Mesenchyme Forkhead 1 (*FOXC2*) plays a key role in metastasis and is associated with aggressive basal-like breast cancers

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The metastatic spread of epithelial cancer cells from the primary tumor to distant organs mimics the cell migrations that occur during embryogenesis. Using gene expression profiling, we have found that the FOXC2 transcription factor, which is involved in specifying mesenchymal cell fate during embryogenesis, is associated with the metastatic capabilities of cancer cells. *FOXC2* expression is required for the ability of murine mammary carcinoma cells to metastasize to the lung, and overexpression of FOXC2 enhances the metastatic ability of mouse mammary carcinoma cells. We show that FOXC2 expression is induced in cells undergoing epithelial-mesenchymal transitions (EMTs) triggered by a number of signals, including TGF-β1 and several EMT-inducing transcription factors, such as Snail, Twist, and Goosecoid. FOXC2 specifically promotes mesenchymal differentiation during an EMT and may serve as a key mediator to orchestrate the mesenchymal component of the EMT program. Expression of *FOXC2* is significantly correlated with the highly aggressive basal-like subtype of human breast cancers. These observations indicate that *FOXC2* plays a central role in promoting invasion and metastasis and that it may prove to be a highly specific molecular marker for human basal-like breast cancers.

epithelial-mesenchymal transition | embryogenesis | Twist and Snail

A number of studies suggest that carcinoma cells often activate a transdifferentiation program termed the epithelial-mesenchymal transition (EMT) to acquire the ability to execute the multiple steps of the invasion–metastasis cascade (1). EMTs occur during various developmental processes, including gastrulation and neural crest formation. During an EMT, epithelial cells lose cell–cell contacts and cell polarity, acquire mesenchymal gene expression, and undergo major changes in their cytoskeleton that enables them to acquire a mesenchymal appearance with increased motility and invasiveness (2–5).

An EMT can be induced by several alternative signaling pathways, notably those involving the cooperation between TGF-β1 signaling with oncogenic Ras or receptor tyrosine kinases (6–10), Wnt (11, 12), Notch (13, 14), and the signaling activated by Hedgehog (15). In addition, certain developmental transcription factors, specifically Snail (16–18), Slug (19, 20), SIP1 (21, 22), E12/E47 (23), Goosecoid (24), and Twist (25), can promote this transition. The expression of some of these transcription factors has been found to be induced during tumor progression (25–30).

We undertook to identify additional genes that function as pleiotropic regulators capable of promoting tumor cell invasion and metastasis. To do so, we used gene expression analysis of mouse mammary metastatic tumor cells exhibiting various degrees of invasive and metastatic competence as described (25). This analysis led us to the identification of the FOXC2 gene, previously termed Mesenchyme Forkhead 1 (MFH-1). Here, we show that FOXC2 is a central mediator of the EMT program and, by acting in this fashion, plays a key role in tumor metastasis. Its preferential expression and subcellular localization in a particularly aggressive subtype of human breast cancer suggests that it contributes directly to the poor clinical outcomes associated with these tumors.

Results

FOXC2 Is Overexpressed in Metastatic Cancer Cells. To identify genes capable of programming cancer invasion and metastasis, we performed gene expression profiling by using primary tumors generated by four murine mammary carcinoma cells lines (67NR, 168FARN, 4TO7, and 4T1) that exhibit differing metastatic abilities (31). The properties of these lines are summarized in Fig. 1*A* and in earlier reports (25, 31). The first of these (67NR) is capable of forming only primary carcinomas, whereas the second (168FARN) can release cells into the blood stream as well; the third (4TO7) can complete the aforementioned steps and generate micrometastases, and the fourth (4T1) can execute all of the steps required for the formation of macroscopic metastases. We performed gene expression profiling (25) and identified 38 genes as being up-regulated and 59 as being down-regulated in 4T1 cells compared with the other three cell lines (67NR, 168FARN, and 4TO7). The up-regulated genes included FOXC2, a winged helix/Forkhead domain-containing transcription factor, which is expressed mainly in mesoderm and mesoderm-derived tissues (32, 33) during embryogenesis and specifies mesenchymal cell fates (34). It is not expressed in any adult tissues except those containing adipocytes (35). We found FOXC2 expression to be up-regulated in the 4T1 cells, the most aggressive of the four mouse mammary tumor cell lines and the only one capable of forming macroscopic lung metastases (Fig. 1 *B–D*).

We examined FOXC2 expression in a panel of established metastatic and nonmetastatic mouse and human tumor cell lines. Five of six metastatic cell lines expressed FOXC2 protein, whereas only two of seven nonmetastatic lines did so. In addition, expression of FOXC2 was not detected in immortalized normal human mammary epithelial cells (HMLE) (Fig. 1*D*). These associations suggested that the expression of FOXC2 was often induced during

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Abbreviations: EMT, epithelial-mesenchymal transition; HMLE, human mammary epithelial cells; shRNA, short hairpin RNA; MDCK, Maden-Darby canine kidney; MMP, matrix metalloproteinase; ER, estrogen receptor.

