# Targeting YB-1 in HER-2 Overexpressing Breast Cancer Cells Induces Apoptosis via the mTOR/STAT3 Pathway and Suppresses Tumor Growth in Mice

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## Abstract

**The Y-box binding protein-1 (YB-1) is a transcription/translation factor that is highly expressed in primary breast tumors where it is consistently associated with poor survival. It induces human epidermal growth factor receptor (*her-2*) along with its dimerization partner *egfr* by directly binding to their promoters. In addition to promoting growth by inducing receptor tyrosine kinases, YB-1 also protects cells against apoptosis through mechanisms that have not been fully revealed. Given this, we addressed whether YB-1 might be an eventual therapeutic target for breast cancer by inhibiting it with small interfering RNAs *in vitro* and *in vivo*. Inhibiting YB-1 suppressed the growth of six of seven breast cancer cell lines that had amplified *her-2* or were triple negative. Importantly, targeting YB-1 induced apoptosis in BT474-m1 and Au565 breast cancer cells known to have *her-2* amplifications. The potential role of signal transducers and activators of transcription 3 (STAT3) was pursued to address the underlying mechanism for YB-1–mediated survival. Inhibition of YB-1 decreased P-STAT3$^{S727}$ but not P-STAT3$^{Y705}$ or total STAT3. This was accompanied by decreased P-ERK1/2$^{T202/Y204}$, P-mTOR$^{S2448}$, and total mammalian target of rapamycin mTOR. Furthering the role of STAT3 in these cells, we show that knocking it down recapitulated the induction of apoptosis. Alternatively, constitutively active P-STAT3 rescued YB-1–induced apoptosis. Finally, targeting YB-1 with 2 different siRNAs remarkably suppressed tumor cell growth in soft agar by >90% and delayed tumorigenesis in nude mice. We conclude that HER-2 overexpressing as well as triple-negative breast cancer cells are YB-1 dependent, suggesting it may be a good therapeutic target for these exceptionally aggressive tumors.** [Cancer Res 2008;68(21):8661–6]

## Introduction

The overexpression of the human epidermal growth factor receptor (HER-2) is clearly associated with one of the most aggressive types of breast cancer (1). Equally challenging are those in the triple-negative or basal-like subtype (1). HER-2 has become a desirable molecular target for breast cancer that has led to the development of therapies designed to inhibit it such as trastuzumab, pertuzumab, and more recently, lapatinib. Thus far, the success of these agents is initially very good; despite this, ~30% of patients do not respond and those that do are often faced with the development of resistance. We have identified a second factor expressed in aggressive types of breast cancer, the Y-box binding protein-1 (YB-1) that induces growth promoting genes such as *her-2, egfr*, proliferating nuclear antigen (*pcna*), *cyclin A*, and *cyclin B* (reviewed in ref. 2). Furthering this, the presence of YB-1 specifically in the nuclear compartment of breast cancer cells is associated with Her-2 based on the examination of primary tumors by immunohistochemistry (3). YB-1 is activated by kinases such as AKT (4) also known to be linked to breast cancer (2). When YB-1 is highly expressed in the mammary gland, transgenic mice develop tumors with 100% penetrance, indicating that it is a *bona fide* oncogene (5). Because YB-1 is commonly expressed in breast cancers (6), we questioned whether they were indeed dependent on it for growth and survival. We therefore inhibited YB-1 using small interfering RNAs as a novel way of potentially blocking the growth of breast cancers. Inhibiting YB-1 suppressed the growth of six of seven breast cancer cell lines that were either triple negative or had *her-2* amplifications. After this, we focused on those that had *her-2* amplifications given the many clinical challenges that currently prevent the successful treatment of this aggressive type of breast cancer.

## Materials and Methods

**Inhibition of YB-1 with small interfering RNAs.** BT474-m1 cells were obtained from MC Hung, M. D. Anderson Cancer Center. SUM149 cells were obtained from Astrand. 184htrt cells were a gift from Dr. J. Carl Barett (National Institute of Health, Bethesda, MA). All other cell lines were purchased from the American Type Culture Collection. The sequence for siYB-1#1 was as previously reported (6), siYB-1#2 was designed using the following sequence (CCACG-CAAUUACCAGCAAAdTdT), and the control siRNA oligonucleotide was (UUCUCCGAACGUGUCACGUdTdT). Each of the cell lines were plated at a density of 1,000 cells per 96-well, transfected with siYB-1#2 (5 nmol/L), and a YB-1 knockdown >75% detected by immunofluorescence after 48 h. Subsequently, tumor cell growth was assessed after 72 h by Hoechst staining as previously describing using the ArrayScan VTI (Cellomics; ref. 7). For the siRNA transfections, where changes in signal transduction were monitored, the cells ($3 \times 10^5$ per 6-well dish to $3.5 \times 10^5$ per 6-well dish) were transfected with 5 nmol/L of control or siRNA oligonucleotides according to the manufacturer's protocol.

