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ORIGINAL ARTICLE

An ErbB-3 antibody, MP-RM-1, inhibits tumor growth by blocking ligand-dependent and independent activation of ErbB-3/Akt signaling

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The ErbB receptors, such as ErbB-1 and ErbB-2, have been intensely pursued as targets for cancer therapeutics. Although initially efficacious in a subset of patients, drugs targeting these receptors led invariably to resistance, which is often associated with reactivation of the ErbB-3-PI3K-Akt signaling. This may be overcome by an ErbB-3 ligand that abrogates receptor-mediated signaling. Toward this end, we have generated a mouse monoclonal antibody, MP-RM-1, against the extracellular domain (ECD) of ErbB-3 receptor. Assessment of human tumor cell lines, as well as early passage tumor cells revealed that MP-RM-1 effectively inhibited both NRG-1β-dependent and -independent ErbB-3 activation. The antagonizing effect of MP-RM-1 was of non-competitive type, as binding of [^{125}I]-labeled NRG-1β to ErbB-3 was not influenced by the antibody. MP-RM-1 treatment led, in most instances, to decreased ErbB-3 expression. In addition, MP-RM-1 was able to inhibit the colony formation ability of tumor cells and tumor growth in two human tumor xenograft nude mouse models. Treatment with the antibody was associated with a decreased ErbB-3 and Akt phosphorylation and ErbB-3 expression in the excised tumor tissue. Collectively, these results indicate that MP-RM-1 has the potential to interfere with signaling by ErbB-3 and reinforce the notion that ErbB-3 could be a key target in cancer-drug design.
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Keywords: ErbB-3 receptor; ErbB-3/Akt pathway; monoclonal antibody; targeted therapy

Introduction

The human epidermal growth factor receptor (EGFR; HER; ErbB) family of receptor tyrosine kinases regulates a large variety of biological processes including cell proliferation, migration, invasion and survival (Gschwind *et al*., 2004). The family includes four members: ErbB-1 (EGFR, or HER-1), ErbB-2 (HER-2), ErbB-3 (HER-3) and ErbB-4 (HER-4). To date, 11 ligands have been reported including EGF, heparin-binding EGF-like growth factor, transforming growth factor-α, amphiregulin, epiregulin, betacellulin and the heregulins (also known as neuroregulins). These ligands bind directly to their cognate receptors, which leads to the formation of receptor homodimers or heterodimers that trigger the activation of multiple signaling pathways (Citri and Yarden 2006), among which the Ras–Raf–MAPK and PI3K–Akt pathways are prevalent. In the last decade, preclinical and clinical studies have demonstrated that a fraction of epithelial cancers are addicted to the signals propagated by ErbB receptors (Hynes and MacDonald 2009). As a consequence, these receptors are nowadays among the most targeted oncoproteins in cancer. Treatment of cancers driven by the HER family of proteins are currently either monoclonal antibodies such as trastuzumab (directed at ErbB-2), cetuximab (directed at ErbB-1), pertuzumab (preventing ErbB-2/ErbB-3 dimerization), or small-molecule tyrosine kinase inhibitors such as gefitinib and erlotinib (ErbB-1 kinase inhibitors) and lapatinib (dual ErbB-2/ErbB-1 kinase inhibitor) (Mendelsohn and Baselga 2006; Hynes 2007; Giampaglia *et al*., 2010; Saxena and Dwivedi 2010). Several other monoclonal antibodies and tyrosine kinase inhibitors directed toward the ErbB family proteins are currently in clinical trials (Hynes and Lane 2005). Although efficacious in a subset of patients, monoclonal antibodies such as trastuzumab or tyrosine kinase inhibitors such as gefitinib, impairing only one ErbB family member, often encounter endogenous or acquired resistance (Romond *et al*., 2005; Mendelsohn and Baselga 2006). This resistance is often associated with compensation by or upregulation of other ErbB family members such as ErbB-3 and ErbB-4 (Bianchi *et al*., 2006; Engelman *et al*., 2007; Karamouzis *et al*., 2007) or increased production of ErbB-1 or ErbB-3 ligands by tumor cells (Ishikawa *et al*., 2005; Zhou *et al*., 2006).

ErbB-3 overexpression has been documented in breast, ovarian and lung cancer and this genetic feature has been correlated with poor prognosis (Yi *et al*., 1997;

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Witton *et al*., 2003; Tanner *et al*., 2006). On activation by heregulins, ErbB-3 dimerizes with ErbB-2 and ErbB-1 to generate potent oncogenic receptor heterodimers (Alimandi *et al*., 1995; Wallasch *et al*., 1995). Within this complex, ErbB-3 preferentially recruits phosphatidylinositol 3-kinase (PI3 K) to its cytoplasmic docking sites thereby regulating cell proliferation and survival (Prigent and Gullick 1994; Sithanandam *et al*., 2005). Interestingly, it has recently been shown that cancer cells escape ErbB inhibition therapy by upregulation of ErbB-3 signaling (Sergina *et al*., 2007; Baselga and Swain, 2009) and that ErbB-3 inhibition abrogates ErbB-2-driven tamoxifen resistance in breast cancer cells (Liu *et al*., 2007). Of interest, recent data have documented that ErbB-3 can be transactivated by MET in lung cancer cells resistant to the ErbB-1 inhibitor, gefitinib (Engelman *et al*., 2007). Thus in view of the crucial role of ErbB-3 in tumor growth and in the development of drug resistance, it is of outmost importance to develop drugs targeting this receptor. As the intrinsic kinase activity of ErbB-3 has been shown to be $\sim$1000-fold weaker than that exhibited by ErbB-1 (Shi *et al*., 2010), thus hampering the use of synthetic tyrosine kinase inhibitors, antibodies directed against ErbB-3 may represent the molecules of choice to disrupt its function. In the present work, we report the functional activity *in vitro* and *in vivo* of a novel murine monoclonal antibody, MP-RM-1, directed toward the extracellular domain (ECD) of ErbB-3. We showed that MP-RM-1 (a) interferes with ligand-dependent and -independent ErbB-3 signaling (b) promotes ErbB-3 downregulation and (c) inhibits the growth of xenografts of tumors with activated ErbB-3–Akt pathway.

Results

MP-RM-1 suppresses ErbB-3/Akt activation

To test the activity of MP-RM-1 on NRG-1β-driven ErbB-3/Akt activation, we first screened a panel of human tumor cell lines for NRG-1β-driven, ErbB-3-dependent Akt activation using the RNAi strategy. A marked impairment of ErbB-3 and Akt phosphorylation was observed in some of the cell lines tested, with the strongest inhibition being observed in MDA-MB-435 human breast cancer cells and IR-8 human melanoma cells (Figure 1a).