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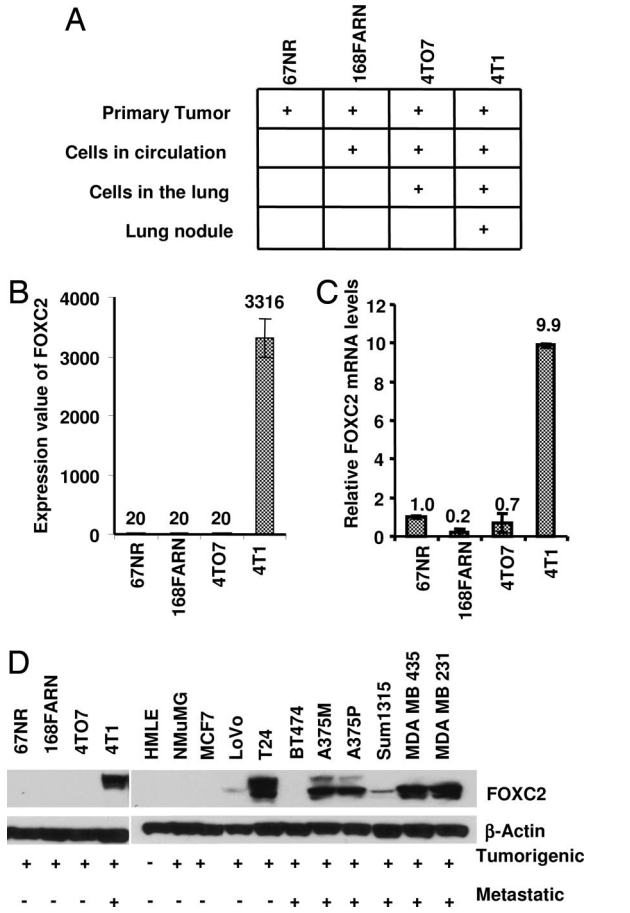

Fig. 1. Increased expression of *FOXC2* in highly metastatic cancer cells. (*A*) Metastatic properties of mouse mammary carcinoma cells used for microarray analysis are represented graphically. (*B*) Expression of *FOXC2* mRNA in primary tumors formed by individual cell lines was measured by microarray analysis. Each bar represents the mean ± SEM. (*C*) Expression of *FOXC2* mRNA was measured by real-time PCR using RNAs isolated from individual tumors formed by the four cell lines. The expression level of FOXC2 in 67NR tumor was used as the baseline. (*D*) Expression of FOXC2 protein was measured in various metastatic and nonmetastatic cell lines, including the four mouse cell lines, by immunoblotting.

