

## Tumor-Initiating Cells of HER2-Positive Carcinoma Cell Lines Express the Highest Oncoprotein Levels and Are Sensitive to Trastuzumab

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**Abstract Purpose:** The existence of tumor-initiating cells in breast cancer has profound implications for cancer therapy. In this study, we investigated the sensitivity of tumor-initiating cells isolated from human epidermal growth factor receptor type 2 (HER2)-overexpressing carcinoma cell lines to trastuzumab, a compound used for the targeted therapy of breast cancer.

**Experimental Design:** Spheres were analyzed by indirect immunofluorescence for HER2 cell surface expression and by real-time PCR for HER2 mRNA expression in the presence or absence of the Notch1 signaling inhibitor (GSI) or Notch1 small interfering RNA. Xenografts of HER2-overexpressing breast tumor cells were treated with trastuzumab or doxorubicin. The sphere-forming efficiency (SFE) and serial transplantability of tumors were assessed.

**Results:** In HER2-overexpressing carcinoma cell lines, cells with tumor-initiating cell properties presented increased HER2 levels compared with the bulk cell population without modification in HER2 gene amplification. HER2 levels were controlled by Notch1 signaling, as shown by the reduction of HER2 cell surface expression and lower SFE following  $\gamma$ -secretase inhibition or Notch1 specific silencing. We also show that trastuzumab was able to effectively target tumor-initiating cells of HER2-positive carcinoma cell lines, as indicated by the significant decrease in SFE and the loss of serial transplantability, following treatment of HER2-overexpressing xenotransplants.

**Conclusions:** Here, we provide evidence for the therapeutic efficacy of trastuzumab in debulking and in targeting tumor-initiating cells of HER2-overexpressing tumors. We also propose that Notch signaling regulates HER2 expression, thereby representing a critical survival pathway of tumor-initiating cells.

Increasing evidence indicates that a small subpopulation of tumor cells, called tumor-initiating cells, is not only responsible for the generation of the phenotypically diverse tumor cell populations, but also capable of long-term self-renewal, therefore supporting the growth and dissemination of cancers (1). Recent studies suggest that tumor-initiating cells might be particularly resilient to therapeutic approaches. For instance, breast tumor tumor-initiating cells seem particularly radio-resistant due to more efficient DNA repair machinery (2), whereas chemotherapy has been reported to select for survival

of tumor-initiating cells in breast carcinomas (3–5). Thus, it has become important to define the sensitivity of this critical subpopulation to current therapies.

About 20% of invasive breast carcinomas show overexpression of human epidermal growth factor receptor type 2 (HER2), and patients with HER2-positive tumors have a decreased overall survival rate (6–8). Recently, a humanized monoclonal antibody (mAb) against the extracellular domain of HER2, trastuzumab, has been approved by the Food and Drug Administration for treatment of patients with invasive HER2-overexpressing breast cancers (9). In this study, we characterized the tumor-initiating cells of HER2-positive carcinoma cell lines and explored the possible mechanism by which the tumor initiating cells of HER2-positive carcinoma cell lines present elevated HER2 expression compared with the bulk cell population. Furthermore, we investigated the sensitivity of tumor-initiating cells derived from HER2-positive carcinoma cell lines to trastuzumab by analyzing the spheres-forming efficiency (SFE) and transplantability of cells derived from tumor xenotransplants treated with this therapeutic agent.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Materials and Methods

**Antibodies and reagents.** The following antibodies were used for Western blotting analysis: anti-HER2 mAb Ab3 (1  $\mu$ g/mL; Calbiochem); anti-phospho-tyrosine clone 4G10 mAb (1  $\mu$ g/mL) and anti-phospho-EGFR (Tyr 1086) polyclonal antibody (0.1  $\mu$ g/mL; Upstate Biotechnology); anti-EGFR 1005 polyclonal antibody (1  $\mu$ g/mL),

## Translational Relevance

Evidence for the existence of tumor-initiating cells in breast cancer points to the likelihood that eradication of these cells is the critical determinant in achieving cure. Thus, changes in therapeutic modalities used in treating human epidermal growth factor receptor type 2 (HER2)-overexpressing tumors have highlighted the need to identify tumor-initiating cells and characterize their sensitivity to such a targeted therapy. In our study of HER2-overexpressing carcinoma cell lines, we provide evidence, for the first time, that trastuzumab can effectively target tumor-initiating cells. In light of the fact that the efficacy of trastuzumab depends on HER2 levels, we show that the results obtained are explained by the higher HER2 expression levels in tumor-initiating cells of HER2-positive tumors compared with the totality of cells forming the tumor. From a clinical perspective, we believe that our study provides a rationale of why and how current trastuzumab therapy exerts its action on HER2-positive breast carcinoma.

anti-HER3 (1  $\mu\text{g/mL}$ ), anti-Bmi1 (H-99) polyclonal antibody (1  $\mu\text{g/mL}$ ), and anti-Oct4 (C-10) mAb (1  $\mu\text{g/mL}$ ; Santa Cruz Biotechnology); anti-phospho-HER3 (Tyr 1289; 0.1  $\mu\text{g/mL}$ ), anti-phospho-p44/42 MAPK (Thr202/Tyr204) polyclonal antibody (0.1  $\mu\text{g/mL}$ ), anti-p44/42 MAPK polyclonal antibody (0.1  $\mu\text{g/mL}$ ), anti-phospho-AKT (Ser473) polyclonal antibody (2  $\mu\text{g/mL}$ ), and anti-AKT polyclonal antibody (0.1  $\mu\text{g/mL}$ ; Cell Signaling); anti-actin clone AC-40 mAb (0.4  $\mu\text{g/mL}$ ; Sigma); anti-Hes-1 polyclonal antibody (1  $\mu\text{g/mL}$ , Chemicon); and anti-human Notch1 intracellular domain polyclonal antibody (4  $\mu\text{g/mL}$ , Rockland).  $\gamma$ -Secretase inhibitor I (GSI) was purchased from Calbiochem, doxorubicin from Pharmacia (Upsala), and trastuzumab from Roche (Basilea). Lapatinib was kindly provided by Dr. Campiglio M. (IRCCS Foundation: National Cancer Institute of Milan);  $17\beta$ -estradiol was from Jenapharm (Jena), and matrigel from BD Bioscience.

**Cell culture.** All parental cell lines were obtained from the American Type Culture Collection and grown according to guidelines.

**Sphere culture.** Spheres were generated from single cells of lines MDA-MB-361, BT-474, ZR-75-1, MCF7, and SKOV-3 seeded at  $10^3$  cells/cm<sup>2</sup> in low-attachment plates (Corning Inc.). Sphere medium has been described (10). To exclude those spheres derived from cellular aggregates instead of division from a single cell, tumor cell lines were stained with PKH26 (red dye; ref. 11) or PKH67 (green dye; ref. 12) before seeding in nondifferentiating culture conditions. After 1 wk, individual spheres homogeneously stained with a single dye were considered clonally derived. Sphere-forming efficiency (SFE) was calculated as the number of spheres (diameter = 60  $\mu\text{m}$ ) formed in 7 d divided by the original number of single cells seeded and expressed as a percentage ( $\pm$  SD; ref. 13). Differentiated cells were obtained from spheres seeded on matrigel for 14 d (14, 15) and tested for their tumorigenic capacity compared with spheres ( $10^6$  versus  $10^3$ ).

**In vivo tumor growth.** Six-week-old nude mice were injected in the mammary fat pad with  $10^6$  MDA-MB-361, MCF7, or ZR-75-1 cells or s.c. with  $10^6$  SKOV-3 cells diluted 1:1 in matrigel:medium (6 mice for group). Tumor growth was evaluated in the presence or absence of 10 mg/kg doxorubicin, administered i.p. at 72 h following tumor cell injection, or 4 mg/kg trastuzumab, administered i.p. twice weekly for 5 wk starting at 72 h following tumor cell injection. Tumors were calibrated twice weekly for 34 d and tumor volume was calculated as  $0.5 \times d_1^2 \times d_2$ , where  $d_1$  and  $d_2$  are the larger and smaller diameters, respectively.

