Oncogenes and Tumor Suppressors

Effects of Simultaneous Knockdown of HER2 and PTK6 on Malignancy and Tumor Progression in Human Breast Cancer Cells

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Abstract

Breast cancer is the most common malignancy in women of the Western world. One prominent feature of breast cancer is the co- and overexpression of HER2 and protein tyrosine kinase 6 (PTK6). According to the current clinical cancer therapy guidelines, HER2-overexpressing tumors are routinely treated with trastuzumab, a humanized monoclonal antibody targeting HER2. Approximately, 30% of HER2-overexpressing breast tumors at least initially respond to the anti-HER2 therapy, but a subgroup of these tumors develops resistance shortly after the administration of trastuzumab. A PTK6-targeted therapy does not yet exist. Here, we show for the first time that the simultaneous knockdown in vitro, compared with the single knockdown of HER2 and PTK6, in particular in the trastuzumab-resistant JIMT-1 cells, leads to a significantly decreased phosphorylation of crucial signaling proteins: mitogen-activated protein kinase 1/3 (MAPK 1/3, ERK 1/2) and p38 MAPK, and (phosphatase and tensin homologue deleted on chromosome ten) PTEN that are involved in tumorigenesis. In addition, dual knockdown strongly reduced the migration and invasion of the JIMT-1 cells. Moreover, the downregulation of HER2 and PTK6 led to an induction of p27, and the dual knockdown significantly diminished cell proliferation in JIMT-1 and T47D cells. In vivo experiments showed significantly reduced levels of tumor growth following HER2 or PTK6 knockdown. Our results indicate a novel strategy also for the treatment of trastuzumab resistance in tumors. Thus, the inhibition of these two signaling proteins may lead to a more effective control of breast cancer.

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Introduction

HER2 is a member of the HER (EGFR/ErbB) receptor family encompassing the 4 HER receptors (HER1–HER4) that are involved in signal transduction (1). Each of these receptors contains an extracellular, a trans-membrane, and an intracellular domain. In response to extracellular stimuli, the HER receptors form a homo- or heterodimer via autop-hosphorylation of the intracellular domains of the receptors (2). Subsequently, the HERs activate many signaling proteins that are linked to cell proliferation and tumor malignancy. The HER receptors are highly involved in breast cancer progression, and for HER2 in particular, there is a large body of experimental and clinical data underlining its significance. Regarding its clinical relevance, HER2 is over-expressed in 25% to 30% of patients with breast cancer and is associated with a poor prognosis (3). In addition to surgery and hormonal and chemo- or radiotherapy, an antibody-based immunotherapy for HER2-positive tumors has been successfully administered in the last decade (4, 5). However, only a third of breast tumors highly overexpress HER2 and therefore may be considered for the anti-HER2–targeted antibody (trastuzumab). Moreover, trastuzumab therapy causes side effects (cardiotoxicity; ref. 6), and after approximately one year of application, the development of resistance is not uncommon (7, 8). Combinations of trastuzumab with the small-molecule tyrosine kinase inhibitor lapatinib targeting EGF receptor (EGFR) and HER2 seem to provide remedy to some extent. Still, also lapatinib, either as a single agent, or in combination with various chemotherapeutic agents, or trastuzumab, could not fulfill the high expectations as initially hoped for (5). HER2 knockdown in vitro led to reduced proliferation and induced apoptosis of HER2-overexpressing breast cancer cell lines (9). In addition, the simultaneous knockdown of HER2 and uPAR (urokinase plasminogen activator receptor) resulted in a higher induction of apoptosis and a stronger inhibition of cell proliferation than the single knockdown of either HER2 or uPAR (10).

Another factor with relevance in tumor progression is PTK6, also known as Brk (breast tumor kinase). This
nonreceptor Src-related tyrosine kinase is highly expressed in up to 80% of breast cancers, as well as in colon and prostate tumors (11, 12). PTK6 was also shown to be expressed in several cancer cell lines (11–13). In the last decade, we and others have identified several interaction partners of PTK6, such as STAT3, (p38) MAPK, Paxillin, and PTEN (14–17). Therefore, PTK6 seems to be involved in several cellular processes, such as proliferation, migration and invasion. PTK6 was also shown to regulate the cell cycle (18). PTK6 knockdown led to a diminished phosphorylation of STAT3, (p38) MAPK, and PTEN, as well as to a reduced migration of breast cancer cells (15, 19).

