). One of the resulting candidate molecules, the Rho-family GTPase Rho-C, directly links metastatic growth to cytoskeleton dynamics and cell motility (Ridley, 2000).

Among the genes whose causative role in invasive and metastatic cell growth has been thus far most convincingly demonstrated is the proto-oncogene *c-Met*, encoding the tyrosine kinase receptor of the hepatocyte growth factor/scatter factor (Comoglio and Trusolino, 2002; Birchmeier *et al*., 2003; Corso *et al*., 2005). *In vitro* and animal studies, as well as molecular analysis of human malignancies, in fact link abnormal activity of c-Met to malignant cell progression.

Although typical features of invasive growth, such as loss of polarity, increased motility, epithelial–mesenchymal transition and mitogenesis are easily induced in responsive cultured cells by HGF(Wells, 2000; Comoglio and Boccaccio, 2001), deregulated HGF/c-Met signaling in transgenic mice leads to aberrant tissue development and to the genesis of histologically distinct neoplasms of both mesenchymal and epithelial origin, including melanomas (Takayama *et al*., 1997), and to metastatic growth (Rong *et al*., 1994). Finally, c-Met activation through either autocrine stimulation or receptor overexpression is a common finding in many human malignant tumors, especially in their advanced stages (http://www.vai.org/vari/metandcancer).

Similar to many other growth factor RTKs, c-Met exerts its oncogenic potential through the deregulated activation of a number of protein phoshorylation-dependent signaling cascades (Blume-Jensen and Hunter, 2001). These include the Grb-2/SOS/Ras pathway linked to cell proliferation and the PI3 Kinase/AkT pathway linked to cell survival and inhibition of apoptosis, but also a complex network of adaptors and transducers connecting c-Met to cytoskeleton dynamics, cell scattering and migration, cell adhesion and invasion (Comoglio and Trusolino, 2002; Birchmeier *et al*., 2003). The molecular organization of such complex motogenic activity requires the association of c-Met with both repulsive coreceptors, called Plexins (Trusolino and Comoglio, 2002), and adhesion molecules, like the $\alpha 6\beta 4$ integrin (Comoglio *et al*., 2003), and the coordinated modulation of the Rho family GTP-ases Rho, Rac and CDC42 (Royal *et al*., 2000).

An emerging theme in signal transduction by growth factor receptors and adhesion molecules is the role of reactive oxygen species (ROS), and of hydrogen peroxide (H_2O_2) in particular, as essential downstream intermediates that modulate cell responses through the transient and reversible oxidation of key intracellular signaling components (Finkel, 1998; Rhee *et al*., 2000). In most cases, similar to the oxidative burst generated by bacterial ligands in activated phagocytes, growth factor- and integrin-triggered generation of ROS involves the membrane assembly of a NADPH-dependent oxidase complex, capable of transferring reducing equivalents to molecular oxygen, with the formation of superoxide anion ($O_2^{-\bullet}$) (Lambeth, 2004); ligand-induced superoxide is subsequently dismutated to H_2O_2 by the cytosolic, Cu- and Zn-dependent suproxide dismutase, also known as SOD1.

Key molecular components of the oxidase complex are cytochromes of the recently described *Nox* family, and the small GTPase Rac-1 (Lambeth, 2004). The Rac/ROS signaling module has been convincingly shown to contribute to mitogenic signaling by PDGF (Sundaresan *et al*., 1995), EGF (Bae *et al*., 1997; Pani *et al*., 2000), and VEGF (Colavitti *et al*., 2002) receptors and by oncogenic H-Ras (Irani *et al*., 1997; Liu *et al*., 2001), and to have a role in the antiapoptotic cascade downstream of neurotrophin receptors (Bedogni *et al*., 2003). Moreover, this signaling cassette is robustly activated by integrins upon cell–matrix interaction (Chiarugi *et al*., 2003; Nimnual *et al*., 2003) and is necessary for optimal focal adhesion assembly and for cell spreading of NIH-3T3 cells. In the current model, many of the above effects are mediated, at least in part, by the inhibitory effect of oxidant species on protein tyrosine phophatases on duty for restraining both the strength and the duration of growth factor and integrin signaling (Xu *et al*., 2002). PTP1B (Lee *et al*., 1998), SHP-2 (Meng *et al*., 2002) and the low molecular weight phosphatase (LMW-PTP) (Chiarugi *et al*., 2001) are among the enzymes for which the biochemical mechanism and the functional relevance of oxidative inactivation during redox signaling have been most convincingly demonstrated.

Although mitogenesis, survival and adhesion certainly require Rac/ROS-dependent signals, a specific role for the above redox pathway in the invasive and metastatic behavior of malignant cells has just started to be addressed (Radisky *et al*., 2005), in spite of invasiveness recapitulating many cellular and molecular aspects of inflammatory processes, in which the involvement of this signaling cascade has been long established. On the other hand, it is conceivable that a redox signaling operates downstream of HGF/SF-R as is the case for many other RTK receptors; by extension, oxidant intermediates generated by Rac may specifically contribute to the pro-invasive and pro-metastatic behavior of malignancies in which c-Met signaling is deregulated.

The present work moved from our initial observation of overexpression of c-Met and elevated release of superoxide in a lung-specific ($B16F_{10}$) metastatic clone of the B16 mouse melanoma (Fidler and Nicolson, 1976), and was designed to assess the potential role of the Rac-ROS pathway in the pro-metastatic signaling downstream of c-Met, within a well-established model of experimental metastasis.

Results

Overexpression of c-Met and elevated release of superoxide anion in a lung-specific clone of B16 melanoma cells

Clones of the B16 murine melanoma with different metastatic capacities and tissue tropism have been originally derived and characterized by Fidler and coworkers (Fidler and Nicolson, 1976; Fidler and Kripke, 1977). The B16 F10 clone forms metastatic colonies with high efficiency almost exclusively in the lungs following direct injection in the blood stream of syngeneic C57Bl6/J mice (Figure 1a); conversely, the original heterogeneous population B16F0 is poorly metastatic, in spite of the two populations having similar growth rates *in vitro* and when transplanted as subcutaneous tumors (Fidler, 1975; and our data not shown).

In order to begin investigating the potential involvement of redox-modulated signaling cascades in the metastatic behavior of B16 cells, we assessed the release of superoxide anion by F0 and F10 cells, using a conventional NBT reduction test. After 1 h of incubation, cell staining by precipitated NBT was significantly more intense in F10 cells, indicating higher steady-state levels of superoxide (Figure 1b). As superoxide scavenging capacity is not different in the two cell lines, as suggested by the comparable expression of the two major superoxide dismutases (SOD1 and SOD2, Figure 1c), the NBT reduction test indicates that an higher amount of superoxide is actively generated in metastatic F10 cells during the assay.

