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IDENTIFICATION OF EPITHELIAL STROMAL INTERACTION 1 AND
EPIDERMAL GROWTH FACTOR RECEPTOR AS NOVEL KRÜPPEL-LIKE
FACTOR 8 TARGETS IN PROMOTING BREAST CANCER PROGRESSION

by

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B.S. Hunan Normal University, 2007

A thesis submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Department of Biomedical Sciences
in the College of Graduate Studies
at the University of Central Florida
Orlando, Florida

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2013

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ABSTRACT

Breast cancer is the major cause of cancer death among women worldwide. Understanding the mechanisms underlying breast cancer progression remains urgent for developing effective treatment strategies to eliminate breast cancer mortality. Our recent studies have demonstrated that Krüppel-like transcriptional factor 8 (KLF8) plays a critical role for breast cancer progression. Other studies have shown that Epithelial stromal interaction 1 (EPSTI1), a recently identified stromal fibroblast-induced gene in non-invasive breast cancer cells and epidermal growth factor receptor (EGFR) are highly overexpressed in aggressively invasive breast carcinomas including triple negative breast cancers. In this thesis project, we demonstrate high co-overexpression of KLF8 with EPSTI1 as well as EGFR in invasive breast cancer cells and patient tumors. We also show that KLF8 upregulates the expression of EPSTI1 by directly binding and activating the EPSTI1 gene promoter, and KLF8 upregulates the expression of EGFR not only by directly activating the EGFR gene promoter but also by preventing EGFR translation from microRNA141-dependent inhibition. Genetic, signaling and animal cancer model analyses indicate that downstream of KLF8, EPSTI1 promotes the tumor invasion and metastasis by activating NF- κ B through binding valosin containing protein (VCP) and subsequent degradation of I κ B α , whereas EGFR promotes tumor growth and metastasis via activation of ERK. Taken together, these data identify EPSTI1 and EGFR as novel

KLF8 targets in breast cancer and suggest that KLF8 may be targeted for new effective treatment of breast cancer.

This work is dedicated to the memory of my father
who was lost to liver cancer.

Jinqiao Li

January 21, 1955 – June 24, 2004

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LIST OF ABBREVIATIONS

5-FU fluorouracil

BLI Bioluminescence imaging

BOP biotinylated oligonucleotide precipitation

ChIP chromatin immunoprecipitation

CoIP Co- immunoprecipitation

CtBP C-terminal binding protein

DCIS Ductal carcinoma in situ

DMEM Dulbecco's Modified Eagle's Minimal Essential Medium

DNA Deoxyribonucleic Acid

EDTA Ethylenediaminetetraacetic acid

EGFR Epidermal Growth Factor Receptor

EGTA Ethylene glycol tetraacetic acid

EMT epithelial to mesenchymal transition

EPSTI1 Epithelial stromal interaction 1

ERK Extracellular Regulated Kinase

FAK Focal Adhesion Kinase

GAPDH Glyceraldehyde -3 Phosphate Dehydrogenase

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H&E Hemotoxylin and Eosin

H₂O₂ Hydrogen Peroxide

hrs Hours

IDC Invasive ductal carcinoma

IFN- α Interferon- α

IHC immunohistochemical staining

ILC Invasive lobular carcinoma

KCL Potassium chloride

KLF8 Krüppel-like factor 8

LCIS Lobular carcinoma in situ

MMP-2 matrix metalloproteinase-2

MMP-9 matrix metalloproteinase-9

MMP-14 matrix metalloproteinase-14

NaCL Sodium chloride

NIH National Institute of Health

NP-40 nonylphenoxyethoxyethanol

PBS Phosphate Buffered Saline

RT Radiation Therapy

SD Standard deviation

TGF β Transforming growth factor β

TNBC Triple-negative breast cancer

VCP Valosin containing protein

WCL Whole cell lysate

CHAPTER 1: GENERAL INTRODUCTION

Cancer

Cancer is a major cause of death and one in 4 deaths in the United States is due to cancer [1]. Cancer death rates decreased by 20% from their peak from 1991 to 2009 because of medical and technological improvement in the early diagnosis and treatment for patients. However, in 2013 alone, over 1.66 million new cases of cancer and 5.8 million cancer deaths are projected to happen in the United States [1]. Consequently, better understanding of the underlying molecular and signaling mechanisms of cancer is critical for developing more effective therapeutic strategies to improve patient survival.

Breast Cancer

Even though the prognosis of breast cancer has significantly improved in recent years, breast cancer still faces a dreary outlook. Breast cancer, while representing about 29% of the new cancer diagnoses for women in the United States, is projected to cause 14% of all cancer related deaths for women [1].

The most common symptom of breast cancer is typically a new lump that feels different from the normal breast tissue. Other possible signs of breast cancer include skin irritation, breast or nipple pain, and swelling of all or part of a breast. Tissue biopsy is the usual way to confirm the diagnosis. Once the cancer is officially diagnosed, treatments are assessed.

Risk factors

Age is the most crucial factor that affects breast cancer risk besides female sex [2]. All women are at risk for breast cancer, and as age increases, the risk increases. Besides age and sex, the lack of childbearing and breastfeeding are known to increase the risk of breast cancer[3]. Risk is also increased by inherited genetic mutations in the BRCA1 and BRCA2 genes[4], a personal or family history of breast cancer, biopsy-confirmed hyperplasia (especially atypical hyperplasia)[5], higher levels of certain hormones including androstenedione, testosterone, androgens and estrogen[6], and obesity[7].

Types of breast cancer

There are several types of breast cancer, ductal carcinoma in situ, lobular carcinoma in situ, invasive ductal carcinoma, invasive lobular carcinoma, and some rare types of breast cancer. In some cases a single breast tumor can be a mixture of several types or be a combination of invasive and noninvasive cancer.

Ductal carcinoma in situ

Ductal carcinoma in situ (DCIS) is noninvasive breast cancer or pre-invasive breast cancer. DCIS is characterized by the uncontrolled proliferation of malignant epithelial cells that are bounded by the basement membrane of the milk ducts. It has been considered as pre-invasiveness because some of the cases can go on to become invasive breast cancers. Currently about 20% of breast cancers that are diagnosed in the United

States are DCIS and most of the women diagnosed at this early stage of breast cancer can be cured[8].

Lobular carcinoma in situ

Lobular carcinoma in situ (LCIS) is an uncommon condition in which abnormal cells are generated in the lobules or milk glands in the breast. LCIS isn't cancer, but being diagnosed with LCIS indicates an increased risk of developing breast cancer[9].

Invasive ductal carcinoma

Invasive ductal carcinoma (IDC) is the most common type of breast cancer. About 80% of breast cancers are invasive ductal carcinomas. IDC is cancer that is grown in the mammary duct and has invaded the surrounding tissue of the breast duct[10]. As time goes on, invasive ductal carcinoma can spread through the lymph nodes and blood to other areas of the body including bone, lung, brain and so on.

Invasive lobular carcinoma

Invasive lobular carcinoma (ILC) is the second most common invasive breast cancer, representing 10-15 percent of diagnosed invasive breast cancers[11]. It is a type of breast cancer that begins in the milk-producing glands (lobules) of the breast. Invasive lobular carcinoma is invasive cancer and the cancer cells can break out of the lobule with the potential to spread to other areas of the body.

Current Treatment Options

Successful treatment of breast cancer means getting rid of the cancer or getting it under control for a prolonged period of time. Because a breast tumor is usually made up of many different kinds of cancer cells, getting rid of all those cells may require different types of therapies. The treatment plan may include a combination of the following treatments: surgery, radiation therapy, chemotherapy, hormonal therapy (anti-estrogen therapy), and targeted therapies.

Surgery

Surgery is usually the first step and the most common form of treatment against breast cancer. Surgery often involves the removal of tumor and surrounding margins. The goal of breast cancer surgery is to remove not only the tumor, but also enough margins to test the invasiveness of the tumor. Although significant improvement has been made in directed systemic therapy after surgery, the locoregional relapses are common and usually lead to the death of the patients[12].

Chemotherapy

Chemotherapy treatment uses the combination of cytotoxic drugs to weaken and kill the cancer cells in the body. It is a systemic therapy, which means the drugs travel in the bloodstream through the entire body. It can kill not only the cancer cells at the original site but also any cancer cells that may have spread to other parts of the patients' body.

Chemotherapy is usually prescribed with other treatment, such as hormonal and targeted therapy. It is also used before the surgery to shrink the tumor for easier and safer removal. The most common chemotherapy drugs used for breast cancer is doxorubicin, paclitaxel and so on[13]. These drugs are sometimes used in a combination with other drugs, like fluorouracil (5-FU) and cyclophosphamide [14].

Radiation therapy

Radiation therapy uses a special kind of high-energy ray to damage cancer cells. It is a highly effective way to destroy any remaining cancer cells that remained in the breast area after surgery [15]. The radiation damages all cells in the path of the ray, both normal and cancer cells. However, because cancer cells are less organized than normal cells, it's harder for them to repair the damage done by radiation. This makes the cancer cells easier to be killed by the radiation than normal cells. It can be done before surgery to shrink the tumor, making the surgery easier and safer to operate.

Hormonal therapy

Hormonal therapy is a form of systemic therapy and most often used as an adjuvant therapy to help reduce the risk of the breast cancer recurrence after surgery. About 60% of breast cancers arising in premenopausal women and 80% of those diagnosed after menopause are estrogen receptor (ER) and progesterone receptor (PR) positive[16]. Estrogen helps the growth of hormone receptor-positive cancer cells. Nowadays, there

are two types of hormone therapy for breast cancer: either stopping estrogen from acting on breast cancer cells or lower estrogen levels. Tamoxifen is a widely used drug for the hormonal therapy. It blocks estrogen receptors in breast cancer cells and prevents estrogen from binding to the receptor and thus shuts down the estrogen induced cell growth signal[17]. It is used for both adjuvant breast cancer treatment and metastatic breast cancer treatment[17]. Aromatase inhibitors (AIs) stop estrogen production in post-menopausal women, have been approved to treat both early and advanced breast cancer. Hormonal treatment is helpful for hormone receptor-positive breast cancers, but it does not help patients whose tumors are hormone receptor negative.

Targeted therapy

Targeted cancer therapies are treatments that target specific characteristics of cancer cells, such as a protein that allows the cancer cells to grow uncontrollably. In this way, we can count hormonal therapy as a targeted therapy. Because it is more specific to certain types of cancer cells, it is less harmful to the normal, healthy cells than chemotherapy. Herceptin is an effective treatment both before and after surgery for people with HER2-positive breast cancer. It binds the HER2 receptors of breast cancer cells and blocks the growth signal for the cancer cells [18, 19]. Olaparib is an inhibitor of poly ADP ribose polymerase (PARP), an enzyme involved in DNA damage repair. By inhibition of DNA damage repair, olaparib causes the apoptosis of cancer cells with DNA

damage after chemotherapy or radiation therapy [20-22]. Lots of targeted reagents are under clinical trial. This may open new directions for breast cancer treatment.

