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KLF8 promotes human breast cancer cell invasion and metastasis by transcriptional activation of MMP9

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Abstract

Epithelial to mesenchymal transition (EMT) and extracellular matrix degradation are critical for the initiation and progression of tumor invasion. We have recently identified Krüppel-like factor 8 (KLF8) as a critical inducer of EMT and invasion. KLF8 induces EMT primarily by repressing E-cadherin transcription. However, how KLF8 promotes invasion is unknown. Here we report a novel KLF8-to-MMP9 signaling that promotes human breast cancer invasion. To identify the potential KLF8 regulation of MMPs in breast cancer, we established two inducible cell lines that allow either KLF8 overexpression in MCF-10A or knockdown in MDA-MB-231 cells. KLF8 overexpression induced a strong increase in MMP9 expression and activity as determined by quantitative real-time PCR and zymography. This induction was well correlated with the MMP inhibitor-sensitive Matrigel invasion. Conversely, KLF8 knockdown caused the opposite changes that could be partially prevented by MMP9 overexpression. Promoter-reporter assays and chromatin and oligonucleotide precipitations determined that KLF8 directly bound and activated the human MMP9 gene promoter. Three-dimensional (3D) glandular culture showed that KLF8 expression disrupted the normal acinus formation which could be prevented by the MMP inhibitor, whereas KLF8 knockdown corrected the abnormal 3D architecture which could be protected by MMP9 overexpression. KLF8 knockdown promoted MDA-MB-231 cell aggregation in suspension culture which could be prevented by MMP9 overexpression. KLF8 knockdown inhibited the lung metastasis of MDA-MB-231 cells in nude mice. Immunohistochemical staining strongly correlated the co-expression of KLF8 and MMP9 with the patient tumor invasion, metastasis and poor survival. Taken together, this work identified the KLF8 activation of MMP9

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Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

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as a novel and critical signaling mechanism underlying human breast cancer invasion and metastasis.

Keywords

KLF8; MMP9; invasion; metastasis; human mammary epithelial cells; human breast cancer

Introduction

Breast cancer survival rate falls from 90% for localized to 20% for metastatic disease. Metastasis requires invasive cells to detach from the localized tumors by EMT, degrade the extracellular matrix (ECM) using proteases including matrix metalloproteinases (MMPs), survive the circulation as circulating tumor cells (CTC), and colonize at distant locations. Understanding of the molecular mechanisms behind each of the steps is essential for targeting metastatic cells at early stage to improve patient survival.

KLF8 is a GT-box (CACCC) binding dual transcription factor that plays a critical role in the regulation of cell cycle progression (Mehta *et al.*, 2009; Urvalek *et al.*, 2010; Wang *et al.*, 2008; Wei *et al.*, 2006; Zhao *et al.*, 2003), transformation (Wang and Zhao, 2007), EMT and invasion (Wang *et al.*, 2007). KLF8 recruits the CtBP co-repressor (van Vliet *et al.*, 2000) to repress or the p300 and PCAF co-activators to activate target gene promoters (Evans and Liu, 2010; Lloyd, 2010; Urvalek *et al.*, 2010; van Vliet *et al.*, 2000; Wang *et al.*, 2007; Wei *et al.*, 2006; Zhang *et al.*, 2005; Zhao *et al.*, 2003). KLF8 expression is promoted by Src and PI3K signaling (Ding *et al.*, 2005; Wang *et al.*, 2008; Zhao *et al.*, 2003) and its transcription is activated by Sp1 (Wang *et al.*, 2008) and KLF1 (Eaton *et al.*, 2008) or inhibited by KLF3 (Eaton *et al.*, 2008). KLF8 is also regulated by post-translational sumoylation (Wei *et al.*, 2006) and localization (Mehta *et al.*, 2009; Rodriguez and Martignetti, 2009). We previously identified KLF8 as a FAK downstream effector (Zhao *et al.*, 2003). FAK is a critical regulator of both EMT and MMPs during breast cancer invasion and metastasis (Zhao and Guan, 2009). Like FAK, KLF8 is highly overexpressed in invasive human cancers including breast cancer (Wang and Zhao, 2007) and promotes breast cancer cell invasion (Wang *et al.*, 2007). However, the mechanisms by which KLF8 promotes human breast cancer invasion have not been investigated.

In this study, we report that KLF8 regulates MMP9 expression by transcriptional activation in human breast cancer cells to promote cell invasion. We also show a strong correlation of KLF8 and MMP9 co-expression with the patient tumor metastasis. Our results support a potentially important role for KLF8 in promoting human breast cancer metastasis.

Results

KLF8 upregulates MMP9 expression and activity to promote cell invasion

Degradation of ECM and vascular basement membrane is required for a cancer cell to invade. MMP9 and MMP2 are among the major proteinases that play such a role. Interestingly, during the invasion of fibroblasts and other cell types, the expression and

activity of these MMPs are found to be up-regulated by FAK (Schlaepfer and Mitra, 2004), the upstream regulator of KLF8.

We first tested if KLF8 is sufficient to promote invasion using our tet-off NIH3T3/KLF8 cells (Zhao *et al.*, 2003). We found that induction of KLF8 expression led to a >3-fold increase in the cell invasiveness (Fig. 1A, left panel, compare I to U). We then determined if KLF8 regulates the MMPs by in-gel zymography. We found that induction of KLF8 expression resulted in an increase in both pro-MMP9 and active MMP9, and only active MMP2 in the cells (Fig.1A, right-top panel, compare I to U). This result suggests that KLF8 regulate MMP9 mainly at the transcriptional level whereas KLF8 activation of MMP2 may be through an indirect mechanism. We have demonstrated that KLF8 promotes the MCF-10A cell invasiveness (Wang *et al.*, 2007). To test if KLF8 also regulates the MMPs in these cells, we performed in-gel zymography using MCF-10A, the non-invasive (MCF-7) and the highly invasive (MDA-MB-231) human breast cancer cell lines (Fig. 1A. right-bottom panel). In agreement with the literature (Rahman *et al.*, 2006; Tsai *et al.*, 2003; Yao *et al.*, 2001), there was little or no expression of MMP9 or MMP2 in MCF-10A-Mock cells (lane 1) (Wang *et al.*, 2007) and MCF-7 (lane 3), but a dramatic increase in the MCF-10A-KLF8 (Wang *et al.*, 2007) (compare lanes 2 to 1) and MDA-MB-231 cells (compare lanes 4 to 3).

