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The Forkhead Box Transcription Factor FOXC1 Promotes Breast Cancer Invasion by Inducing Matrix Metalloprotease 7 (MMP7) Expression*[S]

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Background: FOXC1 is associated with breast cancer aggressiveness and the basal-like breast cancer subtype but the mechanism through which FOXC1 increases aggressiveness has not been elucidated.
Results: FOXC1 induces expression of matrix metalloprotease 7 (MMP7).
Conclusion: The aggressive cancer phenotype imparted by FOXC1 is due, at least in part, to expression of MMP7.
Significance: MMP7 represents a putative target for the treatment of some basal-like breast cancers.

Therapeutic options for treatment of basal-like breast cancers are limited and identification of molecular targets for novel therapies to treat this aggressive cancer is urgently needed. Recently, FOXC1, a forkhead box transcription factor, was identified as a functionally important biomarker of breast cancer aggressiveness and the basal-like breast cancer subtype. However, the mechanism through which FOXC1 controls aggressiveness of basal-like breast cancer remains to be elucidated. Here, we identify matrix metalloprotease 7 (MMP7) as a key downstream effector of FOXC1-mediated invasiveness. Expression of FOXC1 and MMP7 is significantly correlated in breast cancer samples and cell lines at both the mRNA and protein levels. Transient expression of FOXC1 in nontransformed mammary epithelial cell lines resulted in significantly increased expression of MMP7 and an MMP7-dependent increase in invasiveness. In reciprocal experiments, silencing endogenous FOXC1 in basal-like breast cancer cell lines resulted in decreased expression of MMP7 without decreased expression of other matrix metalloproteinases. We also demonstrate that elevated co-expression of *FOXC1* and *MMP7* is an independent predictor of patient outcome in multivariate analyses of two breast cancer patient cohorts. Together, our findings identify MMP7 as a novel mechanism through which FOXC1 may regulate the basal-like breast cancer invasive phenotype and the propensity of these cancers to metastasize. Furthermore, our findings demonstrate for the first time a correlation between MMP7 expression and basal-like breast cancers, suggesting that MMP7 may be a useful therapeutic target for treatment of this disease.

Targeted therapies have improved survival for many women diagnosed with breast cancer. Sex hormone receptor directed therapies have increased survival for women with estrogen receptor and/or progesterone receptor positive breast cancers, whereas treatments that disrupt human epidermal growth factor receptor 2 (HER2)[2] signaling have increased life expectancy of women with HER2 amplified cancers (1–4). However, 15–20% of breast cancers do not express estrogen or progesterone receptors or HER2 (*i.e.* triple negative) and currently have no effective targeted treatment option (5, 6). The majority of triple negative breast cancers can be classified by gene expression profiling as belonging to the basal-like breast cancer (BLBC) subtype (7). These BLBC/triple negative tumors are among the most aggressive breast cancers and tend to be high grade, exhibit pushing boarders and are prone to metastasize (8–10). Identification of the essential regulators of BLBC is a critical step toward developing targeted therapies for this disease.

Members of the forkhead box (FOX) family of transcription factors regulate a wide array of biological processes including development, differentiation, and invasion (11). FOXC1 (Mf1, FKHL7, FREAC3) was originally identified as an important transcription factor that controls development of structures derived from the neural crest and FOXC1 mutations have long been recognized as a primary cause of Axenfeld-Rieger syndrome (12–14). In addition to its roles in normal function and development of the eye and meninges, FOXC1 has recently emerged as a possible master regulator of BLBC. Although one report has demonstrated decreased invasion and metastasis of breast cancer cells in response to FOXC1 expression (15), other groups have reported that FOXC1 increases invasion and metastasis in endometrial and breast cancer models (16–19). Stable overexpression of FOXC1 elicits changes in gene expression indicative of epithelial to mesenchymal transition (EMT) and increases cellular invasion, migration, and proliferation

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[S] This article contains supplemental Figs. S1–S7 and Tables S1–S3.

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[2] The abbreviations used are: HER2, human epidermal growth factor receptor 2; BLBC, basal-like breast cancer; FOX, forkhead box; EMT, epithelial to mesenchymal transition; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; HR, hazard ratio.

(16–19). In addition, FOXC1 expression correlates with the BLBC subtype and predicts poor breast cancer patient outcome (17, 20, 21). A better understanding of the mechanisms though which FOXC1 regulates aggressive cancer phenotypes should lead to the identification of new therapeutic targets for BLBC.

Although it has recently been suggested that FOXC1 may dictate the BLBC phenotype in part through NF-κB signaling (19), the mechanisms utilized by FOXC1 to increase cancer aggressiveness remain to be fully elucidated. Here, we show that transient expression of FOXC1, like the previously reported stable expression of FOXC1 (17, 18), increases invasion of nontransformed mammary epithelial cell lines. However, unlike stable FOXC1 expression, increased invasion imparted by transient FOXC1 expression is not accompanied by increased proliferation, migration, or EMT. These observations suggested that whereas FOXC1 can induce EMT, this transition was not the underlying cause of FOXC1-induced invasion. We postulated that FOXC1 increases cellular invasion through induction of a Matrigel degrading enzyme such as a member of the matrix metalloproteinase (MMP) family. Analyses of publicly available gene expression data sets as well as breast cancer cell line panels revealed a significant correlation between expression of FOXC1 and the matrix metalloproteinase, MMP7 (Matrilysin, PUMP-1). Enforced FOXC1 expression significantly increases MMP7 expression in nontransformed MCF10A and MCF12A cells. Furthermore, the increased invasion of MCF10A cells in response to FOXC1 expression required sustained expression of MMP7. Confirming the link between FOXC1 and MMP7, silencing endogenous FOXC1 in basal-like breast cancer cell lines results in decreased MMP7 expression. Last, high *FOXC1* expression in breast cancers is associated with a significant decrease in patient survival when accompanied by high expression of *MMP7* and elevated co-expression of *FOXC1* and *MMP7* is an independent predictor of patient outcome in multivariate analyses of two breast cancer patient cohorts. Together, these data suggest a novel mechanism though which FOXC1 increases cancer aggressiveness and implicate MMP7 as a putative therapeutic target in BLBC.

EXPERIMENTAL PROCEDURES

Cell Culture—Nontransformed mammary epithelial and breast cancer cell lines were acquired from American Type Culture Collection. MCF10A and MCF12A cells were grown in DMEM/F-12 medium supplemented with 5% horse serum, insulin (0.01 mg/ml), EGF (20 ng/ml), cholera toxin (100 ng/ml), and hydrocortisone (500 ng/ml). BT474, BT549, HCC70, HCC1143, HCC1187, HCC38, MDA-MB-231, and T47D cells were cultured in RPMI media supplemented with 10% FBS. MCF7, MDA-MB-435, MDA-MB-453, and MDA-MB-468 cells were cultured in DMEM supplemented with 10% FBS. SKBR3 cells were cultured in McCoy's 5A media supplemented with 10% FBS. All growth medias contained 1% L-glutamine, 1% penicillin, and 1% streptomycin.

