Inhibition of collagen receptor Discoidin Domain Receptor-1 (DDR1) Reduces Cell Survival, Homing and Colonization in Lung Cancer Bone Metastasis

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Abbreviations:
NSCLC: Non-small cell lung cancer
SCLC: Small cell lung cancer
RTK: Receptor tyrosine kinase
CM: Conditioned medium
i.c.: intracardiac
MMP: Metalloproteinase
SCDC: Single-cell derived colonies
TRAP: Tartrate-resistant acid phosphatase
DDR: Discoidin domain receptor
ECM: Extracellular matrix
i.t.: intratibial
µCT: Micro-Computed Tomography

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Translational Relevance:

In this report, we characterized DDR1, Discoidin domain receptor 1, a collagen receptor and a factor associated with poor clinical outcome in lung cancer, as a relevant mediator of tumor cell survival in bone metastasis. Silencing of DDR1 in lung tumor cells led to increased sensitivity to apoptosis in basal and stimulated conditions. Tumor cells lacking DDR1 also displayed decreased osteoclastogenic and metalloproteolytic activities, key mechanisms required for bone metastasis. In vivo abrogation of DDR1 impaired tumor cell survival in bone metastatic homing and colonization. Thus, it might be possible to use DDR1 as a novel target in combination with chemotherapy to increase the clinical benefit in lung cancer patients with bone metastasis.
ABSTRACT

Purpose: We investigated the role of the collagen binding receptor, discoidin domain receptor-1 (DDR1), in the initiation and development of bone metastasis.

Experimental Design: We performed western blot analysis and phosphorylation status in a panel of human lung cancer cell lines and immunohistochemical analysis in a cohort of 83 lung cancer specimens. Adhesion, chemotaxis, invasiveness, metalloproteolytic, osteoclastogenic and apoptotic assays were performed in DDR1 silenced cells. In vivo, metastatic osseous homing and colonization was assessed in a murine model of metastasis.

Results: DDR1 was expressed in a panel of human lung cancer cell lines and high DDR1 levels in human lung tumors were associated with poor survival. Knock-down (shDDR1) cells displayed unaltered growth kinetics in vitro and in vivo. In contrast, shDDR1 cells showed reduced invasiveness in collagen matrices and increased basal and induced apoptosis in vitro. More importantly, conditioned media of DDR1 knock down cells decreased osteoclastogenic activity in vitro. Consequently, in a model of tumor metastasis to bone, lack of DDR1 showed decreased metastatic activity associated with reduced tumor burden and osteolytic lesions. These effects were consistent with a substantial reduction in the number of cells reaching the bone compartment. Moreover, intratibial injection of shDDR1 cells significantly decreased bone tumor burden, suggesting impaired colonization ability that was highly dependent on the bone microenvironment.
**Conclusions:** Disruption of DDR1 hampers tumor cell survival, leading to impaired early tumor-bone engagement during skeletal homing. Furthermore, inhibition of DDR1 crucially alters bone colonization. We suggest that DDR1 represents a novel therapeutic target involved in bone metastasis.
Introduction

Metastasis is a multistep process that requires compatibility between tumor cells and host tissues, as “the seed in a fertile soil” (1). During this process, cell–cell and cell–matrix receptors have been shown to be crucial for survival in the circulation, engagement in host tissue, and other functions required for thriving efficiently in the target organ (2, 3).

Bone is a frequently preferred site of metastasis for many solid tumors. This tissue-selectivity of tumor cells is favored by the few mechanical constraints offered by the fenestrated capillary bed of the bone marrow. This process is also influenced by chemotactic cues that are highly abundant in the bone stroma, which act as chemoattractants for receptors expressed in tumor cells (4, 5).

 Besides limitations passively imposed by the vasculature, homing to bone is actively driven by specific interactions between tumor and normal cells, and with the extracellular matrix components of the osseous milieu. Interactions between endothelial cell surface molecules and membrane receptors in tumor cells have been shown to mediate this process (6-8). Upon engagement, critical pathways triggered in the tumor, and in the bone microenvironment, modulate the progression of metastasis. For instance, Src activation in lung cancer cells is crucial for tumor cell survival in the bone marrow microenvironment (9), whereas PDGF receptor signaling in the bone marrow is required for proper cell homing and infiltration of lung cancer in the skeleton (10). During bone colonization, tumor cells induce high osteoclastic activity that is exacerbated by paracrine loops between bone-matrix-derived factors and tumor cells (11, 12). In addition, proteolytic degradation at the tumor–stromal/endothelial interface also contributes to the development of osteolytic lesions (13). Thus, elucidation of crucial molecular pathways
activated upon complex cellular interactions that confer selectivity and are required for proper colonization could be of great therapeutic benefit.

