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ORIGINAL ARTICLE

Suppression of breast cancer cell growth by a monoclonal antibody targeting cleavable ErbB4 isoforms

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ErbB4 isoforms mediate different cellular activities depending on their susceptibility to proteolytic cleavage. The biological significance of ErbB4 cleavage in tumorigenesis, however, remains poorly understood. Here, we describe characterization of a monoclonal antibody (mAb 1479) that selectively recognizes the ectodomain of cleavable ErbB4 JM-a isoforms both *in vitro* and *in vivo*. mAb 1479 was used to analyse ErbB4 JM-a expression and ectodomain shedding in a series of 17 matched breast cancer/histologically normal peripheral breast tissue pairs. ErbB4 ectodomain was observed in 75% of tumors expressing ErbB4 but only in 18% of normal breast tissue samples expressing ErbB4. Difference in the relative quantity of ErbB4 ectodomain between normal and tumor tissue pairs was statistically significant ($P = 0.015$). Treatment with mAb 1479 suppressed ErbB4 function by inhibiting ErbB4 tyrosine phosphorylation and ectodomain shedding, and by stimulating ErbB4 downregulation and ubiquitination. mAb 1479 suppressed both anchorage-dependent and -independent growth of human breast cancer cell lines that naturally express cleavable ErbB4 JM-a. These findings indicate that ErbB4 ectodomain shedding is enhanced in breast cancer tissue *in vivo*, and that mAb 1479 represents a potential drug candidate that suppresses breast cancer cell growth by selectively binding cleavable ErbB4 isoforms.
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Introduction

ErbB/HER receptors form a subfamily of receptor tyrosine kinases that includes ErbB1 (also known as epidermal growth factor receptor (EGFR) or HER1), ErbB2 (c-Neu, HER2), ErbB3 (HER3) and ErbB4 (HER4). ErbB1 and ErbB2 are well-established oncogenes and cancer drug targets. They are implicated in the pathogenesis of various epithelial and neural malignancies, and their overactivity is associated with poor patient outcome (Slamon *et al*., 1987; Mendelsohn and Baselga, 2000; Hynes and Lane, 2005). Targeted therapeutics including both monoclonal antibodies and small-molecular-weight kinase inhibitors blocking the functions of ErbB1 and/or ErbB2 have demonstrated therapeutic effect in clinical trials.

The significance of ErbB4 in cancer is poorly understood. Some observations indicate that ErbB4 receptor is downregulated in various cancers, or that its expression is associated with favorable prognostic markers, such as estrogen receptor expression (Srinivasan *et al*., 1998; Witton *et al*., 2003). On the other hand, ErbB4 has been reported to be expressed in high levels in several cancers such as thyroid (Haugen *et al*., 1996), ovarian (Furger *et al*., 1998) and breast cancer (Srinivasan *et al*., 1999), as well as medulloblastoma (Gilbertson *et al*., 1997), and ependymoma (Gilbertson *et al*., 2002). Furthermore, the significance of ErbB4 expression levels for clinical outcome is conflicting (Sundvall *et al*., 2008a).

One of the plausible explanations for contradictory data about the clinical significance of ErbB4 is that four structurally and functionally different isoforms are generated from a single *ErbB4* gene by alternative splicing (Junttila *et al*., 2000, 2003). Two of the isoforms differ in the intracellular cytoplasmic domain (isoforms CYT-1 and CYT-2) and two in the extracellular juxtamembrane region (isoforms JM-a and JM-b) of ErbB4. The extracellular isoform JM-a can be cleaved by tumor necrosis factor-α-converting enzyme (TACE) (Rio *et al*., 2000) whereas the JM-b isoform is proteinase resistant (Elenius *et al*., 1997). Cleavage by TACE triggers a second cleavage of ErbB4 involving γ-secretase activity (Lee *et al*., 2002). As a result the intracellular domain (ICD) is released from the cell membrane and translocates to the nucleus where it may function as a transcriptional regulator (Ni *et al*., 2001; Komuro *et al*., 2003; Määttä *et al*., 2006; Schlessinger and Lemmon, 2006). Consistent with the hypothesis that

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ErbB4 isoforms differ in their function in tumorigenesis, the cleavable ErbB4 JM-a CYT-2 isoform, but not its noncleavable counterpart JM-b CYT-2, demonstrates ligand-independent activity and promotes cancer cell growth (Määttä *et al*., 2006). In addition, localization of an intracellular ErbB4 epitope in the nuclei is associated with shorter survival when compared to localization of ErbB4 at the cell surface (Junttila *et al*., 2005), suggesting that ErbB4 cleavage can regulate tumor progression.

To determine the biological functions of different ErbB4 isoforms in cancer, we screened for isoform-specific anti-ErbB4 antibodies. Here we describe characterization of a monoclonal antibody (mAb) that selectively recognizes cleavable ErbB4 JM-a isoforms predominantly expressed by several cancer types. This antibody, named mAb 1479, suppressed proliferation and colony formation of breast cancer cells *in vitro* by a mechanism involving inhibition of ErbB4 phosphorylation and cleavage and stimulation of ErbB4 down-regulation. The findings indicate that antibodies selectively targeting ErbB4 JM-a may have therapeutic potential. JM-a-specific antibody could also be expected not to cause adverse effects in tissues, such as heart, that exclusively express ErbB4 JM-b.

Results

mAb 1479 selectively recognizes the proteolytically cleavable JM-a isoforms of ErbB4

Twenty-nine hybridoma clones secreting antibodies against the ectodomain of ErbB4 (Supplementary Table 1) were screened for selective recognition of ErbB4 isoforms. COS-7 cells transiently expressing ErbB4 isoforms with alternative extracellular juxtamembrane domains (JM-a CYT-2 or JM-b CYT-2) were analysed. One of the antibodies, mAb 1479, recognized the JM-a isoform, but not the JM-b isoform, when used as the primary antibody in western blotting (Figure 1a). The ErbB4-specific signal was reduced in intensity when samples were boiled before analysis, and totally abolished when samples were boiled as well as subjected to reducing conditions with dithiothreitol (DTT) indicating that mAb 1479 only recognized the native conformation. Under nonreducing conditions, ErbB4 appeared as a band of 150 kDa, close to the expected size of 144 kDa deduced from cDNA (Plowman *et al*., 1993), and under reducing conditions as a band of 180 kDa (compare Figure 1a, bottom panel, lanes 1 vs 7). The specificity of mAb 1479 for JM-a isoform was confirmed by immunofluorescence staining of COS-7 cells expressing hemagglutinin (HA)-tagged ErbB4 isoforms (Figure 1b).