To establish a connection between generation of ROS and other potential upstream and downstream signaling events in B16 cells, the general patterns of protein tyrosine phoshorylation of F0 and F10 cells were also compared by antiphosphotyrosine immunoblot analysis of total protein lysates.

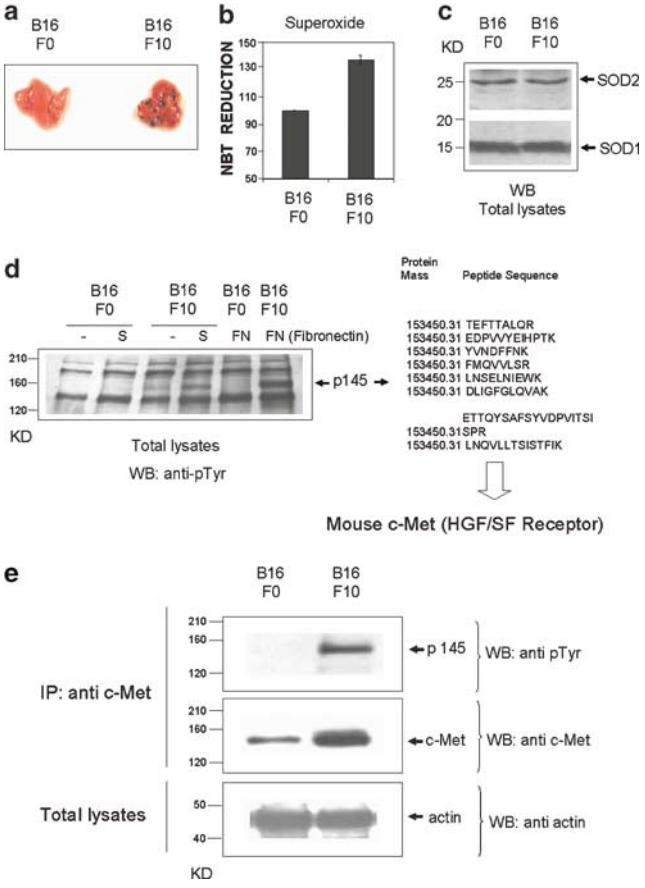

Figure 1 Overexpression of c-Met and elevated release of superoxide anion in a lung-specific clone of B16 melanoma cells. (**a**) Metastatic capacity of the B16 F10 clone. Animals were killed 3 weeks after tail injection of an equal number (150 000) of B16 F0 or B16 F10 cells. (**b**) Superoxide release by F0 and F10 cells. Cells were incubated on plastic in serum-free HBSS for 1 h, in the presence of 2 mg/ml NBT (Sigma). At the end of the incubation, cells were scraped, resuspended in Piridine and boiled for 2 min. Absorbance at 480 nm was measured by an ELISA plate reader. Data are percentage of NBT reduction by F0 cells (mean ± error spreading of duplicate samples). Histogram is representative of several independent experiments. (**c**) Expression of superoxide scavengers in F0 and F10 cells was assessed by Western blot analysis on total protein lysates. Relevant bands are indicated by arrows. Equal protein loading was verified by reversible PonceauS staining of the nitrocellulose filter. (**d**) Antiphosphotyrosine immunoblotting of total protein lysates from resting and FBS or fibronectin-stimulated F0 and F10 cells. A prominent 145 kDa band overrepresented in F10 lysates and induced by serum and by cell adhesion is indicated by an arrow. The band was identified as the product of the c-Met proto-oncogene by tandem mass spectrometry (see Materials and methods). (**e**) Overexpression and constitutive phosphorylation of c-Met in F10 cells. c-Met was immunoprecipitated from F0 and F10 protein lysates. Immunoprecipitates were subjected to either antiphosphotyrosine (top panel) or anti-c-Met (middle panel) immunoblotting. Equal protein content in the two input lysates was verified by antiactin immunostaining (bottom panel).

Although tyrosine phosphorylation profiles appeared roughly very similar in the two cell lines, one prominent 145 kDa phosphoprotein was consistently scored in F10 cells stimulated by either fetal serum or fibronectin, which was much less pronunced in the corresponding F0 lysates (Figure 1d, lanes 2, 4 and 6). Band isolation, followed by tandem (ion trap) mass spectrometry (see Materials and methods), led to the identification of p145 as the product of the murine *c-Met* proto-oncogene.

The identity of p145 as the HGF/SF receptor was further confirmed by anti-c-Met immunoprecipitation from F10 and F0 lysates, followed by either antiphosphotyrosine or anti-c-Met immunoblotting. This analysis revealed that c-Met protein levels are indeed markedly increased in F10 cells with respect to the F0 population (Figure 1e, middle panel) and that the protein is strongly phosphorylated on tyrosine residues in the former cell line (Figure 1e, upper panel). As F10 cells (nor F0) do not produce significant amounts of HGF (data not shown), constitutive Met phosphorylation is likely due to overexpression (leading to spontaneous dimerization) of the receptor (Ponzetto *et al*., 1991).

c-Met overexpression is necessary for the metastatic phenotype of B16F10 cells

Increased activity of c-Met by overexpression, autocrine stimulation or mutation is a frequent finding in metastatic cancer (http://www.vai.org/vari/metandcancer). In the attempt to establish a causal link between elevated expression of the c-Met receptor, generation of oxygen species and metastatic capacity of the B16 F10 melanoma, cells were stably transduced with a lentiviral construct encoding either a Met-specific shRNA, or a scrambled shRNA as a control. In MetshRNA-transduced cells (Met-shRNA F10) both the immature 190 kDa precursor form and the mature 145 kDa form of the receptor were significantly reduced with respect to the scramble-transduced controls (ctrl-shRNA F10), as revealed by anti-c-Met immunoblot analysis (Figure 2A). Importantly, expression levels so obtained were comparable to those observed in the poorly metastatic F0 line. In parallel with the amount of c-Met, steady-state generation of superoxide also was decreased in Met shRNA cells (Figure 2A, b), indicating that deregulated c-Met activity may be directly responsible for abnormal generation of ROS in F10 cells.