To evaluate whether MP-RM-1 could replicate the effect of ErbB-3 silencing, cells were exposed to the antibody for 2 h before stimulation with 10 ng/ml of NRG-1β for 5 min. In both cell types, MP-RM-1 significantly suppressed ErbB-3 and Akt phosphorylation in a dose- and time-dependent manner (Figures 1b–d). This inhibitory effect was paralleled by a decrease of ErbB-3 protein expression (Figure 1b). Interestingly, whereas inhibition of ErbB-3/Akt phosphorylation was observed in cells after a 15-min exposure to the antibody, no effect on ErbB-3 expression was evident within 60 min (Figure 1b). No effect was observed when cells were exposed to a mouse IgG2a isotype control antibody (Supplementary Figure 1a). Additionally, ErbB-3 and Akt phosphorylation was completely inhibited by the $F(ab')_2$ but only partially reduced by the $F(ab')_1$ fragment of the antibody (Supplementary Figure 1b). All together these data indicate that MP-RM-1 significantly suppressed NRG-1β-stimulated ErbB-3/Akt activation, and promoted receptor downregulation, possibly counteracting ligand-driven receptor phosphorylation.

MP-RM-1 prevents ligand-induced ErbB-3 activation and receptor heterodimerization

To study whether the observed suppression of ErbB-3/Akt phosphorylation could be explained by an antagonizing effect of the antibody on NRG-1β-driven receptor activation, MDA-MB-435 cells were co-exposed to 1 ng/ml of NRG-1β and 10 μg/ml of MP-RM-1 for 5 min. In these conditions, ErbB-3 phosphorylation was markedly (more than 50%) suppressed (Figure 2a), indicating that the antibody was able to function as a ligand antagonist. The suppressive effect was extended downstream of the receptor, as there was a dose-dependent inhibition of Akt phosphorylation (Figure 2b). As expected, MP-RM-1 alone did not activate ErbB-3, thus lacking agonistic activity (Figure 2a). To determine whether the antagonizing effect of MP-RM-1 was due to competition of the antibody for NRG-1β binding to the receptor, we performed a cell-based binding assay. For these assays, we selected T47D human breast cancer cells, which displayed a high-specific binding of NRG-1β and MP-RM-1-mediated suppression of receptor phosphorylation (Supplementary Figure 2a). In preliminary assays, radiolabeled and unlabeled NRG-1β gave superimposable results in terms of receptor phosphorylation (Supplementary Figure 2b). In our hand, [^{125}I]-labeled NRG-1β binding was nearly completely abolished by the addition of increasing concentrations of unlabeled NRG-1β (Supplementary Figure 2c, top), In contrast, no effect was seen by the addition of MP-RM-1 (Supplementary Figure 2c, bottom), indicating lack of competition for receptor binding between the antibody and the ligand. Similar results were observed in the DU-145 human prostate cancer and MCF-7 human breast cancer cells (data not shown).

As ErbB-1, ErbB-2, ErbB-3 and ErbB-4 receptor signaling cooperates through a network involving receptor homodimerization and heterodimerization, we examined whether MP-RM-1 could prevent NRG-1β-driven ErbB-3/ErbB-2 dimerization. As shown in Figure 2c, when the ligand and the antibody were simultaneously added to IR-8 cells, formation of heterodimers was completely blocked. Consistently, analysis of whole-cell lysates showed that ErbB-3 and Akt phosphorylation were inhibited by MP-RM-1 (Figure 2c).

MP-RM-1 induces ErbB-3 degradation

Cetuximab has been suggested to suppress tumor growth by facilitating ErbB-1 downregulation (Sunada *et al*., 1986). As ErbB-3 downregulation was observed

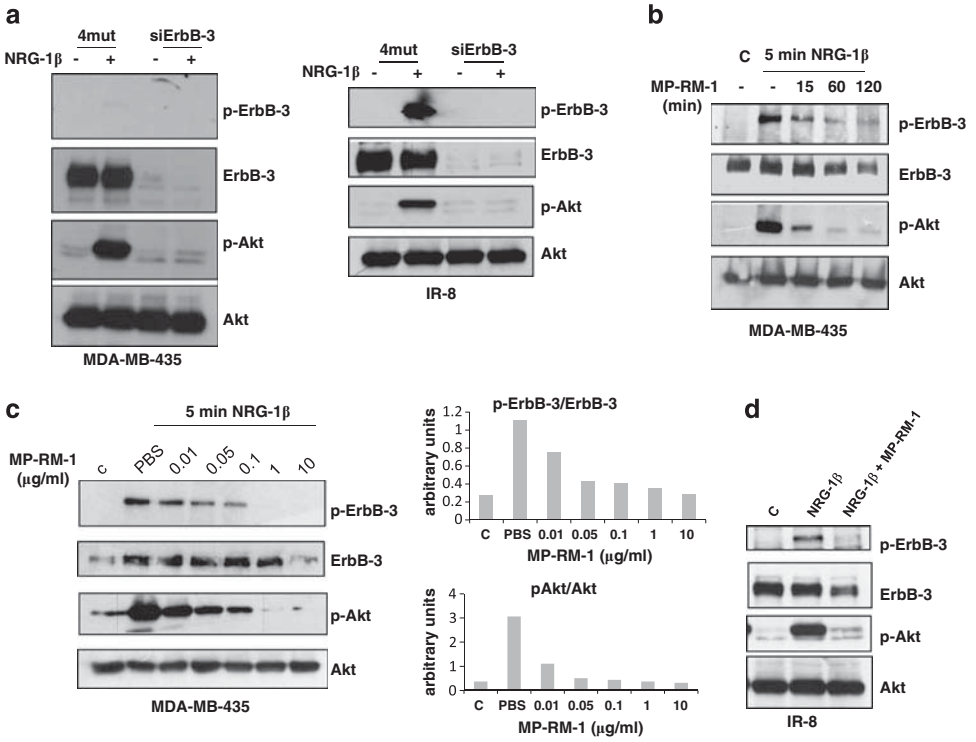

Figure 1 NRG-1β-dependent ErbB-3/Akt activation is inhibited by MP-RM-1. (**a**) pSuper 4Mut and shErbB-3 infected MDA-MB-435 and IR-8 cells were grown in 0.2% FBS RPMI 1640 for 24 h and then stimulated with 10 ng/ml of NRG-1β for 5 min before lysis. Cell lysates where then probed with the indicated antibodies. (**b**) MDA-MB-435 cells were grown in 0.2% FBS RPMI 1640 for 24 h and then incubated with 10 μg/ml of MP-RM-1 for the indicated time before NRG-1β stimulation. Cell lysates were immunoblotted for phosphorylated ErbB-3 and Akt expression levels. The same filter was reprobed with anti-ErbB-3 and anti-Akt antibodies for a loading control. (**c**) MDA-MB-435 cells were grown in 0.2% FBS RPMI 1640 for 24 h and then incubated with the indicated amount of MP-RM-1 for 2 h before NRG-1β stimulation. Cell lysates were immunoblotted for phosphorylated ErbB-3 and Akt expression levels. The same filter was reprobed with anti-ErbB-3 and anti-Akt antibodies for a loading control. Anti-p-ErbB-3 and anti-p-Akt immunoreactivity was quantified and plotted after normalization for total ErbB-3 and Akt immunoreactiviy (right panels). (**d**) IR-8 human melanoma cancer cells were grown in 0.2% FBS RPMI 1640 for 24 h and then incubated with the 10 μg/ml of MP-RM-1 for 2 h before NRG-1β stimulation. Cell lysates were immunoblotted for phosphorylated ErbB-3 and Akt expression levels. The same filter was reprobed with anti-ErbB-3 and anti-Akt antibodies for a loading control.

after 60 min of exposure to MP-RM-1 (Figure 1b), we investigated whether this effect could reflect the antibody-mediated degradation of the receptor. To this end, MDA-MB-435 cells were analyzed for surface expression of ErbB-3 after exposure to the antibody for different times. As shown (Figure 3a), a time-dependent reduction of the membrane expression of ErbB-3 was observed, suggesting receptor internalization. Cell imaging studies showed that MP-RM-1 internalized, as revealed by its co-localization with the early endosome marker, EEA1 (Figure 3b). To clarify whether the internalized MP-RM-1 antibody was still bound to the receptor, an anti-ErbB-3 antibody (C-17) recognizing the *C*-terminal residue, not competing with MP-RM-1, was used to track the receptor. As shown in Figure 3c, tracking ErbB-3 with C-17, while confirming MP-RM-1-induced receptor internalization, documented intracellular co-localization of the two antibodies, indicating that MP-RM-1 was still bound to the receptor.