As members of the ErbB family are homologous, the cross-reactivity of mAb 1479 with other ErbB receptors was assessed by western blotting using NIH 3T3-7d and NR6 transfectants expressing the different ErbB receptors. mAb 1479 gave a strong signal for ErbB4 (JM-a CYT-2) but also recognized a faint band in western analysis of lysates from cells overexpressing ErbB1 (Figure 1c). Thus, to further address the cross-reactivity using a more quantitative assay, a cell-based enzyme-linked immunosorbent assay (ELISA) was carried out. mAb 1479, as well as a positive control anti-ErbB4 antibody (sc-283), clearly bound to NR6 transfectants expressing ErbB4 (JM-a CYT-2) even at the lowest antibody concentration (1:1000; 1.25 μg/ml of mAb 1479; 0.2 μg/ml of sc-283) tested (Figure 1d). However, no binding of mAb 1479 was observed to NIH 3T3-7d cells expressing ErbB1, ErbB2, ErbB3 or the JM-b CYT-1 isoform of ErbB4 even at the highest antibody concentration (1:100; 12.5 μg/ml), whereas the control antibodies anti-ErbB1 (sc-03), anti-ErbB2 (sc-284), anti-ErbB3 (sc-285) and anti-ErbB4 (sc-283) demonstrated binding (1:100; 2 μg/ml) (Figure 1d). These data indicate that mAb 1479 was specific for the JM-a isoform of ErbB4.

mAb 1479 recognizes the shed ectodomain of ErbB4

To address whether mAb 1479 can be used to selectively analyse ErbB4 JM-a isoforms *in vivo*, frozen sections of human kidney and heart were analysed by immunofluorescence staining. These two tissue types were selected as they exclusively express either JM-a (kidney) or JM-b (heart) isoforms (Elenius *et al*., 1997). mAb 1479 specifically stained kidney but not heart tissue (Figure 2a). A positive control antibody against the C-terminal end of ErbB4 (HFR-1) stained both tissues, and no immunostaining was observed when a negative control antibody against a chicken T cell protein (3g6) was used. An antibody against CD44 protein was used as a control for another membrane-anchored protein. The different staining patterns observed for mAb 1479 and HFR-1 in kidney may be explained by the different epitopes they recognize, but more likely by the fact that the ErbB4 molecule is cleaved and its ectodomain shed in kidney tissue, as suggested by localization of ErbB4 ICD in the nuclei of glomerular cells (Supplementary Figure 1). Also in support of ErbB4 cleavage in kidney tissue *in vivo*, western analysis of kidney tissue lysates demonstrated that mAb 1479 recognized two major bands. One migrated at 150 kDa corresponding to the size of full-length ErbB4 under nonreducing conditions (compare Figure 2b, lane 1 vs Figure 1a, lane 1), and the other more prominent one at 100 kDa corresponding to the size of recombinant ErbB4 ectodomain (Figure 2b, lanes 1 vs 2). A third band migrating at $\approx$200 kDa was similar in size to a weak band in the lane with recombinant ectodomain and may represent ectodomain dimers.

To more directly demonstrate that mAb 1479 can recognize the soluble ectodomain of ErbB4, an *in vitro* binding assay was carried out with recombinant ErbB4 ectodomain and mAb 1479. The ectodomain and the antibody formed a complex of 250 kDa that was detected in western analysis (Figure 2c). Moreover, an ELISA with microwell-plate-immobilized ErbB4 ectodomain gave a K_d value of 0.85 nM $\pm$ 0.077 for the interaction with mAb 1479 (Figure 2d). These observations demonstrate that mAb 1479 binds ErbB4 ectodomain with a relatively high affinity.

