Y-Box Binding Protein-1 Induces the Expression of CD44 and CD49f Leading to Enhanced Self-Renewal, Mammosphere Growth, and Drug Resistance

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Abstract

Y-box binding protein-1 (YB-1) is an oncogenic transcription/translation factor expressed in >40% of breast cancers, where it is associated with poor prognosis, disease recurrence, and drug resistance. We questioned whether this may be linked to the ability of YB-1 to induce the expression of genes linked to cancer stem cells such as CD44 and CD49f. Herein, we report that YB-1 binds the CD44 and CD49f promoters to transcriptionally upregulate their expressions. The introduction of wild-type (WT) YB-1 or activated P-YB-1S102 stimulated the production of CD44 and CD49f in MDA-MB-231 and SUM 149 breast cancer cell lines. YB-1–transfected cells also bound to the CD44 ligand hyaluronan more than the control cells. Similarly, YB-1 was induced in immortalized breast epithelial cells and upregulated CD44. Conversely, silencing YB-1 decreased CD44 expression as well as reporter activity in SUM 149 cells. In mice, expression of YB-1 in the mammary gland induces CD44 and CD49f with associated hyperplasia. Further, activated mutant YB-1S102D enhances self-renewal, primary and secondary mammosphere growth, and soft-agar colony growth, which were reversible via loss of CD44 or CD49f. We next addressed the consequence of this system on therapeutic responsiveness. Here, we show that paclitaxel induces P-YB-1S102 expression, nuclear localization of activated YB-1, and CD44 expression. The overexpression of WT YB-1 promotes mammosphere growth in the presence of paclitaxel. Importantly, targeting YB-1 sensitized the CD44high/CD24low cells to paclitaxel. In conclusion, YB-1 promotes cancer cell growth and drug resistance through its induction of CD44 and CD49f.

Introduction

Breast cancer relapse confers a poor prognosis, decreasing survival rates from 80% to 60% when the recurrence is local, and from 80% to 10% for metastatic disease. Relapse is postulated to be mediated by the persistence of cancer stem cells that have survived an initial treatment regimen with radiation (1) and/or chemotherapy (2–4) possibly due to a selective resistance of the cancer stem cells to these agents. Human breast cancer stem cells have been identified as a phenotypically restricted subset of CD44+/CD24−/low cells that form tumors in immunocompromised mice in limiting dilution transplant assays (5). The same CD44+/CD24−/low subset has also been associated with breast tumor mammosphere generation in vitro (3). However, the nature of the essential molecular properties of breast cancer stem cells has remained poorly defined.

Y-box binding protein-1 (YB-1) is a transcription/translation factor that is commonly overexpressed in many cancers, including human breast cancer (40%; refs. 6–8). This elevated expression of YB-1 in human breast cancer correlates with high rates of relapse (6). Targeted overexpression of YB-1 in the mouse mammary gland leads to the development of mammary tumors (9), confirming a role of YB-1 as an oncogene in that tissue. YB-1 is directly phosphorylated, and therefore activated, on its serine 102 site by Akt (10) and even more potently by ribosomal S6 kinase (RSK; ref. 11), a major component of the mitogen-activated protein kinase (MAPK) pathway. Thus, YB-1 is positioned as a key player in the phosphoinositide 3-kinase–S6 kinase (PI3K/Akt and MAPK pathways. YB-1 regulates genes that promote breast cancer cell growth and...
survival, including EGFR (12), Her-2 (erbB2; ref. 7), PI3KCA (13), and the MET (c-Met) receptor (14), and is biologically essential for breast cancer cell growth in vitro (7, 10–12) and in vivo (15). YB-1 expression has also been shown to be associated with drug resistance via the induction of genes such as MDR-1 (16, 17). Consistent with this, inhibition of YB-1 was found to sensitize breast cancer cells to paclitaxel (15), a chemotherapeutic agent commonly used in the clinic to treat advanced breast cancer. To elucidate the transcriptional programming activity of YB-1, we did chromatin immunoprecipitation-on-chip (ChIP-on-chip) assays. This screen revealed a subset of genes known to be active in and important to a number of stem cell populations, including c-KIT, BMI-1, members of the WNT and NOTCH signaling pathways, as well as CD44 and CD49f (also known as α6 integrin; ref. 14). We also found that YB-1 transcripts were present in purified CD44+/CD49f+ subpopulations of primitive human mammary progenitor cells isolated from normal reduction mammmoplasties (14). Taken together, this led us to test the hypothesis that YB-1 plays a key role as an oncogene by transactivating genes associated with a cancer stem cell phenotype.

