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HER3 is required for the maintenance of neuregulin-dependent and -independent attributes of malignant progression in prostate cancer cells

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HER3 (ERBB3) is a catalytically inactive pseudokinase of the HER receptor tyrosine kinase family, frequently overexpressed in prostate and other cancers. Aberrant expression and mutations of 2 other members of the family, EGFR and HER2, are key carcinogenic events in several types of tumors, and both are well-validated therapeutic targets. In this study, we show that HER3 is required to maintain the motile and invasive phenotypes of prostate (DU-145) and breast (MCF-7) cancer cells in response to the HER3 ligand neuregulin-1 (NRG-1), epidermal growth factor (EGF) and fetal bovine serum. Although MCF-7 breast cancer cells appeared to require HER3 as part of an autocrine response induced by EGF and FBS, the response of DU-145 prostate cancer cells to these stimuli, while requiring HER3, did not appear to involve autocrine stimulation of the receptor. DU-145 cells required the expression of HER3 for efficient clonogenicity *in vitro* in standard growth medium and for tumorigenicity in immunodeficient mice. These observations suggest that prostate cancer cells derived from tumors that overexpress HER3 are dependent on its expression for the maintenance of major attributes of neoplastic aggressiveness, with or without cognate ligand stimulation.

Key words: HER3; ERBB3; invasiveness; tumorigenicity; prostate; breast

The 3 transmembrane tyrosine kinases and 1 pseudokinase of the HER family, EGFR/ERBB1, HERs2/ERBB2, HER4/ERBB4 and HER3/ERBB3, are key regulators of the growth, survival, differentiation, motility and invasiveness of many cell types.[1] Their activities are engaged upon binding of polypeptide ligands, of which more than 13 are known in humans.[1] Although all HER ligands are structurally related to EGF, individual ligands preferentially bind to, and activate, specific HERs. Ligand binding induces conformational changes in the receptors, exposing a dimerization surface through which they associate with other HER proteins, forming homodimers or heterodimers.[2] These specific associations transduce additional conformational changes to the cytoplasmic domains of the receptors, eventually exposing and activating their tyrosine kinase catalytic domains.[2] Of the 4 HER proteins, HER2 lacks the capacity to capture ligands, and is constitutively poised to associate, through its dimerization surface, either with the 2nd HER2 molecule, or with the EGFR or HER3 after they adopt active conformations upon ligand binding.[2] The pseudokinase HER3 lacks a tyrosine kinase catalytic center. Binding of NRG-1 by HER3 causes its prompt heterodimerization with HER2 and activation of the kinase activity of the latter, which in turn phosphorylates the HER3 cytoplasmic domain at specific tyrosine residues.[3–5] HER3 can also form heterodimeric complexes with ligand-bound EGFR or HER4[1], resulting in its transactivation. Several of the induced phosphosites on HER3 recruit and activate phosphatidylinositol-3′ kinase (PI3K), directly coupling this receptor to AKT-dependent pathways that promote cell motility, invasion and survival.[3,4]

In breast cancer, amplification of the HER2 gene and consequent overexpression are associated with aggressive tumors.[6] In prostate cancer, HER2 overexpression, although infrequent in primary tumors, is associated with the development of androgen independence and a more aggressive phenotype.[7,8] EGFR is mutated or overexpressed in many tumor types, in particular glioblastoma, lung cancer and pancreatic adenocarcinoma,[9] in which these alterations are essential for growth, survival and invasiveness. Mutations in the kinase domain of HER4 have been reported in some cancers,[10] though their biological or clinical significance is unknown. In contrast, the HER3 gene is rarely amplified in human cancers,[11,12] and activating mutations have not been reported. HER3 is expressed at abnormally high levels in several tumor types, including cancers of the breast, colon, ovary, prostate or pancreas.[13] In primary breast and colon cancers, overexpression of HER3 is associated with overexpression of HER2,[14] suggesting that in these tumors the HER3-HER2 heterodimer functions as an oncogenic unit. In primary, organ-confined prostate cancer, however, overexpression of HER3 is rarely accompanied with increased levels of either HER2 or EGFR.[15]

The importance of the aberrant expression or function of HER2 and EGFR in the growth, maintenance and progression of the tumors harboring these alterations is underlined by the demonstrated clinical efficacy of therapies specifically targeted at these kinases, in the form of neutralizing antibodies such as trastuzumab (Herceptin) and pertuzumab (Omnitarg) for HER2, or cetuximab (Erbitux) for EGFR,[16] of kinase-specific pharmacological inhibitors such as gefitinib (Iressa) and erlotinib (Tarceva) for EGFR, or dual-specific inhibitors such as lapatinib for both HER2 and EGFR.[17] In spite of the remarkable successes of receptor-targeted therapies in cancer, a significant proportion of HER2- or EGFR-positive tumors fail to respond, and resistance to these treatments eventually develops in many cases.[18–20] Aside from toxicity issues, emerging mechanisms that may explain the failure of HER-targeted therapies in some cases include the abnormal activation of HER3, which becomes independent from HER2 or EGFR or insensitive to kinase inhibitors,[21–25] or a surge in the levels of HER3, which can be transactivated in the presence of relatively low levels of HER2.[26] Studies in cellular models have highlighted the relevance of HER3 for the invasive phenotypes of several tumor types,[27,28] and in conferring a survival advantage in

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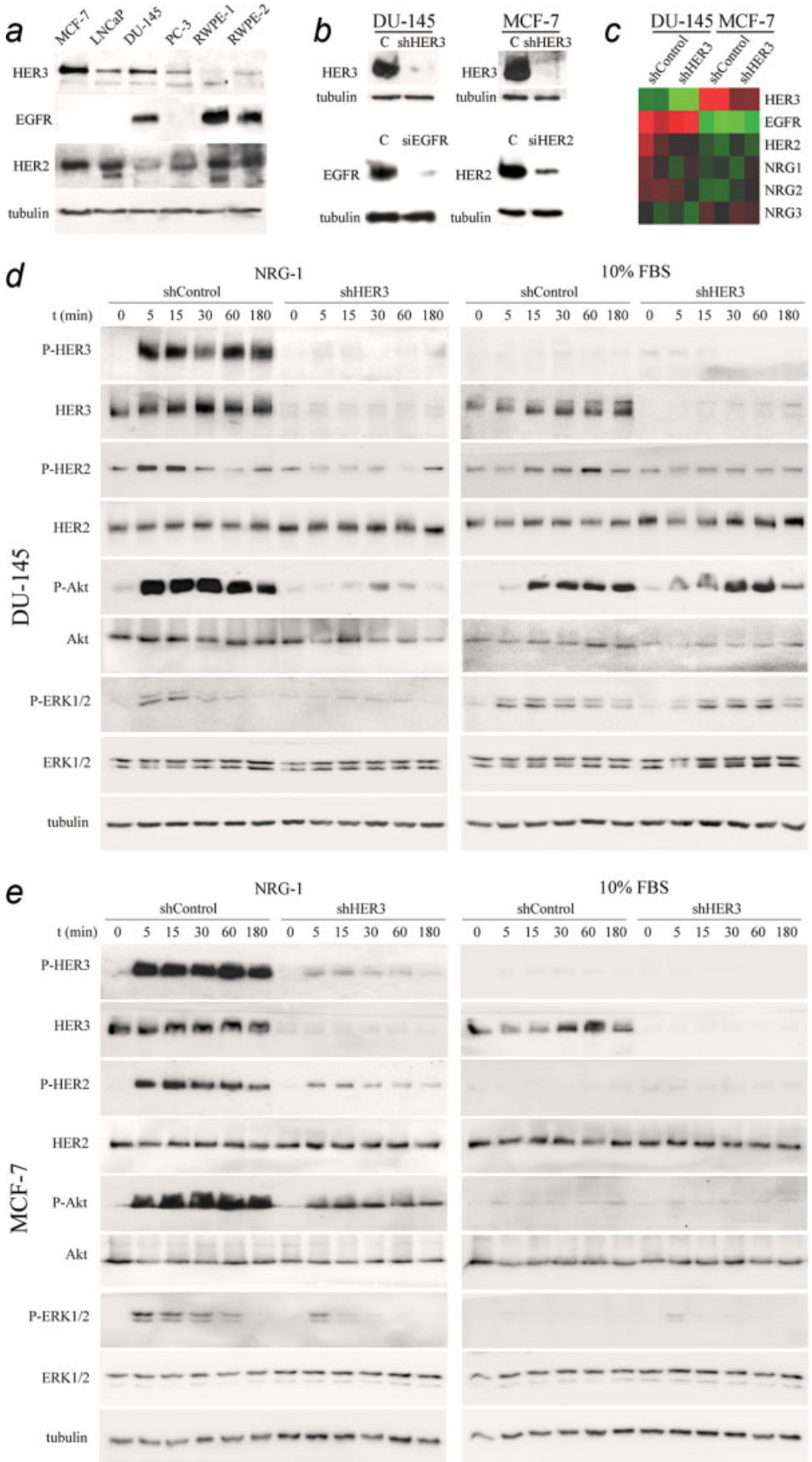
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MCF-7
LNCaP
DU-145
PC-3
RWPE-1
RWPE-2
HER3
EGFR
HER2
tubulin
b
DU-145
C shHER3
HER3
tubulin
MCF-7
C shHER3
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P-HER3
HER3
P-HER2
HER2
P-Akt
Akt
P-ERK1/2
ERK1/2
tubulin
MCF-7