The discoidin domain receptor (DDR) family of receptor tyrosine kinases (RTK) is composed of two members, DDR1 and DDR2, which are characterized by the presence of a domain homologous to the discoidin 1 protein of the slime mold Dictyostelium discoideum (14). DDR1 and DDR2 are activated by different types of collagen and participate in several processes such as cell adhesion, migration, proliferation, and matrix remodeling (15-18). DDR1 is found in highly invasive tumor cells, suggesting its involvement in tumor progression. DDR1 appears to be preferentially expressed in tumor cells (epithelial), whereas DDR2 is expressed in tumor stroma (19).

A unique feature among other RTKs is the fact that DDRs are activated by a major extracellular matrix component, triple-helical collagen (20). Several collagen types activate the DDRs, with different specificities between the two receptors (20, 21). Collagen binding leads to auto-phosphorylation of DDRs with very slow kinetics. In a global survey with phosphoproteomic screening of NSCLC, DDR1 was one of the most phosphorylated RTKs (22). More importantly, DDR1 overexpression is associated with poor prognosis in NSCLC (23, 24). In addition to lung, overexpression of DDR1 has been reported in breast (25) and other types of tumors. However, to date, somatic mutations of DDR1 have been found only in lung cancer (26, 27).

Given the relevance of DDR1 in collagen binding and tumorigenesis, and the osseous abundance of collagen, we investigated its role in a bone metastasis model of lung cancer. At present, few key mediators involved in the early steps of cell–cell and cell–matrix interactions have been characterized. In this study, activation of DDR1 by collagen endowed tumor cells with survival
properties required for effective homing to bone. DDR1 was also required for effective colonization by conferring resistance to apoptosis in the osseous microenvironment. Thus, our findings unveil DDR1 as a crucial receptor that is implicated in bone homing and colonization, and underscore the relevance of DDR1 as a valid therapeutic target.
Results

Expression and significance of DDR1 in lung cancer cell lines and tumors

Expression of DDR1 was investigated by immunoblot analysis in a large panel of lung cancer cells. As shown in Fig. 1A, upregulated DDR1 protein expression levels were found in several cell lines from the panel as compared to normal lung cells (NHBE and BEAS). Most cell lines from large cell carcinoma and adenocarcinoma showed higher levels of DDR1 than in other histological lung cancers. Specific semi-quantitative qPCR for DDR1 isoforms, revealed that the two main isoforms, DDR1a and DDR1b were expressed in all cell lines tested (Sup. fig 1B). Interestingly, phosphorylation of DDR1 was observed upon collagen incubation in most cell lines (Fig. 1B). Phosphorylation status was increased upon collagen incubation with variable intensities in different NSCLC cell lines, but was generally lower or absent in SCLC cell lines.

To study the significance of DDR1 expression in lung cancer, we analyzed the expression levels of DDR1 in a cohort of 83 patients with NSCLC by immunohistochemical analysis. The staining of tumors showed a variable degree of intensities (Fig. 1C, upper panel) (see material and methods). Clinical features are summarized in Table S1. After the stratification of patients by stages, Kaplan-Meier analysis revealed significant differences in patients with stage I. Analysis of untreated patients after surgical resection showed that tumors with high DDR1 levels were associated with poor survival.

Thus, high DDR1 levels in untreated patients after surgery and stage I patients were associated with poor survival (Fig.1C). These findings indicate that high DDR1 levels correlate poor prognosis in lung cancer.
Effects of DDR1 knock down on cell proliferation and apoptosis

We choose the H460 cell line derived from a large cell carcinoma, with the ability to selectively form rapid bone metastasis (13). Of note, no changes of DDR1 protein levels were observed between parental H460 and highly metastatic subpopulation H460M5, obtained after several in vivo passages (Sup. fig. 1C) (13). To dissect the possible contribution of DDR1 in lung cancer bone metastasis, we knocked-down DDR1 levels by lentiviral transduction of two different constructs (shDDR1-58 and shDDR1-87). DDR1 expression levels assessed by quantitative PCR were downregulated by ~78 % in these cells as compared to scramble and vector transduced cells (Fig.2A). The most abundantly expressed DDR1 isoforms, DDR1a and DDR1b, were downregulated with this strategy (Sup. fig 1B).

The qPCR results were validated by western blot analysis. As expected, DDR1 protein was downregulated in both shRNA-transduced cells as compared to scramble and vector cells (Fig. 2B, upper panels). When DDR1 was stimulated by incubation with collagen I for 24 h, we observed a slight increase in protein expression. DDR1 was poorly detected in one of the pools, the cells transduced with shDDR1-58. In cells transduced with shDDR1-87, DDR1 was not detectable by Western blotting (Fig. 2B, upper panels). Consistent with the above results, immunoprecipitation of DDR1 revealed low levels of DDR1 in both silenced sh58 and sh87 cells as compared to control cells (Fig. 2B, lower panels). The blot membranes were reprobed with an anti-phosphotyrosine antibody to detect DDR1 phosphorylation. Upon collagen stimulation, the phosphotyrosine immunoreactive bands were substantially lower in silenced cells as compared to vector and scramble controls (Fig. 2B). These data are consistent with previous findings and indicate that collagen incubation activates DDR1 phosphorylation. Consistent with the lower
DDR1 protein levels, DDR1 phosphorylation was reduced in DDR1 silenced cells as compared to control cells.