Materials and Methods

**Cell lines and culturing.** The human breast cancer cell lines MDA-MB-231, MDA-MB-468, and SUM 149 were purchased and maintained as previously described (13, 14). The cell lines were characterized for CD44 and CD24 expression by flow cytometry (Supplementary Fig. S1A–E). Cell autofluorescence was taken into account.

**Chromatin immunoprecipitation.** ChIP using a polyclonal chicken antibody to precipitate endogenous YB-1 was done as previously described (7). Three primer sets were designed to flank seven putative YB-1 binding sites (ATTG) in the first 2 kb of the CD44 promoter, and similarly, two primer sets flanking eight putative YB-1 binding sites in the first 2 kb of the CD49f promoter were also designed. Details are described in Supplementary Materials and Methods.

**Immunofluorescence assay.** Immunofluorescence assays were done as previously described (13, 14). Antibodies used were rat anti-human and anti-mouse FITC-conjugated anti-CD49f (1:25, clone GoH3, BD Pharmingen), rat anti-human phycocerythrin (PE)-conjugated anti-CD44 (1:100, clone 515, BD Pharmingen), unconjugated rabbit anti-human phospho-YB-1 S102 (P-YB-1 S102; 1:100, clone C34A2, Cell Signaling Technology), and secondary anti-rabbit FITC (1:200; Jackson ImmunoResearch Laboratories, Inc.). Details are discussed in Supplementary Materials and Methods.

**Immunoblotting.** Immunoblotting analyses were done as previously described (7). Antibodies used were as follows: anti-total YB-1 (1:2,000, #ab12148, Abcam; 1:1,000, #2749, Cell Signaling Technology); 1:2,000, a polyclonal antibody designed against the COOH terminus of YB-1, produced by and a generous gift from Dr. Colleen Nelson, University of British Columbia, Vancouver, BC, Canada), anti–P–YB-1 S102 (1:1,000, clone C34A2, Cell Signaling Technology), anti–CD44 (1:500, clone EPR1013Y, Abcam), anti–CD49f (1:1,000, #3750, Cell Signaling Technology), anti–Flag (1:2,000, clone M2, Sigma-Aldrich), anti–total Akt (T-Akt; 1:1,000, #9272, Cell Signaling Technology), anti–pan–actin (1:1,000, #4968, Cell Signaling Technology), and anti–vinculin (1:1,000, clone hVIN-1, Sigma-Aldrich).
Luciferase reporter assay. SUM 149 EV and shYB-1 cells were seeded in a six-well plate (4 × 10^5 cells per well) and allowed to adhere. The cells were then transfected with pGL3b (1 μg) or pGL3b-CD44P (1 μg, Addgene, deposited by Dr. Robert Weinberg, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA; ref. 2) in triplicate using Lipofectamine 2000 (Invitrogen). Cells were lysed 24 h after transfection in passive lysis buffer (Promega) and luciferase activity was measured.

Matrigel semi-fluid basement membrane growth assay. Matrigel basement membranes (BD Biosciences) were added at 40 μL/well in a 96-well plate and incubated briefly at 37°C.
for solidification. MDA-MB-231 and SUM 149 cells in single-cell suspension were added in their respective media at 2 × 10^3 and 5 × 10^3 cells per well. Growth was assessed in photographed fields 7 d after seeding.

**DNA plasmid transfection.** In a six-well plate format, 2 or 4 μg of a Flag:EV, Flag:YB-1 WT, or Flag:YB-1 S102D plasmid were transfected with 5 or 10 μL of Lipofectamine 2000 (Invitrogen), respectively, as specified by the manufacturer’s protocol. Cells were harvested 96 h after transfection.

**Stable transfectant cell lines.** Stable EV, shYB-1, Flag:EV, Flag:YB-1 WT, and Flag:YB-1 S102D cell lines were established by transfecting 4 μg of DNA constructs as described. G418 (400 μg/mL; Calbiochem, EMD Chemicals) was added to the culture media and replaced every 3 to 4 d. Cells were continually split at low density to allow for optimal selection of transfecants with acquired G418 resistance.

