Epigenetic regulation of HGF/Met receptor axis is critical for the outgrowth of bone metastasis from breast carcinoma

Paola Bendinelli1, Paola Maroni2, Emanuela Matteucci1 and Maria Alfonsina Desiderio*1

Our translational research deals with the influence of microenvironment on the phenotype and colonization of bone metastases from breast carcinoma, and on pre-metastatic niche formation. The aim of the present study was to clarify the origin of hepatocyte growth factor (HGF), ligand of Met receptor, the control of the axis HGF/Met by DNA methylation, and its importance for the nexus supportive cells-metastatic cells and for metastasis outgrowth. In bone metastasis of the 1833-xenograft model, DNA methyltransferase blockade using the chemotherapeutic drug 5-aza-2′-deoxycytidine (decitabine) strongly reduced the expression of HGF/Met receptor axis and of E-cadherin, with decrease of metastasis wideness and osteolysis, prolonging mice survival. Thus, DNA methylation events acted as commanders of breast carcinoma cells metastatizing to bone, influencing the epithelial phenotype. HGF emerged as a bone-marrow stimulus, and the exosomes seemed to further HGF to metastatic cells. In fact, decitabine treatment similarly affected some markers of these microvesicles and HGF, indicating that its supply to recipient cells was prevented. Notably, in bone metastasis the hypomethylation of HGF, Met and E-cadherin promoters did not appear responsible for their elevated expression, but we suggest the involvement of hypermethylated regulators and of Wwox oncosuppressor, the latter being affected by decitabine. Wwox expression increased under decitabine strongly localizing in nuclei of bone metastases. We hypothesize a role of Wwox in Met activity since in vitro Wwox overexpression down-regulated the level of nuclear-Met protein fragment and Met stability, also under long exposure of 1833 cells to decitabine. Enhanced phosphoMet and the activity in nuclei, an effect partially prevented by decitabine. Altogether, the data indicated the importance to target the tumor microenvironment by blocking epigenetic mechanisms, which control critical events for colonization such as HGF/Met axis and Wwox, as therapy of bone metastasis.

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The dynamic plasticity of metastases is largely dependent on epigenetic mechanisms and is responsible for opportunistic adaptations, also in the absence of DNA sequence modifications.1–3 Because of the changes of microenvironmental context, metastases need to adapt to the external cues during the various steps of the process from invasion/ dissemination until outgrowth at the secondary site.4 Dysregulation of DNA methylation at CG-enriched regions (known as CpG islands or CGIs), or global genomic hypomethylation, intervenes in the plastic phenotype for successful metastatic process. Aberrant methylation has paramount consequences on tumor biology and for the activation of alternative transcription start sites and the release of pluripotency loci from DNA methylation repression, or (ii) for the blockade of transcription of methylated regions corresponding to oncosuppressors, in epigenetic silencing programs of miRNAs and of relevant adhesion molecules like E-cadherin.5,6 Methylation of CDH1 gene with transcriptional silencing of E-cadherin, an epithelial-specific biomarker, occurs in primary breast carcinoma but not in bone metastasis.6,7

During tumor progression, the aberrant DNA methylation might drive the phenotype switch from epithelial-mesenchymal transition (EMT) to the reverse process MET.8 EMT promotes invasion of primary tumors like breast carcinoma, and dissemination of metastatic cells, while MET contributes to the establishment of distant skeletal metastases.4,5 However, numerous unresolved problems regarding the molecular mechanisms underlying metastasis phenotype still remain.9 Intermediate phenotypic changes between the two end points of epithelium and mesenchymal occur in human carcinomas, each of which is characterized by different patterns of markers and signaling pathways.5 The growth factor network contributes to a metastable or hybrid phenotype in bone metastasis,4 reinforcing the concept of dynamic epithelial-mesenchymal plasticity (EMP).2 Hepatocyte growth factor (HGF) confers to metastatic cells anoikis resistance and survival together with stem-cell characteristics through Wnt-pathway, typical of EMT phenotype, but E-cadherin-intercellular adhesion dependent on HGF would favor proliferation and drug responsiveness.3,10,11