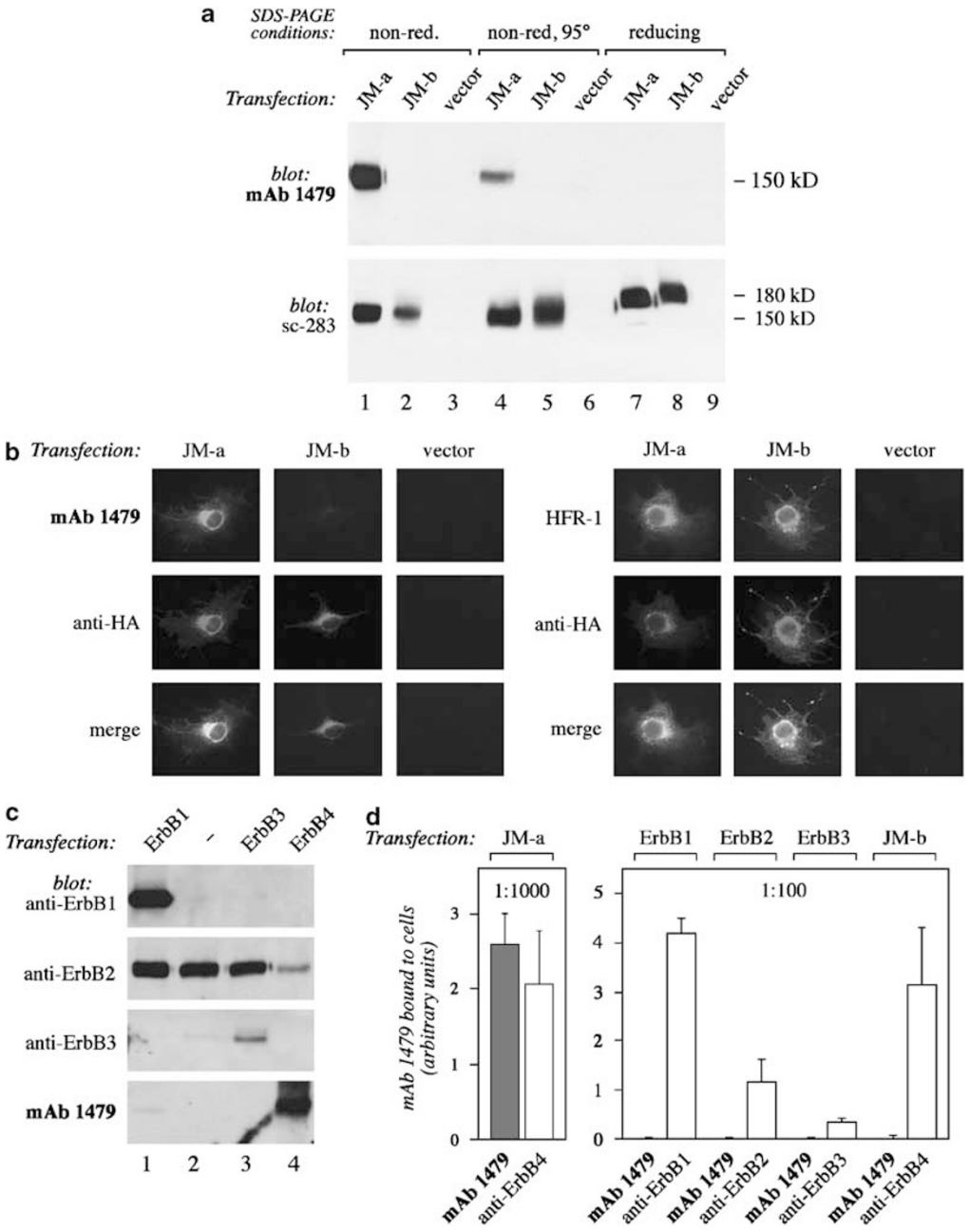

Figure 1 mAb 1479 selectively recognizes the cleavable JM-a isoform of ErbB4. (**a**, **b**) COS-7 cells transiently expressing wild-type (**a**) or HA-tagged (**b**) ErbB4 juxtamembrane isoforms JM-a CYT-2 or JM-b CYT-2 were analysed by western blotting (**a**) or immunofluorescence staining (**b**) using mAb 1479 as the primary antibody. Western analysis was carried out in three conditions representing different degrees of protein denaturation: samples in lanes 1–3 were dissolved in sample buffer without dithiothreitol (DTT) at room temperature (nonreducing), samples in lanes 4–6 were dissolved in sample buffer without DTT but heated for 5 min at 95 °C (nonreducing, 95 °C) and samples in lanes 7–9 were dissolved in sample buffer containing DTT as well as heated for 5 min at 95 °C (reducing). Antibodies against the C terminus of ErbB4 (sc-283 and HFR-1) or against the HA-tag (anti-HA) were used to control transfection efficiency. (**c**) Parental (lane 2) or transfected (lanes 1 and 3) NIH 3T3-7d and NR6 (lane 4) cells expressing different ErbB receptors were analysed by western blotting using anti-ErbB1 (sc-03), anti-ErbB2 (sc-284), anti-ErbB3 (sc-285) or mAb 1479 as the primary antibody. The parental NIH 3T3-7d and NR6 cells express endogenous ErbB2, which was detected in all analysed transfectants with anti-ErbB2. (**d**) Binding of mAb 1479 to NIH 3T3-7d and NR6 transfectants expressing the indicated ErbBs or ErbB4 isoforms was measured using a cell enzyme-linked immunosorbent assay (ELISA) (gray columns). mAb 1479 binding was compared to binding of anti-ErbB1 (sc-03), anti-ErbB2 (sc-284), anti-ErbB3 (sc-285) and anti-ErbB4 (sc-283) (binding of all control antibodies in white columns) in dilutions 1:1000 (left) or 1:100 (right).

ErbB4 ectodomain shedding is enhanced in breast cancer in vivo

Cleavable JM-a isoforms, as well as the TACE enzyme capable of cleaving ErbB4, are overexpressed in breast tumor tissues *in vivo* (Määttä *et al*., 2006). To test whether transformation of histologically normal breast tissue to breast carcinoma is associated with enhanced shedding of ErbB4 ectodomain, 17 matched breast

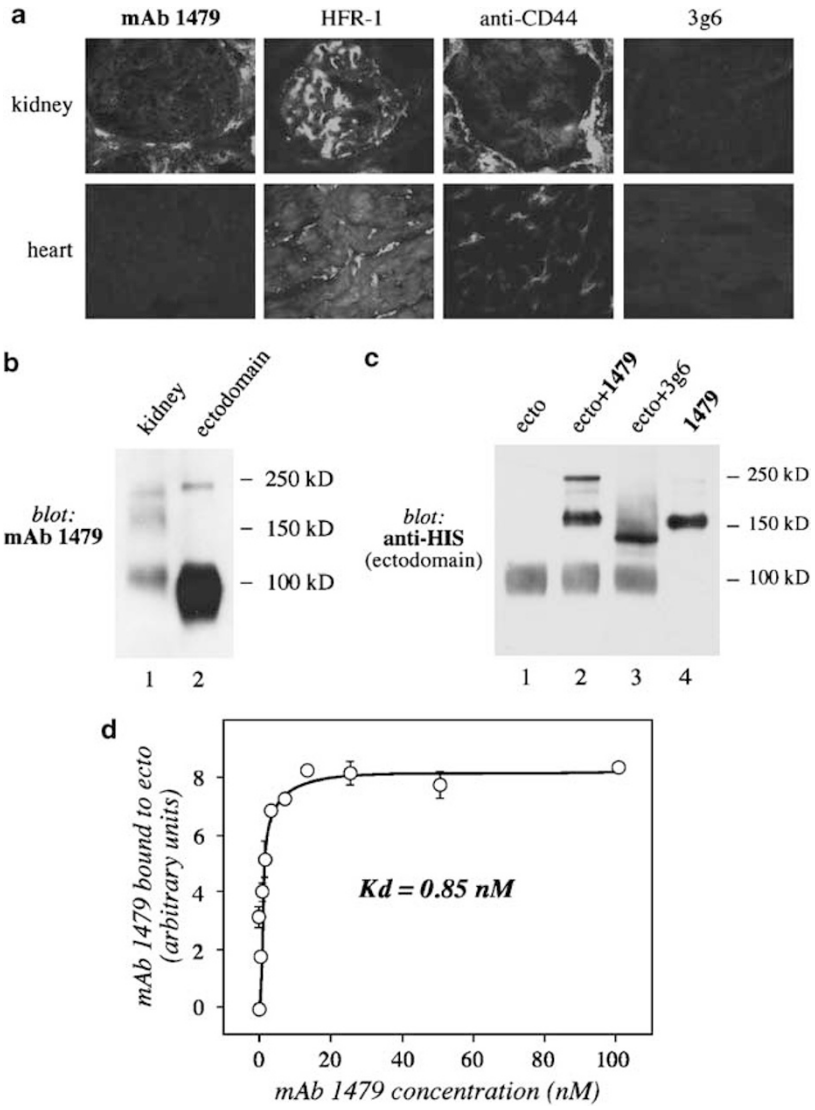

Figure 2 mAb 1479 recognizes the shed ectodomain of ErbB4 *in vivo* and *in vitro*. (**a**) Immunofluorescence staining of frozen tissue sections of human kidney and heart using the following primary antibodies: anti-ErbB4 antibodies mAb 1479 and HFR-1, anti-CD-44 and a negative control antibody 3g6 recognizing a chicken T cell antigen. (**b**) Western blot analysis of human kidney tissue lysate and recombinant ErbB4 ectodomain with mAb 1479. ErbB4 ectodomain is seen migrating at the size of 100 kDa, full-length ErbB4 at 150 kDa and putative ectodomain dimers at $\approx$200 kDa. (**c**) *In vitro* binding assay with mAb 1479 and HIS-tagged recombinant ErbB4 ectodomain. Ectodomain (2 μg) was incubated with 0.5 μg of either mAb 1479 or the negative control antibody 3g6 for 15 min and the formation of a protein complex was visualized by western analysis under nonreducing conditions using an anti-HIS antibody. Free ectodomain is seen migrating at the size of 100 kDa, free antibodies at 150 kDa and the complex formed by the ectodomain bound to the antibody at 250 kDa. (**d**) A nonlinear affinity curve of an enzyme-linked immunosorbent assay (ELISA) measuring mAb 1479 binding to recombinant ErbB4 ectodomain. HIS-tagged recombinant ectodomain (100 ng) was immobilized onto microwells and incubated in the presence of mAb 1479 concentration ranging from 0.195 to 100 nM. mAb 1479 bound ErbB4 ectodomain with a K_d value of 0.85 nM $\pm$ 0.077.

cancer/normal breast tissue pairs were analysed by western blotting with mAb 1479 (Figure 3a). The sample pairs consisted of material from patients from whom both cancer tissue and histologically normal adjacent tissue were available. The western data generated under nonreducing conditions and thus resulting in relatively diffuse signals were scored for the intensities of the 150 kDa signal representing full-length ErbB4 and the 100 kDa signal representing soluble ectodomain. Of 17, 9 (53%) tumor samples demonstrated increased total ErbB4 expression when compared to the matched normal tissue pair. Only one tumor sample (1/17; 6%) had no detectable ErbB4 expression as opposed to six samples of normal breast tissue (6/17; 35%). Similar findings of enhanced ErbB4 protein levels in cancer vs normal tissues were also obtained by immunostaining of frozen sections with mAb 1479 (Figure 3b).

