Osteolytic bone metastasis is hampered by impinging on the interplay among autophagy, anoikis and ossification

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Here we show that the fate of osteolytic bone metastasis depends on the balance among autophagy, anoikis resistance and ossification, and that the hepatocyte growth factor (HGF) signaling pathway seems to have an important role in orchestrating bone colonization. These findings are consistent with the pathophysiology of bone metastasis that is influenced by the cross-talk of supportive and neoplastic cells through molecular signaling networks. We adopted the strategy to target metastasis and stroma with the use of adenovirally expressed NK4 (AdNK4) and Dasatinib to block HGF/Met axis and Src activity. In human bone metastatic 1833 cells, HGF conferred anoikis resistance via Akt and Src activities and HIF-1α induction, leading to Bim isoforms degradation. When Src and Met activities were inhibited with Dasatinib, the Bim isoforms accumulated conferring anoikis sensitivity. The proviability effect of HGF, under low-nutrient stress condition, was related to faster autophagy deactivation with respect to HGF plus Dasatinib. In the 1833 xenograft model, AdNK4 switched metastasis vasculature to blood lacunae, increasing HIF-1α in metastasis. The combination of AdNK4 plus Dasatinib gave the most relevant results for mice survival, and the following molecular and cellular changes were found to be responsible in bone metastasis, we observed a hypoxic condition – marked by HIF-1α – and an autophagy failure – marked by p62 without Beclin-1. Then, osteolytic bone metastases were largely prevented, because of autophagy failure in metastasis and ossification in bone marrow, with osteocalcin deposition. The abnormal repair process was triggered by the dysfunctional autophagy/anoptosis interplay. In conclusion, the concomitant blockade of HGF/Met axis and Src activity seemed to induce HIF-1α in metastasis, whereas the bone marrow hypoxic response was reduced. As a consequence, anoikis resistance might be hampered favoring, instead, autophagy failure and neof ormation of woven bone trabeculae. Mouse survival was, therefore, prolonged by overcoming an escape strategy adopted by metastatic cells by disruption of tumor–stroma coevolution, showing the importance of autophagy inhibition for the therapy of bone metastasis. Cell Death and Disease (2014) 5, e1005; doi:10.1038/cddis.2013.465; published online 16 January 2014

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Systemic signals of primary tumor convert the microenvironment of distant organs into more hospitable sites for metastasis engraftment.¹ For example, breast cancer participates in the formation of premetastatic niche in the bone through systemic release of hepatocyte growth factor (HGF).² We have shown that HGF contributes to bone metastasis plasticity by mediating a metastasis-microenvironment cross-talk via Wnt-β-catenin and Src tyrosine kinase network.²,³ Therefore, bone metastasis does not rely only on Src autonomous program for adapting to demands imposed by foreign tissue at secondary site.⁴

The patient stratification according to HGF/Met receptor expression in breast cancer cells triggering still elusive transduction pathways, centered on Src nuclear activity, and influencing invasive–metastatic phenotype.⁵ Rescue of micrometastasis from quiescence and metastatic colonization requires engagement of Src and focal adhesion kinase downstream of β1-integrin pathway: blockade of this extracellular matrix (ECM)-triggered signal cascade is an important strategy for preventing or treating recurrent metastatic disease.⁶ On the whole, Src inhibition would be relevant as Src family kinases are, on the one hand, involved in regulating the activity of various cell types of the bone marrow stroma,⁷ and on the other hand are implicated in cancer cell proliferation and survival, and in osteoclast differentiation.⁴

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Abbreviations: AdNK4, adenovirally expressed NK4; BLI, bioluminescence imaging; DAS, Dasatinib; ECM, extracellular matrix; HGF, hepatocyte growth factor; IHC, immunohistochemistry; ME, mice bearing bone metastasis; μCT, micro-computed tomography; ROS, reactive oxygen species