FIGURE 1

response to genotoxic damage,[29–31] features that have been associated with advanced neoplasia and the development of resistance to therapy.

Here we report that HER3 promotes, and is required for, the invasiveness of prostate epithelial cells through ligand-specific transactivation by either HER2 or EGFR. We further show that prostate and breast cancer cells require expression of HER3 for optimal motility and clonogenicity *in vitro* and tumorigenicity *in vivo*, and that these functions do not necessarily require stimulation by neuregulins.

Material and methods

Reagents and antibodies

Neuregulin-1β was obtained from Upstate Biotechnology (Lake Placid, NY), recombinant human EGF from Gibco-Invitrogen (Grand Island, NY), AG1478 from Calbiochem (La Jolla, CA), and GM6001 from Biomol (Plymouth Meeting, PA). Antibodies: to EGFR, phospho-EGFR (Tyr1173, monoclonal 9H2), phospho-HER2 (Tyr 1248), HER3 (neutralizing monoclonal H3.105.5) and ERK1/2 from Upstate Biotechnology (Lake Placid, NY); HER3 (polyclonal C-17) from Santa Cruz Biotechnology (Santa Cruz, CA); HER2 (polyclonal) from Biogenex (San Ramon, CA); phospho-HER3 (Tyr1289), phospho-p44/42 (Thr202/Tyr204), Akt and phospho-Akt (Ser437) from Cell Signalling (Beverly, MA); and α-tubulin from Sigma (Alcobendas, Madrid, Spain). Anti-HER2 dimerization blocking antibody 2C4 [32] was a generous gift from Genentech (South San Francisco, CA).

Cell culture

PC-3, DU-145, MCF-7 and HEK293T (American Type Culture Collection, Manassas, VA) were grown in RPMI or DMEM medium supplemented with 10% FBS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 0.1 mM nonessential aminoacids (PAA Laboratories, Linz, Austria). RWPE-2 cells were grown in KSF Medium, supplemented with recombinant human EGF (5 ng/mL) and bovine pituitary extract (50 μg/mL) (Gibco). Medium for HER3 knock-down MCF-7 cells was supplemented with insulin (0.01 mg/mL).

Plasmids and cell transfection

pHER3-Flag and pHER3-GFP[33] were kindly provided by Dr. Philippe I. H. Bastiaens (EMBL, Heidelberg). Control transfections were performed with pEGFP (Clontech, Mountain View, CA). RWPE-2 cells were transfected overnight with Fugene 6 (Roche) in Optimem medium (Gibco-Invitrogen). PC-3 cells were transfected for 8 hr with Lipofectamine (Invitrogen, Costa Mesa, CA).

SiRNA synthesis and transfection

siRNA duplexes were synthesized with the Silencer siRNA kit (Ambion, Austin, TX) and cotransfected with HER3-GFP at 80 nM. Target DNA sequences for siRNA were GGAGCTGCC CATGAGAAAT for EGFR and CAGTCTCCGCATCGTGTAC for HER2. RNA duplexes corresponding to a "scrambled" sequence, without significant similarity to any human transcripts, were used as controls.

Lentiviral shRNA production and transduction

Plasmids for HER3-targeted shRNAs and control pLKO.1-Puro were from Sigma. Plasmids pVSVG and pCMVΔR8.91 for the expression of packaging and envelope proteins were kindly provided by Dr. Luciano di Croce (Center for Genome Research, Barcelona). HEK293T cells were cotransfected with $CaCl_2$/BES buffer, incubated overnight in a 3% CO2 atmosphere, washed and incubated for 48 hr in 10% CO2 with medium containing 2% FBS. Viral particles were concentrated by ultracentrifugation on 20% sucrose gradients, and titrated after infection in the presence of polybrene (8 μg/mL; Sigma). Infected cells were selected with 5 μg/mL puromycin for 3 days and mantained thereafter in medium with 1 μg/mL puromycin. Five shRNA-containing constructs targeting distinct sequences on HER3 were tested, of which 2 were selected for their more effective silencing of HER3. The degree of HER3 silencing of puromicin-selected cells transduced with these 2 shRNAs was tested routinely before performing a particular assay.

Western blotting

Cells were serum-starved overnight, incubated with or without NRG-1 (5 ng/mL) or EGF (25 ng/mL) in cold medium, and lysed (50 mM Tris HCl pH 7.4, 100 mM NaCl, 1% Triton X-100, 10 % glycerol, 100 μg/ml PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM EDTA, 25 mM NaFl, 100 μM Na_3VaO_4, 0.5 μM okadaic acid, 10 mM β-glycerophosphate) at 4°C for 45 min. Samples in Laemmli buffer were boiled for 5 min and analyzed by Western blotting. Protein loading and transfer were monitored by Ponceau-red staining of the membranes and blotting with antitubulin.

Immunoprecipitation

Cell lysates (as above) were precleared by incubation with Sepharose CL4B (Pharmacia, Uppsale, Sweden) for 1 hr, and immunoprecipitated by overnight incubation at 4°C with anti-Flag M2 resin (Sigma). Immune complexes were washed 5 times in lysis buffer, then boiled for 5 min in Laemmli buffer and analyzed by Western blotting.

Wound-healing assays

Cells (2.5×10^5/well) were seeded in 24-well plates, allowed to reach confluent monolayers, and serum-starved for 24 hr. Cell monolayers were treated for 1 h with 1 μM mitomycin C (Sigma), followed by the creation of wounds by using a ≈0.5 mm (outer diameter) plastic pipette tip. For treatment with an anti-HER3 neutralizing monoclonal antibody,[25] a final concentration of 10 μg/mL was added at the moment of wounding, and the treatment repeated 24 h later, maintaining the treatment throughout the experiment. Images were captured and surface areas between leading edges of the monolayers at predetermined wound sites (3 sites per condition, performed in duplicate) were measured using the ImageJ software.

