Interleukin Enhancer-Binding Factor 3 Promotes Breast Tumor Progression by Regulating Sustained Urokinase-Type Plasminogen Activator Expression

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Abstract

Sustained urokinase-type plasminogen activator (uPA) expression is detected in aggressive breast tumors. Although uPA can be transiently upregulated by diverse extracellular stimuli, sustained, but not transiently-upregulated uPA expression contributes to breast cancer invasion/metastasis. Unfortunately, how sustained uPA expression is achieved in invasive/metastatic breast cancer cells is unknown. Here, we show that sustained and transiently-upregulated uPA expression are regulated by distinct mechanisms. Using a collection of transcription factor-targeted siRNAs, we discovered that interleukin enhancer binding factor 3 (ILF3) is required for sustained uPA expression. Two discrete mechanisms mediate ILF3 action. The first is that ILF3 activates uPA transcription by binding to the CTGTT sequence in the nucleotides -1,004~-1,000 of the uPA promoter; the second is that ILF3 inhibits the processing of uPA mRNA-targeting pri-miRNAs. Knockdown of ILF3 led to significant reduction in *in vitro* cell growth/migration/invasion and *in vivo* breast tumor development. Importantly, immunohistochemistry showed that nuclear ILF3, but not cytoplasmic ILF3 staining correlates with elevated uPA level and higher grades of human breast tumor specimens. Nuclear localization of ILF3 highlights the role of ILF3 in sustained uPA expression as a transcription activator and pri-miRNA processing blocker. In conclusion, this study shows that ILF3 promotes breast tumorigenicity by regulating sustained uPA expression.

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Conflict of interest.

The authors declare no conflict of interest.

Keywords

uPA; ILF3; transcription; miRNA

Introduction

The fibrinolytic system, consisting of urokinase-type plasminogen activator (uPA), its receptor (uPAR) and plasminogen activator inhibitors (PAI-1 and PAI-2), is actively involved at multiple stages of tumor progression. Binding of uPA to uPAR triggers the conversion of plasminogen to plasmin and the subsequent activation of metalloproteinases. These events confer tumor cells with the capability to degrade the components of the surrounding extracellular matrix, thus contributing to tumor cell invasion/metastasis (1). uPA-uPAR interaction also elicits signals that stimulate cell migration, cell proliferation/survival and the expression of tumor-promoting genes, thereby further facilitating tumor development (2). uPA level is elevated in various metastatic tumors (3). In breast cancer, high uPA level correlates with tumor aggressiveness and poor patient survival (4). Together with PAI-1, uPA level in breast tumor tissues is used as a prognostic marker (5).

uPA expression can be induced in breast cancer cells by growth factors and phorbol ester (6–8). However, such induction is transient and lasts only a short period of time. AP1- and/or NF-κB-dependent mechanisms have been shown to mediate the transient uPA upregulation (9). On the contrary, invasive/metastatic breast cancer cells exhibit sustained uPA expression (10–11) though uPA level can be further transiently elevated by extracellular stimuli in them (6–8). It is well recognized that cancer cells necessitate sustained, rather than transiently-induced uPA expression for invasion/metastasis (12). Unfortunately, how sustained uPA expression is achieved in invasive/metastatic cancer cells is unknown.

Members of interleukin enhancer binding protein (ILF3) family were originally purified from a protein complex that regulates interleukin-2 transcription (13–15). Members of this family, namely NF90a, NF90b, NF110a and NF110b, are the products of differential splicing of ILF3 transcripts (16). They are identical at the N-terminal and central regions but diverge at their C-terminal regions (17). Through the ability to bind both DNA and RNA (16), members of ILF3 can regulate transcription (15, 18), translation (19–20), mRNA stability (21–22) and primary microRNA (pri-miRNA) processing (23). The observation that ILF3 is overexpressed in nasopharyngeal carcinoma, non-small cell lung carcinoma and ovarian cancer (24–26) indicates that ILF3 is potentially involved in oncogenesis. This possibility is supported by an early study in which ILF3 was shown to promote angiogenesis through the stabilization of VEGF mRNA (21).

The objective of this study is to elucidate the mechanism responsible for sustained uPA expression in breast cancer cells. Using MDA-MB-231 line, we screened a collection of siRNAs that target 1,294 transcription factors. Among identified targets, ILF3 was specifically required for sustained uPA expression in breast cancer cells. Two discrete mechanisms mediate ILF3 action. The first is that ILF3 binds to the CTGTT sequence at nucleotides −1,004~−1,000 of the uPA promoter to activate uPA transcription. The second is

that ILF3 inhibits the processing of uPA mRNA-targeting pri-miRNAs. Moreover, we found that depleting ILF3 suppressed both *in vitro* and *in vivo* breast tumorigenicity. Finally, we showed that enhanced nuclear ILF3 staining correlated with the uPA overexpression and higher tumor grade in breast tumor specimens.

Results

Sustained and transiently-upregulated uPA expression are regulated by distinct mechanisms

Both uPA mRNA and protein were expressed at high levels in invasive, but not in less-invasive breast cancer cell lines (Fig. 1A, 1B and 1C) and knockdown of uPA led to significant reduction in *in vitro* invasion of invasive breast cancer cells (Fig. S1). Interestingly, PMA transiently upregulated uPA level in both invasive (MDA-MB-231, MDA-MB-436) and less-invasive breast cancer cells (T47D) in a similar fashion (Fig. 1D). Treatment with conventional PKC (cPKC) inhibitor G36976 and MEK1/2 inhibitor U0126 prior to PMA stimulation largely abolished PMA-induced uPA upregulation in all three cell lines (Fig. 1E). In contrast, these inhibitors displayed little to slight inhibitory effect on the sustained uPA expression in invasive lines (Fig. 1F). These results indicate that sustained and transiently-upregulated uPA expression may be regulated by distinct mechanisms.

ILF3 is specifically required for sustained uPA expression

To define the mechanism important for sustained level of uPA, we first compared uPA transcription between invasive and less-invasive breast cancer cell lines by performing chromatin immunoprecipitation (ChIP) with RNA polymerase II (RP-II) pAb. Q-PCR showed that uPA promoter sequence was enriched at least 8-fold more in the RP-II ChIPs when compared to that in control IgG ChIPs in invasive breast cancer lines (Fig. 2A); while barely any increase was detected in less-invasive lines (Fig. 2A), indicating that sustained uPA expression is regulated at the level of uPA transcription in breast cancer cells. To identify transcription factor(s) pertinent to sustained uPA expression, MDA-MB-231 cells were individually transfected with a collection of 1,294 siRNAs that target most of known transcription factors in human followed by In-Cell Western to detect uPA (Fig. S2A). Through this screening, 5 siRNAs that target Ets-1, ILF3, c-Jun, NF45 and STAT1 were found to downregulate uPA level (Fig. 2B and Table S1). To validate theses targets, MDA-MB-231 cells were transfected with two distinct siRNAs for each target. Immunoblotting showed that knockdown of ILF3 or NF45 led to over 80% reduction in uPA expression while Ets-1, c-Jun and STAT1 siRNA only moderately reduced uPA level compared to the scrambled siRNA control (Fig. 2C). Similarly, knockdown of ILF3 or NF45 reduced approximately 80% of uPA promoter activity (Fig. S2B). These results suggest that ILF3 and NF45 are the crucial transcription factors for sustained uPA expression.