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In osteolytic bone metastasis from breast cancer, disruption of mineralized matrix and detachment of metastatic cells from ECM occur.\(^9\) Neoplastic cells under duress owing to ECM detachment might undertake autophagy, although the exact role of non-canonical cell death mechanisms have yet to be definitively determined. Oxidative stress is one of the events consequent to ECM detachment, promoting autophagy.\(^{11,12}\) Excess stromal production of reactive oxygen species (ROS) drives the onset of antioxidant defense in adjacent cancer cells, protecting them from apoptosis.\(^{12}\) In fact, tumor cells must overcome both anoikis and necroptosis in order to metastasize.\(^{11}\)

Reconciling several scientific evidences, autophagy becomes more apparent in late stages as tumor cells cope with microenvironmental stress, encountered during progression and metastasis.\(^{13}\) As autophagy has emerged as one of the survival pathways for tumor cells, there is great interest in inhibiting this process for cancer therapy.\(^{14}\)

The aim of the present investigation was to clarify the presence of autophagy in bone metastasis of breast carcinoma, and the critical role of HGF/Met axis influencing the hypoxic response. Very little is known about the role, if any, of hypoxia in regulating anoikis or changes in tissue architecture, in relation to breast cancer progression and treatment. Resistance to detachment-induced anoikis is emerging as a hallmark of metastatic malignancies, mainly because it can ensure anchorage-independent growth and survival during organ colonization. We adopted a strategy to block ligand-activated Met receptor and component(s) of its downstream signaling pathway(s) with NK4, using a bone metastasis model prepared with 1833 clone, derived from MDA-MB231 breast carcinoma cells. NK4 exhibits two distinct biological actions as HGF antagonist and angiogenic inhibitor.\(^{3,16}\) Among them, it prevents lung metastases,\(^{16,17}\) but does not affect proliferation and apoptosis of carcinoma cells.\(^{16}\) In the context of this combination therapy, we targeted Src activity with Dasatinib (DAS), reasoning that NK4 and DAS might be effective in impairing metastatic cell population escaped via Src from selective pressure exerted by the HGF/Met axis blockade.

The cellular processes of angiogenesis and autophagy/ossification were affected by NK4 alone and NK4 plus DAS, respectively. The combined treatment successfully prolonged mice survival preventing metastasis through bone formation coupled to defective autophagy. One of the molecular events causing autophagy failure was hypoxia with HIF-1\(\alpha\) induction due to NK4 effect. In vitro experiments showed that Src activities, both the nuclear HGF-dependent and the HGF-independent, were inhibited by NK4 plus DAS. These pathways were involved in metastatic cell invasiveness, and in anoikis resistance through Akt activity and HIF-1\(\alpha\) induction, as well as Bim degradation.

**Results**

**Blockade of HGF and Src impaired bone metastasis outgrowth.** A xenograft model was prepared with 1833/TGL cells, engineered with a luminescent construct. On the basis of our data on the pivotal role of HGF in bone metastasis,\(^4\) and the hypothesis of Gherardi et al.,\(^5\) regarding Src-mediated selective cell population escape after HGF/Met blockade, we treated 1833 xenograft mice with adenovirally expressed NK4 (AdNK4) in the presence or absence of DAS (Figure 1). The schedule with multiple AdNK4 injections enhanced circulating NK4 levels, with NK4 access to the bone marrow (Supplementary Figures S1a and b).

We monitored metastatic development in real-time exploiting the bioluminescence of mice bearing bone metastasis (ME), and of ME-treated groups (Figure 1a). The injection efficiency for all the mice was controlled by monitoring the bioluminescence signal 1 h after intracardiac injection (Supplementary Figure S2a). To exclude a potential impact of the AdNK4 and DAS pretreatments on extravasation and homing, with an interference with the evaluation of metastasis growth, we normalized the data of bioluminescence with the value obtained at 24 h (Supplementary Figure S2a). The bioluminescence value of AdLacZ control mice was similar to that of ME (Supplementary Figure S2b).