**Apoptosis assays.** BT474-m1 cells were treated with control, siYB-1#1, or siYB-1#2 oligonucleotides for 1 to 4 d and analyzed for P-H2AX$^{S139}$ expression (1:500 dilution; AbCam) by immunoblotting. Annexin V was stained following the manufacturer's protocol (Promega) and the cells were analyzed on a FACSCalibur (BD). Analysis of chromatin condensation, propidium iodidie uptake, and P-H2AX$^{S139}$ were performed as previously described (7). For propidium iodide staining, the cells

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

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were collected after being treated for 4 d with siRNA to YB-1, washed, stained with 30 μg/mL of propidium iodide (Sigma), and suspended in 500 μL of 1% fetal bovine serum–containing PBS before being analyzed on a FACSCalibur (BD).

**Pathway evaluation of apoptosis induction.** BT474-m1 or Au565 cells were treated with siYB-1 for up to 96 h and then lysed in ELB buffer (6), and the proteins were evaluated by immunoblotting using antibodies diluted to 1:1,000 unless otherwise indicated: YB-1 (1:10,000; gift from Dr. Colleen

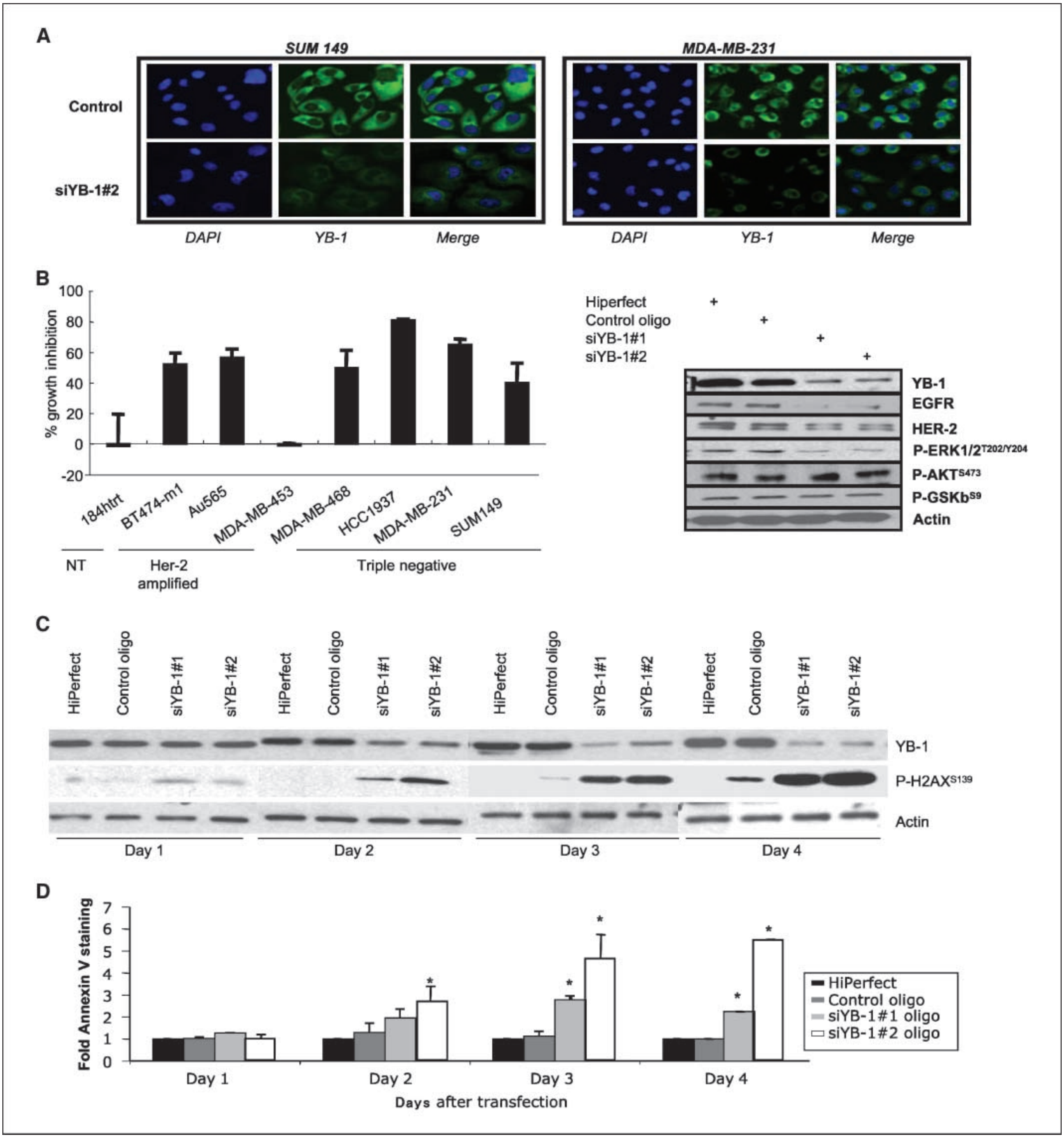


**Figure 1.** Inhibition of YB-1 generally suppresses the growth of breast cancer cell lines and leads to the induction of apoptosis. In a high content screen, YB-1 was silenced for 3 d with siYB-1#2. *A,* the loss of YB-1 expression after treatment with siYB-1#2 was monitored by immunofluorescence. Representative images for SUM149 and MDA-MB-231 cell are shown (magnification, ×10; ArrayScan VTI). *B,* inhibiting YB-1 suppressed the growth of six of seven breast cancer cell lines representing cancers that had *her-2* amplifications or were triple negative. The loss of YB-1 had no effect on the immortalized breast epithelial cell line 184htrt or MDA-MB-453 cells. Silencing YB-1 in the BT474-m1 cells for 72 h also resulted in decreased signaling through the HER-2/EGFR/ERK pathway based on immunoblotting (*B, right*). *C,* BT474-m1 cells were transfected with control or siRNA oligos targeting YB-1 and harvested 1, 2, 3, and 4 days after transfection. Proteins were isolated from the cells, and the lysates were subjected to Western blotting to examine the level of P-H2AX$^{S139}$. Pan-actin was used as loading control. *D,* small interfering RNAs against YB-1 induced Annexin V staining after treatment for 4 d. Changes in Annexin V were monitored by flow cytometry.