Mice inoculated with estrogen-sensitive MCF7 cells or ZR-75-1 cells were treated i.m. with estradiol (0.12 mg/kg) weekly, starting 7 d before

tumor cell injection. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the National Cancer Institute, Foundation IRCCS. Tumors were enzymatically dissociated with 250 U collagenase IV/mg of tumor (Worthington Biochemical Corp), and single-cell suspensions were analyzed *in vivo* for multiple transplantability at different tumor cell dilutions ( $10^3$ - $10^5$  primary cells) and *in vitro* for SFE.

Before every tumor transplant, the percentage of human leukocyte antigen- or HER2-positive cells was evaluated by flow cytometry. Human cells in all tumors analyzed comprised 50% to 60% of total cells.

**Flow cytometry.** Indirect immunofluorescence assay was done on live cells as described (16), using anti-HER2 mAb MGR2, directed against the extracellular domain of HER2 (17). MDA-MB-361, or SKOV-3 cells ( $60 \times 10^6$ ) homogeneously stained with anti-HER2 mAb were sorted into two populations according to low or high HER2 expression by FACS Vantage SE (Becton Dickinson) and analyzed for HER2 expression by immunofluorescence, for tumor-initiating cell markers (by Western blotting), and for *in vitro* SFE.

Aldefluor assay (Stemcell Technologies), used to measure and isolate cells with high aldehyde dehydrogenase (ALDH) activity, was done according to the manufacturer's guidelines. Dissociated single cells were suspended in Aldefluor assay buffer containing the ALDH substrate, bodipy-aminoacetaldehyde, at 1.5  $\mu\text{mol/L}$  and incubated for 40 min at 37°C. To distinguish between ALDH-positive and ALDH-negative cells, a fraction of cells was incubated under identical condition in the presence of a 10-fold molar excess of the ALDH inhibitor, diethylaminobenzaldehyde.

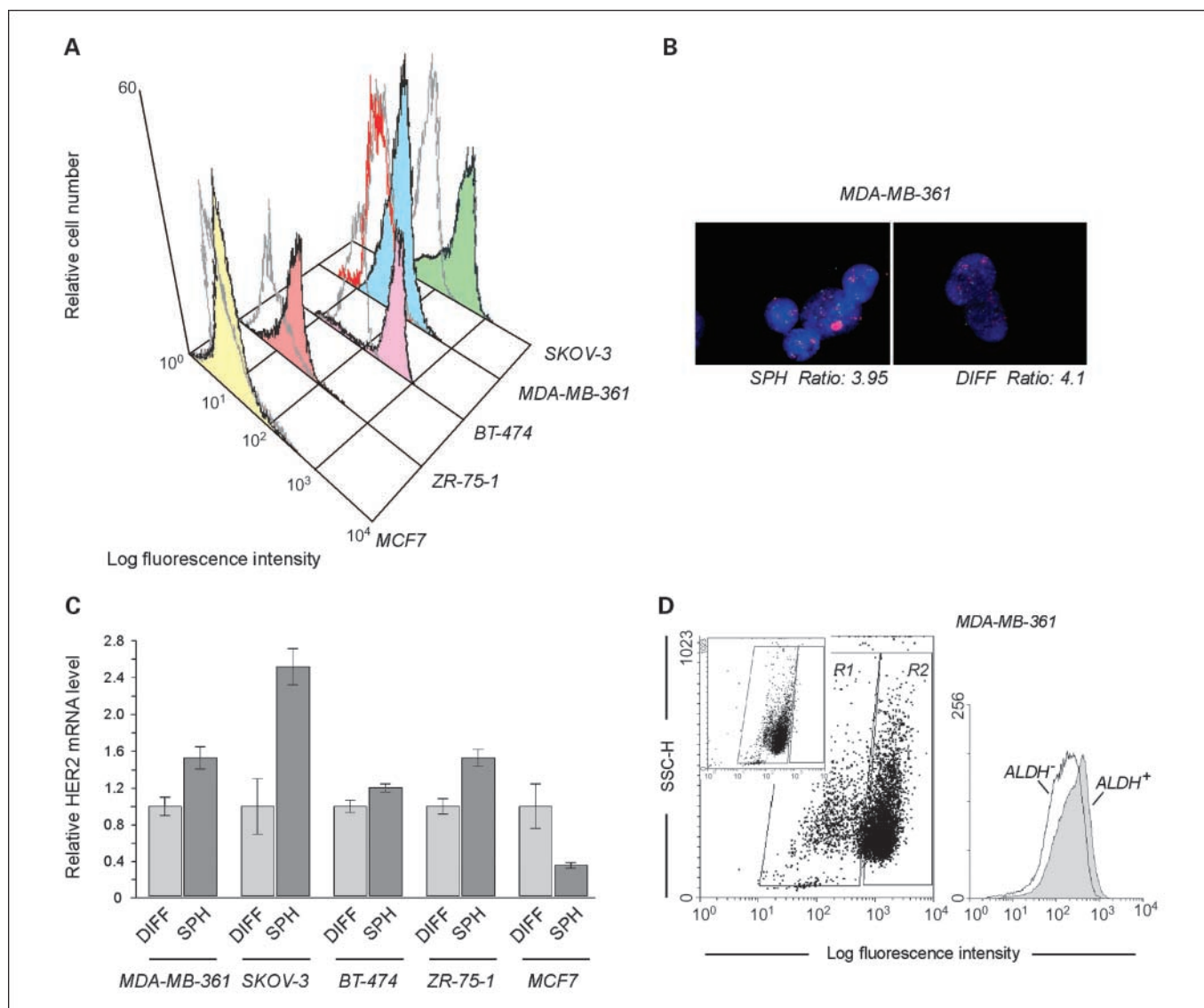
**HER2 fluorescence in situ hybridization.** For all cell lines described, amplification of HER2/neu was detected using the PathVysion HER2 DNA probe kit (Vysis, Inc.). Slides from cell lines were prepared using standard cytogenetic methodologies. Slides were pretreated with  $2 \times \text{SSC}/0$ , 5% NP40 at 37°C for 30 min and codenaturation occurred at 70°C for 2 min and 37°C overnight using Hybrite (Vysis). The fluorescence *in situ* hybridization signals were acquired with an Olympus BX51 microscope coupled to a charge-coupled device camera COHU 4912 (Olympus). The images were analyzed using the Mac Probe software (PowerGene Olympus). The interpretations of signals were done following Vysis manufacturer's protocols.

**Proliferation assay.** Proliferation of residual cells from untreated, doxorubicin-, or trastuzumab-treated MDA-MB-361, SKOV-3, MCF7, and ZR-75-1 was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described (18). Growth index was calculated as optical density of the sample after 3 d of culture divided by the optical density of cells at  $T_0$ .

**RNA extraction and real-time PCR.** RNA was extracted using the Total Quick RNA Cells and Tissue Kit (Talent) according to the manufacturer's protocol. First-strand cDNA was synthesized using the Superscript II RT Kit (Invitrogen) according to the manufacturer's instructions. Amplification primers and probe used for wild-type HER2 have been described (19). The GAPDH gene (endogenous control) was amplified using the Taqman gene expression assay (Applied Biosystems). Data from triplicate samples were analyzed with SDS 2.2 software (Applied Biosystems) and relative HER2 mRNA expression levels were calculated with the  $\Delta\Delta\text{CT}$  method using differentiated cells,  $\gamma$ -secretase inhibitor I (GSI) untreated cells, or cells silenced by a pool of control RNA duplexes as calibrator.

**Biochemical analysis.** Cells were solubilized in lysis buffer as described (16). Whole-cell lysates were resolved in 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Autoradiographic signals of immunoreactive proteins detected by enhanced chemiluminescence (Amersham Pharmacia Biotech) were measured using a Bio-Rad scanning densitometer (Bio-Rad, ChemiDoc/XRS; Bio-Rad). Data were acquired and analyzed using Quantity One version 4.6.1 software.

