Overexpression of LMO4 induces mammary hyperplasia, promotes cell invasion, and is a predictor of poor outcome in breast cancer

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The zinc finger protein LMO4 is overexpressed in a high proportion of breast carcinomas. Here, we report that overexpression of a mouse mammary tumor virus (MMTV)-*Lmo4* transgene in the mouse mammary gland elicits hyperplasia and mammary intraepithelial neoplasia or adenosquamous carcinoma in two transgenic strains with a tumor latency of 13–18 months. To investigate cellular processes controlled by LMO4 and those that may be deregulated during oncogenesis, we used RNA interference. Down-regulation of LMO4 expression reduced proliferation of human breast cancer cells and increased differentiation of mouse mammary epithelial cells. Furthermore, small-interfering-RNA-transfected breast cancer cells (MDA-MB-231) had a reduced capacity to migrate and invade an extracellular matrix. Conversely, overexpression of LMO4 in noninvasive, immortalized human MCF10A cells promoted cell motility and invasion. Significantly, in a cohort of 159 primary breast cancers, high nuclear levels of LMO4 were an independent predictor of death from breast cancer. Together, these findings suggest that deregulation of LMO4 in breast epithelium contributes directly to breast neoplasia by altering the rate of cellular proliferation and promoting cell invasion.

LIM domain | oncogene | proliferation

LMO4 belongs to the LIM-only (LMO) subclass of LIM domain proteins that are defined by two tandem zinc finger domains (1). One of the central functions of the LIM domain is to mediate protein–protein interactions, allowing LMO proteins to act as adaptors for the assembly of multiprotein complexes. LMO proteins have critical roles in normal development. LMO2 is essential for embryonic hematopoiesis and angiogenesis (2, 3), whereas mice lacking both LMO1 and LMO3 die shortly after birth from unknown causes (4). Targeted deletion of *Lmo4* (4, 5) results in perinatal lethality accompanied by failure of neural tube closure and homeotic transformations in the rib cage and cervical vertebrae, suggesting that Lmo4 functions as a modulator of Hox protein function.

Deregulation of LMO expression can lead to oncogenesis. *LMO1* and *LMO2* were originally discovered by their association with recurrent translocations in T cell acute lymphocytic leukemia and subsequently were shown to act as T cell oncogenes in transgenic models (1, 6–9). Remarkably, the *LMO2* gene was ectopically activated by retroviral integration in severe combined immunodeficient patients who developed T cell leukemia after gene therapy (10). LMO4 was initially identified in an expression screen by using serum from a breast cancer patient (11). Moreover, deregulated expression of LMO4 has been demonstrated in a significant proportion of breast carcinomas (12) and, more recently, in cancers of the oral cavity (13). In breast cancer, *LMO4* expression appears to be inversely correlated with estrogen receptor α (ERα) expression (14). LMO4 can act as a negative regulator of mammary epithelial differentiation *in vitro* (12), suggesting that LMO4 may have a role in governing cell proliferation. We have shown that LMO4 forms a complex with the corepressor CtIP and BRCA1 in breast epithelial cells and that LMO4 can repress BRCA1-mediated transcriptional activation (15).

To further explore a role for LMO4 in breast oncogenesis, we generated transgenic mice expressing *Lmo4* under the control of the mouse mammary tumor virus (MMTV) promoter and used RNA interference (RNAi) to investigate the cellular processes affected by LMO4 in breast cancer cells. Mammary hyperplasia, mammary intraepithelial neoplasia (MIN), and/or adenosquamous carcinoma were observed in two MMTV-*Lmo4* transgenic strains, demonstrating that overexpression of LMO4 contributes to mammary tumorigenesis. RNAi revealed that down-regulating LMO4 expression substantially reduces the proliferation, migration, and invasion of breast cancer cells. These findings indicate that LMO4 may induce tumorigenesis by perturbation of cellular proliferation and motility. Further, the observation that high levels of nuclear LMO4 correlate with poor patient outcome suggests that LMO4 may provide an additional marker in breast cancer.

Materials and Methods

Generation and Analysis of Transgenic Mice. The rabbit globin intron (RG-IVS2) was subcloned as a HindIII–EcoRI fragment into HindIII–EcoRI sites of the MMTV LTR expression plasmid (16) to generate the MMTV-RG-SV40 vector to provide a longer intronic sequence for mRNA stability. A HindIII–EcoRV fragment spanning the mouse *Lmo4* coding region was cloned into MMTV-RG-SV40 to generate *MMTV-Lmo4*. A 6-kb *MMTV-Lmo4* fragment was microinjected into C57BL/6 fertilized mouse oocytes, and several transgenic founder mice were identified. Four transgenic lines on a BALB/c background (more than six generations) were further examined. Transgenic mice were identified by PCR analysis using the following SV40-specific primers: 5′-CTCTAGAGGATCTTTGTGAAGG-3′ (forward) and 5′-GGACAAACCACAACTAGAATGC-3′ (reverse). Total RNA was isolated from mammary tissue of transgenic mice by using TRIzol (GIBCO/BRL) according to the manufacturer's instructions, and Northern blot analysis was performed by using 15 μg of total RNA (12). For histology, tissue was fixed in 10% (wt/vol) buffered formalin, dehydrated, and embedded in paraffin, and sections were stained with hematoxylin and eosin.