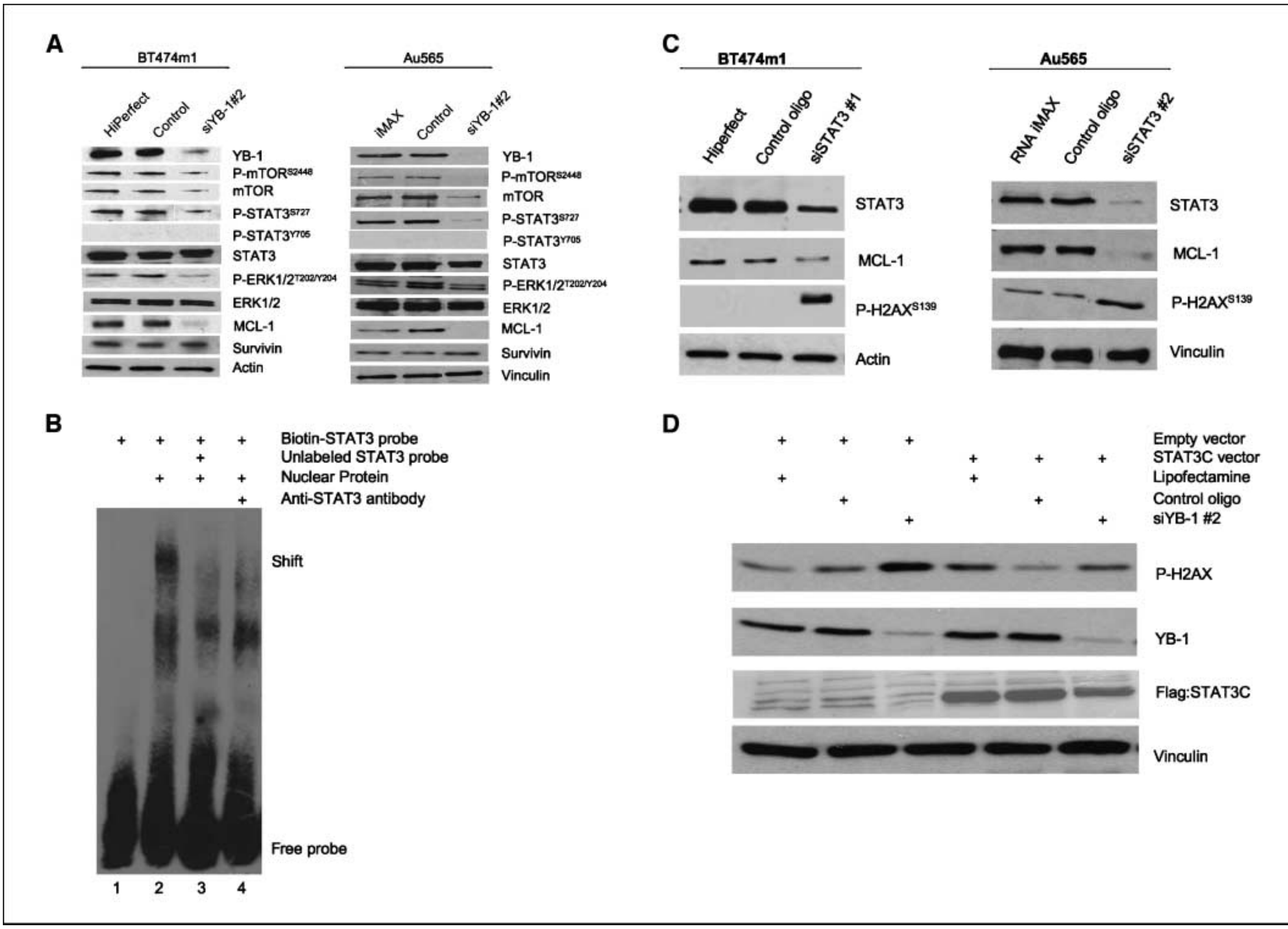


**Figure 2.** Loss of YB-1 decreased signaling through the STAT3 pathway. *A,* loss of YB-1 expression with siYB-1#2 (72 h) was associated with a decrease in P-STAT3$^{S727}$ and MCL-1 relative to the transfection reagent control (*lane 1*) or control siRNA (*lane 2*) in the BT474-m1 cells (*left*). There was no change in total levels of STAT3 or survivin. P-STAT3$^{Y705}$ was undetectable. Actin or vinculin was used as a control for equal sample loading. The extracts were also evaluated for P-mTOR$^{S2448}$, mTOR, and ERK1/2. This was also consistently observed in the Au565 cells (*right*). *B,* nuclear extracts were isolated from BT474-m1 cells to confirm that STAT3 was active in these cells lacking Y705 phosphorylation. In the absence of nuclear protein, no binding was observed (*lane 1*). The addition of nuclear proteins resulted in binding to a MCL-1 promoter sequence containing the STAT3 binding site (*lane 2*), which could be inhibited with cold competitive oligonucleotide (*lane 3*) or an antibody specific to STAT3 (1 μg; *lane 4*). *C,* BT474-m1 and Au565 cells were treated with tranfection reagent, control siRNA (50 nmol/L), or a STAT3 small interfering RNA (50 nmol/L) for 96 h. As a consequence, STAT3 was silenced, MCL-1 decreased, and P-H2AX$^{Ser139}$ was induced in BT474-m1 (*left*) and Au565 cells (*right*). *D,* to examine the possibility that STAT3 could rescue the induction of apoptosis by YB-1 we introduced Flag:STAT3C and siYB-1#2 by cotransfection. The cells were then examined 3 d later for changes in P-H2AX$^{S139}$. The ectopic expression of STAT3C rescued YB-1 induced apoptosis given that P-H2AX$^{S139}$ was reduced.