**GSI treatment.** Cells seeded to grow as spheres ( $10^3$  cells/cm<sup>2</sup>) were treated with 0.1 to 1  $\mu\text{mol/L}$  GSI, a Notch1 antagonist, or an equivalent



**Fig. 1.** HER2 levels in tumor-initiating cells of carcinoma cell lines. **A**, representative fluorescence-activated cell sorting analysis of MCF7, ZR-75-1, BT-474, MDA-MB-361, and SKOV-3 spheres (yellow, red, violet, blue and green areas, respectively), and spheres grown on differentiating conditions (i.e. matrigel; light grey line) or parental cells (red line) by using anti-HER2 antibody. **B**, representative fluorescence *in situ* hybridization analysis of HER2 gene amplification in MDA-MB-361. Ratio between HER2 gene copies (red signals) and chromosome 17 centromeres (green signals) is indicated for spheres (SPH) and spheres cultured in differentiating conditions (DIFF). **C**, evaluation of HER2 mRNA in MDA-MB-361, SKOV-3, BT-474, ZR-75-1, and MCF7 spheres (grey bar) and spheres grown under differentiating condition (light grey bar) by real-time-PCR analysis. Values represent expression of the *HER2* gene normalized to *GAPDH* and relative to *HER2* gene expression levels in differentiated cells used as calibrators. Bars indicate the 95% RQ confidence interval of gene expression in each column. **D**, representative fluorescence-activated cell sorting analysis of MDA-MB-361 cells tested by ALDEFLUOR assay. In the boxed area, cells incubated with ALDH substrate (BAAA) and the specific inhibitor of ALDH, diethylaminobenzaldehyde, were used to establish the baseline fluorescence of these cells (R1) and to define the ALDEFLUOR-positive region (R2). Right side, representative fluorescence-activated cell sorting analysis of HER2 expression of ALDH-positive or ALDH-negative cells.

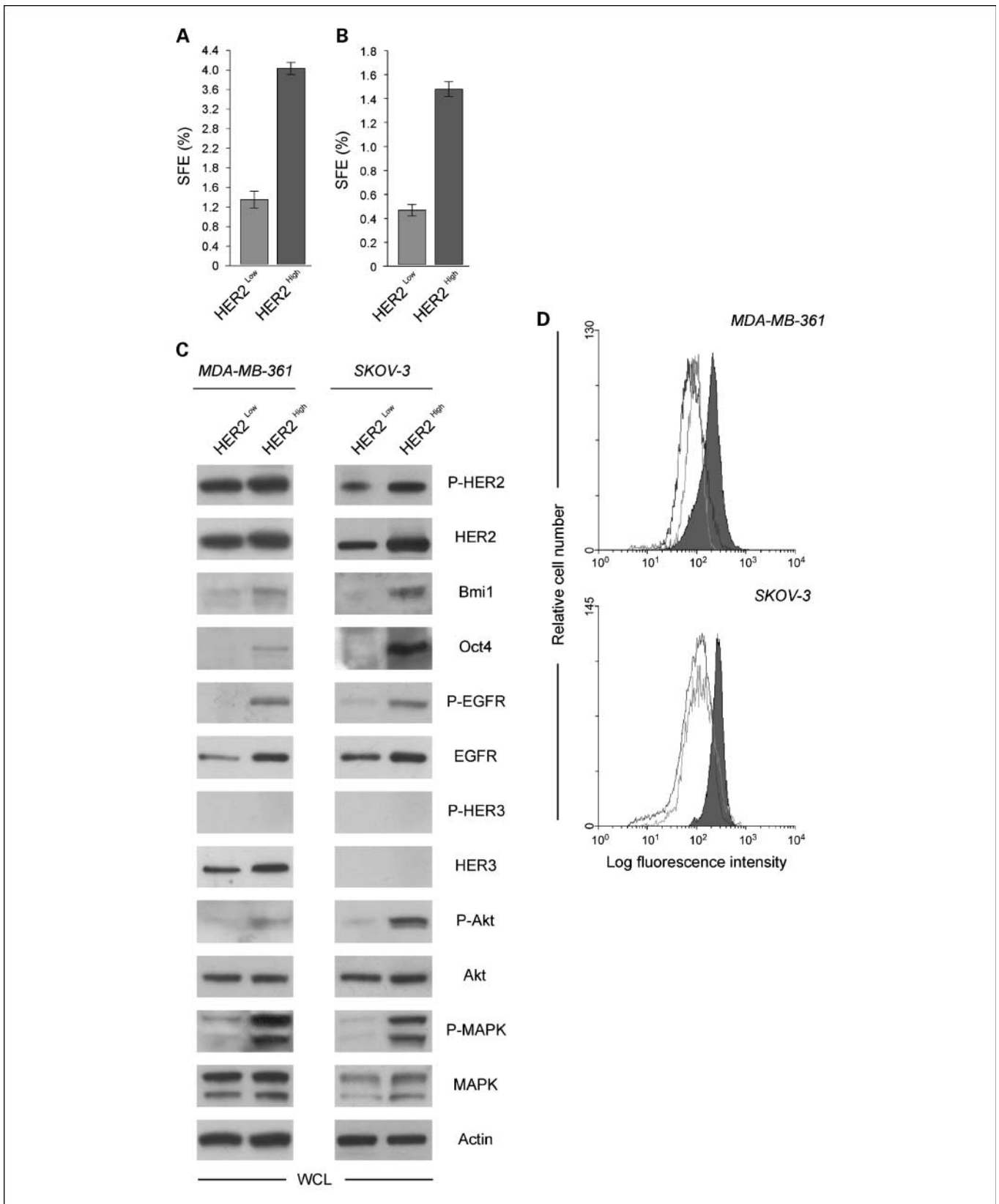
amount of DMSO for 6 h at 37°C. After washing with sphere medium, cells were plated in nondifferentiating conditions and analyzed for SFE 7 d later. Cells were analyzed for HER2 expression by indirect immunofluorescence, for HER2 mRNA expression by RT-PCR, and for Notch1 intracellular domain and Hes1 target gene expression by Western blotting.

**Silencing of Notch1 by siRNA transfection.** Cells seeded in 6-well low-attachment plates (10<sup>3</sup> cells/cm<sup>2</sup>) were transfected with either a small interfering RNA pool for human Notch1 (ON-TARGETplus SMARTpool, Human NOTCH1, final concentration 10-30 nmol/L; Dharmacon) or a pool of control RNA duplexes (On-TARGETplus Non-Targeting Pool, final concentration 10-30 nmol/L; Dharmacon) using 16  $\mu$ l/well of INTERFERin transfection reagent (Polyplus transfection) into 1 mL of

spheres medium. After overnight incubation at 37°C, spheres culture medium was added to a final volume of 2 mL/well. SFE were determined at 5 d posttransfection and spheres were tested for HER2 and Notch1 mRNA levels by real-time PCR. Notch1 and HER2 expression levels were determined by real-time PCR as above described using spheres silenced by a pool of control RNA duplexes as calibrator samples.

**In vitro drug treatment.** MDA-MB-361, BT-474, SKOV-3, ZR-75-1, and MCF7 cells treated or not in suspension with 25 to 50 to 100  $\mu$ g/mL of trastuzumab or with 1 to 10 nmol/L of lapatinib were analyzed for both SFE efficiency and adhesion growth ability using MTT assay after 6 d of culture.

**Statistical analysis.** Data were compared using the Student's *t*-test. Values were expressed as mean  $\pm$  SD; differences were considered



**Fig. 2.** Tumor-initiating cell characteristics in cells expressing the highest HER2 surface levels. *A*, SFE analysis of sorted MDA-MB-361 HER2-Low and HER2-High cells (mean  $\pm$  SD of six replicates). *B*, SFE of sorted SKOV-3 HER2-Low and HER2-High cells (mean  $\pm$  SD of six replicates). *C*, Western blot analysis of whole cell lysates from HER2-Low and HER2-High MDA-MB-361 and SKOV-3 cells for expression of HER2, P-HER2, EGFR, P-EGFR, HER3, P-HER3, AKT, P-AKT, MAPK, P-MAPK, and stem cell markers Bmi1 and Oct4. *D*, representative fluorescence-activated cell sorting analysis of HER2 expression in MDA-MB-361 and SKOV-3 HER2-High spheres (grey area), HER2-High spheres grown on differentiating conditions (light grey line), and parental cells (dark grey line).