Cell Lines and Cell Transduction. BT-549, MCF-7, and MDA-MB-231 cells were maintained in RPMI medium 1640 (GIBCO/BRL) containing 10% FCS. HC11 cells were maintained as described

Abbreviations: siRNA, small interfering RNA; LMO, LIM-only; RNAi, RNA interference; MMTV, mouse mammary tumor virus; MIN, mammary intraepithelial neoplasia; HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor.

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(17). MCF10A(EcoR) breast epithelial cells were kindly provided by J. Brugge and D. Lynch (Harvard Medical School, Boston) (18). Full-length LMO4 cDNA carrying a 5′-FLAG tag was subcloned into the retroviral expression vector pBabe-puro (19), and stable pools of MCF10A(EcoR) transductants were generated (18) by using 2 μg/ml puromycin. MCF10A(EcoR) cells infected with empty pBabe vector provided a control.

RNAi. Cells plated at a density of 0.5 to 1 $\times$ 10^5 cells per well in six-well plates were transfected with 200 pmol of *LMO4*-specific and control small interfering RNA (siRNA) oligoduplexes (Dharmacon Research, Layfayette, CO) by using oligofectamine reagent (Invitrogen) according to the manufacturer's protocols. The sequences of each oligoduplex were as follows: siRNA276, 5′-GUCGAUUCCUGCGAGUGAAdTdT-3′; siRNA376, 5′-GAUCGGUUUCACUACAUCAdTdT-3′; and siRNA276mut, 5′-GUC**C**AUU**U**CU**CG**G**C**GU**U**AAdTdT-3′. The siRNA276mut oligoduplex is based on the sequence of siRNA276 and contains six transversions (bold and underlined).

Cell Proliferation and Differentiation Assays. Proliferation of RNAi-treated cells was analyzed by using the Cell Titer 96 AQueous One solution cell-proliferation assay (Promega) according to the manufacturer's instructions. Cells were replated into a 96-well flat-bottom plate in triplicate wells at a density of 2,500 cells per well. The assay was performed on day 0 to control for cell density and on subsequent days 1–5. For the differentiation assay, RNAi-treated HC11 cells were grown to confluency for 1–2 days and then starved of EGF overnight before the addition of the lactogenic stimulus [10^{-6} M dexamethasone/5 μg/ml insulin/5 μg/ml ovine prolactin (17), kindly provided by C. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases)]. After 48 h, cells were harvested for RNA extraction by using TRIzol; cDNA synthesis and PCR were performed by using primers for β-casein and Hprt as described (12).

Transwell Migration and Invasion Assays. *In vitro* migration and invasion assays were performed by using 24-well, 8-μm pore transwell inserts (Becton Dickinson). Cells were first resuspended in Matrigel (Becton Dickinson) for invasion assays. We seeded 10^6 (migration) or 5 $\times$ 10^6 (invasion) cells in 200 μl of serum-free growth medium in the upper chamber, and 600 μl of medium with or without chemoattractant (2% FCS or 20 ng/ml EGF) was added to the lower chamber. Cells were incubated at 37°C for 5 (migration) or 16 (invasion) h, then fixed in 10% (wt/vol) buffered-formalin and stained with 0.5 μg/ml DAPI (Sigma). Cells on the upper surface were removed with a cotton swab, and migrated cells on the underside were counted (average of 10 fields at a magnification of $\times$40 per transwell).

Western Blotting. Protein extracts were generated by lysing cells in Triton X-100 lysis buffer (12). Proteins were separated by SDS/PAGE; transferred to polyvinylidene difluoride membranes (Millipore); and probed with rat α-LMO4 20F8 (20), α-FLAG (Sigma), or α-tubulin Ab (Sigma).

Immunofluorescence. Cells were plated on coverslips in six-well plates, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X100, and blocked in 5% normal goat serum. Costaining for LMO4 and focal adhesion complexes was performed by incubating with anti-LMO4 (5 μg/ml) (20) and anti-vinculin (1 μg/ml; Sigma) mAbs. Staining was detected with secondary Alexa Fluor 488 and Alexa Fluor 594 Abs (Molecular Probes). Cells were mounted in fluorescent mounting medium (DAKO) and visualized by confocal microscopy (TCS.NT.SP2, Leica, Deerfield, IL).

Patient Cohort. After receiving ethical approval from the St. Vincent's Hospital Campus Human Ethics Committee, we identified a cohort of 194 patients with a diagnosis of invasive ductal breast carcinoma between May 1984 and March 2001. Median follow up was 49.5 months (range, 1.3–135). Archival formalin-fixed, paraffin-embedded pathology specimens were used to construct eight breast carcinoma tissue microarrays (TMAs), which contained up to 45 $\times$ 1.0-mm cores per slide. The pathology of each core on the TMAs was reviewed by a specialist breast pathologist (A.S.F.) before immunohistochemistry.