Transient FOXC1 Overexpression—*FOXC1* cDNA was cloned into pcDNA3.1 (Invitrogen). MCF10A and MCF12A cells were transiently transfected with empty pcDNA3.1 vector as control or pcDNA3.1/*FOXC1* using Lipofectamine 2000 (Invitrogen) per the manufacturer's protocol. Adenoviruses encoding *FOXC1* (AdFOXC1) or *GFP* (AdGFP) as a control were purchased from Welgen. Cells were transduced with 500 multiplicity of infection units of adenovirus unless otherwise noted.

FOXC1 Silencing—Endogenous FOXC1 and MMP7 were silenced with siRNA SMARTpools (Dharmacon). A nontargeting siRNA (sc-44230; Santa Cruz) served as control in all siRNA experiments. Lipofectamine 2000 (Invitrogen) was used as the siRNA delivery agent. In experiments in which MMP7 was silenced in conjunction with FOXC1 overexpression, control or MMP7-targeting siRNA was utilized at a concentration of 200 nM; otherwise transfections were conducted per the manufacturer's recommendations.

FOXC1 was also silenced by shRNAs. Vectors encoding shFOXC1#2 (TRCN0000235692), shFOXC1#3 (TRCN0000235692), shFOXC1#4 (TRCN0000235693), and a control vector encoding an shRNA to luciferase, shluc (SHC007), were purchased from Sigma and used to generate lentiviral particles (Wistar Institute). Cells were transduced at 1 multiplicity of infection in the presence of Polybrene (5 μg/ml) for 24 h. Cells were then allowed to recover in normal growth medium for 48 h at which time RNA was harvested as previously described (22) or cells were selected for stable shRNA expression with puromycin.

Western Blotting and Immunodetection—Generation of protein lysates and Western blotting were performed as previously described (22). FOXC1 antibodies were purchased from Santa Cruz (C-18) or Cell Signaling (number 7415). MMP7 antibodies were purchased from Calbiochem (141-7B2) or R&D Systems (AF907). β-Actin antibody was purchased from Sigma (AC-15). HRP-conjugated secondary antibodies to mouse, rabbit, and goat were purchased from Santa Cruz (sc-2005, sc-2054, and sc-2020).

Quantitative Real-time PCR—Total RNA was extracted and used to generate cDNA as previously described (22). Quantitative real-time PCR was conducted using an Applied Biosystems (ABI) Step-One real-time PCR instrument by the comparative C_t method. *GAPDH* was used as an endogenous control for all experiments. The following ABI gene expression assays were used for quantitative RT-PCR: *FOXC1* (Hs00559473_s1), *MMP7* (Hs01042793_m1), *MMP2* (Hs00234422-m1), *MMP9* (Hs00957562_m1), *SNAI1* (Hs00195591_m1), *SNAI2* (Hs00950344_m1), *TWIST1* (Hs00361186_m1), *VIM* (HS00185584_m1), *FN1* (Hs00365052_m1), *CDH2* (Hs00365052_m1), *CDH1* (Hs00170423_m1), *CCND1* (Hs00277039_m1), and *GAPDH* (Hs99999905_m1). Data were averaged from at least three independent experiments, each conducted in triplicate. Statistical differences in gene expression were assessed by Student's *t* test.

Migration and Invasion Assays—MCF10A and MCF12A cells were transfected and/or transduced 48 h prior to the start of migration or invasion protocols. Migration was assessed using culture well inserts from Costar. Invasion was assayed using Matrigel-coated Transwell chambers (BD Biosciences). For migration experiments, 5×10^4 cells in serum, insulin, and EGF-free media were plated on the upper chamber of the insert. For invasion experiments, 1.25×10^5 cells were seeded in the upper chamber. For invasion and migration experiments, the lower chamber was filled with complete growth medium as a chemoattractant. Migration experiments for MCF10A and

MCF12A proceeded for 24 h. Invasion of MCF10A cells was evaluated at 24 h, whereas the less invasive MCF12A cell line was allowed to invade for 48 h. At the end of the migration or invasion experiment, cells that had migrated/invaded were stained with the Diff-Quik stain kit (BD Biosciences) and photographed under ×10 magnification. Cells were counted in quadruplicate fields of view in duplicate or triplicate membranes. Data were averaged from at least three independent experiments and statistical differences in migration and invasion were determined using Student's *t* test.

Casein and Gelatin Zymography—Cells were transfected as indicated and 48 h later were washed twice with PBS and once with serum-free media followed by 24 h incubation in serum-free media. Gelatin and casein zymography were performed as described previously (22, 23).

Gene Expression Microarray Analysis—Microarray data sets were retrieved from Oncomine (oncomine.org). For co-expression analysis between *FOXC1* and members of the MMP and tissue inhibitor of metalloproteinase (TIMP) families, data sets were pared to those using Affymetrix HG-U133A or U133 Plus 2.0 arrays. The resulting 20 data sets utilized for further analysis are listed in supplemental Table S1. Pearson correlation coefficients between *FOXC1* and members of the MMP and TIMP families were calculated and used to determine covariance of these factors in each data set. The overall significance of covariance between *FOXC1* and each MMP and TIMP family member was determined by Fisher's combined probability test with values <0.05 considered significant.

Survival Analysis—Data from the Sørlie *et al.* (24) and van de Vijver *et al.* (25) data sets were retrieved from Oncomine, stratified by high (upper quartile) or low (remaining three quartiles) expression of *FOXC1* and *MMP7*, and overall survival was analyzed over a five-year period. Kaplan-Meier survival curves were generated and the log-rank test was used for statistical comparison of survival curves between groups. Univariate and stepwise multivariate analyses were carried out using the Cox proportional hazards model. Clincopathological variables included in analysis of the Sørlie *et al.* (24) data set were M Stage, N stage, T stage, grade, and age, whereas N stage was the lone variable available for the van de Vijver *et al.* (25) data set.