**Table 1.** Tumor growth ability

<b>A. Tumor growth of carcinoma HER2-High and HER2-Low cells at different dilutions</b>				
Cell line	HER2 levels	Outgrowths/injections		
		10 <sup>6</sup> injected cells	10 <sup>5</sup> injected cells	10 <sup>4</sup> injected cells
MDA-MB-361	HER2-High	6/6	4/6	3/6
	HER2-Low	6/6	4/6	0/6
SKOV-3	HER2-High	6/6	6/6	4/6
	HER2-Low	6/6	5/6	0/6
<b>B. Serial transplantability of untreated or drug-treated xenotransplants</b>				
Cell line	Treatment	Outgrowths/injections		
		1° passage (10 <sup>6</sup> injected cells)	2° passage (10 <sup>5</sup> injected cells)	3° passage (10 <sup>5</sup> injected cells)
MDA-MB-361	NT	5/5	3/5	2/4
	Doxorubicin	5/5	4/5	3/4
	Trastuzumab	3/5	0/5	-
SKOV-3	NT	5/5	4/5	3/5
	Doxorubicin	5/5	4/5	3/5
	Trastuzumab	3/5	0/5	-
MCF7	NT	5/5	5/5	5/5
	Doxorubicin	5/5	5/5	5/5
	Trastuzumab	5/5	5/5	5/5

Abbreviation: NT, untreated.

significant at  $P \leq 0.05$ . In the real-time PCR analyses, error bars represent the calculated maximum (RQMax) and minimum (RQMin) expression levels that represent 95% confidence interval of the mean expression level (RQ value).

## Results

**HER2 expression in tumor-initiating cells derived from HER2-positive carcinoma cell lines.** An established approach to the isolation of tumor-initiating cells consists in growing cells in suspension under undifferentiating conditions (10). Under this condition, cells grow as undifferentiated spheroids structures (spheres) and tend to express characteristic stem cells markers such as Oct4 and Bmi1 (20–22). This method can be used to assess the self-renewal potential of both mammary gland progenitor cells and tumor-initiating cells.

We selected a panel of breast and ovarian cancer cell lines known to overexpress HER2 and bearing HER2 gene amplification, and compared the levels of HER2 surface expression of spheroid cultures with the corresponding “parental” cell line grown as monolayer or with cells derived from spheres that were differentiated by culturing in matrigel.

Analysis of HER2 cell surface expression in the breast carcinoma lines MDA-MB-361 and BT-474, or in the ovarian carcinoma line SKOV-3, revealed higher HER2 levels in spheres compared with the same cells induced to differentiate or the parental cell lines (2- to 7-fold increase; Fig. 1A).

Similar results were obtained with the breast carcinoma cell line ZR-75-1, which overexpresses HER2 without gene amplification: HER2 expression in spheres was three times higher than in differentiated cells. No differences in HER2 levels were found in MCF7 cells, a cell line expressing low levels of HER2 (Fig. 1A).

We then analyzed HER2 gene copy number in tumor-initiating cells and in their differentiated counterparts by

fluorescence *in situ* hybridization on the MDA-MB-361 cell line (Fig. 1B) and SKOV-3, BT-474, and ZR-75-1 cells (Supplementary Fig. S1). We found that the carcinoma cell lines with HER2 gene amplification showed similar ratio between HER2 gene copies and chromosome 17 centrosomes in tumor-initiating cells and differentiated cells. Similarly, the carcinoma cell line without gene amplification as ZR-75-1 cells (Supplementary Fig. S1C) maintained its HER2 gene property in tumor-initiating cells and differentiated counterparts. RT-PCR analysis indicated that spheres derived from MDA-MB-361, SKOV-3, and BT-474 and ZR-75-1 cells, expressed significantly more HER2 transcripts compared with cells grown under differentiating conditions (Fig. 1C). By contrast, MCF7 spheres showed similar levels of HER2 protein but lower HER2 transcripts levels compared with their counterparts cultured in matrigel.

Our data show that the high expression of HER2 in tumor-initiating cells is transcriptionally controlled and does not depend on increased gene dosage.

Given that previous studies have implicated the ALDH-positive population as possessing stem cell properties (23); we investigated the cell surface expression of HER2 in an ALDH-positive population by fluorescence-activated cell sorting.

We found positive correlation between ALDH activity and HER2 cell surface expression. ALDH-positive cells from MDA-MB-361 cells presented 1.6-fold higher HER2 expression levels than the ALDH-negative population (Fig. 1D). Similar results were obtained with ALDH-positive BT-474 and ZR-75-1 cell lines, which showed twice as much HER2 levels compared with the ALDH-negative population (Supplementary Fig. S2). No differences between aldefluor-positive and aldefluor-negative cells were observed when anti-human leukocyte antigen antibody was tested (data not shown).

In conclusion tumor-initiating cells of our carcinoma cell lines are characterized by the high expression of HER2.

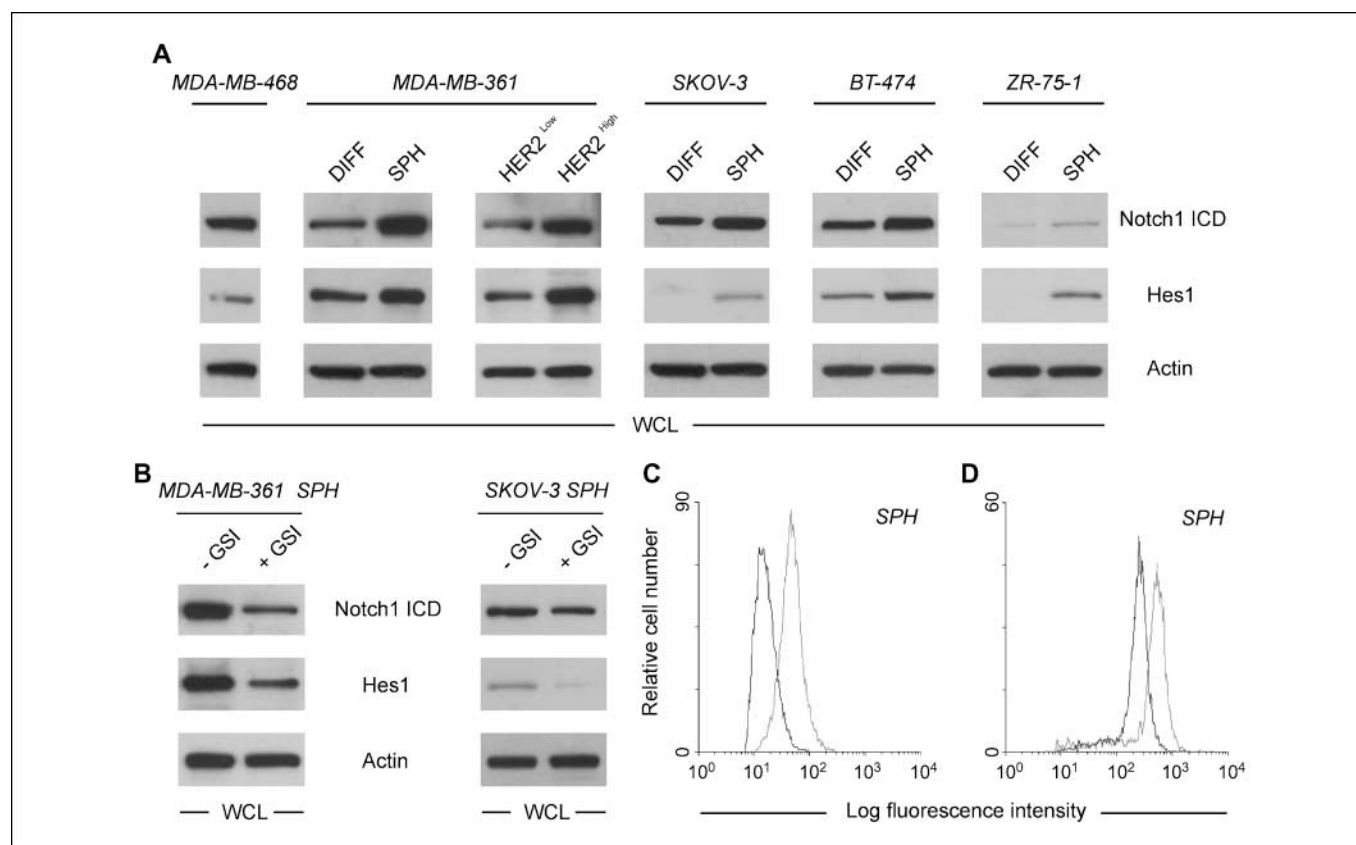
**High HER2 surface levels define tumor-initiating cells in HER2-overexpressing cell lines.** To determine whether HER2 surface expression would define tumor-initiating cells, we sorted cells derived from MDA-MB-361 or SKOV-3, according to membrane HER2 expression levels (defined here as HER2-Low and HER2-High; Supplementary Fig. S3), and we tested SFE in the different populations.