Immunohistochemistry and Scoring. Sections were subjected to target retrieval solution (DAKO) at 100°C for 20 min with a DAKO autostainer. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol, followed by avidin/biotin and serum-free protein blocks (DAKO). Sections were incubated for 30 min with anti-LMO4 mAb (20F8) at 7 μg/ml, followed by biotinylated rabbit anti-rat IgG (DAKO). A streptavidin-biotin peroxidase detection system was used with 3,3′-diaminobenzidine as substrate (DAKO). LMO4 immunostaining was assessed independently by two observers (D.S. and A.S.F.). Scores were given as the percentage of carcinoma cell nuclei staining positive, with an absolute intensity on a scale of 0–4 (0, none; 1, pale; 2, mild; 3, strong homogenous; and 4, intense). The following criteria were used to achieve a positive score for LMO4 overexpression: nuclear intensity, >2 in $>50\%$ of nuclei.

Statistical Analysis. Kaplan–Meier and the Cox proportional-hazards model were used for univariate and multivariate analysis with STATVIEW 5.0 software (Abacus Systems, Berkeley, CA). Death from breast cancer was the end point. The factors that were prognostic on univariate analysis were assessed in a multivariable model to identify factors that were independently prognostic. This analysis was performed sequentially on all patients who had available tissue (n = 159).

Results

LMO4 Induces Mammary Hyperplasia and MIN in *MMTV-Lmo4* Transgenic Mice. To assess the role of Lmo4 as a potential oncogene in the mammary gland, we generated transgenic mice expressing this gene under the control of the MMTV long-terminal repeat. The transgene included rabbit β-globin and SV40 intronic sequences to augment mRNA stability, as well as a polyadenylation sequence (Fig. 1*A*). Analysis of four independent transgenic strains revealed the expected $\approx$2.6-kb transgene transcript in mammary tissue. Moderate transgene expression was detected during midpregnancy, with the highest levels noted during late pregnancy, when the MMTV promoter is most active. Western blot analysis confirmed increased Lmo4 expression during pregnancy and lactation (data not shown). The two strains (strains 34 and 36) that expressed highest levels of the transgene were selected for further study (Fig. 1*B*). The level of transgene-derived *Lmo4* mRNA was estimated to be $\geq$3-fold higher than the endogenous level by Northern blot analysis (data not shown). Neither strain expressed the transgene at appreciable levels in nonpregnant nulliparous mammary glands (Fig. 1*B*), compatible with the hormone-responsive nature of the MMTV-LTR (21). Because luminal cells in the mammary glands of both virgin and pregnant mice already express abundant Lmo4 (12, 20), it was anticipated that high levels of transgene-derived Lmo4, expressed with altered kinetics, would be necessary for the induction of tumors.

No overt phenotypic abnormalities were detected in the mammary glands of nulliparous mice or mice during their first pregnancy by whole-mount and histological analyses. Furthermore, transgenic mice were capable of lactation and their glands were histologically indistinguishable from wild-type mice (data not shown). Given that highest *Lmo4* expression in transgenic animals was achieved during pregnancy, we assessed the capacity of the *Lmo4* transgene to promote mammary tumorigenesis in two strains (strains 34 and 36), both on a BALB/c background,

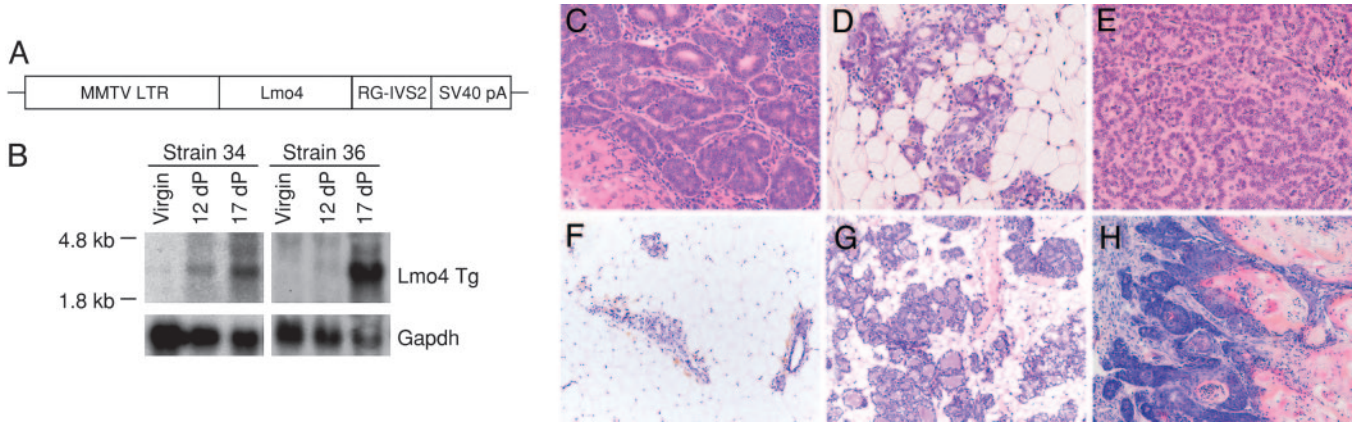

Fig. 1. Overexpression of *Lmo4* in transgenic mice leads to mammary hyperplasia and tumors. (*A*) Schematic representation of the MMTV-*Lmo4* transgene. *Lmo4* cDNA was cloned into the MMTV-RG-SV40 vector. (*B*) Northern blot analysis of total RNA (15 μg) from the mammary glands of transgenic strains 34 and 36. Filters were hybridized with a transgene-specific SV40 probe followed by a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. dP, day pregnant. (*C–E*) Hematoxylin and eosin sections of mammary glands and lung from strain 34, showing high grade MIN (*C*) and acinar hyperplasia (*D*), and alveolar bronchocarcinoma (*E*). (*F–H*) Hematoxylin and eosin sections from strain 36 showing mammary glands from multiparous mice. (*F*) Littermate control. (*G*) Diffuse acinar hyperplasia. (*H*) Adenosquamous carcinoma. [Original magnification: $\times$400 (*C–E* and *H*) and $\times$200 (*F* and *G*).]