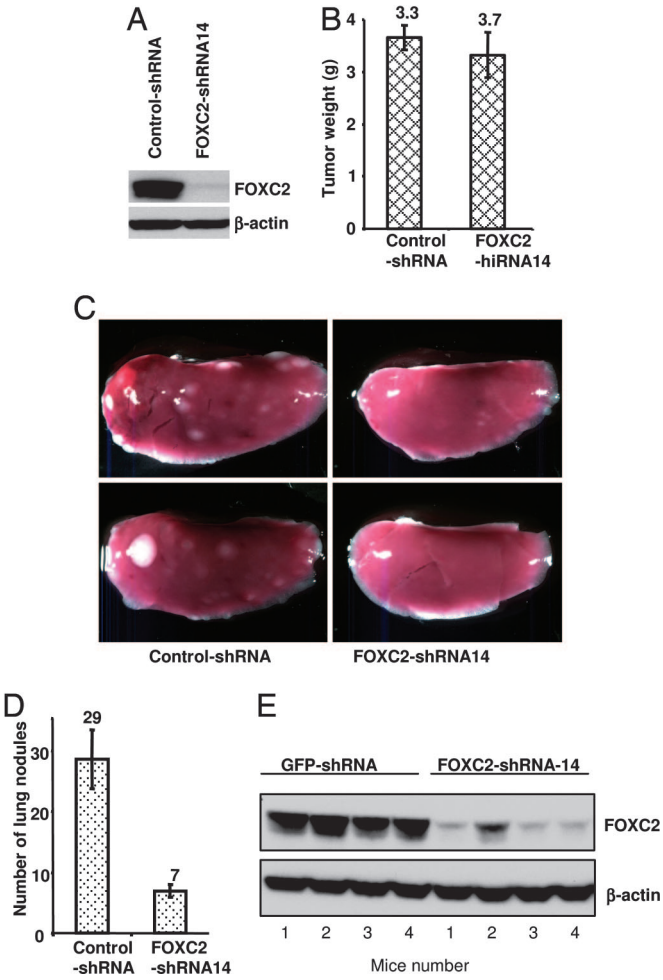

Fig. 2. Contribution of FOXC2 to the metastatic ability of mouse mammary carcinoma cells. (*A*) Expression of FOXC2 protein in cells expressing either the control shRNA or the *FOXC2* shRNA14 is shown. (*B*) *In vivo* growth properties of 4T1 tumors expressing either the control shRNA or the *FOXC2* shRNA14 grown in the mammary glands, harvested 28 days after injection, are shown. The tumor weight was measured and presented as mean ± SEM ($n = 14$). (*C*) Two representative images of lungs harvested from mice carrying 4T1 tumors expressing either the control shRNA (*Left*) or the *FOXC2* shRNA14 (*Right*) are shown. (Magnification: ×0.8.) (*D*) The average number of lung nodules from the mice harboring 4T1 tumors expressing the control shRNA or the *FOXC2* shRNA14 is shown. Each bar represents the mean ± SEM ($n = 14$). (*E*) Expression of FOXC2 protein in 4T1 cells isolated from lung nodules of mice harboring tumor from either 4T1 cells expressing control shRNA or FOXC2 shRNA is shown. β-Actin was used as a loading control.

the course of tumor progression and that it might play a causal role in enabling metastatic dissemination.

FOXC2 Is Required for the Metastatic Ability of Breast Cancer Cells. We set out to determine whether expression of FOXC2 contributes to the metastatic ability of the 4T1 mouse mammary carcinoma cells. To do so, we undertook to suppress FOXC2 expression in these cells by constructing a series of short hairpin RNA (shRNA) oligonucleotides targeting mouse *FOXC2* mRNA. These shRNAs were stably expressed in the 4T1 cells by using lentiviral vectors (36). Of these, the *FOXC2* shRNA14 vector reduced the level of FOXC2 protein expression by >90% (Fig. 2*A*).

To gauge the contribution of FOXC2 to primary tumor formation and metastatic dissemination, we injected 4T1 cells expressing *FOXC2* shRNA14 into the mouse mammary fat pad and examined the resulting primary tumors and lung nodules 28 days later. The *FOXC2* shRNA14-expressing cells formed primary tumors (3.32 ± 0.40 g) similar in size to those arising from 4T1 cells expressing a nonspecific control shRNA (3.66 ± 0.20 g) (Fig. 2*B*). However, suppression of FOXC2 expression reduced the number of metastatic nodules in the lung by 75% (7 ± 1 versus 29 ± 5; $P < 0.001$; Fig. 2 *C* and *D*). A second *FOXC2* shRNA (*FOXC2* shRNA7) suppressed the expression of endogenous FOXC2 partially (by 50%) and also reduced the number of lung nodules (by 40%) (data not shown). Importantly, the few metastatic nodules that eventually did form in the lungs of mice carrying FOXC2 shRNA-expressing primary tumors retained FOXC2 expression, indicating that they arose from cells within the primary tumors in which FOXC2 expression had never been inhibited (Fig. 2*E*). These results indicated that expression of FOXC2 is required for the full metastatic ability of the 4T1 mouse mammary carcinoma cells.

We next tested whether ectopic FOXC2 expression in weakly metastatic cells could promote metastatic nodule formation. To do so, we overexpressed FOXC2 in a weakly metastatic EpRas mouse mammary carcinoma cell line (37) (Fig. 3*A*). Ectopic expression of FOXC2 in EpRas cells did not affect either their proliferation rate *in vitro* or the growth kinetics of the resulting primary tumors at s.c. sites in nude mice (Fig. 3*B*). However, the tumors formed by FOXC2-expressing EpRas cells gave rise to 33 ± 6 metastatic

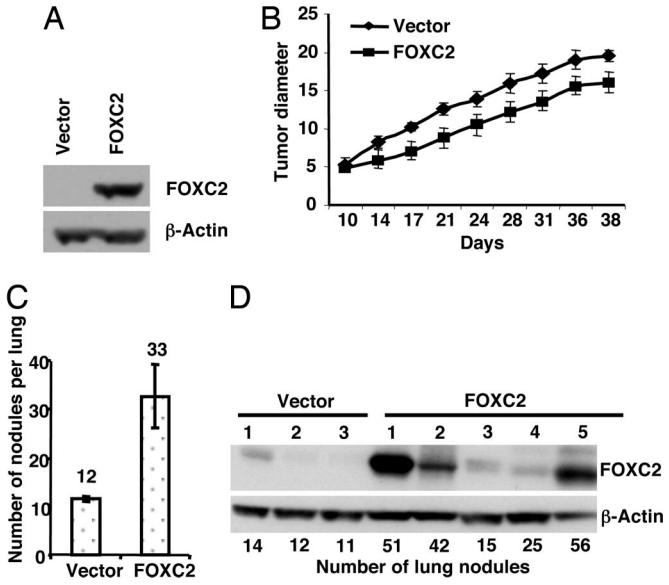

Fig. 3. Effect of *FOXC2* expression on the metastatic behavior of EpRas mouse mammary carcinoma cells. (*A*) Ectopic expression of FOXC2 protein in EpRas cells was analyzed by immunoblotting. (*B*) *In vivo* growth properties of EpRas cells expressing *FOXC2* at the s.c. site of nude mice is shown. The tumor size was measured by their mean diameters ± SEM ($n = 7$). (*C*) The average number of visible lung metastatic nodules in mouse with EpRas cells expressing either the control vector or the FOXC2 is represented as mean ± SEM ($n = 7$). (*D*) The level of FOXC2 protein was assessed by immunoblotting in individual primary tumors formed by EpRas cells expressing either the control vector or FOXC2. The total number of visible lung nodules found in the respective mouse is indicated at the bottom of the gel.

