Cancer Research

The Integrin $\alpha_v\beta_{3\text{-}5}$ Ligand MFG-E8 Is a p63/p73 Target Gene in Triple-Negative Breast Cancers but Exhibits Suppressive Functions in ER^+ and $erbB2^+$ Breast Cancers

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Abstract

The progression from preinvasive lesion to invasive carcinoma is a critical step contributing to breast cancer lethality. We identified downregulation of milk fat globule-EGF factor 8 (MFG-E8) as a contributor to breast cancer progression using microarray analysis of laser capture microdissected (LCM) tissues. We first identified MFG-E8 downregulation in invasive lesions in transgenic mammary tumor models, which were confirmed in LCM-isolated human invasive ductal carcinomas compared with patient-matched normal tissues. *In situ* analyses of MFG-E8 expression in estrogen receptor (ER) positive cases confirmed its downregulation during breast cancer progression and small inhibitory MFG-E8 RNAs accelerated ER^+ breast cancer cell proliferation. MFG-E8 also decreased in $erbB2^+$ human cancers and erbB2 transgenic mice lacking MFG-E8 showed accelerated tumor formation. In contrast, MFG-E8 expression was present at high levels in triple-negative (ER^-, PgR^-, $erbB2^-$) breast cancers, cell lines, and patient sera. Knockdown, chromatin immunoprecipitation, and reporter assays all showed that p63 regulates MFG-E8 expression, and MFG-E8 knockdowns sensitized triple-negative breast cancers to cisplatin treatment. Taken together, our results show that MFG-E8 is expressed in triple-negative breast cancers as a target gene of the p63 pathway, but may serve a suppressive function in ER^+ and $erbB2^+$ breast cancers. Its potential use as a serum biomarker that contributes to the pathogenesis of triple-negative breast cancers urges continued evaluation of its differential functions. *Cancer Res; 71(3); 937–45. ©2010 AACR.*

Introduction

Tumor progression results from accumulation of genetic changes that permit autonomous growth of malignant cells (1). While evaluating the genetics of tumor progression, we identified downregulation of milk fat globule—EGF8 (MFG-E8) mRNA in a microarray analysis of invasive murine tumors (2), but we were puzzled by its original description as a breast cancer antigen (3). A monoclonal antibody raised against human mammary epithelial cells was originally used to demonstrate elevated circulating levels of a 46-kD protein (BA46) in patients with metastatic breast tumors (4). BA46 radioimmune assays accurately monitored tumor burden and the α-BA46 antibody slowed tumor growth in xenotransplantation studies (5, 6). However, cDNA cloning of BA46 revealed that it was the normal breast protein milk fat globule factor 8 (MFG-E8)/lactadherin (7, 8). Importantly, MFG-E8 is the ligand for $\alpha_v\beta_{3\text{-}5}$ integrins (8), which mediates apoptotic cell phagocytosis (9). Likewise, homozygous MFG-E8 loss impairs two mammary developmental stages; its loss blocks both branching morphogenesis (10) and clearance of apoptotic cells during involution (11). Finally, loss of integrins β3, β5, or β3β5 accelerates MMTV–*erbB2* tumor formation (12).

In contrast, microarray studies have shown that MFG-E8 mRNA increases in a diagnostic gene cluster in basal breast cancers (13, 14). p63 gene expression is generally restricted to the basal myoepithelial cell layer of mammary glands and p63/p73 regulation plays a role in the biology of tumors arising from these cells (15). Recent developments in our understanding of BRCA1's functions have suggested new therapeutic strategies incorporating platinum chemotherapy and poly (ADP-ribose) polymerase (PARP) inhibitors for triple-negative tumors, which encompass the basal subtype distinguished by its unique gene signature (16). The use of cisplatin as a targeted therapy is based on findings that BRCA1 defective cells are particularly susceptible to its effects (17). Studies of the p53/p63/p73 protein network provided additional support for this approach. Importantly, both p63 and p73 control of the p53 apoptosis program is a necessary and sufficient

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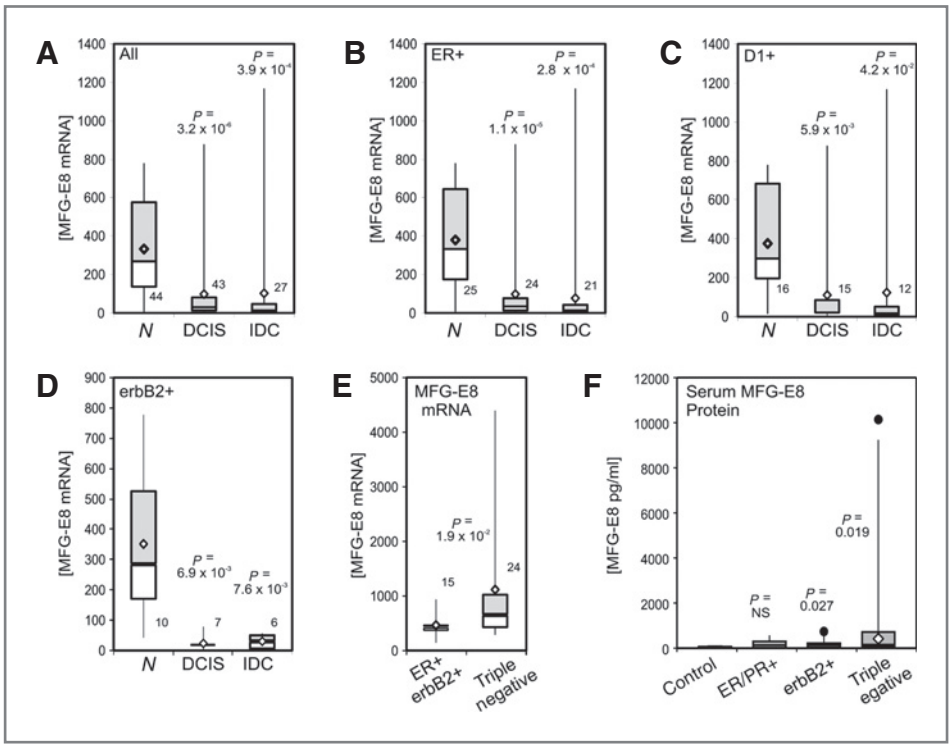