Studies conducted by our group and others showed that HER2 and PTK6 are coexpressed and colocalized, and they directly interact with each other in breast cancer tissue. Furthermore, the HER2 and PTK6 mRNA and protein levels are positively correlated, emphasizing their role as crucial molecules in breast cancer (17, 20–22). In 2004, Tanner and colleagues identified a novel HER2-overexpressing cell line that is intrinsically resistant to trastuzumab and named it JIMT-1 (23). Since then, an appropriate experimental model system has been established to study the possible causes of intrinsic and acquired trastuzumab resistance. In the meantime, several mechanisms for trastuzumab resistance have been hypothesized among others including truncation of extracellular HER2, activation of alternative pathways, loss of the tumor suppressor PTEN, or an interaction with Mucin-4 that potentially inhibits the binding of trastuzumab to HER2 (8, 24, 25). A recent study described survivin (an inhibitor of apoptosis) as indispensable for the survival of HER2-overexpressing and trastuzumab-resistant breast cancer cells (26). However, the underlying mechanisms for the development of resistance are not yet exactly known, and additional therapeutic approaches in breast cancer are strongly needed. To date, no information exists concerning the effects of the simultaneous inhibition of HER2 and PTK6 on tumor cell biology and tumor progression. In addition, in trastuzumab-resistant JIMT-1 cells, this information could give valuable hints towards strategies suited to circumvent the resistance to the clinical trastuzumab-based regimens.

In our present study, we show for the first time the cell line-dependent effects on the deactivation of key proteins and a reduction in migration, invasion, and cell proliferation in vitro following the simultaneous knockdown of HER2 and PTK6 in breast cancer cells. Subsequent xenograft experiments revealed the effectiveness of silencing these signaling proteins in vivo. Therefore, the inhibition of HER2 and/or PTK6 may offer an attractive tool for targeted breast cancer therapy, specifically in a subgroup of patients who develop a resistance to trastuzumab during the course of treatment.

Materials and Methods

Cell culture and transient transfections

The following 2 permanent human breast cancer cell lines were used: T47D and JIMT-1. The trastuzumab-sensitive T47D cell line (HTB-133) was acquired from American Type Culture Collection. It was maintained in RPMI-1640 with GlutaMAX (Life Technologies GmbH) and trastuzumab-resistant cell line JIMT-1 (ACC 589) was acquired from the German Collection of Microorganisms and Cell Cultures. The last cell line authentication was conducted in spring 2012. Therefore, genetic profiles were generated by the company Eurofins (MWG Operon). For this, the PowerPlex 16 System, a multiplex short tandem repeat (STR) system, was conducted. Subsequently, the obtained profiles were introduced into the online STR matching analysis database that is provided by the DSMZ. The cells were maintained in Dulbecco’s modified Eagle’s medium with GlutaMAX (Life Technologies GmbH). Both media were supplemented with human insulin (10 μg/mL, Sigma, St. Louis), 10% FBS (Life Technologies GmbH), 0.25% each of penicillin and streptomycin (Life Technologies GmbH), and also with 0.1% FBS when the serum-independent effects (in the migration, invasion assays, and matrix metalloproteinase (MMP) activation, as described below) were analyzed and the cells were maintained at 37°C in 5% CO2. The medium for JIMT-1 cells was supplemented with trastuzumab (10 μg/mL, Roche Diagnostics).

For transient transfections, 1 × 10^5 breast cancer cells were transfected with small-interfering RNAs (siRNA; 200 pmol) for PTK6 and HER2 knockdown and control siRNAs [positive control: siRNAs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) silencing, and negative control: a non-targeting siRNA as described; ref. 19]. All siRNAs were purchased from Thermo Scientific Dharmacon. Two independent transfections were conducted.