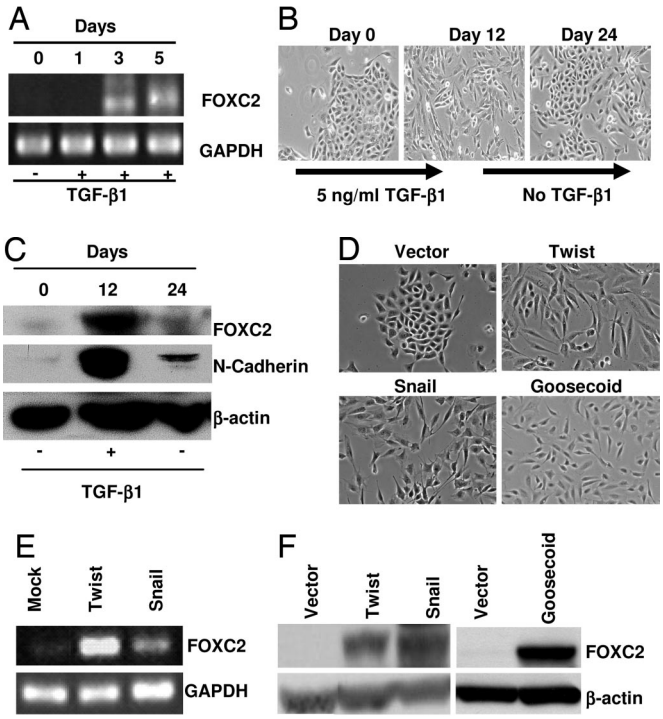

Fig. 4. Induction of FOXC2 by various EMT-inducing signals. (*A*) Expression of *FOXC2* mRNA in EpRas cells treated with TGF-β1 is shown for the indicated number of days. (*B*) Morphologies of HMLEs either untreated or treated with 5 ng/ml of TGF-β1 for 12 days and HMLEs expressing either the control vector, Twist, Snail, or Goosecoid, were revealed by phase-contrast microscopy. (Magnification: ×200.) (*C*) Expression of FOXC2 and N-cadherin was examined before, during, and after the TGF-β1 treatment. (*D*) The morphology of HMLEs expressing either the control vector, Twist, Snail, or Goosecoid was revealed by phase-contrast microscopy. (Magnification: ×200.) (*E*) Expression of *FOXC2* mRNA in the HMLEs ectopically expressing either an empty vector or the indicated EMT-inducing genes by RT-PCR is shown. (*F*) Expression of FOXC2 protein in the HMLEs ectopically expressing either a control vector or the indicated EMT-inducing genes was examined by immunoblotting.

nodules per lung compared with the control cells, which formed only 12 ± 1 nodules per lung in nude mice ($P < 0.02$) (Fig. 3*C*).

We proceeded to examine the expression level of FOXC2 protein in individual primary tumors derived from the control and EpRas FOXC2 cells. Interestingly, the FOXC2 protein in individual primary tumors correlated directly with the number of metastatic nodules generated by such tumors: primary tumors expressing higher levels of FOXC2 protein formed greater numbers of metastatic nodules in the lung (Fig. 3*D*). These results show that FOXC2 can contribute significantly to the metastatic potential of the EpRas mammary carcinoma cells.

EMT-Promoting Signals Induce FOXC2 Expression. Previous work of others (37) had shown that a collaboration of TGF-β1 signals and the activated Ras oncoprotein in the EpRas cells described above causes them to undergo an EMT and thereby promotes their ability to invade and form distant metastases. This result raised the possibility that FOXC2 might operate downstream of TGF-β1 to mediate induction of the EMT program.

Accordingly, we tested whether induction of an EMT in EpRas cells by TGF-β1 treatment affected endogenous FOXC2 expression, which is normally not detected in these cells. Indeed, we found that concomitant with EMT induction, TGF-β1 activated expression of *FOXC2* mRNA in EpRas cells (Fig. 4*A*) by as much as 13-fold. Similarly, treatment of HMLE-immortalized HMLE (25, 38) with TGF-β1 also resulted in an EMT (Fig. 4*B Center*), and concomitant induction of *FOXC2* protein synthesis (Fig. 4*C*, 12 days). Expression of FOXC2 was first detected after 3 days of TGF-β1 treatment and increased in parallel with acquisition of the mesenchymal morphology. Moreover, withdrawal of TGF-β1 from the cells that had undergone EMT resulted in the reversion of most cells to their original epithelial morphology (Fig. 4*B Right*) concomitant reversion of FOXC2 protein expression to its basal level of expression (Fig. 4*C*, 24 days). The slow kinetics of induction of FOXC2 expression by TGF-β1 and subsequent shutdown of FOXC2 expression after removal of TGF-β1 suggests that the *FOXC2* gene is not a direct target of TGF-β signaling pathways.