To verify whether the decreased expression/internalization of the receptor was followed by protein degradation, cells were chased with the protein-synthesis inhibitor cycloheximide (CHX) for 3 or 6 h in the presence or absence of the antibody. As shown in Figure 4a, receptor expression was reduced by approximately eightfold as compared with control (5 vs 40%) after 3 h, whereas it was abrogated after 6 h. The effect of the antibody on ErbB-3 degradation was dose dependent (Figure 4b). Similar results were obtained when intact antibody molecule was replaced by $F(ab')_2$, but not by $F(ab')_1$ (Figure 4a). Notably, cells treated with MP-RM-1, in the absence of NRG-1β and CHX, showed a time-dependent downregulation of ErbB-3, which represents the 'net' effect of the antibody on ErbB-3 protein level (Supplementary Figure 2d).

The ability of MP-RM-1 to induce ErbB-3 degradation was confirmed in SKBr-3 human breast cancer cells (Figure 4c). The effect was specific as it was not observed upon exposure of the cells to the unrelated antibody, that is, the anti-ErbB2 antibody 300G9 (Digiesi *et al*., 1992).

MP-RM-1 suppresses ligand-independent activation of ErbB-3

ErbB-3 has recently been shown to associate with and to be trans-activated by the tyrosine kinase receptor MET in a ligand-independent manner (Engelman *et al*., 2007).

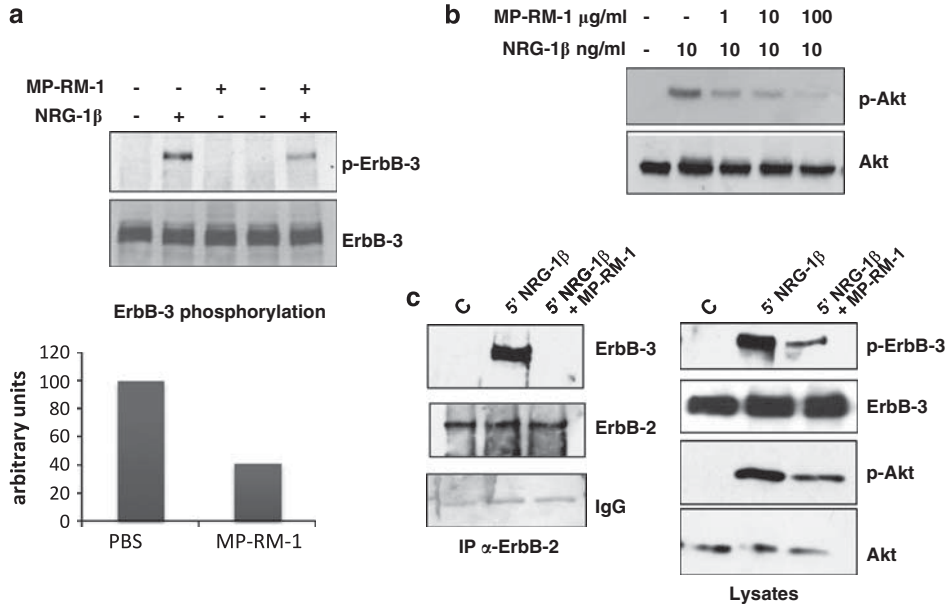

Figure 2 MP-RM-1 impairs ligand-driven ErbB-3 activation and receptor heterodimerization. (**a**) MDA-MB-435 cancer cells were grown in 0.2% FBS RPMI 1640 for 24 h and then simultaneously incubated with 1 ng/ml of NRG-1β and 10 μg/ml of MP-RM-1 for 5 min. Cells were then lysed and analyzed for phosphorylated ErbB-3. The same filter was reprobed with anti-ErbB-3 for a loading control. Densytometric analysis of the western blots is shown. (**b**) MDA-MB-435 cancer cells were grown in 0.2% FBS RPMI 1640 for 24 h and then simultaneously incubated with 10 ng/ml of NRG-1β and the indicated amount of MP-RM-1 for 5 min. Cells were then lysed and analyzed for phosphorylated Akt. The same filter was reprobed with anti-Akt antibody for a loading control. (**c**) IR-8 human melanoma cancer cells were grown in 0.2% FBS RPMI 1640 for 24 h and then simultaneously incubated with 1 ng/ml of NRG-1β and 10 μg/ml of MP-RM-1 for 5 min. Lysates were analysed by immunoblot with the indicated antibodies (right panel) or lysates were immunoprecipitated with an anti-ErbB-2 antibody (300G9) and then probed with an anti-ErbB-3 antibody (left panel). Ponceau staining of 300G9 is shown.

To evaluate whether MP-RM-1 could disrupt MET/ErbB-3 signaling axis, we used the MKN-45 human gastric cancer cells, which exhibit constitutive high levels of MET, ErbB-3 and Akt phosphorylation (Figures 5a–c). Moreover, MKN-45 cells do not express the ErbB-3 ligands, NRG-1β and Betacellulin (Wu *et al*., 2009), indicating the absence of an autocrine loop activating ErbB-3 in these cells. Silencing of ErbB-3, in these cells, markedly inhibited ligand-independent ErbB-3 and Akt phosphorylation (Figure 5a), as did MP-RM-1 in a time-dependent manner (Figure 5b). Interestingly, the MET inhibitor SU11274 paralleled the effect of MP-RM-1, indicating that the MET/ErbB-3/Akt signaling cascade is functionally active in these cells. Notably, MP-RM-1 was as effective as SU11274 or ErbB-3 silencing in inhibiting ErbB-3 and Akt activation (Figure 5c), whereas no effect was seen by treating cells with an antibody to ErbB-2 (trastuzumab) or to ErbB-1 (cetuximab) (Figure 5c and data not shown). Next, we tested the ability of MP-RM-1 to disrupt ErbB-3/MET association in MKN-45 cells. As shown in Figure 5d, exposure of the cells to the antibody caused a nearly complete disappearance of ErbB-3 after immunoprecipitation with an anti-phospho MET antibody. Interestingly, despite the presence of the MET inhibitor, SU1174, exogenous NRG-1β was still able to re-activate the ErbB-3/Akt axis, but not in the presence of MP-RM-1 (Figure 5e). The ability of MP-RM-1 to inhibit ligand-independent ErbB-3/Akt phosphorylation was confirmed in ErbB-2 overexpressing SKBr-3 human breast cancer cells (Supplementary Figure 3a). Taken together these data suggest that MP-RM-1 targets both MET-dependent and ligand-independent ErbB-3/Akt signaling axis.