ILF3 gene products including NF90a/b and NF110a/b are known to regulate gene expression together with NF45 (15, 18–23, 27). We further determined the importance of ILF3 and NF45 in sustained uPA expression by lentivirally introducing two distinct ILF3 or NF45 shRNAs into MDA-MB-231 and MDA-MB-436 cells. Both ILF3 shRNAs not only reduced ILF3 but also NF45 level while depleting NF45 also reduced both uPA and ILF3 levels (Fig.

3A). These results are in line with the earlier study showing that the stability of ILF3 and NF45 proteins requires stable interaction among them (27). The inhibitory effect on uPA expression caused by ILF3 or NF45 knockdown was relatively specific because the level of cyclooxygenase-2 (COX-2), a protein highly expressed in invasive breast cancer cells, was little affected (Fig. 3A). Moreover, PMA transiently upregulated uPA expression in ILF3-knockdown cells in a similar fashion as seen in the respective parental cells (compare Fig. 3B with Fig. 1D). These results suggest that ILF3/NF45 complex is required for sustained uPA expression.

Since our designed ILF3 shRNAs target all splicing isoforms of ILF3, we examined the relevance of individual ILF3 members by expressing shRNA-refractory NF90a, NF90b, NF110a or NF110b in ILF3-knockdown cells either individually or together. Immunoblotting showed that uPA expression was rescued only when all isoforms were expressed in knockdown cells (Fig. 3C). These results coincide with the observation that silencing either NF90 or NF110 led to significant reduction in uPA mRNA as well as protein in MDA-MB-231and MDA-MB-436 cells (Fig. 3D and 3E). Together, these results suggest that both NF90 and NF110 are indispensable for sustained uPA expression.

ILF3 activates uPA transcription

As knockdown of ILF3 resulted in reduced steady-state uPA mRNA level in breast cancer cells (Fig. 3D), we examined the effect of ILF3 depletion on both uPA transcription and mRNA stability in MDA-MB-231 and MDA-MB-436 cells. ChIP with RP-II pAb showed that depletion of ILF3 reduced the uPA promoter occupancy of RP-II by more than 80% (Fig. S3A). Analysis with uPA promoter-containing luciferase reporter plasmid further showed that knockdown of ILF3 decreased luciferase activity to similar extent when compared against negative control (Fig. 4A). In contrast, actinomycin-chasing experiment showed that knockdown of ILF3 did not significantly alter the half-life of uPA mRNA in these cells (Fig. S3B). These results suggest that ILF3 regulates uPA transcription in breast cancer cells.

To identify the region in uPA promoter important for sustained uPA transcription in breast cancer cells, we generated a series of uPA promoter deletion constructs (Fig. 4B). Luciferase activity analysis showed that region between nucleotides $-1,072 \sim -859$ contained sequences essential for sustained uPA promoter activity in invasive breast cancer cell lines (Fig. 4B and S4A). Based on the previously identified consensus ILF3 binding sequence CTGTT (or AACAG in antisense direction) (15, 18), there are 2 consensus sequences at nucleotides $-978 \sim -982$ and $-1,004 \sim -1,000$ in this region (Fig. 4C). Mutagenesis at these two sites and an additional site outside of this region followed by luciferase activity assay showed that only sequence at nucleotides $-1,004 \sim -1,000$ was essential and responsible for uPA promoter activity (Fig. 4C). These results indicate that ILF3 activates uPA transcription through ILF3 consensus sequence at nucleotides $-1,004 \sim -1,000$ in the uPA promoter. This observation is further supported by the observation that ILF3 depletion did not further reduce the luciferase activity of promoter having mutation at nucleotides $-1,004 \sim -1,000$ (Fig. S4B). Subsequently, we also investigated the effect of mutating ILF3- or AP1/NFκB-binding site in PMA-induced uPA promoter activity. PMA greatly elevated luciferase activity

in cells transfected with uPA promoter reporter plasmid containing mutation in ILF3 binding site (Fig. 4D). However, PMA-elevated luciferase activity was not seen in uPA promoter reporter plasmid with mutation in AP1/NFκB-binding site (Fig. 4D). These results show that sustained and transiently-upregulated uPA transcriptions are respectively regulated by ILF3- and AP1/NFκB-dependent mechanisms in breast cancer cells.

To further corroborate transcriptional regulation of uPA by ILF3, we synthesized oligonucleode spanning nucleotides −1,013~−969 of the uPA promoter. EMSA with ^{32}P-labeled oligonucleotides showed that ^{32}P-labeled oligonucleode/protein complex was seen in the nuclear extracts of parental and shRNA control-transduced MDA-MB-231 and MDA-MB-436 cells while it was absent in ILF3-knockdown cells (Fig. 4E). The formation of ^{32}P-labeled oligonucleode/protein complex was diminished by the excess of unlabeled wild-type oligonucleotide but not oligonucleode containing mutation at CTGTT site (Fig. 4F). In parallel, we investigated *in vivo* ILF3-uPA promoter interaction by ChIP using ILF3 pAb. Quantitative PCR (qPCR) with the primers specifically amplifying region containing ILF3 binding site (nucleotides −1,037~−935) showed that this region was enriched more than 10 fold in ILF3 immunoprecipitates over the IgG control (Fig. 4G). However, such enrichment was not detected in ILF3-knockdown cells (Fig. 4G). These results show that ILF3 activates uPA transcription by binding to the CTGTT sequence at nucleotides −1,004~ −1,000 of the uPA promoter.

ILF3 contributes to sustained uPA expression by inhibiting the processing of uPA mRNA-targeting pri-miRNAs

We previously showed that uPA mRNA is the target of miR-193a, miR-193b and miR-181a in breast cancer cells (11). As ILF3 can inhibit the production of miRNA by blocking the processing of pri-miRNA (23), we hypothesized that ILF3 might also regulate sustained uPA expression by inhibiting the processing of pri-miR-193a/193b/181a. To test this possibility, we initially analyzed the levels of pri- and mature miR-193a/193b/miR-181a in both control and ILF3-knockdown MDA-MB-231 cells. Levels of pri-miR-193a/193b/181a were moderately lower in control than those in ILF3-knockdown cells while the levels of their respective mature miRNAs were greatly increased in ILF3-knockdown cells compared to the control (Fig. 5A). Similar results were also observed with MDA-MB-436 cells (Fig. S5). Subsequently, we introduced luciferase reporter plasmid containing 3′-UTR of uPA mRNA into both control and ILF3-knockdown cells. The luciferase activity was reduced more than 40% in ILF3-knockdown cells compared to that of control (Fig. 5B). However, ILF3 knockdown-caused reduction in luciferase activity was largely reversed by the combined treatment of miR-193a/193b/181a inhibitors (Fig. 5C). Luciferase activity was similar with reporter plasmid containing mutations in miR-193/miR-181 seed sequence of the uPA mRNA's 3′-UTR between control and ILF3-knockdown cells (Fig. 5B). The treatment of miR-193a, miR-193b and miR-181a inhibitors, but not the inhibitor of ineffective miR-23b (11), partially restored uPA level in ILF3-knockdown cells (Fig. 5D). Forced NF90b or NF110b expression enhanced uPA levels (Fig. 5E); however, increased uPA levels in cells with forced NF90b and NF110b expression were reduced by the treatment of mixture of miR-193a, miR-193b and miR-181a (Fig. 5E). Taken together, these

results suggest that ILF3 also buttresses sustained uPA expression by inhibiting the processing of pri-miRNAs that target uPA mRNA.

