The Trk family of neurotrophin tyrosine kinase receptors is emerging as an important player in carcinogenic progression in non-neuronal tissues. Here, we show that breast tumors present high levels of TrkA and phospho-TrkA compared to normal breast tissues. To further evaluate the precise functions of TrkA overexpression in breast cancer development, we have performed a series of biological tests using breast cancer cells that stably overexpress TrkA. We show that (1) TrkA overexpression promoted cell growth, migration and invasion in vitro; (2) overexpression of TrkA per se conferred constitutive activation of its tyrosine kinase activity; (3) signal pathways including PI3K-Akt and ERK/p38 MAP kinases were activated by TrkA overexpression and were required for the maintenance of a more aggressive cellular phenotype; and (4) TrkA overexpression enhanced tumor growth, angiogenesis and metastasis of xenografted breast cancer cells in immunodeficient mice. Moreover, recovered metastatic cells from the lungs exhibited enhanced anoikis resistance that was abolished by the pharmacological inhibitor K252a, suggesting that TrkA-promoted breast tumor metastasis could be mediated at least in part by enhancing anoikis resistance. Together, these results provide the first direct evidence that TrkA overexpression enhances the tumorigenic properties of breast cancer cells and point to TrkA as a potential target in breast cancer therapy.


Keywords: neurotrophins; tyrosine kinase receptors; anoikis

Introduction

Breast cancer progression depends not only on primary tumor growth but also on the ability of tumor cells to metastasize to distant sites. Several sets of growth factors and their cognate receptors are known to be significantly involved in the regulation of these processes (Mercurio et al., 2005; Jechlinger et al., 2006; Ursini-Siegel et al., 2007). Thus, disruption of growth factors and receptor signaling is a current strategy for the development of anticancer drugs. So far, several drugs have shown a therapeutic efficiency, such as Herceptin (specific inhibitor of Erb-B2), but its use is limited because only 20–30% of breast cancers overexpress Erb-B2 and less than 30% of patients with Erb-B2 overexpressing metastatic breast cancer respond to Herceptin as a single agent in first-line treatment (Vogel et al., 2002; Sawaki et al., 2004). Identification of other growth factors and their receptors implicated in breast tumor development is therefore essential to improve therapeutic efficiency.

Neurotrophins consist of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and NT-4/5. Neurotrophins bind two classes of receptors, the p75 neurotrophin receptor (p75NTR) and the tyrosine kinase receptors of Trk family (TrkA, TrkB, TrkC). p75NTR binds all neurotrophins with similar affinities (Chao, 1994) and regulates cellular processes through interactions between the cytoplasmic domain of p75NTR and effector molecules (Mukai et al., 2003). The Trk tyrosine kinase receptors activate several signaling pathways that regulate survival and differentiation in neuronal cells (Reichardt, 2006). Although neurotrophin-mediated signaling has been extensively studied in PC12 and neuronal cells, their effects on non-neuronal cells are not fully understood. Accumulating data have demonstrated that NGF and its tyrosine kinase receptor TrkA are involved in tumor growth and the progression of non-neuronal cancers, including medullary thyroid carcinoma (McGregor et al., 1999), lung (Ricci et al., 2001), pancreatic (Zhu et al., 1999), prostatic (Weeraratna et al., 2000) and ovarian carcinomas (Davidson et al., 2003).