Figure 1. Changes in MFG-E8 expression during tumor progression differ among breast cancer subtypes. A–C, box and whisker charts for MFG-E8 mRNA using LCM-isolated tissues from human pathologic specimens where normal, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma components could be isolated from the same patient. The *y*-axis measures MFG-E8 levels in arbitrary units based on a standard curve of MFG-E8 PCR product used in each qRT-PCR run. The patient cohort was originally described in Ma et al. (20). The median value is plotted as the junction of the shaded and empty rectangles. The mean value is plotted as the diamond symbol. A, levels of expression of MFG-E8 comparing all available specimens. B, MFG-E8 expression in the ER^+ patients. C, MFG-E8 expression in patients where a 2-fold or greater increase in cyclin D1 was identified in the same specimen. D, box and whisker charts for fold change in MFG-E8 mRNA using LCM-isolated tissues from human pathologic specimens where normal, DCIS, and invasive ductal carcinoma components could be isolated from the same patient whose tumor was $erbB2^+$ ($N = 10$). E, mRNAs from patients with sporadic basal breast cancers included in a previous study that described their X chromosomal abnormalities were generously provided by the Harvard Breast Cancer SPORE tissue bank. The details of the patients evaluated are described in Richardson et al. (13). We measured MFG-E8 mRNA levels, which are plotted in box and whisker charts as in A–D. F, a commercial MFG-E8 ELISA was used to measure MFG-E8 protein in patient sera. Shown are MFG-E8 protein levels in serum from patients with tumors of the indicated phenotypes plotted using box and whisker plots. $N = 10$ for each tumor phenotype. Differences between ER^+ versus controls were not significant. Differences between control and erbB2 ($P = 0.027$) as well as triple-negative cancers ($P = 0.019$) were significant by Mann–Whitney rank-sum test. (The data were not normally distributed.)

contributor to the effects of cisplatin. Additionally, a recent publication identified consensus p53/p63/p73 binding sites in the *MFGE8* promoter, which control transcriptional responses to p63/p73 in skin (18). Here, we show that MFG-E8 is a potential biomarker for triple-negative breast cancers due to its upregulation by p63/p73, which contrasts with its down-regulation in ER^+ and $erbB2^+$ breast cancers.

Materials and Methods

Animals

MFG-E8 null mice obtained from Barry Shur (Emory University; ref. 19) were crossed with nonmutant MMTV-*erbB2* mice (Jackson Labs). Tumor incidence was evaluated by routine histology or by counting masses in mammary gland whole mounts at 15 months of age. Conditional p63 knockdowns using $p63^{flox}$ mice are described in Supplementary Materials. Animal experiments followed approved standards of the MGH Animal Advisory Committee.

Patient cohorts, laser capture microdissection, RNA isolation and amplification, microarray analysis, and qRT-PCR analysis

The patient cohort used for laser capture microdissection (LCM) specimens was previously described ($N = 36$; ref. 20). Additional $erbB2^+$ patients were added (total $N = 10$). Tumor samples were used to compare MFG-E8 and p63 mRNA levels in the basal versus other tumors that were described in Richardson et al. (13). Unpaired 2-tailed Student's *t* tests were used to determine statistical significance of all data unless otherwise noted.

In situ hybridization

In situ hybridization procedures are described in the Supplementary Methods. MFG-E8 expression was scored by an investigator (D.S.) who was blinded to the estrogen receptor (ER) status of the tumors and who had not performed the *in situ* staining. *In situ* staining was scored as 0 (no staining), + (weak positive), ++ (moderate positive), and +++ (strong positive).