The presence of ILF3 is essential for breast tumorigenicity

To determine the importance of ILF3 in breast tumorigenicity, we first analyzed the effect of ILF3-knockdown on cell growth, cell migration and *in vitro* invasion in MDA-MB-231 and MDA-MB-436 cells. MTT assay showed that cells with ILF3 knockdown grew significantly slower than the control cells (Fig. 6A). Transwell and Matrigel assays further showed that ILF3-knockdown cells were defective in cell migration and *in vitro* invasion respectively (Fig. 6B, 6C and S6). However, soluble single chain uPA (scuPA) partially rescued cell growth, migration and invasion of ILF3-knockdown cells (Fig. 6A, 6B, 6C and S6). These results indicate that reduced uPA expression is at least partially responsible for decreased cell growth, migration and invasion of ILF3-knockdown cells. In the parallel experiment, we also lentivirally introduced each of the ILF3 members into MDA-MB-231 cells. Cells with stably expressed NF90a, NF90b, NF110a or NF110b all exhibited enhanced *in vitro* invasion compared with control cells (empty vector) (Fig. S7), confirming an invasion-promoting role of ILF3 in breast cancer cells.

We next analyzed the effect of ILF3-knockdown in breast tumor development using the well-established orthotopic breast tumor model (28–29). Control or ILF3-knockdown MDA-MB-231 cells (both stably expressing EGFP) were injected into mammary fat pad area of female athymic nude mice and mice were monitored weekly for 7 weeks by measuring fluorescence. Judging by the fluorescence intensity, mice injected with control cells displayed a much greater tumor outgrowth rate than mice injected with ILF3-knockdown cells (Fig. 6D). Tumors were apparent in all 8 mice injected with control cells while significantly smaller tumors were detected in 4 mice injected with ILF3-knockdown cells after 7 weeks (Fig. 6E). As expected, level of uPA was much less in the tumors excised from mice injected ILF3-knockdown cells than those in the control (Fig. 6F). When the frozen sections of lungs from these mice were analyzed, EGFP-expressing cells were detected in lungs of 7 out of 8 control mice but only 1 out 8 mice injected with ILF-3 knockdown cells (Fig. 6G). Identical results were obtained with immunoblotting that determined the presence of EGFP in lung tissue extracts (Fig. 6H). These results show that ILF3 is required for *in vivo* breast tumor growth and metastasis.

The intensity of ILF3 nuclear staining correlates with disease grades and uPA expression of breast tumors

As laboratory studies may not recapitulate clinical breast cancer, we performed immunohistochemistry (IHC) to examine ILF3 level in commercial human tissue arrays that contained 49 breast tumor specimens and 33 normal breast tissues (isolated from area surrounding breast tumors). ILF3 was detected in both cytoplasm and nucleus, and was present in both breast tumors and normal breast tissues (Fig. 7A). There was no significant difference in the level of cytoplasmic ILF3 between breast tumor tissues and normal breast (Fig. 7B). Cytoplasmic ILF3 staining was also not associated with other pathological parameters including age, tumor size (T), lymph node status (N), ER/PR/HER2 status or disease grades (Table. S2).

We next compared the intensity of nuclear ILF3 staining between normal and breast tumor tissues. Nuclear ILF3 staining was strong in 38 of 49 breast tumor specimens but in only 5 of 33 normal breast tissues (77.6% *vs* 15.2%; *P*<0.001) (Fig. 7B). Although the intensity of nuclear ILF3 staining was independent of age, tumor size, ER/PR/HER2 status (Table. 1), it was significantly associated with breast tumor grades (Table. 1). To determine the association between ILF3 and uPA in clinical breast tumor specimens, we also performed IHC to analyze the level of uPA in the same patients' samples. While normal breast tissues were negative or weak for uPA staining, 24 of 49 breast tumor specimens were strong for uPA staining (49.0%, *P* < 0.001) (Fig. 7A). Importantly, all specimens with high uPA level displayed strong nuclear ILF3 staining (R=0.527, *P*<0.001) (Table. S3). To further investigate ILF3-uPA correlation, we analyzed continuous sections from 10 breast tumor tissues. IHC again showed that specimens positive for uPA staining all displayed strong nuclear ILF3 staining while specimens negative for uPA expression exhibited relatively weaker nuclear ILF3 staining although overall cellular ILF3 staining were not significantly different between samples positive and negative for uPA staining (Fig. 7C). These results show a strong correlation between high nuclear ILF3 staining and elevated uPA expression in breast tumor tissues.

Discussion

Sustained high uPA level is detected in both invasive breast cancer cell lines and specimens of aggressive breast tumors (10–11, 30). *In vitro* and *in vivo* studies clearly demonstrate the importance of sustained uPA expression in breast tumorigenicity (31). In the MMTV-PymT transgenic breast cancer model, uPA deficiency has been shown to reduce metastasis by more than 7-fold (32). We have also shown that uPA-targeting siRNAs and miRNAs suppressed breast cancer cell invasion (11). However, how metastasis-pertinent sustained uPA expression is maintained in invasive breast cancer cells is unknown.

In breast cancer cells, uPA can be upregulated by diverse stimuli including growth factors and phorbal ester (6–8). We showed that PMA transiently upregulated uPA expression in both invasive and less-invasive breast cancer cells in cPKC/Erk-dependent mechanism (Fig. 1). However, sustained uPA expression in invasive breast cancer cells is not significantly affected by cPKC or MEK1/2 inhibitor (Fig. 1). This observation led us to hypothesize that distinct mechanisms regulate transient and sustained uPA expression. In this study, we identified ILF3 as a key gene specifically involved in sustained uPA expression in invasive breast cancer cells (Fig. 2, 3 and 4). Early studies have shown that Ets-1, NF-κB and AP1 components regulate uPA expression in breast cancer cells (33–36). These studies were exclusively done in cells under extracellular stimulation. Our observation that Erk activity was required for PMA-induced transient uPA upregulation suggests that Erk may act through these previously identified transcription factors to mediate PMA action, especially Erk signaling pathway has been shown regulate the expression of many of these factors in breast cancer cells (37–39).