Bone marrow is hospitable for breast carcinoma metastases,12 and the microenvironment stimuli may determine the transcription factors and the signaling pathways activated for the EMT-MET switch in metastatic cells.4,13,14
HGF triggers a Twist 1 program in 1833-bone metastatic clone, but not in MDA-MB231-invasive parental breast carcinoma cells: this HGF-dependent epithelial phenotype is transient, because of the secondary activation of TGFβ1-signaling pathway related to ECM and osteolysis. HGF downregulates the nuclear co-factor Wwox leading to phosphoTwist 1 access to nuclei, and the activation of Twist-transcription factor: when Wwox accedes into nuclei, however, unphosphorylated Twist is extruded ending its function.

The so-called pre-metastatic niche can be formed by non-tumorigenic host cells through the secretion of soluble cytokines and growth factors, and by exosomes, but the transfer of HGF from microenvironment-supportive cells to bone metastases is practically unknown. Notably, these small extracellular vesicles of endocytic origin not only transfer information from neoplastic cells to supportive cells, but also they are released by a variety of cell types into the extracellular space or mediate direct cell-cell contacts. The primary carcinoma may instruct the secondary site through exosomes, as in the case of melanoma metastasis educating bone-marrow-derived cells. Exosomes from heparanase rich cells contain HGF, which might influence the aggressive phenotype. Rab GTPases such as Rab5b of early and recycling endosomes are commonly seen in exosomes. Some of the exosomal membrane proteins like LAMP2 are not identified at the surface of the originating cell, consistent with cell-to-cell signaling events mediated by juxtacrine and ectodomain cleavage.

Studies are necessary to evaluate whether breast carcinoma cells methylation-dependent events act as commanders of bone organotropism and whether microenvironment stimuli influence DNA methylation. There is compelling evidence for the deterministic and not stochastic control of gene expression via epigenetic mechanisms, with a balance between pathways intrinsic to metastatic cells and extrinsic cues of the metastasis microenvironment. The high expression of Met receptor in some cancers has been supposed to be consequence of DNA

Figure 1 Effect of dAza on osteolytic metastasis colonization and mice survival. (a) Representative images of three-dimensional reconstruction of μCT, and of bioluminescence. Three xenograft mice per group were analyzed with similar results. The arrows indicate osteolysis. (b) Quantitative data for the bioluminescence signals and the percent of inhibition due to dAza are shown. The data are the means ± S.E. of five mice. ***P < 0.001 versus ME. (c) Survival curve on Kaplan-Meier plots of the data from ME (n = 8) and ME+dAza (n = 12). (d and e) The means of K67 stained cells (percent in five fields) and of multinucleated osteoclasts (TRAP positive in 10 fields) are shown. The assays were done in three xenograft mice. *P < 0.05, ***P < 0.001 versus the ME value at 15 days. ○P < 0.05, ○○P < 0.005 versus the corresponding ME value.
hypomethylation and histone acetylation, but the regulation might be more complex under DNA methyltransferase blockade.25

The aim of the present study in bone metastasis was to clarify the interplay between biological stimuli of the microenvironment and metastasis-methylation state, by examining

Figure 2  dAza affects Met and E-cadherin expression in bone metastasis of the xenograft model. We show representative images of bone metastases from mice treated or not with dAza (xenograft mouse 1 with dAza). Five serial sections were examined for each specimen from three mice at 24 days for ME and at 24–26 days for ME plus dAza obtaining similar results. bm, bone marrow; bo, bone; gp, growth plate; me, metastasis. Scale bar = 120 μm (reported in exemplificative panels × 20 and × 40). Negative controls did not show specific signals
the effect of the blockade of DNA methyltransferases with 5-aza-2'-deoxycytidine (decitabine, dAza) on the survival of xenograft mice, and by considering the patterns of HGF/Met receptor axis and E-cadherin. To this end, the expression of microenvironmental HGF was evaluated together with those of exosomal Rab5b and LAMP2, to test the hypothesis that the