**YB-1 transgenic mouse and mammary gland sectioning.** YB-1 transgenic (1tg 2 line) and wild-type (WT) mice were reared from cryopreserved embryos generated and characterized previously (9), where expression of a human hemagglutinin (HA)-tagged YB-1 cDNA was controlled by the ovine β-lactoglobulin promoter. For our study, histologic sections were obtained from the mammary glands of 6- to 8-mo-old female mice post-lactation after two cycles of mating and nursing. The mice were mated twice and their pups were nursed for 3 wk each time. The YB-1 transgenic and control mice were euthanized at the end of their second lactation (day 20 to 22 after the birth of the pups) and mammary gland tissues were collected. The mammary glands were dipped into Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Finetek USA), frozen, and kept at −80°C. OCT compound–embedded sections were sectioned (6 μm) using a cryostat and placed on Fisherbrand Superfrost Plus glass slides (Thermo Fisher Scientific) and kept at −80°C. The sections were defrosted at room temperature and incubated with PBS for 5 min, fixed with 2% formaldehyde for 20 min, and rinsed twice with PBS. Subsequent immunofluorescence staining and imaging protocol were done as described above. The only exception is that a primary purified rat anti-mouse CD44 antibody (1:100, clone IM7, BD Pharmingen) and a secondary antirat Alexa 546 antibody (Molecular Probes, Invitrogen) were used. A HA-specific antibody (Santa Cruz Biotechnology) was used with immunohistochemistry methods. Details are described in Supplementary Materials and Methods.

**Anticancer drug screen and further evaluation of paclitaxel treatment.** SUM 149 cells were treated with commonly used chemotherapeutic agents at 10 μmol/L and cell viability was assessed as previously described (18). The cells were also stained with anti-CD44-PE (15, clone G44-26, BD Pharmingen) and assessed as previously described (18). SUM 149 cells were also treated with paclitaxel (Sigma-Aldrich) dissolved in DMSO at 10 nmol/L for 48 or 72 h before doing immunoblotting and ChIP as described. MDA-MB-231 Flag:EV and Flag:YB-1 WT cells were treated in monolayer or in the mammosphere assay in the presence of 0 nmol/L or 10 nmol/L of paclitaxel with DMSO as vehicle control for 7 d. MDA-MB-231 cells grown in monolayer had the media changed at day 4 and cells were harvested for immunoblotting on day 7.

**Statistical analysis.** All quantitative data are represented as mean ± SE of three independent experiments. P values were generated using paired Student’s t test, where * denotes P < 0.05, ** denotes P < 0.01, and *** denotes P < 0.001.

**Results**

**YB-1 binds to upstream regulatory regions of the genes encoding CD44 and CD49f and regulates their expression.** Several putative YB-1 binding sites were located 2 kb upstream of the transcriptional start site of the CD44 and CD49f genes, initially identified in a ChIP-on-chip screen (ref. 14; Fig. 1A, top). To validate these results, we did conventional ChIP analyses and provided direct evidence of the ability of YB-1 to bind to the promoters of CD44 and CD49f genes in two human breast cancer cell lines (MDA-MB-468 and SUM 149; Fig. 1A, bottom). Immunohistochemical analysis of SUM 149 cells grown either in monolayer cultures or as xenografts illustrated coincident staining of P-YB-1 S102 and CD44 protein in individual cells (Fig. 1B). Therefore, we first asked whether the expression of CD44 would fluctuate concordantly with changes in activated YB-1 (P-YB-1 S102). Quantification of this association of CD44 expression with the presence of P-YB-1 S102 was shown by isolating the highest and lowest 10% of CD44-expressing viable SUM 149 cells by fluorescence-activated cell sorting (FACS) and costaining with CD44 and P-YB-1 S102 antibodies. There, we observed a 2-fold difference in P-YB-1 S102 levels between the subsets expressing the highest and lowest levels of CD44 (Fig. 1C, top), corresponding to enhanced growth in mammosphere cultures and soft agar (Fig. 1C, bottom).