after three pregnancies. For strain 34, 3 of 13 transgenic mice monitored for 14–18 months exhibited frank tumor nodules, which proved to be MIN (Fig. 1*C*), as defined by the Annapolis guidelines (22). The MIN lesions were multilayered and exhibited nuclear pleomorphism and increased mitotic figures, with two lesions also showing evidence of squamous metaplasia. Mammary glands from two other mice displayed diffuse acinar hyperplasia (Fig. 1*D*) and one of these females developed a bronchoalveolar carcinoma of the lung at 14 months (Fig. 1*E*). For strain 36, four of five multiparous female mice developed multifocal acinar hyperplasia (Fig. 1*G*), with a mean latency of 16 months (range, 14–20) and one of these mice also exhibited

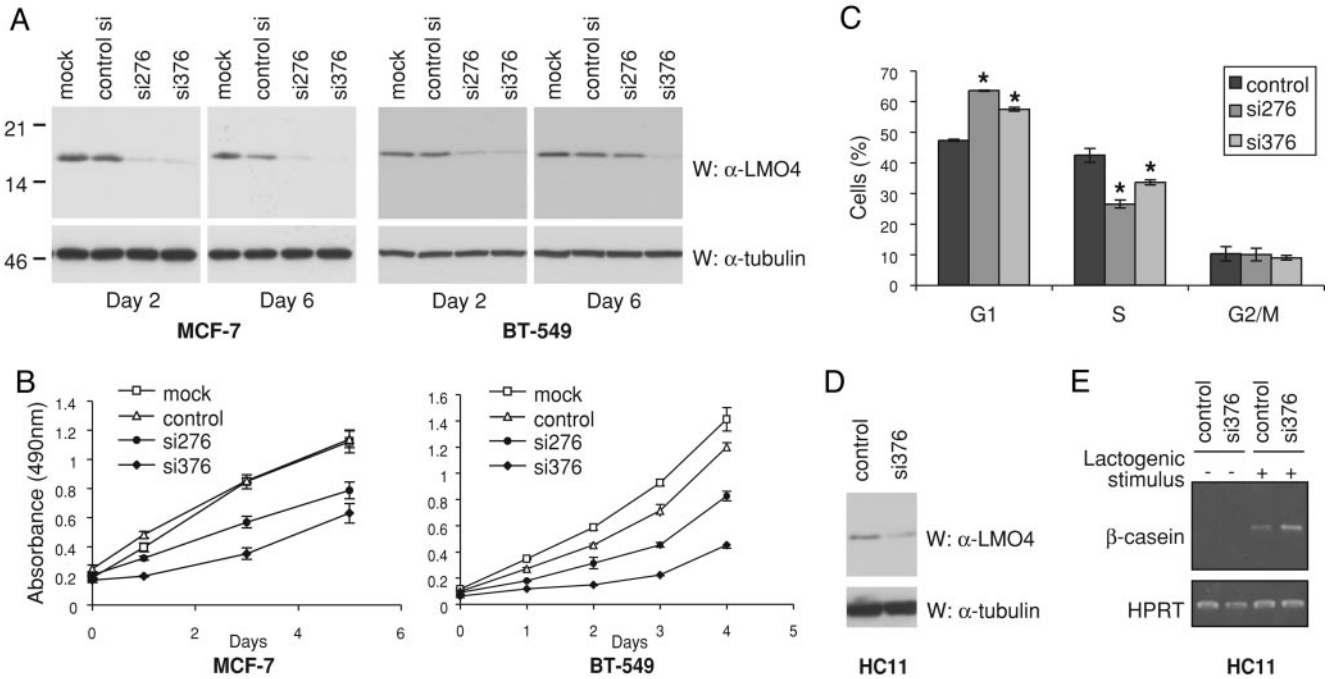

Fig. 2. Down-regulation of LMO4 expression in breast epithelial cells by RNAi inhibits proliferation and augments differentiation. (*A*) Western blot analysis of protein lysates from MCF-7 and BT-549 breast cancer cells transiently transfected with LMO4-specific siRNA276 and siRNA376, compared with control siRNA and mock-transfected cells, 2 and 6 days after transfection by using α-LMO4 (20F8) mAb. Anti-tubulin was used to verify protein loading. (*B*) The proliferation rate of siRNA-transfected MCF-7 and BT-549 cells, and mock-transfected cells, was determined from days 0–5, in three independent experiments. Error bars indicate SEM; $n = 3$. (*C*) Cell cycle defect in LMO4-deficient MCF7 cells. siRNA and mock-transfected cells were fixed in ice-cold 70% ethanol for 24 h before staining with 0.5 μg/ml propidium iodide (PI). FACScan analysis revealed an increase in G_1/G_0 cells and a decrease in S-phase cells in LMO4-deficient MCF-7 cells, compared with control cells, in three independent experiments. Error bars indicate SEM; $n = 3$. *, $P < 0.05$. (*D*) Down-regulation of LMO4 expression in HC11 cells transiently transfected with siRNA376 (or a control siRNA) was confirmed by Western blot analysis of cells harvested 3 days after transfection by using α-LMO4 (20F8) mAb. Anti-tubulin immunoblotting provided a control. (*E*) RT-PCR analysis was performed by using total RNA derived from RNAi-treated HC11 cells that were stimulated with (+) prolactin, insulin, and dexamethasone or unstimulated (−) for 48 h. *β-casein* and *Hprt* were used as markers of differentiation and loading, respectively. At least three independent experiments were performed.