MP-RM-1 inhibits colony formation and growth of tumor xenografts in nude mice

Having demonstrated that MP-RM-1 is able to inhibit ErbB-3/Akt activation, we wondered whether this inhibition could translate into tumor growth inhibition. As illustrated (Supplementary Figure 3b), MP-RM-1 at concentrations of 10 μg/ml significantly inhibited colony formation ability of IR-8 cells as compared with untreated control cultures. Untreated cells produced 26 ± 47 colonies/field, and the colony numbers were suppressed to 15 ± 28 ($P < 0.001$).

To assess the antitumor activity of MP-RM-1 as a single agent *in vivo*, we used IR-8 human melanoma cells and DU-145 human prostate cancer cells, both expressing ErbB-3 and sensitive *in vitro* to the inhibitory effect of the antibody on NRG-1β stimulated ErbB-3/Akt activation (Supplementary Figure 3c), as xenograft tumor models in nude mice. Treatment of the animals with MP-RM-1 at 20 mg/kg resulted in a significant tumor growth delay in both tumor models (Figures 6a and b). In separate experiments, animals harboring IR-8 xenografts (300 mm^3) were treated with a single injection of the antibody (20 mg/kg) and killed 4 h thereafter. A significant reduction of ErbB-3 expression and Akt phosphorylation was seen in tumors from treated

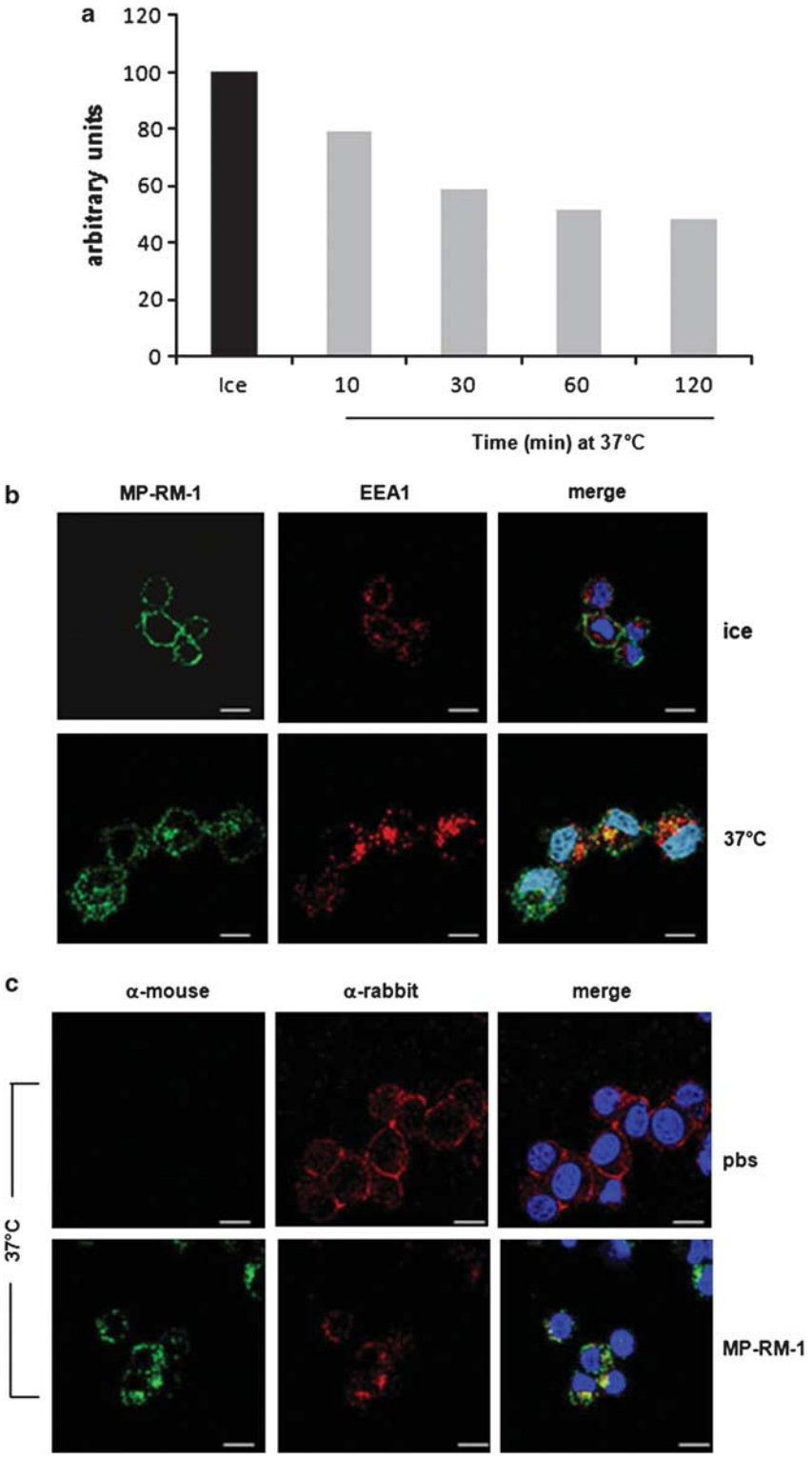

Figure 3 MP-RM-1 reduces surface expression of the receptor and induces ErbB-3 internalization. (**a**) FACS analysis of MDA-MB-435 cells incubated with 10 μg/ml of MP-RM-1 for 30 min on ice and placed back at 37 °C for 30, 60 and 120 min. The histograms represent the percentage of mean fluorescence referred to control cells (maintained 30 min on ice). (**b**) Confocal images of IR-8 cells. After 24 h of serum deprivation, cells were maintained on ice in the presence of 10 μg/ml of MP-RM-1 for 30 min and placed back at 37 °C for 60 min. Cell nuclei are shown in blue. Bars: 10 μm. (**c**) Serum-straved IR-8 cells were maintained in the presence or absence of 10 μg/ml of MP-RM-1 for 30 min on ice and placed back at 37 °C for 60 min. ErbB-3 localization was tracked using a specific rabbit antibody raised against the *C*-terminal residue of the receptor (C-17). MP-RM-1 and C-17 were visualized with an anti-mouse (green) and an anti-rabbit antibody (red), respectively. Cell nuclei are shown in blue. Bars: 10 μm.