Figure 3  Effect of dAza on mouse and human HGF expression in bone metastasis of the xenograft model. We show representative images of bone metastases from mice treated or not with dAza (xenograft mouse 1 with dAza). Five serial sections were examined for each specimen from three mice at 24 days for ME and at 24–26 days for ME plus dAza obtaining similar results. bm, bone marrow; bo, bone; gp, growth plate; me, metastasis. Scale bar = 120 μm (reported in exemplificative panels × 20 and × 40). Negative controls did not show specific signals.

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functionality of Met receptor in metastatic cells might depend on the ligand of mesenchymal origin. In vitro, we examined the molecular mechanisms underlying the expression and regulation of Met receptor by dAza and HGF, and the role played by Wwox on nuclear Met function. dAza is a chemotherapeutic drug directly incorporated in the newly synthesized DNA strands, that is in phase III trial for myeloid monocyte chronic leukaemia, colangiocarcinoma and colon carcinoma, but never tested to fight bone metastasis of women operated for primary-breast carcinoma.

The implication of HGF in the methylation-dependent colonization of bone metastases from breast cancer might have a remarkable significance for therapies based on the blockade of microenvironmental stimuli.

Results

DNA-methyltransferase blockade impaired bone metastasis outgrowth and prolonged mice survival by affecting HGF/Met axis. For the first time we investigated the methylation-dependent control of the couple HGF-ligand-Met receptor in bone metastases from breast carcinoma. Preclinical studies that test innovative therapies targeting the cross-talk microenvironmental HGF-bone metastasis are scarce, and our xenograft model exploits the 90% homology between human and mouse HGF.

We evaluated dAza effectiveness against osteolytic-bone metastasis (ME) in 1833-xenograft model (Figure 1a). By micro-computed tomography (μCT) analysis, performed on day 22 after cell injection, we observed a large reduction of osteolytic-lesion wideness in the hind limbs under dAza (ME+dAza) in respect to ME. The ME+dAza mice survived without changes of osteolyse extent until 34 days, while ME mice were dead. Bioluminescence imaging was consistent with μCT, and in ME+dAza at 34 days, the bioluminescent signal was still lower than that of ME-22 d.

Under dAza, the bioluminescence value in the hind limbs diminished starting from early times (22 days), persisting inhibited until 22 days from 1833-cell injection (Figure 1b). See Supplementary Figure S1 for hematoxylin and eosin staining, and for the number of mice that survived during the observation period.

Kaplan-Meier analysis showed the prolongation of xenograft-mice survival under dAza (Figure 1c); the maximal-death incidence of ME-mice occurred between 22 and 24 days, while dAza prolonged the survival until 38 days. The expression of Ki67 was examined as index of metastatic colonization. At 24 days, Ki67 was strongly positive in ME decreasing of threefold under dAza; Ki67 persisted low in 33–36 days dAza mice (Figure 1d). The mean value of multinuclear (differentiated) osteoclasts (tartrate-resistant acid phosphatase, TRAP positive) decreased in 22–24 days dAza mice compared with ME-mice, consistent with μCT data (Figure 1e). Supplementary Figure S2 reports that dAza treatment by reducing osteoclast differentiation (TRAP-marker) preserved bone volume.

To determine whether HGF/Met axis underwent DNA-methylation control and played a crucial role in bone metastasis outgrowth, we first studied Met pattern by immunohistochemistry under dAza (Figure 2). In 24 days metastasis-bearing mice, a strong Met signal was observed all over the metastatic cells, including nuclei (see inset). Under dAza treatment of mice, Met signal completely disappeared from bone metastases, which seemed also strongly reduced in wideness (see Supplementary Figure S1).