Figure 1 – Effects of HER3 depletion on the growth and signaling of DU-145 and MCF-7 cells. (*a*) Baseline expression levels of HER3, HER2 and EGFR in PC-3, DU-145, and RWPE-2 prostate cell lines, and MCF-7 breast cancer cell line. (*b*) Effective and specific knock-down of HER3 by shRNA transduction of DU-145 and MCF-7 cells and transfection of siRNA duplexes specific for HER2 and EGFR. (*c*) Silencing of HER3 after shRNA transduction, assessed by microarray analysis of control and knock-down DU-145 and MCF-7 cells. In DU-145 cells, the HER3 transcript was silenced by an average of 82.5% and by 74.5% in MCF-7 cells. Shown are also heat maps corresponding to relative transcript levels for neuregulin-1, -2 and -3 in both cell types. NRG-2 is expressed at slightly higher levels by DU-145 cells than MCF-7 cells (average 1.3 fold), and NRG-3 was expressed at higher levels by MCF-7 cells than DU-145 cells (average 1.6 fold), without significant variations between control and HER3 knock-down cells. Lower and more variable levels of NRG-1 transcripts were not significantly different between DU-145 and MCF-7. (*d*) HER3 depletion in DU-145 prostate cancer abrogates the activation of Akt or ERK1/2 by NRG-1, but does not compromise their response to 10% FBS. Control and HER-3-depleted cells were serum-starved for 24 hr and challenged with either NRG-1 (5 ng/mL) or 10% FBS, harvested at the indicated time points, and processed for Western blotting. E, HER3 depletion in MCF-7 breast cancer cells abrogates the activation of Akt or ERK1/2 by NRG-1. Addition of 10% FBS does not stimulate ERK or Akt signaling in either control or HER-3-depleted MCF-7 cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Invasiveness assays

Cells were transfected with either pHER3-GFP or control pEGFP vector (Clontech). The following day, cells were trypsinized, resuspended in 0.1% BSA, and seeded on Transwell chambers (Costar, Cambridge, MA) coated with diluted (1:20 in H_2O) growth factor-reduced Matrigel (Beckton-Dickinson, San Jose, CA). The lower chamber was filled with medium supplemented with chemoattractants (NRG-1 at 25 ng/mL, EGF at 100 ng/mL, or 10% FBS). After 8 (PC-3) or 16 hr (RWPE-2), after removing cells in the upper chamber, cells attached to the membrane in the lower chamber were fixed with cold 4% paraformaldehyde/PBS, counterstained with Hoechst 33258 (Sigma), the membranes excised and mounted on glass coverslips with Mowiol fluorescence stabilizer. An average of 8 microscope fields per condition were captured under a Zeiss Axiophot microscope. Total (Hoechst positive) and GFP-positive cells were scored, normalized for transfection efficiencies and invasion indexes determined as follows:

$$\text{Invasiveness Index} = \left(\left(\frac{\text{Invasive (HER3} - \text{GFP positive/Hoechst positive)}}{\text{Seeded (HER3} - \text{GFP positive/Hoechst positive}} \right) \div \left(\frac{\text{Invasive (GFP positive/Hoechst positive)}}{\text{Seeded (GFP positive/Hoechst positive)}} \right) - 1 \right) \times 100$$

where "Hoechst positive" refers to the total number of cells in each population considered. The "invasive" fraction of cells transfected with either pHER3-GFP or control pEGFP ("GFP") is calculated relative to the total number of seeded cells. The final index is the result of the ratio between the invasiveness of HER3-GFP-positive cells to that of control GFP-positive cells. For DU-145/HER3kd and control DU-145/puro cells, invasiveness was quantitated with CyQuant (Molecular Probes).

Soft agar colony formation

Melted agar (0.6%) in complete medium was placed at the bottom of 12-well plates, allowed to solidify and overlayed with 3×10^3 cells resuspended in 0.4% agar/complete medium. After 3 weeks, wells were fixed with 4% paraformaldehyde, stained with 0.025% crystal violet and colonies $\geq$ 0.1 mm diameter scored.

Tumor xenograft growth

Cells were transduced with lentiviral particles for the bicistronic expression of luciferase and GFP, fluorescent cells selected in a MoFlo sorter (Cytomation, Fort Collins, CO), tested for growth and clonogenicity in soft agar (Supporting Information Fig. 4), and injected (10^6 cells/0.1 mL PBS) into each rear limb of male SCID mice (Charles River Laboratories). Tumor light emission was acquired in an ORCA-2BT instrument (Hamamatsu Photonics, Hamamatsu City, Japan) after intraperitoneal injection of luciferin (16.7 mg/mL; Promega), quantitated and analyzed with Wasabi (Hamamatsu). Values were normalized *versus* initial values for each tumor.

Transcriptional profiling

RNAs were isolated from duplicate samples, amplified, labeled and hybridized to Affymetrix U133 2.0 Plus arrays. Microarray data were RMA normalized,[34] and probes with maximum expression values < 5 were eliminated, resulting in a total of 31,780 probes out of the original 54,675 probeset. To identify differentially expressed genes, we applied Significance Analysis of Microarrays,[35] selecting those genes with a False Discovery Rate (FDR) q value ≤ 0.10.

Real-time RT-PCR

RNAs were retrotranscribed (High capacity cDNA reverse transcription kit, Applied Biosystems) and the level of expression of selected genes was determined by semiquantitative RT-PCR with the Universal Probe Library (Roche) system on a Light Cycler 480 instrument (Roche). Ct values for each transcript were normalized against those for the RPS18 gene.

Results

Effects of HER3 depletion on growth and signaling of prostate and breast cancer cells

Previous studies[15,36] have suggested that most prostate cancers overexpress HER3. We conducted a immunohistochemical survey that confirmed that HER3 is commonly overexpressed in primary prostate cancer (Supporting Information Fig. 1*a*). In contrast, HER2 was never, and EGFR only infrequently, found overexpressed in carcinomatous glands as compared with normal glands in our samples (Supporting Information Table I). In fact, HER2 was barely detectable (<10% of cells with significant staining) in a significant proportion of prostate samples under conditions in which this receptor was readily detectable by immunohistochemistry in other epithelial tissues (Supporting Information Fig. 1 and 2). Although early reports had suggested that primary prostate cancers overexpress HER2 or EGFR,[37–39] more recent studies indicate that their overexpression, and in particular that of HER2, with or without gene amplification, is rarely observed in early-stage primary tumors.[40,41] On the other hand, the overexpression and amplification of HER2 and EGFR in association with tumor progression and the development of androgen independence are well documented.[42–45] This suggests that, in primary prostate adenocarcinoma, HER3 may function mainly in association with EGFR, rather than with its preferred partner HER2.

First, we investigated if stable knock-down of HER3 compromises the growth of prostate and breast cancer cells, with or without added NRG-1 stimulation. For these experiments, we chose the prostate cancer cell line DU-145, which coexpresses relatively high levels of HER3, HER2 and EGFR, and the breast cancer cell line MCF-7, which expresses higher levels of HER2 and HER3, but lower levels of EGFR (Fig. 1*a*). Specific depletion of HER3 was accomplished by lentiviral-mediated transduction and expression of sequence-specific shRNAs. Of 5 shRNAs directed at distinct HER3 target sequences tested, 2 caused consistent and specific knock-down of HER3 by at least 75% of its control levels (Fig. 1*c*), that was maintained for at least 6 weeks for both DU-145 and MCF-7 cells (Fig. 1*b* and 1*c*).

