

# Gefitinib inhibits the ability of human bone marrow stromal cells to induce osteoclast differentiation: implications for the pathogenesis and treatment of bone metastasis

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## Abstract

Significant relief of bone pain in patients with bone metastases was observed in a clinical trial of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib in breast cancer. Osteoclast activation and differentiation are regulated by bone marrow stromal cells (BMSC), a heterogeneous cell compartment that comprehends undifferentiated mesenchymal stem cells (MSC) and their specialized progeny. In this regard, we found that human primary BMSCs express immunoreactive EGFR. Expression of EGFR mRNA and protein was also demonstrated in two human, continuous MSC-like cell lines, HDS-1 and HDS-2 cells. Treatment of HDS cells with EGF produced a significant increase in the levels of activated EGFR which was not observed in the presence of gefitinib. A significant reduction in the basal levels of activation of the EGFR and of Akt was observed in HDS cells following treatment with gefitinib. Treatment of HDS cells with gefitinib produced a significant reduction in the levels of secreted macrophage colony-stimulating factor (M-CSF) and cell-associated receptor activator of NF- $\kappa$ B ligand (RANKL) in both cell lines, as assessed by using specific ELISA and Western blotting techniques. Finally, the ability to sustain the differentiation of pre-osteoclasts of conditioned medium from gefitinib-treated HDS cells was reduced by approximately 45% as compared with untreated HDS cells. These data have demonstrated for the first time that the EGFR regulates the ability of BMSCs to induce osteoclast differentiation and strongly support clinical trials of gefitinib in breast cancer patients with bone disease.

*Endocrine-Related Cancer* (2005) 12 471–482

## Introduction

It has long been established that the epidermal growth factor receptor (EGFR) and its ligands are involved in the pathogenesis of breast carcinoma (Normanno *et al.* 2001). Unfortunately, clinical studies of EGFR tyrosine kinase inhibitors (EGFR-TKIs) as monotherapy in breast cancer patients have shown disappointing results (Normanno *et al.* 2003a, Arteaga & Truica 2004). For example, gefitinib, an EGFR-TKI that was

able to significantly suppress the *in vitro* and *in vivo* growth of breast cancer cells in pre-clinical studies, produced an approximately 10% disease control rate in patients with advanced breast carcinoma (Normanno *et al.* 2004). However, an unexpected phenomenon was observed in one of these trials. Albain *et al.* (2002) enrolled 12 patients with bone metastases and bone pain in their study of gefitinib in breast cancer. Surprisingly, five out of the 12 patients had a significant relief of bone pain, leading to the complete

withdrawal of all scheduled narcotics in several cases. Due to the impressive effects on bone pain palliation, two patients were maintained on gefitinib despite objective progression of the disease. Interestingly, a significant improvement in bone pain has also been reported in a patient enrolled in a different trial of gefitinib in metastatic breast cancer (von Minckwitz *et al.* 2003).

The development and progression of bone metastases are complex phenomena involving tumor cell interaction with different cell types of the bone microenvironment (Roodman 2001). Although it has been shown that breast cancer cells can directly resorb bone, evidence suggests that the main mechanism responsible for bone destruction in cancer patients is tumor-mediated stimulation of osteoclastic bone resorption (Roodman 2001). Osteoclasts represent the specialized progeny of hemopoietic precursors committed to the monocyte/macrophage lineage which, upon appropriate stimuli, fuse by giving rise to mature bone-resorbing cells (Boyle *et al.* 2003). Two main factors that are involved in osteoclast activation and formation have been identified: macrophage colony-stimulating factor (M-CSF), which induces proliferation and differentiation of pre-osteoclast cells, and receptor activator of NF- $\kappa$ B ligand (RANKL) which is involved in fusion and activation of these cells (Boyle *et al.* 2003). Activation of osteoclasts is also regulated by osteoprotegerin (OPG), a soluble decoy receptor of RANKL which functions by sequestering the secreted form of RANKL and, therefore, preventing the binding to its cognate receptor (Boyle *et al.* 2003).

Breast cancer cells are able to synthesize many growth factors and cytokines that can lead to the activation of osteoclasts (Pederson *et al.* 1999). In particular, parathyroid hormone-related protein (PTHrP) is believed to be the main mediator of breast cancer-induced bone resorption (Burtis *et al.* 1990). However, most osteotropic factors act indirectly by binding to accessory cells of the bone marrow microenvironment (Roodman 2001). This heterogeneous cell compartment comprehends specialized endothelial cells, as well as mesenchymal stem cells (MSC), which maintain a level of self renewal and give rise to different specialized connective tissue cells such as 'reticular cells', osteoblasts, chondrocytes, adipocytes and smooth muscle cells (Clark & Keating 1995, Deans & Moseley 2000). MSCs and their progeny are collectively referred to as the bone marrow stromal cell (BMSC) compartment which is known to support hemopoiesis. BMSCs also play a critical role in osteoclast formation. In particular, both marrow stromal cells and osteoblasts have been found to

support osteoclast differentiation (Takahashi *et al.* 1988, Udagawa *et al.* 1989). A recent report has also demonstrated that MSCs play a critical role in bone osteolysis, since MSCs are induced by cancer cells to produce interleukin (IL)-6 which activates osteoclasts (Sohara *et al.* 2005). In this respect, stromal cells and osteoblasts that induce osteoclast formation have been shown to produce M-CSF (Kodama *et al.* 1991). Furthermore, osteotropic factors such as PTHrP induce the expression of RANKL in BMSCs (Horwood *et al.* 1998, Thomas *et al.* 1999). More recently, RANKL has been shown to increase, in BMSCs, the production of IL-6 which, in turn, can up-regulate RANKL and down-regulate OPG in osteoblasts through prostaglandin E2 (Giuliani *et al.* 2004, Liu *et al.* 2004).