Generation of lentiviral constructs for PTK6 and HER2 knockdown

Previously tested siRNAs leading to specific and efficient knockdown of PTK6 and HER2 were used as templates for the generation of corresponding shRNAs. The 5’-3’ sequence of the forward primer for shRNA_PTK6 is: GATCCCCCATTAAAGGTGATCTTCGTTCAAGAGCAGGAAAATCACCTTAATGTTTTTGAAAA, and the sequence of the respective reverse primer is: AGCTTTTCACACGTTAAGCTGTTTTTGAAAA. The 5’-3’ sequence of the forward primer for shRNA_HER2 is: GATCCCCGGTCTCGTCACAAACCAATT CAAGAGTTGGTTGTGAGCGATGAGCTTTTGTGAAAA, and the sequence of the respective reverse primer is: AGGT CTTTCCAAAAACCATGAGTTG AGTTTCTGTCTCTGACGGAGAAATCACCTTAATGTTTTTGAAAA. The sequence of the reverse primer is: AGCTTTTCACACGTTAAGCTGTTTTTGAAAA. The construction of lentiviral vectors was conducted as published previously (27). In addition to specific shRNAs, all of the vectors encoded the GFP gene for visualization and quantification of infections.

Lentiviral transductions of breast cancer cells

A total of 2.5 × 10^5 T47D and JIMT-1 breast cancer cells were infected with lentiviral vectors encoding short hairpin RNAs (shRNA): shRNA_PTK6, shRNA_HER2, the
shRNA_control (lacking the sequence for shRNA), and polybrene (Invitrogen) as described (27). The supernatants were removed 24 hours after infection. Fresh medium was added, and the cells were further maintained for several weeks. The infected JIMT-1 cells were cultivated in parallel with or without the addition of trastuzumab (10 μg/mL, Roche Diagnostics) to ensure the maintenance of resistance. Once per week, a defined fraction of infected cells was used for Western blot analysis to control for knockdown efficiency, whereas the residual fraction was further cultivated. As controls, cells were infected only with the transduction reagents (mock) or with the lentiviral vectors lacking a shRNA sequence, but containing the GFP gene (control vector). The infections were conducted in triplicates. The infection efficiency of T47D and JIMT-1 cells was measured by fluorescence-activated cell sorting as described previously (19).

**Western blot analysis**

For SDS-PAGE and the subsequent Western blot analysis, T47D and JIMT-1 breast cancer cells were treated as described previously (19). The proteins were detected with primary antibodies targeting PTK6 (sc-1188), GAPDH (sc-25778) (Santa Cruz Biotechnology), HER2 (A0485) (DAKO), (phospho, 9554) PTEN (9559), (phospho 4051) Akt (9272), (phospho, 4376) MAPK 1/3, (ERK 1/2; 4695), (phospho 9211) p38MAPK (9212), MMP9 (3852), (phospho-STAT3 (9134), PARP (9532; Cell Signaling Technology), STAT3 (610190), p27 (610242; BD Transduction Laboratories), and tubulin as a loading control (T5168, Sigma). The following peroxidase-conjugated secondary antibodies were used: anti-rabbit (NA934V) and anti-mouse (NA931V; GE Healthcare). All of the bands showing changed intensities were quantified relative to the control bands using the Molecular Imager ChemiDoc XRS and the analysis software Quantity One (Bio-Rad Laboratories).

**WST-1 cell proliferation assay**

Cell proliferation was determined using the water-soluble tetrazolium WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) for the spectrophotometric assay according to the manufacturer’s protocol (05015944001, Roche Diagnostics). T47D and JIMT-1 cells were seeded at a concentration of 10^4 cells per well in a 96-well tissue culture plate. After 48 hours, the WST-1 reagent was added and the cells were incubated for 0.5 to 4 hours at 37°C. The absorbance of the infected and the control cells was measured against a background control using a microplate ELISA reader (Bio-Rad) at 450 nm (reference wavelength at 655 nm). Five independent experiments were carried out. For statistical analysis, the Student t test was used.

**Wound scratch migration assay**

The migration assay was conducted in triplicates under serum-reduced conditions to reduce proliferation (28) as described previously (19) with minor modifications. The wound was scratched into a confluent monolayer 5 weeks postinfection and then documented once a day with phase contrast microscopy on an inverted microscope Evos (AMG). The quantification was conducted using TScratch software (29). All open areas at time point 0 hours were set to 100%, and the open areas at the later time points were calculated in relation to the respective value at 0 hours. For statistical analysis, the Student t test was used.