Nelson, University of British Columbia), P-ERK1/2$^{T202/Y204}$ [Cell Signaling Technology (CST)], P-STAT3$^{S727}$ (CST), P-STAT3$^{Y705}$ (CST), STAT3 (CST), MCL-1 (Santa Cruz Biotechnology), survivin (CST), P-H2AX$^{S139}$ (1:500; AbCam), EGFR (StressGen), HER-2 (AbCam), P-AKT$^{S473}$ (CST), and P-GSTb$^{S9}$ (CST). Vinculin (1:2,000; clone Vin 11-5; V4505 antibody; Sigma) and pan-actin (CST) antibodies were used as loading controls.

The activity of STAT3 was evaluated via gel shift by isolating nuclear proteins from BT474-m1 cells, and a MCL-1 probe (8) was used to detect activity according to our previously reported methods (9). We used 1 μg of STAT3 antibody (Santa Cruz) in the competition experiment. Signal transducers and activators of transcription 3 (STAT3) was silenced using QIAGEN HP validated siRNA in addition to a second siRNA oligonucleotide (10). BT474-m1 and Au565 cells ($3 \times 10^5$ per 6-well dish or $3.5 \times 10^5$ per 6-well dish) were treated with transfection reagent alone, control siRNA (50 nmol/L) or siSTAT3#1 oligonucleotide (50 nmol/L; Dharmacon Research, Inc.) or siSTAT3#2 (50 nmol/L; QIAGEN) for 96 h. For the rescue experiments, Flag:STAT3C (2μg; ref. 11) was cotransfected into BT474-m1 cells ($5.5 \times 10^5$ per 6-well dish) with 5 nmol/L of siYB-1#2 or control siRNA using Lipofectamine 2000 with a DNA/lipofectamine ratio of 1:3, harvested 72 h later and immunoblotted for P-H2AX$^{S139}$, Flag tagged STAT3C (1:2,000 M2 antibody; Sigma) and YB-1 (as described above). Mammalian target of rapamycin (mTOR) mRNA was evaluated using Assay on Demand (Applied Biosystems).