In this study, we investigated the molecular mechanisms that might be involved in the effect of gefitinib on metastases-related bone pain. Since osteoclasts do not express the EGFR and bone pain relief was achieved in patients undergoing tumor progression, it is unlikely that the effects of gefitinib on bone pain are related to any direct activity of this drug on either breast cancer cells or mature osteoclasts (Tanaka *et al.* 1998). We therefore investigated the expression and functional role of the EGFR in MSC-like cells, and the effects of gefitinib on their ability to produce factors that promote and sustain osteoclastogenesis.

## Materials and methods

### Continuous cell lines of MSC-like phenotype

Two continuous stromal-like cell lines, HDS-1 and HDS-2, were established from the bone marrow of two patients with Hodgkin's disease. These spontaneously outgrowing untransformed cell lines do not harbor viral genomes and display a complex phenotypic profile (CD45<sup>-</sup>, CD34<sup>-</sup>, CD31<sup>-</sup>, CD14<sup>-</sup>, vWF<sup>-</sup>, T- and B-cell antigens, smooth-muscle-specific actin<sup>-</sup>, p75 NGF-R<sup>+</sup>, SH2<sup>+</sup>, SH3<sup>+</sup>, SH4<sup>+</sup>, CD10<sup>+</sup>, CD90<sup>+</sup>, CD105<sup>+</sup>, CD106<sup>+</sup>) consistent with pure stromal/MSC-like cells. HDS cells are able to differentiate into adipocyte- and osteoblast-like cells, and to support growth and differentiation of purified human CD34<sup>+</sup> progenitors (D Aldinucci, N Normanno & A Pinto, unpublished observation).

### Cell culture and anchorage-dependent growth assays

HDS-1 and HDS-2 cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Biocrom KG, Berlin, Germany) supplemented with 10%

heat-inactivated fetal calf serum (FCS; Biocrom KG). For the anchorage-dependent growth assays, HDS cells ( $10 \times 10^4$ ) were seeded in 48-well plates in serum-containing medium. After 24 h, the medium was replaced with either serum-containing or serum-free medium, and cells were treated for 3 days with different concentrations of gefitinib (Astrazeneca, Macclesfield, UK). Cells were then trypsinized and counted with an automatic cell counter (Coulter Model Z1; Instrumentation Lab., Milan, Italy).

### Bone marrow biopsies and immunohistochemistry

Bone marrow biopsies were obtained upon informed consent from healthy donors during bone marrow-harvesting procedures for allogeneic transplantation. Immunohistochemical detection of the EGFR in bone marrow biopsies was performed using the anti-EGFR rabbit polyclonal antibody 1005 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as previously described (D'Antonio *et al.* 2002).

### RT-PCR

Total cellular RNA was obtained by the acid guanidine isothiocyanate–phenol–chloroform extraction procedure. RT-PCR analysis for EGFR, transforming growth factor- $\alpha$  (TGF $\alpha$ ) and amphiregulin (AR) expression was performed as previously described (De Luca *et al.* 1999).

### Western blot

Whole protein extracts were prepared and analyzed by Western blotting using the chemiluminescence ECL Western blotting kit (Amersham, Milan, Italy) as previously described (Normanno *et al.* 1996). The following antibodies were used: (a) rabbit polyclonal anti-EGFR antibody 1005 (Santa Cruz Biotechnology); (b) rabbit polyclonal anti-phospho p44/p42 MAP kinase (MAPK; New England Biolabs, Beverly, MA, USA); (c) rabbit polyclonal anti p44/p42 MAPK (New England Biolabs); (d) rabbit polyclonal anti-phospho Akt antibody (New England Biolabs); (e) rabbit polyclonal anti-Akt antibody (New England Biolabs); (f) rabbit polyclonal anti-RANKL FL-317 (Santa Cruz Biotechnology); (g) goat polyclonal anti-actin (Santa Cruz Biotechnology); (h) mouse monoclonal anti-phosphotyrosine PY-20 (BD Biosciences, Milan, Italy). Densitometric analysis of the blots was performed by using the NIH Image 1.62f software (National Institutes of Health, Bethesda, MD).

### EGFR phosphorylation

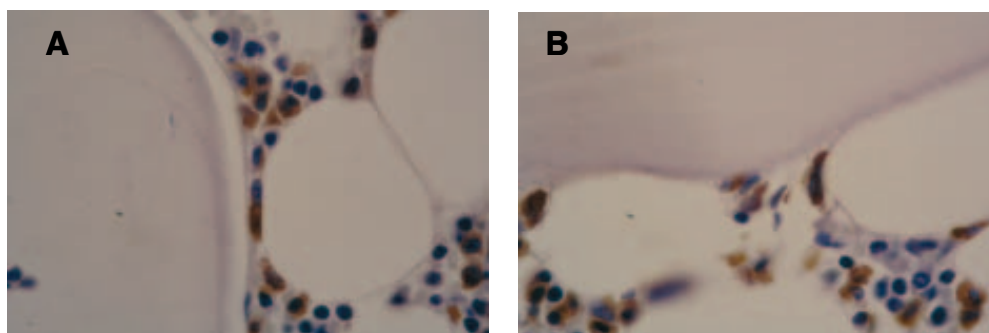
In order to assess the effects of exogenous stimuli on the activation of the EGFR in HDS cells, cell lines were cultured for 48 h in serum-free medium, and subsequently treated with recombinant epidermal growth factor (EGF; 50 ng/ml) (BD Biosciences) for 10 min in the absence or presence of gefitinib (4  $\mu$ M). The concentration of phosphorylated EGFR was measured by using the Active EGFR EIA kit (Takara Biomedicals, Tokyo, Japan) following the manufacturer's instructions, and the concentration was expressed as the amount of phosphorylated EGFR (fmol/ml) per  $1 \times 10^7$  cells. Alternatively, the phosphorylation of the EGFR in EGF-stimulated cells was assessed by using an immunoprecipitation/Western blot technique. For this purpose, cells were incubated at 4 °C with lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 2 mM Na-orthovanadate, 10 mM leupeptin, 100 mM Na-fluoride and 10 mM Na-pyrophosphate), and protein extracts were immunoprecipitated for 12 h using the EGFR 1005 antibody (Santa Cruz Biotechnology) and 40  $\mu$ l protein-A/G sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Precipitates were then separated on 8% SDS-PAGE, transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech) and probed with the anti-phosphotyrosine PY-20 antibody (BD Biosciences). Finally, the effects of gefitinib on the basal levels of activation of the EGFR in HDS cells were investigated by using the Active EGFR EIA kit, as described above. In these experiments, HDS cells were cultured in serum-containing medium for 48 h in the presence or absence of 4  $\mu$ M gefitinib before protein extraction.