Interestingly, signal for ErbB4 ectodomain was observed in 12 (12/16; 75%) of ErbB4-positive tumor

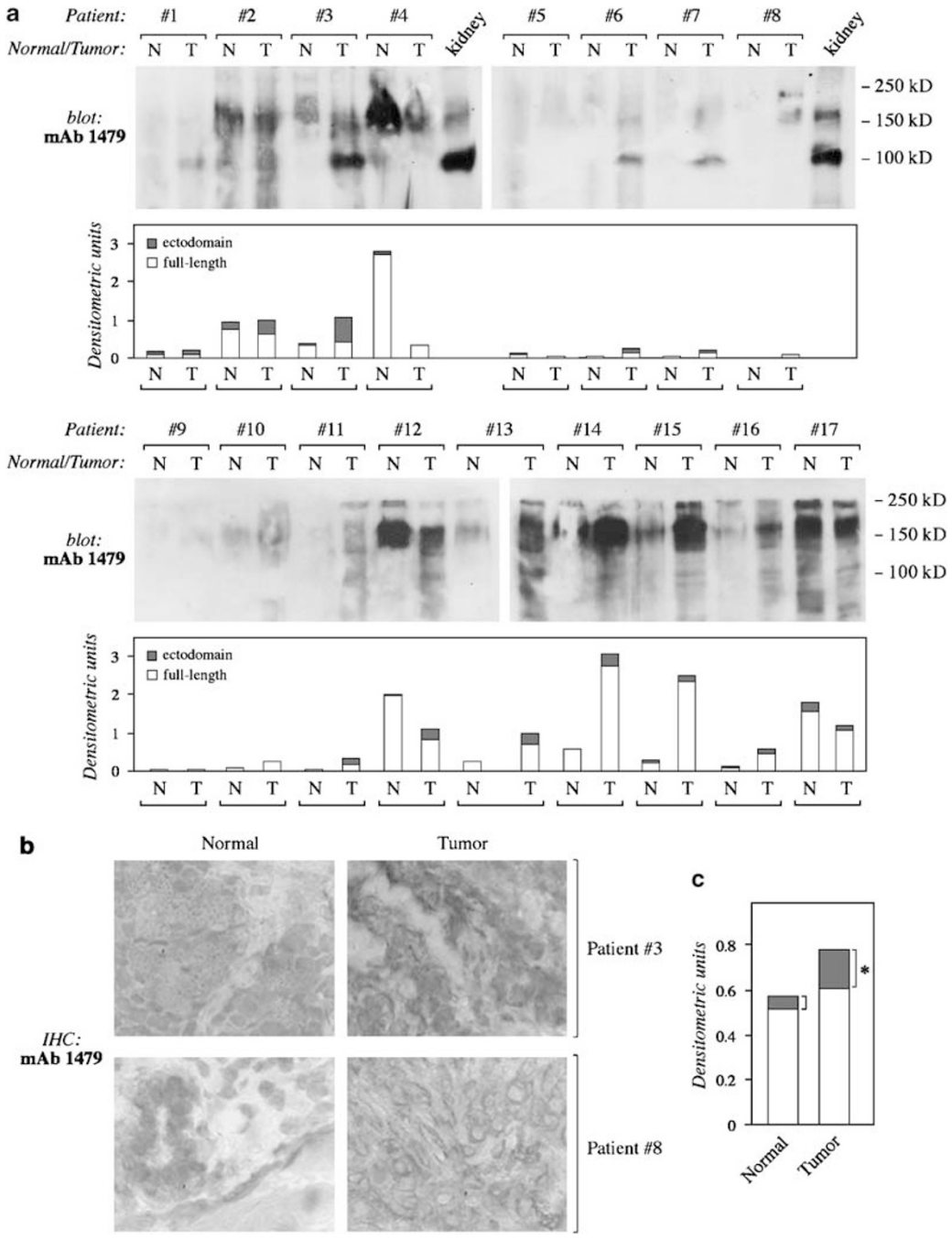

Figure 3 ErbB4 ectodomain shedding is enhanced in breast cancer *in vivo*. (**a**) Western analysis of 17 paired normal breast/breast tumor tissue samples using mAb 1479 as the primary antibody. Densitometric quantitation of the expression levels of 100 kDa ErbB4 ectodomain (gray) and 150 kDa full-length ErbB4 (white) are shown underneath each western lane. (**b**) Immunohistochemical staining with mAb 1479 of normal and tumor tissue sections obtained from patients 3 and 8. (**c**) Mean expression of ErbB4 ectodomain and full-length receptor in the 17 matched normal and breast tumor tissue pairs quantitated by densitometry. *, the fraction of total ErbB4 present as ectodomain was greater within tumor tissues, when compared to the matched normal tissue controls (gray boxes; $P<0.05$; Wilcoxon signed-rank test).

samples but only in 2 ErbB4-positive normal breast tissue samples (2/11; 18%) ($P=0.002$). When the western signals for the 150 kDa full-length receptor and the 100 kDa ectodomain were quantified by densitometry, the ectodomain/full-length receptor ratio was significantly greater within tumor tissues samples when compared to normal tissues ($P=0.015$) (Figure 3c). The higher level of shed ectodomain seen in tumor tissues was not a consequence of more protein being made as the amount of detected 100 kDa ectodomain did not correlate with the total amount of ErbB4 (100 + 150 kDa) in the same sample ($P=0.15$). Taken together, these data indicate that shedding of ErbB4 ectodomain is enhanced during breast cancer

progression, and that mAb 1479 can be used to detect ErbB4 ectodomain shed by human tumor tissue.

mAb 1479 suppresses ErbB4 phosphorylation and cleavage

To analyse the consequences of mAb 1479 binding on ErbB4 function, ErbB4 phosphorylation was measured in MCF-7 breast cancer cells naturally expressing JM-a isoforms. mAb 1479 significantly suppressed ErbB4 phosphorylation stimulated by the ligand neuregulin-1 (NRG-1) (Figure 4a).

Because activation of ErbB4 may also regulate ErbB4 cleavage (Cheng *et al*., 2003), the effect of mAb on both basal and phorbol 13-myristate 12-acetate (PMA)-stimulated ErbB4 cleavage was assessed. Treatment with mAb 1479 significantly decreased the amount of soluble 100 kDa ErbB4 ectodomain shed into the medium of COS-7 transfectants, when compared to nontreated cells or cells treated with mAb 1475 recognizing a different ErbB4 epitope (Figure 4b). No significant effect, however, was observed on the levels of cell-associated 180 or 80 kDa ErbB4 species under the conditions used (Figure 4b), which may reflect the fact that only a minor percentage of total ErbB4 was observed in the medium of COS-7 transfectants expressing relatively high quantities of ErbB4 (data not shown).