**Matrigel invasion assay**

Control or infected JIMT-1 breast cancer cells were seeded at a concentration of 5 × 10^5 cells in serum-reduced medium onto BD BioCoat Matrigel Invasion Chambers (354480, BD Biosciences), inserted in 24-well cell culture plates, and incubated for 24 hours at 37°C. FBS (10%) was used as a chemoattractant in the lower chamber, and the invasion assay was conducted according to the manufacturer’s instructions. After the incubation, noninvading cells were removed with a cotton swab from the apical side of the membrane, and then the invading cells were fixed with methanol and stained with toluidine blue. The invading cells were counted microscopically (Olympus). The assay was conducted after 2 independent infections.

**In vivo experiments in female nude mice**

The animal studies were conducted in accordance with the German and European laws on animal welfare. The treatments were approved by the Bavarian authorities on veterinary issues (file no. 55.2-1-54-2531-118-10). The female athymic nude mice (Crl:NU-Foxn1 nu; originating from Charles River Laboratories) were maintained in a pathogen-free environment. Five-week-old female nude mice were inoculated once into the mammary fat pad with 1 × 10^6 T47D or JIMT-1 cells in 50 μL. The cells were suspended in PBS and mixed with an equal volume of Matrigel (354234, BD Matrigel, BD Biosciences). The tumor size was estimated as the product of the length and width of the xenograft measured once a week with a caliper. The data were collected between day 16 postinjection and day 56 postinjection. Eight weeks postinjection, or when the xenograft reached approximately 1 cm^3, the mice were euthanized. The increases in the tumor size over time were estimated by ANOVA, and the individual size measurements from each day were normalized to the average size on day 23 postinjection for the JIMT-1 cell xenografts or on day 56 postinfection for the T47D cell xenografts. These normalized tumor sizes were tested for differences between the 4 injected cell line approaches using the Mann–Whitney U test. P values that were less than 0.05 were considered to be statistically significant. The median and SEs of the normalized tumor sizes were calculated.

**Results**

The simultaneous knockdown of PTK6 and HER2 in breast cancer cells led to stronger effects in the HER receptor signaling pathway. Before stably downregulating HER2 and PTK6, their expression was transiently downregulated with siRNAs in
the T47D and JIMT-1 cell lines to investigate the effect of simultaneous knockdown. As recently shown by us, specific and efficient siRNAs for PTK6 knockdown were identified as described (19). The siRNAs for HER2 knockdown led to a specific and effective downregulation (Fig. 1A). The simultaneous knockdown of PTK6 and HER2 after 72 hours (using the most efficient siRNAs, respectively) shows that the phosphorylation of MAPK 1/3 (ERK 1/2) at Thr202/Tyr204 and of STAT3 at Ser727 is further reduced compared with the phosphorylation status following single HER2 or PTK6 downregulation (Fig. 1B).

On the basis of these results, for stable and simultaneous knockdown of HER2 and PTK6, the T47D and JIMT-1 cell lines were efficiently infected with lentiviral particles encoding sequences for shRNAs. Because of the stable integration of the sequences into the host genome, the infections led to a stable downregulation of the target proteins. We show the effects of the stable and simultaneous knockdown of PTK6 and HER2 on selected signaling proteins downstream of HER2 and PTK6 (Figs. 2 and 3). Because the results from JIMT-1 cells that were incubated with and without trastuzumab were comparable, we only show the results obtained from cells cultivated with trastuzumab (Fig. 2). In JIMT-1 and T47D cells, the protein expression levels of HER1, HER3, HER4, were not changed (data not shown) following the knockdown of HER2 and PTK6, but the phosphorylation of HER receptor-dependent signaling proteins was affected. Compared with the mock and vector controls, the phosphorylation of MAPK 1/3 (ERK 1/2) at Thr202/Tyr204 in the JIMT-1 cell line was significantly reduced after HER2 (P < 0.001) or PTK6 (P < 0.001) silencing, and in both cell lines, it was significantly decreased due to the combined downregulation (Figs. 2B and 3B). Phosphorylated, and therefore activated, MAPK mediates cellular processes such as proliferation, differentiation, and migration by phosphorylation of its substrates (15). In contrast, p38 MAPK is activated due to environmental stress and is involved in regulating cell death (15). The phosphorylation of p38 MAPK at Thr180/Tyr182 leads to the activation of other MAPK and transcription factors. In T47D and JIMT-1 cells, the simultaneous downregulation of HER2 and PTK6 resulted in a further reduced phosphorylation of this kinase than single knockdown alone (Figs. 2A and 3A). However, in JIMT-1 cells, the dual knockdown significantly reduced the phosphorylation compared with single knockdowns (Fig. 2B). Consequently, antiapoptotic signaling via p38 MAPK may be reduced.