Acute stimulation with NRG-1 potently induced the phosphorylation of HER2, HER3, Akt and ERK1/2 in both cell types, a response that was abolished in cells stably knocked down for HER3 (Fig. 1*d* and 1*e*), attesting to the effectiveness of the HER3 knock-down. While both control and HER3 knock-down DU-145 cells showed strong responses to acute stimulation with 10% FBS, measured as induction of phosphorylation of Akt and ERK1/2 (Fig. 1*d*), MCF-7 cells showed poor signaling in response to the same acute stimulus (Fig. 1*e*). These results suggest that the concentrations of growth factors present in 10% FBS are lower than a threshold necessary to induce the detectable activation of ERK1/2 or Akt in MCF-7 cells in acute stimulation experiments.

Depletion of HER3 compromises neuregulin-dependent and -independent motility of prostate cancer cells

Under standard culture conditions (10% FBS), stable depletion of HER3 caused a slower growth rate of DU-145, although with significant differences with control cells apparent only on relatively long-term cultures (over 5 days). HER3 knock-down caused MCF-7 cells to grow at significantly slower rates than control cells at all times, with obvious differences in growth rates between control and HER3 knock-down cells as early as 24 hr after plating

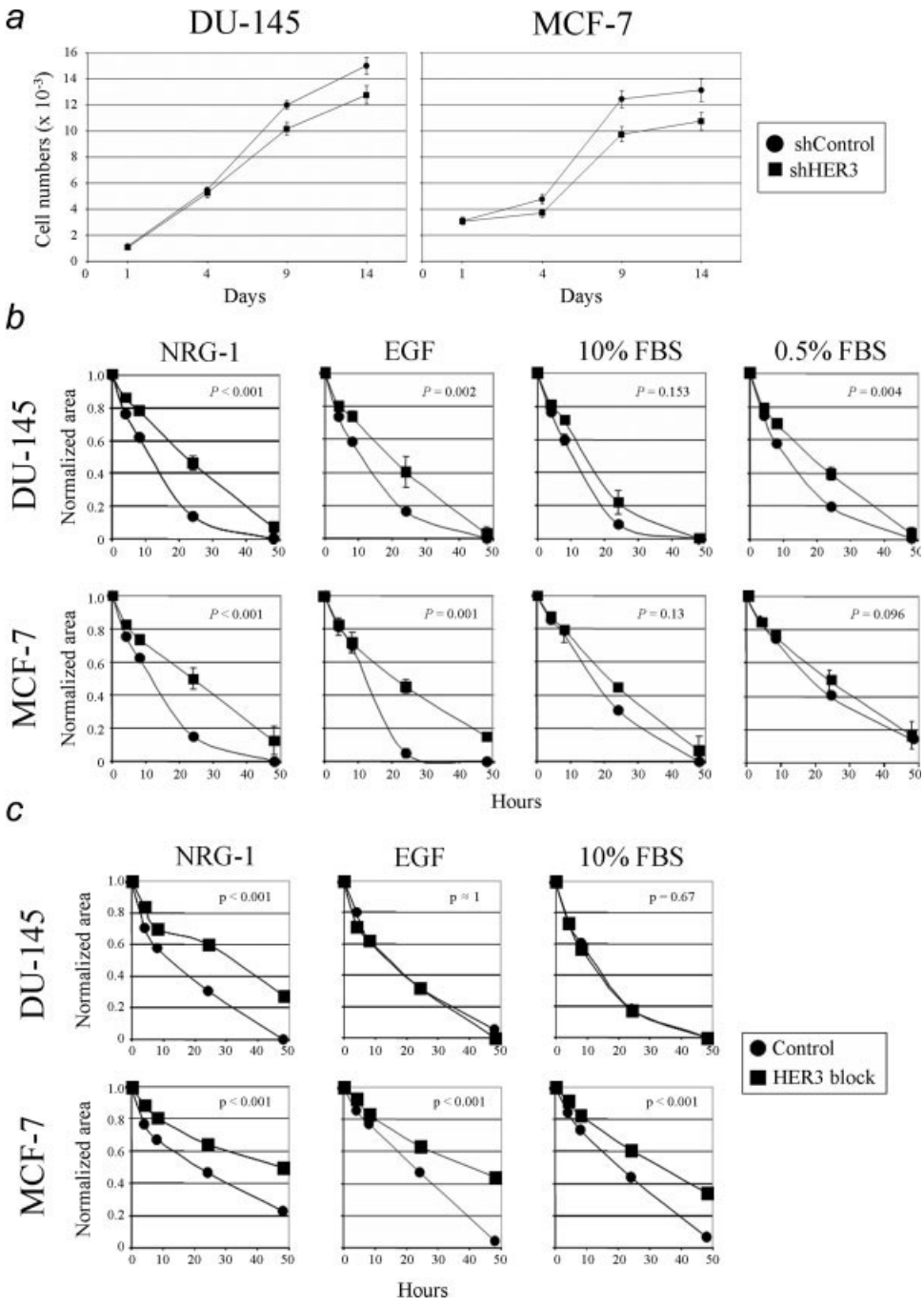

FIGURE 2 – HER3 depletion inhibits the growth and motiliy of prostate and breast cancer cells. (*a*) HER3 depletion inhibits the growth of DU-145 and MCF-7 cells. Cells with integrated lentiviruses for control or HER3-specific shRNA expression were monitored for growth by the CyQuant assay. Cell numbers for each time point were determined in quadruplicate. Error bars correspond to standard deviations for each mean value. (*b*) HER3 depletion inhibits the motility of DU-145 and MCF-7 cells in response to NRG-1, EGF and 10% FBS. Wound-healing migration assays were performed with control and HER3-knocked-down DU-145 prostate and MCF-7 breast cancer cells. Cell monolayers were treated with mitomycin C, wounded with a plastic tip and maintained for the indicated times in medium containing 0.5% FBS, NRG-1 (5 ng/mL), EGF (25 ng/mL) or 10% FBS. Surface areas between the leading edges on either side of the wounds measured, and values were normalized against the surface areas at time 0 for each growth condition. Statistical significance was determined for comparisons between values at 50% migration. (*c*) Autocrine mechanisms are relevant in the stimulation of motility by EGF and FBS of MCF-7 cells, but not of DU-145 cells. Cell monolayers were treated, or not, with a HER3 neutralizing monoclonal antibody (10 μg/mL) in wound-healing experiments, conducted as in (*b*).

(Fig. 2*a*). This suggests a stronger dependence on HER3 of MCF-7 cells as compared with DU-145 cells for growth under chronic stimulation by FBS.