We then established a MCF-10A cell line that expresses inducible KLF8 (10A-iK8) for an in-depth study. The inducible expression was verified by western blotting (Fig. 1B, left panel, top two rows, compare lanes I to U). To determine KLF8 regulation of the MMPs at the message levels, we performed RT-PCR and qRT-PCR and found that KLF8 increased mRNA expression of MMP9 but not MMP2 (Fig. 1B, compare lanes or columns U to I). This is consistent with the change in pro-forms of the MMPs shown in Fig. 1A.

To confirm the role of KLF8 activation of MMPs for the cell invasion, we performed *in situ* zymography using both the 10A-iK8 cells and MG6001 (Fig. 1C). We found that KLF8 expression caused the matrix degradation (compare I+DMSO to U+DMSO) which is MG6001-sensitive (compare I+GM6001 to I + DMSO) and consistent with the cell invasiveness (Fig. 1D, compare columns 3 to 2 and 1).

Taken together, these results suggest that KLF8 activation of MMPs is critical for its promotion of cell invasion.

MMP9 is a direct transcriptional activation target of KLF8

To test if KLF8 regulates MMP9 transcription, we performed prompter (MMP9p) reporter (Lamar *et al.*, 2008) assays. We found that KLF8 activated MMP9p in both NIH3T3 and MCF-10A cells (Fig. 2A, compare the black to white columns). However, the activation domain deficient mutant (mKLF8) (Urvalek *et al.*, 2010) was no longer able to do so (Fig. 2A, compare the gray to black and white columns). These results suggest that KLF8 regulates MMP9p by transcriptional activation.

To test whether KLF8 directly activates MMP9p, we first analyzed the 1.6 kb promoter region and found six GT-boxes (Fig. 2B). To map the promoter region essential for KLF8-

responsive GT-box(s), we generated MMP9p truncation mutants (Fig. 2B). While deletion of the −1.6 kb through −541 bp region only caused a slight decrease in MMP9p activity (compare columns d6, d5–6 or d4–6 to WT), deletion to −359 bp position caused a ~80% decrease (compare columns d3–6 to WT). Further deletion did not have an effect (compare columns d2–6 or d1–6 to d3–6). This result suggests that the region between −541 bp and −359 bp is essential for the promoter activation by KLF8. Interestingly, this region contains a GT-box (GT-box 3).

We disrupted this GT-box to see if it mediates KLF8 activation of MMP9p in the 10A-iK8 cells. Unlike the wild-type promoter (WT) that responded well to KLF8 in a time and thus KLF8 level dependent manner (Fig. 2C, black columns), the mutant promoter (mGT-box 3) completely lost the responsiveness to KLF8 (Fig. 2C, white columns).

To test whether KLF8 interacts with the endogenous MMP9 promoter in the 10A-iK8 cells, we performed ChIP assays (Fig. 2D, top panel). We found that the promoter fragment containing the GT-box 3 was specifically co-immunoprecipitated by anti-KLF8 but not the control IgG from induced (I, compare lanes 3 to 2 and 4) but not uninduced cells (lane 3, compare U to I). These results suggest that KLF8 could bind directly to the MMP9 promoter at the GT-box 3 site.

To see if the KLF8-MMP9p interaction requires the GT-box 3, we performed BOP assays (Fig. 2D, bottom panel). We showed that KLF8 strongly bound to the wild-type (compare lanes 2 to 3) but not the GT-box 3 mutant (mGT) MMP9p fragment (compare lanes 1 to 2 or 3).

Taken together, these results suggest that KLF8 activates MMP9p by directly binding to it at the GT-box 3.

Aberrant upregulation of MMP9 by KLF8 in human breast cancer cells is critical for the cell invasion

For an in-depth understanding of how KLF8 regulates breast cancer cell invasion and metastasis, we also established the 231-K8ikd cell line that expresses inducible KLF8 shRNA. GFP labels all the cells. The induced knockdown of KLF8 was confirmed by RFP expression (Fig. 3A, compare panels f to c), western blotting (Fig. 3B, top panel, compare lanes I to U) and qRT-PCR (Fig. 3B, middle and bottom panels, compare lanes I to U).

Using this cell line, we first tested if KLF8 is required for the MMP mRNA expression. We found that the KLF8 knockdown caused a 3-fold decrease of MMP9 but had no effect on MMP2 mRNA levels (Fig. 3B, middle and bottom panels, compare lanes I to U). In-gel zymography showed that upon KLF8 knockdown, both the pro- and active MMP9 were dramatically reduced, whereas only active MMP2 but not the pro-MMP2 was decreased (Fig. 3C, top panel, compare lanes I to U). MMP9p reporter assay indicated that the MMP9p activity was reduced by 60% when KLF8 was silenced (Fig. 3C, bottom panel, compare lanes I to U).

Consistent with our previous observations using transient KLF8 knockdown (Wang *et al.*, 2007), there was >50% reduction in the cell invasiveness upon the inducible KLF8 knockdown. Importantly, this was correlated with a decrease in MMP9 protein levels (Fig. 3D, compare columns or lanes 2 to 1). To determine the requirement of MMP9 for cell invasion, we transiently silenced MMP9 and found that the cell invasiveness dropped by 40% (Fig. 3D, compare lanes or columns 4 to 3). To test if MMP9 can rescue the cell invasiveness during KLF8 knockdown, we overexpressed MMP9 in the cells and found that the cell invasiveness was recovered regardless of KLF8 knockdown (Fig. 3D, compare columns or lanes 6 to 5). These results suggest that the KLF8 knockdown-mediated decrease in MMP9 mRNA levels was responsible for the decrease in MMP9 protein expression and activity, which in turn limits the cancer cell invasiveness.

In addition, we found that in 3D spheroid culture the normal structure of acini (Debnath *et al.*, 2003a; Lee *et al.*, 2007) formed by the 10A-iK8 cells was disrupted upon induction of KLF8 expression and treatment with GM6001 partially protected this disruption (Fig. S2A). Conversely, the aberrant 3D structures (Carrio *et al.*, 2005; Debnath *et al.*, 2003b; McLachlan *et al.*, 2006; Park *et al.*, 2006) formed by the 231-K8ikd cells was partially reversed by either treatment with GM6001 or induction of KLF8 knockdown (as indicated by the expression of RFP in the cells) and the latter was prevented by MMP9 ectopic expression (Fig. S2B).