Since E-cadherin influences the epithelial phenotype under HGF in bone metastasis, we studied the susceptibility of these adhesion molecules to dAza treatment (Figure 2). Metastatic-E-cadherin signal was downregulated by dAza both at plasma membrane/cytosol level and in nuclei, which were often positive for E-cadherin in ME.

In serial sections, we examined the effect of dAza on HGF of bone microenvironment and metastasis (Figure 3). HGF has a mesenchymal origin, while Met is present in parenchymal cells, and bone marrow stromal fibroblasts and megakaryocytes produce HGF. Using anti-mouse HGF antibody, a strong HGF expression signal was observed in the bone marrow, even if the signal was found only in the bone metastatic cells at cytosol and nuclear levels (Figure 3, inset). We cannot exclude that supportive cells might contribute to HGF content of bone microenvironment and metastasis, and dAza almost completely reduced Met and bone-marrow signals. As expected, the signal obtained with anti-human HGF was scarce in bone metastasis, and it was almost unaffected by dAza (Figure 3).

Together, we would like to underline the paramount importance of dAza-dependent reductions of Met signal in metastases, and of HGF of bone marrow origin both in microenvironment and metastases. These findings confirm the interaction between biological stimuli and epigenetic mechanisms like DNA methylation in bone metastasis colonization. Also, our data are in agreement with the presence of a CpG island in the human Met promoter, and of numerous methylation sites in mouse HGF promoter (Supplementary Figure S3). HGF internalization in bone metastasis indicates a cross-talk between microenvironment and metastasis, but the molecular mechanism(s) are unknown. In the following experiments of the present paper we will clarify some aspects of this point.

Regulation of Met expression by dAza and HGF. Based on the xenograft-model findings, we performed in vitro studies with 1833-bone metastatic clone and parental MDA-MB231 cells to clarify how dAza influenced HGF/Met axis in relation to the cell type. MDA-MB231 cells are invasive, slowly metastasizing in various organs with scarce bone tropism. Met 145 kDa (β subunit of the receptor) was differently affected by dAza exposure in the two cell lines (Figure 4a). After 2-day dAza treatment, Met decreased in 1833 cells and increased in MDA-MB231 cells, but Met-phosphorylation at the catalytic site was vice versa. However, Met protein level and phosphorylation increased concomitantly in 1833 cells exposed to dAza-30 days, versus corresponding control c30, so that the pMet/Met ratio remained constant; in MDA-MB231 cells dAza exposure for 1 day as well as for 30 days enhanced pMet/Met ratios.

Then, we examined Met and its phosphorylation in response to HGF under 2-days dAza, that reduced Met in
1833 cells as observed in vivo (Figure 4b). PhosphoMet augmented under the combination HGF plus dAza, compared to the single treatments. Met protein level decreased under HGF and dAza alone in respect to untreated cells (first lane). In MDA-MB231 cells exposed to the combined treatment, dAza partly reverted the huge inhibitory effect of HGF, even if the pMet/Met ratio was still 50% less than in untreated cells.

We studied the molecular mechanisms underlying these changes of Met protein expression in the two cell lines. In MDA-MB231 cells, the steady-state mRNA and protein levels of Met concomitantly increased both under 2-day dAza treatment and in c30 (Figure 4c). In 1833 cells, the Met mRNA levels were consistent with protein levels under 1 and 2 days dAza, but in dAza-30 days we did not find correspondence (Figure 4c). To evaluate whether long-term exposure to dAza affected the Met protein half-life in 1833 cells, we performed experiments in the presence of cycloheximide (CHX). dAza exposure for 30 days strongly stabilized Met protein, in respect to dAza-1 day. The half-life of Met tripled in dAza-30 days versus c30, explaining the twofold increase in Met protein in this condition (Figure 4d).
In 1833 cells, we deepened the knowledge of the effects of the long-term exposure to dAza: Met and HGF immunofluorescence signals augmented mostly in nuclei under dAza-30 days (Figure 5a). Another key molecular characteristic was the HGF pattern: both the precursor and the 69-kDa subunit of HGF were elevated principally under dAza-30 days (Figure 5b). Since in our experimental conditions the HGF mRNA was undetectable by Northern blot and PCR assays, we hypothesize the provenience of HGF from the culture medium, prepared with 10% FBS, that is known to contain growth factors. This seems in agreement with exogenous HGF origin shown in Figure 3.