Next, we tested the effects of DDR1 knockdown on cell proliferation in vitro and in vivo. There were no significant differences in growth kinetics in cells transduced by shDDR1 as compared to control cells (Fig. 2C, left panel). Similar results were obtained for tumor growth in vivo after subcutaneous injection (Fig. 2C, right panel). Interestingly, DDR1 protein levels were maintained in tumors in vivo (Sup. fig. 2). Moreover, DDR1 phosphorylation status was generally decreased in DDR1 silenced derived tumors, although in some of these tumors DDR1 was somewhat activated possibly due to infiltrating non-tumor cells (Sup. fig. 2).

We also studied the effects of DDR1 on cell apoptosis in basal conditions measured by the accumulation of cleaved poly (ADP ribose) polymerase (PARP). Cells incubated for 24h with collagen type I showed a decrease in cleaved PARP as compared to untreated cells, indicating an anti-apoptotic effect mediated by collagen in these cells (Fig. 2D). Intriguingly, DDR1 knockdown cells were unresponsive to the decrease in cleaved PARP associated with collagen type I treatment.

To verify the anti-apoptotic effect, cells were treated with 100 μM etoposide for 24h to induce 50% apoptosis. DDR1 knockdown cells showed an increased apoptotic rate as compared to control cells, an effect that was not altered by treatment with collagen type I (Fig. 2E). Similar experiment was performed by incubation with TRAIL, an inducer of apoptosis. As shown in fig. 2F, DDR1 knockdown cells showed an increase in apoptosis as compared to control cells. Cells were irresponsible to collagen treatment. Thus, these observations point to a protective function of DDR1 both in basal and stimulated conditions of apoptosis.
Effects of DDR1 in migration and invasiveness

We investigated whether DDR1 had a functional effect in cell migration and invasion. When cells were stimulated with conditioned medium derived from bone-stromal ST2 cells as a chemoattractant, migration was impaired in knocked-down DDR1 cells as compared to control cells. These differences were increased when we supplemented the chemoattractant with collagen type I (Fig. 3A). Similar results were obtained when cells were treated with collagen type I or IV (Sup. fig. 3). Similarly, shDDR1 cells showed a dramatic decrease in invasiveness through collagen type I and IV ($P < 0.001$) as compared to control cells (Fig. 3B). These findings pointed to DDR1 as a factor required for effective migration and invasion.

Reduced invasion has been linked to impaired proteolytic degradation; therefore, we explored whether DDR1 could modify extracellular matrix metalloproteinase (MMP) activity. To explore this possibility, MMP-2 and MMP-9 activity was assessed by zymography (Sup. fig. 4A). Cells with shDDR1-87 showed a significant decreased in metalloproteolytic activity as compared to control cells. MMP-3 and MMP-10 activity was studied by a fluorogenic assay. A dramatic decrease of metalloproteolytic activity was observed in both DDR1 silenced cells (Sup. fig. 4B).

DDR1 on cell–matrix and cell–cell adhesion

To study the effects of DDR1 on cell–matrix adhesion, we cultured the cells on different substrates. DDR1 levels did not show any effect in adhesiveness to collagen type I or type IV (Fig. 3C), fibronectin, vitronectin, hyaluronic acid or control gelatin (data not shown). To study the involvement of DDR1 in the ability of H460 cells to adhere to monolayers of human brain microvascular endothelial cells (HBMECs), we cultured lung tumor cells on a monolayer
of these cells. No changes in adhesion were observed when cells were co-cultured with HBMECs (Sup. fig. 5).

**Effect of DDR1 on osteoclastogenesis**

To examine the role of DDR1 in osteoclastogenesis, conditioned media from DDR1 knockdown cells and control cells treated with collagen type I for 24h were added to mouse bone marrow macrophages to study their ability to induce osteoclast formation. In the presence of macrophage CSF and RANKL, the conditioned medium from shDDR1 cells led to a significant decrease in the formation of multinucleated TRAP$^+$ cells as compared to control cells (Fig. 3D). Similar results were obtained when tumor cells were co-cultured with stromal ST-2 cells. In an initial survey several proosteoclastogenic factors were downregulated in tumor cells as a consequence of DDR1 signaling including: TGF-$\alpha$ and VEGFA (data not shown). These findings indicate that conditioned medium from DDR1 knockdown cells showed a lower osteoclastogenic potential than controls cells.

**Effect of DDR1 in vivo**

Our previous findings suggested the role of DDR1 in cell survival, migration and invasion, which are key cellular functions involved in bone metastasis. Thus, we tested the extent to which DDR1 could participate in the prometastatic activity of lung cancer cells to bone. After intracardiac (i.c.) inoculation of shDDR1, mice ($n=9$ per group) showed a significant decrease in tumor burden from day 7 to day 21, as evaluated by bioluminescence imaging (Fig. 4A). As compared to controls, shDDR1-inoculated mice showed a reduction in bone osteolytic lesions observed by X-ray imaging (Fig. 4B), a decrease in tumor burden (Fig. 4C) assessed by
histological analysis and microcomputed tomography (Fig. 4E). Consistently, trabecular bone volume was higher in animals inoculated with shDDR1 cells (Fig. 4 D). The absence of bone lesions was also associated with a delayed appearance of tumor-induced cachexia (data not shown). Taken together, these data indicate that DDR1 is required for proper prometastatic activity of lung cancer cells to the bone compartment.