Members of ILF3 can regulate gene expression by a variety of mechanisms such as transcription (15, 18), translation(19–20), mRNA stability (21–22) and pri-miRNA processing (23). Interestingly, we have previously shown that uPA expression can also be

regulated at the levels of both transcription and post-transcription (11, 40–42). This study shows that ILF3 regulates sustained uPA expression in two discrete mechanisms. In the first, ILF3 directly facilitates uPA expression by activating uPA gene transcription (Fig. 4). In the second, ILF3 indirectly contributes to sustained uPA expression by suppressing the processing of uPA mRNA-targeting pri-miRNAs (Fig. 5). Our studies thus support a unique bi-modal capability of ILF3 in regulating gene expression.

An earlier study has implicated an important role of ILF3 in tumor progression where ILF3 was shown to promote tumor angiogenesis by stabilizing VEGF mRNA (21). In this study, we showed that silencing ILF3 led to significant reduction in cell growth, migration and *in vitro* invasion; however, the addition of scuPA partially reversed the effects of ILF3-knockdown (Fig. 6). Our findings thus functionally linked ILF3 to sustained uPA expression in breast tumorigenicity. Moreover, we showed that knockdown of ILF3 greatly diminished breast tumor outgrowth and spontaneous lung metastasis in athymic nude mouse model (Fig. 6). Taken together, our study presents strong evidence that ILF3 plays a crucial role in breast tumor progression.

Overexpression of ILF3 was reported in nasopharyngeal carcinoma, non-small cell lung carcinoma and ovarian cancer (24–26). Although we did not detect significant increase in the level of cytoplasmic ILF3 in clinical breast tumor specimens, we observed much stronger nuclear ILF3 staining in breast tumors when compared to normal breast tissues (Fig. 7). Elevated nuclear ILF3 staining correlated with high uPA level and higher tumor grade in breast tumor specimens (Fig. 7, Table. 1 and Table. S3). The nuclear localization ILF3 highlights its role as both transcription factor and pri-miRNA processing suppressor in the process of maintaining sustained uPA expression in invasive breast cancer cells. Our finding also suggests that attention should be paid to the intracellular localization of ILF3 (nucleus *vs* cytoplasm), rather than solely on the level of ILF3 when the relevance of ILF3 to tumor development is investigated.

Our mechanistic study was performed with the established breast cancer cell lines that may not fully simulate clinical setting. However, the consistency seen in mouse model and the excellent correlation between nuclear ILF3 staining and uPA level/tumor grades in clinical specimens argue against any confounding influence derived from our experimental studies. To sum up, our studies suggest that ILF3 promotes breast tumorigenicity by maintaining sustained uPA expression in breast cancer cells.

Materials and methods

ShRNAs/siRNAs

ILF3 and NF45 shRNAs were designed using web-based Block-iT program and subcloned into pLV-mU6-[shRNA]-EF1α-GFP vector (Biosettia). siRNAs specific for NF90 and NF110 were previously described (27). ShRNA/siRNA sequences are included in Supplementary Information.

In-Cell Western to identify transcription factor important for sustained uPA expression

MDA-MB-231 cells were plated at 50,000 cells/well in 96-well plates overnight and then transfected with Dharmacon siGENOME collection that targets 1,294 human transcription factor genes for 3 days followed by fixation. Fixed cells were permeabilized, incubated with uPA mAb/GAPDH pAb and again incubated with Infrared probe-labeled secondary antibodies. The intensities of uPA and GAPDH levels were determined by Odyssey Infrared Imaging System. Each siRNA was tested in triplicates and reduction of more 25% in the staining intensity over cells treated with control siRNA was set as the cut-off point.

Cell growth study

Cell growth was evaluated by MTT assay as previously described (43). Briefly, 5×10^4 cells were seeded into 24-well culture plates and allowed to grow for 1–3 days prior to the addition of MTT. The cell growth rate was determined by reading plates at 560 nm.

Cell migration and In vitro invasion

Cell migration and *In vitro* invasion were evaluated using Transwells and Matrigel invasion chamber respectively as previously described (37). For cell migration, the undersurfaces of Transwells were coated with 10 μg/mL of collagen and 1×10^5 cells were allowed to migrate for 4 h. For *in vitro* invasion, 2×10^5 cells were allowed to invade for 24 h. After migration/invasion, cells on the undersurface of upper units were stained and counted using a phase-contrast microscope.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol, treated by DNase I and reverse transcribed with either SuperScriptase or TaqMan® microRNA reverse transcription Kit (Invitrogen). Generated products were used to measure the levels of uPA mRNA, pri-miRNA181a/193a/193b and miR-181a/193a/193b using the respective TaqMan probes. Level of β-actin mRNA was measured for standardization.

Luciferase Reporter assay

The construction of luciferase reporter plasmids containing human uPA promoter and 3′-UTR has been described previously (11, 41). To map out the region important for sustained uPA promoter activity, sequential deletion was made in uPA promoter by PCR and subsequently inserted into pGL2 vector (Promega). To determine the importance of putative ILF3 consensus sequences in uPA promoter activity, site-directed mutagenesis was performed using QuickChange® site-directed mutagenesis kit (Agilent). To normalize the difference in the transfection efficiencies with various constructs, plasmid containing pGK promoter-driven Renella luciferase gene (pGK-R/Luc, Promega) was included during the transfection. Reporter's firefly luciferase activities were normalized by dividing the values of Renella luciferase activities.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as previously described(41). Nuclear extracts (10μg/reaction) were incubated with ^{32}P-labeled oligonucleotides (1.5×10^5cpm/reaction) and

mixtures were then electrophoresed on a 6% non-denaturing polyacrylamide gel. To determine the specificity of oligonucleotide-nuclear extract complex, up to 40-fold excess of unlabeled oligonucleotides of wild-type or ILF3-binding site mutant were added into the reactions followed by electrophoresis. Oligonucleotide sequences are included in Supplementary Information.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described (44). To determine uPA transcription and *in vivo* ILF3-uPA promoter interaction, chromatin fragments were immunoprecipitated with RP-II and ILF3 pAbs respectively. Rabbit IgG was used as a negative control. uPA promoter sequence enrichment was determined by qPCR using primers amplifying region either near the transcription start site or spanning ILF3-binding site of the uPA promoter. Primer sequences are included in Supplementary Information.

Tumor growth and lung metastasis in nude mice

Animal studies were performed as previously described (37) and were approved by the Georgia Health Sciences University (GHSU) Institutional Animal Care and Use Committee. Briefly, 3×10^6 control or ILF3-knockdown MDA-MB-231 cells (EGFP-expressing) were injected into 4th mammary fat pad of 5-week-old female nude mice (Harlan Laboratory). Starting from 3rd week, tumor growth was monitored by measuring fluorescence in Xenogen IVIS-200 *In Vivo* imaging system. Seven weeks after injection, animals were sacrificed and both tumors and lungs were excised. Collected tumors were homogenized and analyzed for uPA expression. Parts of excised lungs were frozen and sectioned for detecting EGFP-expressing cells and the remaining was analyzed for EGFP by immunoblotting.