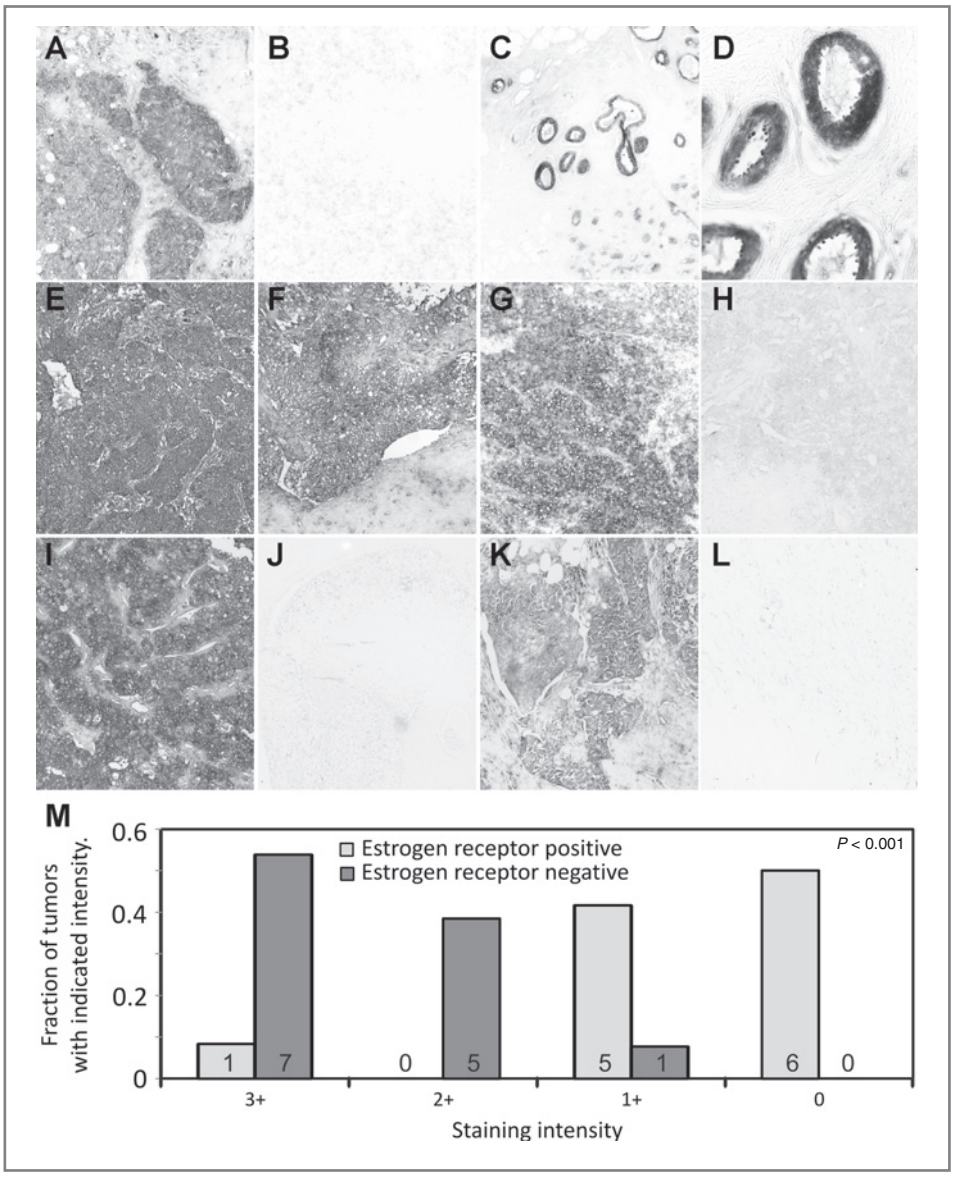

Figure 2. Photomicrographs of *in situ* analyses for MFG-E8 mRNA in human breast cancer samples. *In situ* analyses were performed as described in Supplementary Materials and photographed at 100× magnification (except D). A, labeling with an antisense probe of an ER^- breast cancer. B, labeling with a sense probe of the same tumor as in A. C, labeling of an ER^+ tumor at a junction between normal breast tissue in the top half of the panel and less-intensely labeling tumor in the bottom half of the photograph. D, a higher power magnification (400×) picture of the normal-appearing breast tissue shown in C to demonstrate MFG-E8 labeling of ductal tissue throughout all layers. E, an ER^- tumor scored as 3+. Photographic exposure times for this panel were used in subsequent panels F–L to allow comparison of the relative levels of expression in all other samples. F, the most intensely staining ER^+ tumor scored as 3+. Only this single ER^+ tumor fell in the same range of staining as the ER^- group of tumors. G, an ER^- tumor staining 3+. H, the second most intensely staining ER^+ tumor scored as 1+. I, the median most intensely staining ER^- tumor scored as 3+. J, median most intensely staining ER^+ tumor scored as 0 staining. K, the least intensely staining ER^- tumor scored as 2^+. L, an ER^+ tumor scored as 0 staining. M, *in situ* staining was scored by an investigator (D.S.) who was blinded to the ER status of the tumors and had not performed the actual *in situ* staining. Shown is a graph of the fraction of the tumors staining with various intensities, comparing the ER^+ and ER^- samples. The actual number of tumors staining at each value for each type is shown within the columns of the graph. *In situ* staining was scored as 0 (no staining), 1+ (weak positive), 2+ (moderate positive), and 3+ (strong positive). Comparing specimens staining 2+ or 3+ to those staining 0 or 1+, one ER^+ tumor was among the top 13 MFG-E8-expressing tumors, and one ER^- tumor was among the bottom 12 expressing samples. MFG-E8 expression was highly correlated with the ER^+ status of breast cancers across all staining intensities ($P < 0.001$ by chi-square analysis).

Patient sera and MFG-E8 ELISA

MFG-E8 was measured in stage I–III sera obtained prior to treatment from 10 healthy donors, 10 ER and/or PR^+ patients, 10 $erbB2^+$ patients, and 10 triple-negative patients. Sera were obtained from the Dana-Farber Cancer Institute (DFCI) with support from the NCI Breast SPORE program. Written consent was obtained from all subjects under institutional review board approval. MFG-E8 protein was

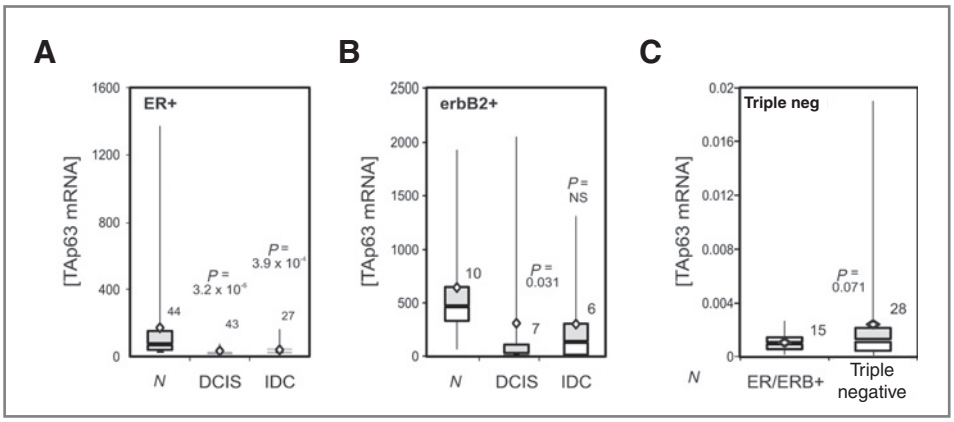

Figure 3. p63 expression uniquely increases during tumor progression in triple-negative breast cancers. A–C, p63 expression changes differ among breast cancer subtypes. Shown are box and whisker charts for the TAp63 isoform mRNA using the tissues described in Figure 1. (Total p63 mRNA measurements gave similar results.) The *y*-axis measures p63 levels in arbitrary units based on a standard curve of 63 PCR product. A, levels of expression of p63 comparing ER^+ specimens. B, p63 expression in the $erbB2^+$ patients. C, differences in p63 expression between $ER^+/erbB2^+$ patients versus patients with basal tumors.

measured using a commercial ELISA (Cusabio Biotech Co.; CSB-E12637h).