As shown in Figures 1b and c, from 13 days from intracardiac cell injection, AdNK4 and DAS alone or in combination reduced metastasis outgrowth to about 90% in both the hind limbs, independent of the projection. At 20 days, the combination of NK4 plus DAS significantly reduced (about 75%) bioluminescence with respect to NK4 alone (about 50% decrease). Considering both the hind limbs and projections, at 20 days NK4 was ineffective (Figure 1c). Bioluminescence values all over the skeleton at 20 days were reduced more after the combined treatment of NK4 plus DAS than after the single treatments (Figure 1d).

**The combined treatment of NK4 plus DAS delayed death and increased the number of surviving mice by reducing osteolytic bone metastasis.** The effectiveness of the combination of AdNK4 plus DAS was further demonstrated by micro-computed tomography (μCT) analysis, showing that osteolysis was largely prevented all over the skeleton (Figure 2a).

ME mice died at 26 days on an average, and the treatments positively but differentially influenced survival (Figure 2b). All DAS-treated animals died between days 32 and 33, whereas NK4 treatment delayed death further by 4 days (Figure 2c). Thus, with respect to ME animals, which by day 26 were all dead, NK4 promoted survival as about 25% of the mice lived up to 36 days. The combined AdNK4 plus DAS treatment was even more effective because 50% of the mice survived until 35 days and died thereafter (day 38), that is 12 days later than ME. The treatments did not affect body weight with respect to ME (Supplementary Figure S2c).

Measurement of HGF and NK4 in the plasma (Figure 2d) showed similar HGF levels in ME between 15 and 20 days, whereas NK4 showed similar values in ME between 15 and 20 days, whereas NK4 treatment delayed death further by 4 days (Figure 2c). Thus, with respect to ME animals, which by day 26 were all dead, NK4 promoted survival as about 25% of the mice lived up to 36 days. The combined AdNK4 plus DAS treatment was even more effective because 50% of the mice survived until 35 days and died thereafter (day 38), that is 12 days later than ME. The treatments did not affect body weight with respect to ME (Supplementary Figure S2c).

As shown in Figures 2e and f, DAS alone seemed less effective than AdNK4 plus DAS, especially considering the total burden.

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Cellular processes and molecular markers affected
in vivo by NK4 plus DAS versus NK4. Considering
that in any case AdNK4 plus DAS prolonged mice survival,
with respect to AdNK4 alone, notwithstanding similar biolumines-
cence signals at 30 days, in the following experiments we
tried to give an explanation examining cellular processes
possibly affected by AdNK4 plus DAS versus the single
treatments (Figures 3–5).

Canonical H&E staining was performed (Figure 3a).
As reported, ME bone marrow cavity of hind limbs was
extensively colonized by metastasis already at 25 days from
1833 cell injection. NK4 (36 days) affected metastasis blood
vessels that switched to wide lacunae. AdNK4 plus DAS (36 days)
gave remarkable alterations of tissue architecture,
because of extensive intercellular void spaces, suggesting
autophagy dysfunction.\textsuperscript{14}

To address this latter possibility, we analyzed the expres-
sion of autophagy-related proteins\textsuperscript{19} (Figure 3b).
Beclin-1 was expressed throughout bone metastatic cells,
including nuclei, and a strong Beclin-1 signal was observed also after AdNK4
treatment. After AdNK4 plus DAS, however, Beclin-1
disappeared, consistent with autophagy failure. The p62
pattern was opposite with respect to that of Beclin-1: absent in
ME, and in ME plus AdNK4, p62 signal appeared after AdNK4

Figure 1 Effects of the inhibitors on bone metastasis outgrowth. (a) Representative bioluminescence imaging (BLI) of two xenograft mice for each experimental group.
(b) Absolute quantitative BLI values and fold variations of BLI for hind limbs in the two projections. The fold variations were calculated versus the ME value, considered as 1.
ME (n = 12); ME + AdNK4 (n = 12); DAS (n = 8); and AdNK4 + DAS (n = 8). Averages ± S.E.M. *P < 0.05, ***P < 0.001 versus corresponding ME value; **P < 0.05 versus AdNK4 value. (d) For all the mice absolute BLI values for the skeleton were calculated, and percent decreases after treatments are reported. Averages ± S.E.M. *P < 0.05,
**P < 0.005 versus corresponding ME value; \textsuperscript{**} P < 0.05 versus AdNK4 value.