Krüppel-like factor 8

Krüppel-like factor 8 (KLF8) belongs to the KLF transcription factor family of proteins. There are 17 KLF family proteins and lots of them have been associated with cancer [23]. Some of the KLF family proteins work as transcriptional repressors, some act as transcriptional activators, while other members work as dual transcription factors by both activating and repressing gene expression. KLF family proteins share highly conserved zinc finger motifs in their DNA binding domains [24].

KLF8 works as a dual transcription factor. It is highly overexpressed in several cancer types including breast and ovarian cancer. It works as an oncogene by promoting cell cycle progression [25], transformation [26, 27], epithelial to mesenchymal transition (EMT) [28-31] and DNA damage response [32]. Besides promoting tumor progression, KLF8 can also work as a key component of the transcription factor network that controls adipocyte differentiation [33].

KLF8's structure

A typical transcription factor usually contains 3 crucial domains for its function: (1) a DNA binding domain to interact with its target gene promoter, (2) a nuclear localization signal (NLS) to translocate the protein into the nucleus, and (3) a transcriptional

regulatory domain to regulate gene expression. In case of KLF8, it has 3 Zinc-finger motifs (highly conserved in the KLF family) in the C-terminus of KLF8 to interact with the target gene promoter at a GT box (CACCC or GGGTG). The two predicted classical nuclear localization signals (NLS) do not regulate KLF8's nuclear translocation [34] but the C-terminal zinc-finger motif 1 and 2 bind to importin, which imports cytosolic proteins into the nucleus. Also the serine 165 and lysine 171 residues of KLF8 are crucial for nuclear localization even though the mechanism is not clear yet [34]. The PVDLS motif of KLF8 can be recognized by the C-terminal binding protein (CtBP) [35] and this interaction will trigger KLF8 transcriptional repression activity. KLF8 can also work as a transcription activator by recruiting co-activator P300 and PCAF to its activation domain containing the core glutamine residues Q118 and Q248. P300 and PCAF then acetylate histones as well as KLF8 itself to activate gene promoters [36].

KLF8's function

KLF8 was originally identified as a transcriptional repressor. KLF8 represses the beta-globin promoter by binding to co-repressor CtBP through the PVDLS repression domain (RD) [36]. KLF8 was then found to be highly unregulated in FAK overexpressing cells. By directly binding to the GT box of cyclin D1, KLF8 activates cyclin D1 promoter and promotes cell cycle progression [25]. By direct repression of KLF4 expression, KLF8 activates cell proliferation and promotes tumor progression [37]. KLF8 can bind to the GT box of the E-cadherin promoter also and this will repress E-cadherin expression and

cause epithelial-mesenchymal transition (EMT) [29]. Matrix metalloproteinase 9 (MMP9) is another KLF8 target gene. KLF8 binds to MMP9 promoter and activates MMP9 expression [30]. Increasing MMP9 activation will break down the extracellular matrix (ECM) proteins and promotes cell invasion. KLF8 also activates MMP14 activity and this will enhance MMP2 activity [38]. Taken together, KLF8 represses tumor suppressor gene expression and promotes oncogene expression and these together enhance tumor progression.

KLF8 and cancer

Several studies have reported the aberrant overexpression of KLF8 in various human cancer types including breast [26, 29, 30, 38], ovarian [27, 39], liver [40, 41], renal [26, 42], brain [43, 44] and gastric cancer [45] as opposed to the undetectable expression in most normal cell or tissue types. In NIH3T3 cells, induction of KLF8 expression enhanced anchorage independent growth, which is a characteristic for oncogenic transformation [26]. KLF8 represses the tumor suppressor gene KLF4 which may also contribute to this transformation [37]. Cancer becomes malignant when cancer cells invade through the extracellular matrix and metastasize to different locations. By enhancing MMP2 and MMP9 activity, KLF8 promotes the degradation of ECM and thus invasion and metastasis [30, 38]. KLF8 also induces the EMT process through repression of E-cadherin, which plays a crucial role in tumor metastasis [29]. KLF8 promotes DNA damage repair and therapeutic resistance in breast cancer cells [32]. The Crucial role of

KLF8 in promoting hepatocellular carcinomas (HCC) cell proliferation has been identified recently in mediating Wnt to β -catenin signaling to activate the transcription of c-Myc, cyclin D1 and Axin1 [40].

Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells lose their cell-cell adhesion, and increase their migration and invasion ability to become mesenchymal cells. EMT process is crucial for lots of developmental processes including the mesoderm formation, neural crest development, heart valve development, and secondary palate formation[46]. Most human solid tumors are carcinomas which originate from various epithelial cell types throughout the body. In order to invade into the surrounding tissue, the carcinomas cells need to lose the cell-cell interaction and acquire the migration and invasion abilities. EMT process actually helps the epithelial tumor cells to acquire motility and lose cell-cell adhesion. Partial loss of E-cadherin induces EMT and has been associated with carcinoma progression and poor prognosis in various human tumors. Also, loss of E-cadherin expression at the membrane will release free β -catenin to translocate to nucleus. Nuclear β -catenin cooperates with T-cell factor 1 (TCF1) to activate MMP14 and further activate MMP2 activity[47]. Active MMP2 and MMP9 can further degrade the extracellular matrix (ECM) and allow the tumor cells to invade through the adjacent tissue. This will further promote invasion and metastasis of carcinomas tumor cells. Our previous studies demonstrated that by repressing E-cadherin

expression, KLF8 works as a strong EMT inducer [29] and promotes breast cancer invasion and metastasis [26, 48]. To further understand the molecular mechanisms by which KLF8 promotes EMT and metastatic progression of breast cancer, we compared the expression profiles genes as well as microRNA between the non-tumorigenic human mammary epithelial MCF-10A cell lines that express and does not express the ectopic KLF8. This thesis project focuses on two of the most highly unregulated genes epithelial stromal interaction 1 (EPSTI1) and epidermal growth factor receptor (EGFR) and one of the most highly downregulated microRNAs microRNA-141 that is a potential negative regulator of EGFR regulation.

Epithelial stromal interaction 1

Epithelial stromal interaction 1 (EPSTI1) was first identified as an induced gene in noninvasive breast cancer cells by co-culture with cancer associated fibroblast (CAF). EPSTI1 is highly unregulated in breast cancer patients' tissue when compared with the normal tissue [49, 50]. EPSTI1 is an interferon response gene and its expression leads to an increase in tumorsphere formation, which is a property associated with cancer stem cell. EPSTI1 expression also promotes the migration ability of breast cancer cells. Most interestingly, ectopic expression of EPSTI1 can replace peritumoral activated fibroblasts by conveying the spread of tumor cells in a tumor environment assay [50]. Also, EPSTI1 expression can increase the expression of EMT inducer slug and twist, and EMT marker

fibronectin, $\alpha 2\beta 1$ integrin. Taken together, these findings suggest EPSTI1 as a potential regulator of breast cancer progression. However, ESPTI1 study remains in its infancy. Whether and how EPSTI1 plays a critical role in breast cancer progression particularly in vivo has been totally unknown.

Epidermal growth factor receptor

Epidermal growth factor receptor (EGFR) is a cell surface receptor that belongs to the epidermal growth factor receptor family. EGFR is a receptor tyrosine kinase with intrinsic tyrosine kinase activity. For many years, it has been believed that EGFR plays limited role in the development and progression of breast cancer. However, recent studies have suggested the crucial roles of EGFR in breast cancer, especially in breast tumor cells survival, proliferation, and invasion.

EGFR structure

Like other EGFR family members, EGFR contains an extracellular region, a transmembrane (TM) domain and a tyrosine kinase domain inside the cell. The extracellular region of EGFR can bind to ligands, which include epidermal growth factor (EGF), transforming growth factor α ($TGF\alpha$), amphiregulin (AR), epigen (EPN), betacellulin (BTC), epiregulin (EPR) and heparin binding EGF-like growth factor (HB-EGF) [51]. The interaction between EGFR and its ligands will stabilize the EGFR extracellular region to an extended conformation. This change will allow EGFR

homodimerize with itself and trigger the autophosphorylation of EGFR intracellular tyrosine kinase activity and thus activate EGFR downstream signaling pathway [52]. In HER2-positive cells, HER2 can stabilize EGFR in a conformation that is ready for dimerization and tyrosine phosphorylation even in the absence of ligand binding, thus contributing to ligand-independent EGFR signaling[53].

EGFR signaling

EGFR signaling is stimulated by the EGFR ligands, whose roles in stimulating EGFR signaling and coupling to biological responses have been well studied [54-56]. These ligands are expressed as membrane proteins and are cleaved by metalloproteinases to release the soluble, mature ligands. These metalloproteinases belong to a disintegrin and metalloproteinase (ADAM) family of membranous proteases. For example, ADAM17 cleaves AREG, EPR, HB-EGF and TGF α [57]. Because cleavage of the ligand precursors is required for the release of soluble, mature ligands, ligand cleavage is also a crucial mechanism in which ligands-induced EGFR signaling can be regulated. Also, the transmembrane ligands stimulate EGFR signaling on adjacent cells through a juxtacrine signaling mechanism that may contribute to the epithelial-stromal interactions related regulation in breast [58]. EGFR ligands usually stimulate EGFR coupling to multiple effectors, including MAPK, PLC γ , PKC, PI3 kinase, Ras, Src, and STAT 3/5 [17, 29]. These effectors are related to increased survival, proliferation, motility and invasiveness in breast cancer progression [56, 59].

Regulation of EGFR gene expression

In general, human cancer cells exhibit elevated levels of EGFR protein, and the mechanisms for elevated EGFR expression include activation of EGFR expression and gene amplification [60-62]. In human breast cancer, EGFR expression also varies widely, but gene amplification is a rare event [63]. Various cellular factors, such as EGF, TGF, and steroid hormones, regulate EGFR expression [64-67] with different mechanisms. For example, EGF stimulates both mRNA and protein levels of EGFR whereas TGF α only triggers the increase of EGFR at mRNA level.

EGFR in breast cancer

EGFR overexpression in primary breast tumors is associated with poor prognosis [68, 69]. Also, high expression level of the EGFR ligands such as AREG, EGF or TGF α is associated with larger and more aggressive tumors [70-72]. EGFR overexpression is also well associated with triple negative breast cancer (TNBC), a type of breast cancer that lacks the expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression. TNBC is usually associated with large tumor size, high tumor grade, EMT, poor survival, and increased frequency of metastasis [73-77]. Because of the lack of targets for therapeutic treatment, TNBC is especially aggressive and hard to cure. Gene expression profiling and immunohistochemical studies have identified that 50 to 70% of TNBC tumors have EGFR expression [78]. Also, low level EGFR expression in TNBC is

correlated with a reduced incidence of metastasis [79]. This suggests the crucial role of EGFR in TNBC and provides new avenues for the treatment of TNBC. Interestingly, a recent study indicated that combination of several EGFR inhibitors significantly promotes the degradation of EGFR and tumor inhibition [80]. Nevertheless, the molecular mechanisms underlying the aberrant upregulation of EGFR expression in breast cancer remain largely uninvestigated.