Both p75NTR and TrkA are expressed in breast cancers (Aragona et al., 2001; Descamps et al., 2001a). Moreover, we have shown that NGF acts as an autocrine growth factor to stimulate cell proliferation and survival (Dolle et al., 2003). Interestingly, NGF cooperates with p185(HER2) to stimulate breast cancer cell growth (Tagliabue et al., 2000), and Tamoxifen, the commonly drug used in hormonotherapy, inhibits the proliferative effects of NGF (Chiarenza et al., 2001). More recently, we have shown that NGF is expressed in the majority of human breast tumors, especially in epithelial cancer cells. Moreover, anti-NGF antibodies and small-interfering RNA against NGF strongly inhibit the tumor growth and metastasis of breast cancer cells xenografted in immunodeficient mice (Adriaenssens et al., 2008).
These *in vitro* and *in vivo* results suggest that NGF may actively be involved in mammary tumor development. In agreement with this hypothesis, high levels of activated TrkA (phospho-TrkA, pTrkA) are observed in breast cancer effusions compared to primary cancers (Davidson et al., 2004). Unfortunately, commonly used breast cancer cell lines express relatively low levels of TrkA compared to tumor biopsies (Descamps et al., 2001a). In this study, we first showed that breast tumor biopsies expressed high levels of TrkA and pTrkA compared to normal biopsies, and then stably transfected TrkA in MDA-MB-231 human breast cancer cells to determine the functional importance of TrkA. Our results show that TrkA overexpression promoted cell growth, migration and invasion *in vitro*. Moreover, TrkA overexpression enhanced tumor growth, angiogenesis and metastasis of xenografted breast cancer cells in immunodeficient mice.

**Results**

**Breast tumor biopsies present high levels of TrkA and pTrkA**

TrkA expression in breast biopsies was first quantified by real-time RT-PCR (Figure 1a). TrkA was expressed in normal biopsies with levels roughly equivalent to those detected in MDA-MB-231 breast cancer cell line. In contrast, the levels of TrkA mRNA in tumor biopsies were about 10- to 140-fold higher than those in normal samples. The high level of TrkA expression in breast tumor biopsies was further confirmed by western blot analysis (Figure 1b). Moreover, pTrkA was detected in the majority of cancer biopsies, whereas it was undetectable in normal ones; no strict correlation was observed between the levels of TrkA and pTrkA in these samples. We then extended our analysis by immunohistochemical staining of breast tissue microarrays (Figure 1c). The levels of these proteins were scored from 0 to 3 (Table 1). Normal tissues expressed no or low levels of TrkA (score \( \leq 1 \)). In contrast, of 37 cancer biopsies, 28 were found to present high levels of TrkA and pTrkA (score \( \geq 2 \)).

**TrkA overexpression promotes cell growth, migration, invasion and survival**

Commonly used breast cancer cell lines express relatively low levels of TrkA compared to tumor biopsies (Descamps et al., 2001a; Figures 1a and b). Moreover, PCR analysis showed that only the TrkAI isoform is expressed in breast cancer cell lines (data not shown), so to determine the functional importance of TrkA overexpression in breast cancer development, we established TrkAI overexpressing MDA-MB-231 breast cancer cells as pools or single clones (Figure 1d). Interestingly, an increase in pTrkA was also observed in TrkA overexpressing cells, indicating that TrkA was activated following its overexpression (Figure 1d). We observed similar results in terms of cell behavior in culture for both the pooled cells and the two selected clones (C1 and C2). To simplify the presentation, only results with the pooled cells are shown (Figure 2). In standard cell culture conditions, TrkA overexpression resulted in accelerated cell proliferation (Figure 2a). After 6 days of culture, the number of TrkA overexpressing cells is about twice that of mock cells. When cell migration was evaluated using Transwell Boyden chambers, more TrkA overexpressing cells were found to migrate to the bottom chamber than did the empty-vector-transfected cells (Figure 2b). Similar results were obtained using the wound-healing method (Figure 2c). The invasive capacities of the cells were assessed using Transwells with filters coated with Matrigel and were also found to be enhanced in TrkA overexpressing cells (Figure 2d). Such an increase in migration and invasion was not due to differences in cell proliferation as no difference in cell number was observed after 24 h of culture in the same conditions (data not shown).