### Flow cytometry

An indirect immunofluorescence assay was performed on live cells using the specific anti-EGFR monoclonal antibody 528 (Santa Cruz Biotechnology). Cells were incubated with antibodies (10  $\mu$ g/ml) for 30 min at 4 °C, washed twice and then incubated with FITC-labeled goat anti-mouse (1:100) (Kirkegaard Perry Laboratories Inc., Gaithersburg, MD, USA) for 30 min at 4 °C. Cells were washed again and re-suspended in phosphate-buffered saline (PBS). Fluorescence was evaluated by a FACScan using LYSIS II software (Becton Dickinson, Mountain View, CA, USA).

### Preparation of conditioned media and ELISA

HDS cells were treated with gefitinib (4  $\mu$ M) for 48 h in serum-containing medium. Cells were then washed



**Figure 1** Immunohistochemical detection of EGFR in two bone marrow biopsies. Magnification  $\times 400$ .

twice with PBS and cultured for 16 h in serum-free medium. The conditioned media were collected, filtered with  $0.22\ \mu\text{M}$  syringe filters and stored in aliquots at  $-80^\circ\text{C}$ . M-CSF and IL-6 concentrations were respectively assessed using the Quantikine Human M-CSF Immunoassay kit and the Quantikine Human IL-6 Immunoassay kit (R&D, Minneapolis, MN, USA), whereas OPG levels were determined using the Osteoprotegerin Assay Kit (Kamiya Biomedical Company, Seattle, WA, USA), following the manufacturers' instructions. The levels of M-CSF and OPG in conditioned media from untreated and gefitinib-treated HDS cells were normalized by mg secreted proteins.

### **In vitro osteoclast differentiation**

Osteoclast precursors (Cambrex, Walkersville, MD, USA) were seeded in eight-well chamber slides (BD Biosciences). Cells were subsequently cultured for 14 days with recombinant RANKL (66 ng/ml) plus recombinant M-CSF (33 ng/ml), or M-CSF alone, in serum-containing osteoclast precursor basal medium (OPBM), as suggested by the manufacturer (Cambrex). Additional wells were incubated for the same period with conditioned media from untreated and gefitinib-treated HDS cells, diluted 1:1 with serum-containing OPBM medium. Cells were then stained for tartrate-resistant acid phosphatase (TRAP) using a specific kit (Sigma-Aldrich, Milan, Italy). The number of multinucleated ( $\geq$  four nuclei) TRAP-positive cells was assessed.

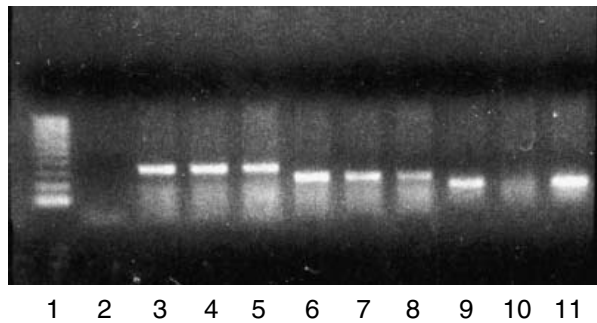
## **Results**

Expression of EGFR has been previously demonstrated in human BMSC strains (Satomura *et al.* 1998). In order to confirm these findings, we performed immunohistochemical analysis for EGFR expression in primary human BMSCs of bone marrow biopsies

from ten healthy bone marrow donors. Expression of immunoreactive EGFR in interstitial stromal cells and in stroma cells lying in the proximity of bone trabecolae was found in all the specimens examined (Fig. 1). Occasional staining of erythroblast precursors was also observed, in agreement with previous findings (Pain *et al.* 1991).

In order to address the functional role of EGFR in BMSCs, we chose HDS-1 and HDS-2 cell lines as our model. These cell lines are untransformed continuous, spontaneously outgrowing MSC-like cell lines that display morphologic, immunophenotypic and functional characteristics consistent with pure stromal/mesenchymal cells. Interestingly, it has been shown that human MSCs positively regulate osteoclastogenesis as undifferentiated progenitor cells, and that osteoclasts in the bone marrow are mostly found close to spindle-shaped MSC-like cells (Mbalaviele *et al.* 1999). Furthermore, the role of MSCs in bone osteolysis has been confirmed in a recent report in which MSCs have been shown to mediate the activation of osteoclasts induced by neuroblastoma cells (Sohara *et al.* 2005). Therefore, HDS cells may represent an ideal model to study the role of stromal cells in osteoclast development and regulation. RT-PCR was used to investigate the expression of EGFR and its ligands in both HDS cell lines. HDS-1 and HDS-2 cells were found to express specific transcripts for both EGFR and  $\text{TGF}\alpha$  (Fig. 2 and Table 1). AR mRNA was detected in HDS-2 cells, but not in the HDS-1 cell line.