MicroRNA

MicroRNAs (miRNAs) are a kind of small non-coding RNA at about 22 nucleotides long. Using the high-throughput sequencing methods, more than 25,000 mature miRNAs have been identified in more than 193 different plant and animal species[81]. RNA polymerase II produces the pri-miRNAs from genomic transcription units or from the intron. Then RNase III enzyme, Drosha, cleaved the long pri-miRNAs into shorter pre-miRNAs. Another RNase III, Dicer, crops the pre-miRNAs into miRNAs. Most of the miRNAs recognize their targets only through the seeding sequence. By binding to 3' untranslated regions (3' UTRs) of targeted mRNAs, miRNAs can either initiate an endonucleolytic cleavage to the mRNA or a translational repression to inhibit gene expression[81]. Researchers have shown that miRNAs have a role in blocking the translation initiation by promoting the deadenylation in poly (A) tail, or by recruitment of cofactors that can interfere with translation. Translation repression is the major mechanism for miRNAs' repression function in mammals[82], while mRNA cleavage is the widespread

mechanism in plants[83, 84]. By repression of gene expression, miRNAs have been reported to regulate lots of diseases including breast cancer progression. Although studies on oncogenic or tumor suppressor roles of microRNAs have advanced rapidly in the past decade, the area of research remains very young and their roles and mechanisms in regulating breast cancer progression remain to be discovered.

In this thesis project, we demonstrate *for the first time* that EPSTI1-to-NFkB and EGFR-to-ERK signaling pathways downstream of KLF8 play a very critical role in the invasion and metastasis of breast cancer. This work opens new avenues for breast cancer mechanistic studies and provide insights for developing novel targeted therapies against mortality associated with aggressive breast cancer.

CHAPTER 2: IDENTIFICATION OF EPITHELIAL STROMAL INTERACTION 1 AS A NOVEL EFFECTOR DOWNSTREAM OF KRÜPPEL-LIKE FACTOR 8 IN BREAST CANCER INVASION AND METASTASIS

Abstract

Krüppel-like factor 8 (KLF8) is a transcriptional factor critical for metastatic progression of breast cancer. Epithelial stromal interaction 1 (EPSTI1), a recently identified stromal fibroblast induced gene in non-invasive breast cancer cells is highly overexpressed in invasive breast carcinomas. The function and regulation of EPSTI1, however, remain largely unknown. In this paper, we report a novel KLF8 to EPSTI1 signaling pathway in breast cancer. Using various expression analyses, we revealed a high co-overexpression of KLF8 and EPSTI1 in invasive human breast cancer cells and patient tumors. Ectopic overexpression of KLF8 in the noninvasive, MCF-10A cells induced the EPSTI1 expression, whereas KLF8 knockdown from the invasive, MDA-MB-231 cells decreased the EPSTI1 expression. Promoter activation and binding analyses indicated that KLF8 promoted the EPSTI1 expression by directly acting on the EPSTI1 gene promoter. EPSTI1 knockdown dramatically reduced the KLF8-promoted MCF-10A cell invasion and ectopic expression of EPSTI1 in the non-invasive, MCF-7 cells is sufficient to induce the cell invasion. Experiments using nude mice demonstrated that the ectopic EPSTI1 granted the MCF-7 cells capability of both invasive growth in the breasts and metastasis to the lungs. Using co-immunoprecipitation coupled with mass spectrometry, we discovered that EPSTI1 interacts with the valosin containing protein (VCP), resulting in

the degradation of I κ B α and subsequent activation of NF- κ B in the nucleus. These findings suggest a novel KLF8 to EPSTI1 to VCP to NF- κ B signaling mechanism potentially critical for breast cancer invasion and metastasis.

Introduction

Krüppel-like factor 8 (KLF8) is a dual transcription factor [25, 27, 29, 30, 32, 35-37, 43, 85, 86] that is highly overexpressed in invasive human cancers and plays an important role in various types of human cancer including breast cancer by promoting the cell cycle progression [25, 26, 34, 36, 39], transformation [26, 27], epithelial to mesenchymal transition (EMT) [28, 29, 85, 87] and DNA damage response [32]. Although substantial progress has recently been made in understanding the regulation of its expression, post-translational modification and nuclear localization [32, 36, 37, 85, 86, 88], the molecular and signaling mechanisms by which KLF8 promotes human breast cancer metastasis remain largely uninvestigated.

Epithelial stromal interaction 1 (EPSTI1) was initially identified as an induced gene in the human breast cancer cell line MCF-7 by co-incubation of the cells with a telomerase (hTERT) – activated human breast fibroblast cell line in a three-dimensional culture [49, 89]. Despite the subsequent studies showing that EPSTI1 is highly overexpressed in breast patient tumor tissues [49, 50] and suggesting that EPSTI1 promotes EMT, tumor

progenitor cell properties and anchorage-independent growth in vitro [49, 50], molecular mechanisms that are responsible for the aberrant high expression of EPSTI1 in the breast cancer cells and whether and how EPSTI1 plays a role in vivo for breast cancer progression have not been reported to date.

In this study, we provide strong evidence showing that EPSTI1 is a novel target of transcriptional activation by KLF8 and plays a critical role in the progression of invasive growth and metastasis of breast cancer through VCP-dependent activation of NF- κ B.

Results

KLF8 and EPSTI1 are highly co-overexpressed in human metastatic breast cancer cell lines and patient tumors.

Our cDNA microarray analysis has demonstrated that EPSTI1 is one of the highly unregulated genes in the 10A-iK8 cells [30] when KLF8 expression is induced as compared to the uninduced cells (submitted elsewhere). In addition, we have also recently shown a co-overexpression of KLF8 and EPSTI1 in the MDA-MB-231 cells [90]. Since both KLF8 and EPSTI1 have been shown to be aberrantly high in breast cancer cells or tissues [26, 29, 30, 49, 50], we asked if EPSTI1 is unregulated in breast cancer cell lines and tumor specimens known to express high levels of KLF8. We first examined the expression of EPSTI1 in a panel of human breast cancer cell lines in which

the KLF8 expression was determined in our previous work [26, 29, 30]. The results clearly indicated that there is a high expression of EPSTI1 at both the mRNA and protein levels in the invasive cell lines including the MDA-MB-231, Hs578T and BT549 cells as compared to the barely detectable expression in the non-invasive cancer cell line MCF-7 and the immortalized non-tumorigenic human mammary epithelial cell line MCF-10A (Figure 1A).

We have recently performed tissue array analysis of KLF8 protein expression in patient tumors and correlated it with the invasive potential of the tumors [30]. To test if expression of EPSTI1 protein is also correlated with that of KLF8 protein and/or the tumor invasive potential in the same set of tissue array, we carried out similar IHC staining for EPSTI1 protein. The result showed that 75% of the KLF8 positive tumors express EPSTI1 (Figure 1B and 1C). Among the KLF8-negative samples, 59% express EPSTI1, suggesting that in the absence of KLF8 other factors could play a role for the upregulation of EPSTI1 (Figure 1C). EPSTI1 expresses in 77% of the invasive tumors but only 20% of the non-invasive tumors (Figure 1D).

These results strongly suggested that EPSTI1 expression is positively regulated by KLF8 in the invasive breast cancer tissues.

EPSTI1 is a direct target of transcriptional activation by KLF8.

We further validated the microarray results by qRT-PCR and western blotting and verified the high expression of EPSTI1 in the 10A-iK8 cells only when the KLF8 expression was induced (Figure 2A, compare lanes 2 with 1). Conversely, when KLF8 was knocked down in the 231-K8ikd cells [30], EPSTI1 expression was significantly reduced (Figure 2A, compare lanes 4 with 3).

To test if EPSTI1 is a KLF8 transcriptional activation target, we first searched the human EPSTI1 gene promoter sequence (EPSTI1p) and found 4 GT-boxes, the potential KLF8 acting sites. We then cloned the EPSTI1p into a luciferase reporter vector (Figure 2B). Co-transfection of the wild-type KLF8 caused more than 3 times increase in the promoter activity in both the HEK293 and NIH3T3 cells. In contrast, the EPSTI1p did not respond to the mutant KLF8 lacking transactivation function [36] (Figure 2C). These results indicated that EPSTI1 is direct transactivating target of KLF8.

To determine if any of the GT-boxes mediates KLF8 activation of the EPSTI1p, we mutated each of the GT-boxes individually and tested their response to KLF8. We found that mutation of the GT-box 1, but none of the other three, prevented the activation of the EPSTI1p by KLF8 (Figure 2D), suggesting that the GT-box 1 is required for KLF8 activation of the EPSTI1p.

To determine the possible interaction between KLF8 protein and the GT-box 1 site of the EPSTI1p, we performed biotinylated oligonucleotide precipitation (BOP) as well as chromatin immunoprecipitation (ChIP) assays. The results demonstrated that both ectopic KLF8 in (Figure 2F) and endogenous KLF8 (Figure 2G) can bind the EPSTI1p in vivo at the promoter region containing the GT-box 1 and the interaction was abolished when the GT-box 1 was disrupted (Figure 2E).

Taken together, these results strongly suggested that KLF8 directly activates the EPSTI1p to upregulate the expression of EPSTI1.

EPSTI1 promotes the cell invasiveness downstream of KLF8.

To determine a potential role of EPSTI1 in the cell invasiveness in the context of KLF8 expression, we first knocked down EPSTI1 from the MDA-MB-231 cells known to express aberrant high levels of KLF8 [26, 29, 30] and examined the cell invasiveness using the matrigel invasion chambers. We found that upon the EPSTI1 knockdown (Figure 3A), the cell invasiveness was drastically reduced as compared to the cells treated with a control siRNA or untreated cells (Figure 3B). To test if EPSTI1 plays this role downstream of KLF8, EPSTI1 was knocked down similarly from the 10A-iK8 cells and the cell response to the induction of KLF8 expression for invasion was examined. We found that the silence of EPSTI1 expression caused approximately 50% reduction in the

cell invasiveness regardless of the KLF8 expression (Figure 3C, compare columns 6 with 4 or 5). These results suggested that EPSTI1 plays a critical role downstream of KLF8 in promoting the cell invasion.

To test if ectopic overexpression of EPSTI1 alone is sufficient to promote the cell invasion, we generated the MCF-10A and MCF-7 cell lines that express inducible EPSTI1 (10A-iE1 and MCF7-iE1, respectively). The cell invasiveness was then compared between the induced and uninduced cells. The results revealed that after EPSTI1 expression was induced (Figure 3D and 3F) the cell invasiveness was significantly enhanced (Figure 3E and 3G, compare columns 4 with 2). These results indicated that EPSTI1 overexpression alone is adequate to promote cell invasion.

EPSTI1 promotes the lung metastasis of breast cancer.