Immunohistochemistry (IHC)

ILF3 and uPA levels in breast tumors and normal breast tissues were evaluated by IHC using anti-ILF3 pAb and anti-uPA mAb on commercial tissue arrays (Shanghai Outdo Biotech) as previously described (45). The array contained 33 normal breast tissues and 49 breast tumor specimens (all were ductal carcinomas of breast). Staining intensity of each sample was given a modified histochemical score (MH-score) that considers both the intensity and the percentage of cells stained at each intensity (46). Samples with below the average MH-score were considered as no/weak staining while samples with above the average MH-score considered as strong staining. To investigate the correlation between ILF3 and uPA expression in breast tumors, continuous sections were made from breast tumor paraffin blocks that were obtained from GHSU tumor bank and subjected to IHC to detect ILF3 and uPA. Experiments involving human tissues were approved by GHSU Human Assurance Committee.

Statistical analysis

Statistical analyses of laboratory studies were performed by Student's *t* test. Chi-square test and Fisher's exact test were used to compare correlation between ILF3/uPA staining and clinicopathological parameters. $P<0.05$ was considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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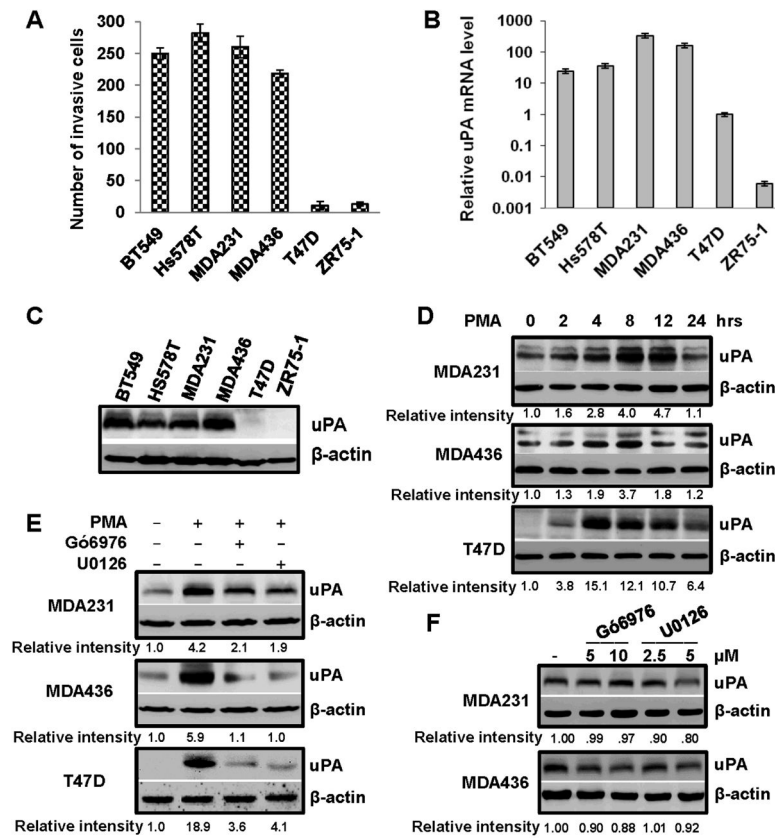
Figure 1. Sustained and transiently-induced uPA expression are regulated by distinct mechanisms

A. In vitro invasion of breast cancer cell lines. Data are the mean ±SE. n=3. *B.* qRT-PCR of uPA mRNA in breast cancer cell lines. Data are the mean ±SE. n=3. *C.* Immunoblotting of uPA protein in breast cancer cell lines. *D.* Cells were stimulated with 100 nM PMA for varying times and then subjected to immunoblotting to detect uPA. *E.* Cells were treated with 10 μM G36976 or 5 μM U0126 for 2 h and then stimulated with 100 nM PMA for 8 h followed by immunoblotting to detect uPA. *F.* Cells were treated with G36976 or U0126 for 1 day and then subjected to immunoblotting to detect uPA.
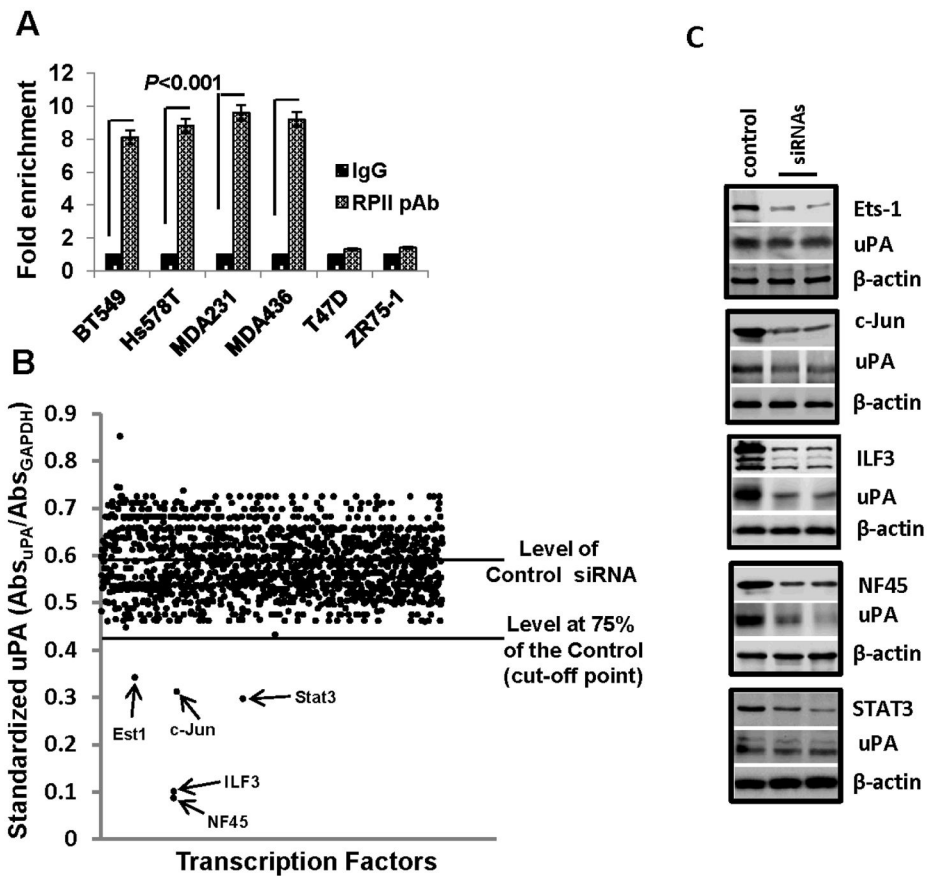
Figure 2. Identifying transcription factors important for sustained uPA expression in breast cancer cells by siRNA-based high-through screening