RESULTS

Transient FOXC1 Expression Increases Invasion without Inducing EMT—Stable overexpression of FOXC1 has been shown to increase cancer cell invasion as well as elicit changes in gene expression that are indicative of EMT (17, 18), yet it is unclear whether EMT is required for the induction of invasion observed with stable FOXC1 expression as well as the association of FOXC1 with breast cancer aggressiveness. We hypothesized that analysis of early changes in gene expression associated with transient overexpression of FOXC1 would uncover immediate downstream transcriptional targets of FOXC1 that induce EMT and may reveal key effectors that increase invasion. To begin to test this hypothesis, we transiently overexpressed FOXC1 in the nontransformed mammary epithelial cell line, MCF10A, utilizing a cationic liposome-based reagent (Fig. 1*A*). Similar to the results reported for cells stably overexpressing FOXC1, transient FOXC1 overexpression causes a significant increase in cellular invasion (4.5-fold; $p < 0.001$) in modified Boyden chamber assays (Fig. 1*B*). Relative migration of MCF10A cells is also significantly increased by transient FOXC1 overexpression (1.4-fold; $p < 0.01$; Fig. 1*C*). However, the increase in migration was not nearly as robust as, and could not fully account for, the increase in invasion. In contrast to previous reports using stable FOXC1 overexpression, transient overexpression of FOXC1 did not increase the expression of genes associated with EMT (Fig. 1*D*). Cells were analyzed for changes in transcription factors known to mediate EMT, including *SNAI1*, *SNAI2*, and *TWIST,* with no significant changes observed. In addition, the expression of *VIM* or *FN1*, two genes frequently up-regulated during EMT whose expression is increased with stable FOXC1 expression (17, 18), were not changed. Finally, a "cadherin switch" (*e.g.* loss of epithelial E-cadherin with a concomitant increase in a mesenchymal cadherin), which is associated with EMT and has been observed with stable FOXC1 expression (17, 18), did not occur.

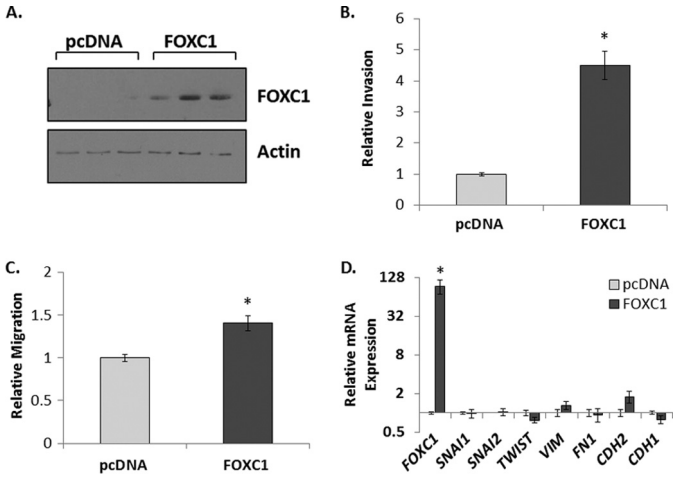

FIGURE 1. **Transient expression of FOXC1 increases invasion of MCF10A Cells.** *A*, representative immunoblots demonstrating transient overexpression of FOXC1 in MCF10A cells transfected with empty vector (*pcDNA*) or vector encoding FOXC1 (*FOXC1*). *B*, relative invasion of MCF10A cells transfected with pcDNA as control or pcDNA/FOXC1. *C*, relative migration of MCF10A cells transfected with pcDNA as control or pcDNA/FOXC1. *D*, relative transcript levels of EMT-related genes normalized to pcDNA control cells with *GAPDH* used as an endogenous control. Data represent at least three independent experiments and are presented as mean ± S.E. *, $p < 0.05$.

Cationic liposome-based transfection agents can influence cell behavior and gene expression (26). To ensure that the apparent lack of EMT in response to FOXC1 was not dependent on the mode of transient transfection, we confirmed these results using adenovirus as a gene delivery agent. Invasion of MCF10A cells was increased following transduction with a FOXC1 encoding adenovirus (4-fold, $p < 0.01$; supplemental Fig. S1, *A* and *B*). In contrast, no significant change in migration (1.6-fold; $p = 0.14$) or changes in EMT-associated gene expression occurred in response to FOXC1 expression (supplemental Fig. S1, *C* and *D*). Last, these data were confirmed using a second nontransformed mammary epithelial cell line, MCF12A. Although MCF12A cells are less invasive *in vitro* than MCF10A cells, a significant increase in the invasive capacity of MCF12A cells occurs in response to FOXC1 overexpression compared with controls in a 48-h invasion assay (4.3-fold; $p < 0.01$; supplemental Fig. S2, *A* and *B*). Again, no significant change in

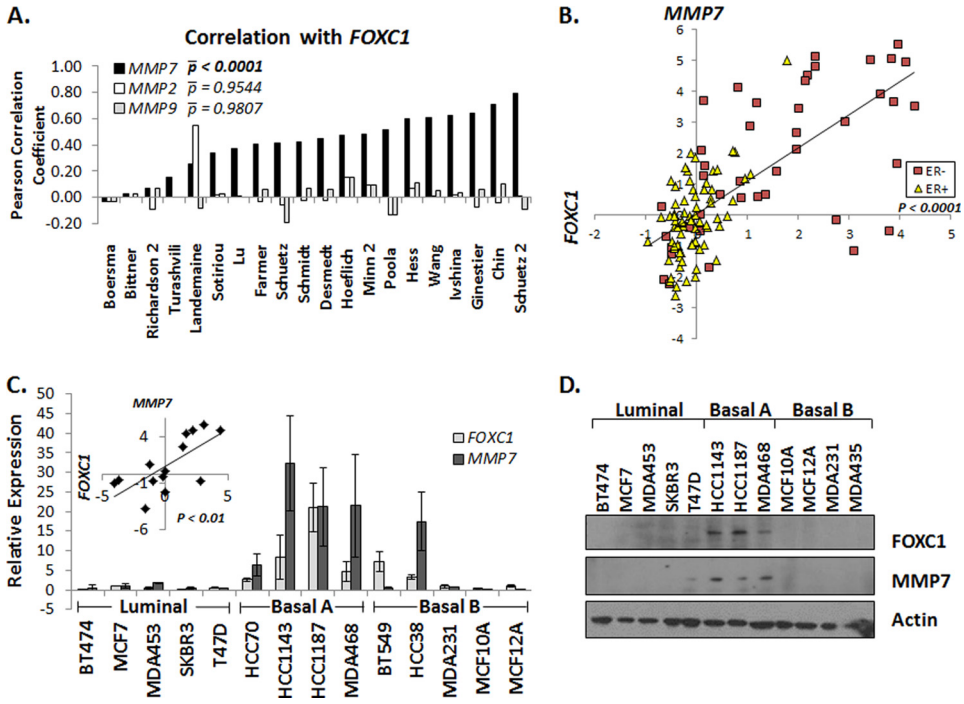

FIGURE 2. **Expression of FOXC1 positively correlates with MMP7 expression in breast cancers and breast cancer cell lines.** *A*, bar graph illustrating the Pearson correlation coefficients for *FOXC1* and *MMP7* (*solid black bars*), *MMP2* (*white bars*), or *MMP9* (*gray bars*) in 20 breast cancer gene expression data sets. Average *p* values (|*p*) were determined using Fisher's method of combined probabilities. *B*, log(2) transformed, median centered data from Chin *et al.* (see supplemental Table 1) was plotted by FOXC1 (*x* axis) and MMP7 (*y* axis) and colored coded according to estrogen receptor (*ER*) status. *C*, relative *FOXC1* (*light gray*) and *MMP7* (*dark gray*) expression in a panel of breast cancer and nontransformed mammary epithelial cell lines. Cell lines are grouped by subtype as defined by Neve *et al.* (27). Data represent at least three independent experiments and are presented as mean ± S.D. Average *FOXC1* (*x* axis) and *MMP7* (*y* axis) values were plotted (*inset*). *D*, representative Western blots demonstrating FOXC1 and MMP7 protein expression in a number of breast cancer and nontransformed mammary epithelial cell lines. *p* values less than 0.05 were considered statistically significant.