It has been shown by others that HER3 is necessary for neuregulin-stimulated motility and invasiveness of breast[28] and lung[27] cancer cells. To address this issue in our cell models, wound-healing motility assays were performed in the presence of mitomycin C. It can be seen that stable HER3 knock-down significantly compromised the motility of both DU-145 and MCF-7 cells (Fig. 2*b*). This defect in motility was also observed in conditions of low

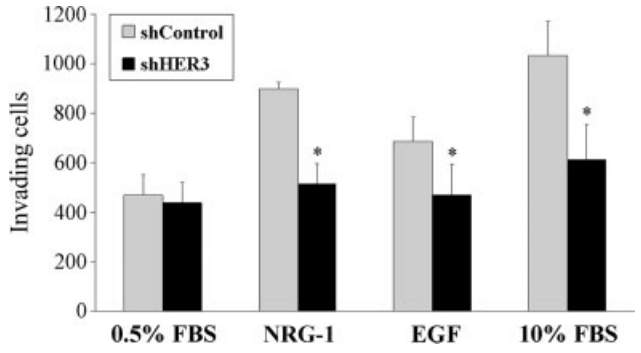

FIGURE 3 – HER3 depletion inhibits the invasiveness of DU-145 cells. Stable knock-down of DU-145 cells inhibits invasiveness in response to NRG-1 (25 ng/ml), EGF (100 ng/ml) or 10% FBS. In the presence of limited concentrations of growth factors (0.5% FBS), control and HER3 knock-down cells showed comparable levels of basal invasiveness. *$p < 0.001$ for $\chi 2$ determinations between control and HER3 knock-down cells. Invasiveness indices for NRG-1, EGF and 10% in control cells were all statistically significant ($p < 0.001$) relative to control cells in 0.5% FBS.

(0.5%) serum concentrations without added NRG-1, but not in the presence of 10% FBS (Fig. 2*b*).

To study if the dependence on HER3 of the motility of these cells could be explained by autocrine neuregulin stimulation, cells were treated in wound-healing assays with a neutralizing antibody that antagonizes the binding of ligand to the ligand-binding site on HER3.[25] This treatment inhibited the motility of both DU-145 and MCF-7 cells in response to NRG-1 (Fig. 2*c*), but not that of DU-145 cells in response to EGF or 10% FBS (Fig. 2*c*). In contrast, the motility of MCF-7 cells in response to EGF or 10% FBS was significantly inhibited by the anti-HER3 neutralizing antibody (Fig. 2*c*). This suggests that DU-145 cells do not respond to EGF or to 10% FBS with a stimulation of HER3 through the autocrine release of HER3 ligands. On the other hand, the inhibition by the HER3 neutralizing antibody of the motile response of MCF-7 cells to EGF or FBS suggests that, in these cells, the requirement for HER3 in these assays is, at least in part, due to an autocrine induction of HER3 ligands by these growth factors. Indeed, transcript quantitation of neuregulin-1, -2 and -3 showed that MCF-7 cells express NRG-3 (Fig. 1*c*). Therefore, the inhibition of the motility of MCF-7 cells as a consequence of HER3 knock-down may result from the combination of the loss of transactivation by EGFR and the block of a HER3 autocrine loop, both constitutive and induced by EGF or 10% FBS.

HER3 regulates neuregulin-dependent and -independent invasiveness of prostate epithelial cells

We next tested if HER3 is required for the invasive response of prostate epithelial cells to NRG-1 and other stimuli. Stable depletion of HER3 severely compromised the invasiveness of DU-145 cells in response to either NRG-1, EGF or 10% FBS, being reduced to basal levels in the absence of growth factors (Fig. 3).

In a reciprocal approach, we assessed the impact of increasing the levels of HER3 on neuregulin-dependent and -independent invasiveness. For these experiments, we used RWPE-2 and PC-3 prostate epithelial cells, because they express lower endogenous levels of HER3 than DU-145 or MCF-7 cells (Fig. 1*a*), and, therefore, we might expect a stronger differential effect upon overexpression of exogenous HER3. We also tested whether PC-3 cells, which have constitutively activated Akt,[46] are susceptible to further induction of this pathway under conditions of high levels of expression of HER3. As expected, Akt is constitutively phosphorylated in resting, unstimulated PC-3 cells. However, the levels of activated Akt were enhanced significantly upon transfection and overexpression of HER3-Flag (Fig. 4*a*). Transfection of the latter form of HER3 permitted more accurate immunoblotting normalization for transfection than transfection of the green fluorescent form used in invasiveness assays (see below). These results suggested to us that the Akt pathway was not activated to saturation in PC-3 cells,[46] and that we might expect significant functional consequences from the additional activation of this pathway resulting from the overexpression or activation of HER3 in these cells.

To assess their invasive potential, RWPE-2 or PC-3 cells were transfected with pHER3-GFP, seeded on Matrigel-coated porous membranes in the top chamber and challenged with growth factors present in the bottom chamber. As a control, cells were transfected with pEGFP, for the expression of GFP alone, and subjected to the same treatments. Internal controls for each experimental condition were provided by the populations of cells that were not detectably transfected with HER3-GFP or pEGFP. Therefore, in this approach, invasiveness is normalized both for transfection efficiency and for nonspecific effects potentially associated with the expression of green fluorescent protein.

In the presence of either NRG-1 or complete medium (containing EGF and bovine pituitary extracts for RWPE-2; 10% FBS for PC-3) as growth factors, expression of HER3-GFP significantly enhanced the invasiveness of both RWPE-2 and PC-3 cells (Fig. 4*b*). Expression of HER3-GFP also enhanced the invasiveness of RWPE-2 cells in response to EGF alone (Fig. 4*b*, left panel). Our population of PC-3 cells expresses relatively low levels of EGFR (Fig. 1*a*) and, consistently, the invasiveness of PC-3 cells expressing HER3-GFP was not enhanced significantly by EGF relative to control cells (Fig. 4*b*, right panel). This enhanced invasiveness was abolished completely by the metalloprotease inhibitor GM6001 (50 μM; Fig. 4*b*). Zymographic analysis of cell supernatants indicated that transfection and overexpression of HER3 activated gelatinases in PC-3 cells (Supporting Information Fig. 3), which suggests that overexpression of HER3 may be sufficient to activate metalloproteases in these cells, although autocrine mechanisms may be involved in these cells, as we have shown for MCF-7 cells (see above). The fact that NRG-1 further stimulated the invasiveness of HER-3 overexpressing cells suggests that other enzymatic activities may be additionally induced by NRG-1, not readily detected by gelatin-based zymography.

To determine the ligand and heterodimer specificity of the enhanced invasiveness of HER3-GFP-positive cells, EGFR or HER2 were specifically depleted with siRNAs (Fig. 1*b* and 4*c*). Depletion of HER2 abolished the invasive response to NRG-1 of HER3-GFP-expressing RWPE-2 cells, whereas maintaining an intact invasive response to EGF (Fig. 4*c*, left panel). Conversely, depletion of EGFR abolished the invasive response to EGF of HER3-GFP-expressing RWPE-2 cells, without affecting their response to NRG-1 (Fig. 4*c*, left panel). Depletion of HER2 in PC-3 cells also prevented its HER3-GFP-dependent invasive response to NRG-1 (Fig. 4*c*, right panel), and also to 10% FBS (Fig. 4*c*). Therefore, HER3-dependent invasiveness of prostate epithelial cells stimulated by either NRG-1 or FBS requires HER2, and that stimulated by EGF requires EGFR. These experiments also demonstrate the high degree of specificity of the knockdowns achieved by our RNAi tools for each of the three HERs under study.