We also found ectopic expression of any one of the three EMT-inducing transcription factors (Twist, Snail, or Goosecoid) led, as anticipated, to an EMT in the HMLE (24, 25) (Fig. 4*D*) and, interestingly, to induction of both *FOXC2* mRNA (Fig. 4*E*) and protein expression (Fig. 4*F*). Together, these results demonstrate that FOXC2 expression is induced by a variety of EMT-inducing agents and suggest that FOXC2 is commonly involved in a diverse set of EMT programs, possibly by organizing the mesenchymal state of cells, as suggested by its expression in mesoderm-derived tissues during embryogenesis (32, 33).

FOXC2 Promotes Mesenchymal Differentiation as Part of the EMT Program. To test whether FOXC2, on its own, is sufficient to induce the EMT program, we ectopically expressed FOXC2 in Maden-Darby canine kidney (MDCK) epithelial cells, which have been widely used to study epithelial cell biology (Fig. 5*A*). In fact, ectopic FOXC2 expression in these cells caused them to shed their cuboidal epithelial morphology and acquire instead a morphology reminiscent of mesenchymal cells (Fig. 5*B*).

At the biochemical level, *FOXC2* expression elicited expression of mesenchymal markers typically induced during an EMT (Fig. 5

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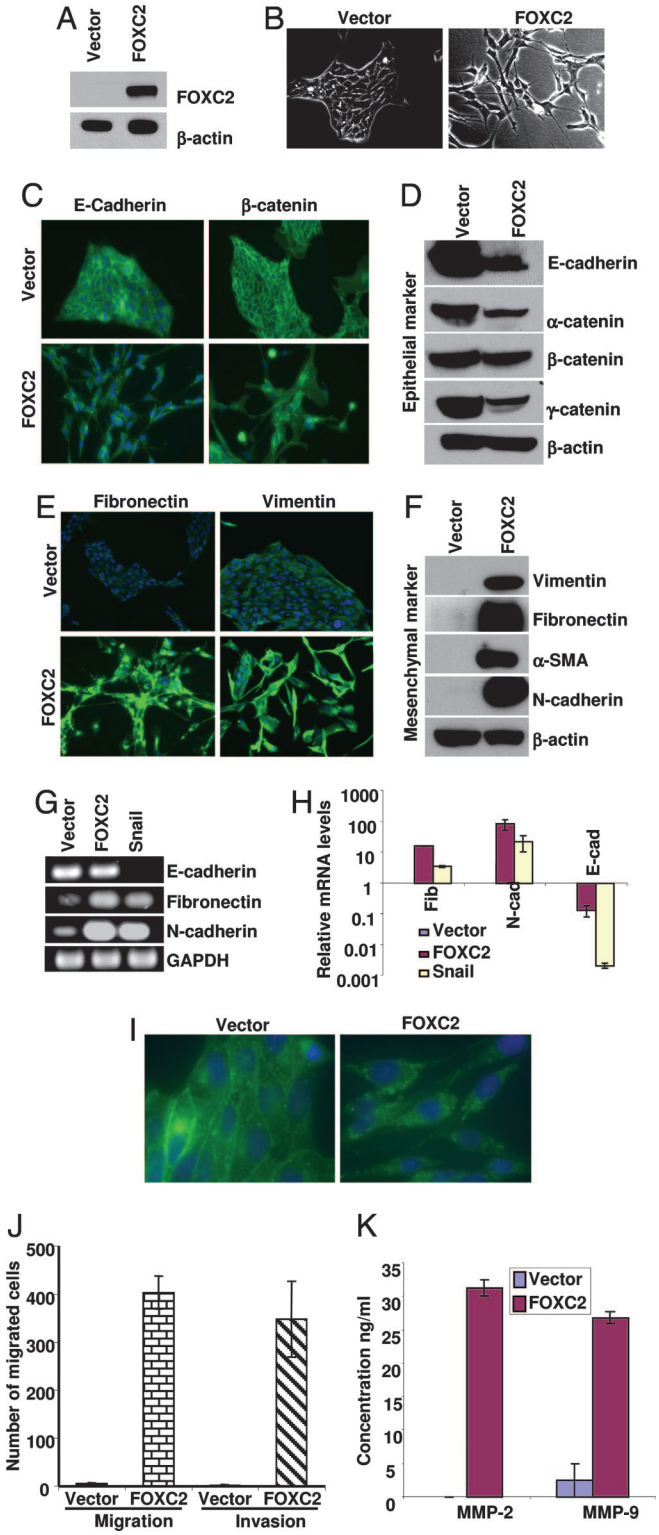