A. Cells were subjected to ChIP with control IgG or RP-II pAb. DNA eluted from immunoprecipitates was analyzed by Q-PCR with primers amplifying region near the transcription start site of the uPA promoter. Data are the mean ±SE. n=3. *B.* MDA-MB-231 cells were transfected with siRNAs targeting individual transcription factors followed by In-cell western as described in Material and Methods. Level of uPA was standardized by dividing the Absorbance of uPA with the absorbance of GAPDH. *P*<0.05. *C.* MDA-MB-231 cells were seeded in 6-well plates for overnight and then transfected with 2 Dharmacon-supplied sequence-specific siRNAs for each target. After 3 days, cells were lysed and cell lysates were subjected to immunoblotting to detect the respective targ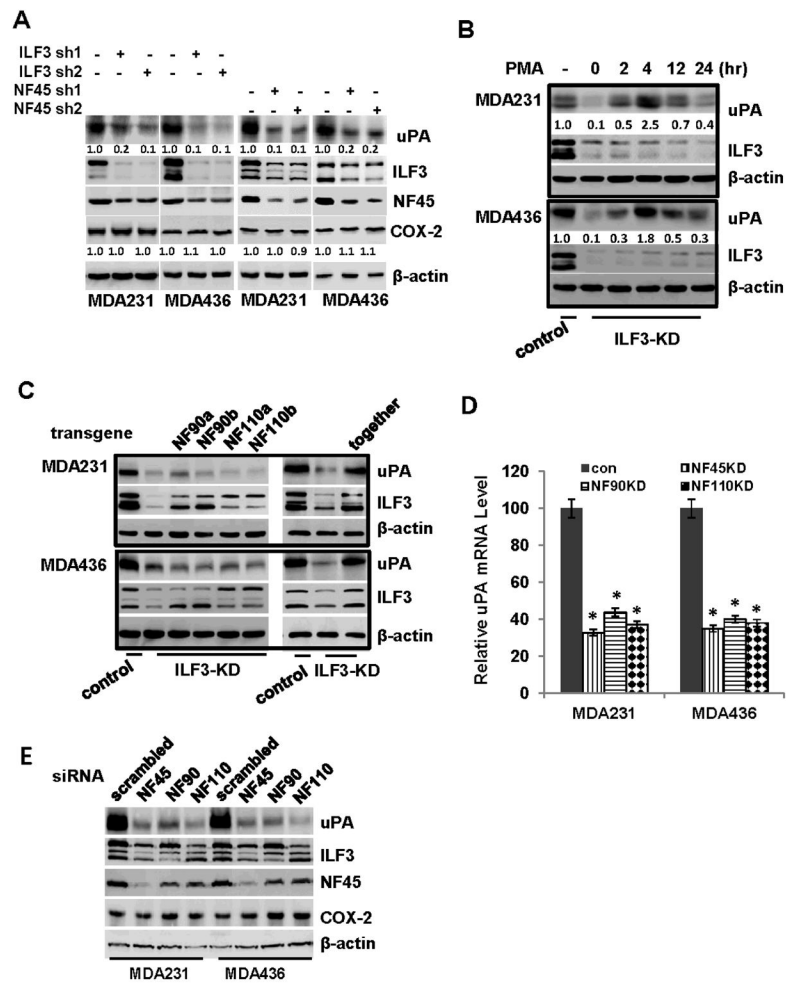et antigens, uPA and β-actin with the respective antibodies. *C.* MDA-MB-231 cells were seeded in 6-well plates for overnight and then transfected with sequence-specific siRNAs for each target. After 3 days, cells were lysed and cell lysates were subjected to immunoblotting to detect the respective target antigens, uPA and β-actin with the respective antibodies.

Figure 3. ILF3 is specifically required for sustained uPA expression
A. Control, ILF3 or NF45 shRNA-transduced cells were subjected to immunoblotting to detect uPA, ILF3, NF45, COX-2 and β-actin. *B.* ILF3-knockdown cells were stimulated with 100 nM PMA for varying times and then subjected to immunoblotting to detect uPA, ILF3 and β-actin. *C.* shRNA-refractory NF90a, 90b, 110a or 110b were expressed in ILF3-knockdown cells either individually or in together and then analyzed for uPA expression by immunoblotting. *D.* QRT-PCR was performed to measure uPA mRNA in cells that were treated with NF45, NF90 or NF110-specific siRNA for 3 days. Data are the mean ±SE. n=3. *, $P<0.005$ *vs* control. *E.* Immunoblotting was performed to detect uPA in cells that were transfected with NF45, NF90 or NF110-specific siRNA.
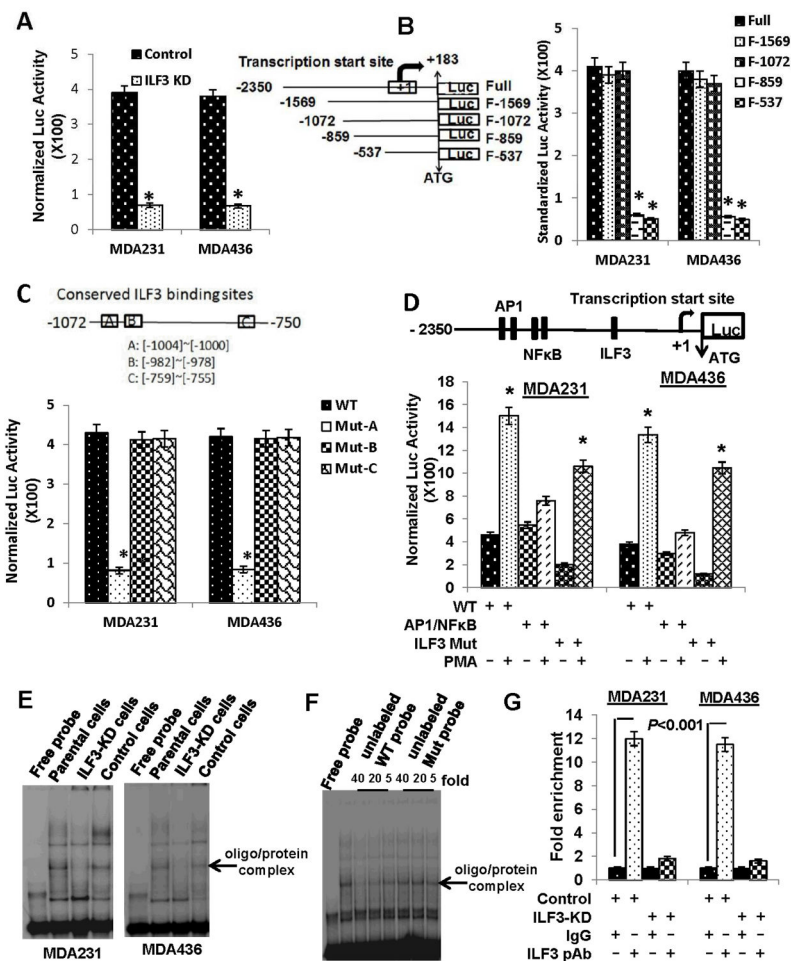
Figure 4. ILF3 is specifically involved in sustained uPA transcription