To test whether the decrease in metastatic lesions was due to a lower number of metastatic tumor cells thriving in bone, mice (n=6 per group) were inoculated i.c. with shDDR1 and control cells, according to the regimen presented in Fig. 5A. At day 5, cells in the hind limbs were isolated by bone marrow flushing. Conspicuous single-cell-derived colonies (SCDCs) that were derived from each animal were counted. The number of SCDCs was dramatically decreased in hind limbs of animals inoculated with shDDR1 as compared to control-injected animals, suggesting that cells with knock down levels of DDR1 had an impaired ability to home and survive in the bone compartment.

We performed a complementary experiment using H727 cells with low endogenous DDR1 expression levels. Cells retrovirally overexpressing DDR1 levels and mock-transduced cells were i.c. inoculated in mice (n=5 per group). At day 15 post-inoculation, animals were sacrificed and cells were isolated from the hind limbs. The number of SCDCs was dramatically increased in mice inoculated with DDR1-overexpressing cells suggesting that DDR1 conferred an overt ability of bone homing (Sup Fig 6).

Next, the contribution of DDR1 to cell growth and colonization within the osseous compartment was assessed by intratibial injection (i.t.). Bioluminescence imaging showed marked differences between shDDR1 groups as compared to control inoculated mice at day 7 post-injection (Fig. 5B). Histological imaging of tibias at day 7 post-injection showed a marked
decreased in osteolytic lesions in shDDR1-injected mice as compared to control animals. Consistently, image analysis of histological sections of hind limbs showed a decreased tumor area in shDDR1-injected mice as compared to controls. Although the tumor–bone interface was decreased in shDDR1 animals, there were no obvious differences in the number of TRAP$^+$ multinucleated cells at tumor–bone interface at day 7 under all conditions (Fig. 5C).

We performed an immunohistochemical analysis of cleaved caspase-3 in osseous tumor sections at day 7 post injection. An increased number of caspase-3 positive cells were found in shDDR1 tumors as compared to control inoculated mice (Fig. 5D).

Taken together, these data suggest that DDR1 is required for effective homing and colonization in the bone compartment.
Discussion

In the present study, we showed that the receptor tyrosine kinase DDR1 was a crucial component for bone metastasis in lung cancer cells. DDR1 mediated prosurvival signals upon cell–matrix interactions \textit{in vitro}. Consequently, abrogation of DDR1 levels resulted in marked tumor cell apoptosis \textit{in vivo}, severe reduction in bone metastasis, and increased animal survival. Thus, our approach unveiled a crucial role of the collagen receptor DDR1 in the bone metastatic process and its contribution to osseous homing and colonization.

Importantly, these effects were highly dependent on the host microenvironment, because tumor growth was unaffected when cells were subcutaneously injected, independent of DDR1 levels. In contrast, survival of knocked down DDR1 cells in bone, upon direct intratibial injection, was severely impaired \textit{in vivo}. These findings acquired a marked relevance during bone colonization, where lack of DDR1 prevented cells from adapting and surviving in this foreign “soil”. Even during the early events of bone homing, decreased survival upon tumor cell–bone matrix interactions could contribute to explain our \textit{in vivo} observations. The collagen-rich microenvironment of bone could trigger the non-cell autonomous effects related to cell–matrix engagement, in agreement with \textit{in vitro} findings. Moreover, we cannot discard the possibility of higher anoikis in DDR1 knock-down circulating tumor cells, given the presence of fibrillar collagen in the serum. Consequently, this could lead to low numbers of DDR-1 knock-down cells reaching the bone. Further studies may be required to substantiate this speculation.

In addition to these findings, DDR1 could also mediate other functions, because silenced DDR1 cells showed decreased osteoclastogenic activity \textit{in vitro}. It is possible that DDR1-collagen interactions could activate other pathways, leading to the release of pro-osteoclastogenic factors. Of note, despite the marked \textit{in vitro} abrogation of osteoclastogenesis,
the number of multinucleated TRAP+ cells observed at the tumor–bone interface was similar between DDR1 knock-down and control groups. Although technically undetected, a subtle delay in osteoclast formation \textit{in vivo} could still be compatible with our findings. This delay might contribute to the deferred appearance of the osteolytic lesions observed in DDR1 knock-down injected mice. Alternatively, non-cell autonomous effects mediated by the bone microenvironment could compensate and lead to normal numbers of osteoclasts \textit{in vivo}, which clarifies the apparent discrepancy with our \textit{in vitro} results. Thus, these findings support the notion that DDR1 signaling upon collagen interaction acts through a dual mechanism that promotes tumor cell survival and osteoclastogenesis, which favors early steps of bone metastatic homing and colonization. 