In conclusion, both Met and HGF expression were highly sensible to DNA methyltransferase blockade, depending on the time of exposure and the microenvironment conditions. The reduction of HGF in the microenvironment

![Figure 5](image)

**Figure 5** dAza effects on cellular Met distribution and HGF protein levels. (a) the dAza treated and control 1833 cells on coverslips were probed with anti-Met (green) or anti-HGF (red) antibody. The nuclei were stained with DAPI (blue). The images, taken at ×400 magnification, are representative of experiments performed in triplicate. Scale bar = 120 μm. (b) Representative images of Western blots repeated three times are shown; vinculin was used for normalization. The numbers at the bottom indicate the fold variations versus the first lane, considered as 1.
might favor the Met-dependent downregulation by dAza. Therefore, the HGF/Met axis can be suggested as target of dAza to prevent metastasis colonization, as observed in the xenograft model.

Met activity in nuclei under HGF, and Wwox regulatory role. Because of the presence of Met in nuclei, that underwent HGF regulation in xenograft-mice metastases and in 1833-metastatic cells, we evaluated some molecular mechanisms responsible for Met nuclear translocation and activity, and for the downregulation of Met in vivo under dAza (Figure 6 and 7). In nuclei of 1833 cells Met is a 60 kDa fragment;10 exogenous Wwox in tumors is known to suppress the activity of tyrosine-kinase transcription factors by cytosol sequestering,35,36 but the role of Wwox in bone metastasis has never been investigated.

As shown in Figure 6a, dAza-30 days reduced Wwox protein level. The Met access into nuclei in a phosphorylated form was favored by short-term and long-term dAza exposure, compared to the respective controls (Figure 6b). This was in agreement with the immunofluorescence findings for Met in dAza30 versus c30 (Figure 5a). Also, Figure 6b shows that exogenous Wwox strongly interfered with the nuclear shuttle of Met in the presence of 2 days or 30 days dAza as well as under basal conditions. WWOX expression vector (e.v.) tripled Wwox-protein level in cytosol and nuclei, increasing also its phosphorylation (Figure 6c). Thus, ectopic Wwox was able to interfere with dAza effect.

To clarify how WWOX e.v. interfered with Met protein enhancement under dAza-30 days, we performed experiments with CHX (Figure 6d). dAza-30 days stabilized Met protein showing a half-life of 6 h versus 2 h in c30; the blockade of new protein synthesis in cells transfected with WWOX e.v. in the presence of dAza (CHX) further reduced proportionally Met protein half-life.

Importantly, we report the immunohistochemistry assay of Wwox (Figure 7a). Enhancement of Wwox expression (+++) was observed under dAza throughout the metastatic cells including the nuclei; Wwox signal was practically absent in nuclei of ME, the latter presenting Wwox signal in the cytosol (++).

Altogether, dAza data indicated an important role of Wwox access into nuclei of metastatic cells, which might reduce the level of Met also by destabilizing the protein. The scheme summarizes the changes of molecular players under long-term exposure to decitabine in bone metastases (Figure 7b).
Effect of HGF on nuclear Met, and microenvironmental origin of HGF in bone metastasis.