A. Control and ILF3-knockdown cells were transfected with uPA promoter reporter plasmid and then analyzed for luciferase activity. Data are the mean ±SE. n=3. *, $P<0.001$ *vs* control. **B.** Cells were transfected with reporter plasmids containing various uPA promoter deletion mutants for 1 day and then analyzed for luciferase activity. Data are the mean ±SE. n=3. *, $P<0.001$ *vs* Full-length. **C.** Cells were transfected with reporter plasmids containing mutation at various CTGTT sites for 1 day and then analyzed for luciferase activity. Data are the mean ±SE. n=3. *, $P<0.001$ *vs* wild-type (WT). **D.** Cells were transfected with reporter plasmids containing mutation in ILF3 or AP1/NFκB-binding site for 1 day and then stimulated with 100nM PMA for 6 h followed by the analysis of luciferase activity. Data are the mean ±SE. n=3. *, $P<0.001$ *vs* no PMA. **E.** Nuclear extracts of control or ILF3-knockdown cells were incubated with ^{32}P-labeled oligonucleotides spanning nucleotides −1,013~−969 of the uPA promoter (wild-type). The reaction mixtures were subjected to EMSA. **F.** Nuclear extract of MDA-MB-231 cells were incubated with ^{32}P-labeled wild-type oligonucleotide in the presence of 5 to 40-fold of excess of unlabeled wild-type or mutant oligonucleotides and then subjected to EMSA. **G.** Cells were subjected to ChIP with ILF3 pAb. Q-PCR was performed using primer set that amplifies region spanning nucleotides −1,037~−935 of the uPA promoter. Data are the mean ±SE. n=3.
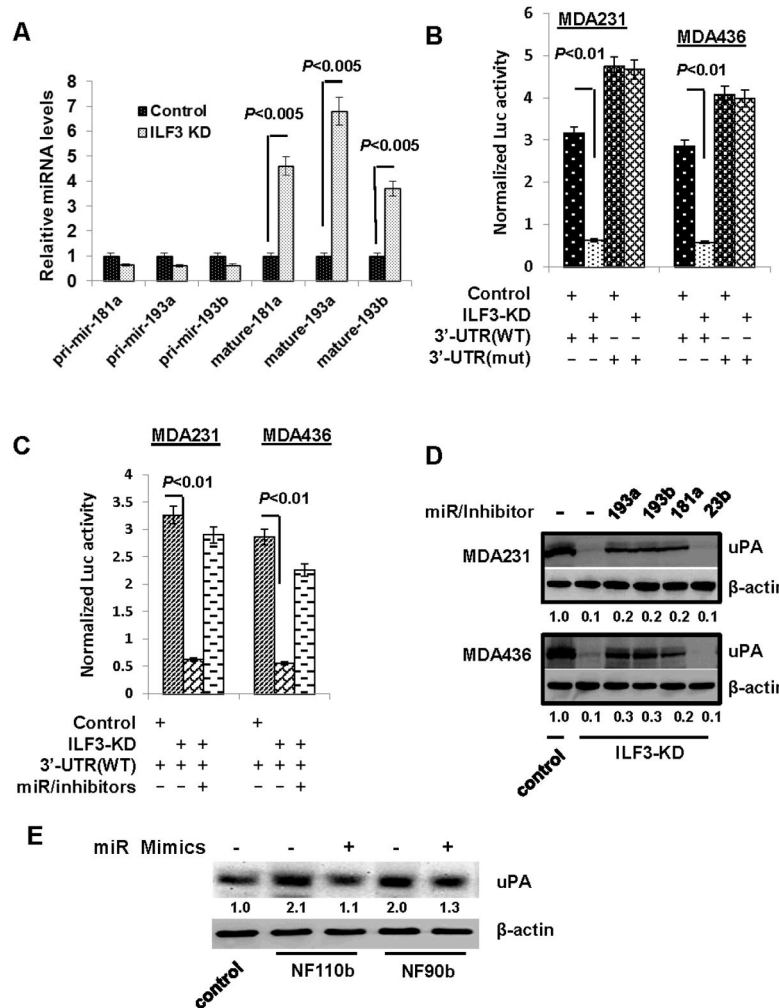
Figure 5. ILF3 depletion enhances the production of uPA mRNA-targeting miRNAs
A. QRT-PCR of primary and mature miR-193a, miR-193b and miR-181a in control and ILF3-knockdown MDA-MB-231 cells. Data are the mean ±SE. n=3. **B.** Cells were transfected with reporter plasmids containing wild-type or mutant uPA 3′-UTR and then analyzed for luciferase activity. Data are the mean ±SE. n=3. **C.** uPA 3′-UTR reporter plasmid was cotranfected into ILF3-knockdown cells with 30 pmole miR-193a/miR-193b/miR-181a inhibitors and then analyzed for luciferase activity. Data are the mean ±SE. n=3. **D.** ILF3-knockdown cells were treated with 30 pmole miR-193a, miR-193b, miR-181a or miR-23b inhibitor and then subjected to immunoblotting to detect uPA. **E.** MDA-MB-231 cells were lentivirally transduced with vector encoding NF90b or NF110b for 3 days and then transfected with 30 pmole of miRNA mimic mixture (miR-193a, miR-193b and miR-181a) for another 3 days. Cells were lysed and cell lysates were subjected to immunoblotting to detect uPA.