We then determined the anoikis resistance of cells on poly-HEMA-coated wells that effectively inhibit cell attachment (Figures 2e and f). TrkA overexpressing cells could survive and proliferate as large spheroid aggregates in suspension, resulting in a twofold increase in cell numbers by 36 h of culture. Of note, during different culture times, the viability of TrkA overexpressing cells remained superior or similar to that at the beginning of the experiment whereas mock cells underwent rapidly cell death. Morphological analysis after Hoechst staining revealed that after 72 h of culture, more than 60% of mock cells were apoptotic whereas less than 5% of TrkA overexpressing cells were apoptotic (data not shown). Moreover, TrkA overexpressing cells formed more colonies than did mock cells in soft agar assay (Figure 2g). This reinforced the fact that TrkA overexpression bypasses the need for anchorage. Finally, TrkA overexpression enhanced the resistance of cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (Figure 2h).

Surprisingly, although exogenous NGF further stimulated cell growth in soft agar, migration and invasion, it did not modify anoikis resistance either in mock or in TrkA overexpressing cells. Although we have previously shown that NGF can stimulate the growth of breast cancer cells both *in vitro* and *in vivo* by an autocrine loop (Dolle et al., 2003; Adriaenssens et al., 2008), we then determined whether the effects of TrkA overexpression were due to the activation of the autocrine loop. PCR analysis revealed no modification of NGF expression in TrkA overexpressing MDA-MB-231 cells compared to that of mock cells (data not shown). Neutralizing anti-NGF antibody partially inhibited the migration of TrkA overexpressing MDA-MB-231 cells, but had no effect on anoikis resistance; similar results were obtained in MCF-7 breast cancer cells (Supplementary Figure 1). These data suggest that NGF produced by breast cancer cells is efficient in stimulating TrkA-induced migration but not TrkA-enhanced anoikis resistance. Together, whatever the involvement of NGF, TrkA overexpression increased cell growth, invasion and survival in breast cancer cells.
**PI3K-Akt and MEK MAP kinase are required for TrkA overexpression-promoted migration and anchorage-independent growth**

Activation of signaling pathways such as PI3K-Akt and mitogen-activated protein (MAP) kinases in TrkA overexpressing cells was first determined by western blot analysis. Higher levels of phospho-Akt (pAkt), phospho-ERK (pERK) and phospho-p38 (pp38) MAP kinases were observed in TrkA overexpressing cells than in mock cells (Figure 3a; Supplementary Figure 2A–C).
Inhibition of TrkA phosphorylation by K252a strongly reduced the levels of pAkt, pERK and pp38 MAP kinases without modifying the expression of corresponding proteins. Similar results were obtained when cells were cultured in suspension (Supplementary Figure 2D). This implies that overexpressed TrkA was functional in signal transduction by activating PI3K-Akt and ERK/p38 MAP kinases. Furthermore, pharmacological inhibitors of TrkA, PI3K-Akt and ERK MAP kinase more strongly reduced the migration and anoikis resistance of TrkA overexpressing cells than that of mock cells (Figures 3b and c; Supplementary Figure 3A–D). Colony formation of TrkA overexpressing cells was also diminished in the presence of inhibitors of TrkA and ERK/p38 MAP kinases (Figure 3d). However, cells did not survive in soft agar whatever the levels of TrkA in the presence of LY294002 or Akt inhibitor III (Figure 3d), confirming the crucial function of PI3K-Akt in colony formation in soft agar (Nakanishi et al., 2002).