In addition, Akt is activated by phosphorylation at Thr308 and Ser473, regulating survival and apoptosis by inhibiting the target proteins (30). The single and the simultaneous knockdowns of HER2 and PTK6 led to a reduced phosphorylation of Akt at Ser473 in both cell lines (Figs. 2 and 3). In particular, in T47D cells, compared with PTK6-depleted cells, the dual knockdown significantly reduced the phosphorylation (Fig. 3B). The phosphorylation status at Thr308 was not changed (data not shown).
STAT3 is an additional signaling protein that mediates cell growth, survival, differentiation, and gene expression via phosphorylation at Tyr705 followed by dimerization and translocation to the nucleus and DNA binding (31). STAT3 that is phosphorylated at Ser727 promotes gene expression. Following combined downregulation in JIMT-1 cells, we observed a slightly diminished phosphorylation of STAT3 at Ser727 (Fig. 2). In T47D cells, the phosphorylation at this site was significantly reduced in HER2-depleted cells ($P = 0.002$) and also significantly decreased in cells depleted of both compared with the PTK6 knockdown alone (Fig. 3B). The phosphorylation of STAT3 at Tyr705 was not analyzed because this phosphorylation site was marginal detectable in the cells.

The phosphatase PTEN is a tumor suppressor that negatively regulates the phosphoinositide-3-kinase (PI3K)/Akt pathway, and PTEN phosphorylation at Ser380/Thr382/383 lessens its activity as a tumor suppressor (32). In both cell lines, only the simultaneous silencing of HER2 and PTK6 significantly reduced the phosphorylation of PTEN in comparison with controls and single knockdowns (Figs. 2 and 3).

The simultaneous knockdown of PTK6 and HER2 enhanced p27 expression and significantly reduced cell proliferation

The induction of apoptosis as a consequence of HER2 and PTK6 knockdown was investigated on the basis of PARP cleavage using Western blot analysis. PARP was only cleaved when the breast cancer cells were incubated for 24 hours with staurosporine, an unspecific kinase inhibitor that induces apoptosis (positive control), and not after PTK6 and HER2 downregulation (Fig. 4A).

We also analyzed the expression of cell-cycle proteins depending on HER2 and PTK6 protein levels. The protein expression of cyclins and cdk2 following single and simultaneous knockdown of HER2 and PTK6 was not affected in T47D and JIMT-1 cells (data not shown).
However, compared with the mock and vector controls, in both cell lines, an induction of the cell-cycle inhibitor p27 was observed because of HER2 and PTK6 knockdown (Fig. 4B).

We examined the proliferation of T47D and JIMT-1 cells by analyzing the enzymatic cleavage of tetrazolium salts into formazan (WST-1 assay). This reaction can be proceeded in metabolically active cells, and the amount of formazan dye is directly associated with the amount of viable cells (33). Compared with control cells (mock), the number of cells following HER2 knockdown is significantly reduced (T47D cells: \( P < 0.008 \), JIMT-1 cells: \( P = 0.004 \); Fig. 4C). It is also reduced when PTK6 is silenced (T47D cells and JIMT-1 cells: \( P < 0.001 \), respectively), and following simultaneous knockdown, the number of metabolically active cells is additively diminished (T47D cells and JIMT-1 cells: \( P < 0.001 \), respectively). The differences between HER2-depleted cells and cells depleted of both proteins (T47D cells: \( P < 0.001 \), JIMT-1 cells: \( P = 0.04 \)), were also statistically significant (Fig. 4C).