The requirement for functional heterodimers between HER3 and HER2 or EGFR in this ligand-specific invasive response was further substantiated by the use of HER-selective inhibitors. Incubation of RWPE-2 or PC-3 cells with 2C4 (20 μg/mL), a monoclonal antibody that prevents heterodimerization of HER2,[32] completely abolished the invasive response of HER3-GFP-positive cells to NRG-1, while maintaining intact their response to EGF (Fig. 4*d*). Incubation of RWPE-2 cells with the EGFR-specific kinase inhibitor AG1478[47] inhibited the enhanced invasiveness of HER3-GFP-positive cells to EGF, but not to NRG-1 (Fig. 4*d*, left panel). These results also indicate that, for the experimental

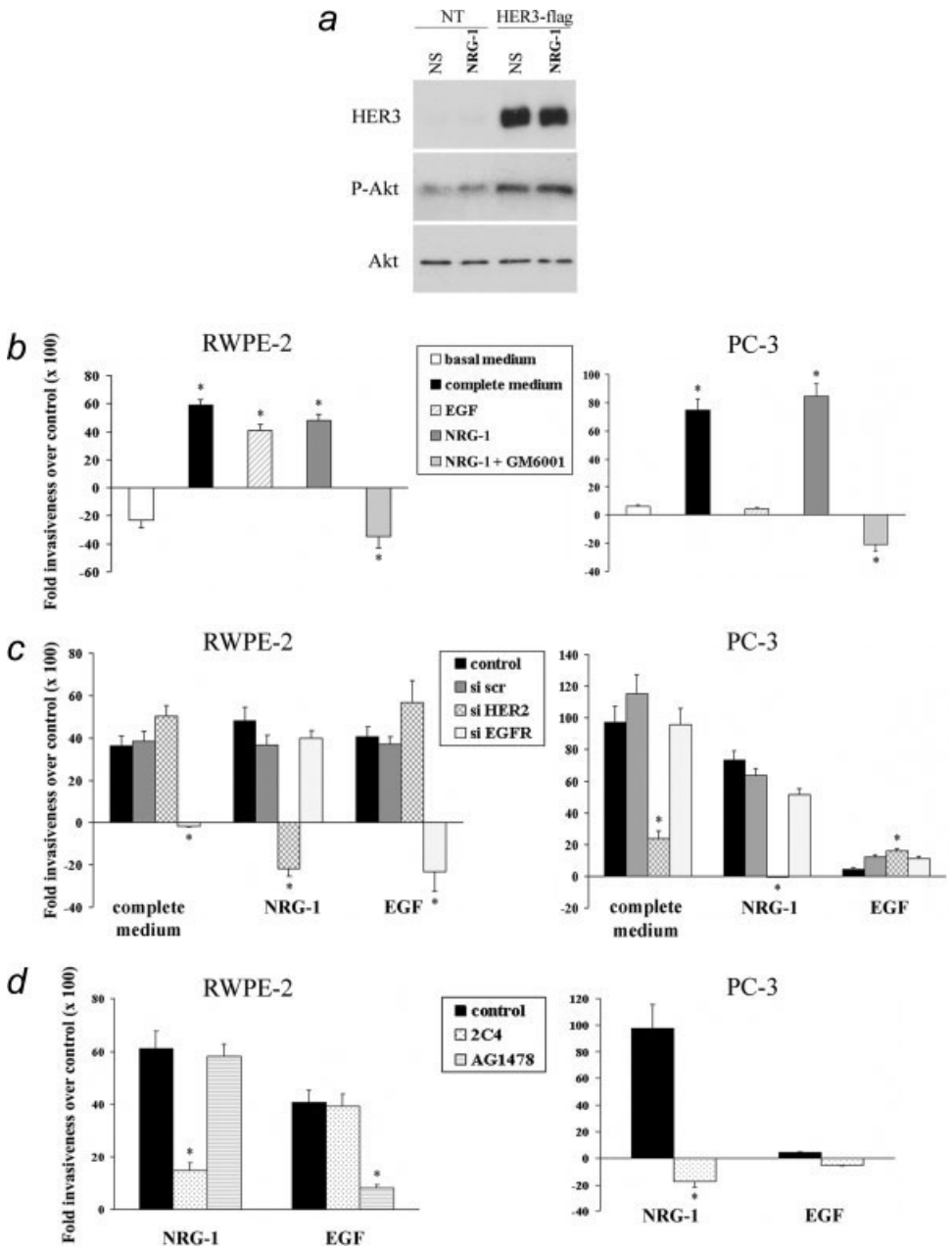

Figure 4 – Expression of exogenous HER3 enhances ligand-induced invasiveness of prostate epithelial cells. (*a*) PC-3 cells have constitutively active Akt that is further activated by transfection with HER3. Extracts from untransfected (NT) or HER3-Flag-transfected PC-3 cells were analyzed by Western blotting with antibodies to phospho-Akt (Ser437), total Akt, or HER3. (*b*), HER3-dependent invasiveness of RWPE-2 cells is promoted by NRG-1 and EGF, and that of PC-3 cells by NRG-1. Cells transfected with HER3-GFP or GFP (control) were challenged either with basal medium (without growth factors); complete medium, containing EGF and bovine pituitary extract (RWPE-2 cells, left panel) or 10% FBS (PC-3 cells, right panel); EGF (25 ng/ml); NRG-1 (5 ng/ml). The broad-range metalloprotease inhibitor GM6001 (50 μM) abolished the HER3-dependent invasiveness promoted by NRG-1. (*c*) Effects on the invasiveness of RWPE-2 cells of the selective depletion of HER2 and EGFR by RNAi. The HER3-dependent invasiveness of RWPE-2 and PC-3 cells promoted by NRG-1 requires HER2, and that of RWPE-2 cells promoted by EGF requires EGFR. RPWE-2 cells (left panel) depleted of HER2 or EGFR and PC-3 cells (right panel) depleted of HER2 were transfected with HER3-GFP and the HER3-dependent invasiveness assessed when challenged with complete medium, NRG-1 or EGF. (*d*) HER3-dependent invasiveness in response to NRG-1 requires dimerization with HER2, and that promoted by EGF requires a catalytically active EGFR. HER3-GFP-transfected RWPE-2 (left panel) and PC-3 (right panel) cells were pretreated for 30 min with either the HER2 heterodimerization blocking antibody 2C4 (20 μg/mL) or the EGFR kinase inhibitor AG1478 (2.5 μM), and assayed for HER3-dependent invasiveness in response to NRG-1 or EGF. The two inhibitors were present in the upper and lower Transwell chambers throughout the assay.

conditions and assays used here, AG1478 and 2C4 specifically target EGFR and HER2, respectively, without evidence of significant cross-target effects.

Whether HER2 or EGFR partner with HER3 in the invasive function of the latter should depend on the available ligand. Thus, in RWPE-2 cells EGF induced the phosphorylation of both EGFR and HER3, but not HER2 (Supporting Information Fig. 4*a*), whereas NRG-1 induced a rapid phosphorylation of HER2 and HER3, but not EGFR (Supporting Information Fig. 4). This is consistent with the fact that both NRG-1 and EGF enhance the HER3-dependent invasive response of cells that also express HER2 and EGFR.