migration (1.3-fold, $p = 0.36$) or EMT-associated gene expression was detected (supplemental Fig. S2, *C* and *D*). Furthermore, transient expression of FOXC1 in MCF10A or MCF12A cells did not induce the fibroblast-like morphology typically observed with EMT (data not shown). Stable expression of FOXC1 has been shown to increase cell proliferation (17, 19). To determine whether changes in cell number could account for the increase in invaded cells, we analyzed changes in cell number of MCF10A and MCF12A cells transduced with *GFP* control or *FOXC1* expressing adenovirus over a 6-day period. Transient expression of FOXC1 in MF10A cells significantly ($p < 0.05$ at day 6) reduced the number of viable cells when compared with cells transduced by control virus (supplemental Fig. S3*A*) indicating that the increase in invasion imparted by FOXC1 expression was not due to an increase in cell number. In MCF12A cells, transient expression of FOXC1 had no effect on cell number (supplemental Fig. S3*B*), further supporting the conclusion that transient FOXC1 expression in MCF10A and MCF12A directly increases invasion of breast epithelial cells.

FOXC1 Expression Correlates with Expression of MMP7—The observation that FOXC1 significantly increases invasion through Matrigel without increasing the cell number or robustly increasing migration suggested that transient FOXC1 expression increases expression and/or activity of an enzyme capable of degrading Matrigel such as members of the MMP family. To determine whether FOXC1 expression increases expression of one or more MMP or decreases expression of a member of the TIMP family, we evaluated correlations between expression of *FOXC1* and 20 members of the MMP family along with four TIMPs by analyzing 20 publicly available human breast cancer gene expression microarray data sets (supplemental Table S1). To avoid platform-associated variance, all of the data sets included in the meta-analysis used Affymetrix U133A or U133 Plus 2.0 arrays. Twenty different datasets were used in this analysis, comprising >2,300 breast cancer samples. Although stable expression of FOXC1 *in vitro* has been shown to increase expression of the gelatinases MMP2 and -9 (20), no significant correlation between *FOXC1* and either *MMP2* or *MMP9* was observed across the datasets ($p = 0.9544$ and $p = 0.9807$, respectively; Fig. 2*A*). In contrast, we found that *MMP7* was the only MMP or TIMP family member whose expression was significantly correlated with the expression of *FOXC1* ($p < 0.0001$; Fig. 2*A* and supplemental Table S2). Further analysis of individual data sets revealed not only a significant correlation between *FOXC1* and *MMP7* expression, but also that both *FOXC1* and *MMP7* were preferentially expressed in ER-negative breast cancers (Fig. 2*B* and supplemental Fig. S4*A*). Expression of *FOXC1* and *MMP7* was also significantly correlated ($p < 0.01$) in a panel of 14 breast cancer cell lines and nontransformed mammary epithelial cell lines (Fig. 2*C*). The correlated expression of *FOXC1* and *MMP7* in breast cancer cell lines was further confirmed in two public gene expression array datasets (27, 28) (supplemental Fig. S4, *B* and *C*). Furthermore, expression of FOXC1 and MMP7 proteins is highly correlated in 14 breast cancer and nontransformed mammary epithelial cell lines (Fig. 2*D*). Notably, the breast cancer cell lines that exhib-

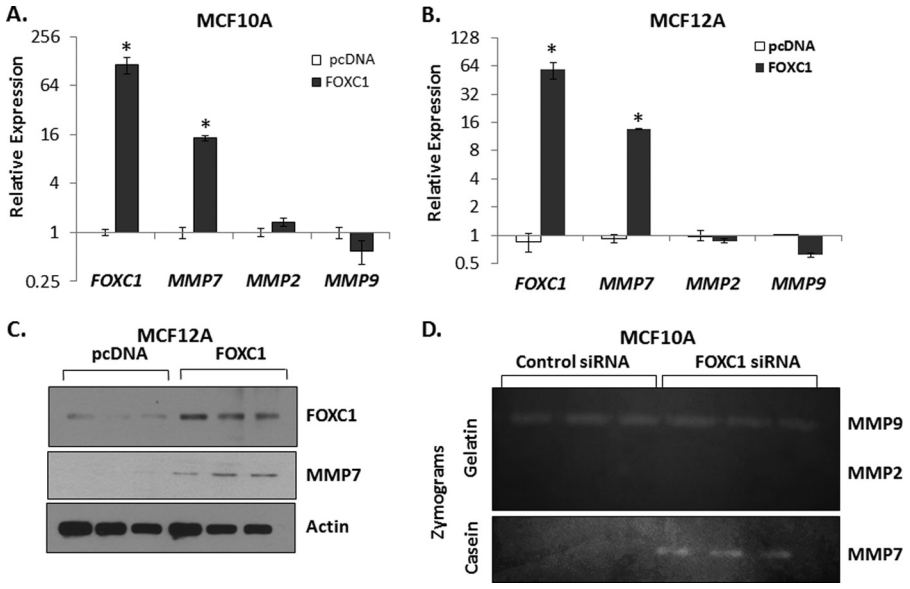

FIGURE 3. **Transient overexpression of FOXC1 in MCF10A or MCF12A cells results in increased expression of MMP7.** Transcript levels of *FOXC1*, *MMP7*, *MMP2*, and *MMP9* in MCF10A (*A*) or MCF12A (*B*) cells transiently transfected with empty vector (*pcDNA*) or vector encoding FOXC1. Data are relative to pcDNA; *GAPDH* was used as an endogenous control. Data summarize three independent experiments and are presented as mean ± S.D. *C*, representative Western blots demonstrating expression of FOXC1 and MMP7 in MCF12A cells transiently transfected with control (*pcDNA*) vector or vector encoding FOXC1. *D*, representative gelatin or casein zymograms demonstrating the relative levels of MMP9, MMP2, or MMP7 in conditioned media from MCF10A cells transfected with empty vector (pcDNA) or vector encoding FOXC1. *, $p < 0.05$.

ited the highest expression of FOXC1 and MMP7 are those classified as basal A, whereas luminal and basal B/mesenchymal-like breast cancer cells lines showed low or moderate expression of FOXC1 and MMP7 (27, 28). Basal A cell lines are most like BLBC, whereas basal B lines are more similar to the rare claudin-low breast cancer subtype. Of note, there are several conflicting reports regarding the relative expression level of the FOXC1 protein in breast cancer cell lines (15, 17, 29). To exclude the possibility that the data presented herein was associated with poor antibody specificity, these data were confirmed using additional antibodies to FOXC1 and MMP7 (supplemental Fig. S4*D*). Together, these data demonstrate that FOXC1 and MMP7 expression is highly correlated in breast cancer tissues as well as cell lines.