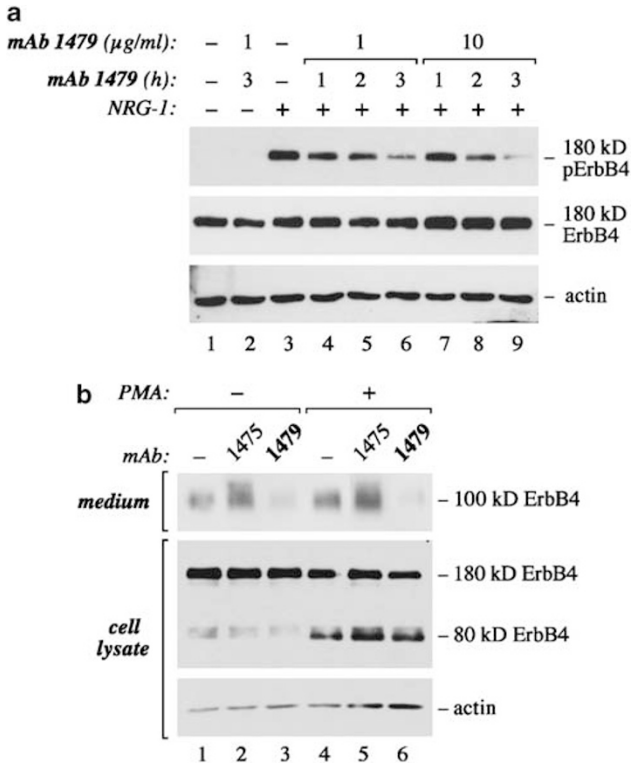

Figure 4 mAb 1479 suppresses ErbB4 phosphorylation and cleavage. (**a**) MCF-7 breast cancer cells were stimulated for 1, 2 or 3 h with 0, 1 or 10 μg/ml mAb 1479 before a 15 min stimulation with 50 ng/ml neuregulin-1 (NRG-1), and analysed for ErbB4 tyrosine phosphorylation using a phospho-specific antibody against pTyr1284 of ErbB4. The membrane was reblotted with anti-ErbB4 (Abcam) and anti-actin. (**b**) Shedding of 100 kDa ErbB4 ectodomain into the culture medium of COS-7 transfectants expressing ErbB4 JM-a CYT-2 was analysed by western blotting with an anti-ErbB4 antibody mAb 1464 after stimulating the cells for 24 h with 1 μg/ml mAb 1479 or a control antibody mAb 1475. Total cell lysates from the same experiment were analysed by western blotting with anti-ErbB4 (Abcam) and anti-actin.

mAb 1479 is efficiently internalized and promotes ErbB4 downregulation

Cetuximab has been suggested to suppress tumor growth by facilitating EGFR downregulation (Sunada *et al*., 1986). mAb 1479 was observed to become rapidly depleted from the culture media of cells expressing ErbB4 but not from media of vector control cells (data not shown). To address whether this was due to ErbB4-mediated internalization of the mAb, COS-7 cells expressing ErbB4 JM-a were treated with mAb 1479 and analysed by confocal microscopy to visualize the subcellular localization of the antibody. After 5 min of incubation, mAb 1479 was predominantly detected on the cell surfaces (Figure 5a). However, in 2 h the antibody had been internalized to the cytosol and partially colocalized with green fluorescent protein (GFP)-tagged Rab5, a marker of early endocytic vesicles (Figure 5a). No cytoplasmic localization of mAb 1479 was observed in cells expressing the JM-b isoform (Figure 5a). Internalization of mAb 1479 did not require ErbB4 kinase activity as mAb 1479 was efficiently internalized also when cells expressing a kinase-dead ErbB4 JM-a construct (K751R) were analysed (Figure 5a).

mAb 1479 also enhanced ubiquitination of cleavable JM-a ErbB4 isoforms (Figure 5b) suggesting that, in addition to being internalized through basal ErbB4 turnover, mAb 1479 may also actively stimulate ErbB4 endocytosis into degradative vesicles. Consistent with previous observations about differential association of the ErbB4 isoforms with the ubiquitin ligase Itch (Sundvall *et al*., 2008b), ubiquitination of the CYT-1 isoform was more efficient than ubiquitination of the CYT-2 isoform (Figure 5b).

To address whether the efficient internalization of mAb 1479 into cells expressing ErbB4 associated with stimulation of ErbB4 downregulation, MCF-7 cells expressing moderate levels of endogenous ErbB4 were cultured for up to 72 h in the presence of mAb 1479 and the total levels of full-length ErbB4 were analysed by western blotting (Figure 5c). Treatment with mAb 1479, but not with the control antibody 3g6, significantly decreased the ErbB4 steady-state levels. The effect was already seen at the 2-h time point and lasted for 72 h.

mAb 1479 suppresses proliferation and colony formation of breast cancer cells

MTS assays measuring the amount of viable cells were carried out with human breast cancer cell lines. mAb 1479 significantly suppressed the proliferation of T-47D ($P = 0.0014$) and MCF-7 ($P = 0.047$) cells (Figure 6a). The effect was similar or more potent when compared to the anti-ErbB2 antibody 2C4, the mouse monoclonal