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plus DAS treatment with prevalent nuclear positivity. Ubiquitin-containing p62 bodies are targeted by autophagy. Negative controls performed without specific antibodies did not give positive staining.

An elevated Beclin-1 signal, marker of autophagy, was also observed in metastasis stroma in the presence or absence of AdNK4 (Supplementary Figure S3). As shown in Figures 4a and b, AdNK4 plus DAS caused a surprising effect also at the medullary cavity level, where H&E and osteocalcin stainings evidenced trabeculae of woven bone similar to that observed in primary ossification. Of note, osteocalcin signal was present in metastatic cells, possibly due to osteomimicry. Negative controls performed without specific antibodies did not give positive staining. A scheme accounting for bone matrix organization at metastatic site is shown (Figure 4c). Altogether, the combination NK4 plus DAS appeared to interfere with metastasis autophagy, and caused woven bone deposition. The latter process might counteract the metastasis-driven alteration(s) of osseous physiological turnover.

Even if the AdNK4 group did not undergo autophagy failure, differently from AdNK4 plus DAS group (Figure 3), the latter
group at 30 days still presented bioluminescence signal in metastases, at an intensity similar to that of AdNK4-treated animals (Figures 2e and f). Autophagy failure in the AdNK4 plus DAS group determines an intercellular discontinuity, but the metastatic cells seemed to maintain the luciferase construct during the time of observation. Growth was likely to be arrested, but the neoplastic cells were not shed from metastasis at 30 days in both AdNK4 plus DAS and AdNK4 groups, leading to similar bioluminescence all over the skeleton.

To further clarify the cellular and molecular mechanisms involved, immunohistochemistry (IHC) was performed with anti-CD31, to evaluate the structure of blood vessels (Figure 5a). In metastatic tissue (me) of ME group, the blood vessels were imperfect but appeared still delimited by endothelial cells, marked by CD31. After AdNK4 injections in ME, we observed blood lacunae without the endothelial lining.

We then evaluated the expression of HIF-1α, which is a critical player in the cellular response to hypoxia (Figure 5b). In the ME group, HIF-1α was principally expressed in the bone marrow, whereas metastasis showed a lower HIF-1α signal throughout the cell, localizing mostly in the cytosol. AdNK4 alone or in combination with DAS modified HIF-1α pattern: the HIF-1α signal was higher in bone metastasis than in bone marrow. In the ME plus AdNK4 group, the HIF-1α signal in bone marrow appeared only in the endothelial lining of blood vessels.

Negative controls performed in the absence of primary antibody did not show specific signals (Figures 5a and b).

Our data regarding the markers of autophagy and hypoxia indicated that: (1) positivity for p62/HIF-1α, without Beclin-1, meant autophagy failure in AdNK4 plus DAS group; (2) HIF-1α/Beclin-1 positivity without p62 might suggest a hypoxic condition due to NK4-dependent blood lacunae formation, with possible loss of anoikis resistance (Supplementary Figure S4).

Effects of NK4 and DAS in vitro on signaling pathways and invasiveness triggered by HGF. In 1833 cells exposed to HGF, we explored the signaling pathways that
might be affected by the treatments. In Figure 6, HGF-dependent time courses of Met phosphorylation at the catalytic site and of the canonical downstream pathways are reported. HGF rapidly (within 5 min) and strongly (hundred folds) enhanced the ratios phospho-Met (pMet)/Met and phospho-Akt (pAkt)/Akt. Then, these ratios underwent a diminution, but persisted at high levels for almost 60 min. At earlier times after HGF treatment, Src and ERK1/2 phosphorylation tripled and doubled, respectively.