The expression of EGFR protein in HDS cells was then assessed by Western blot analysis (Fig. 3A). Both cell lines were found to express EGFR, with the HDS-2 cells showing levels of protein that were five- to tenfold higher than in the HDS-1 cell line. The expression of EGFR on the cell membrane of HDS cells was confirmed by flow cytometry. High levels of membrane-bound EGFR were detected in HDS-2 cells following



**Figure 2** Expression of EGFR, TGF $\alpha$  and AR mRNAs in HDS cells and in GEO colon carcinoma cells. Lane 1, marker 100 bp ladder; lane 2, negative control; lane 3, EGFR expression in HDS-1 cells; lane 4, EGFR expression in HDS-2 cells; lane 5, EGFR expression in GEO cells; lane 6, TGF $\alpha$  expression in HDS-2 cells; lane 7, TGF $\alpha$  expression in HDS-1 cells; lane 8, TGF $\alpha$  expression in GEO cells; lane 9, AR expression in HDS-2 cells; lane 10, AR expression in HDS-1 cells; lane 11, AR expression in GEO cells.

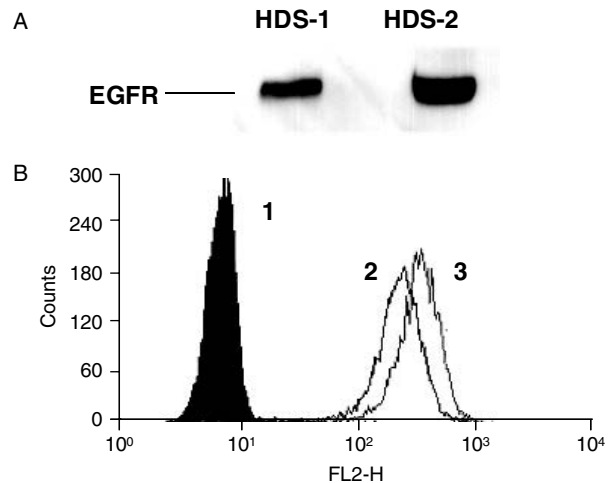
detachment of cells from plastic with either PBS-EDTA or trypsin (Fig. 3B).

To address whether EGFR expressed in HDS cells is functionally active, we cultured HDS-1 and HDS-2 cell lines in serum-free medium for 48 h. HDS cells were subsequently treated with recombinant EGF in the presence or absence of gefitinib. The levels of phosphorylation of EGFR were assessed using an ELISA kit specific for the activated form of EGFR, as described in the Materials and methods. HDS cells cultured in serum-free medium showed very low levels of EGFR phosphorylation (Fig. 4A). Treatment with EGF produced a significant increase in the levels of activated EGFR in both cell lines. Such an increase was not observed when HDS cells were incubated with 4  $\mu$ M gefitinib prior to treatment with EGF. These results were confirmed by using an immunoprecipitation/Western blot technique in HDS-2 cells (Fig. 4B). No phosphorylation of EGFR was detectable in serum-starved HDS-2 cells. Treatment with EGF produced a significant increase in EGFR phosphorylation that was completely suppressed by gefitinib.

When HDS-1 cells were grown in serum-containing medium, treatment for 3 days with concentrations of gefitinib up to 8  $\mu$ M did not produce any significant

**Table 1** Expression of EGFR and its ligands in HDS cells

	HDS-1	HDS-2
EGFR	+	+
TGF $\alpha$	+	+
AR	-	+

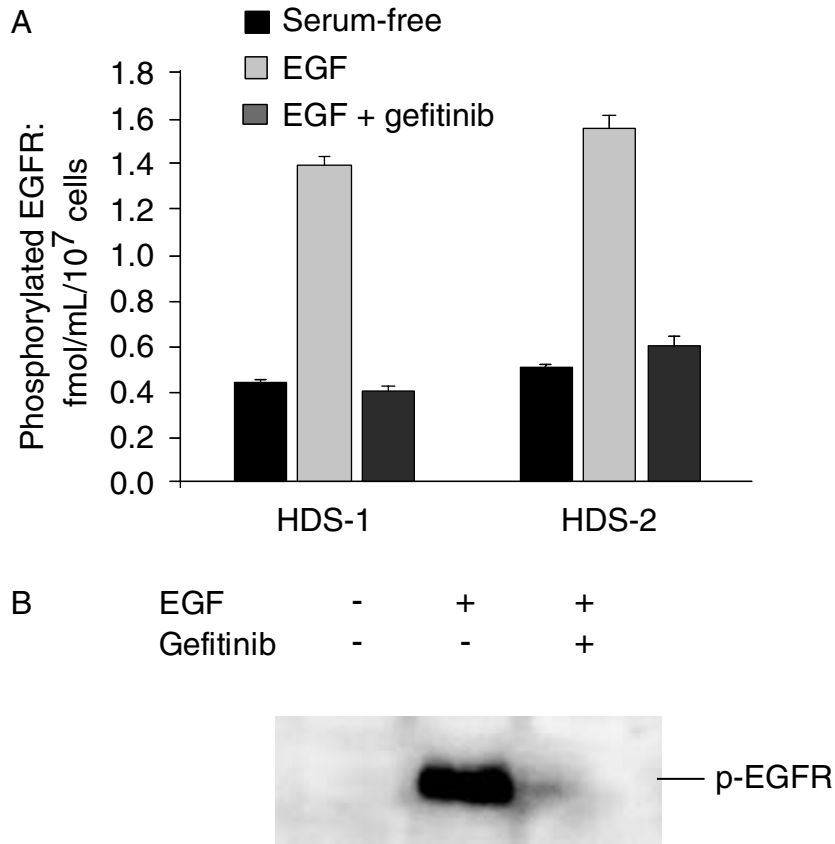


**Figure 3** EGFR expression in HDS cells. (A) Western blot analysis in HDS-1 and HDS-2 cells and (B) flow cytometry in HDS-2 cells. Peak 1, HDS-2 cells detached with trypsin and stained with an irrelevant control IgG; peak 2, cells detached with PBS-EDTA and stained with the anti-EGFR 528 antibody; peak 3, cells detached with trypsin and stained with the anti-EGFR 528 antibody.