We next asked whether YB-1 is a determining regulator of CD44 and CD49f expression in breast cancer cells by knocking down YB-1 expression in MDA-MB-231 and SUM 149 cells using three siRNA oligonucleotides designed to target YB-1 transcripts specifically. The result was a decrease in the transcript and protein levels of CD44 and CD49f (Fig. 2A and B). To obtain stable inhibition of YB-1 expression, SUM 149 cells

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**Figure 3.** YB-1 overexpression induces CD44 and CD49f expression. A, transient, incremental exogenous Flag:YB-1 WT and Flag:YB-1 S102D expression in MDA-MB-231, MDA-MB-468, and SUM 149 cells was achieved via plasmid transfections, visualized by immunoblotting with a Flag antibody, and affected CD44 and CD49f transcript levels were measured by quantitative real-time PCR experiments. Vinculin served as loading control. B, MDA-MB-231 cells stably expressing Flag:EV, Flag:YB-1 WT, and Flag:YB-1 S102D were analyzed for long-term Flag, CD44, and CD49f expression by immunoblotting. C, CD44 and CD49f protein expression was induced by Flag:YB-1 S102D in SUM 149 cells. D, BGL-YB-1/HA and WT counterpart mammary glands were collected from 6- to 8-mo-old female mice post-lactation, frozen in OCT compound, and sectioned. Slides were stained with primary CD49f-FITC antibody (green) or unconjugated CD44 with secondary Alexa 546 Fluor antibody (red) or unconjugated P-YB-1 S102 with secondary antirabbit FITC antibody and DAPI. Representative images of cells from eight mice are shown. Insets, enlarged photographs of the CD44 and CD49f membrane-localized staining. Lactating mammary glands from the same mice were stained for HA by immunohistochemistry techniques.
YB-1 Regulates CD44 and CD49f

A

Molecular Dynamics of YB-1

B

MDA-MB-231
Flag Lines
EV WT S102D

CD44
CD49f
Flag-YB-1
T-YB-1
Actin

C

SUM 149

EV

CD44
CD49f
CD44, CD49f
CD44, CD49f, DAPI

D

WT
YB-1 TG

HA

P-YB-1

P-YB-1

CD49f
CD44, DAPI
CD44, DAPI

iii

iv

iii

iv
were transfected with anti-YB-1 shRNAs. This produced sustained decreases in CD44 and CD49f transcript and protein levels as compared with control cells transfected with an empty vector (EV; Fig. 2C). Stable knockdown of YB-1 transcripts also resulted in a decreased expression of CD104 (β1 integrin), a β-chain partner of CD49f (Fig. 2B and C). Further, YB-1 shRNA–expressing cells showed a 50% decrease in CD44 promoter activity (Fig. 2D), suggesting YB-1 as a significant regulator of CD44.

Conversely, when we forced a transient elevation in the expression of either Flag:YB-1WT or a constitutively active Flag:YB-1S102D, increases in CD44 and CD49f transcript levels were obtained in both cases, with the largest increases resulting from the constitutively active YB-1 construct (Fig. 3A). A similar result was seen when these constructs were stably expressed in the same cells (Fig. 3B). Importantly, we confirmed that a heightened expression of CD44 and CD49f was concomitantly induced in the same transfected YB-1–overexpressing cells (Fig. 3C). Likewise, expression of YB-1 in immortalized breast epithelial cells, which typically have very little of this protein, caused a measurable increase in CD44 mRNA and protein (Supplementary Fig. S2). To functionalize the effect in breast cancer cells, we determined that MDA-MB-231 cells that stably express Flag:YB-1WT or Flag:YB-1S102D have a greater affinity for the CD44 ligand hyaluronan (Supplementary Fig. S3).