As shown in Figure 7a, in total extracts NK4 treatment abrogated phosphorylative activation of Met and Akt observed 5 min after HGF exposure without significantly affecting Src and ERK1/2 phosphorylation. Conversely, DAS treatment reduced HGF-mediated activation of Met, Akt and ERK1/2, but completely abolished Src activation either in the presence or absence of HGF. The experiments have been repeated on five serial sections for each specimen from three xenograft mice with similar results. For (a) and (b), me, metastasis; bo, bone; bm, bone marrow. (c) A Scheme of osseous formation after AdNK4 plus DAS treatment is shown.

To assess some of the biological consequences of NK4 plus DAS treatment, we examined the invasiveness of 1833 cells through Matrigel. As shown in Figure 7d, NK4 reduced the basal cell motility to about 30%, while preventing the HGF-induced invasiveness to about 50%. DAS, either alone or combined with NK4, decreased by more than 90% the HGF-dependent cell invasion.

Figure 7e summarizes a model whereby NK4 exerted an inhibitory effect on Src phosphorylation at the nuclear level, whereas the blockade of HGF/Met axis at the plasma membrane reduced pAkt. This led us to suppose the existence of an alternative HGF-independent pathway for Src activation. DAS was effective in inhibiting pSrc in the presence or absence of HGF.

Central role of Src in anoikis resistance of 1833 cells exposed to HGF, and interaction with autophagy. In vivo in ME xenograft model, microenvironment stimuli like HGF maintain metastatic cell survival and metastatic tissue architecture.² Thus, we examined whether HGF was involved in viability and anoikis resistance of 1833 cells, as well as the role played by Src and Akt.

In 1833 cells grown on an antiadhesive substratum, HGF pretreatment enhanced viability that was largely dependent on Akt activity (Supplementary Figure S5a). The time course...
of Akt phosphorylation was evaluated at earlier and later times after HGF (Supplementary Figure S5b).

Under the non-adhesive condition, we also examined the effect of 36-h pretreatment with HGF in the presence or absence of DAS: at various times thereafter, we evaluated cell viability with MTT assay, and Beclin-1 steady-state protein levels (Figures 8a and b). In these experiments, we intended to correlate stress, due to 48 h starvation, and autophagy under our non-adhesive experimental conditions. At the end of the pretreatment, cell viability was 90% (considered as 1); HGF pretreatment unaffected viability until 24 h, while decreasing thereafter, and the concomitant DAS exposure strongly counteracted the prosurvival effect of HGF starting from 12 h (40% decrease) until 72 h (95% decrease) (Figure 8a). Beclin-1 was downregulated by HGF pretreatment faster than by HGF plus DAS pretreatment (Figure 8b).

In the xenograft model, DAS (33 days) had marginal or no effects on bone metastatic tissue architecture, examined by H&E staining (Figure 8c).

As shown in Figure 8d, under HGF with or without DAS the marker of anoikis Bim was analyzed, and different isoforms were detected. BimEL, strong in control-starved cells, was degraded under HGF. Also BimL and BimS were less expressed after HGF exposure. DAS addition led to progressive BimEL, BimL, and BimS accumulation. HGF biphasically enhanced Akt phosphorylation, consistent with the time-course data of Supplementary Figure S5b, that was almost completely prevented by DAS cotreatment. HIF-1α was studied in extracts from nuclei, where it has its function, and we observed HIF-1α induction by HGF (Figure 8d). HIF-1α is a key player of hypoxia-induced anoikis.

Figure 8e summarizes the signaling pathways downstream of HGF/Met, including a small ROS burst involved in anoikis resistance, and the role of DAS in anoikis triggering.

**Figure 5** Analysis of vascular changes and hypoxic consequences caused by AdNK4 alone or combined with DAS in xenograft mice. Representative immunostaining images: (a) for CD31 and (b) for HIF-1α; magnifications are reported in the insets. Arrows indicate endothelial cells. The experiments have been repeated on five serial sections for each specimen from three xenograft mice with similar results. me, metastasis; bl, blood lacunae; bv, blood vessel; bo, bone; bm, bone marrow.
Discussion
For the first time we have shown the occurrence of autophagy in the human bone metastasis xenograft model, evidenced through the opposite expression of the markers Beclin-1 and p62, consistent with adaptation to stress posed by foreign tissue. As p62 is an autophagy substrate, defects of autophagy cause p62 accumulation. The role of autophagy during cancer progression depends on tumor type, context and stage. In bone metastasis from breast cancer, and in supportive fibroblasts, autophagy...
seemed to function as a survival mechanism under the various forms of microenvironment duress. In fact, osteolysis and ECM detachment occurred, and the remarkable HIF-1α signal also indicated hypoxic changes. 23 The microenvironmental features stimulate several adaptative responses in metastatic cells, such as activation of antioxidative transcription factors HIF-1 and NF-κB,22,26 and the release of antiapoptotic stimuli such as HGF.27,28