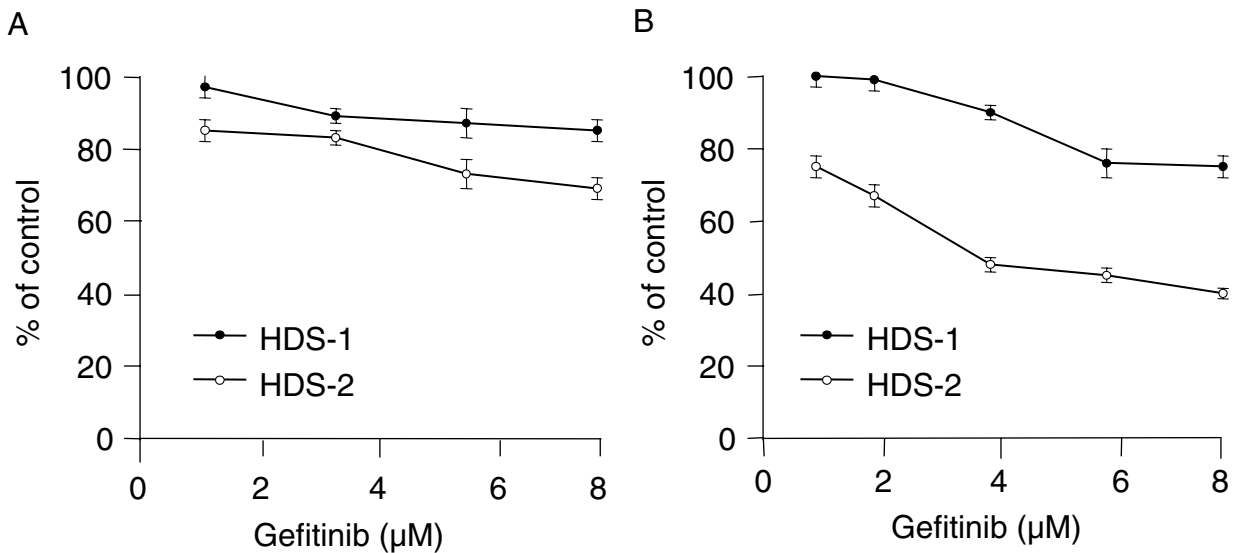
effect on their cell proliferation (Fig. 5A). An approximately 25–30% growth inhibition was observed in HDS-2 cells treated with gefitinib at concentrations ranging between 6 and 8  $\mu$ M in the presence of serum (Fig. 5A). A more significant growth inhibition was observed when HDS-1 and HDS-2 cells were switched to serum-free medium following treatment with gefitinib (Fig. 5B). In particular, the growth of HDS-2 cells was inhibited by approximately 50% following treatment at gefitinib concentrations ranging between 4 and 8  $\mu$ M.

The effects of gefitinib on the basal levels of activation of EGFR and on its downstream signaling in HDS cells were assessed. Treatment with gefitinib (4  $\mu$ M for 48 h) produced a significant reduction in the basal levels of EGFR phosphorylation in both HDS cell lines cultured in the presence of serum (Fig. 6). In agreement with these findings, a significant reduction in the levels of activation of Akt was observed in both HDS cell lines following treatment with gefitinib (Fig. 7). A slight reduction in the levels of activation of p42/p44 MAPK was also observed in gefitinib-treated HDS-1 cells, but not in HDS-2 cells (Fig. 7).

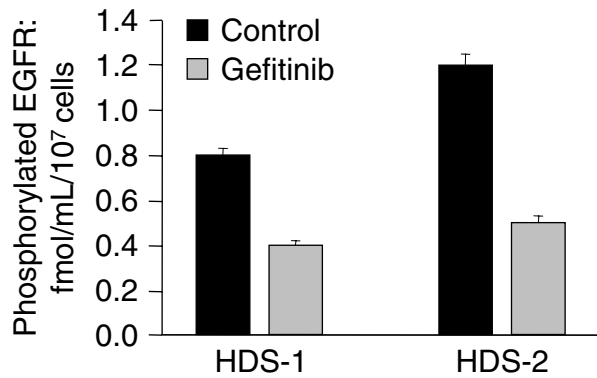
We then investigated the effects of gefitinib treatment on the ability of HDS cells to produce osteoclastogenic factors. Both HDS cell lines produced M-CSF as assessed using a specific ELISA kit (Fig. 8A). Treatment of HDS cells with gefitinib (4  $\mu$ M) for 48 h produced a significant reduction in



**Figure 4** Effects of EGF treatment on the activation of EGFR in HDS cells in the presence or absence of gefitinib. (A) Levels of phosphorylated EGFR were detected with a specific ELISA in both HDS cell lines and (B) immunoprecipitation/Western blot analysis in HDS-2 cells. p-EGFR, phosphorylated EGFR.