When subjected to the experimental metastasis assay, Met-silenced cells formed a significantly lower number of large (>2 mm) lung colonies (16.2 ± 9.5 versus 2.4 ± 2.3 per mouse, $P < 0.05$), compared to control cells overexpressing the HGF receptor, although with some variability among different animals (Figure 2B). This finding is in full agreement with a previous report of loss of invasiveness by Met-silenced cancer cells (Shinomiya *et al*., 2004). As Met shRNA cells represent a polyclonal population, and metastases generated by Met-silenced cells were GFP-negative and positive for antiphospho-Met immunostaining (not shown), we assume that those colonies arose from cells which had escaped c-Met downregulation. Interestingly, intrinsic cell proliferative and survival capacity was not affected by the downregulation of c-Met, as indicated by the superimposable profiles of growth on plastic depicted in Figure 2C.

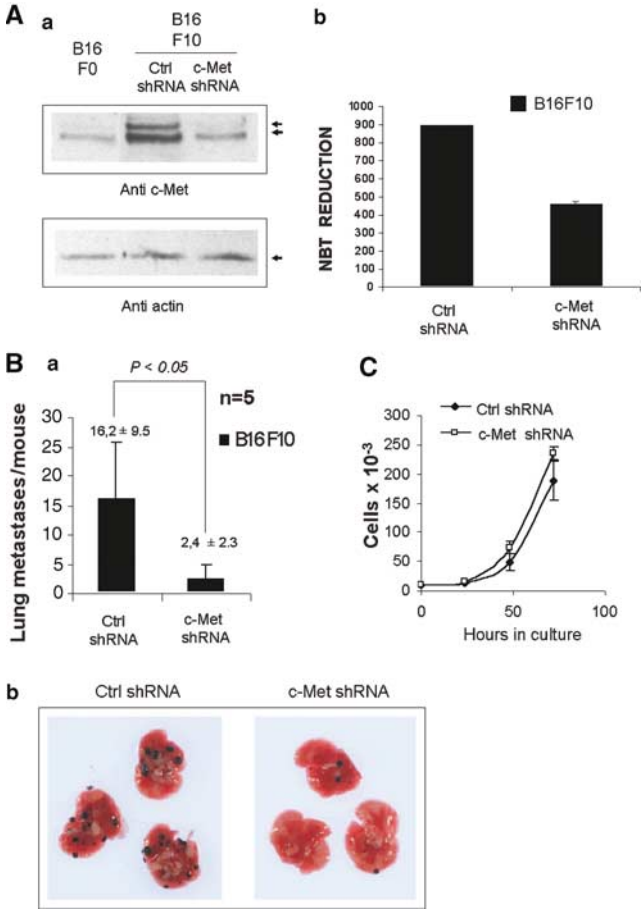

Figure 2 c-Met overexpression is necessary for the metastatic phenotype of B16F10 cells. **A** (**a**) shRNA-mediated downregulation of c-Met in F10 cells. Met expression, although not completely abolished, is significantly reduced to a level which approaches that observed in the F0 population. p145 HGF/SF-R and its precursor p190 (visible only in the middle lane) are marked by arrows. (**b**) Superoxide release by Ctrl- and Met-shRNA F10 cells. Decreased generation of $O_2^{\bullet}$ parallels lower c-Met expression in MetshRNA cells. Values are arbitrary units of absorbance. Data are mean $\pm \ominus$ error spreading of duplicate samples. Figure representative of two independent experiments. **B** (**a**) Impaired metastatic capacity of F10-Met shRNA cells. Groups of five animals each were injected with either Mock- or Met-silenced cells. Lung metastases were scored 3 weeks later. Average number of metastases per mouse ($\pm$s.d.) is indicated on top of the columns. The figure is representative of two independent experiments. *P*-value was determined by a directional Mann–Whitney rank test. (**b**) Examples of lungs from mice injected with either control- or Met-silenced B16 F10 cells. Very few large metastases are generated by Met-shRNA cells, likely due to the emergence of clones escaping oncogene downregulation. (**C**) Downregulation of c-Met does not affect exponential growth of B16F10 cells *in vitro*. Met- and Ctrl-shRNA cells were seeded at identical density (1000/cm^2) on plastic (12-well plates) and counted every 24 h. Note that the curves are superimposible. Values are mean $\pm$ error spreading of duplicate wells.

These findings therefore indicate that elevated expression of c-Met is necessary to maintain both the prooxidant activity and the metastatic phenotype of B16F10 cells, whereas it is dispensable for cell proliferation *in vitro*.

Activation of the small GTPase Rac-1 by c-Met in B16 melanoma cells

The HGF/SF receptor exerts its biological activities by coupling with a multiplicity of intracellular signaling components (Trusolino and Comoglio, 2002; Birchmeier *et al*., 2003) including members of the Rho-like family of small GTPases. Among these G proteins, Rac-1 is part of the NADPH oxidase complex and is involved in the ligand-dependent generation of ROS (Pani *et al*., 2000; Colavitti *et al*., 2002). Prompted by the above evidence linking c-Met expression to the generation of superoxide anion in B16 cells, we evaluated the activity of Rac-1 in the same set of cell lines. In F0 and F10 cells, GTP loading of Rac-1 (an indirect parameter for Rac activation) clearly correlated with high c-Met expression and with the cell metastatic capacity (Figure 3A, a), being significantly higher in the F10 clone. Accumulation of Rac-GTP in F10 cells is not due to increased expression of the GTPase (Figure 3A, a, lower panel), but rather reflects functional activation by one or more upstream signals. In both F0 and F10 populations, Rac GTP loading was further increased upon cell exposure to HGF, confirming the capacity of c-Met to activate this transducer (Figure 3A, b). Moreover, in F10 and F0 cells engineered to express lower or higher amounts of c-Met, respectively, Rac activity mirrored the expression level of the HGF receptor (Figure 3B and C). Taken together, these observations indicate that Rac-1 is a downstream target of c-Met in the B16 cell model, and by extension that the activity of this GTPase may have a role in determining the metastatic capacity of B16 cells.

Rac-1 couples c-Met to a redox-signaling cascade

The concomitance of c-Met overexpression, elevated activity of Rac-1 and increased release of superoxide in B16 F10 cells suggests that these three components may lie along the same oncogenic cascade. In order to further evaluate whether a redox-signaling circuitry operates downstream of the activated HGF/SF receptor, B16 cells were loaded with a cell-permeant fluorescent probe (dichlorofluorescein-diacetate (DCF-DA)) capable of detecting the intracellular release of peroxides (Pani *et al*., 2000), and left untreated or stimulated with an exogenous hepatocyte growth factor. Cell fluorescence was then determined by flow cytometry. As indicated in Figure 4a, intracellular peroxides, in keeping with initial observations on the release of superoxide anion, are higher in F10 than in F0 cells. Unfortunately, a similar comparison could not be made between ctrl-shRNA and Met-shRNA F10 cells, because of the constitutive, bright green fluorescence of these GFP-transduced cells.