Fig. 5. Effects of *FOXC2* expression in MDCK cells. (*A*) Overexpression of FOXC2 protein by retroviral infection in MDCK cells was examined by immunoblotting. (*B*) Phase-contrast images of MDCK cells expressing either the control vector (*Left*) or FOXC2 (*Right*) are shown. (Magnification: ×200.) (*C*) MDCK cells expressing either the control vector (*Upper*) or FOXC2 (*Lower*) immunostained with antibodies recognizing E-cadherin (*Left*) or β-catenin (*Right*) are shown. The green signal represents the staining of corresponding protein, and the blue signal represents the nuclear DNA staining by DAPI. (Magnification: ×200.) (*D*) Immunoblotting of epithelial markers, including E-cadherin, α-catenin, β-catenin, and γ-catenin in MDCK cells expressing either the control vector (*Left*) or the FOXC2 (*Right*) is shown. (*E*) MDCK cells expressing either the control vector (*Upper*) or FOXC2 (*Lower*) immunostained with antibodies recognizing fibronectin (*Left*) or vimentin (*Right*) are shown. The green signal represents the staining of the corresponding protein, and the blue signal represents the nuclear DNA staining by DAPI. (Magnification: ×200.) (*F*) Immunoblotting of mesenchymal markers, including vimentin, fibronectin, α-smooth muscle actin, and N-cadherin in MDCK cells expressing either the control vector (*Left*) or the FOXC2 (*Right*) is shown. (*G* and *H*) The level of expression of E-cadherin, fibronectin, and N-cadherin mRNA in MDCK cells expressing control vector, FOXC2, or Snail was measured by RT-PCR (*G*) or real-time PCR (*H*). (*I*) Immunofluorescence images of MDCK cells expressing the control vector (*Left*) or the FOXC2 stained with antibodies against E-cadherin (*Right*). The green signal represents the staining corresponding to E-cadherin, and the blue signal represents the nuclear DNA staining by DAPI. (Magnification: ×1,000.) (*J*) Migration and invasion assay used MDCK cells expressing either the control vector or FOXC2. The migration and invasion ability is presented as total number of cells migrated to the bottom chamber. Each bar represents the mean ± SEM of samples measured in triplicate, and each experiment was repeated at least three times. (*K*) Expression of MMP2 and MMP9 was measured by ELISA; the data represent the level of expression in FOXC2 and the vector-infected control cells. Each bar is the average of the triplicate samples, and each experiment was repeated twice.

E and *F*). Significantly, however, the preexisting expression of epithelial cell-specific proteins was only partially reduced (Fig. 5 *C* and *D*). Moreover, expression of the mRNA encoding E-cadherin, a central effector of the epithelial cell phenotype, was only partially reduced by the expression of *FOXC2* (Fig. 5 *G* and *H*). This behavior contrasted starkly with the known behavior of Snail, Slug, SIP1, Goosecoid, and Twist, all of which strongly reduce E-cadherin expression (16, 17, 21, 24, 25, 39). In fact, the E-cadherin protein that continued to be synthesized in the *FOXC2*-expressing cells was delocalized from the plasma membrane to the cytoplasm, ostensibly the cytosol (Fig. 5*I*). Hence, unlike other embryonic transcription factors studied to date, FOXC2 is able to effectively program only the second half of the EMT transdifferentiation program, that involving the induction of mesenchymal traits.

We also performed Boyden-chamber transwell assays and determined that ectopic expression of *FOXC2* in MDCK cells rendered them to become highly migratory (Fig. 5*J*) and invasive (Fig. 5*J*). Cancer cells use secreted matrix metalloproteinases (MMPs) to degrade the nearby extracellular matrix (40). As gauged by ELISA, we found MMP2 and MMP9 to be induced to ≈31 and 26 ng/ml, respectively, in response to the expression of *FOXC2* in MDCK cells (Fig. 5*K*) in comparison with control MDCK cells, which do not express any detectable MMP2 and express very low levels of MMP9 (≈2.5 ng/ml). These observations reinforced the notion that FOXC2 can serve as an organizer of mesenchymal differentiation during an EMT and can potentially program malignancy-associated cellular traits.