MDA-MB-361 HER2-High cells, which represented 10% of the total cell population, showed 3-fold higher SFE than their HER2-Low counterpart (10% of total cells; Fig. 2A). Similarly, SKOV-3 HER2-High cells (11.21% of total cells) had 3-fold higher SFE than the HER2-Low cells (10.76% of total cells; Fig. 2B). These data suggest that cells presenting the highest HER2 levels contain a larger fraction of tumor-initiating cells. In line with the enrichment in tumor-initiating cells present in the HER2-High population, Western blotting analysis of the sorted MDA-MB-361 and SKOV-3 populations showed that HER2-High cells expressed the stem cell markers Oct4 and Bmi1, whereas HER2-Low cells were negative (Fig. 2C). HER2-High cell populations of both MDA-MB-361 and SKOV-3 cell lines expressed higher levels of total and active receptor tyrosine kinases as HER2 and HER1, compared with HER2-Low cells. Only MDA-MB-361 HER2-High cells showed detectable levels of HER3 but not phosphorylated in Y1289. In addition, the activation of AKT and MAPK was significantly higher in

the HER2-High compared with the HER2-Low cells, whereas the total protein levels were similar in the two populations. When the MDA-MB-361 HER2-High or the SKOV-3 HER2-High cells were grown under differentiating conditions (i.e. in matrigel), HER2 cell surface expression decreased to the levels measured in the parental cells (Fig. 2D), therefore identifying the HER2-High subset as capable of recapitulating the heterogeneity of the parental cells. Overall, these data suggest that overexpression of HER family members provides survival and proliferation signals in tumor-initiating cells.

Next, we analyzed the tumorigenicity of the HER2-High cells compared with the HER2-Low cells by injecting different cell doses in nude mice and then scoring for the development of tumors.  $10^4$  MDA-MB-361 and SKOV-3 HER2-High cells were able to give rise to tumors (3 of 6 mice and 4 of 6 mice with tumor, respectively), whereas no tumor growth was observed in the 6 mice injected with the HER2-Low population. However, the two populations showed similar tumor growth abilities when  $10^6$  or  $10^5$  cells were inoculated (Table 1A). Thus, HER2-High cells are enriched in tumor-initiating cells.

**The Notch1 pathway and HER2 expression in tumor-initiating cells.** Because breast stem cell properties are tightly regulated by Notch1 signaling (24), and the activation of Notch1 signaling has been reported to increase HER2 transcription (25, 26), we analyzed Notch1 signaling in our tumor-initiating cells.



**Fig. 3.** Role of Notch signaling on HER2 expression of tumor-initiating cells. **A**, Western blot analysis of whole cell lysates from MDA-MB-361 HER2-High/HER2-Low populations, MDA-MB-361, SKOV-3, BT-474, and ZR-75-1 spheres, and each sphere grown under differentiating conditions for Notch1 intracellular domain and Hes1 expression. MDA-MB-468 whole cell lysates were used as a positive control for Notch1 intracellular domain and Hes1 expression. **B**, Western blot analysis of whole cell lysates from untreated or GSI-treated MDA-MB-361 and SKOV-3 spheres for Notch1 intracellular domain and Hes1 expression. **C**, immunofluorescence analysis of HER2 expression in untreated (light grey line) or GSI-treated (grey line) MDA-MB-361 and SKOV-3 spheres (SPH).

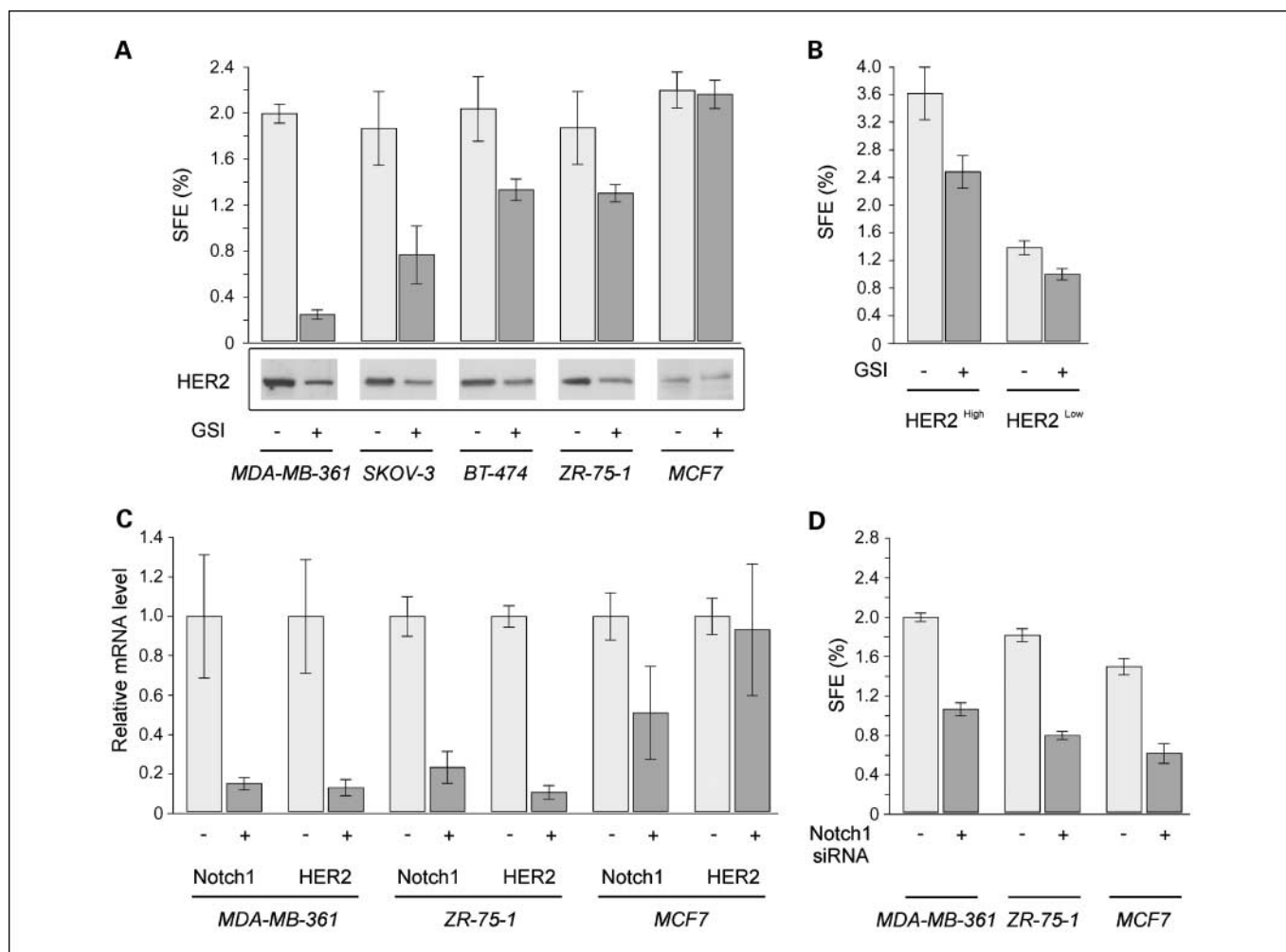
We assayed for the presence of the active form of the Notch1 receptor, the Notch1 intracellular domain, in spheres propagated in suspension or in spheres grown under differentiating conditions (Fig. 3A). Spheres derived from MDA-MB-361, SKOV-3, BT-474, and ZR-75-1 expressed higher levels of Notch1 intracellular domain compared with the spheres grown on matrigel (from 1.6 to 1.3 folds). Hes1, a known Notch1 target gene (27, 28), was up-regulated in the spheres of the four cell lines, thus confirming the activation of Notch1 in tumor-initiating cells (Fig. 3A).