We have recently demonstrated that KLF8 promotes breast cancer lung metastasis [27, 30]. To see if EPSTI1 plays a similar role, we injected the MCF7-iE1 cells (uninduced or induced) or the parental cells into the tail vein and followed up the lung metastasis by bioluminescent imaging (BLI) (Figure 4A – FC), stereomicroscopy (Figure 4A), as well as histological analyses (Figure 4D). We found that the lung metastases began to be detectable by BLI at week 6 or week 7 and became significantly more obvious at week 8 even by surgical microscopy (Figure 4A – 4C). Notably, the lung metastases were formed

by the MCF7-iE1 cells, at an incidence of 86%, only when EPSTI1 expression was induced (I), whereas the uninduced cells (U) or the parental cells (P) did not form any detectable lung metastasis (Figure 4A - 4C). The human origin of the metastases (indicated by GFP expression) and the effective induction of EPSTI1 expression in the metastases were verified by the H&E and IHC staining (Figure 4D). These results supported the notion that EPSTI1 serves as one of the driving forces for the lung metastasis of breast cancer.

EPSTI1 promotes tumor invasion in the breast.

To determine how EPSTI1 expression affects the tumor progression in the breast, we injected the MCF7-iE1 cells or the parental cells orthotopically into the mammary fat pad and examined the tumor growth and invasion (Figure 5). We found that the tumors became detectable between week 3 and week 4 and grew at the same rate and incidence across the groups regardless of EPSTI1 expression (Figure 5A – 5C). Interestingly, however, the histological analyses indicated that the tumors formed by the MCF7iE1 cells with the induction of EPSTI1 (I) have invaded the surrounding adipose tissue of the host mice, which did not occur to the tumors formed by the uninduced MCF7-iE1 cells (U) or the parental cells (P) (Figure 5D). These results suggested that EPSTI1 plays a role primarily in promoting the tumor invasion rather than proliferation.

EPSTI1 interaction with the N-terminal half of VCP is critical for the cell invasion.

Molecular mechanisms behind EPSTI1 have not been studied to date. To begin with, we performed CoIP coupled with mass spectrometry to identify potential EPSTI1 interacting proteins. One of the strongly interacting candidate proteins identified was VCP (Figure 6A and 6B), a primary function of which is to target proteins for degradation [91]. Truncation mutagenesis of VCP indicated that the amino terminal 187 residual stretch (VCP-N187) contains an EPSTI1 binding domain or motif (Figure 6C). To determine the relevance of this VCP region to the function of EPSTI1, the VCP-N187 peptide was co-expressed with EPSTI1 in the MCF7iE1 cells (Figure 6D). We found that the expression of this peptide inhibited the cell invasiveness induced by EPSTI1 (Figure 6E). This result suggested that the EPSTI1-VCP interaction is critical for the cell invasion and the VCP-N187 peptide reduced the cell invasiveness probably by competing with the endogenous VCP for EPSTI1 interaction.

EPSTI1 interaction with VCP is critical for the activation of NF- κ B.

To further understand the signaling mechanisms downstream of the EPSTI1-VCP interaction, we tested if the expression of VCP target proteins is altered by the overexpression of EPSTI1. One of the affected proteins was I κ B α whose protein levels were significantly decreased in both the 10AiE1 and MCF7-iE1 cells upon induction of

the EPSTI1 expression (Figure 7A). And this change was rescued by overexpression of the VCP-N187 peptide (Figure 7A, compare lanes 3 or 6 with 2 or 5) (see Figure S2A for quantitative data).

We then examined the effect of EPSTI1 expression on the subcellular expression of NF- κ B subunits p65 and p50. We found that EPSTI1 expression caused a dramatic increase in the levels of both subunits in the nucleus (Figure 7B, compare lanes 2 with 1) and decrease in the cytoplasm (Figure 7B, compare lanes 4 with 3) (see Figure S2B and S2C for quantitative data).

We then determined the transactivation activity of NF- κ B by co-expressing EPSTI1 with an NF- κ B-responsive promoter reporter, and found that EPSTI1 expression caused a 3 – 4 times increase in the reporter activity (Figure 7C).

Taken together, these results suggest that EPSTI1 plays a critical role in the degradation of I κ B α and subsequent activation of NF- κ B.

Discussion

This study identified EPSTI1 as a novel transcriptional target of KLF8 and a critical signaling effector downstream of KLF8 for the invasive growth and lung metastasis of human breast cancer (Figure 8). In this signaling model of cancer progression, the

aberrant high expression of KLF8 in the cancer cells ensures the high levels of EPSTI1 expression through activation of transcription of EPSTI1 by KLF8. EPSTI1 then recruits VCP by an unknown mechanism to I κ B α to subsequently induce the degradation of I κ B α , resulting in the nuclear translocation of NF- κ B to activate certain target genes associated with tumor invasion and metastasis.

We have recently demonstrated that the aberrant co-overexpression of KLF8 and EPSTI1 plays a critical part for the invasive growth and drug resistance of breast tumor [90]. Our cDNA microarray analysis (submitted to elsewhere) identified EPSTI1 as one of the most highly unregulated genes by KLF8 in the 10A-iK8 cells and this result was verified at both mRNA and protein levels by various means (Figure 1 and 2). In addition, we have correlated the cooverexpression of KLF8 and EPSTI1 with aggressive potential of the patient tumors (Figure 1 and Table S1). Furthermore, we demonstrate that the KLF8-binding consensus site at the EPSTI1p is highly conserved during evolution (Figure S1). Lastly, KLF8 and EPSTI1 share some common targeted cellular functions such as EMT. These lines of evidence, taken together with the earlier reports [49, 50, 89], strongly suggests that EPSTI1 is likely a true target of transcriptional activation by KLF8 and the KLF8 to EPSTI1 signaling pathway potentially associated with the tumor microenvironment is critical for breast cancer progression.

EPSTI1 was initially identified due to the induction of its expression in breast cancer cells by cocultured stromal fibroblasts [49], suggesting that an extracellular factor(s)

responsible for the induction of EPSTI1 may be released by the stromal fibroblasts. Our results suggest that this extracellular factor(s) could induce EPSTI1 via KLF8-EPSTI1. Interferon- α (IFN- α) has been associated with the upregulation of EPSTI1 in breast and ovarian cancer tumors [50, 92, 93].

TGF- β and Wnt play a role for the induction of KLF8 expression in cancers [25, 26, 28, 29, 40, 85]. These data indicate that these stromal factors may act upstream of KLF8-EPSTI1 pathway in breast cancer. Epithelial cells are converted to stromal fibroblast-like cells during EMT. The fact that both KLF8 and EPSTI1 can induce EMT [29, 50] raises a possibility that the KLF8EPSTI1 signaling induces EMT, causing release of the stromal factor(s) that in turn stimulates the expression of KLF8 and EPSTI1 via an autocrine loop. Experiments are in progress to test these interesting possibilities.

Our recent studies have demonstrated that KLF8 not only promotes invasive tumor growth and metastasis but also plays a role in the growth of tumor sizes of breast cancer [30] (submitted to elsewhere). In contrast, EPSTI1 appears to promote tumor invasion and metastasis only (Figure 5 and 6) and, unlike KLF8, does not appear to play a role in the regulation of cell proliferation (data not shown). These results suggest that EPSTI1 contributes primarily to the invasion/metastasis-promoting function of KLF8.

Our results show that VCP-mediated activation of NF- κ B plays an important role downstream of KLF8-EPSTI1 signaling (Figures 6-8). Consistently, the VCP-NF- κ B

signaling plays a similar role also in the metastasis of osteosarcoma [94]. VCP can potentially target many proteins for degradation [91]. Similarly, NF- κ B targets a wide array of genes associated with a broad range of cellular functions contributing to tumor progression [95]. How EPSTI1 interaction regulates VCP-dependent degradation of proteins and how this regulation affects the protein targeting profiles of VCP? In addition to VCP, what other potential EPSTI1 interacting proteins also contribute to the tumor invasion and metastasis and by what distinct mechanisms? What subgroup of NF- κ B target genes are involved downstream of EPSTI1 for the invasive and metastatic progression of breast cancer? Although beyond the scope of this report, it is obvious that these interesting questions need to be answered by future investigations.

In summary, this work provides a novel insight into the mechanisms of metastatic progression of breast cancer and opens a new avenue in the research of both KLF8 and EPSTI1. The KLF8 to EPSTI1 to VCP signaling identified here could generally apply to other types of cancer associated with aberrant overexpression of KLF8 and even non-cancerous diseases or disorders associated with expression of the EPSTI1-VCP complex. More investigations are worth of pursuing to understand the details in the signaling pathway as a potentially therapeutic target.

Materials and Methods

Reagents and cell culture.

Antibodies. Antibodies used for western blotting were HA-probe (F-7) mouse monoclonal Ab (sc-7392), HA-probe (Y-11), rabbit polyclonal Ab (sc-805), c-Myc (9E10) mouse monoclonal Ab (sc-40), c-Myc (C-19) rabbit polyclonal Ab (sc-788), β -actin (C4) mouse monoclonal Ab (sc-47778), I κ B α (FL) rabbit polyclonal Ab (sc-847), NF- κ B p65 (C-20) rabbit polyclonal Ab (sc-372), Sp1 (H-225) rabbit polyclonal Ab (sc-14027), and vinculin (7F9) mouse monoclonal Ab (sc-73614) (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), EPSTI1 rabbit polyclonal Ab (HPA017362) (Sigma-Aldrich Co., St. Louis, MO, USA), and HRP-conjugated donkey anti-mouse IgG (715-035-150) or anti-rabbit IgG (711-035-152) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Antibodies used for CoIP were Anti-HA mouse monoclonal (IP0010) and Anti-c-Myc rabbit polyclonal Ab (IP0020) Immunoprecipitation Kit (Sigma-Aldrich Co., St. Louis, MO, USA). Antibodies used for IHC or tissue array were EPSTI1 rabbit polyclonal Ab (HPA017362) (Sigma-Aldrich Co., St. Louis, MO, USA); GFP mouse monoclonal Ab (sc-101525) and HA-probe (F-7) (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) and Peroxidase substrate kit (DAB) (SK-4100) (Vector laboratories Inc., Burlingame, CA, USA). Antibodies used for BOP and ChIP assays were HA probe (F-7) and KLF8 rabbit polyclonal Ab (8477), respectively as previously described [25, 29, 30].