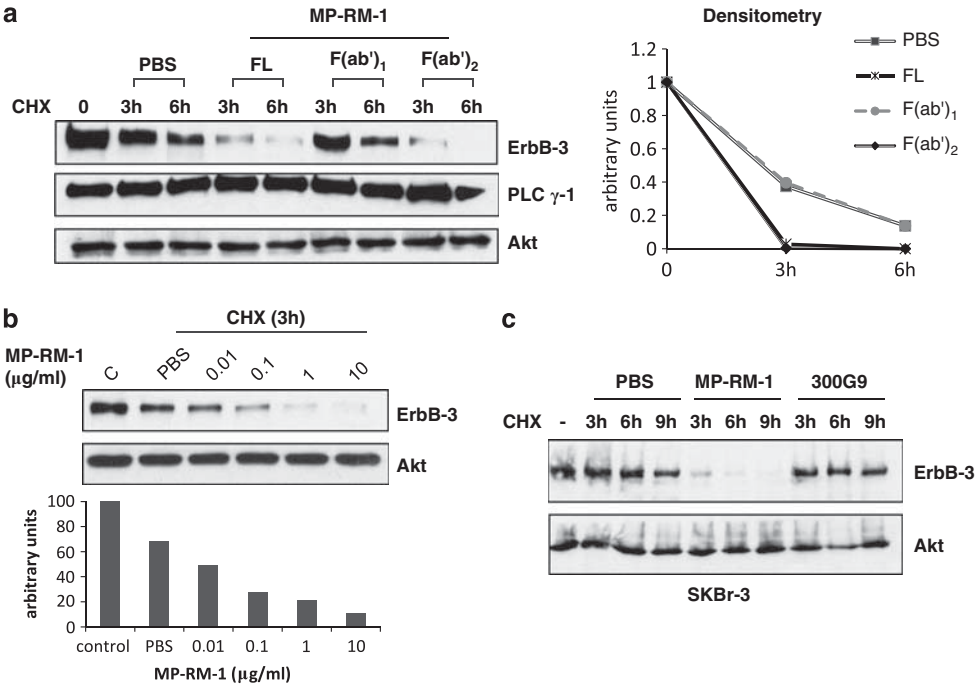

Figure 4 MP-RM-1 induces ErbB-3 degradation. (**a**) MDA-MB-435 human breast cancer cells were grown in 0.2% FBS RPMI 1640 for 24 h and then exposed for 3 h and 6 h to CHX in the presence or not of full length (FL) MP-RM-1, $F(ab')_1$ or $F(ab')_2$ truncated forms. Cell lysates were immunoblotted for ErbB-3 and Akt expression levels. The same filter was reprobed with anti-PLCγ-1 antibody for a loading control. Densitometric analysis of the western blots is shown. (**b**) IR-8 cancer cells were grown in 0.2% FBS RPMI 1640 for 24 h and then incubated with protein-synthesis inhibitor cycloeximide (CHX) for 3 h in the presence or absence of increasing amount of MP-RM-1 antibody. Lysates were then probed with the indicated antibodies. Anti-ErbB-3 immunoreactivity was quantified and plotted after normalization for total Akt immunoreactiviy, data are expressed as % over control. (**c**) SKBr-3 cancer cells were grown in 0.2% FBS DMEM for 24 h and then exposed for the indicated time to CHX in the presence or absence of MP-RM-1 or 300G9 antibodies. Cell lysates were immunoblotted for the indicated antibodies.

animals as compared with untreated controls, indicating that MP-RM-1 targets ErbB-3/Akt pathway *in vivo* (Figure 6c). Moreover, tumors from treated animals displayed a significant lower cell proliferative activity (as measured by Ki-67 staining and cell mitotic index) than controls (35.8 vs 57.9, $P=0.048$, and 6.6 vs 9.5, $P=0.017$, respectively) (Figure 6d and Supplementary Figure 4).

MP-RM-1 inhibits ErbB-3 activation in primary tumor cells cultures

The results presented above indicate that MP-RM-1 is a potent suppressor of ErbB-3/Akt activation in established tumor cell lines. To investigate the effects of MP-RM-1 on early *in vitro* passage human tumor samples, we evaluated NRG-1β-driven ErbB-3/Akt phosphorylation in tumor cells obtained from pleural or peritoneal effusions of eight patients with either breast or ovarian cancer. Treatment with the antibody resulted in a reduction of ErbB-3/Akt phosphorylation accompanied by receptor downregulation in two patients, a reduction of ErbB-3 phosphorylation but not of Akt in four, and no effect in the remaining two. The suppressive effect on ErbB-3 phosphorylation was always associated to a parallel effect on receptor expression. Representative examples of antibody-sensitive, partially sensitive and insensitive cells are illustrated in Figures 7a–c, respectively.

Discussion

ErbB-3 is highly expressed in many human tumor types and has been variably associated with poor prognosis (Yi *et al*., 1997; Tanner *et al*., 2006; Reschke *et al*., 2008). Moreover, recent studies suggest that breast cancers exposed to ErbB-1 or ErbB-2 targeted therapies escape this inhibition by persistent activation of ErbB-3 and the PI3-K–Akt pathways (Baselga and Swain 2009), suggesting that agents targeting ErbB-3 could provide a novel and promising approach toward the treatment of some cancers. As ErbB-3 lacks elevated kinase activity (Shi *et al*., 2010), antibodies targeting the extracellular domain of the receptor represent molecules of choice to disrupt its function.

In the present paper, we characterized the mouse monoclonal antibody, MP-RM-1 recognizing the ECD of ErbB-3. Application of this antibody to cancer cells suppressed ErbB-3/Akt activation in different cancer cell lines naturally expressing ErbB-3. MP-RM-1 also efficiently promoted downregulation of total cellular ErbB-3 protein level. The effect of the antibody was not limited to long-term established tumor cell lines, but it was also evident, although at a variable extent, in tumor cells freshly obtained from malignant effusions of patients with breast and ovarian cancer, suggesting MP-RM-1 as a suitable candidate for clinical development.