Cell culture, RNAi and cisplatin sensitivity

T47D, MCF7, ZR-75-1, BT474, SKBR3, HCC 1937, MDA-MB468, MB231, HCC1143, BT20, and HS578T cells were grown in conditions indicated by the supplier (ATCC). Secreted MFG-E8 was measured in culture supernatants 24 hours after changing media by using the commercial ELISA.

A published lentiviral small inhibitory RNA for p63 (shp63; ref. 15) was transduced, and total RNA and protein were harvested 3 days later for MFG-E8 regulatory studies (15).

To study antiproliferative effects of MFG-E8, MFG-E8 (Ambion; ID numbers 1436, 1531, and 1621) and scramble (Dharmacon D-1205-20) siRNAs were transfected using Oligofectamine (Invitrogen). Ten wells of 96-well plates were seeded at 6,000 cells per well for each treatment condition for MTT assays performed 72 hours after transfection. Two hours after siRNA transfection, RGD blocking peptide (7) or its control peptide from ENZO Life Science was added to the cell culture medium to a final concentration of 2 μg/mL. For cisplatin sensitivity the same siRNAs were transfected, cells were harvested 48 hours after transfection in varying doses of cisplatin (Sigma-Aldrich), and MTT assays were performed as previously described (15).

Western blots

Antibodies used for standard Western blots included anti-MFG-E8 MAB2767 (R&D Systems), anti-p63 (H-129; Santa Cruz Biotechnology Inc.), and anti-actin MAB1501R (Chemicon).

Chromatin immunoprecipitation and luciferase reporter assays

We performed p63 chromatin immunoprecipitation (ChIP) experiments as detailed in Supplementary Materials. MFG-E8 reporter plasmids were provided by Dr. I. Katoh (Ikawa Laboratory, RIKEN, Wako, Japan; ref. 18) and luciferase reporter assays are detailed in Supplementary Materials.

Results

MFG-E8 expression decreases during tumor progression in ER^+ and $erbB2^+$ breast cancers but is increased in triple-negative breast cancers

Comparing mRNA expression changes between invasive tumors and preinvasive mammary tissues, we initially identified MFG-E8 as a uniformly downregulated gene during tumor progression in murine transgenic *erbB2*−, *ras*−, and *cyclin D1*−induced tumors (Supplementary Fig. S1A; ref. 2). An analysis of available SAGE expression data suggested that similar changes also occur in human cancers (Supplementary Fig. S1B). We therefore evaluated MFG-E8 expression changes in LCM-isolated human specimens where the patient's own normal and neoplastic tissues could be directly compared (20; ref. Fig. 1). We found that MFG-E8 mRNA decreased 3.3-fold from normal to ductal carcinoma *in situ* (DCIS) and from normal to invasive ductal carcinoma (IDC) in a mixed breast cancer population (Fig. 1A). This result contrasted with the original description of MFG-E8 as the breast cancer antigen BA46 so we next considered whether MFG-E8 changes might differ among different breast cancer subtypes.

We first found that MFG-E8 decreased 3.9-fold in ER^+ DCIS and 4.8-fold in ER^+ IDC samples (Fig. 1B). This was especially true for cyclin $D1^+$ tumors (Fig. 1C). A similar decrease in MFG-E8 expression was found in LCM-isolated $erbB2^+$ tumors during tumor progression from normal to DCIS to IDC (Fig. 1D). Published microarray data suggested that basal and BRCA-mutated breast cancers likely had increased MFG-E8 mRNA levels (Supplementary Fig. S2), which might explain the original description of MFG-E8 as a metastasis-associated tumor marker in this poor prognosis tumor class. We therefore assessed MFG-E8 in these BRCA1 associated and sporadic basal cancers (13), finding a significant increase in MFG-E8