To determine whether elevated YB-1 expression would have a similar effect on the expression of CD44 and CD49f in primary mammary epithelial cells in vivo, we evaluated the mammary glands of 6- to 8-month-old transgenic mice expressing human HA-tagged YB-1 cDNA under the control of the ovine β-lactoglobulin promoter (9). To confirm transgene expression, we stained the tissues for HA and showed that YB-1WT expressing cells were treated with control, CD44, or CD49f siRNAs, were also examined for their ability to form colonies in soft agar subsequent to the gene knockdowns. MDA-MB-231 Flag:EV, Flag:YB-1WT, and Flag:YB-1S102D cells, as well as Flag:YB-1S102D cells expressing cells to grow in soft agar and this was again blocked when the expression of either CD44 or CD49f was silenced (Fig. 4C). The ability of MDA-MB-231 cells to proliferate and form extensive structures in Matrigel cultures was also inhibited when the cells were pretreated with CD44, CD49f, or YB-1 siRNAs (Fig. 4D). These findings show a critical role of YB-1 in enabling breast cancer cells to grow in three-dimensional assays in a manner that is dependent on the downstream upregulation of CD44 and CD49f. Notably, the expressions of CD44 and CD49f seem to be interdependent in these cell lines, suggesting a functional association.

Paclitaxel-treated SUM 149 cells show increased expression of YB-1 and CD44. The preceding findings intimate that the increased expression of YB-1 associated with breast cancer relapse in patients might be due to a selection of cells showing an increased expression of CD44. To investigate this...
possibility, we screened a panel of commonly used anticancer drugs (all at 10 μmol/L) for their ability to kill SUM 149 cells in vitro in parallel with an assessment of the proportion of CD44High cells present 72 hours later. All compounds killed >80% of the input cells and the percentage of CD44High cells among the survivors was consistently higher than that in the control cells (Supplementary Table S1). The most marked effect in this regard was obtained with paclitaxel treatment, which produced an almost 10-fold increase in the proportion of viable CD44High cells (Supplementary Table S1). Next, we determined that paclitaxel at a much lower and more clinically relevant concentration of 10 nmol/L was able to promote nuclear expression of P-YB-1S102 (Supplementary Fig. S5). Likewise, the chemotherapeutic agent cisplatin had the same effect (Supplementary Fig. S5). Related to these findings, paclitaxel increased YB-1 binding to the CD44 promoter after 48 hours (Fig. 5A), which also induced P-YB-1S102, correlating with activation of the RSK pathway based on higher levels of P-RSKS221(S227) and a second substrate, P-GSK3βS9 (Fig. 5A). The induction of this pathway then led to increased CD44 after 72 hours (Fig. 5B). In MDA-MB-231 cells stably expressing Flag:YB-1WT, after 10 nmol/L paclitaxel treatment, there was a further increase in CD44 expression and a greater amount of Flag:YB-1WT detected per cell on the whole (Fig. 5C). Further, the Flag:YB-1WT-transfected MDA-MB-231 cells formed more mammospheres in the presence of paclitaxel (10 nmol/L) as compared with control transduced cells (Fig. 5D). Finally, we sorted the SUM 149 cells for CD44High/CD24Low by FACS (Fig. 6A) and confirmed enrichment by immunofluorescence, comparing CD44High sorted cells to unsorted cells for CD44 expression (Fig. 6B). The CD44High/CD24Low cells were then transfected with YB-1 siRNAs for 48 hours and treated with paclitaxel (10 nmol/L) for 24 hours. Notably, the CD44High/CD24Low cells treated

Figure 5. Paclitaxel treatment preferentially selects for breast cancer cells with elevated P-YB-1S102 expression. A, treatment of SUM 149 cells with 10 nmol/L paclitaxel for 48 h increased YB-1 binding to the CD44 promoter based on ChIP. The same cells were harvested for immunoblotting to detect P-RSKS221(S227), P-YB-1S102, total YB-1, and P-GSK3βS9. Vinculin served as loading control. B, SUM 149 cells were treated with 10 nmol/L paclitaxel for 72 h to examine CD44 levels. C, in MDA-MB-231 cells, where EV and YB-1 expression vector were stably overexpressed, cells were treated with 10 nmol/L paclitaxel for 7 d and harvested for immunoblotting to assess CD44, exogenous Flag:YB-1, and endogenous total YB-1 levels. Endogenous total YB-1 served as loading control. D, MDA-MB-231 EV- and YB-1-overexpressing cells were grown in mammosphere cultures in the presence of DMSO or 10 nmol/L paclitaxel for 7 d. Bar graph represents the percent of spheres obtained in the paclitaxel-treated cultures as compared with DMSO for each cell type.
with the scrambled control were resistant to paclitaxel; however, if YB-1 was silenced, the growth of this tumor-initiating cell population was significantly attenuated based on crystal violet staining (Fig. 6C) and quantification of cell viability by Hoechst33342 staining (Fig. 6D).