FOXC2 Is Specifically Overexpressed in Highly Aggressive Basal-Like Breast Cancers. We undertook to determine whether *FOXC2* expression plays a role in the development of various subtypes of human mammary carcinomas. To do so, we performed immunohistochemistry with a FOXC2-specific antibody on tissue microarrays containing 117 samples of human invasive breast carcinomas of various subtypes and a set of normal human breast tissue sections. In fact, FOXC2 protein was not detected in most cells of normal breast ducts and lobules obtained from reduction mammoplasties, with the exception of a small percentage of basal epithelial cells (Fig. 6*A*). In stark contrast, FOXC2 expression was readily detectable by immunohistochemistry in 85% of invasive breast carcinomas. Among these tumors, the immunoreactivity pattern ranged from absent (Fig. 6*B*), to faint cytoplasmic staining (low) in 52% of the cases (Fig. 6*C*), moderate cytoplasmic staining in 37% (Fig. 6*D*), to strong cytoplasmic and/or nuclear staining (high) in 10% (Fig. 6 *E* and *F*). Further analysis of these data revealed that FOXC2 expression was associated with several adverse prognostic

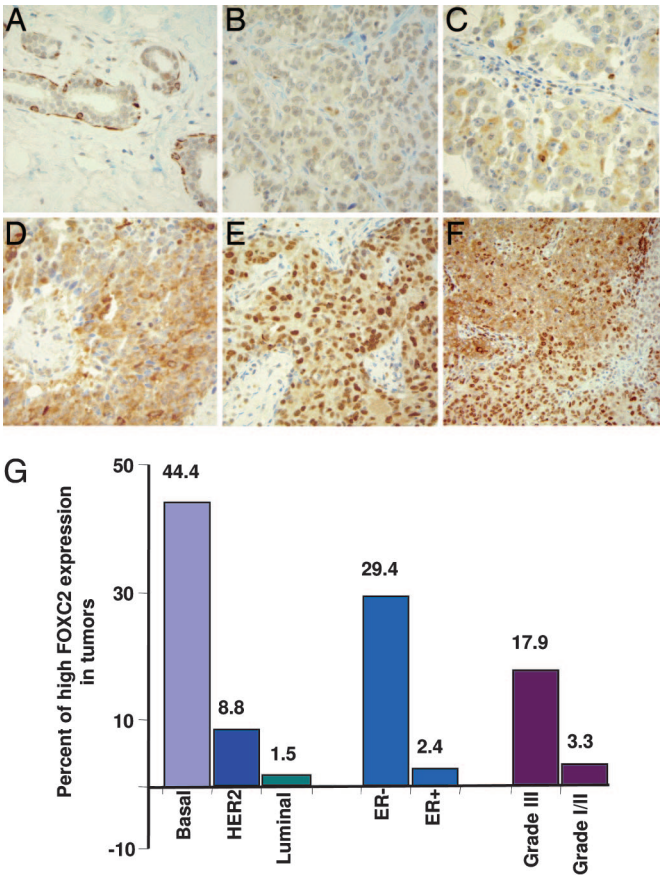

Fig. 6. Expression of *FOXC2* in human basal-like breast cancers. Shown are representative immunohistochemical images of human breast cancer tissue microarrays stained with the monoclonal anti-human FOXC2 antibody. (*A*) Normal human breast tissue. (*B*) Negative tumor staining. (*C*) Weak cytoplasmic staining. (*D*) Strong cytoplasmic staining. (*E*) Strong nuclear staining. (*F*) Strong nuclear and cytoplasmic staining. (Magnification: ×400.) (*G*) The percentage of human breast cancer samples with high level of FOXC2 expression in the respective tumor subtypes is shown.

markers, including estrogen receptor (ER) negativity ($P < 0.001$) and high tumor grade ($P < 0.002$) (Table 1).

Most interestingly, high levels of FOXC2 expression were associated with the aggressive, basal-like subtype of invasive ductal breast cancers ($P < 0.0001$) (Fig. 6*G* and Table 1) (41–46). As shown in Fig. 6*G*, 44% of basal-like tumors exhibited high levels of FOXC2 expression, whereas only 9% of HER2+ tumors and 2% of the far more common luminal ER+ subtype of tumors showed high expression of FOXC2 protein (Fig. 6*G* and Table 1). Only a few distinct molecular markers have been identified to date that are uniquely associated with basal-like breast cancers (45, 46). FOXC2 expression therefore may prove to be a valuable diagnostic marker for this subtype.

Table 1. Correlations of FOXC2 staining with tumor subtypes

Tumor type	Total no. of cases	No. with high-level expression for FOXC2	Percentage with high FOXC2 expression	*P value*
Grade I/II	61	2	3.3	0.990000
Grade III	56	10	17.9	0.001260
ER+	83	2	2.4	1.000000
ER−	34	10	29.4	0.000003
Luminal-ER+	65	1	1.5	1.000000
HER2	34	3	8.8	0.481000
Basal	18	8	44.4	0.000001

The percentage of samples with high level of FOXC2 expression in individual tumor subtypes and the corresponding *P* values are shown.