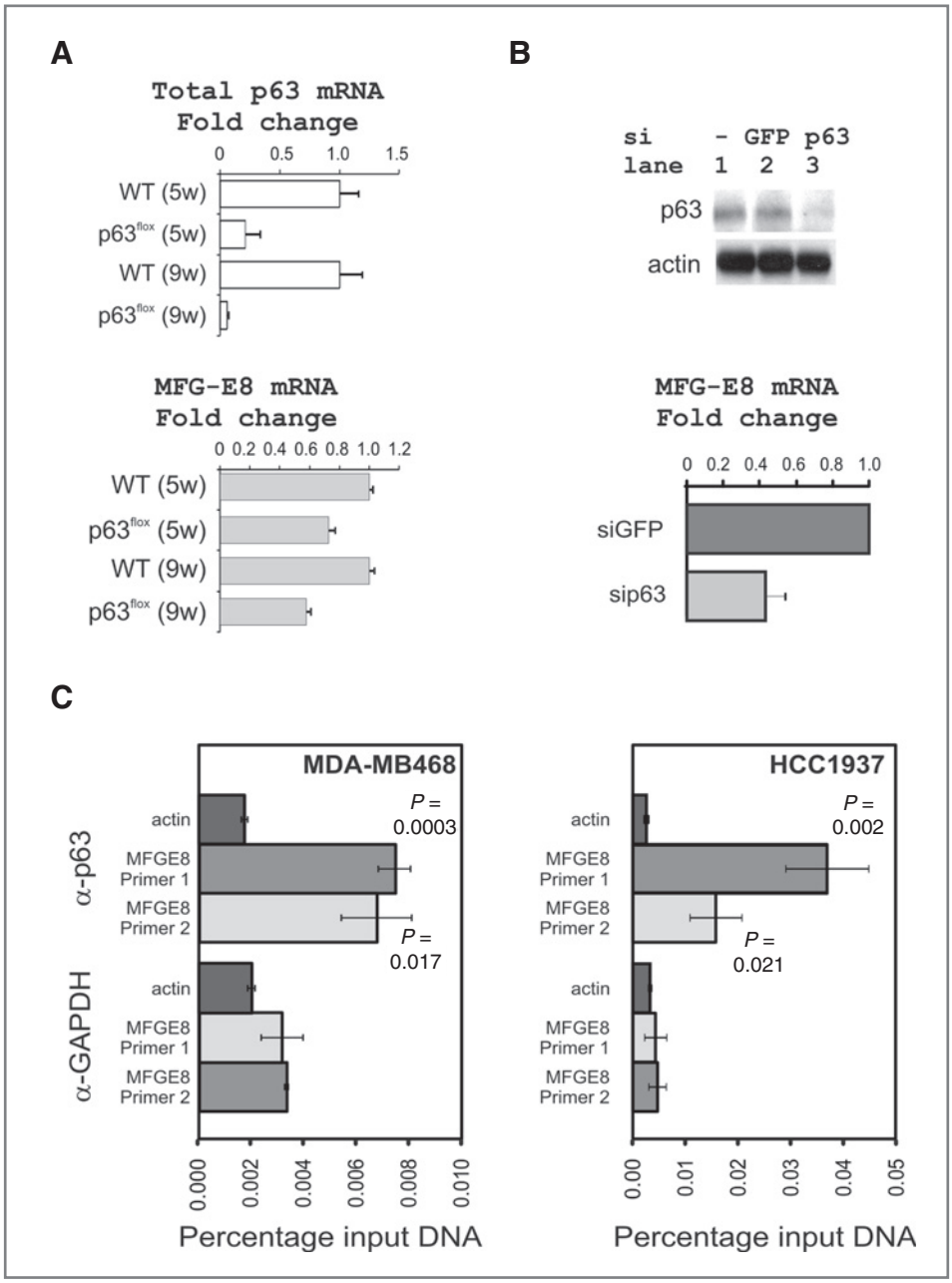

Figure 4. Conditional mammary p63 loss, RNAi, and ChIP link p63 function to MFG-E8 expression control. A, p63 was conditionally knocked down in mouse mammary myoepithelial cells using *K14CreERTam/p63^{flox}* mice. Shown are the resulting decreases in p63 and MFG-E8 mRNA (normalized to GAPDH) in mouse mammary glands 5 and 9 weeks after knockdown. MFG-E8 changes are significant ($P = 2.99 \times 10^{-4}$ at 5 weeks and $P = 6.42 \times 10^{-4}$ at 9 weeks.) B, decreased MFG-E8 mRNA in cells expressing a shp63 lentiviral construct. MDA-MB-468 cells were treated as described (15). Protein was harvested and analyzed by Western analyses 72 hours after infection. Tested cells included untreated controls (–), and cells transduced with shGFP (GFP) and shp63 (p63). An actin loading control is shown. Total mRNA was analyzed from the shGFP- and shp63-transduced cells. Shown is the mean and standard error for 4 independent samples comparing the fold change in the shp63-expressing cells to the shGFP-expressing cells. C, we performed ChIP-PCR for p63 binding as described in Supplementary Materials. Anti-p63 ChIP-PCR signals are plotted as a percentage of the total input DNA for each reaction. We measured α-p63 ChIP DNA in triple-negative MDA-MB468 and HCC1937 cells. Shown are the mean and standard deviation for signals at the actin promoter (negative control) versus reported p63 binding sites. Binding at the PUMA promoter was used as positive control (not shown).

levels in them compared with ER$^+$ and erbB2$^+$ tumors (Fig. 1E). In addition, we compared MFG-E8 protein in sera from 10 controls, 10 ER$^+$, 10 erbB2$^+$, and 10 triple-negative breast cancer patients (Fig. 1F). Control sera contained 318 ± 96 pg/mL of MFG-E8. MFG-E8 levels increased slightly to 1,700 and 1,730 pg/c in ER$^+$/PR$^+$ and erbB2$^+$ patient sera, respectively. In contrast, a mean of 3,900 pg/mL of MFG-E8 was found in sera from triple-negative breast cancer patients. This increase was due in large part to 4 of the 10 patients whose MFG-E8 ranged from 3,000 to 50,700 pg/mL, markedly exceeding levels seen in the other tumor types.

In situ hybridization confirms that MFG-E8 downregulation is highly correlated with ER$^+$ breast cancer

To delineate cell types expressing MFG-E8 and to validate the results in ER$^+$ breast cancers, we used *in situ* analyses using specimens from a separate cohort of breast cancer patients (Fig. 2, ref. 21). We found high-level expression of MFG-E8 in an ER-negative breast cancer (Fig. 2A) where a sense control probe showed no hybridization (Fig. 2B). Moreover normal-appearing breast tissues showed robust MFG-E8 expression, which was decreased in adjacent tumor tissue in