Consistent with many reports on a variety growth-factor responsive cell systems (see Introduction), exposure of B16 cells to HGF elicits the ligand-dependent generation of intracellular peroxides, more evident in the F0 population (Figure 4a), in a fashion that is clearly inhibited by overexpression of a Rac-1 dominant-negative mutant (RacV12N17, Figure 4b and relative insert).

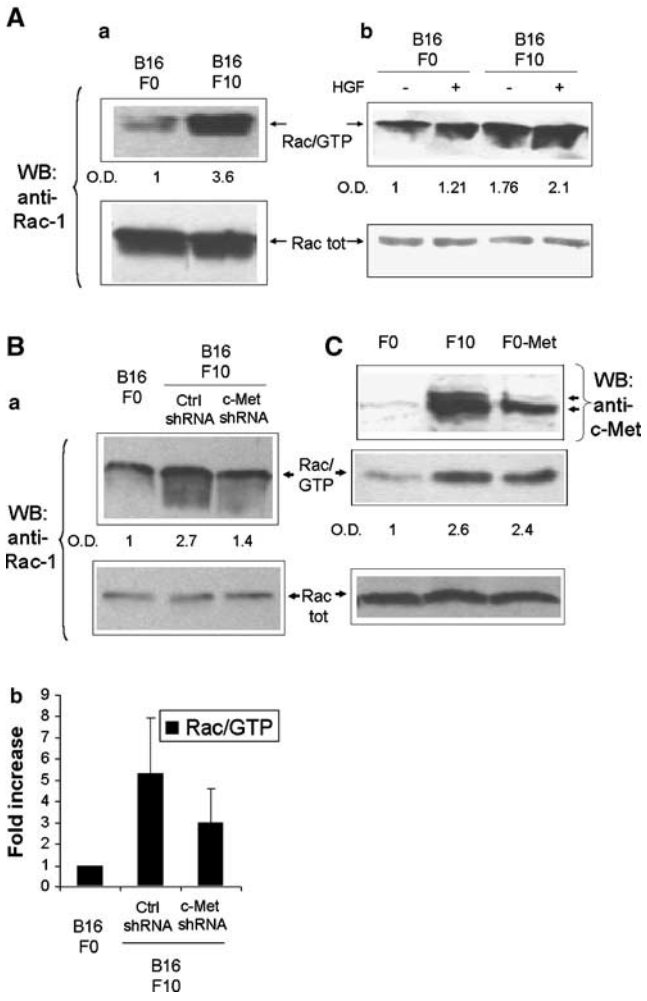

Figure 3 Activation of the small GTPase Rac-1 by c-Met in B16 melanoma cells. **A** (**a**) Elevated activity (GTP loading) of Rac-1 in F10 cells. Rac-1 GTP loading in F0 and F10 protein lysates was determined as described (Sander *et al*., 1998). Differences in Rac activity in the two cell populations closely mirror the expression level of c-Met and the amount of released superoxide. (**b**) Activation of Rac-1 by HGF in F0 and F10 cells. Small but reproducible increases in Rac GTP loading were observed in both F0 and F10 cells exposed for 15 min to 50 ng/ml HGF. Numbers under the PAK pull-down panels are optical densities (o.d.) referred to the rac/GTP bands. Bands were quantified by a Biorad Gel.Doc image analyzer, using the Quantity.One software. The amount of total Rac-1 is equal in F0 and F10 cells, and is not affected by HGF (**a** and **b**, lower panels). (**B** and **C**) Manipulation of c-Met expression in F10 cells leads to a parallel modification in the basal activity of Rac-1. Rac GTP loading appears significantly decreased in Met-silenced F10 cells (**B** (**a** and **b**)) and increased in F0 cells engineered to overexpress the HGF-R (**C**) Neither manipulation affects the amount of total Rac-1 in the input. Band intensity values for Rac/GTP are indicated. In **B** (**b**), histogram columns are mean ± error spreading of two independent experiments, and indicate GTP-Rac band intensity relative to F0 cells. Relevant protein bands are indicated by arrows throughout the figure. Two protein species, p145 and its precursor p190, are recognized by the anti-c-Met antiserum.

Thus, although with some limitations, these findings clearly imply that the intracellular content of peroxides (most likely H_2O_2) in our cell model mirrors the expression/activity of c-Met, and is directly tuneable by HGF in a Rac-1-dependent manner.

To begin elucidating the signaling potential of c-Met-dependent oxidant species, we first determined whether differences observed with the fluorescent probe corresponded to a different oxidation status of intracellular protein targets. To this end, protein lysates of untreated or HGF-stimulated F0 and F10 cells were immunoblotted with an antibody capable of detecting the formation of protein-glutathione adducts, a reliable marker of oxidative stress (Caplan *et al*., 2004). As indicated in the panel of Figure 4c, cell content of glutathiolated proteins parallels the fluorimetric detection of ROS, being constitutively higher in c-Met overexpressing cells and further inducible by exogenous HGF. Cell exposure to the strong pro-oxidant diamide was used as a positive control for inducible protein glutathiolation.

Optimal activation of ERKs in response to many growth factors, including PDGF (Sundaresan *et al*., 1995) and VEGF (Colavitti *et al*., 2002), requires the transient generation of ROS. To further evaluate the involvement of oxygen species in signal transduction by HGF/SF, we assessed the phosphorylation of ERKs in response to the factor, in antioxidant conditions. As shown in Figure 4d, c-Met coupling to ERKs was significantly blunted by removal of oxygen species (compare lane 4 with lanes 5 and 6), obtained by pre-incubating cells (B16 F0) with two different, highly effective superoxide/peroxide scavengers (Rong *et al*., 1999). This finding suggests that ERK activation by c-Met requires ROS, at least in the present experimental system. Importantly, antioxidants neither compromised cell viability (not shown) nor affected the intracellular content of total ERKs (Figure 4d, lower panel).