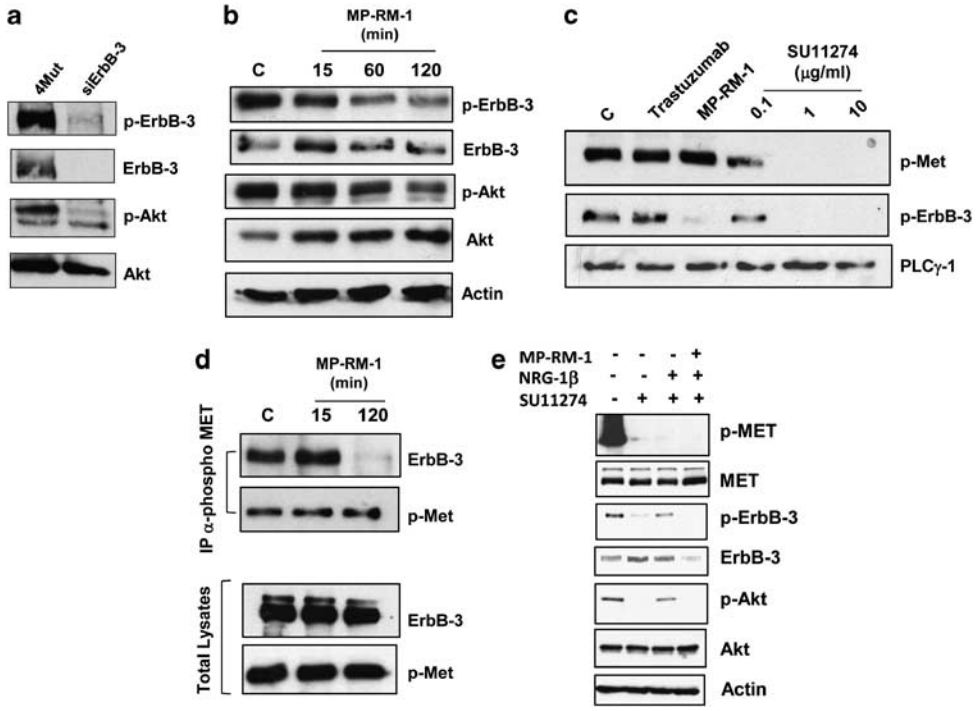

Figure 5 MP-RM-1inhibits ligand-indipendent activation of ErbB-3. (**a**) pSuper 4Mut and shErbB-3 infected MKN-45 cancer cells were grown in serum-free DMEM for 24 h and then lysed for immunoblot analysis. (**b**) MKN-45 cells were grown in serum-free DMEM for 24 h and then exposed to MP-RM-1 for the indicated time. Lysates were analyzed by immunoblot for phosphorylated ErbB-3 and phosphorylated Akt. The same filter was reprobed with the indicated antibodies for a loading control. (**c**) MKN-45 cells were grown in serum free DMEM for 24 h and then incubated for 2 h with trastuzumab (10 μg/ml), MP-RM-1 (10 μg/ml) or increasing amount of MET inhibitor SU11274. Lysates were analyzed by immunoblot with the indicated antibodies. (**d**) MKN-45 cells were grown in serum-free DMEM for 24 h and then incubated with 10 μg/ml of MP-RM-1 for 15 and 120 min. Lysates were analysed by immunoblot with the indicated antibodies (lower panel) or immunoprecipitated with an anti-phosphorylated MET antibody and then probed with an anti-ErbB-3 antibody (upper panel). (**e**) MKN-45 cells were grown in serum free DMEM for 24 h and then incubated with 1 μg/ml of SU1174 alone or together with 10 μg/ml of MP-RM-1. After 2 h, NRG-1β (10 ng/ml) was added, as indicated.

Suppression of receptor activation and promotion of its downregulation have previously been demonstrated to mediate the antitumor activity of therapeutic antibodies targeting ErbB receptors. The anti-ErbB-1 antibody cetuximab inhibits ligand-mediated phosphorylation of ErbB-1 by directly competing with ligand binding to ErbB-1 (Prewett *et al*., 1998). The anti-ErbB-2 monoclonal antibody pertuzumab sterically hinders recruitment of ErbB-2 into ErbB/ligand complexes impairing the formation and activation of ErbB-2-containing dimers (Franklin *et al*., 2004). In this context, our *in vitro* experiments (Figure 2) suggest that MP-RM-1 does antagonize ligand-driven activation of ErbB-3 and prevents ErbB-2/ErbB-3 dimerization. The mechanism underlying these antagonizing effects of MP-RM-1 is not yet understood, but it does not involve competition with receptor ligand, as the antibody was unable to compete with NRG-1β for binding to ErbB-3. Another interesting feature of MP-RM-1 lies in its ability to block the activation of MET-dependent ErbB-3/Akt signaling (Figure 5). This is particularly important at the light of evidence that cells becoming resistant to the ErbB-1 inhibitors display constitutive activation/amplification of MET, which in turn re-activates ErbB-3/Akt axis (Engelman *et al*., 2007).

The effect of MP-RM-1 on tumor growth was also assessed in this study. The antibody exerted a significant tumor growth inhibitory activity *in vitro* by decreasing the anchorage-independent growth of IR-8 human melanoma cells. Moreover, when given as a single-agent therapy, it led to a significant growth delay of xenografts of melanoma (IR-8) and hormone refractory prostate cancer (DU-145). Of note, tumors from MP-RM-1-treated animals displayed a decreased ErbB-3/Akt phosphorylation and ErbB-3 expression, suggesting that the antibody targets ErbB-3/Akt signaling pathway also *in vivo*. The therapeutic activity on melanoma is of particularly relevance, as this cancer which is often resistant to currently available therapies, frequently overexpresses ErbB-3 (Reschke *et al*., 2008) and MET (Natali *et al*., 1993). An increased ErbB-3 expression has also been documented in prostate cancer as compared with normal prostate, and its induction has been observed after progression to hormone independency (Chen *et al*., 2010). Thus, potential future therapeutic application of MP-RM-1 includes both these neoplasms. In this respect, it is noteworthy that the $F(ab')_2$ form of MP-RM-1 is sufficient to inhibit ligand-driven activation of ErbB-3 and to promote receptor downregulation, implicating that the Fc region of the antibody is not necessary for its activity (Figure 4a and Supplemen-

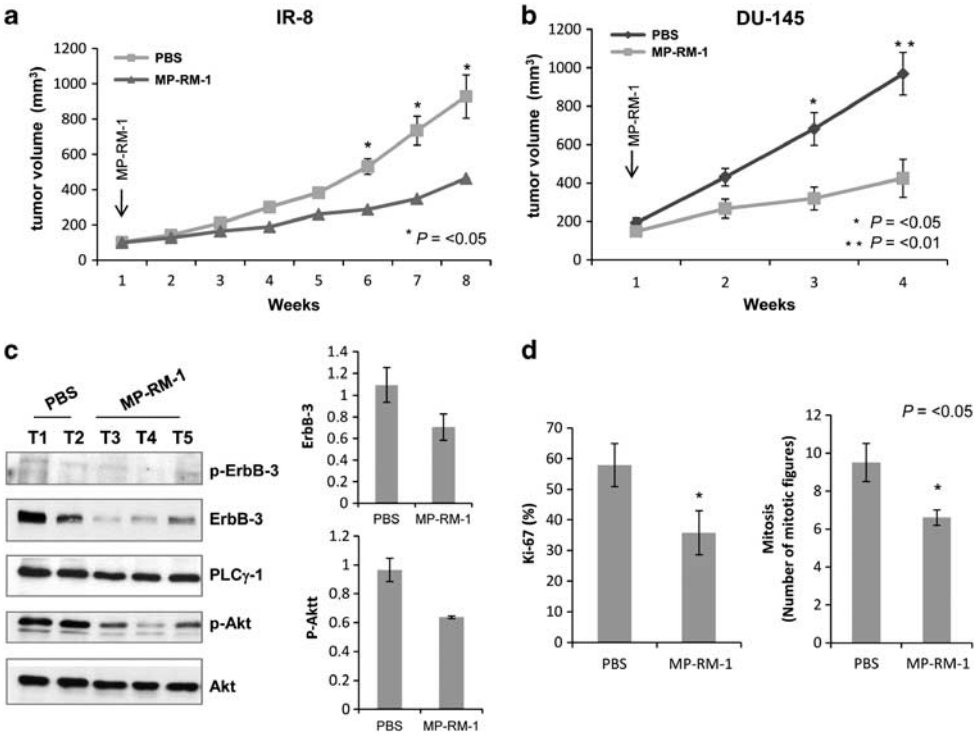

Figure 6 MP-RM-1 reduces the expression of ErbB-3 and tumor cell division. Exponentially growing IR-8 (**a**) and DU-145 (**b**) cells were injected into the right flank of the recipient mice. Tumor growth was assessed, as described in Materials and methods section. (**c**) Western blotting analysis of tumor lysates of IR-8 xenografts excised after 4 h of treatment with 20 mg/kg MP-RM-1 or PBS. (**d**) The mitotic activity, as assessed by Ki-67 immunohistochemical staining and mitotic figure counts, is lower in tumors from mice receiving MP-RM-1 than in controls.