We further clarified the regulation of Met function in nuclei. Exogenous HGF increased Met-GFP access to nuclei only in 1833 bone-metastatic cells (see merge) (Figure 8a); starved 1833 cells appeared almost round-cuboid (epithelial phenotype) and MDA-MB231 triangular (mesenchymal phenotype), consistent with previous data.4 Noteworthy, the transactivating activity of Met increased in response to HGF in 1833 clone, but not in MDA-MB231 cells (Figure 8b), and dAza partly prevented this stimulatory effect (Supplementary Figure S4).

In 1833 cells, HGF increased the protein level and phosphorylation state of endogenous-nuclear Met fragment, and Met phosphorylation persisted elevated for all the observation period (Figure 8c). The Met fragment was present also in nuclei of MDA-MB231 cells, but it was less phosphorylated than in 1833 cells, undergoing downregulation under HGF treatment (Figure 8c). The latter finding is consistent with confocal analysis of MDA-MB231 cells showing Met in nuclei.35

The results regarding the effect of exogenous HGF on Met in 1833 cells, in particular the HGF-dependent regulation of COOH-Met levels and activity, and the intracellular increase of HGF under 30-days dAza, led us to clarify the microenvironmental origin of HGF (Figure 8d). Similar to HGF, the two exosome markers LAMP2 and Rab5b were present at high levels in bone marrow and metastatic cells. Under dAza, not only HGF signal practically disappeared from the two metastatic compartments, but also LAMP2 signal strongly diminished. A relevant reduction of Rab5b expression was shown in metastatic and supportive cells.

Our data indicated that DNA methylation state affected HGF content of bone metastases, consistent with LAMP2 and Rab5b expression, leading to suppose that exosomes might
 originates from supportive cells permitting HGF access to recipient cells in the metastatic bulk with redistribution in various compartments.

Discussion

Host cells through the release of biological stimuli like HGF seem to support metastasis colonization, as testified by
DNA-methyltransferase blockade with decitabine (dAza) which hampered the HGF (mouse)/Met (human) axis. Notably, the microenvironmental HGF bound Met at plasma-membrane level and in nuclei of metastatic cells. Consequently, under dAza the mice survival was prolonged due to the reduction of metastatic cell proliferation, with decreases of metastasis wideness and osteolysis. Our data were consistent with HGF production by bone marrow cells, and with an extensive autoactivation of the HGF precursor stored in the stroma via proteolysis. An HGF-dependent downregulation of miR-27b and upregulation of the target ST14/matriptase is proposed. Since HGF substitutes for M-CSF to support osteoclastogenesis, decitabine treatment might intervene halting this mechanism and reducing osteolysis in bone metastasis. HGF is known to trigger TGFβ1-signaling pathway also involved in osteoclast activation and to activate Twist, which facilitates establishment of osteolytic metastases through miR-10b.

Interestingly, DNA-methyltransferase blockade reduced HGF access to metastatic cells, and various molecular events might be responsible. HGF was likely to be supplemented to metastatic cells by exosomes, which might originate from supportive cells like megakaryocytes presenting high HGF levels. In fact, in bone-metastatic tissue under decitabine the exosome marker LAMP2 and the mouse HGF expression concomitantly decreased. The exosomes may influence the methylation state of metastasis and microenvironment in different manners: many of the proteins and miRNAs contained in exosomes are regulators of DNA methyltransferases or of cytidine deaminase, an enzyme involved in DNA-initiative demethylation. As a consequence, Met and other oncoproteins might be reduced in bone metastasis of the xenograft model by direct or indirect mechanisms. Moreover, LAMP2 promoter has numerous putative sites for metabolism, participating in LAMP2-protein expression in bone metastatic tissue, LAMP2 might be responsible for the formation of exosomes, implicated in juxtacrine communication within metastasis area, but also for autophagy activity being LAMP2 present in the lysosome membranes. A LAMP2-mediated degradation process cannot be excluded under our experimental conditions, since autophagy is prometastatic by fuelling nutrients and supplying energy. Rab5b intervenes in the early endosomal phase that precedes exosome formation, and was largely affected by DNA-methyltransferase blockade.