As ERK activation by HGF is necessary for anchorage-independent cell growth (Zeng *et al*., 2002), a hallmark of malignancy, we tested whether colony formation by F10 cells suspended in soft agar was also impaired by antioxidants. As indicated in Figure 4e, cell proliferation in agar was drastically reduced by both antioxidant compounds, already in the low micromolar range (20 μM). Although an effect of EUKs also on mitogenic signals independent of hepatocyte growth factor receptor (HGF-R) cannot be excluded in this experiment, the result, together with the phosphorylation studies depicted in Figure 4d, supports the role of ROS as critical intermediates in the pro-malignant cascade triggered by c-Met.

Involvement of the Rac-ROS cascade in pro-metastatic signaling by c-Met

Since overexpression of c-Met is essential for the lung-specific metastatic phenotype of B16F10 cells (Figure 2B), this cell line can be exploited as a model for Met-dependent metastatic growth *in vivo*. Activation of Rac-1 and generation of ROS by Met in B16 cells suggest that oxidative signals may have a role in cell metastatic switch, a hypothesis that calls for experimental verification.

In order to directly address the involvement of the Rac-ROS pathway in the metastatic behavior of B16F10 cells, a number of genetic manipulations were brought about with the scope of either disrupting or amplifying the above redox-signaling cascade.

In a current model (Figure 5B), the weak oxidant superoxide, generated by Rac-dependent oxidases in response to growth factor stimulation, is efficiently converted into the stronger oxidant and signaling molecule H_2O_2, which in turn targets relevant intracellular transducers such as phosphatases and transcription factors (Lambeth, 2004).

We blocked the Rac-ROS pathway either at an upstream level, by means of the Rac dominant-negative mutant RacV12N17 (Colavitti *et al*., 2002), or by directly targeting H_2O_2 via overexpression of its specific scavenger enzyme Catalase (Figure 5A). In both cases, cell manipulation lead to a significant and comparable decrease in cell metastatic capacity. Notably, a similar effect of Rac inhibition on experimental metastasis had already been reported in a similar model utilizing breast cancer cells (Bouzahzah *et al*., 2001).

In a complementary approach, we overexpressed the copper/zinc-dependent superoxide dismutase, an H_2O_2-generating enzyme, with the aim of elevating the intracellular content of peroxide. As indicated in Figure 5A, a, this treatment substantially increased metastasis formation by B16F10 cells, in comparison to the mock-transfected control. Importantly, overexpression of SOD1 was nearly ineffective in F0 cells (not shown) but, somehow surprisingly, restored the metastatic capacity of Met-silenced F10 cells (Figure 5A, b). These findings, while underscore the potential pro-metastatic action of H_2O_2, suggest that peroxide may be not only necessary, but, at least in part, sufficient, if in the right cell context, to signal metastatic growth downstream of c-Met.

Discussion

The main findings of the present work can be summarized as follows: (a) Overexpression of c-Met plays a causative role in the metastatic behavior of a lung-specific clone derived from the B16 mouse melanoma. (b) In this cell type, c-Met activates a Rac-1-dependent oxidative cascade that contributes to intracellular signaling by HGF. (c) Manipulation of the redox-signaling cascade downstream of c-Met modulates metastatic growth of B16F10 cells, underscoring the relevance of this signaling branch and opening the possibility of this pathway being exploited as a target for new antimetastatic therapies.

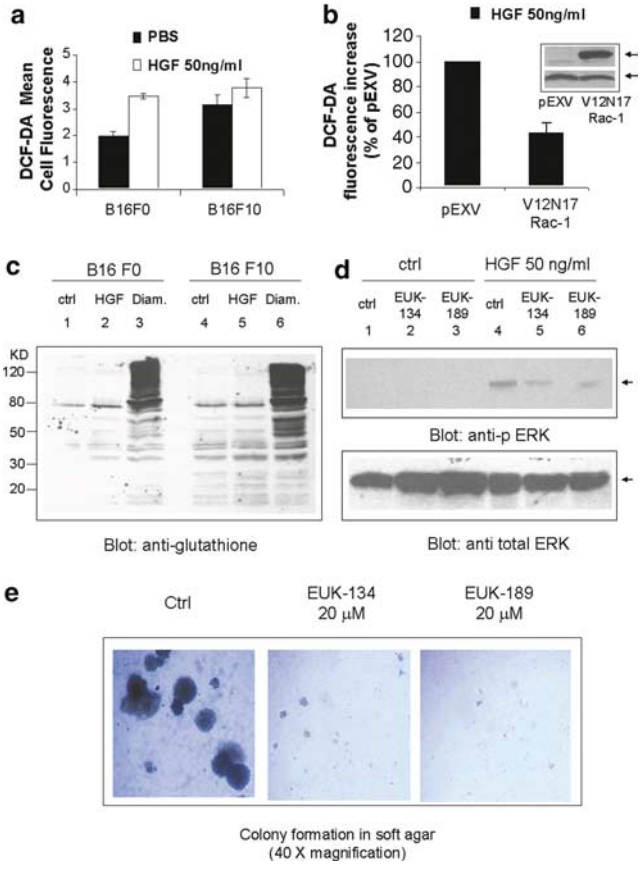

Figure 4 Rac-1 couples c-Met to a redox-signaling cascade. (**a** and **b**) Rac-dependent generation of intracellular peroxide in B16 cells stimulated by HGF. (**a**) B16 F0 and B16 F10 cells were treated with 50 ng/ml HGF/SF in serum-free HBSS for 15 min, or left untreated. During the last 5 min of stimulation, DCF-DA was added at 20 μg/ml. After one wash in PBS cells were tripsynized and immediately subjected to flow cytometry. F10 cells show a poorer response to the stimulus, likely due to a constitutive activation of the Rac-ROS cascade. (**b**) F0 cells were transiently transfected with a dominant-negative mutant of Rac-1 (V12N17Rac) or the corresponding empty vector (pEXV), selected for 48 h in puromycin and further processed as in (**a**). Inhibition of Rac 1 attenuates oxidative response to HGF/SF. Values are expressed in percentage of the fluorescence increase (HGF stimulated–unstimulated) registered in control (pEXV) cells. Numbers are mean ± error spreading of duplicate samples. Figure representative of at least two independent experiments. (**c** and **d**) Oxidant-dependent signaling downstream of c-Met. (**c**) Accumulation of glutathiolated proteins in B16 cells exposed to HGF. Total protein lysates from cells treated as indicated were resolved on SDS–PAGE and immunoblotted with a glutathione-specific monoclonal antibody (Virogen). Note that changes in protein glutathiolation in F0 and F10 cells closely mirror the intracellular content of intracellular peroxide, shown in panel a. (**d**) Optimal phosphorylation of ERKs in response to HGF requires ROS. B16 F0 cells were pretreated for 30 min with 100 μM of the synthetic antioxidants EUK-134 and EUK-189, or their vehicle (PBS) alone, before stimulation with HGF/SF. ERK activation was monitored by anti-phospho-ERK immunoblotting on the total protein lysates. The same nitrocellulose filter was subsequently reprobed with an anti-ERK antiserum, as a loading control. Antioxidants used here were not toxic, at least in the timeframe of the experiment. The phospho-ERK and total ERK bands are indicated by arrows. (**e**) Micromolar concentration of catalytic antioxidants drastically decrease anchorage-independent growth of F10 cells. Cells were seeded at low density (5000/10 cm^2) in complete medium containing 0.3% agarose, in the presence of 20 μM of either antioxidant, or without addictions (Ctrl). Colonies were photographed 2 weeks later under the phase-contrast microscope. For graphical reasons, HGF/SF was indicated simply as *HGF* throughout the figure.