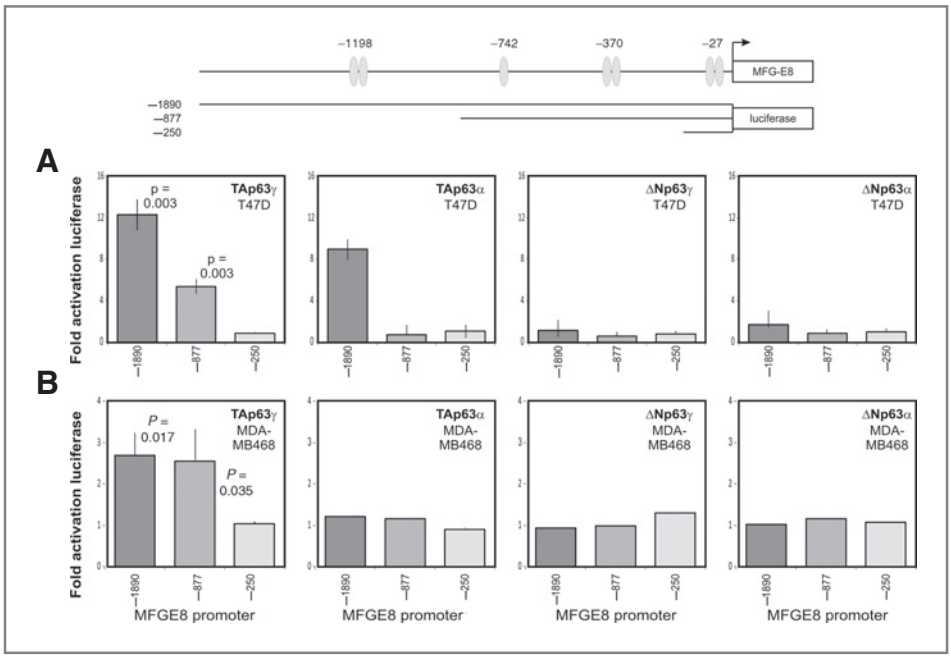

Figure 5. Regulation of the MFG-E8 promoter by p63 isoforms in luciferase reporter gene assays. A diagram of luciferase reporter constructs containing different proportions of the MFG-E8 promoter is shown. Gray ovals indicate p63 binding sites. The reporter constructs were cotransfected with expression constructs containing p63 isoforms in combination with pCMV-Renilla luciferase. The ratio of firefly luciferase values in the presence and absence of the p63 expression constructs (normalized to the internal Renilla standard) were calculated for each condition. Shown are the means and standard errors for three replicas at each point. A, the results for the four different p63 isoforms in T47D cells. B, the results for the four different p63 isoforms in MDA-MB468 cells.

an ER^+ breast cancer (Fig. 2C). We confirmed that MFG-E8 is expressed in both juxta-lumenal and myoepithelial cells in normal ductal tissues (Fig. 2D), as previously described (10). We then performed our *in situ* analyses using 13 randomly selected ER^- and 12 ER^+ breast cancers to evaluate the significance of our observations. Characteristic *in situ* results for ER^+ versus ER^- tumors are demonstrated in Figure 2E–L. One ER^+ tumor was among the top 13 MFG-E8-expressing tumors, and one ER^- tumor was among the bottom 12 expressing samples, showing that low MFG-E8 expression is highly correlated with the ER^+ status of breast cancers (Fig. 2M, $P < 0.001$ by chi-square analysis).

p63 regulation of MFG-E8 expression

Abnormalities in p63/p73 regulation are an important feature of triple-negative (basal) breast cancers (15). Recently, MFG-E8 was found to be a p63/p73 target gene in skin cells (18). To determine whether p63/p73 regulatory changes in triple-negative breast cancer cells might account for their increased MFG-E8 levels, we compared p63 levels in LCM-isolated normal, DCIS, and IDC breast tissues in ER^+, $erbB2^+$, and triple-negative cancers (Fig. 3A–C). mRNA for the transcriptionally active (TA) form of p63 (TAp63) increased in triple negative but decreased in ER^+ and $erbB2^+$ cancers.

We next evaluated the effect of conditional loss of p63, finding that 90% knockdown of p63 in myopithelial cells in $p63^{flox}$ mice targeted by a K14 Cre recombinase system decreased mammary MFG-E8 levels by 40% (Fig. 4A). We then used p63 siRNA to knock its expression down in a triple-negative breast cell line. The p63 siRNA knockdown caused a 2.5-fold decrease in MFG-E8 mRNA (Fig. 4B). Using two triple-negative cell lines, we performed anti-p63 ChIP experiments, finding that p63 is present in the p63 binding region in the MFGE8 promoter in triple-negative breast cancers (Fig. 4C). We compared p63 binding at the MFG-E8 promoter to actin promoter binding as a negative control. As a positive control, we found that p63 bound to the *PUMA* promoter as previously described (not shown; ref. 15).

Finally, we used a previously described luciferase reporter assay (18) that reports activity at −1,198, −742, −370, and −27 p63 MFG-E8 promoter binding sites in T47D (Fig. 5A) and in MDA-MB468 cells (Fig. 5B). Reporter activity increased 12.3-fold in response to cotransfection with TAp63γ at the upstream site and 5.3-fold at the −877 site in T47D cells. This changed to 9-fold for TAp63α at the upstream site. p63 cotransfections had similar but lesser effects in MDA-MB468 cells.

MFG-E8 RNAi stimulates ER^+ breast cancer cell proliferation, and MFG-E8 loss accelerates tumor formation in erbB2 transgenic mice

Since MFG-E8 is the ligand for integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ that promotes apoptosis (22–24), we evaluated effects of MFG-E8 RNAi on cell proliferation in ER^+ breast cancer cells (Fig. 6A and B). We also compared the effect of the RGD peptide that blocks integrin–ligand interactions (7) on siMFG−E8-induced cell proliferation. MFG-E8 knockdown increased cell numbers for ER^+ breast cancer cell lines and the RGD peptide blocked this stimulation.

These data identified potentially antiproliferative effects of MFG-E8-integrin β3/β5 signaling in ER^+ breast cancer cell lines. Such antiproliferative effects have been shown *in vivo* for the integrin β3/β5 receptor by acceleration of tumor formation in transgenic MMTV-*erbB2* mice lacking integrins β3 and/or β5 (12). To assess MFG-E8 *in vivo*, we crossed MFG-E8 knockout mice to *erbB2* transgenic mice. Several tumors