Overexpression of FOXC1 Induces MMP7 Expression—The significant correlation between FOXC1 and MMP7 expression in breast cancer samples and cell lines and the ability of FOXC1 overexpression to induce invasion suggested that FOXC1 regulates the expression of MMP7. To test this possibility, FOXC1 was transiently overexpressed and the impact on MMP7 expression was assessed. Transient overexpression of FOXC1 resulted in a significant increase in *MMP7* mRNA in both MCF10A (14.5-fold; $p < 0.05$) and MCF12A (13.6-fold; $p < 0.05$) cells (Fig. 3, *A* and *B*), whereas *MMP2* and *MMP9* expression were unaffected by overexpression of FOXC1. MMP7 protein expression was also increased in response to FOXC1 overexpression in both MCF10A and MCF12A cells (Fig. 3*C* and data not shown). Zymography utilizing gelatin as a substrate for MMP2 and MMP9 or β-casein as an MMP7 substrate revealed that FOXC1 overexpression increases the amount of MMP7 protein in MCF10A- or MCF12A-conditioned media, but does not increase the amount of secreted MMP2 or MMP9 (Fig. 3*D* and data not shown). We confirmed that FOXC1 overexpression significantly increased the amount of MMP7 detectable in the conditioned media of MCF12A cells by ELISA (19.4-fold; $p < 0.05$; supplemental Fig. S5*A*). These data were further confirmed using adenoviral-mediated FOXC1 overexpression (supplemental Fig. S5, *B* and *C*). Last, the ability of FOXC1 to increase MMP7 expression was dose-dependent (supplemental Fig. S5*D*).

Increased Invasion Imparted by FOXC1 Expression Requires Induction of MMP7—To determine whether the increase in invasion imparted by FOXC1 expression in MCF10A and MCF12A cells is dependent upon increased expression of MMP7, we overexpressed FOXC1 while blocking the induction of MMP7. This was accomplished by transducing MCF10A cells with control adenovirus encoding GFP or adenovirus encoding FOXC1 and simultaneously transfecting the cells with a nontargeting siRNA or siRNAs targeting *MMP7* (Fig. 4*A*). Transfection with siRNA to *MMP7* resulted in a 3.8-fold reduction ($p < 0.01$) in *MMP7* expression compared with the nontargeting siRNA. More importantly, the siRNA targeting *MMP7* significantly mitigated the increase in *MMP7* expression that occurs in response to FOXC1 overexpression (3.5-fold decrease compared with AdFOXC1 + Control siRNA; $p < 0.01$). As shown above, transduction of MCF10A cells with FOXC1-expressing adenovirus resulted in a significant increase in invasion (Fig. 4*B*). However, MMP7 silencing significantly inhibited the increase in invasion imparted by FOXC1 overexpression and the extent of this inhibition was comparable with the ability of the MMP7 siRNA to block the induction of *MMP7* expression (Fig. 4*B*). Combined, these results demonstrate that FOXC1 expression imparts a more invasive phenotype and that this increase in invasion is due, at least in part, to the increase in MMP7 expression that results upon FOXC1 overexpression.

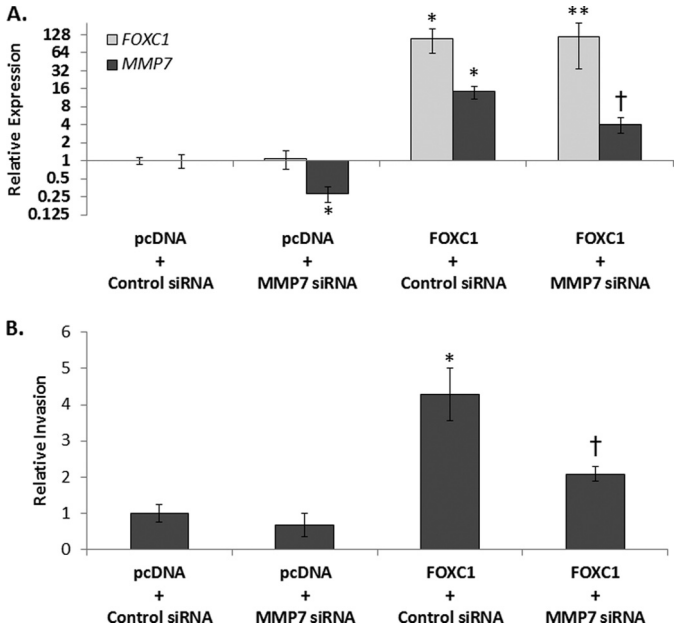

FIGURE 4. **MMP7 is required for increased invasion of MCF10A cells overexpressing FOXC1.** *A*, *FOXC1* (*light gray*) or *MMP7* (*dark gray*) transcript levels in MCF10A cells transfected with empty pcDNA vector or FOXC1 vector in combination with control siRNA or siRNA targeting MMP7. *GAPDH* was used as endogenous control. Data are relative to the pcDNA + Control siRNA-treated cells. *B*, relative invasion of MCF10A cells transfected with empty pcDNA vector or FOXC1 vector in combination with control siRNA or siRNA targeting *MMP7*. Data are relative to the pcDNA + Control siRNA-treated cells. Data summarize at least three independent experiments and are presented as mean ± S.D. *, $p < 0.05$ compared with pcDNA + Control siRNA-treated cells. **, $p < 0.05$ compared with pcDNA + MMP7 siRNA-treated cells but not significantly different from FOXC1 + Control siRNA-treated cells. †, $p < 0.05$ compared with either pcDNA + MMP7 siRNA or FOXC1 + control siRNA-treated cells.