Stronger reduced migration and invasion of breast cancer cells due to the simultaneous knockdown of PTK6 and HER2

To further determine the effects of HER2 and PTK6 silencing in breast cancer cells, the migration and invasion of the cells were analyzed in vitro. JIMT-1 cells were seeded into 6-well plates, then a wound was scratched into the confluent monolayer and the cells were observed for the following 72 hours under serum-reduced conditions, as exemplarily shown (Fig. 5A). Although the control JIMT-1 cells almost overgrow the open area [mock (6%) and control vector (5%); Fig. 5B], the open area of HER2-depleted cells was at 39% (\( P = 0.037 \)), of PTK6-depleted cells was at 42% (\( P = 0.008 \)) indicating a significantly reduced migration. Moreover, the simultaneous HER2 and PTK6 knockdown
further reduced the cell migration, as indicated by an area that remained more than 52% opened ($P=0.003$, Fig. 5B) but it is not significant compared with single knockdowns. Because of a very strong HER2 knockdown effect in the T47D cell line, these cells (approaches with HER2-depleted and cells depleted of both: HER2 and PTK6) did not become confluent and as this is a prerequisite for the scratch assay, the cells could not be used for the migration assay. Furthermore, the invasion of JIMT-1 cells was also reduced in vitro due to the combined silencing of HER2 and PTK6 when compared with single downregulation of the respective proteins (Fig. 6A). Indeed, the values were statistically not significant, but a clear tendency was observed. In the control experiments, the amount of invasive cells was approximately 60%, 40%, and 20%, respectively (Fig. 6A).

To confirm the outcome of reduced invasion, we analyzed the cleavage, and therefore the activation, of MMP9 by immunoblotting using supernatants of JIMT-1 cells that were cultivated in serum-reduced medium. MMPs mediate the digestion of the extracellular matrix and play an important role in wound healing, tumor invasion, angiogenesis, and carcinogenesis (34). The cleavage of the precursor protein into the active MMP is required for invasion (34). In comparison with controls (mock and control vector), the cleavage, and thus the activation of MMP9 (illustrated by the band at 84 kDa), is diminished when PTK6 and both proteins are downregulated (Fig. 6B). Here, we only show the results obtained with JIMT-1 cells because T47D cells are less invasive and express lower levels of MMP9.
Significantly reduced in vivo tumor growth following HER2 and PTK6 knockdown

Female nude mice were inoculated with control cells (T47D cells and JIMT-1 cells infected with the control vector), and with T47D or JIMT-1 cells that were depleted of HER2, PTK6, and with cells containing the combined knockdown. The median tumor size (normalized to the average on day 23 postinjection) in JIMT-1 xenografts was significantly reduced following PTK6 knockdown (32.7–25.9 mm² = −21%; P = 0.0003), HER2 knockdown (32.7–29.4 mm² = −10.2%; P = 0.0008), and the combined PTK6 and HER2 knockdown (32.7–29.3 mm² = −10.4%, P = 0.0007; Fig. 7). Concerning T47D cell xenografts, the median tumor size was estimated at the end of the experiment (day 56 postinjection) due to a slower proliferation of the T47D cells. In all approaches, the reduction of xenograft size of HER2- and PTK6-depleted T47D cells was statistically significant (P ≤ 0.0001; Fig. 7). In comparison with control xenografts (10.2 mm²), the size of HER2-depleted xenografts was reduced to approximately 5.2 mm² (−49%), of PTK6-depleted xenografts to approximately 8.7 mm² (−15%) and the size of xenografts depleted of both were reduced to approximately 6.4 mm² (−37%).

Discussion

A possible approach for a targeted therapy for breast cancer is the anti-HER2 treatment by means of using the humanized antibody trastuzumab in HER2-overexpressing tumors. However, a percentage of them become resistant within a year of administration of the antibody (7, 8). Therefore, novel biomarkers and targets are needed for additional tailored therapies in breast cancer. One expedient concept to circumvent the development of resistance to breast cancer therapy would be simultaneous targeting of different, but physiologically related, targets within already validated cancer-related signaling pathways. Nevertheless, to date, studies that examine and confirm the advantageous effects of simultaneous knockdown of such target pairs are still scarce, although some groups have reported stronger or synergistic effects following the simultaneous knockdown of uPAR and HER2, uPAR and MMP9, or uPA and uPAR (10, 35, 36).
In previous studies, we have shown the association of HER2 with PTK6 in breast cancer (19, 20, 22). These studies led us to investigate the effects of simultaneous knockdown of HER2 and PTK6. To show stronger effects in regards to the reduction of tumor progression, we simultaneously knocked down HER2 and PTK6 in T47D cells and in trastuzumab-resistant JIMT-1 cells. This inhibition may offer an attractive approach for directed therapy of resistant breast tumors.