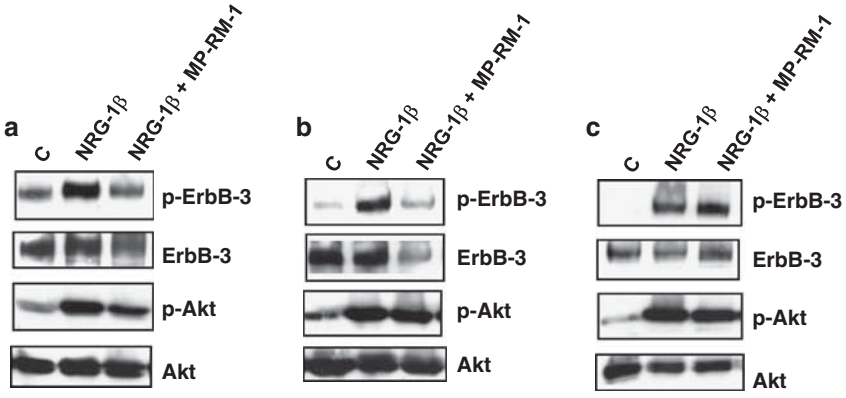

Figure 7 MP-RM-1inhibits NRG-1β -induced ErbB-3/Akt axis in patient-derived cancer cells. Short-term cultured patient-derived carcinoma cells were serum starved for 24 h and then incubated with 10 μg/ml of MP-RM-1 for 2 h before NRG-1β stimulation. Cells were then lysed and analyzed for phosphorylated ErbB-3 and Akt. The same filter was reprobed with anti-ErbB-3 and anti-Akt and for a loading control. Examples of antibody-sensitive (**a**), partially sensitive (**b**) and insensitive (**c**) cells are shown.

tary Figure 1b). Because of the delivery advantage and larger volume of distribution of $F(ab')_2$ compared with whole IgG molecule, and these data showing antibody internalization (Figure 3 and Supplementary Figure 1c), MP-RM-1 could be considered as an excellent vehicle to deliver toxins or drugs to cancer cells expressing ErbB-3. Finally, differently from what observed in transformed cells, ErbB-3 membrane expression as detected with MP-RM-1 was never observed in normal human tissues. On these substrates, the antibody produced a cytoplasmic staining pattern confined to few cell lineages and notably absent from the cardiac muscle (Natali *et al*., in preparation), thus suggesting low levels of toxicity once the antibody is administered to humans.

The therapeutic potential of a fully human monoclonal antibody targeting ErbB-3, MM-121, has recently been reported (Schoeberl *et al*., 2009, 2010). The antibody proved to effectively inhibits ErbB-3

phosphorylation across multiple cancer cell lines including lung, ovarian, prostate, renal, breast and colon, and it is currently in clinical development. Although both antibodies appear to exert inhibitory functions *in vitro* and *in vivo,* MM-121 displays a remarkable higher affinity (Kd, 0.76×10^{-9} m vs 3.27×10^{-8} m). This suggests that a higher affinity may not be a necessarily desirable feature of antibodies to be used as antitumor agents *in vivo*. In fact, according to the 'binding-site barrier' hypothesis, macromolecular ligands could be prevented from penetrating tumors by the very fact of their successful strong binding to the target receptor (Weinstein and van Osdol 1992). Furthermore differently from MM-121, MP-RM-1 is capable of inhibiting both ligand-dependent and -independent activation of ErbB-3–Akt pathway, which may potentially expand the therapeutic use of this antibody. Finally, no $F(ab')_2$ activity has been documented for MM-121 antibody.

In conclusion, our data indicate that MP-RM-1 has the potential to interfere with ErbB-3/Akt activation and point out new possibilities for therapeutic intervention.

Materials and methods

MP-RM-1 generation

Four-weeks-old Balb/c mice were immunized by intraperitoneal injection of live NIH3T3 cells transfected with the human ErbB-3 receptor-coding sequence. After 7 days, mice were given an additional intraperitoneal injection of the immunogen. After additional 7 days, mice were boosted intravenously with the immunogen, and spleens were removed for cell fusion 3 days later. Somatic cell hybrids were prepared by fusion of immune splenocytes with the murine non-secreting myeloma cell line NS-1. Hybridoma supernatants were screened by ELISA and selected on the basis of differential reactivity with the cell surface LTR-ErbB3 NIH/3T3 transfected cells, but not with LTR-neo NIH/3T3. All ErbB3-positive hybridoma cell cultures were cloned twice by limiting dilution and further characterized. Potential cross-reactivity of hybridoma supernatants with other ErbB receptors were evaluated by a cell-based ELISA and immunoprecipitation using NIH3T3 cells transfected with individual human ErbB receptor-coding sequences as recombinant antigen source. NIH3T3 cells transfected with expression vectors for human coding sequences of ErbB family receptors have previously been described (Di Fiore *et al*., 1987a, 1987b; Kraus *et al*., 1993; Baulida *et al*., 1996). Of $\sim$1000 hybridoma clones screened, one, MP-RM-1, recognized the ECD of ErbB3 but not that of the other ErbB family members (ErbB-1, ErbB-2 and ErbB-4), when used in a cell-based ELISA and immunoprecipitation. Using surface plasmon resonance, the dissociation constant (Kd) for MP-RM-1 was determined to be 3.27×10^{-8}m.

The hybridoma murine cell line producing MP-RM-1 antibody was deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and designated DSM ACC3018.

Materials and constructs

Sequence targeting the human ErbB-3 mRNA (Lee-Hoeflich *et al*., 2008) was subcloned into pSuper vectors. Control vector pSuper 4Mut contains a four-point mutated sequence unable to target the human ErbB-3 mRNA (Sala *et al*., 2008). Silencing of ErbB-3 was obtained by using pSuper retro-based vectors to express stable RNA (shRNA). Retroviral stocks were generated as described (Anastasi *et al*., 2005). Antibodies were as follows: phosphorylated ErbB-3 (Tyr^{1289}), phosphorylated Akt (Ser^{473}), Akt, PLCγ1, phosphorylated MET ($Tyr^{1234-1235}$) from Cell Signaling (Boston, MA, USA); C-17 ErbB-3 and MET from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Actin from Sigma-Aldrich (St Louis, MO, USA); anti-ErbB-2 antibody 300G9 was obtained as described in Digiesi *et al.* (1992) rhodamine[^{125}I]-labeled phalloidin from Sigma; Draq5 from Alexis; Yo-Pro dye from Invitrogen (Carlsbad, CA, USA). MET inhibitor SU11274 was purchased from Biaffin GmbH & Co KG (http://www.proteinkinase.de).

Cell lines

MDA-MB-435, T47D and SKBr-3 human breast cancer cells, DU-145 human prostate cancer cells were purchased from ATCC. The cutaneous melanoma cell line IR-8 (Leonetti *et al*., 1996) was kindly provided by Dr Carlo Leonetti ('Regina Elena' National Cancer Institute, Rome, Italy). Gastric carcinoma cell line MKN-45 (Rege-Cambrin *et al*., 1992) was kindly provided by Dr Sergio Anastasi ('Regina Elena' National Cancer Institute, Rome, Italy). MDA MB 435, DU-145 and IR-8 cell lines were grown in RPMI 1640 medium, SKBr-3 and MKN-45 were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin and incubated in 5% CO2 at 37 °C.