Taken together, these results suggest that KLF8 activation of MMP9 transcription is essential for maintaining MMP9 expression and activity in the breast cancer cells and the cell invasiveness possibly by disrupting the normal and maintaining the abnormal glandular structure.

KLF8-MMP9 axis is critical for the cell disaggregation in suspension

It is implicated that single cell migration is important for breast cancer cell invasion, circulation and metastasis (Giampieri *et al.*, 2009) and single CTC circulation is also critical for extravasation (Balzer *et al.*, 2009; Whipple *et al.*, 2008).

To test if KLF8 plays a role in maintaining the non-aggregated status of CTC-mimicking suspended breast cancer cells, we analyzed the aggregation ability of the 231-K8ikd cells (Fig. 4). We found that the cell aggregation was induced within 30 minutes in suspension culture under induced but not uninduced conditions (Fig. 4A and 4B, compare 5 to 2 or 6 to 4). This result was verified by an independent spheroid formation approach (Fig. 4C and 4D, compare I to U). In this assay, the cells in which KLF8 was not knocked down (U, labeled by GFP only) grew sparsely. In contrast, when KLF8 knocked was induced (I, labeled by both GFP and RFP) the cells tended to aggregate together to form spheroids. To test if MMP9 plays a role in this regulation by KLF8, we performed rescue experiments and found that MMP9 overexpression prevented the spheroid formation regardless of KLF8 knockdown (Fig. 4C and 4D, compare I+MMP9 and I).

These results suggest that the KLF8-MMP9 axis plays an important role for single cell invasion during breast cancer metastasis.

The aberrant overexpression of KLF8 is crucial for the lung metastasis

All the results described above pointed out a possibility that KLF8 expression is potentially important for the progression of tumor metastasis. To test this possibility, the 231-K8ikd cells were injected into nude mice through tail veins and the lung metastasis of the cells was examined (Fig. 5). Knockdown of KLF8 resulted in approximately 75% reduction in the metastasis (Fig. 5, compare I with U).

This result clearly demonstrates the critical role of KLF8 in the regulation of the breast cancer metastasis.

Aberrant co-overexpression of KLF8 and MMP9 is correlated with patient tumor metastasis

The above results support a possibility that KLF8 could promote MMP9 expression in human breast tumors and thus tumor invasion and metastasis. To test this, we examined the co-expression of KLF8 and MMP9 in human breast tumor tissues by IHC using two tissue arrays from different sources. One of the arrays consists of 15 duplicates of breast tumor specimens with matched normal tissue counterparts (IMH-371, Fig. 6A and 6B and Supplemental Table B). The other is composed of 75 specimens representing normal through aggressive breast cancer tumors (BR1503, Fig. 6C and Supplemental Table C). As expected, KLF8 was predominantly localized in the nuclei whereas the MMP9 showed both cytoplasmic and extracellular presence (Fig. 6A and Fig. S3). Among all specimens of the small group examined, 47% were positive for both KLF8 and MMP9 (Supplemental Table B). Importantly, the KLF8+/MMP9+ tumors were associated with a significantly higher metastasis rate (Fig. 6B) and poor patient survival (Supplemental Table B). Similarly, among all the large set of 75 samples of normal tissue, benign tumor and malignant cyctosarcoma phyllodes, intraductal and invasive ductal carcinoma (Supplemental Table C), there was a statistically significant association of expression of KLF8 with MMP9 expression ($p<0.001$) (Fig. 6C1). KLF8 and/or MMP9 expression was present almost exclusively in invasive ductal carcinoma where 35 of 60 invasive ductal samples showed one or both compared to 1 of 15 samples of tissue other than invasive ductal carcinoma (in this one case it was MMP9 expression alone). Thus there was a statistically significant association of KLF8, MMP9, or both expression with the invasive potential ($p<0.001$) (C3). Indeed, in the 60 invasive ductal carcinoma samples only, there were 25 samples showing expression of both KLF8 and MMP9 and their expression was again statistically significantly associated with each other ($p<0.001$) (C2).

Collectively, these results strongly suggest that KLF8 activation of MMP9 transcription and expression plays a critical part in promoting human breast cancer invasion and metastasis.

Discussion

In this report, we identified the KLF8 regulation of MMP9 as a novel mechanism for human breast cancer cell invasion. Firstly, we showed that KLF8 promotes MMP9 transcription and activity in human breast cancer cells. Secondly, we demonstrated that the MMP activity is required for KLF8-induced cell invasion, the invasive 3D glandular architecture and the loss of cell-cell aggregation capability in suspension. Thirdly, we identified a strong correlation

of the co-expression of KLF8 and MMP9 with the patient tumor metastasis. This work has revealed a potentially significant role for KLF8 in promoting human breast cancer metastasis and shed a new light on the underlying molecular mechanisms.

This study identified MMP9 as a novel target of transcriptional activation by KLF8. This was demonstrated by 1) that KLF8 directly binds *in vivo* to the MMP9 promoter at the GT box and 2) both the KLF8 binding site and activation domain are required for its activation of the MMP9 promoter (see Fig. 2). Notably, both this GT-box and the KLF8 activation domain are well conserved across species (see Supplemental Figure) (Urvalek *et al.*, 2010), suggesting a broad significance of this important KLF8 regulation of MMP9. Our finding that KLF8 activates MMP9 transcription is also supported by our gene expression profiling studies using the 10A-iK8 cells (data not shown). It is known that MMP9 message stability is also aberrantly regulated in invasive breast cancer (Iyer *et al.*, 2005; Lamar *et al.*, 2008; Yan and Boyd, 2007). The message stability is controlled primarily by its 3′-UTR. Our MMP9 promoter reporter, however, (see Fig. 1) does not contain the 3′-UTR-encoding template and there is no evidence that KLF8 might regulate 3′-UTR of any gene. Therefore, it is unlikely that KLF8 also plays a role in stabilizing MMP9 message.