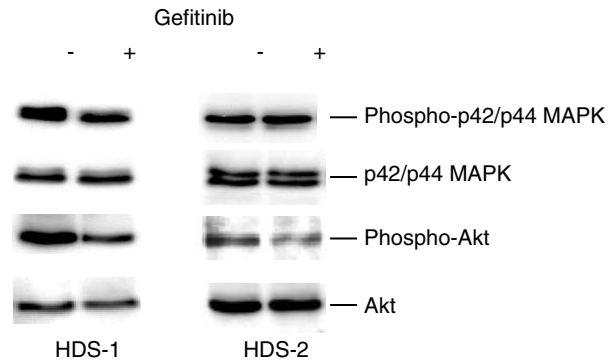
**Figure 5** Effects of gefitinib on the growth of HDS cells cultured in the (A) presence or (B) absence of serum.



**Figure 6** Effects of gefitinib on the basal levels of activation of EGFR in HDS cells cultured in serum-containing medium. Cells were treated with 4  $\mu$ M gefitinib for 48 h in serum-containing medium.

the levels of M-CSF secreted by both cell lines ( $P < 0.001$ ; Student's *t*-test). Analogously, Western blot analysis showed that the levels of cell-associated RANKL were significantly reduced in HDS cells following treatment with gefitinib compared with untreated cells (Fig. 8D and E) ( $P < 0.05$ ; Student's *t*-test). Interestingly, no significant changes were observed following gefitinib treatment in the ability of HDS cells to secrete the RANKL decoy receptor OPG (Fig. 8B). Although IL-6 mRNA was detected in both HDS cell lines, the levels of IL-6 secreted by HDS-1 cells were below the threshold of sensitivity of the kit that we used (data not shown). In HDS-2 cells, treatment with gefitinib produced an approximately 22% reduction in the levels of secreted IL-6 (Fig. 8C). This difference was statistically significant ( $P < 0.05$ ; Student's *t*-test). Gefitinib therefore had selective effects on the ability of HDS cells to produce factors involved in osteoclastogenesis: a significant inhibitory effect on M-CSF and RANKL production, a marginal inhibitory effect on IL-6 and no effect on OPG.

Finally, we evaluated whether treatment with gefitinib might affect the ability of HDS cells to induce osteoclast differentiation. For this purpose, osteoclast precursors were incubated with either RANKL + M-CSF or M-CSF alone for 14 days, as described in the Materials and methods. When cultured in the presence of M-CSF, osteoclast precursors were able to form a limited number of multinucleated/TRAP-positive cells, i.e. mature osteoclasts (Fig. 9B and E). Following treatment with both M-CSF and soluble RANKL, a significant increase in the number of mature, multinucleated and TRAP-positive osteoclasts was observed (Fig. 9A and E). Culture of osteoclast precursors in the conditioned medium of HDS-2