Western blotting analysis revealed that MDA-MB-361 HER2-High cells, expressed 3-fold more Notch1 intracellular domain and 2-fold more Hes1 protein compared to HER2-Low cells (Fig. 3A).

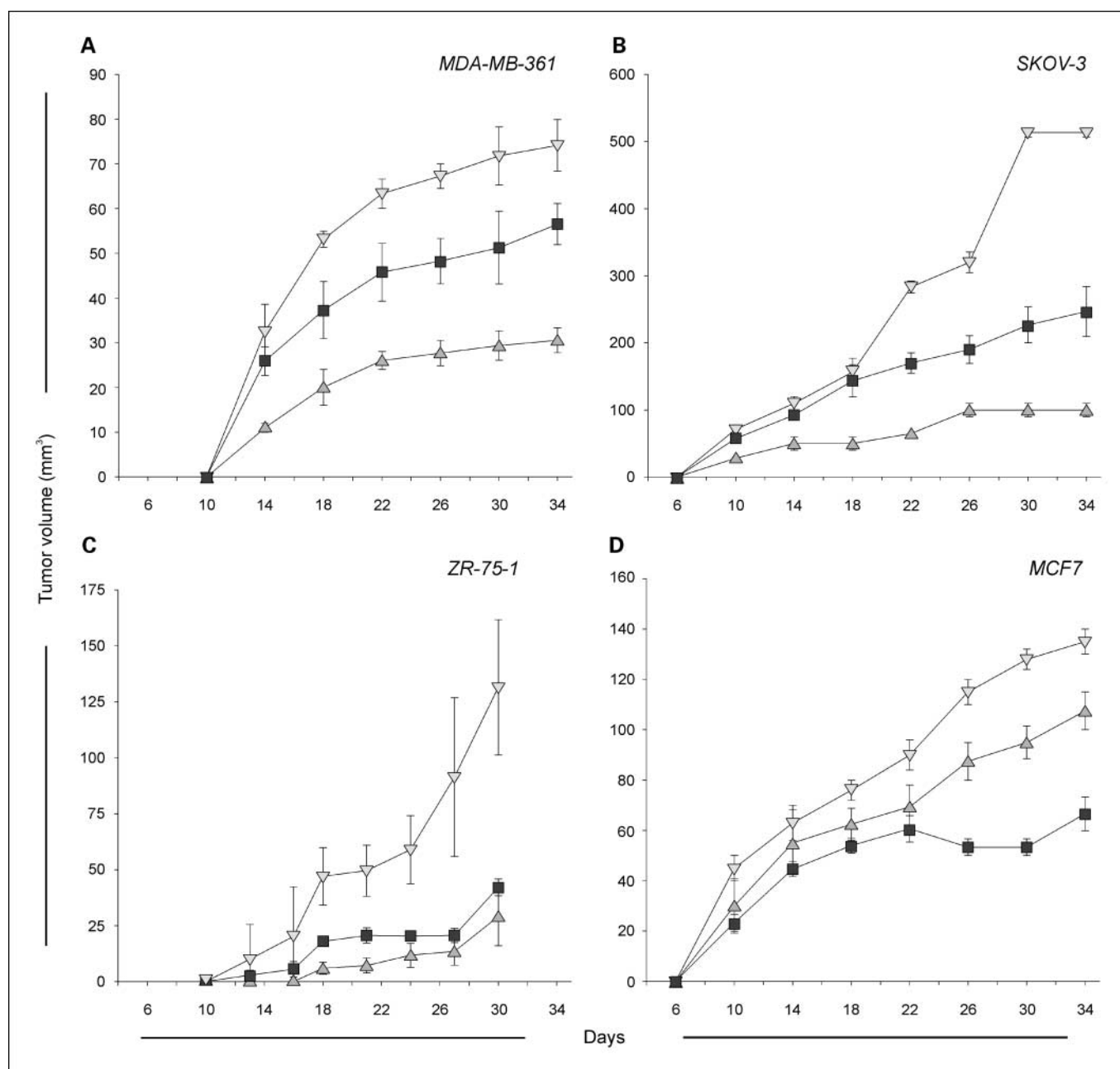
To analyze the role of Notch1 pathway in regulating HER2 expression in tumor-initiating cells, we treated our cell lines with GSI, in order to block Notch1 intracellular domain formation and translocation into the nucleus (29). GSI

treatment, which blocked both Notch1 intracellular domain formation and Hes1 expression in spheres (Fig. 3B), reduced the HER2 cell surface expression on MDA-MB-361 and SKOV-3 spheres by 70% and 50%, respectively (Fig. 3C), with no effect on HER2 expression in spheres grown on differentiating conditions (Supplementary Fig. S4). Accordingly, GSI treatment reduced the HER2 cell surface expression on the MDA-MB-361 HER2-High population by 7-fold whereas the reduction of HER2 expression on the HER2-Low population was minimal: mean fluorescence intensity of  $483.36 \pm 324.41$  versus  $69.03 \pm 75.28$  and  $139.66 \pm 64.04$  versus  $106.08 \pm 58.56$ , respectively.

Inhibition of Notch1 intracellular domain formation led to a significant decrease in HER2 mRNA levels in the spheres formed by our four cell lines (Supplementary Fig. S5). Western blotting analysis showed that GSI treatment down-regulated HER2 expression in all carcinoma cell lines (Fig. 4A, boxed area).



**Fig. 4.** Effect of Notch1 inhibition. *A*, SFE analysis of untreated (light grey bar) or GSI-treated (grey bar) MDA-MB-361, SKOV-3, BT-474, ZR-75-1, and MCF7 spheres (values are mean  $\pm$  SD of six replicates); in the bottom boxed area, Western blot analysis of whole cell lysates from untreated or GSI-treated MDA-MB-361, SKOV-3, BT-474, ZR-75-1, and MCF7 spheres for HER2 expression. *B*, SFE of MDA-MB-361 HER2-High/HER2-Low populations untreated (light grey bar) or treated with GSI (grey bar). *C*, real-time PCR analysis of Notch1 and HER2 mRNA in cell lines treated with a pool of control RNA duplexes (light grey bar) or Notch1 small interfering RNA (grey bar). Values represent expression of target genes normalized to *GAPDH* relative to target gene expression levels in cells treated with a pool of control RNA duplexes as calibrator samples. Bars indicate the 95% RQ confidence interval of gene expression in each column. *D*, SFE analysis of carcinoma cell lines silenced by a pool of control RNA duplexes (light grey bar) or by Notch1 small interfering RNA (grey bar).



**Fig. 5.** Effect of trastuzumab on tumor growth of HER2-overexpressing xenotransplants. Tumor volume (mean  $\pm$  SD) of MDA-MB-361 (A), SKOV-3 (B), ZR-75-1 (C), and MCF7 (D) untreated ( $\nabla$ ), doxorubicin- ( $\square$ ), or trastuzumab-treated ( $\Delta$ ) transplants in nude mice. Data are representative of three experiments.