We have shown that KLF8 inhibits the E-cadherin transcription during EMT (Wang *et al.*, 2007). We noticed that this EMT induction took several days although the E-cadherin mRNA began to decrease much earlier (data not shown). This observation suggests that the early repression of E-cadherin transcription by KLF8 is not sufficient to induce the EMT and destruction of the pre-existing E-cadherin protein is also essential. The shedding product of E-cadherin ectodomain, i.e., the soluble E-cadherin, has been found in the blood and urine of metastatic cancer patients (Cristofanilli, 2006). Recent studies have demonstrated that the shedding is done by MMP9, MT1-MMP or MMP2 in lung or skin cancer cells during EMT (Covington *et al.*, 2006; Dwivedi *et al.*, 2006; Symowicz *et al.*, 2007). On the other hand, treatment with the soluble E-cadherin has been shown to in turn upregulate the MMPs (Nawrocki-Raby *et al.*, 2003) and promote the cell invasion (Symowicz *et al.*, 2007). Additionally, overexpression of full-length E-cadherin can reduce MT1-MMP expression and MMP2 activity in the cells (Ara *et al.*, 2000; Nawrocki-Raby *et al.*, 2003). Our expression profiling data showed that MMP9 and MT1-MMP are upregulated within 24 hours of KLF8 induction in the 10A-iK8 cells (data not shown). These results support an interesting possibility that during the early stage of KLF8-induced EMT, the pre-existing E-cadherin protein is downregulated by this shedding mechanism and the KLF8-activated MMPs may play a role for this potentially novel mechanism for the initiation of invasion at the early stage of breast cancer metastasis. Alternatively, KLF8 could use proteolytic degradation (Janda *et al.*, 2006; Maeda *et al.*, 2006; Yang *et al.*, 2006) to destroy the pre-existing E-cadherin proteins. Nevertheless, the potentially mutual regulation between E-cadherin and the MMPs may represent a novel way by which KLF8 promotes the initiation of human breast cancer invasion by regulating both target gene transcription and protein stability. Using our inducible expression cell lines, we are vigorously testing this interesting possibility.

Single CTC circulation and extravasation is critical for breast cancer metastasis (Giampieri *et al.*, 2009). The microtubule-based microtentacles formed on the surface of single CTCs

are essential for the cells to adhere to and penetrate through the blood vessel endothelial layer during breast cancer cell extravasation (Whipple *et al.*, 2008). Importantly, microtentacle formation is closely associated with vimentin (Whipple *et al.*, 2008), a hallmark of EMT. Consistently, the disaggregation of the 10A-iK cells was clearly induced and maintained in suspension by KLF8 (Fig. 4). Since KLF8 has been shown to strongly induce EMT (Wang *et al.*, 2007) and the KLF8-induced disaggregation depends upon MMP9 (Fig. 4B), it is plausible to speculate that the KLF8-to-MMP9 signaling may play an important role in the formation and maintenance of single breast cancer CTCs and microtentacles to promote distant metastasis.

The tumor cell disaggregation could lead to either difficult cell movement resulting in reduced metastasis or increased efficiency of colonization and thus increased metastasis. Our results have clearly demonstrated that KLF8 is critical for the lung metastasis (Fig. 5). Therefore, it is likely that KLF8-promoted single cell circulation helps the cell metastasis by enhancing the metastatic colonization.

We have previously demonstrated that KLF8 downregulation of E-cadherin is critical for breast cancer cell invasion (Wang *et al.*, 2007). Here we show that KLF8-mediated upregulation of MMP9 is also critical. In addition, the aberrant elevation of KLF8 is highly correlated with both the loss of E-cadherin (Wang *et al.*, 2007) and the overexpression of MMP9 (Fig. 6). These results strongly suggest that the downregulation of E-cadherin and upregulation of the MMP(s) by KLF8 are two important signaling arms required for maximizing KLF8-promoted breast cancer metastasis. Experiments are in progress to test this important possibility.

In summary, we have identified MMP9 as a novel target for transcriptional activation by KLF8 and demonstrated that the KLF8 regulation of MMP9 is critical for human breast cancer cell invasion. We have also shown that KLF8 expression is important for human breast cancer cell metastasis. Our results suggest a potentially significant role for KLF8 in the metastatic progression of human breast cancer. To date, MMP inhibitors have been proven less effective than expected in clinical trials (Coussens *et al.*, 2002). Unlike MMP9 which is expressed in normal human tissue, the expression of KLF8 is barely detectable in normal epithelial cells (Wang and Zhao, 2007; Wang *et al.*, 2007). In addition to MMP9, KLF8 also targets E-cadherin. Therefore, KLF8 may represent a novel favorable intervention target against breast cancer metastasis.

Materials and Methods

Cell culture and reagents

MCF-10A (Debnath *et al.*, 2003a; Wang *et al.*, 2007) and the tet-off NIH 3T3/KLF8 cells were previously described (Zhao *et al.*, 2003). The lung-prone MDA-MB-231 variant 4175 expressing thymidine kinase-GFP-luciferase (TGL) was a kind gift from Dr. Joan Massagué (Kang *et al.*, 2005; Minn *et al.*, 2005). These cells were maintained in either DMEM/F-12 or DMEM with 10% FBS. To construct pLVUT-tTR-KRAB-KLF8, we replaced GFP with HA-KLF8 in the vector (Szulc *et al.*, 2006). To construct pLVCT-tTR-KRAB-shKLF8, we cloned the shRNA duplex (Supplemental Table) into pLVTHM downstream of H1 promoter

and then transferred the H1-shKLF8 casette into pLVCT-tTR-KRAB (Szulc *et al.*, 2006) and replacing the GFP with mCherry (RFP). The human MMP9 expressing lentiviral vector and MMP9 promoter reporter were previously described (Iyer *et al.*, 2005; Lamar *et al.*, 2008). To generate the MCF-10A line that expresses inducible KLF8 (10A-iK8) and the MDA-MB-231 line that expresses inducible KLF8 shRNA (231-K8ikd), the pLVUT-tTR-KRAB-KLF8 or pLVCT-tTR-KRAB-shKLF8 lentiviruses were used to infect the MCF-10A or 4175 cells and positive cells were cloned. The cell lines were maintained under uninduced (U in the absence of doxycycline or Dox) or induced (I in the presence of Dox) conditions depending upon the experimental requirement.

In-gel zymography

The MMP activities were assayed as described (Jorda *et al.*, 2005). Briefly, 5×10^5 cells in a 6-well plate were cultured in serum-free medium for 16–24 h, and the conditioned medium was separated on an SDS-PAGE gel containing 1 mg/ml gelatin. The gel was washed with buffer I (Tris-HCl [pH 7.5] and 2.5% Triton X-100), incubated overnight in buffer II (150 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris-HCl [pH 7.6]) at 37°C and stained with Coomassie blue. The clear bands indicate where MMPs degraded gelatin.