**Soft agar assays.** YB-1 was silenced as described above in the BT474-m1 and MDA-MB-453 cells, and 24 h later, the cells were plated into soft agar. Colony formation was performed as previously described (4). Briefly, BT474-m1 ($1 \times 10^4$/6-well dish) or MDA-MB-453 ($1.5 \times 10^5$/6-well dish) cells were added to a 1:1 mixture of 2× DMEM and 0.6% agarose (Invitrogen), and the colonies were counted 28 d later.

**Inhibition of YB-1 *in vivo*.** BT474-m1 cells were transfected with 5 nmol/L of control or siRNA oligonucleotides for 24 h, harvested, and washed twice with HBSS (Invitrogen). They were then mixed ($1 \times 10^6$ cells) with Matrigel (BD) at a 1:1 ratio yielding a total volume of 200 μL, which was injected s.c. into the right lower hind flank of 6 female *nu/nu* mice per treatment group. Tumor growth was measured using calipers and body weight was measured twice a week. Differences in tumor incidence were evaluated using the Student's $t$ test. Tumors from the termination of the study (week 3) were evaluated for YB-1 protein levels.

## Results and Discussion

Initially, we silenced YB-1 with siRNA in a panel of breast cancer cell lines and cell growth was evaluated 96 hours later using a high content screening (HCS) platform (Fig. 1*A*, representative images of YB-1 knockdown). Seven breast cancer cell lines were screened that had *her-2* amplifications or were triple negative. Silencing YB-1 with siYB-1#2 inhibited the growth of the Her-2 overexpressing BT474-m1 and Au565 cells by ~50% (Fig. 1*B*), yet the 184htrt-immortalized breast epithelial cells and MDA-MB-453 cells were insensitive to the effect of YB-1. Beyond this, YB-1 knockdown also suppressed the growth of triple-negative breast cancer cell lines by 40% to 80% (Fig. 1*B*). To understand the underlying reason for growth suppression in the HER-2 overexpressing models of breast cancer, we show that inhibiting YB-1 in the BT474-m1 (Fig. 1*B, right*) and Au565 cells (data not shown) decreased HER-2, EGFR, and ERK1/2 signaling, whereas there was no effect on the P-AKT$^{S473}$ or P-GSK-3β$^{S9}$ pathway. For unknown reasons, siYB-1 failed to inhibit the ERK pathway in MDA-MB-453 cells, which may explain why their growth was also not suppressed (data not shown). Over a 1- to 4-day time course with siYB-1 the BT474-m1 cells underwent apoptosis that began after 2 days based on the induction of P-H2AX$^{S139}$ and increased Annexin V staining