Besides cell functions that are altered in the context of tumor–matrix interactions, DDR1 may also participate via cell–cell adhesion in the early stages of the metastatic process. DDR1 could promote metastatic spreading through collective cell migration at the primary site (28). These effects could be relevant in metastasis but require further proof in experimental models. 

The fact that cell–matrix interactions elicit signals that modulate cell behavior represents a recurrent theme in metastasis. Stiffness and topography of the extracellular matrix (ECM) surrounding cells exert an important role in local invasion and tumorogenesis (29, 30). It is tempting to speculate that DDR1 receptor could participate in the signaling mediated by the extracellular matrix. Similarly, other receptors such as integrins interacting with collagen, also involved in the ECM signaling, have been shown to also mediate pro-survival effects. A variety of integrins such as \( \alpha_\nu\beta_3 \) or \( \alpha_5\beta_1 \) interact with circulating collagen fragments (tumstatin and endostatin, respectively). These interactions promote antitumor effects in endothelial cells,
leading to tumor cell apoptosis (31, 32). Moreover, a β1 integrin inhibitory antibody promotes apoptosis in breast cancer cells (33).

At present, the signaling pathways elicited by DDR1 upon cell–matrix interaction remain elusive, but pro-survival effects directed by DDR1 have been shown to be mediated at least in part by functional interaction with Notch 1 (34). Activation of this pathway through Jagged-1 has been shown to induce osteolytic bone metastasis (35). In other tumor cells, DDR1 contributes to survival by activation of nuclear factor-κB and its downstream effectors cyclooxygenase-2 and X-linked inhibitor of apoptosis protein (36). In hepatoma cells, DDR1 has been shown to increase migration and invasion (37). Thus, it is tempting to speculate that DDR1 could also be implicated in other tumor types metastasizing to bone.

Our findings are in agreement with the implication of DDR1 as a direct p53 transcriptional target acting as survival effector in cells exposed to genotoxic stress (38). Taken together, these results support the therapeutic benefit of DDR1 blockade and the proof-of-concept for successful synergism with radiotherapy and conventional chemotherapy. These combinatorial regimens might offer greater efficacy with less adverse effects than conventional treatments.

In summary, our results demonstrated the requirement for the DDR1 signaling pathway for effective engagement of lung cancer cells into the bone microenvironment. Blocking this interaction dramatically decreases cell survival, invasion, proteolytic and osteoclastogenic degradation of bone. All these effects are probably responsible for the significant impairment of homing, bone metastatic colonization, and increased overall survival. Moreover, this novel component represents a key target for effective treatment of bone metastasis.
MATERIALS AND METHODS

Patients and tissue samples

The biological material used was formalin-fixed paraffin-embedded tissue sections of tumor lung. Samples were stored in their respective biobanks. A nonsmoker was defined as someone who had smoked less than 100 cigarettes in his or her life.

A series of 83 patients diagnosed with NSCLC who underwent surgical resection at the Clínica Universidad de Navarra, Pamplona, Spain, between 2000 and 2009 were included in this study. The inclusion criteria were NSCLC histology, complete resection of the primary lung tumor, no previous malignancy within the 5 years previous to surgery and no neo-adjuvant chemotherapy. Relapse time was calculated from the date of surgery to the date of detection of recurrence. Survival status was verified and updated in May 2011. A total of 47 patients were treated with surgery and 36 with surgery followed by adjuvant treatment (chemotherapy alone or radiotherapy and concomitant chemotherapy), according to pathologic findings within the resected tumor sample. The median follow-up for this cohort was 45 months. The median time of progression (PD) was 36 months. The 73.5% patients did not reach 5 years survival time with the disease. Patients who died from other causes unrelated to lung tumors were not included in the survival study. Supplementary Table 1 summarizes the characteristics of the cohort.

Immunohistochemistry

Immunohistochemistry (IHC) staining was performed in formalin-fixed paraffin-embedded tissue sections. After microwave antigen retrieval with citrate buffer (10 mM, pH 6), incubation with anti-DDR1 antibody (AdDSerotec; dilution 1:75) was carried out overnight at 4 °C. Detection was performed with ENVISION HRP system (Dako, Glostrup, Denmark). The peroxidase activity was visualized with diaminobenzidine. The specificity of the antibody was
assessed by Western blot analysis of proteins from lung tissue and siRNA technology. Negative controls were also performed by omission of the primary antibody or incubation with an isotype control antibody. Two observers evaluated the samples independently and blinded in regard to the clinico-pathological characteristics of the patients. The extension of the staining was scored as percentage of positive cells (0-100%) and the intensity of staining was assessed compared with a known external positive control (1+, mild; 2+, moderate and 3+, intense labeling) as previously described (39). Tissues expressing different levels of DDR1 were included in each immunohistochemical run to unify the possible intensity discordance. Discordant independent reading was resolved by simultaneous review by both observers. A final score was obtained by multiplying the intensity and the extension values (range, 0–300). The upper quartile of the score in each series was defined as “high” and the lower quartile was defined as “low”. REMARK criteria were followed throughout all the study (40).