In situ zymography

The 10A-iK8 cells were grown for 72 h under U or I conditions, 10^6 cells were washed with DMEM and replated on DQ™ collagen fluorescein conjugate (Molecular Probes) coated cover glass and cultured with serum-free media for 16 h. The fluorescence release resulting from MMP cleavage of the matrix was visualized by microscopy. The MMP inhibitor GM6001 was included in some cultures.

Quantitative real-time PCR (qRT-PCR) and western blotting

These assays were done essentially as previously described (Wang *et al.*, 2007). See primer information in Supplemental Table. Anti-MMP9 (1:1000) was from Cell Signaling.

Promoter reporter assays, chromatin immunoprecipitation (ChIP) and biotinylated oligonucleotide precipitation (BOP)

These assays were performed essentially as previously described (Wang *et al.*, 2007). For reporter assays, cells were transfected with reporter constructs with transient or induced expression of KLF8. For ChIP asays, the 10A-iK8 cells were cultured under uninduced or induced conditions for 3 days and processed for the analyses. For BOP assays, the 10A-iK8 cells were cultured with Dox for 3 days and cell lysates were processed for analysis. See oligouncleotide information in Supplemental Table.

Three dimensional (3D) culture

3D cell culture was performed as previously described (Debnath *et al.*, 2003a). The 10A-iK8 cells were propagated in DMEM/F-12 with FBS and other supplements. Single cells in medium containing 5% Matrigel were seeded at a density of 5×10^4 cells/cm^2 on a Matrigel-coated well. The 231-K8ikd cells in DMEM plus 10% FBS were seeded at 2×10^4

cells/cm^2. GM6001 was included in some experiments. In other experiments, the cells were infected with lentiviruses for 2 days to overexpress MMP9 prior to transfer to 3D culture.

Cell aggregation analysis

Trypsin-singularized cells were incubated in suspension (1×10^6 cells/ml) for various periods of time, a 50 μl volume of cells were carefully transferred to a cover slide. For spheroid formation in hanging drops, 2×10^4 cells were incubated in a 50 μl drop of complete medium hanging on the inner side of the lid of 12-well plate for 12 h. After the incubation, the drop was pipetted five times with a 200-μl standard tip and spread on a cover slide. Images of more than five random fields from at least three independent cultures were taken by phase contrast or fluorescent microscopy. Single cells or spheroids that contain at least 30 cells were counted.

Immunohistochemistry (IHC)

Human breast cancer tissue arrays (IMH-371 from IMGENEX and BR1503 from US Biomax) sections were baked for 1 h at 62°C for rehydration and microwaved in 0.01 M sodium citrate for 5 min for antigen retrieval. After incubated in 3% H_2O_2 for 6 min, the sections were serum-blocked for 30 min, incubated overnight at 4°C with first antibodies in PBS and subsequently with biotin-labeled secondary antibodies for 30 min, followed by a peroxidase-labeled avidin-biotin complex (Vector Laboratories, Burlingame, CA) for 30 min. The sections were developed in 3,3-diaminobenzidine tetrahydrochloride for 2 min and counterstained with hematoxylin for 4 min. The stained sections were dehydrated, treated with xylene, and mounted for microscopy. Positive staining was displayed in brown color.

Matrigel invasion assays

The tet-off 3T3/KLF8, 10A-iK8 or 231-K8ikd cells grown under uninduced or induced conditions for 24–48 h were assayed as previously described (Wang *et al.*, 2007). In some experiments, transient MMP9 overexpression, knockdown or inhibition with GM6001 was included. The invasion rates were normalized to the uninduced cells.

Bioluminescence imaging analysis of lung metastasis

All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Female Balb/c nude mice (Taconic) 4–6 weeks old were used for all xenografting studies. For lung metastasis formation, 2×10^6 viable cells were washed and harvested in 0.1 ml PBS and subsequently injected into the lateral tail vein. Five or six weeks after injection mice were anaesthetized using isoflurance and injected intraperitoneally with 150 mg/kg of D-luciferin (15 mg/ml in PBS). Imaging was completed between 2 and 5 min after injection with a Xenogen IVIS system coupled to Living Image acquisition and analysis software. For BLI plots, photon flux (photons/sec/cm^2/steradian) was measured with a region of interest (ROI) drawn around the bioluminescence signal encompassing the thorax of the mouse in a prone position. A background value was subtracted that was obtained from a luciferin-injected control mouse.

Statistical analysis

Summary data is presented as mean +/− the SD with a minimum of three observations per group. Unpaired, paired or single sample Student's t-test with the Bonferroni correction for the multiple comparisons was applied as appropriate. The two by two table for human data was analyzed by Fisher's Exact Test. Significance was determined by the alpha level of 0.05

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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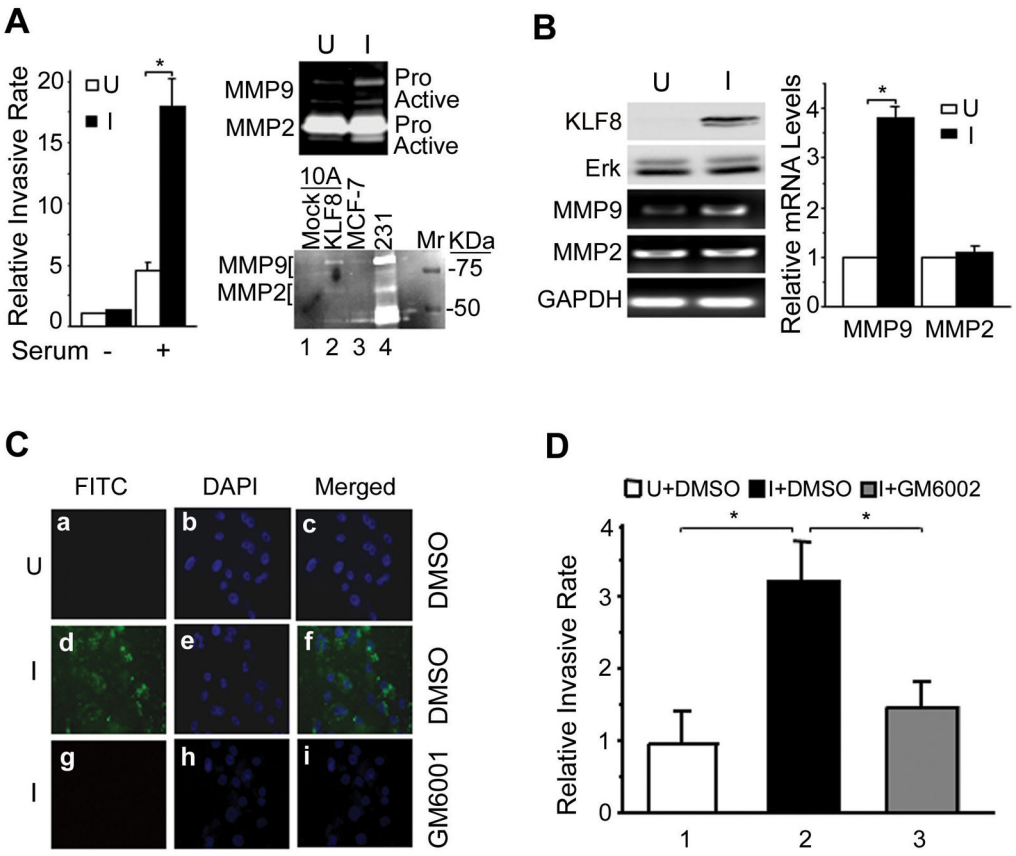


Figure 1.