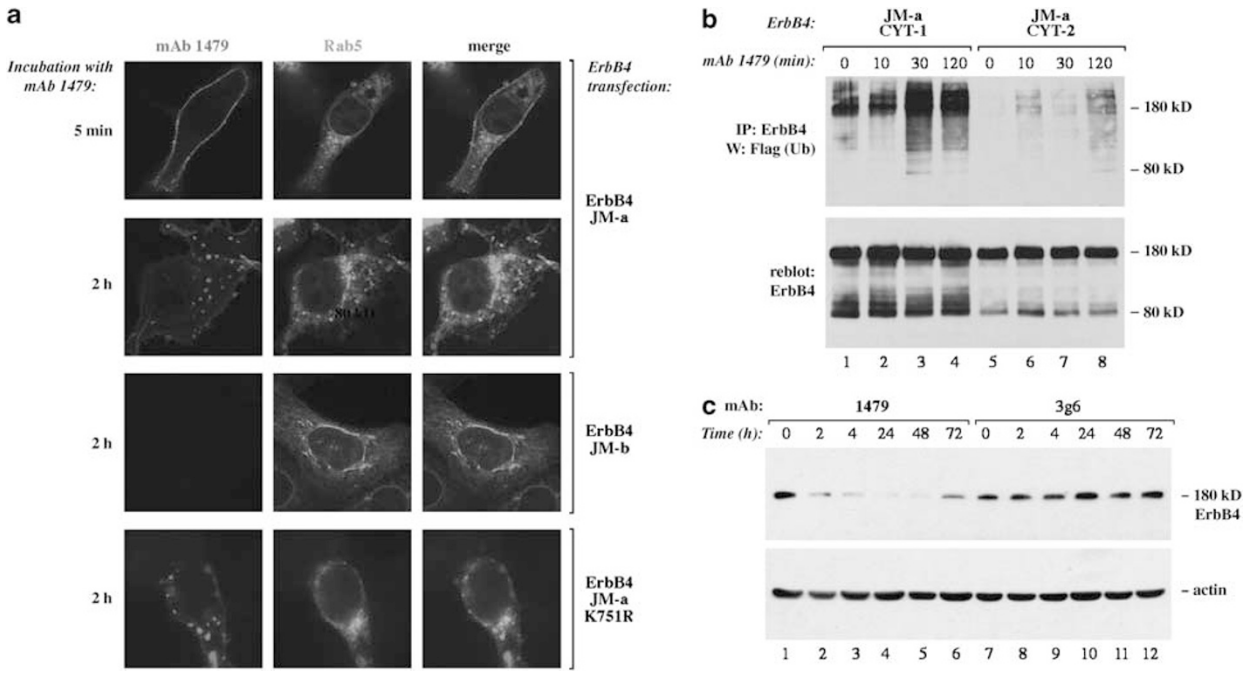

Figure 5 mAb 1479 is efficiently internalized and stimulates ubiquitination and downregulation of ErbB4. (**a**) COS-7 cells transiently expressing ErbB4 isoforms JM-a CYT-2 or JM-b CYT-2, or the kinase-dead K751R-mutated version of JM-a CYT-2 together with GFP-tagged Rab5, were treated for 5 min or 2 h with 1 μg/ml mAb 1479. Colocalization of ErbB4 with Rab5-positive early endosomal vesicles was analysed by confocal microscopy. (**b**) COS-7 cells transiently expressing JM-a CYT-1 or JM-a CYT-2 together with Flag-tagged ubiquitin were treated for 0, 10, 30 or 120 min with 2 μg/ml mAb 1479 and analysed for ErbB4 ubiquitination by anti-ErbB4 immunoprecipitation with HFR-1 followed by western blotting with an anti-Flag antibody. Loading of ErbB4 protein was controlled by reblotting with anti-ErbB4 (Abcam) antibody. (**c**) MCF-7 cells were treated with 1 μg/ml mAb 1479 or the negative control antibody 3g6 for the indicated periods of time. Steady-state ErbB4 protein expression levels were analysed by western blotting with anti-ErbB4 (Abcam) antibody and loading was controlled with anti-actin.

from which the clinically developed pertuzumab is a humanized derivative (Franklin *et al*., 2004). Moreover, mAb 1479 significantly suppressed the anchorage-independent growth of both T-47D ($P<0.001$) and MCF-7 ($P=0.002$) cells in soft agar colony formation assays (Figure 6b and c).

Discussion

To better understand the cancer biology of the specific ErbB4 isoforms and to address their potential as drug targets, we characterized an isoform-specific mouse mAb against the JM-a isoform of ErbB4. This antibody, mAb 1479, selectively recognized the proteolytically cleavable isoforms of ErbB4 both *in vitro* and *in vivo*. mAb 1479 was capable of detecting the intact full-length receptor as well as the soluble cleavage product that is released into the culture medium or extracellular space of tissues. Application of this antibody to cancer cells suppressed proliferation and colony formation of two breast cancer cell lines that naturally express cleavable ErbB4 isoforms.

Characterization of an antibody that recognized the cleaved ErbB4 ectodomain in human samples enabled us to analyse ErbB4 shedding *in vivo* in cancer tissues. Western analysis data indicated that the release of soluble ErbB4 ectodomain was increased in breast cancer samples when compared to normal tissues. The relatively small number of patient samples did not allow us to statistically analyse the prognostic significance of ErbB4 shedding. However, our recent observations that nuclear localization of an intracellular ErbB4 epitope associates with unfavorable clinical outcome when compared to membranous ErbB4 expression (Junttila *et al*., 2005) suggest that enhanced ErbB4 cleavage is associated with poor survival. The functional role of soluble ErbB4 ectodomain *in vivo* has not been addressed but shed ErbB4 ectodomain may trap ErbB4 binding ligands (Gilmore and Riese, 2004), or even function as a ligand itself by signaling through membrane-anchored precursor isoforms of NRG-1 (Bao *et al*., 2003; Iivanainen *et al*., 2007). The mechanism leading to enhanced shedding is currently not known but may involve breast cancer-associated upregulation of expression and activity of TACE (Borrell-Pages *et al*., 2003; Määttä *et al*., 2006).

mAb 1479 suppressed both ErbB4 tyrosine phosphorylation and ErbB4 cleavage. Both of these mechanisms have previously been demonstrated to mediate the anti-tumor activity of therapeutic antibodies targeting extracellular domains (ECDs) of other ErbB receptors. The anti-ErbB1 antibody cetuximab inhibits ligand-mediated phosphorylation of ErbB1 by directly competing with ligand binding to ErbB1 (Prewett *et al*., 1998). The anti-ErbB2 mAb pertuzumab sterically hinders recruitment of ErbB2 into ErbB/ligand complexes

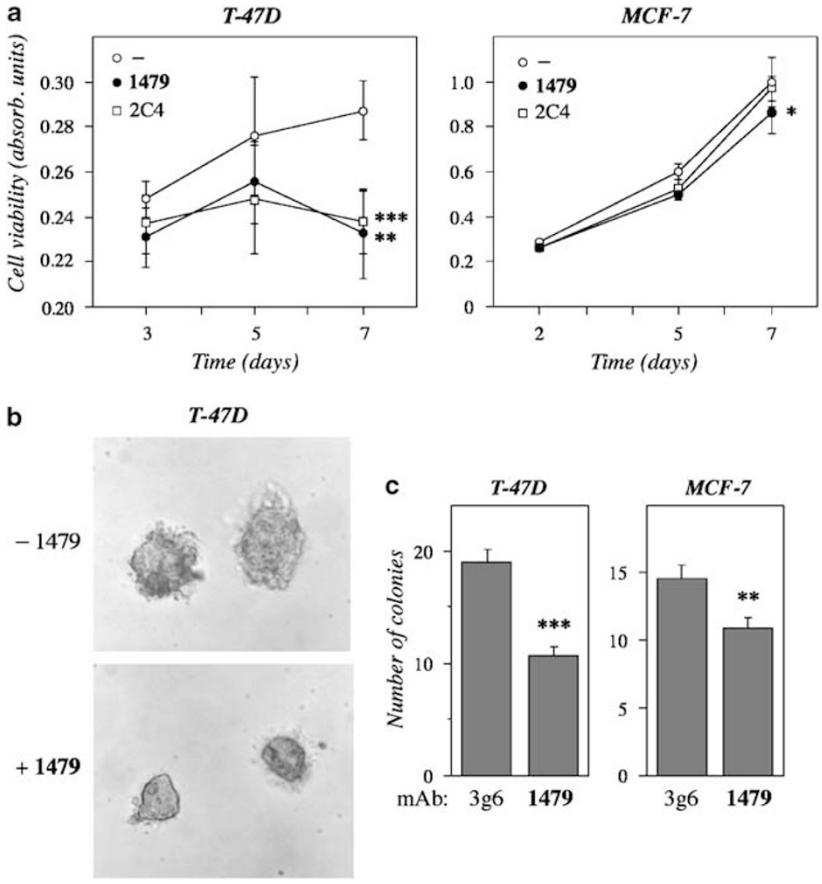

Figure 6 mAb 1479 suppresses proliferation and anchorage-independent growth of breast cancer cells. (**a**) T-47D and MCF-7 cell lines were treated with 1 μg/ml of mAb 1479 or the positive control antibody 2C4 for the indicated periods of time. The number of viable cells was estimated by MTS assays. The antibodies significantly reduced the number of viable cells at the 7-day time point (*$P<0.05$; **$P<0.01$; ***$P<0.001$; Student's *t*-test; five independent experiments carried out in triplicates). (**b**) Representative images of colonies of T-47D cells growing in soft agar after 14-day treatment with or without 1 μg/ml mAb 1479. (**c**) Quantitation of the soft agar growth. Colonies over the size of 8 cells per ×20 field at the 14-day time point were calculated from 10 microscopic fields per experiment carried out three times. mAb 1479 treatment significantly (**$P<0.01$; ***$P<0.001$; Student's *t*-test; three independent experiments carried out in triplicates) reduced the number of colonies at the 14-day time point when compared to the control antibody 3g6. Mean and standard deviations are shown.

affecting the formation and activation of ErbB2-containing dimers (Agus *et al*., 2002; Franklin *et al*., 2004). However, our preliminary *in vitro* experiments with recombinant proteins suggest that mAb 1479 does not compete with NRG binding to ErbB4 or interfere with NRG-induced ErbB4 dimerization.