Prompted by preliminary results in which the transient and simultaneous downregulation of HER2 and PTK6 revealed to be more efficient involving the reduced phosphorylation of HER2-associated signaling proteins, we stably silenced HER2 and PTK6 in 2 human breast cancer cell lines. MAPKs, STAT3, Akt, and PTEN are involved in tumorigenic processes, and reduced phosphorylations of these signaling proteins may more strongly inhibit cell proliferation, migration, and antiapoptotic pathways (14, 15, 30, 32). Consequently, the tumor cell growth regulated by these proteins may be reduced. In the JIMT-1 cell line, the effects concerning reduced phosphorylation of MAPK 1/3 (ERK 1/2) were the most extensive and compared with single knockdowns, the simultaneous silencing of both proteins resulted in a further statistically significant reduction of phosphorylation. Our results are in agreement with previous studies showing decreased phosphorylations of (p38) MAPK (MAPK 1/3, ERK 1/2) following PTK6 knockdown (15).

Regarding the STAT3 activation at Ser727, we observed a slightly reduced phosphorylation after simultaneous knockdown in JIMT-1 cells, whereas in T47D cells, a HER2 knockdown-dependent phosphorylation was observed. Usually, PTK6 is involved in the phosphorylation of STAT3 at Tyr705 (31), but phosphorylation at this site was not detectable in JIMT-1 and T47D cells. Because the phosphorylation of STAT3 at Ser727 is affected by the MAPK pathway (37), we assume that there is an indirect regulation of STAT3 at Ser727 via the MAPK that was explicitly reduced following the combined silencing. Interestingly, due to siRNA-mediated downregulation, the phosphorylation of STAT3 at Ser727 was already reduced following the single HER2 or PTK6 knockdown. We hypothesize that the...
long-term downregulation causes a time-dependent adaptation of the cells and compensates for the absent phosphorylation at Ser727 by activation of alternative kinases. The reduced phosphorylation of Akt at Ser473 in particular after HER2 and simultaneous silencing suggests a HER2-dependent regulation. This result correlates with a study presented by Knuefermann and colleagues (38), showing Akt activation through the HER2/PI-3K pathway in breast adenocarcinoma cells. In addition, study conducted by Ostrander and colleagues showed no change in the phosphorylation of Akt following PTK6 knockdown (15). The phosphorylation, and therefore the inactivation of the tumor suppressor PTEN, was significantly reduced only when both proteins were silenced in combination. Here, we also assume a time-dependent compensation regulated by other kinases when HER2 or PTK6 were stably knocked down. According to our observations, the effects following the dual knockdown may be based on the inhibition of HER2-associated pathways, and additionally, by diminished PTK6-regulated signaling involved in EGFR and insulin-like growth factor-1 receptor pathways (e.g., refs. 21, 39).

Although the simultaneous knockdown led to a reduced activation of several signaling proteins involved in tumorigenesis, an induction of apoptosis was not observed. It seems that even a combined knockdown of HER2 and PTK6 was not powerful enough to induce apoptosis neither in T47D nor in JIMT-1 cells. Our results regarding the additive effects due to simultaneous knockdown are in agreement with previous studies showing stronger effects following combined silencing of other target proteins (10, 35, 36, 40). However, RNAi of HER2 and uPAR together, with the addition of trastuzumab, induced apoptosis in breast cancer cells (10). This outcome may be explained by the application of different cell lines, for example, SKBR-3 or BT474, which are sensitive to trastuzumab in contrast to JIMT-1 cells, and by the fact that the proliferation in these cell lines is more dependent on HER2 expression levels than in JIMT-1 cells (9, 10).