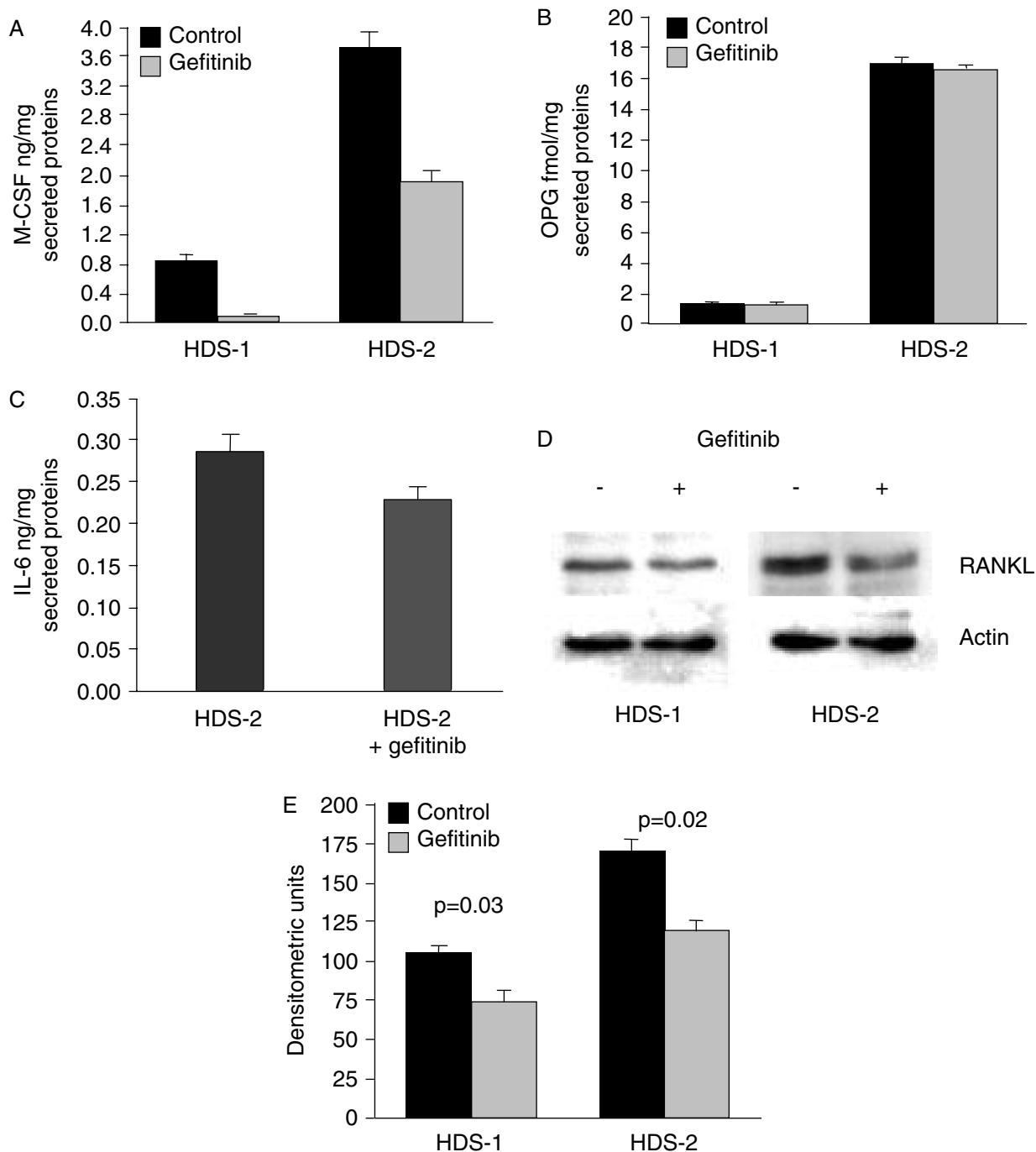


**Figure 7** Western blot analysis for the expression of the total and activated forms of Akt and p42/p44 MAPK in gefitinib-treated and untreated HDS cells. Cells were treated as indicated in Fig. 6.

cells induced the formation of a significant number of mature osteoclasts (Fig. 9C and E). This phenomenon was also observed for the conditioned medium of HDS-1 cells, although to a lesser extent (Fig. 9E). These findings are in agreement with the observation that HDS-1 cells produce lower levels of M-CSF and RANKL compared with HDS-2 cells (Fig. 8). Culture of osteoclast precursors in the conditioned media of HDS cells pretreated for 48 h with gefitinib resulted in an approximately 45% reduction in their ability to induce osteoclast differentiation (Fig. 9D and E). In particular, a reduced number of large multinucleated cells and an increase in the number of immature osteoclasts was observed (Fig. 9D). This difference was statistically significant ( $P < 0.001$ ; Student's *t*-test).

## Discussion

The present study is the first to demonstrate that the EGFR pathway regulates the production of pro-osteoclastogenic factors in human MSC-like cells and their ability to induce osteoclast differentiation. MSC is the main cell type involved in osteoclast physiology, both promoting their development from CD34+ hemopoietic progenitors and inducing their terminal differentiation into bone-resorbing cells (Mbalaviele *et al.* 1999). Our data therefore strongly support the hypothesis that the effect of gefitinib on metastatic bone pain is due to its ability to interfere with MSC-regulated osteoclast-mediated bone resorption. Since the majority of human primary breast carcinomas have been demonstrated to produce high levels of EGFR ligands such as TGF $\alpha$  and AR, it is conceivable that activation of EGFR in MSC by tumor-derived EGF-like growth factors might occur in the majority of breast cancer patients (Normanno *et al.* 2001).

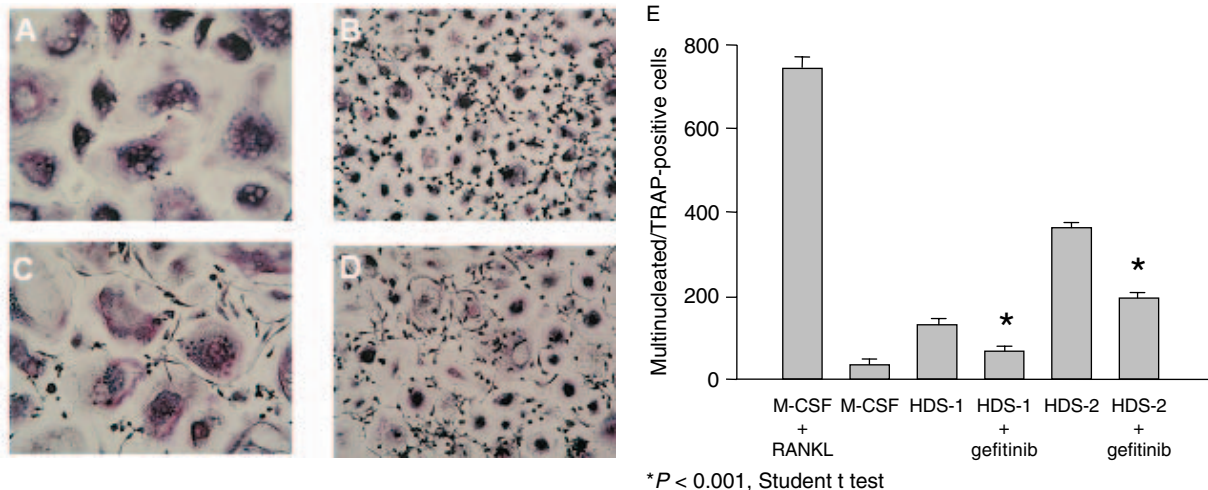


**Figure 8** Levels of secreted (A) M-CSF, (B) OPG and (C) IL-6 in the conditioned medium from untreated and gefitinib-treated HDS cells. (D) Western blot analysis for RANKL and actin expression in untreated and gefitinib-treated HDS cells. (E) Densitometric analysis of the blots for RANKL shown in (D). The densitometric values were normalized for the relative content of actin.

Several previous findings are in agreement with our hypothesis that the EGFR pathway has an important role in regulating the ability of MSCs and their

progeny to induce osteoclast formation and activation. Although osteoclasts have been shown to lack EGFR expression, addition of recombinant TGF $\alpha$  to long-term





**Figure 9** Induction of osteoclast differentiation. Pre-osteoclasts were incubated for 14 days with (A) RANKL + M-CSF, (B) M-CSF, (C) conditioned media from untreated HDS-2 cells or (D) conditioned media from gefitinib-treated HDS-2 cells. Cells were then stained for TRAP. Magnification  $\times 200$ . (E) Number of multinucleated/TRAP-positive cells.