**TrkA overexpression accelerates tumor growth by enhancing cell proliferation and angiogenesis**

MDA-MB-231 cells were subcutaneously injected into severe combined immunodeficiency (SCID) mice. All animals formed a tumor at the injection site, but the tumor growth rates were quite different: tumors formed by TrkA overexpressing cells were palpable 2 weeks after injection and attained a size of 2 cm³ at about 5 weeks after injection. In contrast, empty-vector-transfected cells formed palpable tumors with a latency of 5 weeks and too 10 weeks to form tumors of similar size (about 2 cm³) (Figure 4a). Primary tumors from MDA-MB-231 TrkA overexpressing cells maintained high levels of TrkA and pTrkA as revealed by immunohistochemical staining (Figure 4b). Similar results were obtained with another xenograft model T47-D human breast cancer cells that are less tumorigenic (Supplementary Figure 4). To determine whether apoptosis, cell proliferation and angiogenesis were modified in tumors formed by TrkA overexpressing cells, we then performed terminal transferase dUTP nick-end labeling (TUNEL) assay, immunostaining of proliferating cell nuclear antigen (PCNA) and von Willebrand factor (vWF), which are markers of apoptosis, cell proliferation and angiogenesis, respectively. No significant differences in TUNEL-positive cells were observed between tumors formed by mock and TrkA overexpressing cells. In contrast, about twofold increases in the number of PCNA- and vWF-positive cells were found in TrkA overexpressing tumor sections (Figure 4c). These data indicated that accelerated tumor growth was due to the enhanced proliferation of TrkA overexpressing cells and increased angiogenesis.

**TrkA overexpression enhances tumor metastasis**

To evaluate metastasis in vivo, we generated MDA-MB-231 cells (overexpressing TrkA or not) stably expressing green fluorescent protein (GFP). The GFP-positive cells exhibited the same levels of TrkA and similar in vitro properties (proliferation, migration and survival) as their parental counterparts (data not shown). Metastatic potentials of the GFP-positive cells were then first evaluated after subcutaneous injection into SCID mice (Figures 5a and b; Supplementary Figure 5). More and bigger metastatic foci were found in the lungs, liver and brain of mice that received TrkA overexpressing cells (Figure 5a). Quantification of GFP-positive cells using fluorescence-activated cell sorting (FACS) showed that TrkA overexpression induced two- and threefold increase in the metastatic burden in lungs and liver, respectively (Figure 5b, Table 2). Interestingly, only three animals among eight bearing empty-vector-transfected cells developed brain metastases with a low tumor burden, as metastatic (GFP-positive) cells were found to be inferior to 0.2%. In contrast, nine out of nine animals receiving TrkA overexpressing cells developed brain metastases with an average tumor burden 25-fold higher than control animals. To evaluate the abilities of cells to metastasize independently of primary tumor formation and intravasation, we injected cells into the lateral tail veins of SCID mice. After 8 weeks, the mice were killed and the metastatic (GFP-positive) cells in the lungs were quantified. As shown in Figure 5c, a significant increase in metastatic cells was observed in the lungs of mice that received TrkA overexpressing cells, suggesting that TrkA overexpression also affects the later steps of metastasis.

The metastasis-promoting effect of TrkA overexpression was also observed by using another breast cancer cell line T47-D known to be tumorigenic but not metastatic (Supplementary Figure 4C).
TrkA overexpressing cell lines isolated from lung metastases were more resistant to anoikis.

To get insight into the mechanisms of TrkA-driven metastasis, we isolated and cultured TrkA overexpressing cells derived from the lungs of nine animals separately to evaluate their behavior in terms of growth, migration and resistance to TRAIL-induced apoptosis, as well as resistance to anoikis. As shown in Figure 6a, the levels of TrkA and pTrkA in recovered metastatic TrkA overexpressing cells were slightly higher than those of cells before injection. No modifications in cell growth, migration and resistance to TRAIL-induced apoptosis.
apoptosis were observed (Figures 6b–d). In contrast, the recovered metastatic TrkA overexpressing cells exhibited enhanced anoikis resistance compared to parental TrkA overexpressing cells. This enhanced anoikis resistance was specific to metastatic cells as no difference was observed between cells derived from primary tumors and their parental cells before injection (data not shown). More importantly, TrkA inhibition with K252a totally abolished the anoikis resistance of these metastatic cells (Figure 6e). Taken together, these results suggested that the enhanced anoikis resistance could be at least partially responsible for the increased metastatic capacity of TrkA overexpressing cells.