For the first time, we demonstrated the interaction between HGF and epigenetic mechanisms controlling gene expression, important for metastasis phenotype. It might be easier to prevent bone metastasis colonization by modifying the HGF-dependent MET (epithelial) phenotype—testified by the decrease of E-cadherin expression under decitabine—rather than to change the genotype. The plasticity of bone metastasis largely depends on phenotype adjustment, and Twist expression also diminishes at the invasive front of bone metastases in the 1833-xenograft model under dAza.

Commonalities between in vivo and in vitro conditions regard the indirect effect of dAza on HGF: the lack of HGF mRNA in 1833 cells excluded dAza effect on the promoter, while the hampering of HGF supply can be hypothesized. In agreement, exogenous HGF seemed to play a pivotal role by reverting the inhibitory effect of dAza on Met protein level and nuclear COOH-Met activity.

Wwox accumulation seemed critical for the nuclear COOH-Met decrease under long-term exposure to dAza in the xenograft mice, probably because it prevented the access to nuclei as shown in vitro. Similarly, Wwox prevents the nuclear translocation of Twist. In addition, we observed that Wwox reduced the half-life of cellular Met, further explaining why in the xenograft model, the Met signal diminished under dAza. Thus, the discrepancy between in vivo and in vitro data was only apparent, because in 1833 cells dAza actually decreased Wwox preventing Met destabilization, while in the xenograft mice dAza accumulated Wwox. The regulation of Met expression is complex, and the effect of decitabine cannot be explained by hypomethylation of Met promoter in bone metastasis. In various types of tumors, regulatory sequences intervene in Met expression such as the antisense L1-Met, that is hypermethylated and is inversely correlated with the transcript and the protein level of Met and with metastasis; also the promoter of long non-coding RNA gene 3 (IntRNA Meg3) is hypermethylated favoring Met expression, while under decitabine Met downregulation occurs.

Only in 1833 cells but not in parental MDA-MB231 cells, HGF activated the cytosol-nuclear shuttle of COOH-Met fragments and the Met-receptor transactivation. Nuclear Met might transactivate genes for bone metastatization under HGF stimulus, and the decrease of HGF due to decitabine was likely to influence the phenotypic changes. As shown in our previous preclinical study, an effective anti-metastatic treatment was the combination NK4 plus dasatinib, inhibitors of HGF and Src activity. The interactive crosstalk between Met and EGFR/HER2/VEGFR is responsible for Met resistance to tyrosine-kinase receptor targeted therapies, and the lack of standardized biomarkers predictive for patient response reduces the benefits. We add insight that new effective approaches would target the microenvironment of bone

**Figure 8** HGF treatment on Met protein and activity in nuclei, and effect of dAza on the expression of mouse HGF, LAMP2 and Rab5b in bone metastasis of the xenograft model. (a) cells were transfected with Met-GFP on coverslips, and exposed to HGF under starvation. The nuclei were stained with DAPI (blue). The images, taken at x 400 magnification, are representative of experiments performed in triplicate. (b) the cells were co-transfected with the construct containing Met fused with Gal4 DNA-binding domain, and the reporter plasmid encoding Firefly luciferase controlled by Gal4-responsive promoter (Gal4Luc). The data are the means ± S.E. of three independent experiments performed in triplicate. *P<0.05, **P<0.005 versus Gal4Luc transfected cells. (c) representative images of Western blots performed with nuclear extracts from cells exposed to HGF; the experiments were repeated three times, and B23 was used for normalization. The numbers at the bottom indicate the fold variations versus the first lane, considered as 1. (d) we show representative images of bone metastases from mice treated or not with dAza (xenograft mouse 1 with dAza). Five serial sections were examined for each specimen from three mice at 24 days for ME and at 24–26 days for ME plus dAza with similar results. bm, bone marrow; me, metastasis; bo, bone. Scale bar = 120 μm (reported in exemplificative panels × 20 and × 40). Negative controls did not show specific signals.
emetases, formed by bone marrow cells and a combination of biological components.49