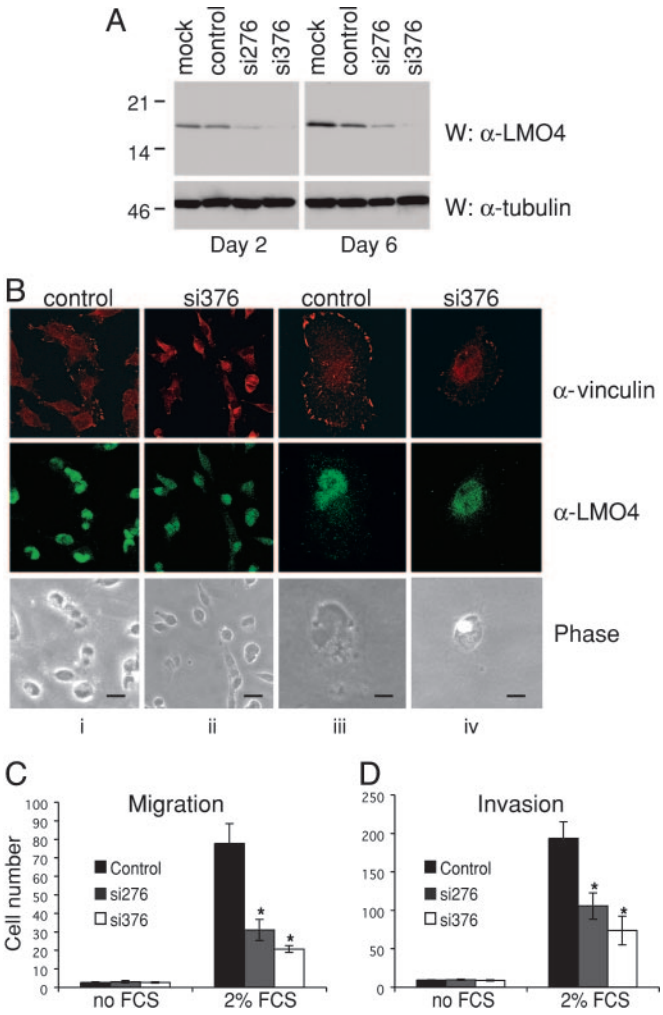

Fig. 3. Reduced LMO4 expression impedes the migration and invasion of breast cancer cells. (*A*) Western blot analysis of protein lysates from MDA-MB-231 breast cancer cells transfected with siRNA276, siRNA376, control siRNA, or mock-transfected cells, 2 and 6 days after transfection by using α-LMO4 (20F8) mAb. Anti-tubulin provided a control. (*B*) Focal adhesions in MDA-MB-231 cells transfected with siRNA376 or a control siRNA were visualized by indirect immunofluorescence by using α-vinculin mAb. Costaining with α-LMO4 (20F8) mAb confirmed the reduction in LMO4 expression in siRNA376-transfected cell nuclei. [Scale bars, 20 (*i* and *ii*) and 8 (*iii* and *iv*) μm.] (*C*) The number of migrating MDA-MB-231 cells transfected with either LMO4-specific siRNA276 or siRNA376, or a mutant siRNA, was determined by counting 10 random fields in each of three independent experiments. Error bars indicate SEM; $n = 3$. (*D*) The number of MDA-MB-231 cells, transfected with siRNA276, siRNA376, or a mutant siRNA, capable of invading Matrigel was determined for three independent experiments, as in *C*. Error bars indicate SEM; $n = 3$.