Plasmids. The mammalian expression vectors pKH3 (HA-tagged) and pHAN (Myc-tagged), pKH3-KLF8 and pKH3-mKLF8 were previously described [36]. SFG-nTGL encoding the tkGFP-Luciferase fusion reporter protein was a kind gift from Dr. V. Ponomarev [96]. The TetO-FUW-OSKM [97] (# 20321), FUW-M2rtTA [98] (#20342), VCP (wt)-EGFP [99] (# 23971), pLVTHM [100] (#12247) were purchased from Addgene (Cambridge, MA, USA). The ORFEXPRESSTM-shuttle hEPSTI1V2 vector encoding the human EPSTI1 cDNA (GC-T1743) was purchased from GeneCopoeia Inc. (Rockville, MD, US). The CH17-13N15 BAC clone containing the human EPSTI1 gene promoter (EPSTI1p) was purchased from Children's Hospital Oakland Research Institute (CHORI) (Oakland, CA, USA). The NF- κ B (2) luciferase reporter vector (LR0052) and the control vector pTL-Luc (LR0000) were purchased from Affymetrix Panomics (Santa Clara, CA). The pKH3-EPSTI1 and pHAN-EPSTI1 plasmids were generated by PCR using the ORFEXPRESSTM-shuttle hEPSTI1V2 as template and the hEPSTI1-RV-F and hEPSTI1V2-RI-R primer set, cutting the PCR product by EcoRI and EcoRV and ligating it into the pKH3 or pHAN vector between the SmaI and EcoRI sites. TetO-FUW vector was recovered from TetO-FUW-OSKM by EcoRI digestion and self-ligation. TetO-FUW-RFPEPSTI1 was made by multistep PCR linking mCherry, the T2A and HA-EPSTI1 cDNAs together and ligating it into TetO-FUW. The multistep PCR was done using a mCherry encoding plasmid as template and the Kozak-RFP-F/T2A-RFP-R and Kozak-RFP-F/T2A-R primer sets, the pKH3-EPSTI1 as the template and the T2A-HA-

F/pKH3-R and T2A-F/pKH3-R primer sets, and the two PCR product mix as template and the Kozak-RFP-F/pKH3-R primer set. The end PCR product was cut with EcoRI and inserted into the EcoRI site of TetO-FUW. The pGL3bhEPSTI1p reporter vector was made by PCR using the CH17-13N15 BAC clone as the template and hEPSTI1p-F/hEPSTI1p-R primer set, cutting the PCR product with HindIII and inserting it into the HindIII site of PGL3b vector. GT-box specific mutants of the promoter were constructed by site-directed mutagenesis PCR [101] using the pGL3b-hEPSTI1p vector as the template and each of the mutant GT-box (mGT) primer sets along with the hEPSTI1p-F/hEPSTI1p-R primer set. The pHAN-VCP, pHAN-VCP-N187 and pHAN-VCP-dN187 plasmids were constructed by PCR amplifying the human VCP cDNA from the VCP (wt)-EGFP vector using the primer set of hVCP-BHI-F/hVCP-BHI-R, hVCP-BHI-F/hVCP-187-BHI-R, and hVCP-188-BHI-F/hVCPBHI-R, respectively and cloning into the BamHI site of pHAN. To construct pLVTHM-nGL vector, the tk fragment was removed from the SFG-nTGL by deletion PCR using the dT-F/dT-R. The resulting SFG-nGL was then used as template for PCR using the nGL-F/nGL-R primer set. The PCR product was cut with SpeI and inserted between the PmeI (blunted) and SpeI sites of pLVTHM. Correctness of the constructs was confirmed by insert orientation check with restriction digestion, DNA sequencing, and/or protein expression analysis. See primer sequences in Table S2.

Cell lines. The HEK293 and NIH3T3 [101-103], MCF-10A, MCF-7, MDA-MB-231, Hs578T and BT-549 [26, 29], and the KLF8-expressing Tet-on MCF-10A (10A-iK8) and the KLF8 shRNA expressing Tet-on MDA-MB-231 (231-K8ikd) [30, 32] cell lines were described previously. These cells were maintained in DMEM/F-12 or DMEM with 10% fetal bovine serum or calf serum. MCF-10A and MCF-7 cell lines expressing the nGL (MCF-10A-nGL and MCF-7-nGL) were generated by infecting the cells with the pLVTHM-nGL lentivirus followed by selection of GFP expressing cells by fluorescent microscopy. To generate the Tet-on MCF-10A and MCF-7 lines that express inducible EPSTI1 (10A-iE1 and MCF7-iE1), the TetO-FUW-RFP-EPSTI1 and FUW-M2rtTA lentiviruses were generated as previously described [30] and used to co-infect the MCF-10A-nGL or MCF-7-nGL cells. Infected cells were seeded into 96-well plate at a density of 1 cell per well. Positive clones were selected by transient induction of RFP expression coupled with fluorescent microscopy. These Tet-on inducible cell lines were maintained under uninduced (U, in the absence of doxycycline) or induced (I, in the presence of doxycycline) conditions depending on the experimental requirement as previously described [25, 29, 30, 32, 101, 102]. Doxycycline hydrochloride was purchased from Sigma-Aldrich Corp. (D3072, St. Louis, MO, USA).

Quantitative real-time PCR (qRT-PCR), western blotting and co-immunoprecipitation (CoIP).

These assays were done as previously described [30]. Cells and antibodies used were described above. Sequences of the PCR primers are listed in Table S2. Sub-cellular fractionation was described previously [39]. Specifically, after two washes with 1× PBS and equilibration for 5 min with 0.5 ml of 0.5 mM EDTA in PBS, cells were scraped off and centrifuged ($4,500 \times g$, 2 min, 4°C). The cell pellets were re-suspended in 0.4 ml of low-salt HEPES buffer (10 mM HEPES [pH 7.8], 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) for 15 min, lysed in 20 μ l of 10% NP-40, and centrifuged ($10,000 \times g$, 30 s, 4°C) to obtain the cytosolic supernatant. The pellet will be re-suspended in 50 μ l of high salt buffer (840 mM NaCl, 40mM HEPES [pH 7.8], 2 mM EDTA, 2M EGTA, and 40% glycerol) for 30 min and centrifuged ($10,000 \times g$, 5 min, 4°C) to obtain the nuclear extract.

Affinity precipitation coupled with mass spectrometry, RNA interference, and Matrigel invasion assays.

These assays were performed as previously described [29, 30, 88]. The pKH3- and pHAN-EPSTI1 plasmids and antibody beads were described above. The human EPSTI1 specific ON-TARGET plus SMART pool siRNAs (L-015094-01) and control siRNAs

(D001810-0X) were purchased from Thermo Scientific, Dharmacon (West Palm Beach, FL, USA). The siRNAs were delivered into the cells by Oligofectamine-mediated transfection according to manufacturer's instructions (Invitrogen, Grand Island, NY, USA). BD BioCoat™ Matrigel™ Invasion Chambers (354480, BD Biosciences, San Jose, CA, USA), crystal violet (C3886, Sigma-Aldrich Co., St. Louis, MO, USA) and a Zeiss AXIO Observer A1 microscope (Carl Zeiss Inc., Thornwood, NY, USA) were used for the invasion assays.

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

These assays were performed as previously described [29, 103]. The plasmids encoding the wild-type or mutant human hEPSTI1p, the NF- κ B(2) luciferase reporter and the control vector, HEK293, NIH3T3, 10A-iK8 and 231-K8ikd cell lines were described above. Sequences of the ChIP primers and BOP oligos for the human EPSTI1p are described in Table S2.

Bioluminescence imaging (BLI) analysis of tumor growth and metastasis.

Female ovariectomized athymic nude mice (4-5 weeks old, 7 mice per group, Hsd:Athymic NudeFoxn1nu, Harlan Laboratories, Indianapolis, IN, USA) were used for

all the xenograft studies. The mice were housed and maintained in specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institute of Health. IACUC protocols were approved by the Institutional Animal Care and Use Committee. Human care of the mice was thoroughly considered. A 17β -ESTRADIOL pellet (NE-121, Innovative Research of America, Sarasota, FL, USA) was s.c. implanted. One day later, 10^6 of the MCF7-iE1 or MCF7-nGL cells were washed and harvested in 0.05 – 0.1 ml PBS and injected into either the mammary fat pad injection or lateral tail vein. The mice were fed with diet (Bioserve, Frenchtown, NJ, USA) supplemented with doxycycline or Dox Diet (S3888) to induce the EPSTI1 expression in the cells in vivo or with the Control Diet not containing doxycycline (S4207). After injection, tumor growth or lung metastasis was monitored daily or weekly visually and/or by BLI. For BLI, mice were anaesthetized and injected i.p. with 150 mg/kg of Dluciferin (15 mg/ml in PBS) (LUCK-1, Gold Biotechnology, Inc., St. Louis, MO, USA). BLI was then completed between 2 and 5 min using a Kodak In Vivo Imaging System coupled to Molecular Imaging Software (Carestream, Rochester, NY, USA) followed by X-ray imaging. Photon flux (photons/s/cm² per steradian) was measured with a region of interest drawn around the bioluminescence signal

encompassing the thorax or the mammary fat pad. A background value was obtained from a D-luciferin-injected control mouse and subtracted.

Hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining.

The mammary tissues and the lungs were surgically collected, fixed in formaldehyde solution (F8775, Sigma Aldrich Co., St. Louis, MO, USA), embedded in EM-400 Embedding Medium Paraffin (3801320, Leica Microsystems Inc., Buffalo Grove, IL, USA), and sectioned with the Manual Rotary Microtome for Routine Sectioning (Leica RM2235, Leica Microsystems Inc., Buffalo Grove, IL, USA). H&E and IHC staining was performed as previously described [27]. Human breast cancer tissue array with invasive data (BR1503, US Biomax, Rockville, MD, USA) was analyzed as previously described [27, 30]. Large Volume Myer's Hematoxylin (AMH100420), Eosin Y (17372-87-10) and Xylene (X3P-1GAL) were purchased from Thermo Fisher Scientific (West Palm Beach, FL, USA). The 3, 3-diaminobenzidine tetrahydrochloride (868272-85-9) was purchased from Sigma-Aldrich Co., (St. Louis, MO, USA). GFP and EPSTI1 antibodies were described above. The number of tumor nodules on the surface of the whole-mounted lungs or GFP-positive nodules across three independent whole lung sections were counted for each mouse by microscopy. Microscopy was done using Leica DFC295

microscope for sectioned tissues or surgical microscope (LeicaL2) for whole mount tumors or lungs (Leica Microsystems Inc., Buffalo Grove, IL, USA).

Statistical Analysis.