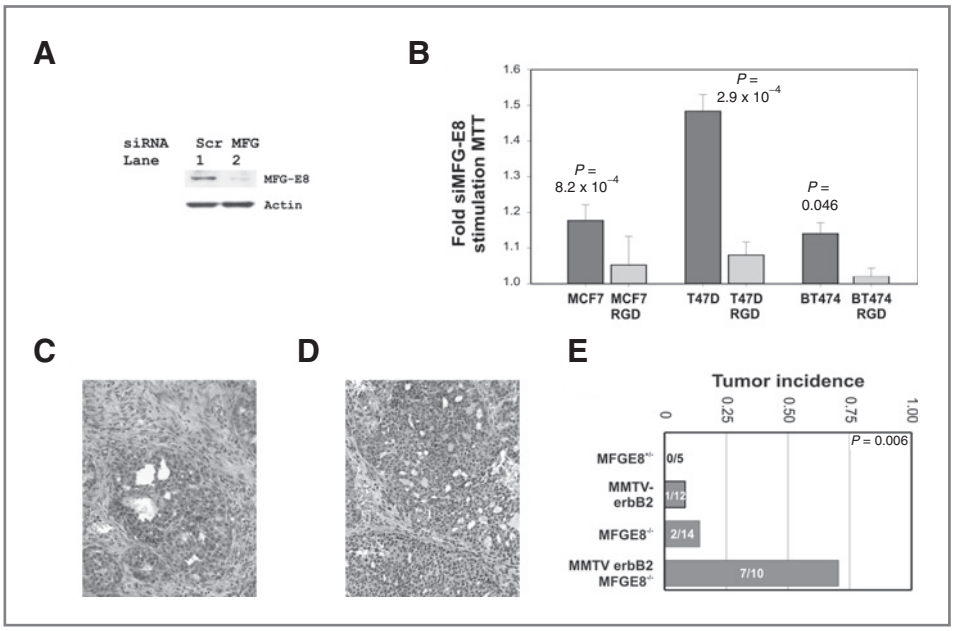

Figure 6. MFG-E8 is antiproliferative in estrogen receptor positive breast cancer cells, and its loss accelerates tumor formation in erbB2 transgenic mice. A, T47D cells were transfected with an siRNA oligonucleotide for MFG-E8 (*MFG*) or a scrambled control siRNA (*Scr*), and MFG-E8 and actin levels were analyzed 48 hours later using standard immunoblots to validate the siRNA. B, three breast cancer cell lines were transfected with scramble and MFG-E8 siRNAs. They were grown for 72 hours in the absence and presence of the RGB peptide that competitively inhibits ligand binding to α and β integrins and harvested for the MTT assay. The fold difference in proliferation was obtained by dividing the MTT measurement in the MFG-E8 siRNA samples by the MTT measurement in the scrambled oligonucleotide samples. Plotted are the means and standard deviations for each cell line. C and D, we crossed MFG-E8 null mice with homozygous MMTV-erbB2 transgenic mice that express the nonmutated form of erbB2 in their mammary tissues. Resulting MFG-E8$^{+/-}$ mice were then backcrossed to the MFG-E8$^{-/-}$ mice and mice of 4 genotypes were identified using PCR-based genotyping: nontransgenic-MFG-E8$^{+/-}$, erbB2 transgenic-MFG-E8$^{+/-}$, nontransgenic-MFG-E8$^{-/-}$, and erbB2 transgenic-MFG-E8$^{-/-}$. Mice were evaluated weekly for palpable masses and scored as positive upon appearance of a tumor. Shown are photomicrographs of standard hematoxylin and eosin stained tumors that arose in compound erbB2/MFG-E8$^{-/-}$ transgenic mice before 15 months of age (40×). The tumors are high-grade invasive ductal carcinomas that display a predominant solid pattern with focal gland formation. E, at 15 months of age, all remaining mice were sacrificed and mammary gland whole mounts were prepared. Tumors identified either in the whole mounts or in the mice that developed overt palpable tumors were scored as positive. We plotted the incidence of tumor formation for each genotype of mice and indicate the tumor number/number of mice evaluated within the bars of the graph. ($P = 0.006$ by Fisher's exact test.)

developed in *erbB2*$^{+}$/MFG-E8 null mice before 1 year of age, but none developed in either the *erbB2*/MFG-E8$^{+/-}$ or mice that were solely MFG-E8 null (Fig. 6C and D). However, erbB2-induced tumor onset was relatively slow in these crosses compared to the usual kinetics of tumor formation in inbred erbB2 mice. Consequently, we sacrificed remaining mice at 15 months of age and evaluated tumor formation using whole mounts to assess tumor incidence at 15 months of age (Fig. 6E). Tumor incidence caused by the MMTV-nonmutant-*erbB2* transgene on its own was lower than published experiments using inbred strains. However, mice that were erbB2^{+} and MFGE8 null developed tumors at 3 to 7 times the rate seen in mice that were singly *erbB2*$^{+}$ or MFG-E8 null alone.

Triple-negative cell lines have higher levels of MFG-E8 expression and knockdown of MFG-E8 increases chemosensitivity to cisplatin

We also evaluated MFG-E8 protein levels in 10 breast cancer cell lines (Fig. 7A top). High levels of MFG-E8 expression were only seen in triple-negative breast cancers. We measured secreted MFG-E8 levels in culture supernatants, finding that secreted MFG-E8 matches intracellular expression (Fig. 7A bottom). We evaluated the functional significance of these changes by knocking MFG-E8 down in two triple-negative breast cancer cells. The MFG-E8 siRNA effectively knocked its protein levels down (Fig. 7B), which led to increased sensitivity of both triple-negative breast cancer cell lines to the inhibitory effects of cisplatin (Fig. 7C and D).

Discussion

BA46/MFG-E8 was first identified using an antibody (BA46) that could monitor metastatic breast cancer burden in patient samples. Subsequently, MFG-E8/lactadherin physiologic role was shown in mammary development, complicating the earlier findings. Here, we clarify potentially opposing roles MFG-E8 may play in breast cancers of different types. We first show that MFG-E8 is downregulated in ER^{+} and erbB2^{+} breast cancer (Figs. 1and 2) where MFG-E8 is antiproliferative (Fig. 6). In contrast, we found that MFG-E8 is expressed at high levels in the basal/ triple-negative set of breast cancers