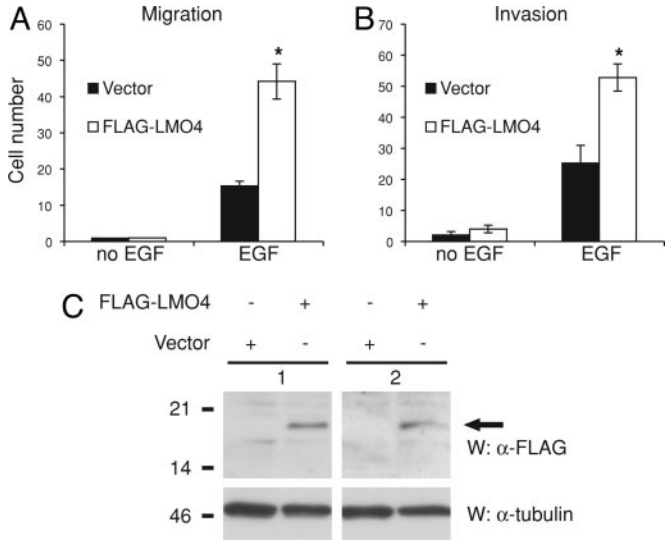

Fig. 4. LMO4 overexpression augments mammary epithelial cell migration and invasion. (*A*) The motility of MCF10A(EcoR) cells, stably transduced with a FLAG-*LMO4* retroviral construct or vector alone (pBabe-puro), was determined by counting the number of migrated cells in 10 random fields from each of four experiments. Error bars indicate SEM; $n = 4$. (*B*) The number of MCF10A(EcoR) cells expressing either FLAG-*LMO4* or vector alone (pBabe-puro), capable of invading through Matrigel, was determined in four experiments, as described for *A*. Error bars indicate SEM; $n = 4$. (*C*) Western blot analysis confirmed the expression of FLAG-LMO4 in the transductants. Lysates from cells expressing FLAG-LMO4 or empty vector were subjected to SDS/PAGE and immunoblotted with mouse α-FLAG mAb. Anti-tubulin was used to control for protein loading.

an adenosquamous carcinoma (Fig. 1*H*) at 18 months. Hyperplasia was also observed in a third transgenic strain. In contrast, mammary glands from 13 nontransgenic littermate mice that underwent three pregnancies did not develop hyperplasia or tumors during a similar follow-up period of 14–19 months (Fig. 1*F*). In addition to these findings, lymphoproliferative disease or lymphomas were observed in four transgenic mice and one wild-type mouse, presumably reflecting MMTV promoter activity in lymphoid cells (21) and aging of the animals. Although Lmo4 is not a potent oncoprotein, these data demonstrate that it can contribute to mammary tumorigenesis.

Down-Regulation of LMO4 Expression by siRNA Inhibits Proliferation of Breast Epithelial Cells. To determine whether LMO4 regulates the proliferation of breast cancer cells, we used RNAi to down-regulate LMO4 expression. Two breast cancer cell lines, MCF-7 and BT-549, were transiently transfected with siRNA oligoduplexes. LMO4-specific siRNAs (siRNA276 and siRNA376) effectively suppressed LMO4 expression in both cell lines, whereas a control mutant siRNA had no effect, nor did mock transfection (Fig. 2*A*). LMO4 expression was down-regulated rapidly, with low levels evident at day 1 after transfection, consistent with the short half-life (< 2 h) of this protein (data not shown). A reduction in LMO4 protein levels was observed up to day 6 after transfection in both cell lines with siRNA376, although this was not the case for siRNA276 in BT-549 cells (Fig. 2*A*). The LMO4-specific siRNAs substantially reduced the proliferation of both MCF-7 and BT-549 breast cancer cells, whereas the control siRNAs had no effect (Fig. 2*B*). No increase in apoptosis was observed upon transfection with LMO4-specific siRNAs (data not shown). FACScan analysis of transfected MCF-7 cells revealed that down-regulation of LMO4 expression led to cell cycle changes. An increase in the proportion of G_1/G_0 cells and a corresponding decrease in S-phase cells was evident in three independent experiments with a P value of <0.05, whereas the control siRNA had no effect (Fig. 2*C*). Thus, LMO4 appears to promote S-phase entry of breast epithelial cells, providing evidence for a role in cell proliferation.

To examine the effect of LMO4-specific RNAi on mammary differentiation, we used mouse mammary epithelial cells as there is no suitable human counterpart line to study differentiation. HC11 cells have the ability to differentiate into milk-producing cells upon treatment with a lactogenic stimulus (17). Down-regulation of LMO4 expression mediated by siRNA376 (Fig. 2*D*) was found to augment β-casein mRNA levels compared with cells transfected with the control siRNA (Fig. 2*E*). These data are consistent with the notion that LMO4 maintains the proliferative rather than differentiative state of mammary epithelial cells.

Reduced LMO4 Expression Impedes the Migration and Invasion of Breast Cancer Cells. To examine the role of LMO4 in cell motility and invasion, we used RNAi to down-regulate LMO4 expression in

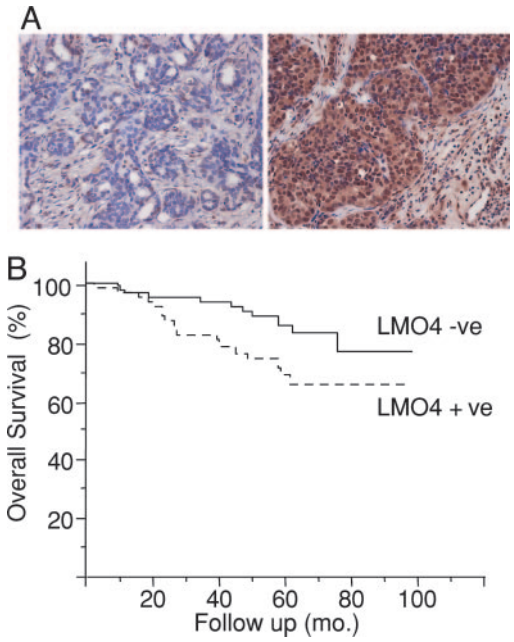

Fig. 5. Overexpression of LMO4 in breast cancer is a predictor of poor clinical outcome. (*A*) LMO4 immunostaining was performed on tissue microarrays containing archival breast tumor specimens using α-LMO4 (20F8) mAb. Representative images of tumor specimens displaying low (*Left*) and high (*Right*) levels of LMO4 expression are shown. (*B*) Kaplan–Meier curves for overall survival (OS) of 159 breast cancer patients according to LMO4 nuclear staining. High LMO4 expression was significantly associated with decreased OS in univariate ($P = 0.023$) analysis.