A minimum of three observations per group was conducted. Data is presented as mean +/- the standard deviation (SD). Unpaired, paired or single sample Student's t test with the Bonferroni correction for the multiple comparisons was applied as appropriate. The tissue array data was analyzed by χ^2 -test. Significance was determined by the alpha level of 0.05.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

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Figures and Table

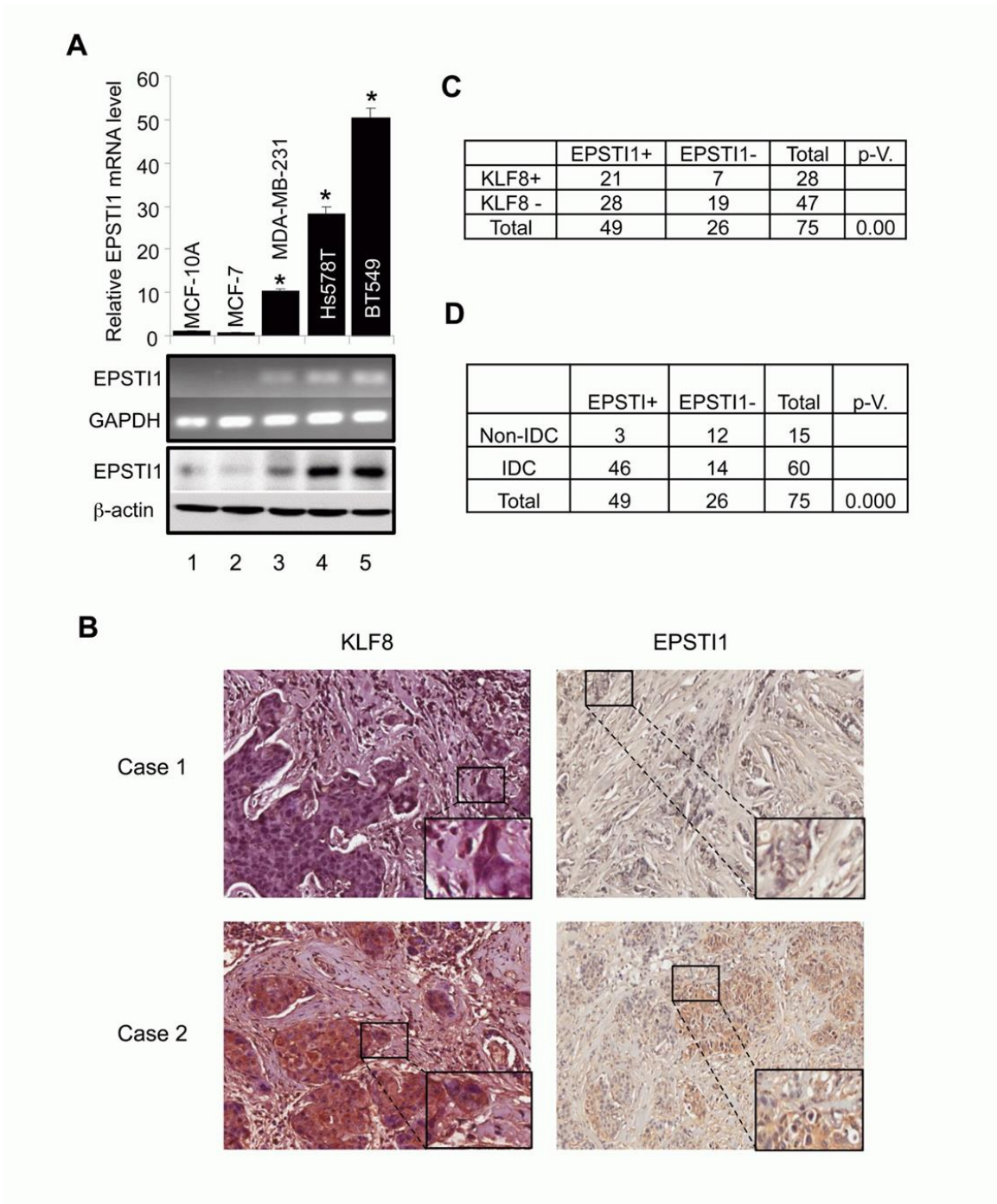


Figure 1. Correlated upregulation of KLF8 and EPSTI1 expression in human metastatic breast cancer cell lines and patient tumors.

Figure 1. Correlated upregulation of KLF8 and EPSTI1 expression in human metastatic breast cancer cell lines and patient tumors.

(a) Aberrant high expression of EPSTI1 in invasive breast cancer cells is well correlated with KLF8 expression. Total RNA and protein lysate were prepared from sub-confluent cells for qRT-PCR (Top panel, *P < 0.01 compared to column 1), semi-quantitative RT-PCR (middle panel) or western blotting (bottom panel) analysis of EPSTI1 expression.

(b-e) Positive correlation of protein expression between EPSTI1 and KLF8 in metastatic breast cancer patient tumors. IHC staining of KLF8 or EPSTI1 (brown) in the human breast cancer tissue array containing specimens in duplicate from 75 patient tumors or normal tissues was performed. Images representing a sample negative for both KLF8 and EPSTI1 (case 1) or positive for both KLF8 and EPSTI1 (case 2) were shown in (b). Correlation of EPSTI1 and KLF8 expression was shown in (c). Positive correlation of EPSTI1 expression with the invasive potential was outlined in (d). Statistical significance was determined by χ^2 -test. (See Supplemental Table 1 for details.)

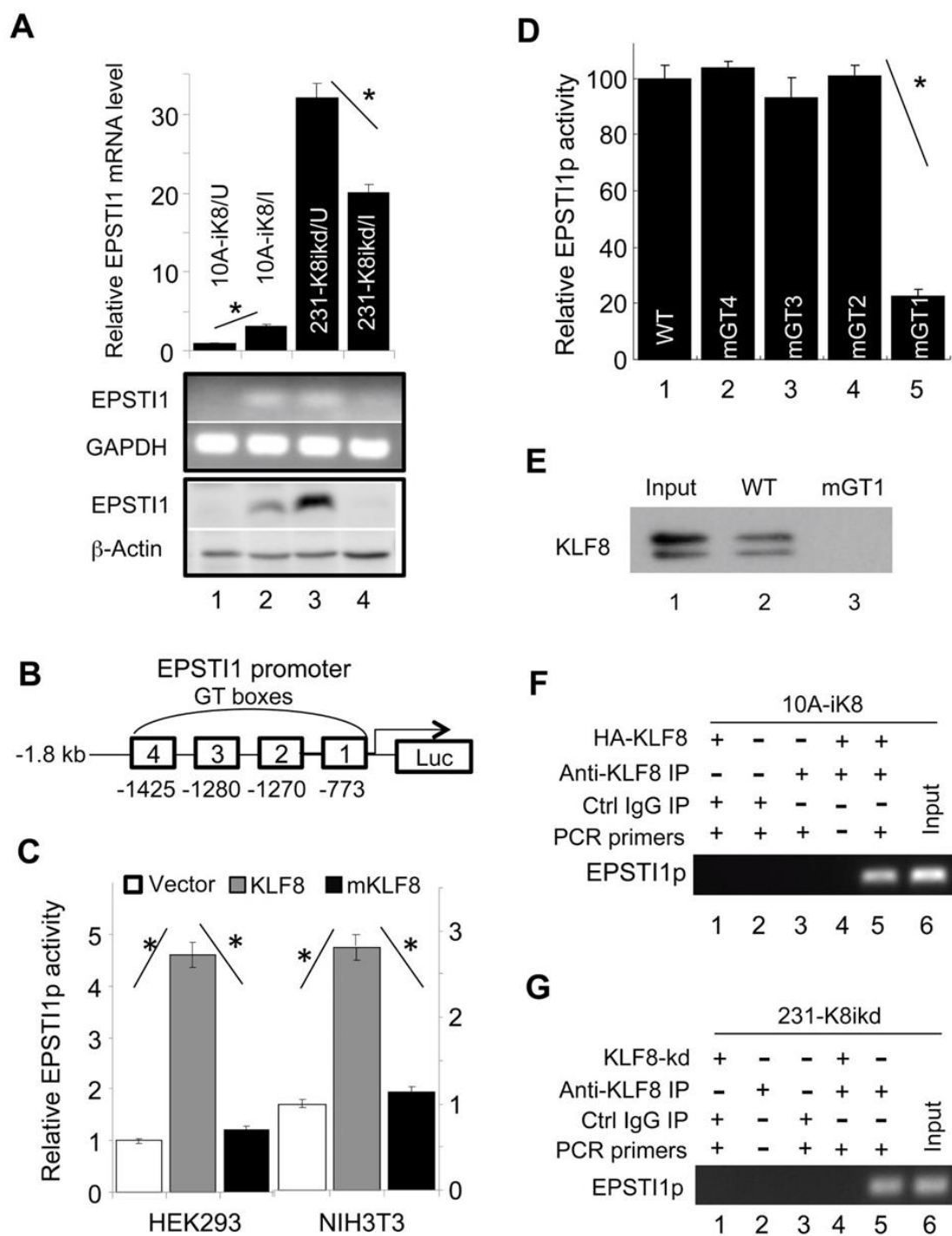


Figure 2. KLF8 upregulates EPSTI1 expression at the transcriptional level.

Figure 2. KLF8 upregulates EPSTI1 expression at the transcriptional level.

(a) KLF8 expression is both sufficient and necessary for EPSTI1 expression. The 10A-ik8 and 231-k8ikd cells were grown under induced (I) or uninduced (U) conditions for 48 h. Analyses of EPSTI1 expression were performed similarly as described in Figure 1A. (b and c) KLF8 activates the EPSTI1 gene promoter (EPSTI1p). The human EPSTI1p was cloned into a luciferase reporter plasmid (b) and co-transfected into the indicated cells with wild-type KLF8, its transactivation-defective mutant (mKLF8) [36] or the control vector plasmid for 16 h. The reporter activity was determined as described in the Material and Methods (c, *P < 0.01). (d) The GT-box 1 (GT1) is required for the activation of EPSTI1p by KLF8. The GT-boxes were individually mutated (CACCC to TCTCA). The wild-type (WT) or mutant (mGT) reporters were used for similar reporter assay. *P < 0.01. (e) KLF8 directly binds EPSTI1p at the GT-box 1 site. HEK293 cell lysate containing HA-KLF8 and oligos spanning the wild-type GT1 (WT) or its mutant (mGT1) were used for BOP assay as described in the Material and Methods. (f and g) KLF8 binds EPSTI1p in vivo. The 10A-iK8 or 231-K8ikd cells were cultured under the uninduced (U) or induced (I) conditions for 72 h. ChIP assay was done as described in the Material and Methods.