Discussion

We first showed that TrkA was overexpressed in human breast cancer biopsies compared to normal breast tissues with levels of pTrkA also being higher in breast cancer biopsies. TrkA can be activated following mutation, overexpression or binding to NGF. On one hand, no mutations have been found either in breast cancer biopsies or in breast cancer cell lines including MDA-MB-231 and MCF-7 (unpublished personal data). On the other hand, the majority of breast cancers express NGF (Adriaenssens et al., 2008) that may activate TrkA in an autocrine manner. To determine the incidence of TrkA overexpression in breast cancer biology, we stably transfected MDA-MB-231 breast cancer cells with the full-length TrkA isoform cDNA (the only TrkA isoform found in these cells). We showed that TrkA overexpressing cells (pooled and separated clones) exhibited increased cell proliferation, migration, invasion as well as survival. These results were further confirmed using two other prototypic breast cancer cell lines MCF-7 and T47-D that are known to be less tumorigenic. However, we had previously shown that upon NGF stimulation, endogenous TrkA is only involved in cell proliferation but not in survival of breast cancer cells (Descamps et al., 2001b), this may be explained by the fact that a relatively low level of endogenous TrkA is not sufficient to activate survival pathways. Accordingly, in TrkA overexpressing cells, tyrosine receptor signaling pathways were found to be constitutively activated, as revealed by the high levels of pAkt, pERK and pp38. Moreover, both PI3K-Akt pathway and ERK MAP kinase were necessary for TrkA-stimulated biological effects such as migration, anoikis resistance and colony formation in soft agar. p38 MAP kinase only seemed to be implicated in colony formation of TrkA overexpressing cells.

TrkA overexpression not only promoted cell growth in vitro but also accelerated primary tumor growth.
in vivo in SCID mice. The enhanced primary tumor growth was found to be associated with increased cell proliferation and angiogenesis. These data together with our previous findings showing that inhibition of TrkA by the tyrosine inhibitor K252a efficiently inhibits tumor growth and angiogenesis (Adriaenssens et al., 2008) prompted us to suggest a model in which relatively low endogenous TrkA and NGF are sufficient to sustain tumor growth by an autocrine loop. However, this loop is probably suboptimal as TrkA overexpression enables stronger signaling, resulting in enhanced tumor growth and angiogenesis. NGF has already been shown to stimulate angiogenesis (Cantarella et al., 2002). In addition, a correlative analysis suggests a function for NGF in ovarian cancer angiogenesis (Davidson et al., 2003). It is not yet known if NGF can exert a direct and/or indirect action, as NGF can also induce the expression of pro-angiogenic factors such as VEGF (Campos et al., 2007). Clearly, it needs to be clarified how TrkA overexpressing tumor cells can stimulate tumor angiogenesis, but our present findings show that TrkA impact in breast carcinogenesis may also encompass angiogenesis.

Another important finding of our work is the increased metastatic ability of TrkA overexpressing cells. Metastasis is a complex process consisting of multiple steps. These steps include growth of the primary tumor, growth of vessels (blood vessels and lymphatics) in and around the tumor, intravasation, transport, arrest of tumor cells and formation of secondary tumors at distant sites. Acquisition of resistance to anoikis may allows the survival of cancer cells during systemic circulation and facilitate tumor dormancy or metastasis in distant organs (Frisch and Screaton, 2001; Rennebeck et al., 2005). In support of this concept, it has been reported that overexpression of TrkB renders normal intestinal epithelial cells anoikis resistant and highly tumorigenic (Douma et al., 2004; Geiger and Peeper, 2007). Here, we showed overexpression of TrkA rendered breast cancer cells more resistant to anoikis. In addition, TrkA overexpressing cells derived from lung metastases were more resistant to anoikis than their parental TrkA overexpressing cells. These correlations strongly suggested that the increased metastatic capacity of TrkA overexpressing cells could be at least in part due to the enhanced anoikis resistance. It is also reasonable to presume that the increase in lung metastasis due to TrkA overexpression after tail vein injection was relevant with increased capacity of anchorage-independent growth. To our knowledge, this is the first report of TrkA being involved in anoikis resistance, which extends our understanding of the function of TrkA in breast tumor development. Surprisingly, we found that anoikis resistance was independent of NGF action, as neither exogenous NGF nor neutralizing anti-NGF could modify anoikis resistance in both control and TrkA overexpressing cells. It is unclear whether overexpressed TrkA can act in an intracrine manner or through a direct interaction with other proteins such as adhesion molecules. Indeed, it has been reported that E-cadherin-mediated suppression of anoikis is associated with ligand-independent activation of Erb-B1 (Shen and Kramer, 2004). Studies to address these questions are now under way.