Immunochemistry

Proteins were solubilized in HNTG buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM EGTA). Immunoblotting was performed as described in Fiorini *et al*. (2002). Proteins from blots were quantified using Image J software program (http://rsbweb.nih.gov/ij/).

Fluorescence-activated cell sorting analysis

For ErbB-3 surface expression analysis MDA-MB-435 cells have been incubated on ice in the presence of 1 μg/ml of MP-RM-1 for 30 min and then returned to 37 °C for the indicated time, harvested and stained with a fluorescent goat anti-mouse secondary antibody. After staining procedures, samples were analyzed by flow cytometry using a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Finally, data were analyzed using CELLQuest 3.2.1.f1 software (Becton Dickinson).

Confocal microscopy

Microscopy was performed as described in Piccolo *et al*. (2002) using a Bio-Rad MRC-1024 confocal system (BioRad Laboratories, Hercules, CA, USA). Briefly, cells has been plated in 22×22 coverslips and grown in 0.2% FBS RPMI 1640 for 24 h. Thereafter, cells were incubated or not with 10 μg/ml of MP-RM-1 for 30 min on ice and placed back at 37 °C for 60 min. At the end of the experiment, cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton phosphate-buffered saline (PBS) and then stained with fluorescently labeled secondary antibodies as indicated. Alexa-Fluor-568 monoclonal antibody labeling kit was purchased from Molecular Probes (Invitrogen). Rhodamine-labeled phalloidin and Draq5 (or Yo-Pro dye) were used to visualize actin cytoskeleton and nuclei, respectively. No further processing of the images was done except for changes in brightness/contrast to better visualize the data.

[^{125}I]-labeled NRG-1β-binding studies

NRG-1β zwas [^{125}I]-labeled using the IODO-GEN kit according to the supplier's protocol (Thermo Fisher Scientific,

Illkirch Cedex, France). Specific activity was about 15 μCi/μg. Cells were plate at 5×10^4 in 12-well plates, serum starved for 24 h and then incubated with 10 ng/ml of [^{125}I]-labeled NRG-1β in binding medium (BM; that is, DME containing 0.2% bovine serum albumin and 20 mM Hepes, pH 7.5) at 4 °C for 60 min in the absence or presence of NRG-1β and MP-RM-1. The free [^{125}I]-labeled NRG-1β was eliminated washing three times with ice-cold PBS. The cells were lysed in 0.3 ml of lysis buffer (1% sodium dodecyl sulfate and 10 mM NaOH), and the radioactivity was measured by a gamma counter.

Soft-agar colony formation assay
For the anchorage-independent growth assay, 15×10^3 IR-8 cells were suspended in 0.3% agarose medium containing RPMI 1640 10% FBS and layered onto a 2 ml bed of 0.5% agarose in a six-well plate dish. Dishes were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. A measure of 10 μg/ml MP-RM-1 or PBS was added to cells every alternate day and the number of colonies was determined (under light microscopy) after 10 days.

In vivo *tumor growth*
Athymic CD-1 nu/nu mice (7-weeks old) and BALB/c mice were purchased from Charles River Laboratories and maintained under specific pathogen-free conditions with food and water provided *ad libitum*. The animals health status was monitored daily. Procedure involving animals and their care were conducted according to the institutional guidelines in compliance with national and international laws and policies.

IR-8 and DU-145 xenografts were generated by s.c. into the right flank of 3×10^6 IR-8 cells and 5×10^6 DU-145 cells in 200 μl PBS, respectively. When xenografts were palpable, animals were divided into two groups (10 animals each) selected to provide a similar range in tumor size in each group. One group received i.p. injection twice weekly of 20 mg/kg MP-RM-1 in PBS, whereas the other received PBS only (control group). Tumor volume was monitored every day by a caliper and volumes were calculated using the following formula: tumor volume (mm^3) = (length × width^2)/2. At the end of the study, mice were anesthetized with isoflurane inhalation before cervical dislocation. Tumors were excised and snap frozen for immunohistochemical analysis or analyzed for western blotting as previously described (Sala *et al*., 2008).

Tumor mitotic index and Ki-67 expression analysis
Formalin-fixed and paraffin-embedded tissues from xenografts DU-145 human prostate cancer cells of untreated mice ($n = 7$) and treated mice ($n = 8$) were sectioned at 5 μm and stained with Hematoxylin and Eosin (H&E). For the evaluation of the mitotic figures, the sections were scanned at low magnification (× 5). Four high-power fields (× 40; 0.17 mm^2 field) of tumor with the greatest number of mitotic figures were then selected and used for the statistical analysis. Tissue microarrays (TMA) were also constructed by extracting 2-mm diameter core of hystologically confirmed DU-145 human prostate cancer cells areas from each original mouse paraffin block and re-embedding these cores into gridded paraffin blocks using a TMA precision instrument (Beecher Instruments, Sun Prairie, WI, USA). TMA sections were incubated—after antigen retrieval by thermostatic bath at 96 °C in 10 mM/l citrate buffer, pH 6 for 40 min—with the anti-Ki-67 monoclonal antibody MIB-1 (Dako, Glostrup, Denmark) for 30 min at room temperature. The immunoreactions were revealed by a streptavidin-biotin-enhanced peroxidase system (Super Sensitive Link-Label IHC DetectionSystem; BioGenex, Space, Milan, Italy). Positive and negative controls were included for each antibody and in each batch of staining. Mean differences in mitotic count and Ki-67 expression were compared with the use of the paired *t* test. SPSS Version 15.0 (SPSS, Chicago, IL, USA) was used and $P < 0.05$ was considered statistically significant.

Immunohistochemistry
Frozen normal human adult tissues belonged to the archivial bank of CINBO and were collected and used according to the Ethic Committee guidelines. Sections (4 μm) were fixed in absolute acetone for 10 min and after air drying either immediately processed or stored at −20 °C with no loss of immune reactivity for at least 6 months. For indirect immunoperoxidase stain, sections were incubated overnight with MP-RM-1 antibody (50 μg/ml). The immune reactivity was detected by using the EnVision Peroxidase Detection System. Specimens using aminoethylcarbazole as chromogen and finally counterstained with Mayer's hematoxylin for nuclear stain. The experiments included appropriate negative and positive controls. Immunohistochemical findings were scored as follows: negative, when no stain or dubious weak reactivity was observed, 1+ when the stain was homogenous of moderate intensity, 2+ when the stain was homogenously intense. Scores were carried out by two independent readers.

Patient-derived carcinoma cells
Freshly collected tumor cells from pleural and peritoneal effusions appearing in patients free from therapy were propagated *in vitro* for a limited number of passages using RPMI 1640 medium supplemented with 10 % decomplemented FBS, 2 mm L-glutamine at 37 °C in a 5% $C0_2$/95% air atmosphere. Tumor cells harvesting and processing before *in vitro* growth as well immuno phenotyping, were carried out as described (Mottolese *et al*., 1988).

Statitstical analysis
Mean volumes of treated and untreated xenografts were compared using an unpaired *t* test (Student's *t* test) considering as statistically significant a *P*-value <0.05 (CI 95%). Mean differences in mitotic count and Ki-67 expression were compared with the use of the paired *t* test. SPSS Version 15.0 (SPSS) was used and $P < 0.05$ was considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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