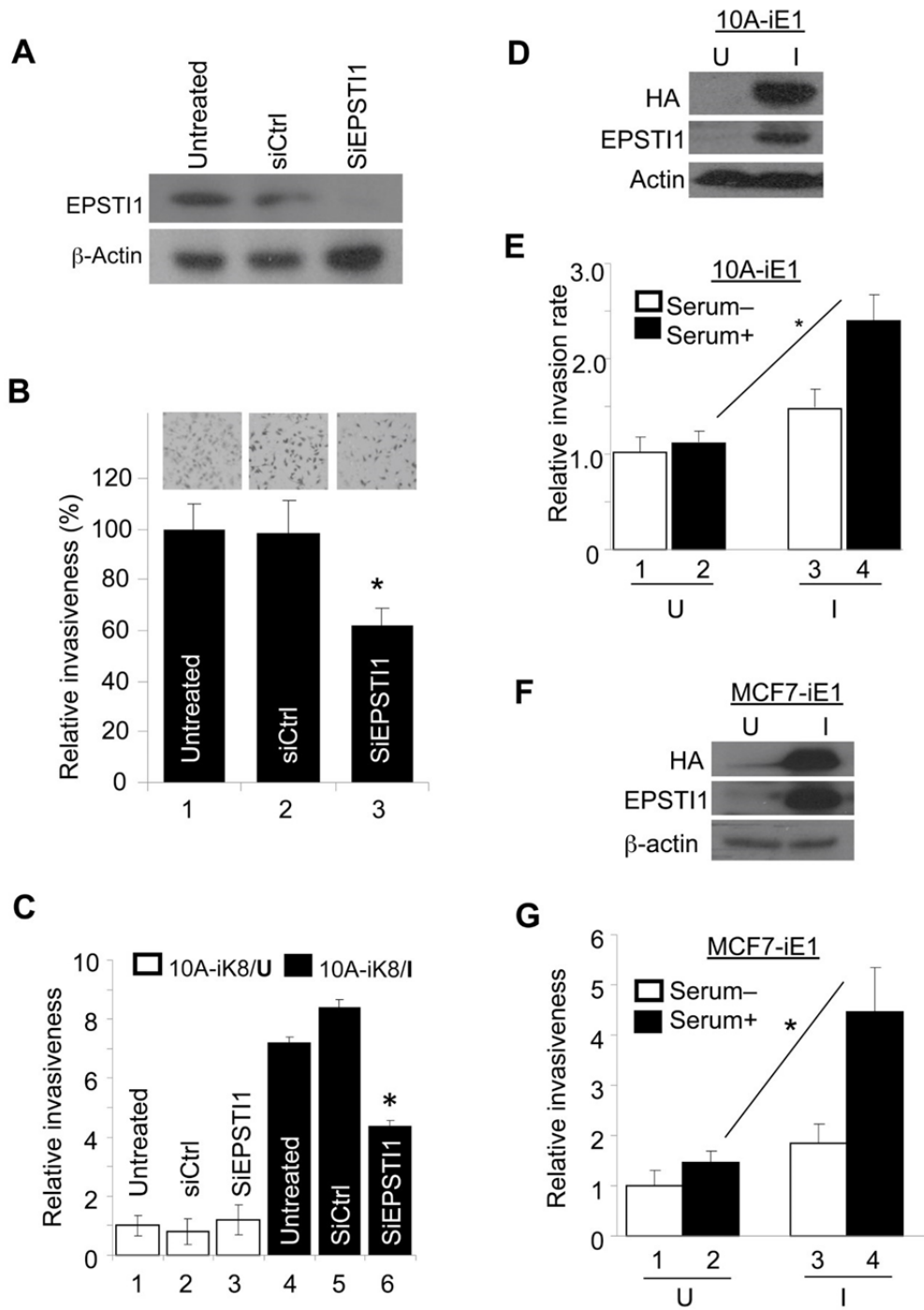


Figure 3. EPSTI1 promotes the cell invasiveness downstream of KLF8.

Figure 3. EPSTI1 promotes the cell invasiveness downstream of KLF8.

(a and b) EPSTI1 knockdown decreases the invasiveness of MDA-MB-231 cells. The cells were transfected with siRNA for 72 h. A fraction of the cells was prepared for western blotting (a). Another fraction of the cells was used for Matrigel invasion for 16 h (b) as described in the Material and Methods. Data was normalized to the untreated cells. *P < 0.05. (c) EPSTI1 knockdown inhibits KLF8promoted invasiveness. The 10A-iK8 cells were cultured under uninduced (U) or induced (I) conditions for 48 h and then treated with or without the indicated siRNA for 72 h. Matrigel invasion was done similarly. Data was normalized to the uninduced, untreated cells. (d-g) EPSTI1 overexpression is sufficient to promote invasiveness. The 10A-iE1 and MCF7-iE1 cells were generated as described in the Material and Methods. After grown under U or I conditions for 24 h, the cells were prepared for western blotting (d, f) and invasion assay (e, g). The invasion data were normalized to the U and serum-free conditions. *P < 0.05. All the data are representatives of at least three independent experiments in duplicate.

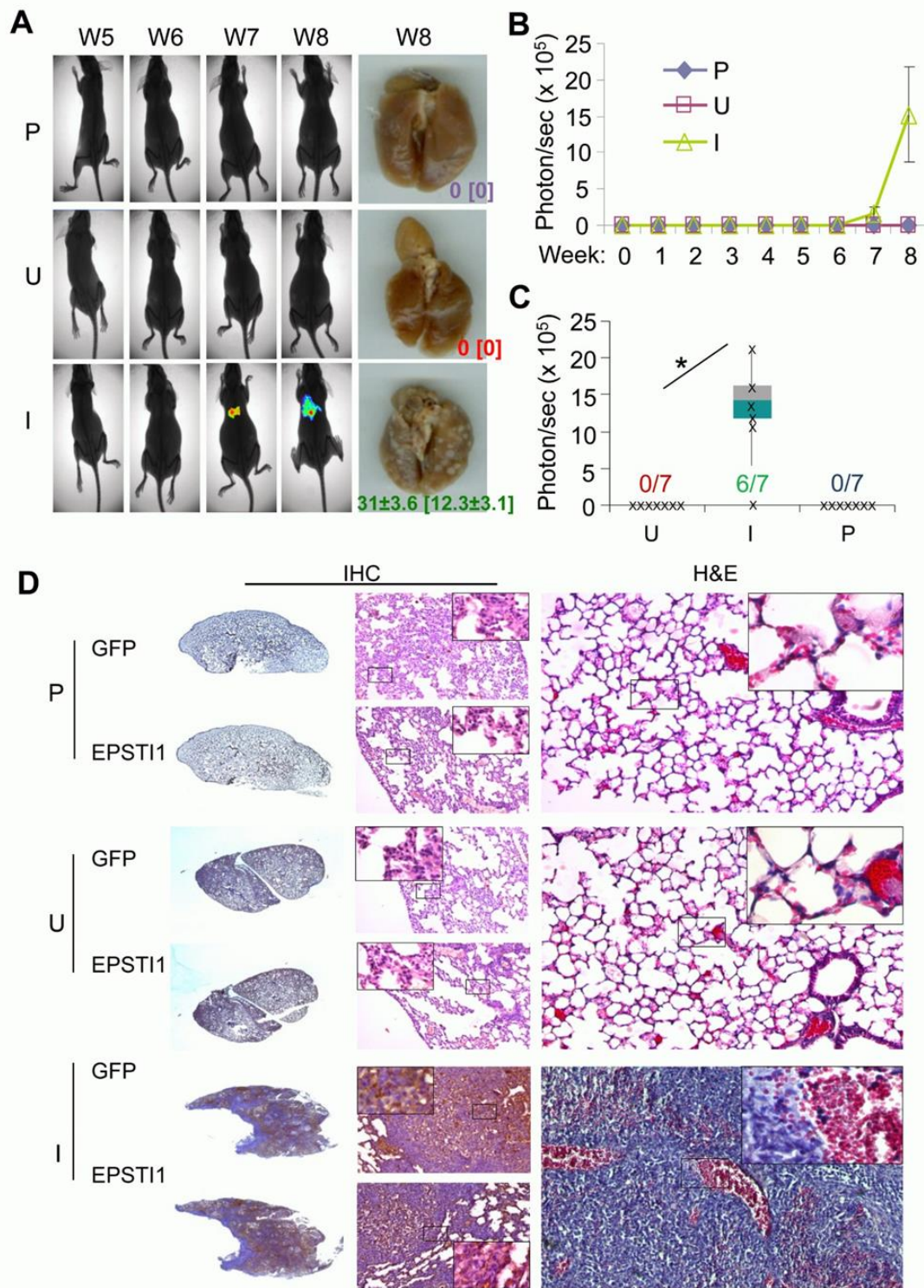


Figure 4. EPSTI1 promotes the lung metastasis of breast cancer.

Figure 4. EPSTI1 promotes the lung metastasis of breast cancer.

The MCF7-iE1 cells or the parental cells (P) were injected into the tail vein. The mice injected with MCF7-iEPSTI1 cells were fed with the Dox Diet (I, EPSTI1 induction) or Control Diet (U, no EPSTI1 induction). The mice injected with the parental cells were fed with normal food. The lung metastasis was followed up for 8 weeks by BLI or stereomicroscopy at week 8 as described in the Materials and Methods. (a) Representative images Representative images with average number and standard deviation of metastatic tumor nodules per mouse visible on the surface of the lungs or stained positive for GFP in the tissue section shown in the brackets. (b) Quantitative results of the volumes of the metastasis. (c) Metastatic incidence at week 8. * $P < 0.05$. (d) H&E and IHC staining with GFP or EPSTI1 antibody confirming the human origin of the lung metastases and the effective induction of EPSTI1 expression in the tumors.

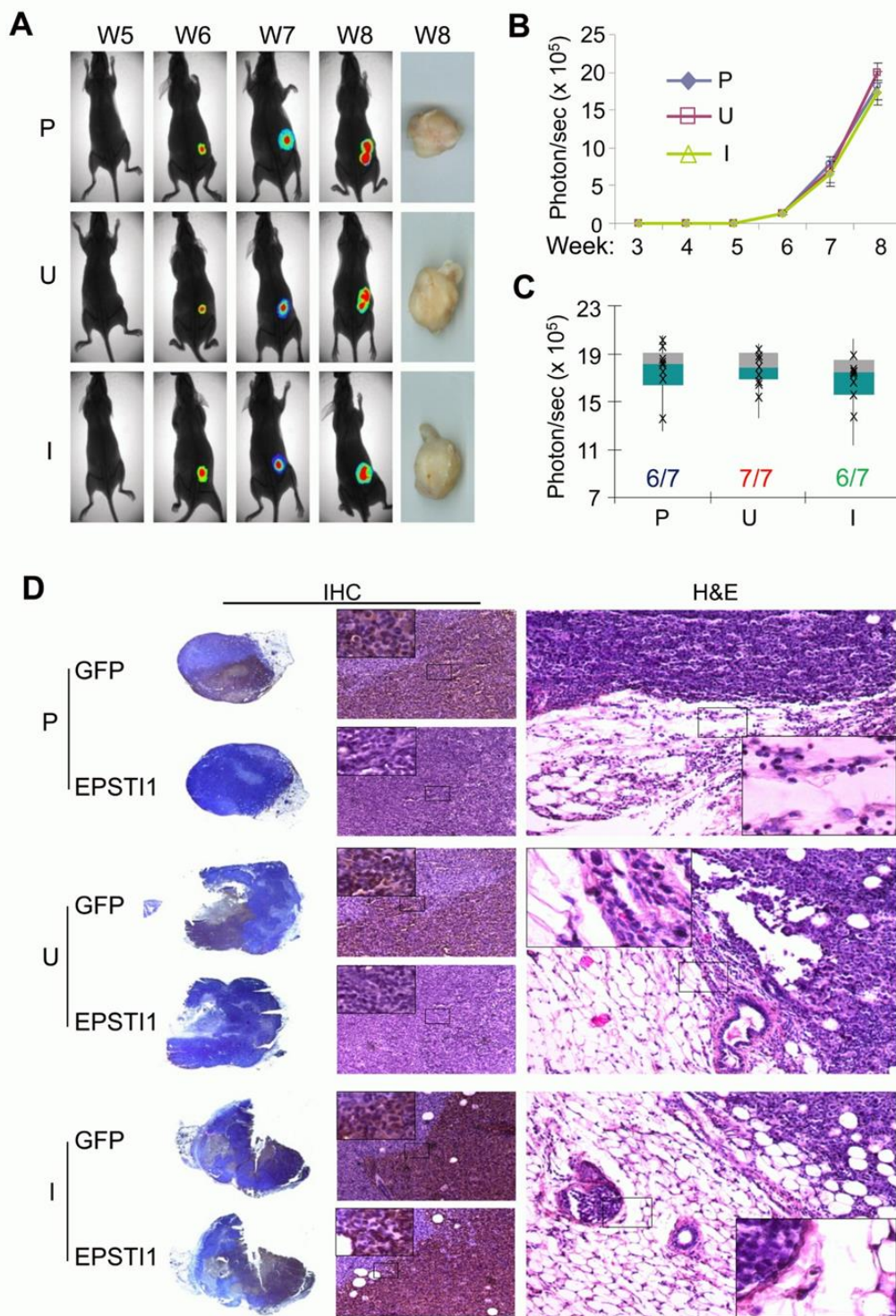


Figure 5. EPSTI1 promotes tumor invasion at the orthotopic site.