HGF is critical both locally in the bone microenvironment,22,23 due to paracrine (stroma cells) and autocrine (metastasis) production, and systemically because of the elevated levels in the bloodstream. Competitive inhibition of HGF ligand with NK4 might target Met receptor function on more than one cell type, such as metastasis and bone cells. Osteoclasts are also responsive to HGF.29

The therapeutic strategy, consisting in the combined treatment of AdNK4 plus DAS, inhibited in bone metastatic tissue both the HGF/Met signaling cascade and the total Src activity, hampering escape strategies of metastatic cells through Src-dependent rescue pathway(s). Mice survival

Figure 7 Cell signaling and migration after exposure to HGF in the presence or the absence of the inhibitors. (a) Representative images of western blots performed with total protein extracts are shown. We report fold variations of the ratios of phosphorylated/unphosphorylated proteins, calculated after densitometric evaluation. Vinculin was used for normalization. Averages ± S.E.M. of three independent experiments. *P < 0.05, **P < 0.005, ***P < 0.001 versus starved untreated cells. (b) Nuclear extracts from treated cells were immunoprecipitated with anti-Src antibody; western blots and histograms with fold variations of pSrc/Src values are shown. The experiments were repeated three times with similar results. **P < 0.005 versus starvation value; ΔΔΔP < 0.001 versus HGF value. (c) Nuclear extracts from transfected cells were analyzed by western blot, which was hybridized with anti-HGF antibody. B23 was used for normalization. The experiments were repeated three times with similar results. (d) Matrigel invasion assay was performed. Averages ± S.E.M. of three independent experiments performed in triplicate. *P < 0.05 versus starved untreated cells. ΔP < 0.05, ΔΔΔP < 0.005 versus HGF-treated cells. (e) Schematic representation of in vitro data.
was prolonged for about 12 days, and osteolytic metastases were prevented all over the skeleton. The efficacy of NK4 plus DAS strategy against osteolytic metastasis might be explained by the different cellular processes affected. In the metastasis bulk, NK4 plus DAS caused autophagy failure, and woven bone was observed in the bone marrow. The rescue processes of the bone that resembled primary ossification, with osteocalcin deposition in association with defective autophagy, could be important for prolonging mice survival. The higher effectiveness of the combined treatment of NK4 plus DAS than NK4 alone might be related, in any case, to the hypoxic condition in metastasis versus stroma, implicated in autophagy failure.

One of the principal findings of our study was, therefore, the abnormal repair effect of the combined treatment. Enhancement of osteocalcin, a non-collagenous bone matrix protein, created a circuit to impair the outgrowth of bone metastasis also impinging on autophagy. Thus, the cancer tissue organization field theory, related to ECM composition, might be applied. Our hypothesis is that the therapeutic impairment of paracrine tumor–stroma cell interaction promoted pro-osteogenic changes. Arrested metastatic cells cannot disturb anymore the differentiation of osteoprogenitors. We propose that the blockade of the angiogenic role of HGF and of Src multiple functions, might affect various key steps for colonization. The combination of NK4 plus DAS not only affected the network of events, responsible for HGF-triggered chemoinvasion, but was also likely to impact on exit from dormancy, which is known to require Src activity, and on stromal autophagy, a new protective mechanism for bone metastasis, besides allowing escape from antiangiogenic therapies. Altogether, the combined treatment of NK4 plus DAS was more effective than the single treatments.