While the cell-cycle protein expression (cyclin A, cyclin D1, cyclin E and cdk2; data not shown) was not affected following downregulation, the expression of the cell-cycle inhibitor p27 was clearly elevated in T47D and JIMT-1 cells when HER2, PTK6, and both were knocked down. This may result in a G1 arrest and therefore in a reduced cell-cycle progression. This outcome corresponds to the significantly pronounced decrease in the percentage of viable cells following the combined downregulation of HER2 and PTK6. Our results are in accordance with a previous study showing regulation of p27 expression by PTK6 in MDA-MB-231 breast cancer cells through the activity of its transcription factor FoxO3a (18). Another study reported an epigallocatechin-3 gallate-induced inhibition of growth in HER2-positive breast cancer cells via repression of FoxO3a and induction of p27 (41). However, PTK6 silencing seemed to influence the trastuzumab-resistant JIMT-1 cell proliferation more than HER2 knockdown. In T47D cells, the HER2 knockdown impaired the cell proliferation more than the PTK6 downregulation.

The stronger effects due to simultaneous RNAi were also underpinned by reduced migration and invasion of JIMT-1 cells in vitro, correlated with a reduced activation of MAPK 1/3 (ERK 1/2), and MMP9. Both proteins are also involved in cell motility, wound healing, and invasion (15, 34). Migration and invasion are complex processes, and both highly contribute to the malignancy of tumor cells (42). Although each single silencing statistically reduced JIMT-1 cell migration and the simultaneous approach further diminished the migration, in comparison with single knockdowns, it was not statistically significant. We suggest that the single silencing effect, in particular the PTK6 RNAi, was so strong that the dual knockdown does not lead to significant changes anymore. However, the migration and invasion of JIMT-1 cells seemed to be more affected by the depletion of PTK6 than of HER2.

To show the effect of HER2 and PTK6 silencing in vivo, we carried out animal experiments with female immunosuppressed nude mice. The tumor growth was significantly reduced following HER2, PTK6, and simultaneous knockdown. Interestingly, in JIMT-1 cells, PTK6 downregulation seemed to have a greater impact on reducing tumor growth than HER2 RNAi, whereas in T47D cells, the HER2 knockdown stronger impaired the cells in vitro. These results partly reflect our observations from the previously conducted in vitro assays. This outcome might be related to the different genotypic features of the respective cell line, namely that the survival of the JIMT-1 cells is not as strongly regulated by HER2 protein expression, as it may be in other HER2-overexpressing and HER2-dependent cell lines, for example, SKBR-3 or BT474 (9, 10, 43). In addition, the strong effect of PTK6 knockdown may also be associated with the fact that PTK6 is expressed in approximately 80% of breast tissues (13), and therefore, it may be an additional target in particular considering the therapy of trastuzumab-resistant breast cancers. Furthermore, although the HER2 expression in T47D cells is not very high, the HER2 knockdown in this cell line has a greater impact than the PTK6 RNAi. This indicates a highly HER2-dependent proliferation which may also be correlated with the trastuzumab-sensitivity of T47D cells (data not shown). Consistent with our findings, some research groups showed that overexpression of HER2 and PTK6 in vitro and in vivo increases breast cancer tumorigenesis (21, 44, 45). Accordingly, when HER2 was downregulated, the cell proliferation of JIMT-1 breast cancer cells was also reduced in vivo (9, 46). Concerning PTK6 knockdown in vivo only, investigations in the murine small intestine are described and show a contrary role compared with its role in breast cancer (47, 48).

Unexpectedly, although the reduction of tumor growth in vivo following single PTK6 or HER2 knockdown was significantly reduced in both cell line xenografts, the combined approach led to an intermediate reduction of the tumor size and not to an additive one. Here, we hypothesize that in contrast to the positively infected cells of the combined knockdown approach (which significantly reduced the cell proliferation), the minimal number of noninfected cells,
which have a survival benefit, may strongly prolifera-
te during the experiment duration of up to 8 weeks.

In summary, our experiments for the first time provide
evidence that the knockdown of HER2 and/or PTK6 can
strongly inhibit tumor progression through the significan-
tly reduced activation of key proteins that are linked to tumor-
genesis, and via diminished migration, invasion, and cell
proliferation leading to significantly decreased tumor
growth. Consequently, our results show that the inhibition
of HER2 and/or PTK6 proteins represent an attractive tool
in targeted breast cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N. Ludya, M. Schmitter, H. Höffler
Acquisition of data (provided animals, acquired and managed patients, provided
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