HGF/Met axis and Wwox are critical molecular players regulated by DNA methyltransferases, which are important for epithelial phenotype and colonization of bone metastasis from breast carcinoma. The therapeutic approaches targeting the nexus microenvironment–DNA methylation of metastatic cells, with consequent defects in the biological stimuli and adhesive molecules, are promising to prevent colonization of osteolytic bone metastases affecting also the pre-metastatic niche formation.45

Materials and Methods

Cells. 1833-bone metastatic clone, the parental MDA-MB231 breast carcinoma cells, and the 1833-retrovirally transduced with HSV1-1k/SGF/firefly luciferase (1833/TGL) were kindly obtained from Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, USA). The cells, routinely maintained in DMEM containing 10% FBS (Sigma-Aldrich, Saint Louis, MO, USA), were used after 2 or 3 passages in culture.17

Xenograft model preparation. Animal studies were carried out according to the Institutional Guide for Care and Use of Laboratory Animals, and with International Laws. The 1833/TGL cells were injected in the heart of anesthetized nude/nu mice; one animal group (ME, n = 8) received only the 1833/TGL cells; a second group (ME+dAza, n = 12) received 1833/TGL cells concomitant with 5 μg/g of dAza i.p., that was given once a week until the suppression.17,46 The animals were killed to prevent the suffering.

Immunohistochemistry. Bone samples were fixed, decalcified and serial sections were prepared.11 Immunostaining was performed with anti-Met (C-12, 1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse HGF (1:200) (R&D Systems, Minneapolis, MN, USA), anti-human HGF (H487, 1:200) (Immuno-Biological Laboratories, Aramachi, Takasaki-Shi, Gunma, Japan), β-catenin (1:100) (BD Transduction Laboratories, Lexington, KY, USA), anti-ΔNp73 (ABL-93, 1:50) (Santa Cruz Biotechnology), anti-Rab5b (A-20, 1:50) (Santa Cruz Biotechnology), anti-LAMP2 (2:2 kb) kindly given by R. Zarnegar (University of Pittsburgh, PA). Reverse transcription was performed using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Monza, Italy). Gene expression was measured using TaqMan Gene expression assay (ThermoFisher Scientific; HGF, assay ID HS00300159_m1).46 GAPDH was used as internal control to normalize the mRNA levels.

Immunofluorescence assay. The cells (4 × 10^6) on coverslips were probed with anti-Met or anti-vHGF antibody (1:50). The images were collected under Eclipse 80i, Nikon Fluorescence microscope at ×400 magnification.43

Transfection with Met fluorescent constructs. The cells on coverslips were transfected with 200 ng/well of Met-expression vector, last set with GFP.35 Subconfluent cells were transfected with Met (full-length Met cDNA) kindly given by L. Schmidt (National Cancer Institute, Frederick, MD, USA). Starved cells were treated with 100 ng/ml HGF for 24 h. The images were collected under Eclipse 80i, Nikon Fluorescence microscope at ×400 magnification.44 To measure Met activity, the cells in 24-well plates were co-transfected with 200 ng of the construct containing Met fused with Gal4BD, and with 200 ng of p35-38HIV-Luc (Gal4Luc), encoding Firefly luciferase controlled by a Gal4-responsive promoter; these cells were exposed not to HGF for 1 day.14 The fluorescence was performed with a DNA: 

Statistical analysis. The statistical analysis of the densitometric and luciferase values was performed by analysis of variance. The survival data of xenograft mice were analyzed by Kaplan-Meier method and the Log-rank (Mantel-Cox) test. P < 0.05 was considered significant.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

Conception and design: MAD; development of methodology: MAD, PB and EM; acquisition of data: PM, EM and P.B.; analysis and interpretation of data: MAD, PB and PM; writing of the manuscript: MAD; administrative, technical, or material support: MAD and PM; study supervision: MAD

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