However, given the complexity of metastatic process, cancer cells must acquire a series of traits that enable them to overcome the multiple barriers erected by
normal tissues. Apart from the enhanced anoikis resistance, the increased abilities of proliferation, migration and invasion may also potentiate metastasis of TrkA overexpressing cells. Therefore, more detailed quantitative analysis in vivo as recently reported (Hedley et al., 2008) is required to see the exact impact of TrkA overexpression in the numerous steps of metastatic process. Nevertheless, our findings provide primary explanations for the observation that pleural effusions of breast cancers present high levels of pTrkA compared
to corresponding primary tumors (Davidson et al., 2004). These results are not in agreement with our previous report in which TrkA mRNA expression predicted improved overall survival (Descamps et al., 2001a). This difference may be attributed to the fact that the levels of TrkA protein especially in its activated form

Table 2  Effect of TrkA overexpression on incidence and severity of metastasis

<table>
<thead>
<tr>
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<th>GFP-positive cells (%)</th>
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<tbody>
<tr>
<td></td>
<td>&lt;0.2</td>
<td>0.2–0.5</td>
<td>0.5–1</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Liver</td>
<td>2/8</td>
<td>4/8</td>
<td>1/8</td>
<td>1/8</td>
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<tr>
<td>Lung</td>
<td>6/8</td>
<td>2/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>Brain</td>
<td>3/8</td>
<td>0/8</td>
<td>0/8</td>
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Mice injected with mock cells (eight animals) or with TrkA overexpressing cells (nine animals) were killed when the average volume of primary tumors approached 2 cm³. The liver, lungs and brain of each mouse were recovered and digested with trypsin, collagenase XI and hyaluronidase for 1h at 37 °C to obtain individual cells. The percentage of GFP-positive cells was then analysed by FACS.

Figure 6  Properties of lung metastatic TrkA overexpressing cells. Lung metastatic TrkA overexpressing cells were recultivated (nine cultures) from the lungs of nine mice subcutaneously injected with TrkA overexpressing cells. (a) Immunoblots of TrkA and phospho-TrkA (pTrkA) from pooled metastatic cells. (b) Growth assay on standard culture plastic. (c) Migration assay using Transwells. (d) Resistance to terminal transferase dUTP nick-end labeling (TRAIL)-induced apoptosis. (e) Anoikis resistance of cells treated or not with K252a. *P<0.01, metastatic versus parental TrkA overexpressing cells.
cannot be compared with the levels of mRNA. Another explanation may relate to the cohort studied. Indeed, we analysed tumors from patients who presented variable disease stages, with highly variable clinical outcomes, whereas the cohort used by Davidson et al. (2004) was uniformly composed of patients who developed stage IV disease at some point along the clinical course, and who, despite long disease-free periods in some cases, all suffered tumor-related death.

In conclusion, we show that TrkA is overexpressed in breast carcinoma. We also provide the first direct evidence that TrkA overexpression in breast cancer cells enhances their tumorigenic properties. Our findings imply that the targeting of TrkA signaling in breast cancers is of potential interest. Given the correlation of anoikis resistance and metastatic potential, suppression of anoikis resistance would contribute to limiting metastasis and could be useful in combination with treatments that directly target primary tumor growth.