KLF8 upregulates the expression and activity of MMP9 and promotes cell invasion. *A*, KLF8 promotes NIH3T3 cell invasion and upregulates MMP9 in both NIH3T3 and MCF-10A cells. Induced (I) and uninduced (U) Tet-off NIH3T3/KLF8 cells were examined for invasion through Matrigel (Left panel). The MMP activities in the media from either the same 3T3/KLF8 cells (right-top panel) or the indicated normal or cancer cell lines (right-bottom panel) were examined by in-gel zymography after 3–5 days of incubation. *B*, induction of MMP9 mRNA expression by KLF8. The 10A-iK8 cells were grown under I or U conditions for 48 h. Whole cell lysates or total RNA were prepared for western blotting with anti-HA using Erk as a loading control, or RT-PCR and qRT-PCR using GAPDH as an internal control (left panel). *C*, KLF8 activates MMPs *in situ*. The 10A-iK8 cells were cultured under U (a~c) or I conditions with DMSO (d~i) or 20 μM of GM6001 (g~i) in the medium and processed for *in situ* zymography. *D*, KLF8-induced invasion is blocked by the MMP inhibitor. Matrigel invasion assays were conducted using the 10A-iK8 cells described in B. *$p < 0.05$.

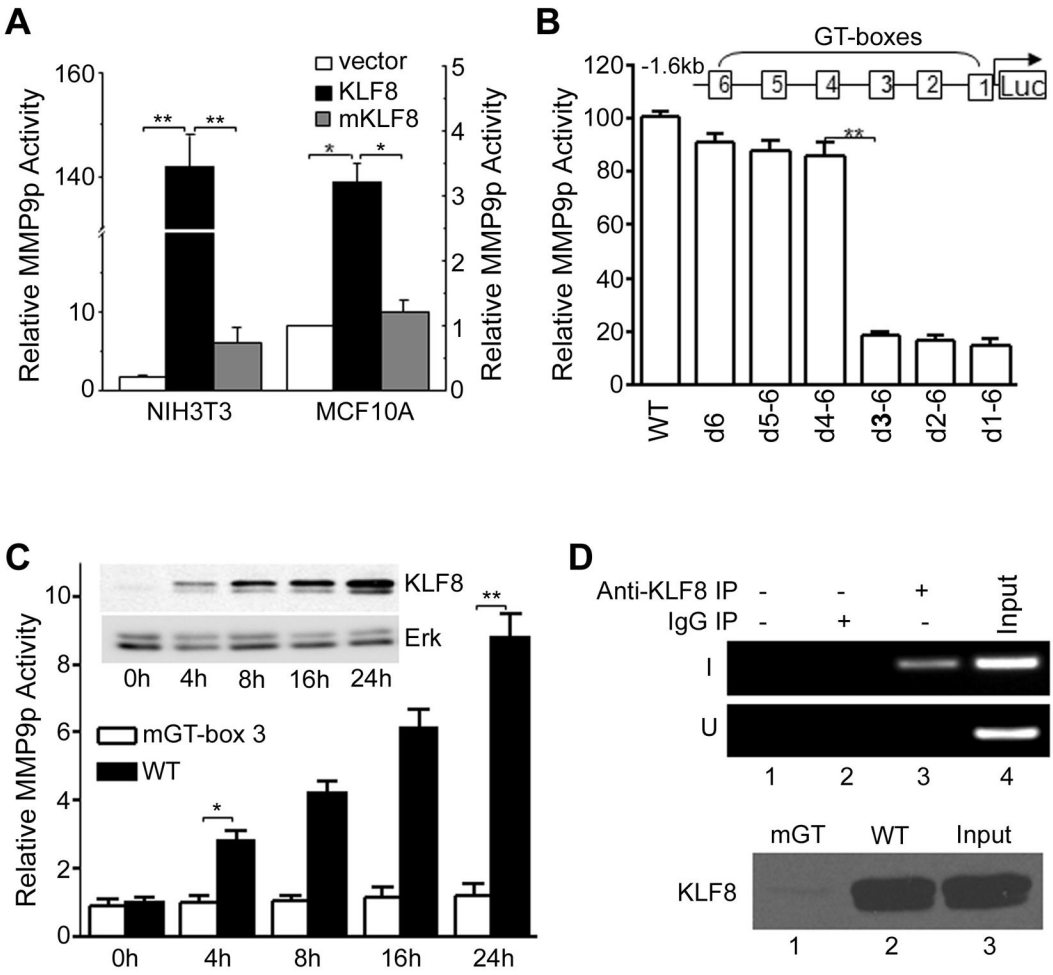


Figure 2.

The GT-box located at the −449 bp of MMP9 promoter is the primary mediator of the activation of MMP9 transcription by KLF8. *A*, KLF8 activates MMP9 promoter (MMP9p) in both NIH3T3 and MCF-10A cells. Reporter activity was performed as described in Materials and Methods. *B*, The minimal KLF8-responding MMP9p region contains three GT-boxes. A serial MMP9p mutants (e.g., d6) were co-transfected KLF8 and luciferase activities were measured after 16 h. *C*, The GT-box at −449 bp is crucial for MMP9p activation by KLF8. Uninduced 10A-iK8 cells were transfected with the −1.6 kb MMP9p (WT) or its mutant (mGT-box 3, GGGTG to TGAGA mutation). After a 16 h, the KLF8 expression was induced for indicated periods of time as confirmed by western blotting (inset) and luciferase assays were performed. *D*, KLF8 directly binds to the endogenous MMP9p at the GT-box 3. The 10A-iK8 cells were grown under U or I conditions for 72 h prior to ChIP (top panel) or BOP (bottom panel) assays as described in Material and Methods. WT, wild type and mGT, GT-box 3 mutated oligos. * $p < 0.05$ and ** $P < 0.01$.