Silencing Endogenous FOXC1 Results in Decreased MMP7 Expression—Basal A breast cancer cell lines (27) exhibit the highest expression of both FOXC1 and MMP7 (Fig. 2). We next determined if FOXC1 is necessary for MMP7 expression in these cells. Transient silencing of FOXC1 by siRNA in HCC1187 cells, the basal A line with the highest level of endogenous *FOXC1* mRNA and the third highest level of *MMP7* mRNA, reduced FOXC1 mRNA expression by 2.5-fold ($p < 0.05$). In response to this change in FOXC1 expression, *MMP7* mRNA was also decreased to a similar extent (2.86-fold; $p < 0.05$) (Fig. 5*A*). At the protein level, FOXC1 silencing resulted in a 70% decrease in FOXC1 and 90% decrease in MMP7 expression (Fig. 5*B*). In contrast, silencing of FOXC1 in HCC1187 cells did not result in decreased expression of either *MMP2* or *MMP9* although a modest but significant increase in *MMP2* mRNA expression was observed (1.6-fold; $p < 0.05$; Fig. 5*A*). Furthermore, silencing of FOXC1 in HCC1187 cells resulted in decreased levels of secreted MMP7 as detected by casein zymography, whereas no changes in MMP2 or MMP9 could be detected by gelatin zymography (Fig. 5*C*). We confirmed that HCC1187 cells treated with siRNA against FOXC1 secreted less MMP7 into conditioned media by ELISA (1.75-fold decrease; $p < 0.05$; Fig. 5*D*). Finally, we confirmed these data using shRNAs directed to FOXC1 to exclude off-target effects of the siRNA. Transient transduction of HCC1187 cells with three different shRNAs to FOXC1 resulted in significantly decreased *FOXC1* expression 72 h post-transduction with commensurate significant decreases in *MMP7* expression (supplemental Fig. S6*A*).

To confirm that the requirement for sustained expression of FOXC1 to maintain MMP7 expression in basal-like breast cancer cells was not cell line dependent, we transiently silenced FOXC1 in two additional basal A breast cancer cell lines: HCC1143 and MDA-MB-468. FOXC1-targeted shRNAs decreased *FOXC1* expression in these cells 72 h after transduction with corresponding decreases in *MMP7* expression (supplemental Fig. S6, *B* and *C*). Furthermore, stable silencing of FOXC1 in MDA-MB-468 cells resulted in significant reductions in both *FOXC1* and *MMP7* expression (supplemental Fig. S6*D*). These data confirm that FOXC1 consistently plays an important role in maintaining elevated MMP7 expression in basal-like breast cancer cells.

Silencing of FOXC1 has previously been shown to decrease viability or proliferation in a number of cell types (29, 30). In agreement with these previous findings, FOXC1 silencing in basal A breast cancer cell lines resulted in a substantial decrease in cell number compared with control treated cells (data not shown). This prohibited the utilization of these lines in invasion assays, but suggests that FOXC1 plays multiple roles in regulating the behaviors of basal breast cancer cells.

High FOXC1 Expression in Combination with High MMP7 Expression Predicts Poor Breast Cancer Patient Outcome—Expression of FOXC1 correlates with the BLBC subtype and poor prognosis in breast cancer patients (17, 20, 21). Given the significant functional correlation between FOXC1, MMP7, and invasion in our *in vitro* findings, we postulated that the association between *FOXC1* expression and poor prognosis in breast cancer patients might be explained by the association between *FOXC1* and *MMP7* expression. To test this hypothesis we obtained gene expression data from a well studied breast cancer patient cohort, Sørlie *et al.* (24) and stratified patient samples into groups based upon high (upper quartile) or low (lower three quartiles) expression of *FOXC1* and *MMP7*. This revealed that high *FOXC1* expression alone was associated with poor outcome in this dataset ($p < 0.05$; supplemental Fig. S7*A*), whereas high *MMP7* expression alone trended toward, but was not significantly associated with, poor outcome ($p = 0.094$; supplemental Fig. S7*B*). We next divided patients into four groups based on the pairwise expression of either high or low *FOXC1* and high or low *MMP7*. Kaplan-Meier estimates revealed a significant difference in overall survival between the four resulting groups ($p < 0.01$; Fig. 6*A*). When the $FOXC1^{high}/MMP7^{high}$ group was removed from the analysis, the Kaplan-Meier curves for the remaining three groups were not statistically different ($p = 0.935$). Importantly, direct comparison of survival curves for the $FOXC1^{high}/MMP7^{high}$ and $FOXC1^{high}/MMP7^{low}$ groups revealed that the $FOXC1^{high}/MMP7^{high}$ group had a significantly worse outcome ($p < 0.05$; Fig. 6*B*) than the $FOXC1^{high}/MMP7^{low}$ group. These findings were confirmed using a gene expression dataset from a second, larger patient cohort reported by van de Vijver *et al.* (25). In this group of patients we found that, whereas there was a trend between high *FOXC1* expression and outcome, elevated expression of neither *FOXC1* nor *MMP7* alone was significantly associated with poor

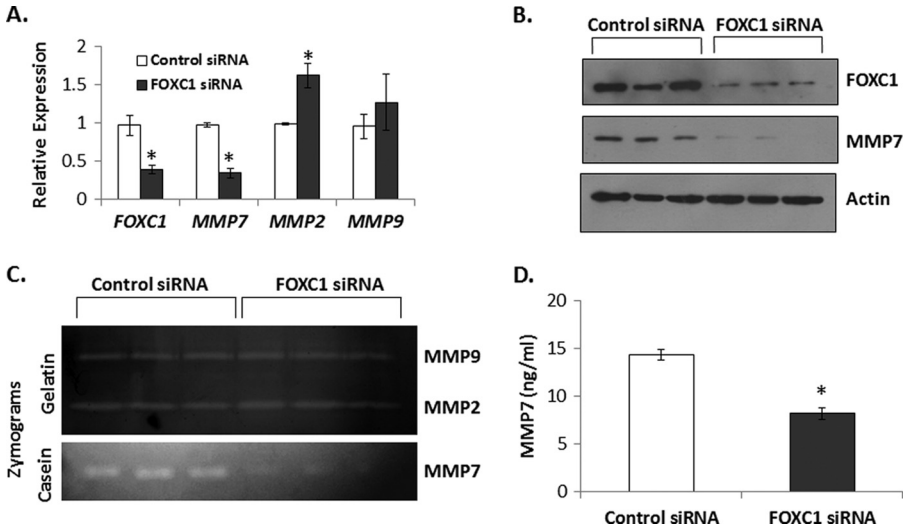

FIGURE 5. **Silencing of FOXC1 reduces MMP7 expression in the basal-like breast cancer cell line HCC1187.** *A*, transcript levels of *FOXC1*, *MMP7*, *MMP2*, and *MMP9* in HCC1187 cells transfected with nontargeting control siRNA or siRNA targeting *FOXC1*. Data are relative to control siRNA-transfected cells; *GAPDH* was used as endogenous control. Data summarize three independent experiments and are presented as mean ± S.D. *B*, representative Western blots demonstrating expression of FOXC1 and MMP7 in HCC1187 cells transiently transfected with control siRNA or siRNA targeting *FOXC1*. *C*, representative gelatin (*upper panel*) or casein (*lower panel*) zymograms demonstrating the relative levels of MMP9, MMP2, or MMP7 in conditioned media from HCC1187 cells transfected with control siRNA or siRNA targeting *FOXC1*. *D*, conditioned media from HCC1187 cells transfected with control siRNA or siRNA targeting *FOXC1* was used in an ELISA for MMP7. Bar graph represents mean ± S.D. for three independent experiments. *, $p < 0.05$.