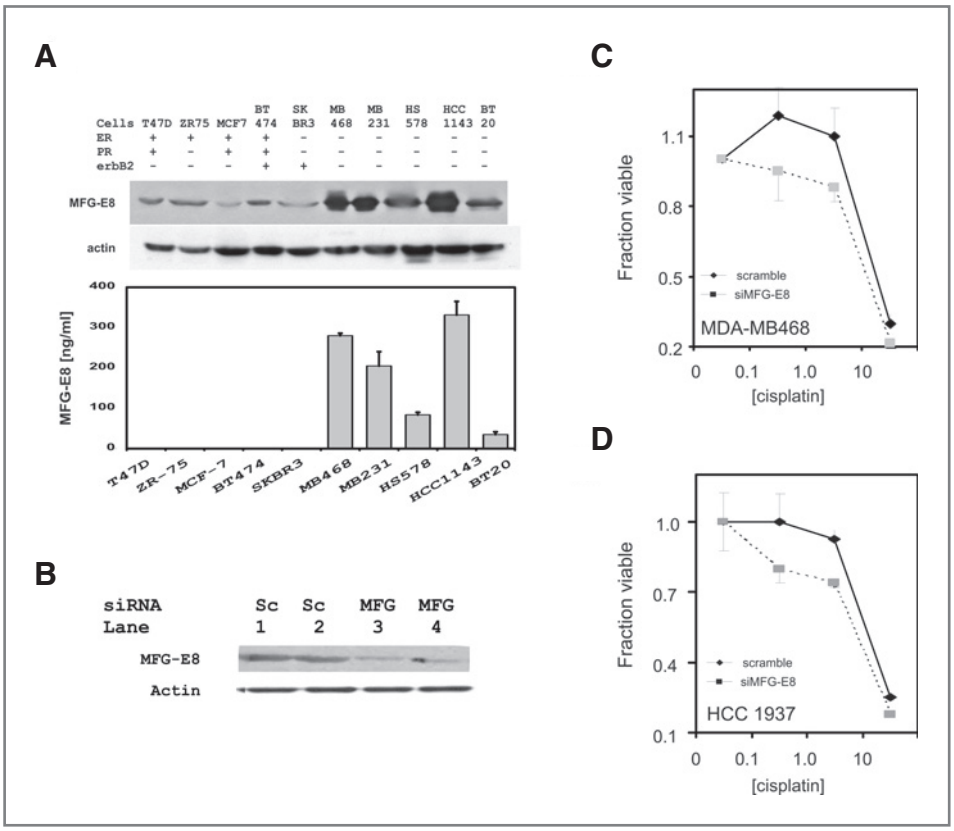

Figure 7. MFG-E8 expression in breast cancer cell lines and role of MFG-E8 in the DNA damage response in triple-negative breast cancers. A, MFG-E8 immunoblots for a variety of breast cancer cell lines whose ER, PR, and erbB2 status are known. Cell lysates were harvested and Western blots were performed for MFG-E8. A commercial MFG-E8 ELISA was then used to measure MFG-E8 protein in the supernatants of media from the same cell lines. Supernatant media were harvested after 24 hours of incubation and ELISAs were performed according to kit instructions. The mean and standard error are shown for three replicas harvested from each cell line, which are plotted as ng/mL of MFG-E8 in the conditioned media. B, an MFG-E8 immunoblot of cells transfected using scramble (Sc) and siMFGE8 (MFG) shows effective MFG-E8 knockdown. An actin control is shown. C and D, dose–response curves (MTT cell viability assay) of cells expressing the scramble (sc) or siMFGE8 (MFG) after treatment with cisplatin at the indicated concentrations (μM). Error bars show SD for 10 experiments. C, MDA-MB-468; and D, HCC-1937 cells. $P < 0.01$ for each point on the curves.

where it apparently functions as a functional target gene of the p63/p73 pathway. We present evidence that p63 directly regulates MFG-E8 in those cells (Figs. 3–5). These descriptions of MFG-E8 suggest that its interactions with its $\alpha_v\beta_{3,5}$ receptor offer new insights for the diagnosis and treatment of different breast cancer types.

We started by investigating gene regulation during breast cancer progression. We were surprised to find MFG-E8 decreases in mouse models and in human breast cancers, given its initial description. This was especially surprising since we separately found that estradiol induces MFG-E8 and inhibitors of erbB2 downregulate its expression (not shown). Evidently these effects are secondary to regulation by p63 given its control of MFG-E8 as shown in Figures 3–5. Decreases in MFG-E8 that accompany decreased p63 in ER^+ or $erbB2^+$ tumors may provide a selective advantage in these cancer types to evade immune clearance during tumor progression since MFG-E8 acts as the ligand for the phagocytic clearance pathway (9).

In contrast, MFG-E8 expression was increased in a different set of tumors--the triple-negative subset. These tumors are the most refractory to treatment, and recent studies are investigating novel treatment strategies for them. By evaluating microarray databases, a triple-negative–specific association with MFG-E8 expression in breast cancer was readily apparent. We confirmed this increased expression using cell lines and in a patient cohort, thus showing that the initial descriptions of BA46 potentially focused on patients with this type of tumor. We confirmed reports that p63/p73 regulates MFG-E8, and we showed this is functionally significant since an MFG-E8 siRNA increased cisplatin sensitivity. These results suggest that antagonists of MFG-E8-integrin signaling should be investigated in triple-negative breast cancers and further suggest that circulating MFG-E8 levels might be used to monitor the clinical response of some patients where p63 dysregulation is a major feature of their triple-negative breast cancer. MFG-E8 levels vastly exceeded those seen in any other tumor patient in three patients (3,000, 5,000, and 50,000 pg/mL). While these patients represent only 30% of the triple-negative patients tested, markers such as alpha fetoprotein and carcinoembryonic antigen are useful when found in similar proportions of patients with other cancers. One erbB2

tumor patient had a serum level of 3,670 pg/mL. This level could be due to misclassification, to a mixed tumor phenotype, to co-occurrence of another disease with elevated MFG-E8 levels such as systemic lupus erythematosus (25) or to secretion by inflammatory cells in the tumor itself. Importantly, healthy donors exhibited no MFG-E8 levels higher than 366 pg/mL, again suggesting that further explorations of MFG-E8 levels as a serum tumor marker are warranted.

Taken together, our results show that MFG-E8 joins a large group of ligands with context-specific functions in cancer. For example, these dual roles are not unlike the dual functions of transforming growth factor-β. Our results provide additional evidence that triple-negative breast cancers are phenotypically distinct from other breast cancers and these interactions suggest that integrins might be a druggable target whose ligands need to be evaluated in context as integrin-related therapies are advanced in the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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