**Knock-down constructs**

Lentiviral vectors containing short hairpin for DDR1 were obtained from shRNA Mission (Sigma). To obtain viral particles, packaging cells were transfected with 8 µg of plasmid by calcium phosphate method. Empty, and scramble vectors were used to obtain mock and scramble infective viral particles. Two days after transfection, supernatants were centrifuged for 10 min at 600 x g and filtered through 0.45 µm pore cellulose acetate filter. For transduction, cells were seeded at 1 x 10^5 and incubated overnight with viral supernatants in the presence of 4µg/mL polybrene (Sigma). Forty-eight hours post-infection, cell populations were incubated in medium containing the appropriate antibiotic for two additional weeks. Antibiotic resistant pools were expanded and frozen at each cell passage.
Quantitative real-time RT-PCR analysis

Measurement of DDR1 gene expression was performed with quantitative reverse-transcribed polymerase chain reaction (RT-PCR) in an ABI PRISM 7500 device (Applied Biosystems, Foster City, CA) using Taqman® as detection dye. Expression levels were normalized with β2-microglobuline as an endogenous control. Target cDNA was quantified using standard curve method. Primer sequences used for real-time PCR were as follows: 5´GCTCCCTGTGTCCTCCAAT 3´ and 5´ TGGCTTCTCAGGCTCCATA 3´.

Western blotting

Cells were stimulated with 10 µg/ml collagen type I for 24 hours and then lysed with RIPA-buffer supplemented with protease inhibitors (Sigma, St. Louis, MO). Protein lysates were subjected to 6% SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad) and incubated with a polyclonal anti-DDR1 to the carboxy-terminus (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) and PARP antibody (Cell Signalling Technology). Bands were developed with enhanced chemiluminescence (ECL) system (Amersham Bioscience, UK). The blots were reprobed with GAPDH antibody.

Collagen-induced DDR1 -Autophosphorylation

The assay was performed as described previously in detail (41). Cells were incubated with serum-free medium for 16 h and then stimulated with collagen I at 10 µg/ml or 0.1M acetic acid for 2 h at 37 °C. Cells were lysed in 1% Nonidet P-40, 150 mm NaCl, 50 mm Tris, pH 7.4, 1 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, 50 µg/ml aprotinin, 1 mM sodium orthovanadate, 5
mM NaF. Cell lysates were centrifuged at 15000 g for 15 min at 4º C and supernatants were separated and subjected to immunoprecipitation with rabbit anti-DDR1 antibody (Santa Cruz) overnight at 4º C. Immune complexes were isolated with protein A conjugated Sepharose beads (GE Healthcare). The immunoprecipitates were washed 4 times with lysis buffer. The proteins were analyzed by SDS-PAGE on 7.5% polyacrylamide gels followed by blotting onto nitrocellulose membranes. The blots were probed with mouse anti-phosphotyrosine antibody, followed by sheep anti-mouse Ig horseradish peroxidase. Detection was performed by enhanced chemoluminescence Plus (Amersham Biosciences).

To strip the blots, membranes were incubated in antibody stripping solution (Alpha Diagnostic International, San Antonio, Texas) for 10 min at room temperature. The blots were reprobed with goat anti-DDR1 antibodies followed by rabbit anti-goat Ig-horseradish peroxidase.

**In vitro assays**

**Chemotaxis assays** were performed with conditioned medium from murine BM stromal ST-2 cells as a chemoattractant placed in the lower compartment of an 8-μm pore Boyden chamber. Treatments were added to the lower wells together with conditioned medium. Cells (3.5× 10^4 per well) in serum-free medium were seeded in the upper compartment in 24-well plates (Costar, High Wycombe, UK). After 9 h, cells in the top chamber were wiped with a cotton swab, and cells in the lower compartment were fixed and stained with crystal violet. The number of migratory cells was evaluated with a computerized image analysis system, AnalySIS® (Soft Imaging System GmbH, Münster, Germany).
For the **invasion assays**, $2 \times 10^5$ cells were seeded in each well; twice for each condition in serum-free medium. The upper chamber was precoated with 0.5 µg/µL Matrigel (Sigma), dried at room temperature for 5 h, or with 50 µg/mL collagen type I (Inamed, Fremont, CA, USA), and dried at 37°C overnight. Conditioned medium from ST2 was added to the lower wells. The experiment was performed for 24 h, when inserts were collected, cells in the upper chamber were removed with cotton swabs, and cells in the lower chamber were fixed in 4% formalin, and stained with 0.4% crystalviolet. Pictures were taken using an inverted photomicroscope. Cells from 6 random fields per insert were counted.

**Adhesion** experiments were performed according to previously published protocols (42). Substrates used were fibronectin, hyaluronic acid, collagen type I and gelatin, and plastic and BSA were used as controls.