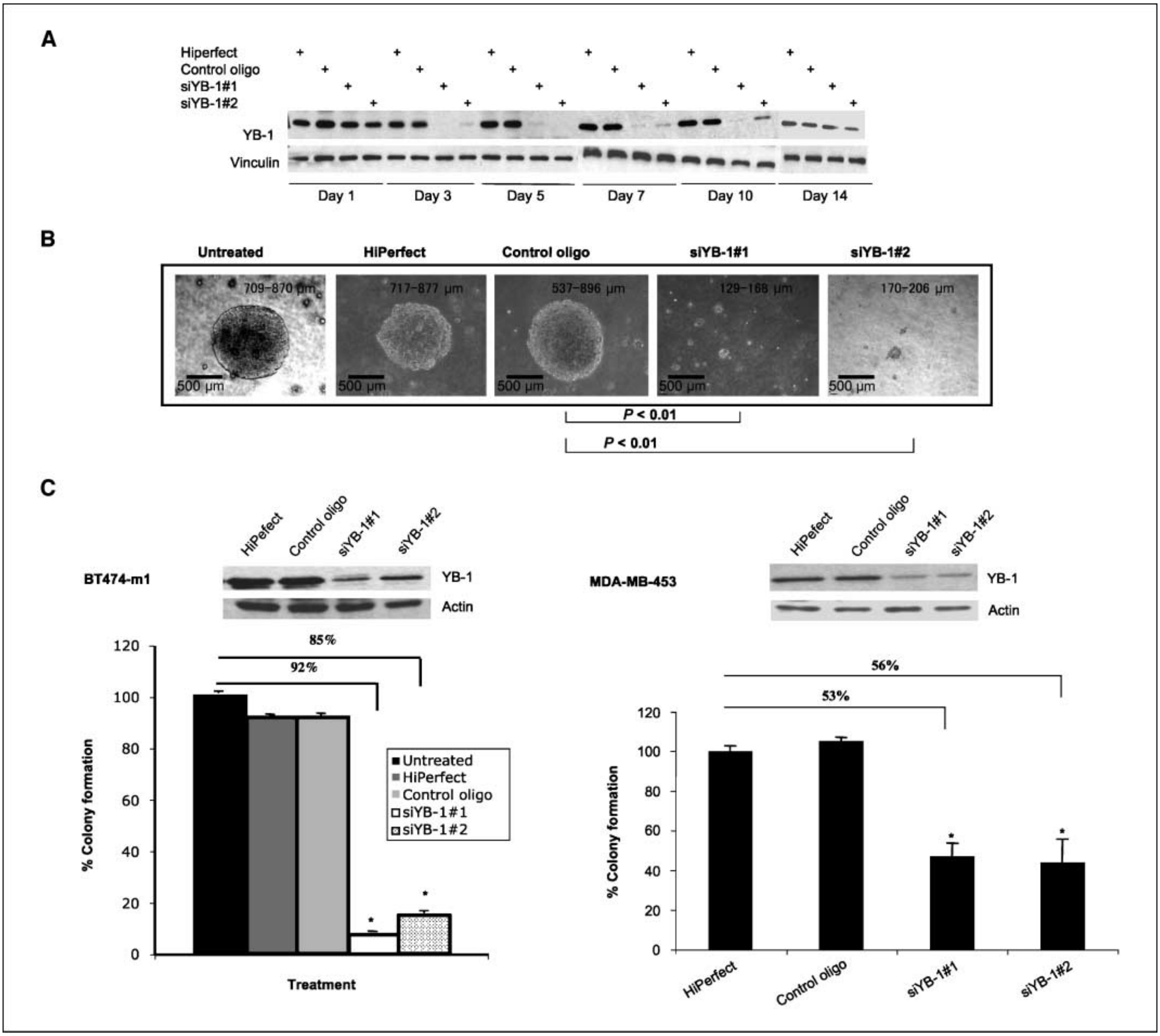


**Figure 3.** Inhibition of YB-1 suppresses tumor growth in soft agar. *A,* time course study (1–14 d) was conducted to examine the long-term stability of the YB-1 siRNA oligonucleotides. Vinculin antibody was used as loading control. YB-1 inhibition was sustained out to 10 d with some detectable inhibition still at 14 d. *B,* the effect of knocking down YB-1 was evaluated in soft agar. BT474-m1 cells were treated with siYB-1 for 24 h and colony growth was assessed after 28 d. Photomicrographs of representative colonies were taken. YB-1 inhibition decreased the size of colonies compared with the scrambled control. *C,* BT474-m1 and MDA-MB-453 cells were treated with YB-1 siRNA, and anchorage-independent growth of the cells was assessed by soft agar assays. Quantification of the number of colonies is shown in the bar charts. On average the BT474-m1 cells produced larger colonies, i.e., >300 to 800 µm and 10 to 12 could be counted per field. The MDA-MB-453 cells developed smaller colonies of ~50 µm in size but were more numerous. On average, there were 40 to 50 colonies per field. *, treatment was significantly different from the controls based on Student's *t* tests.