Notably, our results are the first to report a therapeutic function of NK4 against breast cancer bone metastasis, prolonging mice survival, when compared with DAS. NK4
induced the formation of blood lacunae lacking endothelial lining, at a difference with metastatic blood vessels, and might prevent metastasis adhesion. NK4 is known to impair endothelial cells by binding to perlecain and inhibiting, therefore, the fibronectin assembly. In ME plus AdNK4 group, the blood lacunae increased HIF-1α/hypoxia in metastasis, whereas HIF-1α disappeared in the stroma/bone marrow. We cannot exclude that the stroma protective function towards anoikis in metastasis became defective, Beclin-1 persisting elevated.

High doses of ROS under hypoxia maintain elevated expression of HIF-1α, and are also responsible for Src activity inhibition and mitochondria dysfunction. However, different roles are played by a small burst of ROS downstream of growth factor receptors, such as Met stimulated by HGF. ROS at low levels might be important for anoikis resistance in 1833 cells exposed to HGF.

The existence of multiple signaling pathways regulating anoikis is likely to be critical for neoplastic cell survival. Bim exists in various isoforms, generated by alternative splicing. In 1833 cells, known to produce more transforming growth factor-β than HGF, with pro- and antiapoptotic functions, BimL and BimL were elevated. Transforming growth factor-β increases Bim expression levels and enhances the proapoptotic function by downregulating Akt and ERKs. BimL supports acidification of lysosomes that may associate with autophagyc vesicles. The 1833 cells were susceptible to autophagy under low nutrient (stress) condition.

In the present paper, we show that the anoikis function of BimL was regulated by Akt activity downstream of HGF. In response to HGF, persistent Akt phosphorylation seemed to be involved in the post-transcriptional regulation of Bim via ubiquitination and proteasomal degradation, attenuating its apoptotic function. In addition, the prosurvival role of HGF did not implicate autophagy under stress and non-adhesive conditions.

Notwithstanding the known acquisition of resistance to anoikis of carcinoma cells during metastatic progression, and suppression of anoikis under hypoxic conditions in an HIF-1α/Src-dependent manner, our results allow new insights into the etiology of the molecular mechanisms granting anoikis resistance of bone metastatic cells, in the absence of proper adhesion and under HGF stimulus. Concomitant DAS exposure of 1833 cells, through suppression of pMet/Src/Akt pathways, converging on Bim phosphorylation, abolished its degradation finally leading to the accumulation of BimL, principal mediator of anoikis. Met activity is known to be controlled by phosphorylation also via Src.

HGF-dependent Src activity was observed only in nuclei, consistent with previous data. DAS prevented Src activation dependent on HGF/Met axis. For example, Src activity is controlled by prostaglandin E2 downstream of cyclooxygenase 2, critical enzyme in 1833 cells. DAS delayed the growth of metastasis during the first period of observation of the xenograft model: eventually and consistently with previous data. DAS prevented Src activation and inhibited autophagy under stress and non-adhesive conditions.

Similarly, Src dominant-negative or DAS treatment impairs bone metastasis after parental MDA-MB231 cells implant in the tibia. In conclusion, the demonstration that tumor cells – surviving to antiangiogenic therapies through autophagy – exhibit increased invasiveness fits with our data obtained with NK4 in the presence of HGF in vitro (Figure 7 and Supplementary Figure S4) and in vivo (Figure 3). Autophagy inhibition could prevent invasion, occurring after antiangiogenic therapy, by disrupting it at an early stage, which may be more effective than targeting invasion directly. Akt activity itself downstream of HGF/Met is implicated in tumor angiogenesis by increasing vascular endothelial growth factor secretion, and by mediating the expression of nitric oxide and angiopeptins. The meshwork of molecular and cellular mechanisms, underlying tumor–stroma interaction and bone metastasis, may be successfully targeted with therapeutic interventions, exploited to prevent late recurrence of breast cancer patients by inhibiting autophagy. On the basis of preclinical evidence, autophagy inhibition is currently being investigated as a way of modulating the response to cancer therapies in patients.
ME = AdLacZ (n = 6); ME = DAS (n = 8); and ME = AdNK4 + DAS (n = 8). The adenoviral vectors AdNK4 and AdLacZ (control) were administered intravenously 1 day before 1833/TGL cells, and then every 5 days. AdNK4 is a replication-deficient adenovirus vector, based on human adenovirus type 5 with E1 and E3 deletions, that expresses human NK4 gene under the transcriptional control of the cytomegalovirus early promoter and enhancer.\(^3\)