MDA-MB-231 cells, an invasive breast cancer cell line. Transfection with either of the LMO4-specific siRNAs profoundly reduced LMO4 expression in MDA-MB-231 cells, whereas the control siRNA did not (Fig. 3*A*). Transfection of MDA-MB-231 cells with siRNA276 and siRNA376 resulted in a 2.5- and 3.8-fold decrease in cell motility, using transwell migration assays in the presence of 2% FCS, compared with that for control cells (Fig. 3*C*). Next, we examined invasion of these cells through an extracellular matrix (Matrigel) in a modified Boyden chamber, which is an assay that has been shown to correlate with *in vivo* metastatic potential (23). siRNA276 resulted in a 1.8-fold decrease in the invasion of MDA-MB-231 cells, whereas siRNA376 elicited a 2.6-fold decrease (Fig. 3*D*). These findings indicate that LMO4 regulates the motility and invasiveness of breast cancer cells. To determine whether LMO4 influences focal adhesions, we assessed vinculin distribution in siRNA-transfected MDA-MB-231 cells by indirect immunofluorescence. Focal adhesions appeared to be less prominent in MDA-MB-231 cells transfected with siRNA376, compared with cells transfected with a control siRNA (Fig. 3*B*). Costaining with anti-LMO4 mAb (20F8) confirmed a substantial decrease in LMO4 levels in the nuclei of siRNA376-transfected cells (Fig. 3*B*).

LMO4 Overexpression Stimulates Mammary Epithelial Cell Migration and Invasion. To further investigate the role of LMO4 in cell motility and invasion, we transduced human breast epithelial MCF-10A cells harboring the murine ecotropic receptor, MCF-10A(EcoR), with a LMO4-expressing retrovirus. MCF10A(EcoR) cells expressing FLAG-*LMO4* showed a 2.9-fold increase in migration when EGF was used as the chemoattractant, compared with cells infected with a control virus (Fig. 4*A*). Moreover, a 2.1-fold increase in the number of invasive cells was observed for MCF10A(EcoR) cells transduced with pBabe-FLAG-*LMO4*, relative to control cells (Fig. 4*B*). Expression of FLAG-LMO4 was confirmed by Western blot analysis using α-FLAG Ab (Fig. 4*C*). Thus, either down-regulation or overexpression of LMO4 markedly affects the motility of mammary epithelial cells and their ability to invade an extracellular matrix *in vitro*.

Table 1. Univariate analysis of clinicopathological parameters

Parameter	Cohort, %	HR (95% CI)	*P* value
Tumor size (>20 mm)	42	3.83 (1.74–8.43)	0.0008
Axillary lymph node involvement	54	5.40 (2.06–14.18)	0.0006
High grade	52	7.45 (2.59–21.42)	0.0002
ER-positive	71	0.19 (0.09–0.41)	<0.0001
PR-positive	66	0.29 (0.14–0.60)	0.0009
Her2-positive	15	2.40 (1.01–5.66)	0.047
LMO4-positive	43	2.38 (1.12–5.04)	0.023

Although the cohort comprised 194 patients, it was only possible to score 159 samples for LMO4 because of tissue loss from the tissue microarrays. Therefore, all analyses were on these 159 samples. ER and PR status were not available for two samples.

LMO4 Overexpression in Breast Cancer Correlates with Poor Clinical Outcome. We have reported in a small series of 60 cases that LMO4 protein is overexpressed in >50% of primary breast cancers (12). To further investigate the relationship between LMO4 expression and clinicopathological features of the disease, we performed immunohistochemical analysis on tissue from a cohort of 194 primary breast cancer patients of known clinical outcome.

LMO4 overexpression, as defined in *Materials and Methods*, was apparent in 68 of 159 (42.7%) ductal carcinomas from which tissue was available (Fig. 5*A*). In a univariate Cox proportional-hazards regression analysis, high nuclear LMO4 expression was significantly associated with decreased patient survival ($P = 0.023$), as were other well established markers of outcome [i.e., tumor size; tumor grade; axillary lymph node status; and ER, progesterone receptor (PR), and HER2 status] (Table 1). In a multivariate analysis incorporating these parameters, only axillary lymph node status [hazard ratio (HR), 4.02; 95% confidence interval (CI), 1.4–11.5; $P = 0.009$] and LMO4 nuclear overexpression (HR, 2.27; 95% CI, 1.01–5.11; $P = 0.048$) were independent predictors of death from breast cancer (Table 2). Kaplan–Meier analysis confirmed a significant relationship between LMO4 overexpression and patient survival (Fig. 5*B*). Interestingly, most (15/23) tumors overexpressing HER2, an adverse prognostic marker, also overexpressed LMO4 (Fisher's exact test; $P = 0.0247$). However, HER2-positive tumors only represented 23% of LMO4-positive tumors (64 samples).