The anti-ErbB2 antibody trastuzumab is an example of a therapeutic anti-ErbB antibody that blocks both basal and 4-aminophenylmercuric acetate-induced cleavage of ErbB2, thus preventing the formation of constitutively active truncated receptor fragments (Molina *et al*., 2001). Structural data of a trastuzumab cocrystal together with ErbB2 ectodomain indicates that trastuzumab binds ErbB2 at the extracellular juxtamembrane domain within the membrane-proximal cysteine-rich domain IV (Cho *et al*., 2003). Although the exact binding site of mAb 1479 within ErbB4 ectodomain was not determined, the specific interaction of mAb with the JM-a isoforms, but not with JM-b isoforms, suggests that the epitope resides at or in vicinity of the JM-a-specific stretch of 23 amino acids, also in cysteine-rich domain IV. These findings imply that mAb 1479 may interfere with ErbB4 cleavage by a mechanism similar to that of trastuzumab. Whether the mechanism involves blocking an interaction of ErbB4 with the ErbB4-cleaving enzyme TACE that cleaves the receptor at the JM-a-specific domain (Elenius *et al*., 1997; Rio *et al*., 2000; Cheng *et al*., 2003) is currently not known.

mAb 1479 also efficiently stimulated downregulation of total cellular ErbB4 protein levels. ErbB4 downregulation was associated with enhanced ErbB4 ubiquitination, and endocytosis of mAb 1479 into Rab5-positive early endosomal vesicles. It has recently been demonstrated that ErbB4 ubiquitination by the E3 ubiquitin ligase Itch targets ErbB4 to a degradative pathway involving Rab5-positive vesicles (Omerovic *et al*., 2007; Sundvall *et al*., 2008b). Tumor-inhibitory antibodies targeting ErbB2 may also induce internalization and degradation of ErbB2 by recruiting c-Cbl and enhancing receptor ubiquitination (Klapper *et al*., 2000). One of the implications of efficient internalization is that mAb 1479 could also be considered as a vehicle to target

antibody-conjugated toxins (Carter, 2001) to cancer cells expressing ErbB4 JM-a.

Taken together, our data suggest that selective targeting of ErbB4 JM-a isoforms by mAb 1479 may suppress growth of other tumor types with demonstrated ErbB4 JM-a overexpression (Paatero and Elenius, 2008), or tumors with somatic ErbB4 mutations, such as lung cancer (Ding *et al*., 2008). The mechanism by which this antibody regulates cell behavior is dependent of ErbB4 JM-a expression by the target cell, and probably involves inhibition of ErbB4 phosphorylation and cleavage, as well as downregulation of the levels of functional cell-surface protein. Importantly, selective inhibition of the JM-a isoforms would be specifically targeted to the growth-promoting ErbB4 isoform (Määttä *et al*., 2006), and would lack unnecessary toxicity to normal tissues, such as heart, that exclusively express ErbB4 JM-b (Elenius *et al*., 1997). The development of a predictive or prognostic marker for ErbB4 JM-a expressing tumors could be facilitated by our observation that ErbB4 JM-a-positive tumors efficiently shed ErbB4 ectodomain that is recognized by mAb 1479.

Materials and methods

Anti-ErbB4 antibodies

Specific oligonucleotides were synthesized on the basis of the ErbB4 cDNA sequence (Plowman *et al*., 1993). Total cellular RNA was extracted from MDA-MB-453 cells and used as a template in reverse transcriptase–PCR (RT–PCR) to generate the human ErbB4 ECD coding sequence. Antigen purification was accomplished utilizing anti-gD affinity chromatography (Paborsky *et al*., 1990). Balb/c mice were immunized using standard protocols.

Commercially available antibodies used included HFR-1 (Neomarkers, Fremont, CA, USA), anti-ErbB4 (Abcam, Cambridge, UK) and sc-283 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Tissues

Frozen sections of normal human heart and kidney were obtained from a 2-year-old male child who died of an electric shock. Seventeen snap-frozen tissue sample pairs representing human breast cancer and histologically normal peripheral tissue from the same patient were kindly provided by Dr Manolo M Morente, Spanish National Tumour Bank Network, Spanish National Cancer Centre (CNIO), Madrid, Spain. Use of all tissue samples was approved by an Institutional Review Board.

ErbB4 plasmid constructs and transfection

The expression plasmids pcDNA3.*1ErbB4JM-aCYT-1*, pcDNA3.1*ErbB4JM-aCYT*-2, pcDNA3.1*ErbB4JM-bCYT-2*, pcDNA3.1*ErbB4JM-aCYT-2-HA* and pcDNA3.1*ErbB4JM-bCYT-2-HA* (Määttä *et al*., 2006; Sundvall *et al*., 2007) were used to transiently express ErbB4 isoforms with or without C-terminal HA epitope tags. To generate kinase-dead ErbB4 construct, the putative ATP-binding site within the kinase domain of ErbB4 was mutated (K751R) in pcDNA3.1*ErbB4JM-aCYT-2* using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) to produce pcDNA3.1*ErbB4JM-aCYT-2-K751R*. To generate HIS-tagged pcDNA3.1*ErbB4ECD-HIS*, ErbB4 ectodomain encoding sequence was derived from full-length pcDNA3.1*ErbB4JM-aCYT-1* (Määttä *et al*., 2006) by PCR using 5′-primer TTGGTACCGCACCATGAAGCCGGCGACAGGAC and 3′-primer TTATCTCGAGTTAGTGATGGTGATGGTGA TGTTGTGGTAAAGTGGAATG. Cells plated on 24-well plates (4×10^4) or 6-well plates (1.5×10^5) were transfected with 0.5–1 μg of appropriate plasmid using FuGENE 6 transfection reagent (Roche, Mannheim, Germany).

Production of recombinant extracellular domain of ErbB4

HIS-tagged ErbB4 ectodomain was purified from conditioned medium of HEK293 EBNA cells transfected with pcDNA3.1*ErbB4ECD-HIS* by immobilized metal chelate affinity chromatography (GE Healthcare, Chalfont St Giles, UK).

Immunoprecipitation and western blot analyses

To study isoform specificity of monoclonal anti-ErbB4 antibodies, COS-7 cells were transiently transfected with pcDNA3.1*ErbB4JM-aCYT*-2, pcDNA3.1*ErbB4JM-bCYT-2* or pcDNA3.1 vector and lysed in lysis buffer 24 h later. The lysates were either boiled or not boiled at 95 °C for 5 min in sample buffer with or without DTT and analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting, as previously described (Kainulainen *et al*., 2000). ErbB expression in NIH 3T3-7d (Zhang *et al*., 1996) and NR6 (Sundvall *et al*., submitted) transfectants was analysed by western blotting under nonreducing conditions using 1:1000 dilution of anti-ErbB1 (sc-03), anti-ErbB2 (sc-284), anti-ErbB3 (sc-285) (all stocks 0.2 mg/ml; Santa Cruz Biotechnology) or mAb 1479 (stock 1.25 mg/ml).