Discussion

The EMT program has been found to provide carcinoma cells with many of the phenotypes required to execute multiple steps of the invasion–metastasis cascade (2, 3, 47–51). The fact that FOXC2 expression is induced by a large number of known regulators of the EMT program, notably the Twist, Snail, and Goosecoid transcription factors as well as TGF-β1, suggests that *FOXC2* is involved in a diverse array of EMT programs. One key functional trait of *FOXC2* sets it clearly from other EMT-inducing transcription factors to date: these others, notably *Twist* (25), *Snail* (16–18), *Slug* (19, 20), *E12/E47* (23), *Goosecoid* (24), and *SIP1* (21), show a potent ability to repress E-cadherin expression either directly or indirectly. In contrast, *FOXC2* does not affect E-cadherin mRNA levels, but instead redirects the E-cadherin that continues to be synthesized from the plasma membrane to the cytoplasm. Indeed, the present evidence suggests that these other EMT regulators suppress epithelial gene expression and induce *FOXC2* expression, delegating to *FOXC2* the task of inducing the mesenchymal component of their EMT programs.

Ectopic expression of FOXC2 promotes mesenchymal differentiation in MDCK cells, but induces cell death in a variety of epithelial cell types, including MCF7, T47D, and NMuMG carcinoma cells as well as MCF10A and HMLE-immortalized mammary epithelial cells. In contrast, overexpression of *FOXC2* did not affect the growth or survival of HMLEs that had previously undergone an EMT induced by Twist (unpublished observations). These observations, when taken together, indicate that the abrupt activation of mesenchymal differentiation without a coordinated, well programmed loss of epithelial properties is detrimental to most epithelial cells. Moreover, when cells that have undergone an EMT induced by these factors are deprived of FOXC2 expression they undergo cell death (data not shown). Hence, FOXC2 expression must be carefully coordinated with the expression of one or more of these other transcription factors to ensure cell survival.

The genetic and epigenetic alterations conferring the aggressive phenotypes of breast cancers of basaloid subtype have been obscure. Strikingly, in the preliminary survey conducted here, elevated expression of FOXC2 is observed in almost half of these basaloid tumors, but is present only in <2% of the far more common ductal carcinomas of the luminal type. The present functional characterizations of FOXC2 suggest that it contributes directly to the aggressive clinical behavior of these carcinomas. We previously reported that expression of Twist correlates with invasive lobular breast carcinoma (25). These observations, and those of others (4, 16–20, 22, 25), reinforce the notion that various types of human cancers opportunistically up-regulate normally latent, pleiotropically acting embryonic transcription factors to acquire many of the cellular phenotypes that are needed to execute the invasion–metastasis cascade.

Materials and Methods

Cell Cultures. The mouse mammary tumor cell lines (67NR, 168FARN, 4TO7, and 4T1), human mammary epithelial cell line, immortalized with SV40 large T antigen and catalytic subunit of telomerase (HMLE), and MDCK cell lines were maintained as described (25). MCF7, LoVo, NMuMG, T24, BT474, MDA MB 435, and MDA MB 231 were obtained from the American Type Culture Collection (Manassas, VA) and grown per American Type Culture Collection recommendations. A375P and A375M cells (a gift from Isaiah J. Fidler, M.D. Anderson Cancer Center, Houston, TX) were maintained in MEM supplemented with vitamins, sodium pyruvate, L-glutamine, nonessential amino acid, and 10%

FBS. EpRas cells (a gift from Ernst Reichmann, University of Zurich, Zurich, Switzerland) were maintained in DME supplemented with 8% FCS, pen/strep, and buffered Hepes, pH 7.0 and 500 μg/ml G418. HMLEs expressing Snail and Twist have been described (25). For TGF-β1 treatment, the HMLEs were cultured in DME/F12 media (1:1) supplemented with insulin, EGF, hydrocortisone, and 5% calf serum and treated with 5 ng/ml TGF-β1 for the indicated time. The EpRas cells were treated with 1 ng/ml of TGF-β1 for the indicated period.

Plasmids. The human *FOXC2* cDNA was subcloned into the pBabe-Puro vector. The pBp-Snail, pBp-Twist, and pWZL-GSC have been described (24, 25). The shRNA-expressing U6 lentivirus system was provided by Richard Iggo (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) (36). The *FOXC2* shRNA14-targeting sequence is CCACACGTTTGCAACCCAA; *FOXC2* shRNA7-targeting sequence is GGACGAGGCTCTGTCGGAC. The U6 promoter with *FOXC2* shRNA14 and *FOXC2* shRNA7 insert was subcloned into pSP108-PURO. A control shRNA oligo, which does not match any known mouse coding cDNA, was used as control.

Metastasis Assay. EpRas-pBp and pBp-FOXC2 cells were generated by infecting EpRas cells with a retroviral vector expressing FOXC2 or the control vector and selected in 6 μg/ml of puromycin. Cells (5×10^5) were injected s.c. into female nude mice. The primary tumor weight was monitored periodically. Five to six weeks later, the mice were killed and analyzed for the number of metastatic nodules in the lung under the dissection microscope. All mouse experiments were performed in accordance with relevant guidelines and regulations. See supporting information (SI) *Text* for additional methods.

Statistical Analysis. Data are presented as mean ± SEM. When two groups were compared, we used Student's t test. $P < 0.05$ was considered significant. Expression of FOXC2 in a subset of human breast cancer samples was analyzed by using the cumulative hypergeometric distribution and χ^2 test.

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