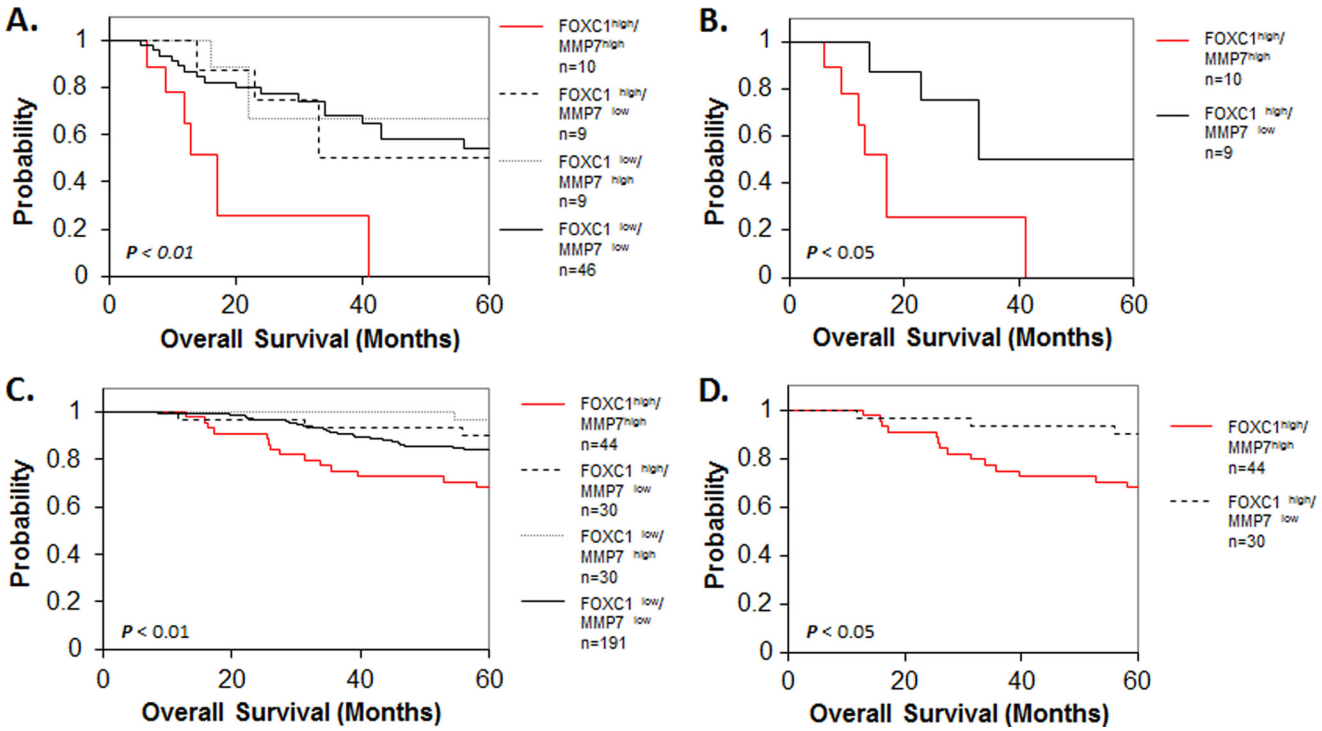

FIGURE 6. **High *FOXC1* in combination with high *MMP7* is associated with decreased breast cancer patient survival.** Kaplan-Meier curves of overall survival using data from the Sørlie *et al.* (24) (*A* and *B*) or van de Vijver *et al.* (25) (*C* and *D*) datasets. Samples were stratified into four groups by high (upper quartile) or low (lower three quartiles) *FOXC1* and *MMP7* expression (*A* and *C*). Survival curves of *FOXC1*^{high}/*MMP7*^{high} and *FOXC1*^{high}/*MMP7*^{low} groups were directly compared in *B* and *D*. *p* values less than 0.05 were considered statistically significant.

outcome when stratified by high (upper quartile) *versus* low (lower three quartiles) expression ($p = 0.063$ for *FOXC1* and $p = 0.29$ for *MMP7*, respectively; supplemental Fig. S7, *C* and *D*). However, after stratifying this cohort into four groups based on the expression of both *FOXC1* and *MMP7*, a significant difference in overall survival between the four resulting groups was revealed ($p < 0.01$, Fig. 6*C*). If the FOXC1^{high}/MMP7^{high} group was removed from the analysis, there was no statistical difference between survival times of the remaining three groups ($p = 0.145$). Furthermore, direct comparison of the survival curves from the FOXC1^{high}/MMP7^{high} and FOXC1^{high}/MMP7^{low} groups confirmed that the FOXC1^{high}/MMP7^{high} group had a significantly worse outcome ($p < 0.05$, Fig. 6*D*) than did patients from the FOXC1^{high}/MMP7^{low} group. Inter-

TABLE 1
High combined expression of *FOXC1* and *MMP7* is an independent predictor of poor patient outcome in the Sorlie *et al.* (24) dataset

Univariate Analysis			95% Confidence Interval	
Variable	*P* value	Hazard Ratio	Lower	Upper
High *FOXC1*	0.0264	2.4163	1.6377	3.1949
High *MMP7*	0.1009	1.8946	1.1312	2.6581
High *FOXC1* and *MMP7*	0.0009	4.4180	3.5451	5.2909
T Stage	0.0419	1.8278	1.2469	2.4088
Grade	0.0081	1.5554	1.2283	1.8825
Age	0.3746	1.1174	0.8724	1.3623
N Stage	0.4390	1.2056	0.7321	1.6790
M Stage	0.7320	1.4179	-0.5809	3.4167

Multivariate Analysis			95% Confidence Interval	
Variable	*P* value	Hazard Ratio	Lower	Upper
High FOXC1 and MMP7	*0.0003*	*6.3588*	*5.3454*	*7.3721*
T Stage	0.0088	2.4154	1.7557	3.0751

estingly, high expression of *MMP7* in the absence of elevated *FOXC1* was not significantly associated with poor outcome in either patient cohort suggesting that MMP7 may be an important mediator of breast cancer aggressiveness only within the context of basal-like breast cancers, or when it is expressed in combination with FOXC1.