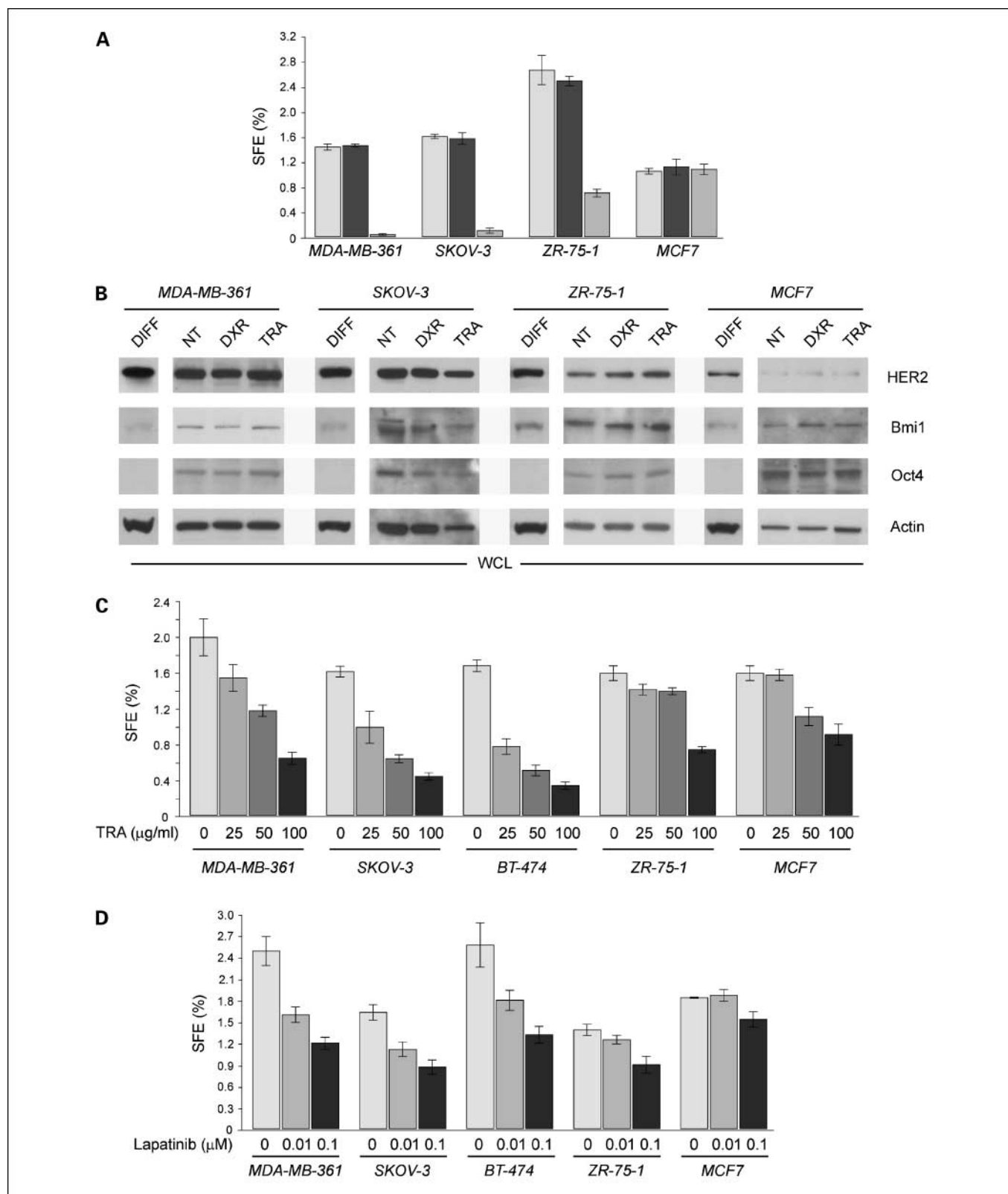
Most remarkable was the 90% reduction in SFE of GSI-treated MDA-MB-361 cells ( $P < 0.0001$ ; Fig. 4A). Similar results were obtained in GSI-treated SKOV-3 cells (60% reduction in SFE,  $P = 0.0095$ ), in GSI-treated BT-474 (34% reduction in SFE,  $P = 0.0305$ ), and ZR-75-1 (30% reduction in SFE,  $P = 0.0434$ ). By contrast, GSI treatment did not affect either SFE or HER2 transcript in MCF7 cells. Strikingly, SFE resulted 5 times higher on the HER2-High compared to the HER2-Low MDA-MB-361 population and GSI treatment affected the SFE of both populations (Fig. 4B).

To validate these observations, we silenced Notch1 in MDA-MB-361, ZR-75-1, and MCF7 cells and analyzed SFE. Notch1 small interfering RNA treatment, found to reduce significantly

the expression of Notch1 transcripts and HER2 mRNA level, decreased by 50% to 60% the SFE of MDA-MB-361 and ZR-75-1 cells (Fig. 4C). Our data suggest that Notch1 signaling regulates HER2 transcription in HER2-overexpressing breast carcinoma tumor-initiating cells, therefore affecting their self-renewal properties. In HER2-negative cells, Notch1 silencing did not affect HER2 mRNA expression, but was still effective in SFE reduction (Fig. 4D), suggesting that Notch1 but not HER2 is implicated in the self-renewal of these tumor-initiating cells.

**Drug sensitivity of tumor-initiating cells present in HER2-overexpressing carcinoma cell lines.** Because tumor-initiating cells derived from HER2-overexpressing cell lines expressed





**Fig. 6.** Effect of trastuzumab and lapatinib on tumor-initiating cells of HER2-overexpressing cell lines. *A*, SFE of untreated (light grey bar), doxorubicin- (black bar), or trastuzumab-treated (grey bar) xenotransplants of MDA-MB-361, SKOV-3, ZR-75-1, and MCF7 cells. SFE was calculated from 6 mice/group as mean  $\pm$  SD. *B*, Western blot analysis of spheres from untreated (NT), doxorubicin (DXR)-, or Trastuzumab (TRA)-treated xenotransplants of MDA-MB-361, SKOV-3, BT-474, ZR-75-1, and MCF7 cells for expression of HER2 and the stem cell markers Bmi1 and Oct4. Markers expression was compared with spheres recovered from xenotransplants and then grown under differentiating conditions (DIFF). *C*, SFE analysis of MDA-MB-361, SKOV-3, BT-474, ZR-75-1, and MCF7 cells untreated (light grey bar) or treated with trastuzumab at 25 (grey bar), 50 (dark grey bar), or 100  $\mu$ g/mL (black bar) cells. *D*, SFE analysis of MDA-MB-361, SKOV-3, BT-474, ZR-75-1, and MCF7 cells untreated (light grey bar) or treated with lapatinib at 0.01 (grey bar) or 0.1  $\mu$ mol/L (black bar).

high levels of HER2 protein, we asked whether trastuzumab, the humanized monoclonal antibody directed against HER2, would be more effective than conventional chemotherapeutics, like doxorubicin, in targeting tumor-initiating cells. To this end, we s.c. injected cells derived from HER2-overexpressing cell lines into the mammary fat pad or in the flank of nude mice and monitored tumor development in mice treated with trastuzumab or doxorubicin at doses that would partially inhibit tumor growth. We found that both trastuzumab and doxorubicin inhibited MDA-MB-361 (Fig. 5A), SKOV-3 (Fig. 5B), and ZR-75-1 (Fig. 5C) tumor growth to different extents, whereas, as expected MCF7 (Fig. 5D) xenografts were more resistant to these treatments.

The presence of tumor-initiating cells in drug-treated tumors was analyzed by measuring the ability of residual cells to form spheres when grown in suspension. We found that the SFE of trastuzumab-treated xenografts derived from HER2-overexpressing cell lines was significantly lower than that of doxorubicin-treated or untreated tumors. By contrast, the SFE of MCF7 xenografts were not affected by doxorubicin or trastuzumab treatment (Fig. 6A). To exclude that the differences in SFE efficiencies would simply reflect a generalized reduction in cellular viability, cells derived from xenografts of all the experimental groups were also cultured in adherent condition and cell growth was monitored by MTT assay. We found that all treated xenograft-derived tumor cells showed comparable growth when cultured in adhesion (Supplementary Fig. S6A).