**Osteoclast differentiation**

Non-adherent bone marrow mononuclear cells were isolated from femurs and tibias of 3- to 6-week-old wild-type Balb/C (Harlan Iberica, Spain). Cells were cultured on 100 mm dishes overnight in α-MEM (Invitrogen) media supplemented with 10% fetal bovine serum (Gibco), and penicillin/streptomycin (Gibco). Non-adherent bone marrow mononuclear cells were collected and erythrocytes were lysed with Red Blood Cell Lysis Buffer (Bioscience) 5 min. at 37°C. Cells were seeded in 48 Well Plates at 2x106 cells/mL per well, were grown for 24 hours on α-MEM supplemented in the presence of 20 ng/mL recombinant mouse M-CSF (R&D Systems) and of 40 ng/mL human sRANKL (Peprotech) to generate osteoclast precursors (hereafter also called BMM). Conditioned media were added to BMMs for 6 days.
and subsequently the cells were subjected to TRAP staining (Sigma, St Louis, MO), according to the manufacturer’s instructions.

**Osteoclast number**

TRAP was performed by histochemical analysis using Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich, Barcelona, Spain). TRAP positive multinucleated osteoclasts were identified at the interface between tumor and cortical bone, and counted at 400 x magnification. Eight to ten fields per bone of each animal were quantified. Data are shown as a percentage of osteoclasts counted into the tumor-bone interface’s area per each group.

**In vivo assays**

Female athymic nude mice (Harlan Iberica, Spain) were maintained under specific pathogen-free conditions. For intracardiac injection (i.c.), cells were seeded at 50% confluence one day before injection. Cells were gently washed with PBS, detached, and resuspended at 2×10^6 cells/ml in sterile PBS. All cell suspensions showed ≥95% cell viability, otherwise cells were discarded. Mice (n=8/group) were anaesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg of body weight) before injection. An exact amount of 100µl containing 2×10^5 cells were injected into the left cardiac ventricle of the 4-week old mice (Harlan Iberica, Spain) using a 29-G needle syringe. All the animals were sacrificed according to the approved protocols of the Local Animal Committee (University of Navarra, protocol 003-04).

For intratibial injection (i.t.), cells were suspended at 10×10^6 cells/ml in sterile PBS. Mice (n=6/group) were anaesthetized before injection. Five µl containing 2.5×10^5 cells were
injected into the tibia’s bone marrow through femoro-tibial cartilage of the 6-week old mice (Harlan Iberica, Spain) using a Hamilton syringe.

**Isolation of Single Cell-derived colonies (SCDC) from long bones**

Metastatic cells were isolated from bone marrow of lower limbs. Long bones were excised, and cleaned of all soft tissues. Marrow cells were released by “flushing”, introducing 5-10 ml of α-MEM medium containing 2 x penicillin/streptomycin with a 27 gauge needle in the distal epiphysis through the bone marrow compartment. Cell clumps were disaggregated by passing medium containing cells through 27 G needle syringe. Cells were plated in 10 or 15 cm dishes expanded for 5 days in medium containing 0.4 mg/ml G-418. This procedure was performed separately for each femur and tibia of 7 mice per group. SCDCs were counted under light microscope after crystal violet staining.

**Statistical analysis**

Statistical analysis was performed using SPSS 15.0. To study differences in proliferation rates, invasion and migration assays, differences in metastatic area, number of osteoclasts, SCDC number, adhesion to cell monolayers or precoated wells, caspase-3 staining and MMP activity, data were analyzed by parametric test (Anova) or non-parametric homologue Kruskal–Wallis test depending on data distribution. Multiple comparisons were studied with Dunnet’s test or U Mann-Whitney adjusted by Bonferroni test. Kaplan-Meier curves were performed to study the survival rate. Values were expressed as means ± SEM and statistical significance was defined as p<0.05 (*), p<0.01 (**), and p<0.001 (***)
Figure Legends

**Figure 1.** A. Western blot analysis of DDR1 in lysates of normal lung epithelial and lung tumor cell lines using specific anti-DDR1 antibody. B. Immunoprecipitation of DDR1 from cell lysates of human tumor cell lines, after cell incubation with collagen I (+) or vehicle control (-) for 2 hours at 37°C. The blots were probed with anti-phophotyrosine antibody and subsequently reprobed with anti-DDR1. C. Upper panel, Representative immunohistochemical staining of human lung tumors expressing different DDR1 levels. Bar=50 µm. Lower panels, Kaplan-Meier curves of survival for high and low levels of DDR1 assessed by immunohistochemistry.