We next performed COX regression analysis on the Sørlie *et al.* (24) data set to assess the independent prognostic ability of combined *FOXC1* and *MMP7* expression (Table 1). As expected, univariate analysis showed that high (upper quartile) *FOXC1* expression in combination with high *MMP7* expression was a significant predictor ($p = 0.009$) of poor outcome with the highest hazard ratio (HR) of the factors examined (HR = 4.418). High *FOXC1* expression alone ($p = 0.026$; HR = 2.416), T Stage ($p = 0.042$; HR = 1.828), and Grade ($p = 0.008$; HR = 1.555) were also significantly associated with outcome in univariate analysis (Table 1). In stepwise multivariate analysis, high combined expression of both *FOXC1* and *MMP7* was found to be a significant ($p = 0.0003$) independent predictor of poor outcome (HR = 6.3588) (Table 1). In a second patient cohort (van de Vijver *et al.* (25)) we confirmed that high expression of both *FOXC1* and *MMP7* was a significant predictor of poor outcome in both univariate and multivariate analyses ($p = 0.002$; HR = 2.6674), whereas neither high *FOXC1* expression ($p = 0.0667$; HR = 1.7393) nor high *MMP7* expression ($p = 0.2924$; HR = 1.388) alone were predictive of outcome in this dataset (supplemental Table S3). Together, these findings suggest a novel mechanism through which FOXC1 increases cancer cell invasion and that the relationship between FOXC1 and MMP7 has functional consequences on breast cancer patient outcome.

DISCUSSION

Improved treatment options for women with BLBC/triple negative breast cancer will require the identification of targetable molecules that are functionally responsible for the proliferation, survival, and metastasis of these cancers. Here, working downstream of FOXC1, a transcription factor known to correlate with the BLBC subtype, we identified MMP7 as a possible therapeutic target in BLBC treatment. Although previous studies have shown that FOXC1 increases invasion and metastasis of cancer cells, its mechanism of action is not fully understood. The increased invasive capacity imparted by FOXC1 in previous studies could be attributed to induction of EMT by FOXC1 expression (17, 18). However, the studies reported herein demonstrate that FOXC1 can increase the invasive capacity of mammary cell lines in the absence of EMT. Furthermore, whereas FOXC1 has previously been associated with increased expression of MMP2 and MMP9, we utilized meta-analysis of 20 gene expression datasets to demonstrate for the first time that a different MMP family member, MMP7, significantly correlates with FOXC1 both *in vitro* and in human breast cancer samples, whereas neither MMP2 nor MMP9 are associated with FOXC1. MMP7 is an established instigator of aggressive behavior in a number of cancer types including colorectal tumors (31–36). A role for MMP7 in breast cancer aggressiveness has been investigated (37–39) but to our knowledge this is the first report of a critical role for MMP7 in BLBC. Thus, MMP7 may be a promising druggable target for the treatment of at least a subset of BLBC.

Our studies identified a number of cell lines with high endogenous expression of FOXC1 and MMP7. Interestingly, the cell lines with the greatest FOXC1 and MMP7 expression were basal A breast cancer lines (27). These cell lines have gene expression patterns that most closely correlate with BLBC (27, 28). Hence, basal A breast cancer cell lines such as HCC1187 may serve as superior models than basal B lines such as MDA-MB-231 for understanding BLBC and the roles of FOXC1 and MMP7 in this disease. For this reason, we utilized basal A breast cancer cell lines in our current study. In addition, we found that FOXC1 was sufficient to induce expression of MMP7 in non-transformed mammary epithelial cells. Of note, enforced expression of FOXC1 did not robustly induce MMP7 expression in models of other breast cancer subtypes, including luminal (MCF-7 and T47D) or basal B (MDA-MB-231) (data not shown). Several previous studies have overexpressed FOXC1 in luminal or basal B breast cancer cell lines such as MCF7 or MDA-MB-231 to gain insight into the role of FOXC1 in breast cancers. However, these cell lines may not be ideal models in which to study the role of FOXC1 in BLBC. It is postulated that luminal, basal-like, and claudin-low breast cancers originate from different mammary cell populations (40, 41) and therefore may retain some of the epigenetic imprinting of their cells of origin. As a result, key transcriptional targets of FOXC1, including those that dictate BLBC aggressiveness such as *MMP7*, may be inaccessible to FOXC1 in the chromatin of luminal or basal B breast cancer cells. In support of the possibility that the *MMP7* promoter may be inaccessible to FOXC1 in luminal and basal B cell lines, this promoter is silenced by hypermethylation in a number of pancreatic cancer cell lines (42). With these possible epigenetic shortcomings in mind we choose to utilize nontransformed mammary epithelial cell lines, rather than luminal or claudin-low breast cancer cell lines, in our FOXC1 overexpression experiments. Unlike breast cancer cell lines, nontransformed mammary epithelial cell lines such as MCF10A cells retain a level of plasticity allowing them to form acinar structures with apico-basal polarity in three-dimensional culture (43). The inherent luminal-basal plasticity of these cells may explain their ability to increase MMP7

expression in response to FOXC1. Future studies are necessary to determine whether the MMP7 promoter is methylated in a subtype-dependent manner in breast cancer cells and if it is directly or indirectly regulated by FOXC1. Intriguingly, the MMP7 proximal promoter contains a single consensus binding element for FOXC1 as well as numerous binding sites for other forkhead box transcription factors suggesting a direct role for FOXC1, as well as other forkheads, in MMP7 regulation. Supporting a role for forkheads in the regulation of MMP7 expression, a polymorphism that results in a novel binding site for FOXA2 in the MMP7 promoter was recently shown to result in FOXA2-dependent up-regulation of MMP7 expression (44).

Last, we demonstrated that the association between *FOXC1* and *MMP7* expression has important implications in breast cancer patient outcome. FOXC1 has previously been shown to correlate with a poor outcome in a number of patient cohorts (17, 20, 21), and our analyses of survival data provide novel insight into the mechanism underlying this correlation. We analyzed the association between combined *FOXC1* and *MMP7* expression with patient outcome and found that the correlation between high *FOXC1* and poor outcome may depend, in part, upon high *MMP7* expression. High *FOXC1* expression was associated with significantly worse patient outcome when accompanied by elevated *MMP7* in our analyses. Furthermore, combined expression of *FOXC1* and *MMP7* identified a unique population of breast cancers that convey poor patient survival in multivariate analyses. Together, these data demonstrate a novel role for MMP7 in the increased aggression imparted by FOXC1 expression. More importantly, these data suggest that inhibiting MMP7 may be a therapeutic strategy in treatment of BLBC.

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