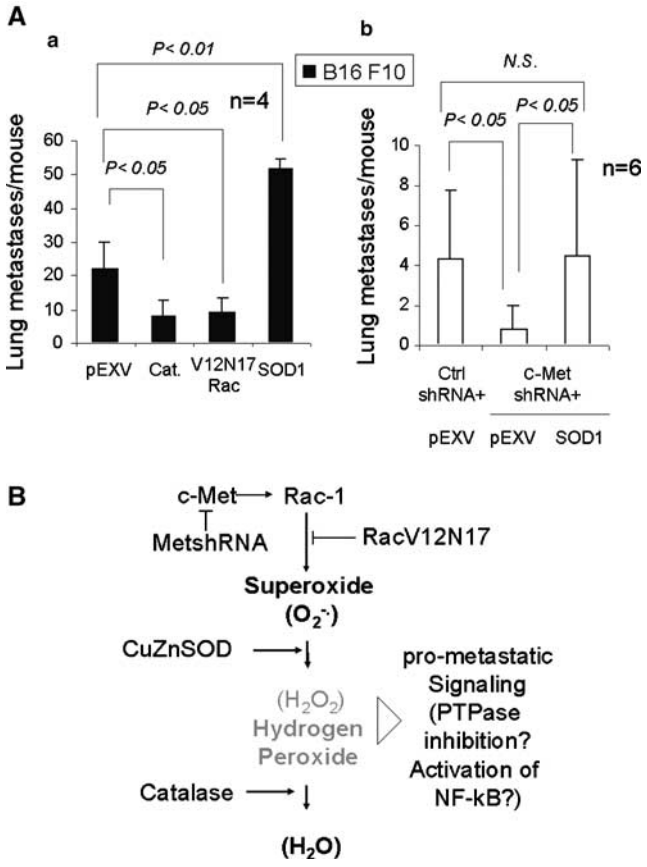

Figure 5 Involvement of the Rac-ROS cascade in pro-metastatic signaling by c-Met. (**A**) Genetic manipulation of the Rac-ROS pathway downstream of c-Met affects cell metastatic growth. (**a**) B16F10 cells were transfected as desribed in Materials and methods with the indicated constructs, selected in puromycin and subjected to the experimental metastasis assay. Lung metastases were scored 3 weeks later. A significant reduction in the average number of metastases per mouse resulted either by inhibition of Rac activity (V12N17Rac) or by increased peroxide scavenging (Catalase), whereas overexpression of copper/zinc SOD markedly enhanced cell metastatic capacity. Data are mean $\pm$ s.d. of lung metastases in individual mice ($n=4$/group). *P*-values were determined by subjecting the indicated paired populations to a directional Mann–Whitney rank test. Figure is representative of two independent experiments. (**b**) Effect of SOD1 overexpression on metastatic capacity of Met-silenced cells. Ctr-shRNA and Met-shRNA F10 cells were transiently transfected with the pEXV-SOD1 or the corresponding empty vector, and injected in mice as in (**a**). After 2 weeks, superficial lung metastases >2 mm were counted under the stereomicroscope. Regain of metastatic capacity by Metshrna cells overexpressing SOD1 was significant ($P<0.05$) by the directional Mann–Whitney rank test ($n=6$). The yeild of lung metastases was overall low in this particular experiment. (**B**) Flow-chart depicting a model for redox-signaling downstream of c-Met, and its impact on metastatic growth in light of the current knowledge and the results presented above. Experimental evidence points to a major role for hydrogen peroxide as a key pro-metastatic signaling molecule, likely through oxidative modifications of phosphatases, such as LMW-PTP, and transcription factors. Implications of this models are discussed in the text.

Deregulated activity of the HGF/SF receptors plays a pivotal role in the invasive and metastatic conversion of malignant cells, in both human and experimental settings. Here we provide evidence that overexpression of c-Met is necessary for the metastatic growth of a lung-specific variant of the B16 mouse melanoma. Involvement of c-Met in this popular model of experimental metastasis had not been investigated so far, with the exception of one study reporting c-Met overexpression in sublines selected for liver-specific metastatic capacity (Rusciano *et al*., 1995). Although elevated expression of c-Met is required for B16F10 cells to colonize the lung, we have at the moment no evidence that this molecular event is sufficient to drive B16 metastatic growth. Preliminary experiments with F0 cells forced to overexpress c-Met (Figure 3C) failed to demonstrate increased metastatic capacity of this population to form lung metastases (not shown). One possible interpretation of this finding is that additional signaling changes are required for a fully metastatic phenotype in our model. Although we have excluded that this 'second signal' resides in autocrine secretion of HGF (data not shown), it is possible that the metastatic growth capacity, but not the homing specificity of F10 cells, relies on Met signaling. This possibility goes along with previous reports on the expression of lung-specific adhesion molecules on the surface of this cell subline (Zhu *et al*., 1991). In support of the latter hypothesis is also the finding that metastatic capacity can be restored by overexpression of SOD1 only in Met-silenced F10, but not in non-metastatic F0 cells (Figure 5A, b and data not shown). In this scenario, Met could be required for promoting tissue invasion and/or ectopic growth once cells have reached and bound the endothelium of the lung.

Although these points certainly need to be clarified, data presented strongly indicate that experimental lung metastases formation by B16F10 cells is overall a c-Met-dependent process, and suggest, by extension, that this widely used model can be utilized for mechanistic studies on pro-metastatic signaling by the HGF/SF receptor.