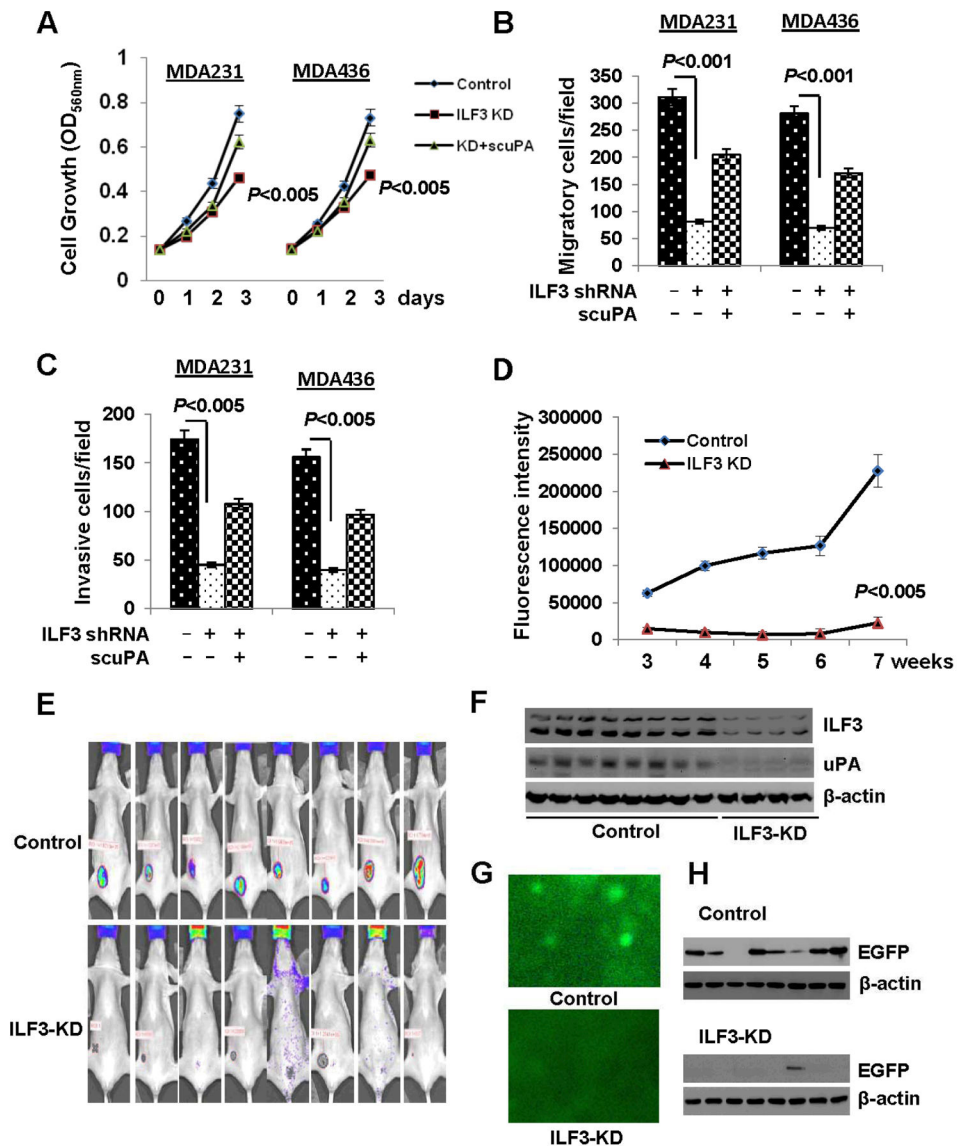
Figure 6. ILF3 is required for breast tumor progression
A. MTT assay to analyze cell growth of control and ILF3-knockdown cells in the absence or presence of 4 nM scuPA. Data are the mean ±SE. n=3. *B.* Transwell assay to analyze cell migration of control and ILF3-knockdown cells. Data are the mean ±SE. n=3. *C.* Matrigel invasion assay to evaluate *in vitro* invasion of control and ILF3-knockdown cells. Data are the mean ±SE. n=3. *D.* Control or ILF3-knockdown MDA-MB-231 cells were injected into nude mice. Tumor growth was monitored by measuring fluorescence intensity weekly starting from 3rd week after injection. Data are the mean ±SE. n=8. *E.* Tumors were imaged in Xenogen system after 7 weeks. *F.* Tumors were homogenized and then analyzed for ILF3 and uPA by immunoblotting. *G.* Lungs excised from sacrificed mice were viewed under a fluorescence microscope. *H.* Aliquots of excised lungs were analyzed for EGFP by immunoblotting.
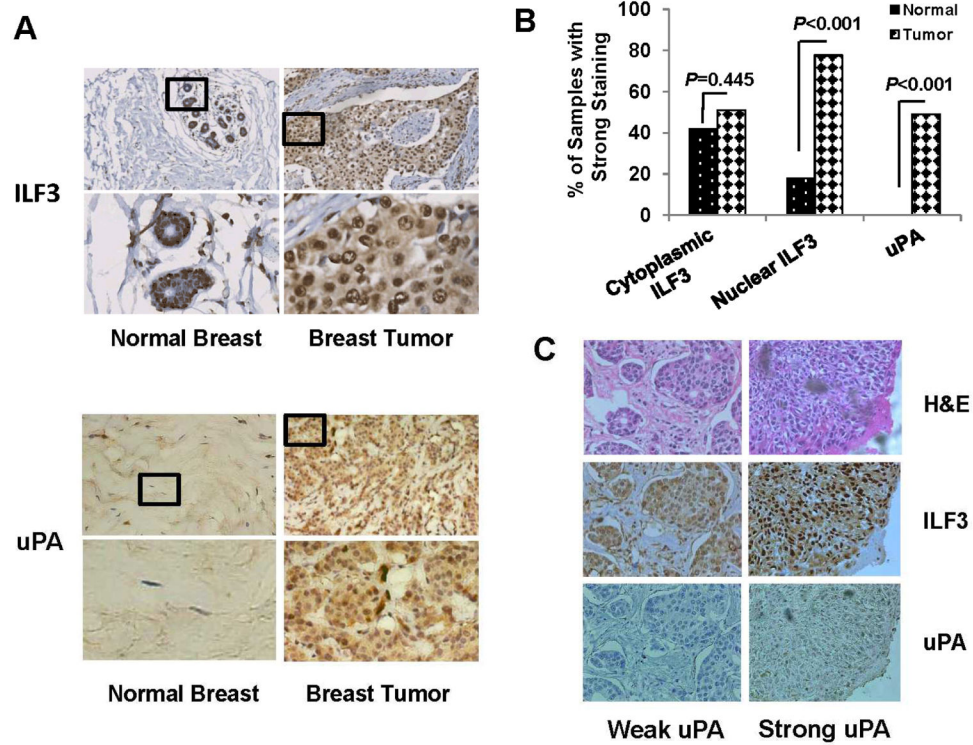
Figure 7. Nuclear ILF3 staining correlates with uPA level and disease grades of breast tumor specimens

A. IHC of ILF3 and uPA in normal breast and breast tumor tissues. Upper panel magnification, ×200; lower panel magnification, ×800. *B.* Percentage of tissues displaying strong cytoplasmic ILF3, nuclear ILF3 and uPA staining. *C.* IHC of ILF3 and uPA on continuous breast tumor sections. Magnification, ×400.

Table 1

Correlation between Nuclear ILF3 Staining and Clinicopathological Parameters of Breast Tumor Specimens

n=49	Nuclear ILF3 Staining		
	no/weak[1]	Strong[2]	*P* value
Age			
<50	4	7	
50	7	31	0.398
T			
T1+T2	10	31	
T3+T4	1	7	0.784
N			
N0	8	14	
N1–N3	3	24	0.078
Grades			
I	8	6	
II	3	21	
III	0	11	**0.001**
ER			
+	6	25	
−	5	13	0.744
PR			
+	8	32	
−	3	6	0.672
HER2			
+	6	25	
−	5	13	1.00

[1] denotes samples with MH-scores below the average MH-score.

[2] denotes samples with MH-score above the average MH-score.