Interestingly we found that levels of the stem cell markers Oct4 and Bmi1 were higher in xenotransplant-derived spheres than in the same spheres grown under differentiating conditions, thus suggesting that spheres derived from xenografts had tumor-initiating cell properties (Fig. 6B).

To functionally assess the tumor-initiating cell properties of xenograft-derived cells, residual cells were serially transplanted at different cell doses. Serial transplant analysis of MDA-MB-361 and SKOV-3 xenografts showed that cells from control or doxorubicin-treated tumors were transplantable multiple times, whereas cells derived from trastuzumab-treated tumors could not be transplanted into a secondary recipient at any of the cell doses tested (Table 1B). MCF7 cells derived from untreated, trastuzumab-, or doxorubicin-treated tumors showed similar transplantation abilities (Table 1B). In addition, we studied the effect of trastuzumab on SFE and on the growth ability of the differentiated counterparts. Treatment of MDA-MB-361, SKOV-3, and BT-474 cells caused a dose-dependent inhibition of SFE (Fig. 6C), whereas growth in differentiating conditions was only marginally affected (Supplementary Fig. S6B).

We also found that lapatinib, an inhibitor of HER2 and HER1 tyrosine kinase activity, at doses able to decrease phospho-HER2 protein levels almost by 80% in MDA-MB-361, SKOV-3, BT-474 and ZR-75-1 carcinoma cell lines (data not shown), decreased by 30% to 50% the ability of these cells to form spheres depending on the drug concentration (Fig. 6D). Of note, lapatinib treatments had mild effects on the growth ability of the differentiated counterparts (Supplementary Fig. S6C). In MCF7 cells 0.01  $\mu\text{mol/L}$  lapatinib affected both SFE and growth ability in differentiating conditions.

Therefore, our data strongly suggest that anti-HER2 therapeutics, especially trastuzumab, can effectively target tumor-initiating cells derived from HER2-overexpressing tumor cell lines.

## Discussion

The present study provides evidence that in HER2-positive carcinoma cell lines, cells presenting the highest levels of the HER2 oncoprotein showed higher frequencies of tumor-initiating cells. In fact, not only cells grown as spheres or positive for ALDH activity presented higher levels of HER2, but subpopulations of cells with elevated surface expression of HER2 (HER2-High cells) also showed increased SFE compared with the bulk population of cells. HER2-High cells also had the highest ability to grow *in vivo* in a xenotransplantation animal model that remains the gold standard assay for testing functional stem cell properties.

The mechanism controlling HER2 expression levels in the tumor-initiating cells does not depend on variation of HER2 gene copies, but rests, at least in part, in Notch1-dependent regulation, a molecule previously shown to increase HER2 transcription and to regulate breast stem cell survival (30), as shown by the reduction of HER2 cell surface expression together with SFE following inhibition or silencing of Notch1 signaling. The lack of effect of Notch1 inhibitor on HER2 expression in HER2-overexpressing cells grown in differentiating conditions could be explained by the significantly lower levels of activated Notch1 in these cells. Also, the higher HER2 expression levels found in cells presenting ALDH activity support a role of this oncoprotein in the maintenance of the putative breast tumor-initiating cells. In analogy with several genes, including Notch, Hedgehog, Wnt, and ras, initially identified as oncogenes but shown subsequently to be involved in tumor-initiating cell pathways of self-renewal and survival (3, 31, 32), HER2 might also represent a relevant molecule in cancer stem cells. Similarly, Korkaya et al. (33) found that increased HER2 expression in normal mammary epithelial cells or in breast carcinoma cell lines enhances the tumor-initiating cell component driving tumorigenesis, invasion, and metastasis. Although no HER2 increase was observed in MCF7 spheres compared with cells grown under differentiation conditions by fluorescence-activated cell sorting, it is possible that a Notch1-dependent increase in HER2 on tumor-initiating cells was below the limits of detection in this low-HER2-expressing cell line.

Moreover the therapeutic activity of trastuzumab seems to extend its action on putative cancer stem cells of HER2-positive carcinoma cell lines and the effect is due mainly to the elevated HER2 expression in tumor-initiating cells compared with the other bulk tumor cells. Indeed, the lack of serial transplantability of residual cells derived from trastuzumab-treated HER2-overexpressing xenografts indicates that tumor cells growing in trastuzumab-exposed mice were depleted of tumorigenic cells. Higher sensitivity to trastuzumab or lapatinib of tumor-initiating cells compared with differentiated counterparts evidenced *in vitro*, confirms *in vivo* findings and suggests that stemness markers (i.e. Bmi1 and Oct4) might be predictive of tumor cell sensitivity to trastuzumab. The activity of the trastuzumab antibody in breast carcinoma patients is directly proportional to the level of HER2 present on tumor cell membrane (34). Thus, our observation of higher HER2 expression in tumor-initiating cells of HER2-overexpressing carcinoma cell lines compared with the same cells grown on matrigel explains why trastuzumab is so efficient in inhibiting the tumorigenicity of HER2-positive carcinoma cells. HER2-High cells contained a larger fraction of tumor-initiating cells,

the only population able to give rise to tumor growth at very low dilutions. In light of these results, it is even clearer why the four clinical trials involving more than 10,000 cases of HER2-positive breast carcinoma patients (9) revealed that trastuzumab represents a success in the field of targeted antineoplastic agents. More importantly, the tumor-initiating cells of breast carcinoma cell lines present higher levels of active receptors of HER family with consequent increased survival and proliferation signaling mediated by these receptors. Signaling mediated by HER family receptors drives the stem cell pool, i.e. regulating the stem/progenitor cell populations, as also supported by significant SFE reduction of breast carcinoma cell lines by inhibitor of HER1 and HER2 kinase activity at doses ineffective on differentiated cells.

In the clinical context, it is important to note that HER2 levels in tumor-initiating cells of breast carcinoma overexpressing HER2 but without gene amplification were also higher compared with total tumor cells, with levels reaching those of cells showing gene amplification. This suggests the usefulness of trastuzumab in treating these types of tumors by virtue of its ability to bind and block the cells responsible for tumor aggressiveness. In fact, benefits for disease-free survival after trastuzumab adjuvant therapy have been observed in patients with breast tumors negative for HER2 overexpression (based on fluorescence *in situ* hybridization criteria) or with less than 3+ staining intensity (based on HercepTest immunohistochemical scoring; ref. 35).

Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and transplantability of untreated and doxorubicin-treated tumors indicates that a drug able to reduce tumor volume by 50% kills both tumor-initiating cells and bulk tumor cells. Because doxorubicin sensitivity of HER2-positive breast carcinoma cells

does not directly depend on the receptor molecule but instead on the higher proliferation of HER2-positive cells compared with negative cells (36), it is possible that in our xenotransplant model consisting in proliferating tumor cells, tumor-initiating cells also proliferate and thereby become sensitive to the drugs' action. Such a possibility would not be inconsistent with the recent findings (3, 5) that breast cancers from patients who received neoadjuvant chemotherapy or mice genetically manipulated for developing mammary tumors are substantially enriched for self-renewing cells. Indeed, in already established tumors, as those tested by Yu and coworkers or Shafee and coworkers, tumor-initiating cells representing the nondividing cells within the tumor bulk population resulted resistant to drug treatment.

Although the relevance of our results obtained with tumor-initiating cell-enriched cells from carcinoma cell lines awaits confirmation in studies using tumor cells obtained from breast tumor patients, we provide evidence of the efficacy of trastuzumab in inhibiting tumor-initiating cells of HER2-positive tumors. Moreover, our finding that HER2 expression is up-modulated in tumor-initiating cells in a Notch1-dependent manner in addition to gene amplification suggests the potential for new therapeutic modalities targeting this stem cell marker pathway.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Tumor-Initiating Cells of HER2-Positive Carcinoma Cell Lines Express the Highest Oncoprotein Levels and Are Sensitive to Trastuzumab

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