Discussion

Here, we provide evidence that LMO4 is an important regulator of breast epithelial cell proliferation and invasion and that it can act as a mammary oncoprotein in a transgenic mouse model. Enforced expression of *Lmo4* in the mammary glands of transgenic mice recapitulates the high levels of Lmo4 that occur in ≈50% of primary breast cancers. The development of mammary hyperplasia and MIN in MMTV-*Lmo4* transgenic mice supports the notion that

Table 2. Multivariate analysis of clinicopathological parameters

Parameter	HR (95% CI)	*P* value
Tumor size (>20 mm)	1.51 (0.59–3.83)	0.389
Axillary lymph node-positive	4.02 (1.40–11.5)	0.009
High grade	3.03 (0.96–9.59)	0.059
ER-positive	0.28 (0.07–1.12)	0.072
PR-positive	1.16 (0.31–4.38)	0.822
HER2-positive	1.54 (0.61–3.89)	0.367
LMO4-positive	2.27 (1.01–5.11)	0.048

Lmo4 has a causative role in the pathogenesis of breast cancer. Diffuse acinar hyperplasia was observed in 15% and 80% of mice in transgenic strains 34 and 36, respectively. MIN or adenosquamous carcinoma arose in ≈20% of mice (4 of 18 mice) with long latency, indicating that Lmo4 is not a potent oncogene. Nevertheless, D-type cyclins (D1 and D2) and cyclin E also induce carcinomas in transgenic mice with long latency and/or a low incidence (24–26). For example, tumors arise in 10% and 19% of cyclin E (24) and cyclin D2 (25) transgenic mice, respectively. Interestingly, when *Lmo4* was fused to the repressor *engrailed* in a transgenic model, no hyperplasia or tumors were apparent (26). The RNAi studies reported here suggest that overexpression of Lmo4 contributes to breast oncogenesis by deregulating proliferation and increasing cell motility.

LMO4 appears to be necessary but not sufficient for mammary tumorigenesis, similar to that observed for several transgenic oncogene models. The latency of mammary lesions in *Lmo4* transgenic mice was 13–18 months, indicating that additional mutations were required. It is particularly relevant that other Lmo family members elicit tumorigenesis with long latency. Overexpression of the *Lmo1* or *Lmo2* gene in transgenic mice induces clonal T-cell leukemia after several months. All tumors in *Lmo1* transgenic animals have an immature phenotype, and *Lmo2* transgenic mice exhibit a marked accumulation of immature double negative T cells, indicating that a block in differentiation has occurred in these mice (1, 8).

LMO4 functions as a positive regulator of cellular proliferation and a negative regulator of differentiation. Down-regulation of LMO4 expression in breast cancer cell lines by RNAi led to markedly reduced proliferation, accompanied by a partial G_0/G_1 block. Therefore, we examined expression of cell cycle regulators, including cyclins D1 and E, retinoblastoma protein (pRb), p21, and p27 in these cells. Although changes were evident in the case of cyclin D1 and p27, they were not always consistent. Overall, the data indicate that LMO4 is unlikely to regulate the cell-cycle machinery. Rather, deregulation of LMO4 levels may lead to an uncoupling of proliferation and differentiation. Consistent with this proposal, RNAi-mediated knock-down of Lmo4 expression in HC11 cells augmented differentiation. Conversely, overexpression of LMO4 in mammary epithelial or neuroblastoma cells blocked their maturation (12, 27). These data, in combination with RNAi-mediated inhibition of proliferation, imply that LMO4 functions to maintain the undifferentiated state and that the absolute levels of LMO4 may govern the rate of proliferation. Interestingly, the dosage of the *Drosophila LMO* gene *Beadex* has been established to be critical for wing formation, indicating that the stoichiometry of LMO-containing complexes is an important parameter (28). Similarly, the stoichiometry and composition of LMO4-multimeric complexes in normal breast epithelial cells is likely to be perturbed by even small changes in protein concentration, resulting in profound effects on cell homeostasis.

LMO4 has a role in regulating the motility and invasiveness of breast epithelial cells. We have demonstrated that increased LMO4 levels promote migration and invasion, whereas a reduction in LMO4 expression is inhibitory to these processes. Although there was no apparent change in the actin cytoskeleton of siRNA-transfected MDA-MB-231 cells (data not shown), down-regulation of LMO4 levels led to a consistent decrease in the number of focal adhesions. Increased cell invasion has been linked to an increase in focal adhesions. However, there are examples in which fewer focal adhesions have been observed in more invasive cells, such as cells overexpressing LIM-kinase (29). These findings underscore the complexity of the process of cell migration.

The influence of LMO4 on cell proliferation, migration, and invasion has implications for the role of this protein in breast cancer progression. Indeed, high nuclear expression of LMO4 in primary invasive breast carcinomas was significantly associated with patient survival. Although there was a significant association between LMO4 and HER2 overexpression, ≈75% of patients with high LMO4 levels were not HER2-positive. These data raise the possibility that LMO4 may provide an additional marker in breast cancer and that it represents a potential target for therapeutics. Defining the molecular pathways that are regulated by LMO4 is an essential step toward dissecting the mechanisms through which LMO4 influences the development and progression of breast carcinomas.

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