Although activation of the small GTPase Rac-1 has already been reported as part of the motogenic program triggered by c-Met (Royal *et al*., 2000), the possibility that the HGF/SF receptor couples to a Rac-dependent oxidative cascade had not been investigated so far. Rac-dependent redox signals have an established role not only in cell proliferation and survival (Joneson and Bar-Sagi, 1998; Bedogni *et al*., 2003), but also in adhesion, detachment and shape changes, secretion of metalloproteases and epithelial–mesenchimal transition (Kheradmand *et al*., 1998; Werner and Werb, 2002; Chiarugi *et al*., 2003; Radisky *et al*., 2005), all responses integral to the invasive and metastatic behavior of malignant cells; activation of the Rac-ROS cascade by c-Met therefore appears to be biologically plausible. Interestingly, a redox-sensitive component has been recently identified in the signaling pathway triggered by plexins (Ventura and Pelicci, 2002), a family of molecules known to associate and cooperate with c-Met during invasive growth (Trusolino and Comoglio, 2002). This component, named MICAL (for Molecule Interacting with CAS-Ligand), is necessary for semaphorin-induced neurite collapse in primary neurons, and harbors a

FAD-binding domain suggestive of a potential involvement in electron exchange reactions (Terman *et al*., 2002). This evidence further supports the notion that ROS and redox changes may have an important role in cell repulsion and scattering triggered by c-Met and its associated coreceptors. In this perspective, the possibility that generation of ROS triggered by HGF is mediated by Met-associated plexins and/or integrins is currently under investigation.

In line with the idea that oxidants participate in intracellular signaling by c-Met are also the inhibitory effects of antioxidant compounds on HGF-induced phosphorylation of ERK 1/2 and on anchorage-independent cell growth, as presented in Figure 4d and e. Although not directly addressed in this paper, it is conceivable that ROS signal to ERKs is mediated by the oxidative inhibition of one or more protein tyrosine phosphatases. In fact, several PTPs have been previously reported to associate with the HGF/SF receptors (Villa-Moruzzi *et al*., 1998; Maroun *et al*., 2000; Palka *et al*., 2003) and to modulate its downstream signaling pathways. Moreover, preliminary evidence suggests physical association between c-Met and the low molecular weight protein tyrosine phoshatase (LMW-PTP; data not shown), an enzyme exquisitely sensitive to redox modulation by growth factors and integrins (Chiarugi *et al*., 2003; Nimnual *et al*., 2003), and able to restrain ligand-dependent activation of MAP kinases (Chiarugi *et al*., 2003).

In a simple model, inhibition of Met-specific PTPs by ROS generated in response to receptor activation may create a feed-forward loop that amplifies downstream oncogenic signaling. Along similar lines, PTP inactivation may mediate ligand-dependent transactivation of HGF-R by H_2O_2, as it was recently reported in tumor cells stimulated with EGF (Fischer *et al*., 2004). These hypotheses still wait for experimental verification.

Experiments shown in Figure 5 clearly indicate that the integrity Rac-ROS signaling pathway is crucial in a cell model of lung-specific, Met-driven metastatic growth. Although the finding that Rac-1 blockade inhibits experimental metastasis is not completely surprising (Bouzahzah *et al*., 2001), data on metastasis modulation by peroxide-producing (CuZnSOD) or -scavenging (Catalase) enzymes add novel information to the picture, directly implicating ROS and redox signaling in the metastatic process. In this respect, of particular interest is the evidence, presented in Figure 5A, b, of a regain of metastatic capacity by Met-shrna F10 cells overexpressing CuZn SOD, which suggests that peroxide could be able, at least in some circustances, to recapitulate the all pro-metastatic signal downstream of Met. It should be noted that both Met and intracellular superoxide are reduced but not eliminated in Met-silenced cells (Figure 2A, a and b). In this context, increased conversion to superoxide by SOD overexpression may amplify residual Met signaling up to a critical threshold necessary for metastases to form. Alternatively, SOD1 may promiscously bring to metastatic potential other superoxide-generating oncogenes different from Met and normally insufficient to drive fully metastatic growth.

We can only speculate on the cellular mechanisms whereby, and the phase(s) of tumor dissemination at which ROSs exerts their pro-metastatic effects. We have recently shown that Trk-A triggered H_2O_2 in PC12 cells prevents apoptosis by inducing activation of the cAMP responsive element binding protein (Bedogni *et al*., 2003). Importantly, the closely related receptor Trk-B has been recently identified in a genomewide phenotypical screen as a metastasis determinant that prevents tumor cell anoikis in the very early phase of metastatic spreading (Douma *et al*., 2004). Thus, ROS generated by c-Met may promote tumor cell survival in the bloodstream.

Alternatively, ROS may simply foster cell proliferation in established metastatic colonies, or contribute to cell adhesion-migration during extravasation. It is interesting to note that the Rac-ROS signaling module has also been implicated in oxygen sensing (Fu *et al*., 2000), and may therefore have a specific role in directing circulating malignant cells towards the lungs along a chemotactic gradient of pO_2.

It is at present difficult to sort among the above possibilities, in view of the fact that our experimental design, in which the number of visible, full-grown metastases is used as a read-out of cell metastatic potential, does not discriminate between differences in local cell growth and changes in cell capacity to reach and invade the target tissue. The fact that experiments shown in Figure 5A are made with transiently transfected populations and not with clones selected for stable protein expression may represent an initial hint of an early role of ROS-dependent signals during the establishment of metastasis. This issue clearly requires further and specific investigation.

Notwithstanding the above limitations, evidence that ROS directly contribute to Met-dependent metastatic growth opens a novel perspective in the complex correlation between oxygen radicals and malignancy, and suggest new possibilities of antioxidant-based therapeutic intervention, complementary to the search for Met-inhibitory compounds (Corso *et al*., 2005).

Materials and methods

Animals

Male C57Bl6/J mice (6–8 weeks old) were obtained from the animal facility of Universita' Cattolica del Sacro Cuore. Animals were maintained in temperature-controlled rooms with a 12 h light/dark cycle. All experimental procedures involving mice complied with the Guidelines of the Italian National Institute of Health, and were approved by the Institutional Ethical Committee.

Cell lines

B16 F0 and B16 F10 mouse melanoma cell lines were obtained from the American Type Culture Collection (ATCC). Cells were routinely propagated in standard DMEM (4.5 g/l glucose) (Eurobio, France) containing 10% fetal calf serum (Sigma).