For western analysis of ErbB4 protein in breast tissue, 80 μm sections of frozen tissue blocks were cut, homogenized and lysed in lysis buffer. Aliquots of lysates equivalent to 50 μg of total protein were analysed by western blotting with mAb 1479 under nonreducing conditions.

To study the effect of mAb 1479 on ErbB4 phosphorylation, MCF-7 cells were starved overnight in RPMI without serum and treated with or without 1 or 10 μg/ml of mAb 1479 for different time points before 15 min stimulation with 50 ng/ml NRG-1 (R&D, Minneapolis, MN, USA). Cell lysates were analysed by western blotting with a phospho-ErbB4 (Tyr1284) antibody (Cell Signaling, Beverly, MA, USA). The membranes were reblotted with anti-ErbB4 (Abcam), and an anti-actin antibody (Santa Cruz Biotechnology).

To study ErbB4 ubiquitination, COS-7 cells were transiently transfected with plasmids encoding full-length JM-a CYT-1 or JM-a CYT-2 together with Flag-tagged ubiquitin (Katz *et al*., 2002), stimulated for 10, 30 or 120 min with or without 2 μg/ml mAb 1479, and analysed by ErbB4 immunoprecipitation followed by anti-Flag western blotting, as previously described (Sundvall *et al*., 2007).

To study ErbB4 downregulation, 3.5×10^5 MCF-7 cells were plated on six-well plates and 1 μg/ml of either mAb 1479 or control antibody 3g6 (kindly provided by Dr Sirpa Jalkanen, University of Turku, Turku, Finland) was added into the culture medium for 0–72 h. ErbB4 protein level was analysed by western blotting with a monoclonal anti-ErbB4 antibody (Abcam).

Immunofluorescence staining

COS-7 cells were grown on coverslips and transfected with pcDNA3.1*ErbB4JM-aCYT-2-HA*, pcDNA3.1*ErbB4JM-bCYT-2-HA* or pcDNA3.1 vector control. The cells were fixed with methanol, stained with anti-ErbB4 (HFR-1) or anti-HA

(Roche) in 1:100 dilution, followed by incubation with Alexa Fluor 488 goat anti-mouse or Alexa Fluor 568 goat anti-rat (both from Molecular Probes, Leiden, the Netherlands) in 1:250 dilution.

In vitro *binding assay*
Recombinant HIS-tagged ErbB4 ectodomain (2 μg) was incubated with or without 0.5 μg of mAb 1479 or 3g6 for 15 min. Complex formation between the ectodomain and other proteins was visualized by western blotting with anti-penta HIS antibody (Molecular Probes) under nonreducing conditions.

ELISA of mAb binding to cells expressing ErbB4 or to recombinant ErbB4 ectodomain
Cell ELISA was carried out with NIH 3T3-7d and NR6 cells stably expressing ErbB1, ErbB2, ErbB3, ErbB4 JM-a CYT-2 or ErbB4 JM-b CYT-2. Cells were plated (3×10^4 cells per well) on white flat bottom 96-well plates (Perkin Elmer, Zaventem, Belgium). Cells were fixed and permeabilized with methanol and stained with mAb 1479, anti-ErbB1 (sc-03), anti-ErbB2 (sc-284), anti-ErbB3 (sc-285) or anti-ErbB4 (sc-283) in 1:100 dilution followed by incubation with HRP-conjugated goat anti-mouse or goat anti-rabbit (1:1000; Santa Cruz Biotechnology). Luminescence signal was obtained using a chemiluminescence substrate (Thermo Scientific, Rockford, IL, USA) and measured by TECAN Ultra (Tecan Nordic AB, Mölndal, Sweden).

To measure binding of mAb 1479 to the ErbB4 ectodomain, 100 ng of the HIS-tagged recombinant ectodomain was coated on white flat bottom 96-well plates in a buffer containing 100 mM $NaHCO_3$ (pH 9.6). The plates were washed with washing solution (0.1% Tween in phosphate-buffered saline), and blocked with blocking solution (5% milk and 1% bovine serum albumin in TBST (0.88% NaCl, 10 mM Tris-HCl, pH 7.5, 0.05% Tween)) following incubation with different concentrations of mAb 1479 or a negative control antibody (3g6; 100 nM) diluted in blocking solution. Luminescence was measured as above and the background signal of 3g6 was subtracted before a nonlinear curve analysis with OriginPro7 (OriginLab Corporation, Northampton, MA, USA).

Immunohistochemistry
Frozen sections (5 μm) were stained using 20 μg/ml of primary antibodies mAb 1479, anti-ErbB4 (HFR-1), anti-CD44 (Hermes-3; kindly provided by Dr Sirpa Jalkanen) or 3g6, and secondary antibodies Alexa Fluor 488 goat anti-mouse (1:200) or HRP-conjugated goat anti-mouse (1:100; Santa Cruz Biotechnology). For peroxidase staining, sections were treated with diaminobenzidine peroxidase substrate (Vector Laboratories, Burlingame, CA, USA) followed by staining with hematoxylin.

Analysis of ErbB4 shedding
COS-7 cells transiently expressing JM-a CYT-2 were treated for 24 h in serum-free medium with or without 1 μg/ml mAb 1479 or the control mAb 1475 before stimulating ErbB4 cleavage with or without 100 ng/ml PMA (Sigma-Aldrich, St Louis, MO, USA) for 45 min (Vecchi *et al*., 1996; Elenius *et al*., 1997). Ectodomain shedding was analysed from 60 μl samples of culture medium by western blotting with the anti-ErbB4 mAb 1464. Cell lysates were analysed by western blotting with the anti-ErbB4 antibody (Abcam) and anti-actin.

mAb 1479 internalization
COS-7 cells were grown on coverslips and transfected with pcDNA3.1*ErbB4JM-aCYT*-2, pcDNA3.1*ErbB4JM-bCYT-2* or kinase-dead pcDNA3.1*ErbB4JM-aCYT*-2*K751R* and Rab5a-GFP (Gomes *et al*., 2003). The cells were treated for 5 min or 2 h with 1 μg/ml of mAb 1479. Cells were fixed with methanol and stained with Alexa Fluor 568 goat anti-mouse. Images were obtained by LSM 510 Meta confocal microscope (Carl Zeiss Inc., Thornwood, NY, USA)

MTS proliferation assay
Cells were starved overnight and plated (1.5×10^4 per well) on 96-well plates in RPMI containing 5% charcoal-stripped fetal calf serum (FCS) and 1 μg/ml mAb 1479 or 10 μg/ml 2C4 (Genentech Inc., South San Francisco, CA, USA). The number of viable cells was estimated with CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA).

Anchorage-independent growth assay
Bottom layers consisting of 2 ml RPMI, 0.5% Bacto agar and 10% FCS were applied on six-well plates. After solidification of the bottom layers, top layers consisting of 30 000 cells per well in 1.2 ml RPMI, 0.33% Bacto agar and 10% FCS with or without 1 μg/ml mAb 1479 or the control antibody 3g6 were applied. Cells were incubated for 14 days at 37 °C. Colonies larger than eight cells were counted under microscope.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)