Materials and methods

Human breast biopsies and animals
Normal breast tissues were obtained from individuals treated by mastectomy at the Hospital of Lille (France) and breast carcinoma specimens were obtained from the Clinique du Croisé Laroche (Lille, France). All these samples were collected with institutional safety review board approval. Breast cancer tissue microarrays (Cliniscience, Montrouge, France) included 10 normal breast samples and 37 tumoral breast samples (3 infiltrating lobular carcinomas, 2 in situ ductal carcinomas, 32 infiltrating ductal carcinomas). Animal studies were performed in accordance with the Animal Care and Use Committee Procedures and Guidelines of the Institut Pasteur de Lille (Lille, France).

Cell culture, transfection and generation of TrkA overexpressing cancer cells

The MDA-MB-231 breast cancer cell line was obtained from the American Type Culture Collection. Cells were maintained in Eagle’s minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) and stably transfected with TrkA cDNA and/or GFP cDNA as described in the Supplementary data.

Quantitative real-time PCR and western blot analysis

The protocols were detailed in the Supplementary data. TrkA isoforms were determined by reverse-transcriptase (RT)–PCR using specific primers as described (Tacconelli et al., 2004).

Assays of cell behavior in culture

Protocols were detailed in the Supplementary data (cell growth, migration, matrigel chemo-invasion, wound-healing assay, anoikis assay, soft agar assay for colony formation, apoptosis analysis). Specific inhibition with pharmacological inhibitors was performed with 10 nM K252a, 15 μM LY294002, 15 μM Akt inhibitor III, 10 μM U0126 or 10 μM p38 inhibitor II, all from Calbiochem, San Diego, CA, USA. Control cells were treated with DMSO at a 1:1000 dilution. The concentrations used were based upon the absence of toxicity in the MDA-MB-231 cells, which was determined by cell proliferation assay in serum-free medium for 48 h.

Tumor growth in SCID mice

Female SCID mice (6-week old) were purchased from the Charles River Laboratories (France) and acclimatized for at least 2 weeks. MDA-MB-231 mock and TrkA overexpressing cells were harvested and resuspended in phosphate-buffered solution before subcutaneous injection into the flanks (2 × 10^6 cells per flank) of 8-week-old SCID mice. The tumor volume was determined every week by measuring the length (l) and width (w) and then calculating the volume as \( \frac{\pi lw^2}{2} \).

Immunohistochemistry and TUNEL staining

TrkA and pTrkA staining was performed using tissue microarrays according to the manufacturer’s instructions (Cliniscience). Apoptotic cells were detected by the TUNEL assay in situ cell death detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer’s instructions. For detailed protocols, see the Supplementary data.

Spontaneous and experimental metastasis assays

To measure spontaneous metastasis, we subcutaneously injected GFP-positive cells into the flanks (2 × 10^6 cells per flank) of 8-week-old SCID mice as described above. Once the average tumor size approached 2 cm^3, the mice were killed and their lungs, liver and brain were removed for further analysis. The organs were cut into two parts: one half for histological analysis, the other half was digested with enzymes (2.5 mg/ml trypsin, 0.5 mg/ml collagenase XI, 0.5 mg/ml hyaluronidase) for 1 h at 37°C before quantification of the metastatic cells (GFP-positive) by FACS analysis. Cultured GFP-positive MDA-MB-231 cells were used as a positive control. Cells derived from the lungs, liver and brain of a mouse injected with GFP-negative MDA-MB-231 cells were used as a negative control. To recultivate cancer cells derived from primary tumors and lungs, we washed enzymatic digests once in 10 ml EMEM 10% FCS and seeded in cell culture dishes in EMEM 10% FCS with 800 μg/ml hygromycin for 2–3 weeks. Selected tumor cells were confirmed after examination of GFP expression under fluorescent microscopy.

To measure experimental metastasis, we injected 5 × 10^5 GFP-positive cells into the lateral tail veins of 8-week-old SCID mice. After 8 weeks of injection, the mice were killed and metastatic cells in their lungs were quantified as described above.

Statistical analysis

Statistical differences were determined with two-tailed Student’s t-tests. All P-values were two sided. P < 0.01 was considered as statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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