**Discussion**

Here, we present YB-1 as the first oncogene identified that induces both CD44 and CD49f. YB-1 uses CD44 and CD49f to promote self-renewal, mammosphere growth, soft-agar colony formation, and drug resistance in breast cancer cells. The YB-1/CD44/CD49f relationship was also evident in the mammary glands of YB-1 transgenic mice as well as in immortalized normal breast epithelial cells. These findings have broad implications given the important role for CD44 in mediating tumor initiation and cancer relapse. It also gives us some insight into why YB-1 may be so highly associated with breast cancer recurrence as previously reported (6). Consistent with this idea, we showed that CD44 cells were resistant to chemotherapy whereby the agents stimulate YB-1 to bind to the CD44 promoter. Furthermore, we report that eliminating YB-1 in the CD44 high/CD24 low population can improve cellular response to paclitaxel.

We question whether YB-1 somehow hijacks normal breast stem cells during neoplastic progression. This extends our initial finding that YB-1 is detectable in primary mammary progenitor cells from women who have undergone reduction mammoplasties (14). In the study presented herein, we show that inducing YB-1 in immortalized breast epithelial cells (HTRY) causes an increase in CD44 mRNA and protein. Consistent with this, both CD44 and CD49f are selectively expressed on normal stem cells in the breast and other tissues (21–24). We therefore propose that early activation of YB-1 during mammary tumorigenesis involves a primitive cell population.

![Figure 6. Targeting YB-1 suppresses the growth of CD44 high/CD24 low cells. A, SUM 149 cells were analyzed by FACS and the CD44 high/CD24 low cells were collected. B, the sorted cells fractions were profiled by immunofluorescence for CD44 enrichment and compared with the unsorted SUM 149 cells as well as the CD44 low/CD24 high cells. C, the CD44 high/CD24 low cells were treated with 20 nmol/L YB-1 siRNAs for 48 h and exposed to 10 nmol/L paclitaxel for 24 h. YB-1 had an inhibitory effect on the growth of the CD44 high/CD24 low cells when exposed to paclitaxel based on crystal violet staining. D, cell viability was quantified using Hoechst33342 staining and the average number was taken relative to the control treated with scrambled siRNA plus paclitaxel. YB-1 silencing was confirmed by immunoblotting.](image-url)
CD44 is a widely recognized marker of many cells with cancer-initiating (5, 25–27) and, in some cases, metastatic activities (28–30). As such, understanding its regulation has implications in tumor cell invasion, escape from apoptosis, and drug resistance. CD44 couples to a number of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), c-Met receptor, and CD49f to stimulate signal transduction (14, 31). It also partners with the oncogene RHAMM to promote signaling through the extracellular signal–regulated kinase-1/2 pathway whereby CD44 stimulates tumor cell invasion (32, 33). Importantly, CD44 expression is thought to confer drug resistance by directly inducing downstream stem cell–associated and/or drug-resistant genes, including Nanog and MDR-1, through its interaction with hyaluronan (34–36). This is attributable to activation of the PKC and signal transducer and activator of transcription-3 (STAT3) signaling pathways (34–36). Notably, as CD44 expression was increased, cells were less sensitive to paclitaxel due to STAT3 activation and consequent upregulation of MDR-1. YB-1 expression has already been reported to be associated with chemoresistance perhaps through the regulation of MDR-1 (16, 17). Bridging these studies is the evidence for the role of YB-1 in invasion (13) that may also be attributed to CD44 and CD49f. It is foreseeable that it induces other genes associated with cancer stem cells as well. We reported that YB-1 also regulates the stem cell–associated Met receptor (c-Met), EGFR, and Her-2 (erbB2; refs. 7, 14), signaling partners of CD44 and CD49f (37–40), suggesting a cooperative complex on the surface of tumor-initiating cells that activates downstream pathways such as the PI3K-Akt (40–42) and MAPK pathways (refs. 33, 43; Supplementary Fig. S6). The crosstalk between these receptors and their downstream effectors provide interesting opportunities for future therapeutic possibilities. We conclude that YB-1 inhibition may be a new approach to eliminating tumor-initiating cells and, by extension, possibly cancer recurrence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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