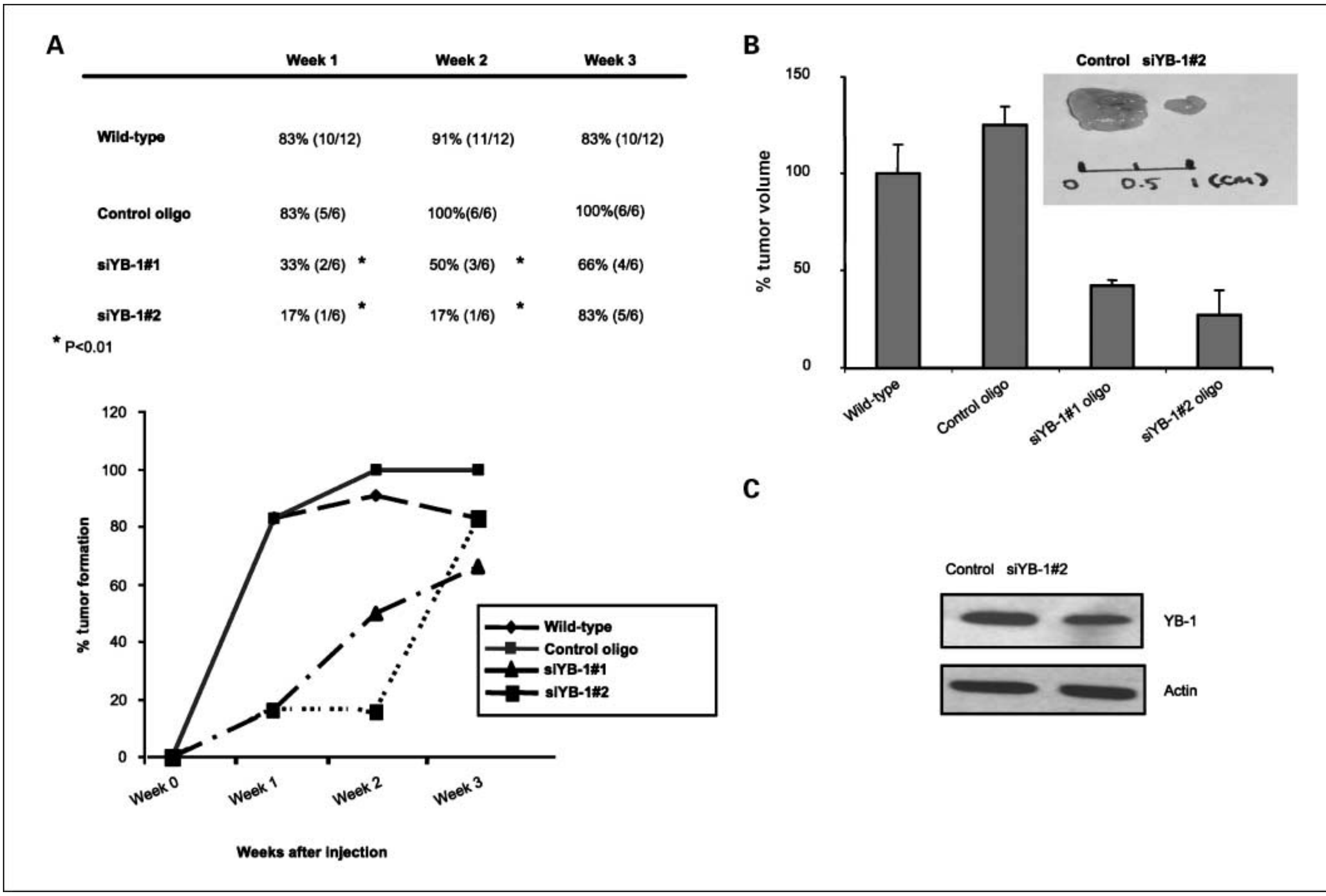


**Figure 4.** Inhibition of YB-1 suppressed the development of tumors in mice. *A,* BT474-m1 cells were transfected with control oligo, siYB-1#1, or siYB-1#2 for 24 h, and then the cells were injected s.c. into nu/nu mice. The incidence of tumor formation was on average 83% in the mice treated with either the wild-type cells or the scrambled control oligonucleotide in the first week. Conversely, tumor incidence was only 33% or 17% when mice were injected with cells transfected with siYB-1#1 or siYB-1#2, respectively ($P < 0.01$). Similarly, the loss of YB-1 expression suppressed tumor formation in the second week as well ($P < 0.01$). *B,* by the third week, tumors developed in the mice; however, they were remarkably smaller in size in the animals that were injected with cells transfected with siYB-1#1 or siYB-1#2. Compared with the control oligonucleotide, the tumors that arose in siYB-1#1 or siYB-1#2 were ~50% to 67% smaller ($P < 0.01$), respectively. Significance was determined using a Student's *t* test. *, $P < 0.01$. *C,* the re-expression of YB-1 was confirmed in the small tumors that eventually arose in the cells transfected with siYB-1#2.

(Fig. 1*C–D*). In support of this, cells treated for 3 days with siYB-1#2 had enhanced chromatin condensation, propidium iodide uptake, and P-H2AX$^{S139}$ at the cellular level shown by HCS in both BT474-m1 and Au565 cells compared with the scrambled control (Supplementary Fig. S1*A–B*). Treating BT474-m1 cells for 4 days with siYB-1#1 or siYB-1#2 increased propidium iodide uptake as much as 30-fold based on flow cytometry (Supplementary Fig. S1*C*). Because YB-1 inhibition altered the apoptotic threshold of the BT474-m1 cells as indicated above, we examined the possibility that this would enhance their sensitivity to Taxol. To this effect, we showed that silencing YB-1 improved the growth inhibitory effect of Taxol (0.1–10 nmol/L) on the BT474-m1 cells compared with cells treated with the control oligonucleotide (Supplementary Fig. S1*D*). Our studies therefore indicate that breast cancers that express high levels of HER-2 as well as those that are triple negative depend on YB-1 for growth and survival. Furthermore, combining YB-1 inhibition with Taxol improves cell killing.

To investigate the underlying mechanism for siYB-1 induced apoptosis, we queried the STAT3 prosurvival pathway (12). This was a candidate pathway because it was previously reported that STAT3 is phosphorylated at S727 in breast cancer cells (13) as an event downstream of HER-2 (14) and its dimerization partner EGFR (15), which are both transcriptionally regulated by YB-1 (6). Yet a link between YB-1 and STAT3 has not been previously documented. Inhibiting YB-1 in BT474-m1 or Au565 cells for 3 days decreased P-STAT3$^{S727}$ and its downstream gene MCL-1; however, P-STAT3$^{Y705}$ was undetectable and total STAT3 was unaffected (Fig. 2A). The STAT3 downstream gene survivin did not change. The reduction in P-STAT3$^{S727}$ correlated with decreased signaling through P-ERK1/2$^{T202/Y204}$ and P-mTOR$^{S2448}$. Surprisingly, total mTOR was also decreased (Fig. 2*A*). In addition, after YB-1 knockdown, the decrease in mTOR protein levels were noted in other cell lines, namely SUM149 (triple-negative breast cancer) and SF188 (pediatric glioblastoma) cells (Supplementary Fig. S2*A–B*). However, mTOR transcript level did not decrease after YB-1 inhibition (Supplementary Fig. S2*C–D*), ruling it out as a direct transcriptional target. Additional studies are under way to investigate whether YB-1 regulates the rate of mTOR translation or sustains its protein stability. Independent of how mTOR is altered by YB-1, it could have an important effect on the STAT3 pathway because it is known to phosphorylate STAT3$^{S727}$ (16).