human marrow cultures, which contain MSCs, stromal cells, osteoblasts and osteoclast precursors, has been shown to stimulate the formation of bone-resorbing osteoclasts (Takahashi *et al.* 1986, Tanaka *et al.* 1998). In addition, the ability of TGF $\alpha$  and EGF to indirectly stimulate bone-resorbing activity has long been described (Tashjian *et al.* 1985, Ibbotson *et al.* 1986). Several previous studies have also shown expression of EGFR in BMSCs and in osteoblast-like cells, as well as biological effects of EGF and/or TGF $\alpha$  in osteoblasts (Ibbotson *et al.* 1986, Drake *et al.* 1994, Sodek *et al.* 1995, Satomura *et al.* 1998). For example, EGF and TGF $\alpha$  caused a concentration-dependent inhibition of alkaline phosphatase activity in osteoblast-like cells (Ibbotson *et al.* 1986). Also, EGF has been found to up-regulate the expression of mRNA for osteopontin in osteoblasts; osteopontin is a bone matrix protein that is synthesized by osteoblastic cells and mediates osteoclast adherence to the bone matrix (Sodek *et al.* 1995). This finding suggests that activation of EGFR regulates the ability of osteoblasts to affect osteoclast function at different levels. Interestingly, PTH induced an increase in the levels of expression of EGFR in osteoblast-like cells, suggesting that PTH-induced growth of osteoblasts might be mediated by EGFR (Drake *et al.* 1994). Since expression of EGF has been demonstrated to occur in osteoclasts (Symons 2003), it is also conceivable that paracrine circuits involving EGFR and its ligands are operating between osteoclasts, osteoblasts and, as shown for the first time by our data, MSCs.

We found that HDS-2 cells express higher levels of EGFR as compared with the HDS-1 cell line. In this respect, it has been previously described that, while not absolutely predictive of osteogenic capacity, non-bone-forming human BMSCs express higher levels of EGFR as compared with bone-forming strains (Satomura *et al.* 1998). However, both HDS cell lines retained the ability to differentiate into osteoblast-like cells (D Aldinucci, N Normanno & A Pinto, unpublished observation). These two multi-potent cell lines might therefore represent slightly different stages of initial differentiation in specialized bone marrow populations.

A previous report demonstrated that genistein, an inhibitor of both topoisomerase II and protein TKIs, was able to down-regulate the production of RANKL mRNA in ST2 mouse stromal cells, and to reduce the ability of ST2 cells to induce osteoclast differentiation in a co-culture assay (Yamagishi *et al.* 2001). However, inhibitors of topoisomerase II such as amsacrine or etoposide, but not the EGFR-TKI inhibitors tyrphostin 25 or lavendustin A, were able to block ST2-induced osteoclast differentiation (Yamagishi *et al.* 2001). In this regard, the expression and function of EGFR, as well as the effects of the above-mentioned TKIs on the activation of EGFR and of its downstream signaling, have not been investigated in the ST2 cell line. In addition, in this report ST2 cells were treated with the TKIs for a short time (6h) before co-culture with pre-osteoclasts for 8 days. Such a short incubation might have been sufficient for the activity of

topoisomerase inhibitors, but not for TKIs. Furthermore, a recent report has demonstrated that BMSCs express ErbB-2, and that treatment with herceptin produces a significant reduction in the ability of human bone marrow stromal cell lines to produce vascular endothelial growth factor (Corsini *et al.* 2003). We and other research groups have previously demonstrated that breast cancer cells that express ErbB-2 are highly sensitive to gefitinib (Moasser *et al.* 2001, Moulder *et al.* 2001, Normanno *et al.* 2002, Campiglio *et al.* 2004). In these cells, treatment with gefitinib produced a significant reduction in the activation of EGFR, ErbB-2 and ErbB-3 by inducing the formation of EGFR/ErbB-2 and EGFR/ErbB-3 inactive dimers (Moasser *et al.* 2001, Moulder *et al.* 2001, Anido *et al.* 2003, Normanno *et al.* 2003b, Campiglio *et al.* 2004). This phenomenon has been shown for gefitinib and might not occur with other non-quinazoline TKIs that have different characteristics (Normanno *et al.* 2003b). For example, quinazolines, but not other TKIs, have been shown to induce the formation of inactive dimers with intermediate ligand-binding affinity in cells overexpressing EGFR (Lichtner *et al.* 2001). Since preliminary findings have demonstrated that HDS cells express both ErbB-2 and ErbB-3 (N Normanno, unpublished observation), the response of MSC-like cells to gefitinib might also be related to the ability of this compound to disrupt the cross-talk between EGFR and the other ErbB receptors.

We also found that gefitinib produces a significant reduction in the levels of secretion of M-CSF in MSC-like cells. Previous *in vitro* studies have demonstrated that M-CSF is involved in tumor invasion and formation of metastasis of breast cancer cells (Sapi *et al.* 1996, Lin *et al.* 2001). Expression of M-CSF receptor has been also shown in human primary breast carcinomas (Tang *et al.* 1992, Maher *et al.* 1998, Kluger *et al.* 2004). In particular, the expression of this receptor was more frequent in lymph node-positive tumors compared with lymph node-negative tumors, suggesting that the M-CSF receptor might be involved in local invasion and metastasis (Kluger *et al.* 2004). In this regard, the ability of gefitinib to reduce the production of M-CSF in MSCs might, in part, explain its effects on the pathogenesis of bone metastases, and ultimately on bone pain.

In conclusion, our results have clearly suggested that activation of EGFR in MSCs is involved in the generation and progression of bone metastases, and strongly support clinical trials of gefitinib in breast cancer patients with metastatic bone disease. Our findings have also highlighted the importance of studying the effects of anti-EGFR agents in non-cancer cell types of

the neoplastic microenvironment that might be involved in tumor growth and progression.

## Acknowledgements

This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC) (national and regional grants), and from Ministero della Salute (ICS-167/RF2002) to NN. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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