The B16 F0-Met and B16 F10-Met shRNA cell lines were derived from the parental populations by lentiviral cell transduction. For Met shRNA-mediated silencing, lentiviral particles were produced in 293 packaging cells transfected with packaging plamids together with the pCCLsin. PPT.hPGK.GFP.Wpre lentiviral vector, carrying, under the H1 promoter, the required sequence for synthesizing Met-targeted shRNAs (5′-GTCATAGGAAGAGGGCATT-3′) or a control-scrambled shRNA (5′-AGTCTACATGCTCA CACTT3′).

Antibodies, reagents and plasmids

The following antibodies were used in the present study: anti-c-Met (rabbit polyclonal), antiphosphotyrosine (mouse monoclonal 4G10), and anti-SOD2 (rabbit polyclonal) from Upstate Biotechnology; anti-c-Met (mouse monoclonal), antiphospho-ERK (rabbit polyclonal), antitotal ERK (rabbit polyclonal), and anti-Rac-1 (mouse monoclonal) from Cell Signaling Technology; antiactin (goat polyclonal) and anti-*myc*-tag (mouse monoclonal 9E10) from Santa Cruz Biotechnology; anti-SOD1 (goat polyclonal) from Calbiochem; antiglutathione (mouse monoclonal) from Virogen; horseradish peroxidase (HRP)-conjugated goat anti-mouse antiserum from Amersham Pharmacia; HRP-conjugated goat anti-rabbit antiserum from Biorad.

Chemicals and reagents were purchased from Sigma if not differently stated. The peroxide-sensitive fluorescent probe DCF-DA was from Molecular Probes. Glutathione-Sepharose 4B matrix for the purification of the GST-CRIB fusion protein was from Amersham Pharmacia.

The superoxide/peroxide catalytic scavengers EUK-134 and EUK-189 were kindly provided by Dr George Tocco (Eukarion Inc., MD, USA).

PEXV-based expression constructs for V12 (constitutively active) and V12N17 (dominant negative) Rac-1 were kindly provided by Dr A Hall (UCL London, UK); pLNX-Catalase was a gift from Dr Toren Finkel (National Institute of Health, Bethesda, USA). CuZnSOD-Neo was created by cloning a full-length human SOD1 cDNA (ATCC) in the *Eco*RI site of the PcDNA3 expression vector (Invitrogen).

The *Escherichia coli* BL21 bacterial strain transformed with the IPTG-inducible GST-PAK-CD (PAK-CRIB domain) fusion protein was a generous gift from Dr J Collard (Amsterdam, the Netherlands).

Proteomics

Protein identification by tandem mass spectrometry was performed by ProtTech Inc. In brief, each protein gel band was in-gel digested with trypsin (Promega) and the resulting peptide mixture was subjected to tandem mass spectrometry. A Finnigan ion trap mass spectrometer LCQ coupled with a HPLC running a 75 μM ID C18 column was used. MS/MS spectra were used to search protein databases from GenBank with the ProtTech's ProtQuest software suite. The output from database search was manually analysed and validated by ProtTech scientists.

Cell transfection

B16 cells (150 000 in six-well plate) were cotransfected with 0.8 μg of each expression construct and 0.2 μg of pBabe/puro, using the EFFECTENE reagent (Quiagen) according to the manufacturer's recommendations. Transfection efficiency was routinely of 75–85% based on cell fluorescence following transfection of a CMV-EGFP expression construct (PROMEGA). This percentage was further increased by a 48 h selection in 2.5 μg/ml puromycin.

Colony formation assay in soft agar. Cells were resuspended at low density (5000 cells/ml/10 cm^2) in complete medium containing 0.3% agarose and seeded in six-well plate on top of a solid layer of complete medium/0.6% agarose. Antioxidants (EUK-134 and EUK-189), where necessary, were present from the beginning of the assay in both the lower and the upper agarose layer. Colony formation was assessed and colonies photographed 2 weeks later under the phase-contrast microscope.

Experimental metastasis assay

Mice (6–8 weeks old) were injected in the tail vein with 150 000 B16 cells in 200 μl of saline. Mice were killed 2–3 weeks later, dissected and carefully inspected for the presence of black metastatic foci throughout the body. Lungs were removed and macroscopic colonies (>2 mm) counted under the stereomicroscope by two of the authors.

Measurement of ROS

Release of superoxide anion by adherent cells was quantified by a standard nitro-blue tetrazolium (NBT) reduction test, as described (Chiarugi *et al*., 2003). Briefly, cells (100 000/cm^2) were incubated in Hank's balanced salt solution (HBSS) containing 2 mg/ml NBT (Sigma). After 2 h at 37°C, supernatants were removed and cells lysed in isopropanol/0.04 M HCl. Following low-speed centrifugation, pellets were dissolved in pyridine at 100°C, and the absorbance at 570 nm quantified by an ELISA plate reader (Biorad).

Intracellular peroxides were assessed by loading cells with DCF-DA (20 μg/ml for 10 min) in HBSS (Pani *et al*., 2000). Mean green cell fluorescence (FL-1) was then determined by flow cytometry, using a COULTER flow cytometry equipped with a 488 nm Argon laser lamp.

Rac-1 activity assay

Rac GTP loading was evaluated according to Sander (Sander *et al*., 1998). Briefly, GTP-loaded Rac-1 was pulled down from total protein lysates using a Sepharose-bound GST-PAK-CRIB fusion protein. Washed precipitates were boiled, subjected to SDS–PAGE, transferred onto nitrocellulose and quantified by anti-Rac-1 immunoblotting.

Quantification of HGF in cell supernatants

HGF secretion by B16 (F0 and F10) cells was evaluated on serum-free cell supernatants, 48 h after medium replacement, using the AN'ALYZA Immunoassay kit (TECHNE) according to the manufacturer's recommendations. Fresh serum-free medium and medium conditioned by the HGF-producing MRC-5 cell line (human embryonic fibroblasts) were included in the analysis as negative and positive controls, respectively.

Miscellaneous

Cell lysis, immunopecipitations and Western blotting were performed according to standard procedures (Pani *et al*., 2000). For the evaluation of protein-glutathione adducts, cells were lysed in the presence of the thiol blocking reagent *N*-ethyl maleimide (NEM, 50 mM, Sigma), in order to avoid post-lysis oxidations.

Statistics

Pairs of data sets were compared by directional Mann–Whitney rank test, where indicated. This non-parametric alternative to Student's *t*-test was preferred in view of the high variability and the non-normal distribution of values

within samples. The threshold of significance was set at a value of $P<0.05$. Calculations were performed with the web-based VassarStats Software (http://faculty.vassar.edu/lowry/webtext.html).

Abbreviations

HGF-R, hepatocyte growth factor receptor; ROS, reactive oxygen species.

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