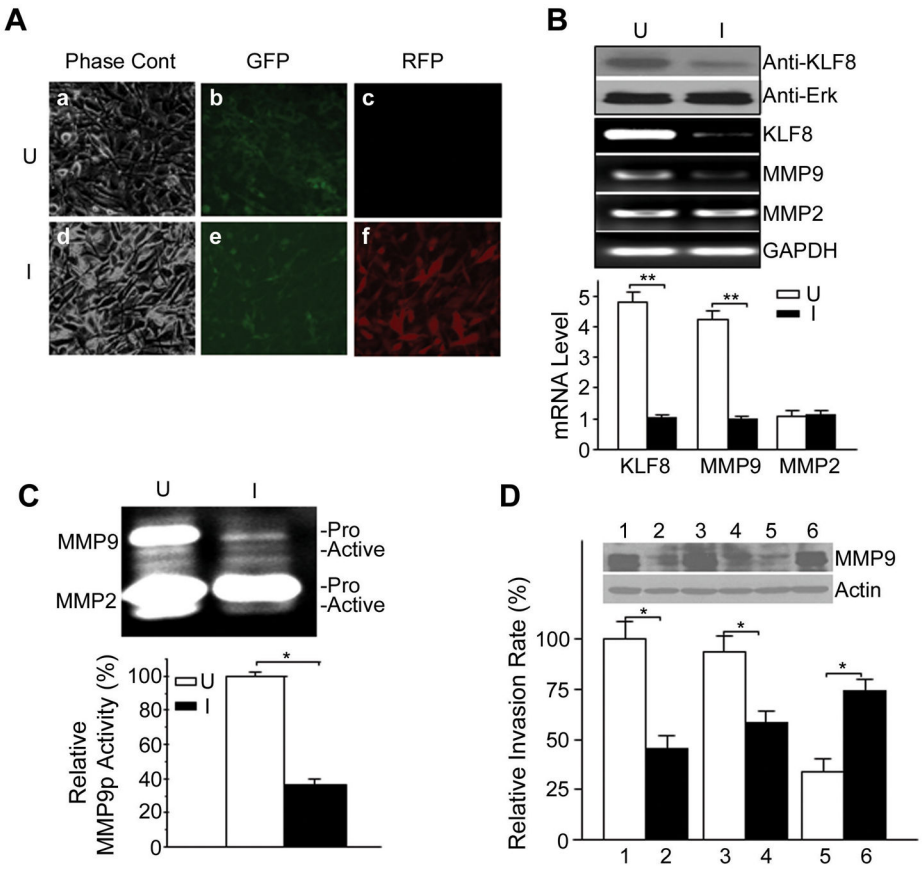


Figure 3.

Knockdown of KLF8 causes a decrease in the expression and activity of MMP9 and cell invasion. *A*, 4175TGL cells expressing inducible KLF8 shRNA (231-K8ikd). Images of the cells grown under U (a–c) and I (e–f) conditions. GFP labels all the cells whereas RFP represents those that express KLF8 shRNA. *B*, KLF8 knockdown blocks the expression of MMP9 mRNA. The 231-K8ikd cells were grown under the indicated conditions for 72 h. Western blotting and RNA analysis were conducted as in Fig. 1B. *C*, KLF8 knockdown inhibits enzymatic and promoter activities of MMP9. 231-K8ikd cells were grown for 72 h under the indicated conditions and processed for in-gel zymography (top panel) and promoter reporter assays (bottom panel) as in Fig. 1A and 2B, respectively. *D*, KLF8 knockdown-mediated inhibition of invasion can be prevented by overexpressing ectopic MMP9. The 231-K8ikd cells were maintained under the indicated conditions with or without MMP9 knockdown or overexpression for 72 h prior to Matrigel invasion assays. MMP9 protein was monitored by western blotting (inset). 1, U; 2, I; 3, U + control siRNA; 4, U + MMP9 siRNA; 5, I + Vector control; 6, I + MMP9 overexpression. *p< 0.05 and **p< 0.01.

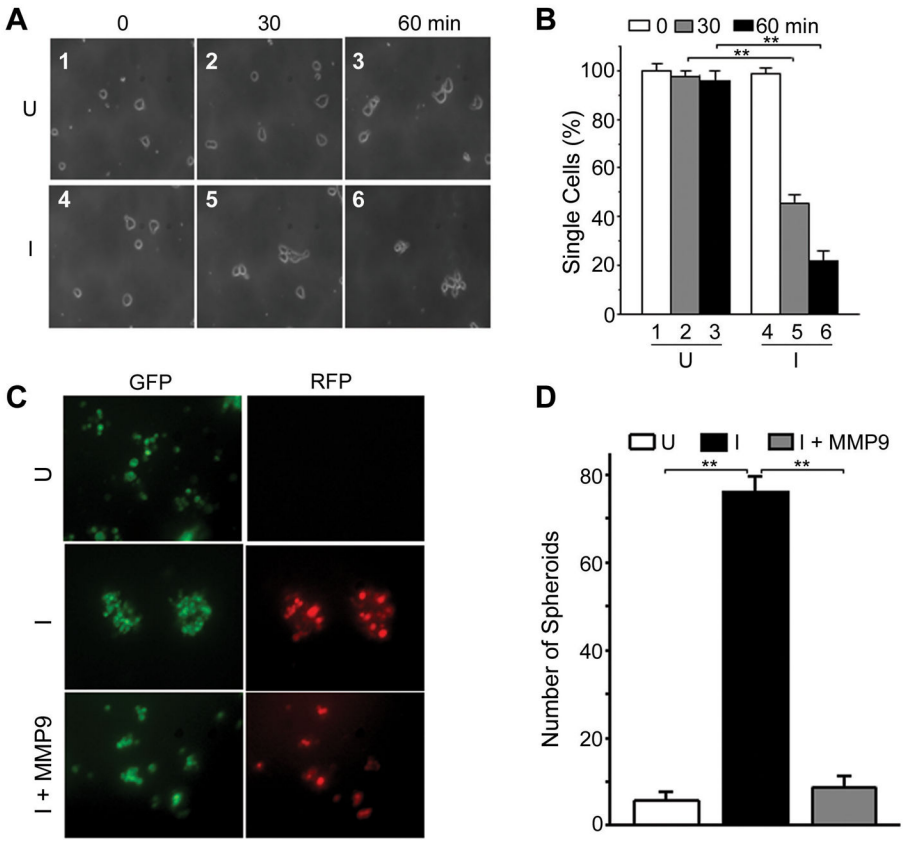


Figure 4.