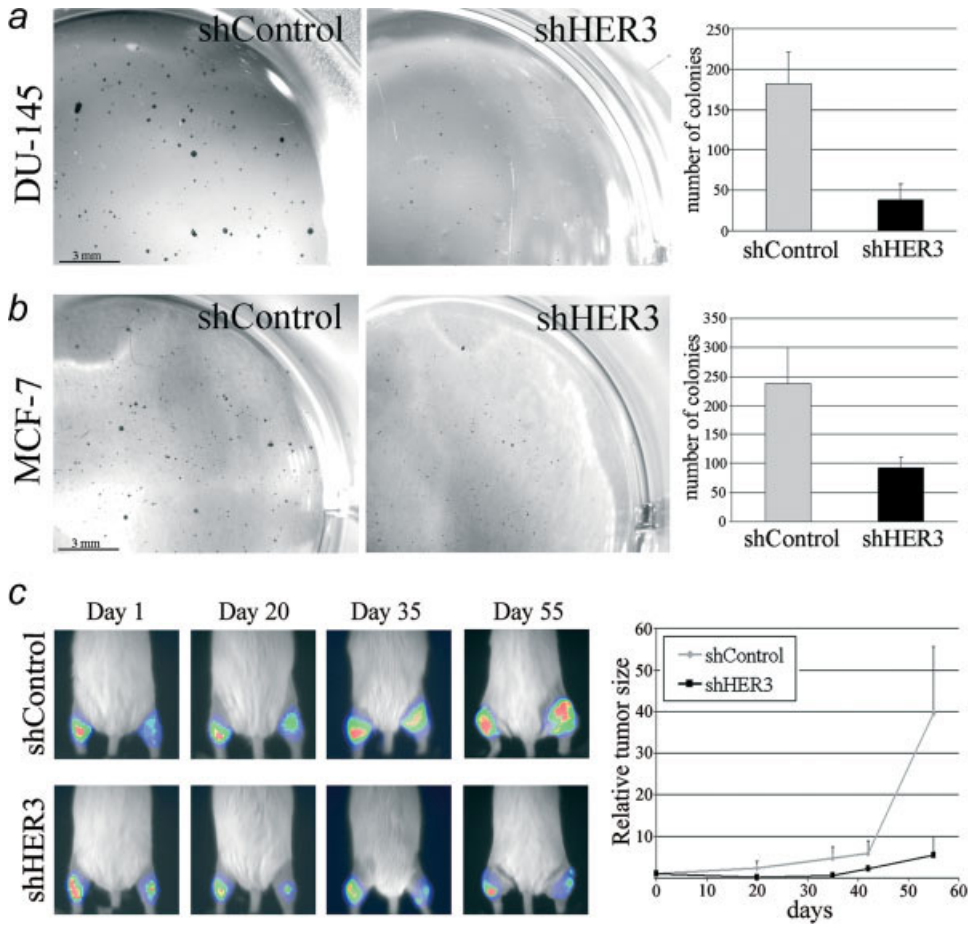

FIGURE 5 – HER3 depletion inhibits the *in vitro* clonogenicity of DU-145 and MCF-7 cells, and the *in vivo* tumorigenicity of DU-145 cells. knock-down of HER3 in DU-145 (*a*) or MCF-7 cells (*b*) strongly inhibits their capacity for colony formation in soft agar. The graphs represent mean values from 3 independent experiments. Error bars represent standard deviation. (*c*) The growth in SCID mice of HER3 knock-down DU-145 cells is severely impaired compared with control cells. Cells constitutively expressing firefly luciferase were implanted by intramuscular injection in SCID mice, and their growth monitored by luminescence quantitation at the indicated time points. Values in each point are averages of normalized photon counts from 6 tumors from 3 mice. Error bars represent standard deviation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Depletion of HER3 inhibits the clonogenic and tumorigenic capacities of prostate cancer cells

Studies by others have shown that signaling through HER2 sustains self-renewal of breast cancer and embryonic stem cells.[48] We thus studied if HER3 is similarly required for self-renewal and colony formation in soft agar. Depletion of HER3 abolished the clonogenic potential of both DU-145 and MCF-7 cells under chronic exposure to 10% FBS (Fig. 5*a* and 5*b*). These *in vitro* results were paralleled by *in vivo* growth experiments, showing that stable depletion of HER3 caused a significant inhibition of tumorigenesis of DU-145 cells in immunodeficient mice (Fig. 5*c*). The control and HER3 knockdwown DU-145 cells used for the mouse xenograft experiments expressed the firefly luciferase gene and displayed *in vitro* phenotypes comparable to their parental cells that do not express luciferase (Supporting Informaton Fig. 5). We conclude that expression of HER3 in DU-145 prostate cancer cells is necessary for their self-renewal and tumorigenicity.

Depletion of HER3 compromises transcriptional programs in prostate and breast cancer cells under standard growth conditions

The above observations suggested a requirement for HER3 for the growth, motility and invasiveness of DU-145 prostate cancer cells that may not be based on autocrine release of HER3 ligands. They also suggested that in MCF-7 breast cancer cells, autocrine mechanisms may play a significant role in the dependence of these cells on HER3 for the expression of similar phenotypes.

Transcriptional profiling showed that stable HER3 depletion caused a profound change in the transcriptional programs of either DU-145 or MCF-7 cells, under standard growth conditions. In DU-145 cells, HER3 depletion caused a statistically significant decrease in the expression levels of 1,110 genes, and a significant increase in 5,193 genes (Supporting Information Table II). In MCF-7 cells, HER3 depletion caused a significant decrease in the levels of 1,280 genes and an increase in 230 genes (Supporting Information Table III). There was a limited overlap between the two cell types in terms of HER3-dependent changes in gene expression, suggesting that the HER3-dependent, probably a reflection of cell-type specific effects of HER3 silencing, and the possible autocrine stimulation of MCF-7 of the residual HER3 molecules that the cells may continue to express after RNAi knock-down. Nevertheless, a common set of genes showed significant HER3-dependent changes in expression in both cell types, including the downregulation in HER3-depleted cells of the genes for the transcription regulators SOX9, JUND and CNOT6, the membrane receptors EPHA4 and NOTCH2, the cell cycle regulators CDC27 and RCBTB1, or the growth factors HDGFRP3 and INHBA (Fig. 6*a*). Conversely, HER3 depletion caused in both DU-145 and MCF-7 cells the upregulation of genes for tumor suppressor proteins such as TXNIP, SASH1 and FAS, but also the antiapoptotic protein BCL2 (Fig. 6*b*). The transcript levels for sev-