After cytokine stimulation, STAT3 is phosphorylated at Y705 leading to cooperation with S727 for maximal transcriptional activation (17). However, Notch signaling leads to P-STAT3$^{S727}$ in the absence of P-STAT3$^{Y705}$ (18). Similarly, P-STAT3$^{S727}$ is essential for the survival of macrophages in the absence of P-STAT3$^{Y705}$ (8). Given our data, we suspected that STAT3 was able to protect the BT474-m1 and Au565 cells from apoptosis upon phosphorylation at S727, although it seems that Y705 was not required. In support of this, we confirmed the absence of Y705 phosphorylation by comparing the BT474-m1 cells to the MDA-MB-231 cells, which are known to express high levels of P-STAT3$^{Y705}$ (data not shown; ref. 19). We determined STAT3 was indeed active by isolating nuclear proteins from BT474-m1 cells and then probing an MCL-1 promoter sequence using gel shift assays. Nuclear isolates bound to the MCL-1 promoter, which was inhibited with unlabeled oligonucleotide or by preincubating the nuclear extracts with an antibody to STAT3 (Fig. 2*B, lanes 1–4*). Similar to the effect of siYB-1, inhibition of STAT3 expression using siRNA decreased expression of MCL-1 protein and thereby increased P-H2AX$^{S139}$ (Fig. 2*C, left* and *right columns*, respectively). Finally, transfection of a constitutively activated form of STAT3 (STAT3C; ref. 11) rescued the cells from siYB-1#2–induced apoptosis based on reduced P-H2AX$^{S139}$ (Fig. 2*D*). Taken together, we concluded that YB-1 engages the STAT3 prosurvival pathway to protect breast cancer cells from apoptosis.

Given this, we addressed whether inhibiting YB-1 could suppress the tumorigenic potential of HER-2 overexpressing breast cancer cells by examining growth in soft agar and then in mice. To do so, we showed that inhibition of YB-1 with siYB-1#1 and siYB-1#2 silenced the expression of the target protein for up to 14 days (Fig. 3*A*). In soft agar, YB-1 inhibition prevented colony growth by 85% to 92% compared with the control (Fig. 3*B* and *C, left column*). Au565 cells responded similarly (data not shown). Colony formation was inhibited in the MDA-MB-453 cells but to a lesser degree (∼50%; Fig. 3*C, right column*). Finally, we characterized the tumor growth of BT474-m1 cells in mice and determined that >80% of the mice develop tumors within 1 week (Fig. 4*A*), making this a convenient model for studying the effect of relatively short-lived siRNAs *in vivo*. We therefore transfected the cells with siYB-1#1 or siYB-1#2 for 24 hours and then injected 1 million cells into the hind flank of nude mice. Inhibition of YB-1 suppressed tumor formation throughout the first 2 weeks ($n$ = 6; Fig. 4*A*). By the third week, smaller but detectable tumors were apparent in four of six and five of six mice injected with siYB-1#1 and in siYB-1#2–treated cells, respectively (Fig. 4*A–B, inset*, representative images of tumors). As we suspected, the small tumors that developed by the third week, from the cells pretreated with siYB-1, re-expressed YB-1 indicating that the siRNA was no longer active (Fig. 4*C*). Taken together, our data shows that inhibiting YB-1 disrupts the tumor initiating potential of HER-2 overexpressing breast cancer cells likely via sensitizing the cells to apoptosis by interfering with the STAT3 pathway.

It is noteworthy that in a recent study the PTEN/mTOR/STAT3 pathway reportedly promotes the growth of tumor-initiating cells in breast cancer cells (20). In that study, STAT3 was specifically phosphorylated at S727 based on reverse phase array profiling. Importantly, inhibition of STAT3 with siRNA or a small molecule called IS3 295 selectively blocked the growth of these cells and perturbed the establishment of tumors in mice (20). Because we find that inhibiting YB-1 interferes with this pathway, it seems reasonable that it could also suppress the growth of tumor initiating cells by altering this network.

To conclude, these studies provide preclinical rationale for targeting YB-1 in HER-2 overexpressing or triple-negative breast cancers, setting forward the idea that it may be a good molecular target across different tumor subtypes.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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