**Figure 2.** Effects of knockdown of DDR1 levels on proliferation and apoptosis. A. Gene expression levels of DDR1 were downregulated by using two lentiviral transduced shRNAs targeting different two different sequences (-58 and -87), in the H460 cell line. B. Upper panel, Western blot analysis of DDR1 in shDDR1, scramble-transduced and parental cells. Cells were treated with collagen type I (10µg/mL) for 24 h to activate DDR1. shDDR1–87 achieved the best knock-down effect. Lower Panel, Immunoprecipitation of DDR1 from cell lysates of cells incubated with (+) or without (-) collagen for 2h. The blots were probed with anti-phophotyrosine antibody and subsequently reprobed with anti-DDR1 antibody. (C- and C+ denote negative and positive controls). C. Left panel, Cell proliferation was assessed by MTT assay in H460 cells. Right panel, Tumor growth after subcutaneous injection of tumor cells shows no differences. D. Apoptosis was studied in presence/absence of collagen type I by PARP cleavage. The slight protection against apoptosis conferred by collagen type I was ablated in shDDR1 cells. E. Etoposide induced apoptosis in the presence/absence of collagen type I was
prominent in the absence of DDR1 receptors. Cells were incubated with 100 µM etoposide. F. TRAIL (5 ng/mL) incubation for 3 h induced a marked apoptosis in shDDR1 cells as compared to control and scramble cells.

**Figure 3.** Effects of knockdown of DDR1 levels on adhesion, invasion and chemotaxis. A. Chemotactic assay of shDDR1 cells induced by conditioned medium (CM) derived from ST-2 cells treated or untreated with collagen type I showed a significant decrease in cells with complete abrogation of DDR1 levels as compared to scramble cells. B. Invasion assay with collagens type I and IV of shDDR1 cells as compared to scramble and control cells showed decreased invasion in shDDR1 transduced cells. C. Adhesion assay of shDDR1 and control cells to collagen types I and IV showed no differences. D. Osteoclastogenesis assay was performed treating DDR1 knockdown and control cells with collagen type I for 24h. CM of tumor cells induced a significant decrease in the number of multinucleated TRAP+ cells. The experiment was repeated three times with similar results.

**Figure 4.** Effect of DDR1 in bone metastasis in vivo. A. Cells with knocked down DDR1 levels or scramble and vector-transduced cells were inoculated into the left cardiac ventricle of athymic nude mice. (Top) Representative bioluminescence images. (Bottom) Quantification of photon flux at day 7 post-inoculation. B. Quantification of osteolytic bone area of X-ray imaging at day 12 post-inoculation. C. Tumor burden assessment in histological sections. D. Quantification of bone volume over tissue volume (% BV/TV) at day 12 postinjection. E. Representative images of X-ray, microcomputed tomography scans, and histological sections (H&E stained) showing the dramatic decrease in bone metastasis in knocked down DDR1 cells.
**Figure 5.** Ablation of lung cancer bone metastatic homing and colonization by knock down of DDR1 levels. **A.** Experimental regimen of bone homing assay showing that, after i.c. inoculation, mice were sacrificed on day 5, and hind limbs were flushed and cells isolated and cultured. *Top,* Representative images of bioluminescence in hindlimbs. *Bottom.* A dramatic decrease in single cell-derived colonies (SCDC) derived from shDDR1 cells was observed. Statistical differences were observed between control and shDDR1 cells (*P* < 0.05). **B.** Experimental regimen of bone colonization assay after intratibial injection of cells. *Left.* Representative photon flux images, X-ray and H&E histological sections of tumor-bearing mice. *Right, upper panel,* Quantification of bioluminescence (representatives images are in the left panel) showed a decrease in shDDR1-injected animals. *Middle panel,* Quantification of osteolytic lesions in shDDR1-injected animals showed a decreased tumor burden in the metaphysis. *Lower Panel,* Bone tumor burden was quantified by image analysis at day 7 post-injection. **C.** TRAP$^+$ multinucleated cells were counted at the tumor–bone interface. **D.** Expression of cleaved caspase-3 was studied at day 7 in the tumor area. Representative images are shown.
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REFERENCES


Figure 1

(A) Western Blot analysis of DDR1 and GAPDH expression in NSCLC and SCLC cell lines.

(B) Immunoblot analysis of collagen, P-Tyr, and DDR1 in different cell lines.

(C) Immunohistochemical staining of low and high DDR1 expression in tissue samples.

Graphs showing the survival analysis of non-treated patients, stage I patients, and non-treated stage I patients.

Valencia et al. Figure 1
Figure 3

A. Area of Migration (%)

B. Invaded Area (%)

C. Abs (595-700nm)

D. Osteoclast area/Total area %
Figure 4

A

Normalized photon flux (p/s)

Day 7 after injection

Vector Scramble sh58 sh87

B

Log Metastatic Area/Total Bone Area (%)

Vector Scramble shDDR1-58 shDDR1-87

C

Bone Tumor Burden (%)

Vector Scramble shDDR1-58 shDDR1-87

D

% BV / TV

Vector Scramble shDDR1-58 shDDR1-87

E

Image
Figure 5

A. i.c. H460

B. i.t. H460

C. Graph showing Total Number SCDGx

D. Graph showing TRAP+ Tumor-Bone Interface and Caspase-3 Expression

Valencia et al. Figure 5
Inhibition of collagen receptor Discoidin Domain Receptor-1 (DDR1) Reduces Cell Survival, Homing and Colonization in Lung Cancer Bone Metastasis

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