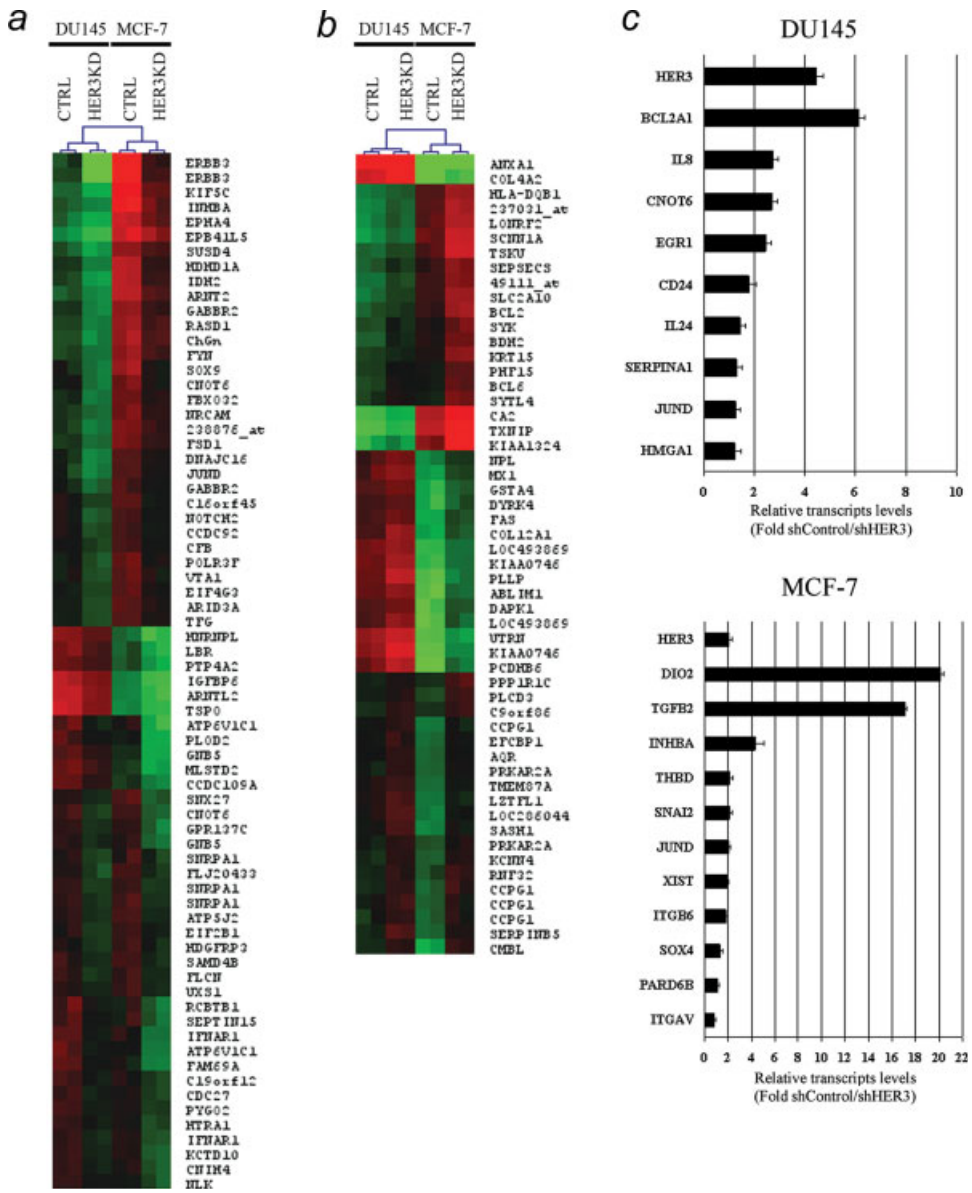

FIGURE 6 – A neuregulin-independent, HER3-dependent transcriptional program in prostate and breast cancer cells. Micorarray transcriptional profiling of control and HER3 knock-down DU-145 and MCF-7 cells, grown in standard growth conditions. (*a*) Genes that are most significantly downregulated by stable HER3 knock-down in both DU-145 and MCF-7 cells knocked down for HER3. (*b*) Genes that are most significantly upregulated by stable HER3 knock-down in both DU-145 and MCF-7 cells. Two independent experimental samples were used for each microarray analysis. After filtering and normalization (Material and Methods), genes with statistically significant differences in expression levels between sample groups were selected by SAM (FDR q value ≤ 0.10), and samples and genes were grouped by means of hierarchical clustering. For the purpose of graphical representation, genes were ordered according to their FDR q values for control *versus* shHER3 comparisons for each cell type. (*c*) Real-time RT-PCR transcript quantitation for several of the genes with the most significant differential expression by microarray analysis between control and HER3-knock-down cells. Each transcript was quantitated in triplicate, and values expressed as fold levels of HER3-knock-down *versus* control cells. Error bars indicate standard deviations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

eral of these genes were quantitated by real-time RT-PCR, confirming their differential expression in DU145 or MCF-7 cells under conditions of suppression of HER3 expression (Fig. 6*c*). This common set of genes modulated by HER3 depletion in both DU-145 cells and MCF-7 cells might reflect a HER3-dependent and neuregulin-independent transcriptional program common to both cell types.

Discussion

The HER3-HER2 dimer is a potent mediator of ligand-induced growth,[1,49] and mediates neuregulin-dependent invasiveness in cells that express high levels of HER2.[27,28] The dependence on HER3 for these functions has been adscribed to its role as the neuregulin-binding subunit within the heterodimer. Our observations suggest that HER3 may exert functions in prostate cancer cells that may not require neuregulin-dependent signaling. Our study was prompted by the observation that primary prostate cancer cells frequently overexpress HER3, and only infrequently EGFR or HER3, suggesting that the excess of HER3 molecules in these tumor cells may function in partnerships with either of the 2 other HER family members.

We have found that HER3 overexpression confers an increased invasive capacity in response to either NRG-1 or EGF, and that

HER3 is required for the invasive response of prostate epithelial cells to these 2 ligands. The invasive functions of HER3 required its association with, and transactivation by, either HER2 or EGFR, which occurred in response to NRG-1 or EGF, respectively. Therefore, transactivation of HER3 by its receptor kinase partners is a critical requirement for an invasive behavior of prostate epithelial cells in response to different HER ligands, and possibly also to unrelated stimuli. It has been shown by others that the activation of HER3 by signals that are not directly related to the HER ligand-receptor system plays critical roles in the aberrant activation of the PI3K pathway.[22]

Although depletion of HER3 abolished signaling induced by acute stimulation with NRG-1 in both DU-145 and MCF-7 cells, it did not affect the induction of ERK1/2 or activation of Akt by FBS in DU-145 cells. This suggests that the inhibitory effect of HER3 depletion on the motility, clonogenicity and tumorigenicity of these cells is not due to a general compromise in signaling. On the other hand, the lack of detectable activation of HER3 in response to acute treatment with FBS in MCF-7 cells, together with a strong effect on the growth of these cells as a consequence of HER3 knock-down and a block in migration by a HER3 neutralizing antibody in response to FBS suggests that, in these cells, autocrine stimulation with neuregulins is a relevant factor that explains the HER3 dependence of these cells for FBS-induced motility and growth.

Recent studies have shown that HER2 and neuregulin contribute to the maintenance of self-renewal and pluripotency of embryonic stem cells,[48] and that HER3 is instrumental for the clonogenic and tumorigenic properties to several types of cancer cells.[50,51] We have also found that the *in vitro* motility and clonogenicity of DU-145 and MCF-7 cells were severely compromised after HER3 knock-down. In the case of DU-145 cells, the stimulation of their motility by EGF was not inhibited by treatment with a HER3 neutralizing antibody, which further supports the hypothesis that the motile response of these cells to EGF is mainly mediated by receptor transactivation, and not by the induction of autocrine signaling through HER3 in response to other growth factors. Collectively, our observations with DU-145 prostate cancer cells suggest that HER3 expression, likely in partnership with EGFR, is sufficient to enable the expression of the genes and biochemical networks that maintain the motile, invasive and self-renewal properties of prostate cancer cells, even in the absence of cognate ligand stimulation. In contrast, an autocrine release of HER3 ligands may explain at least part of the dependence of MCF-7 breast cancer cells on HER3 for their growth, motile and invasive phenotypes.

These observations lend further support to the consideration of HER3 as a significant therapeutic target in tumors in which this receptor is expressed at high levels, including tumors in which HER2 or EGFR are not concomitantly overexpressed. HER3 could be targeted either singly, or as an adjunct to therapies aimed at other HERs. For example, the development of certain forms of resistance to therapies aimed at inhibiting HER2 or EGFR has been associated with the concomitant inappropriate activation of HER3.[22,24] To overcome these resistances, anti-HER2 and anti-EGFR therapies could be complemented with therapeutic approaches aimed at more than one HER,[17] directed specifically at HER3,[25] or at downstream regulators. That HER3 knock-down causes sensitization to radiotherapy[30] or to antiestrogens[52] also provides grounds for additional therapeutic combinations.

Acknowledgements

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