KLF8 activation of MMP9 is required for maintaining the non-aggregation status of breast cancer cells in suspension. *A & B*, KLF8 knockdown promotes cell aggregation. The 231-K8ikd cells were grown under U or I conditions on a dish for 3 days. The cells were trypsinized, washed, re-suspended in whole medium and incubated with rotation. At the indicated time points, 50 μl of cells were carefully transferred to a hemocytometer for microscopic imaging (A) and single cell counting (B). KLF8 knockdown was monitored by fluorescent microscopy of RFP expression as shown in C. *C & D*, KLF8 knockdown promotes spheroid formation which can be prevented by overexpressing ectopic MMP9. The 231-K8ikd cells were grown as in A & B with or without ectopic expression of MMP9. Equal numbers of the cells in 50 μl were used for 'hanging drop' culture as described in the Materials and Methods. After 24 h the fluorescent images were taken where GFP labeled all the cells and RFP displays only those with KLF8 knockdown (C) and spheroids containing at least 30 cells were counted (D). Data represent the mean +/− SD for at least three independent experiments. ** $p < 0.01$.

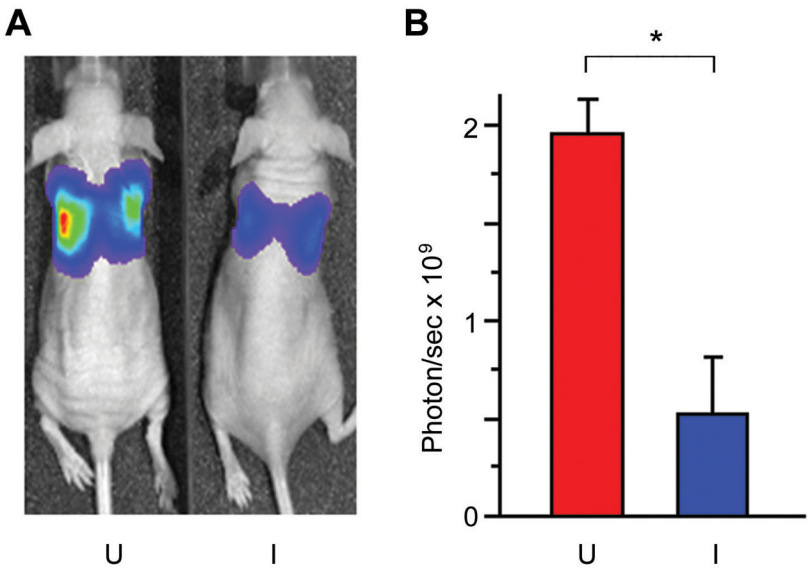
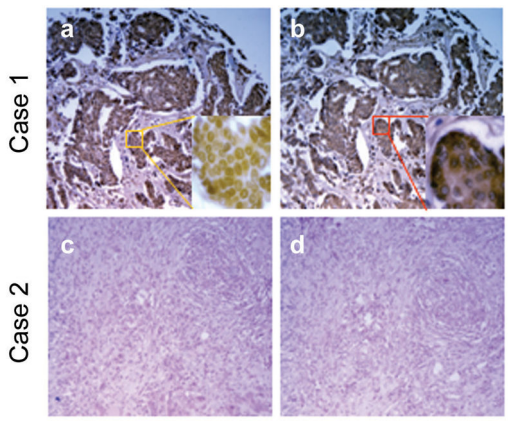


Figure 5.

KLF8 knockdown suppresses lung metastasis of breast cancer. 4175TGL-KLF8ikd cells were injected through the tail vein. Mice were fed with food containing (I, KLF8 knockdown induced) or without doxycycline (U, KLF8 knockdown uninduced) for six weeks. Mice were anaesthetized and injected with D-luciferin followed by bioluminescence imaging analysis of the lung metastasis as described in Materials and Methods. *A*, Representative images of the lung metastasis. *B*, Quantitative results for the volumes of the lung metastasis. *, $p < 0.01$.

A



B

	% Metastatic Lymph Nodes 0-50	>50	Total	p-V.
K8+/M9+	3 (20%)	4 (27%)	7 (47%)	
Non-K8+/M9+	8 (53%)	0 (0%)	8 (53%)	
Total	11 (73%)	4 (27%)	15 (100%)	0.026

C1

	K8+	K8-	Total	p-V.
M9+	25	8	33	
M9-	3	39	42	
Total	28	47	75	0.000

C2

	K8+	K8-	Total	p-V.
M9+	25	7	32	
M9-	3	25	28	
Total	28	32	60	0.000

C3

	K8- or M9-	K8+, M9+ or Both	Total	p-V.
Non-IDC	14	1	32	
IDC	25	35	28	
Total	28	32	60	0.000

Figure 6.

Positive correlation between KLF8 and MMP9 expression in metastatic breast cancer patient tumors. *A*, MMP9 positive and negative surgical specimens of human breast tumors (IMH-371) were subject to IHC co-staining as described in Materials and Methods for the expression of KLF8 (a, c) and MMP9 (b, d). The data represent 15 duplicates of breast tumor specimens with matched normal tissue counterparts (see Supplementary Table B). Case 1 and case 2 represent positive and negative staining, respectively, for both KLF8 and MMP9. The zoom-ins of the indicated areas are shown in the insets. *B*, Summary of the correlation. The correlation between the aberrant KLF8/MMP9 co-overexpression and the percentage of metastatic lymph nodes was analyzed by Fisher's exact test. K8, KLF8; M9, MMP9; p-V., p-Value. *C*, Similar data obtained from another set of 75 human tumor

specimens (BR1503) (See Supplemental Table C). Four more cases positive for both KLF8 and MMP9 were shown in Supplemental Fig. S3. Association of expression of KLF8 with MMP9 expression in all the specimens (C1), or of KLF8 expression with MMP9 expression in invasive ductal carcinoma of only (C2), or of either KLF8, MMP9, or both expression with invasive intraductal carcinoma (C3) was analyzed by Chi square test. A p-value of less than 0.05 was set as the criterion for statistical significance.