The recombinant virus was grown in the HEK293 cells, and then purified and concentrated with Adeno-X Max Purification Kit (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). Titers of viral stocks were determined by plaque assays using the HEK293 cells. AdLacZ adenoviral vector, which expresses the LacZ gene, was prepared as reported above, and was used as control vector.

The DAS group of animals received DAS (50 mg/kg per day) concomitantly with the intacardiac injection of DAS-pretreated 1833/TGL cells, and daily thereafter.\(^4\)

Firefly D-luciferin (150 mg/kg) was given intraperitoneally under anesthesia, and metastasis formation was monitored at Transgenic-Operative Products s.r.l. (Lodi, Italy) as luminescence signals by Optical Imaging, using Xenogen IVIS 200 System (Caliper Life Sciences, Hopkinton, MA, USA), and photon emission was quantified with Living-Image Software (Caliper Life Sciences). Acquisition time for bioluminescence at the beginning of observation time points (1 and 24 h) was 5 min; for the following observations, the acquisition time was reduced to 1 min in accordance with signal strength, to avoid saturation.

Ventral and dorsal projections were examined. For normalization of the data, regions of interest (ROI) over all the skeleton were defined, and the corresponding bioluminescence values were evaluated at 24 h in ME exposed or not to the treatments. We verified that the different groups had similar bioluminescence value at ROI level. Then, bioluminescence signals of subsequent images for each animal were normalized in accordance with signal strength, to avoid saturation. Ventral and dorsal projections were reconstructed using Slicer 3D software.

ELISA. HGF and NK4 were evaluated in the plasma of AdNK4 injected xenograft mice by Quantikine Immunoassay (R&D System).

**Westen blot, immunoprecipitation and Matrigel invasion assays.** Total (100 μg) and nuclear (50 μg) protein extracts were used for western blots. The antibodies and their dilutions were as follows: anti-HGF (1:200), anti-Met, anti-β-catenin, anti-Src, anti-p-Src, anti-Akt, anti-p-Akt, anti-α2, anti-α5, anti-pERK1/2 (1:1000), anti-Bim (2 μg/ml), anti-HIF-1α (1:200), and anti-Beclin-1 (1:500). For immunoprecipitation, 500 μg of nuclear proteins from 30 μm HGF-treated cells, in the presence or absence of the inhibitors, were probed with 3 μg of anti-Src antibody, followed by immunoblotting with the antibody for anti-Src (1 μg/ml) or anti-pSsrc (2 μg/ml). For invasion assay, breast cells were seeded in the upper chambers of BD Biocoat Cellware (Becton Dickinson Labware); HGF was present for 20 hours in lower chambers during incubation. Ten fields under ×200 magnification were randomly counted, and the number of the cells in each field was counted and averaged.

**Immunohistochemistry.** Femurs and tibiae from the five groups of xenograft mice were fixed and decalcified, \(^2\) except for CD31 staining, performed on frozen sections fixed in formaldehyde. \(^2\) Serial sections were stained with H&E or were immunostained with the following antibodies: anti-Beclin-1 (1:400), anti-STOML1 (1:150), anti-osteocalcin (5 μg/ml), anti-CD31 (1:50) and anti-HIF-1α (1:50). Nerve fibers were detected with anti-Sec-8 antibody. Nuclei were stained with DAPI. Cells were observed at ×400 magnification under fluorescence microscopy.\(^2\)

**Statistical analysis.** The statistical analysis was performed by analysis of variance, with the exception of the survival data of xenograft mice, which 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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**Supplementary Information** accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)