Figure 5. EPSTI1 promotes tumor invasion at the orthotopic site.

The MCF7-iE1 or parental (P) cells were injected into the mammary fat pad. The mice were fed as described in Figure 4. BLI analysis and stereomicroscopy of the tumors (a-c) and H&E or IHC analyses (d) were performed similarly as described in Figure 4.

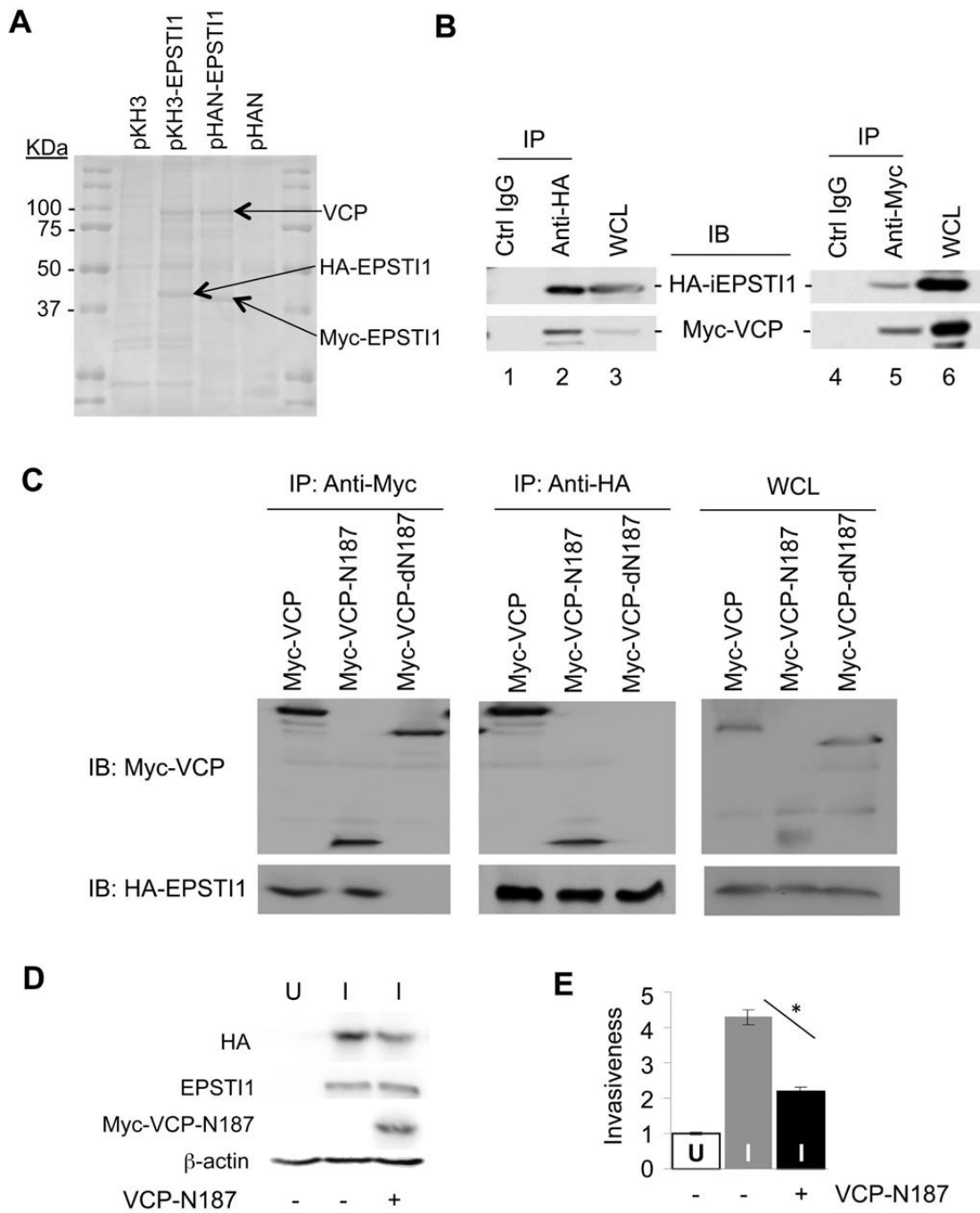


Figure 6. EPSTI1 interaction with the 1-187 aa region of VCP (VCP-N187) is critical for cell invasion.

Figure 6. EPSTI1 interaction with the 1-187 aa region of VCP (VCP-N187) is critical for cell invasion.

(a) Mass spectrometry identified VCP as an EPSTI1 interaction protein. Plasmid encoding HA- or Myc-tagged EPSTI1 (pKH3-EPSTI1 or pHAN-EPSTI1) or the control vector was transfected into HEK293ft cells for 24 h, followed by anti-HA or anti-Myc CoIP and subsequent mass spectrometry as described in the Materials and Methods. (b) CoIP verification of interaction between EPSTI1 and VCP. The HEK293ft cells were cotransfected with pKH3EPSTI1 and pHAN-VCP for 24 h and prepared for CoIP and western blotting. WCL, whole cell lysate. (c) VCP-N187 is where EPSTI1 binds. The full-length VCP, its N-terminal 187 residual fragment (N187) or its C-terminal half (dN187) was co-transfected and Co-IP were followed similarly as described in b. (d and e) VCP-N187 is crucial for EPSTI1 to promote the cell invasion. The MCF7-iE1 cells were grown under U or I conditions for 24 h. The I-cells were then transfected with the VCP-N 187 peptide or the control vector for another 24 h prior to western blotting (d) or Matrigel invasion assay (e, *P < 0.05).

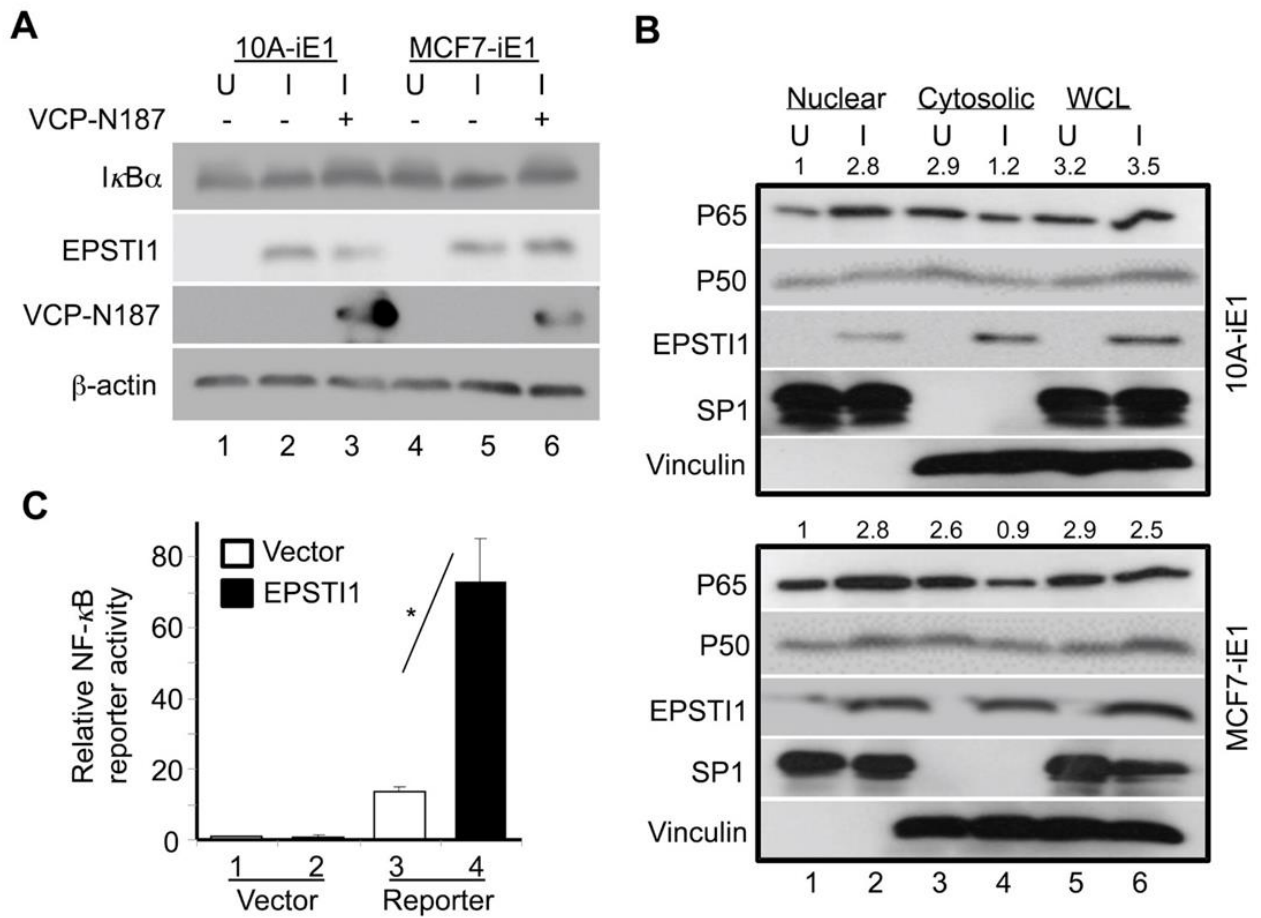


Figure 7. EPSTI1 interaction with VCP is critical for the activation of NF- κ B.

Figure 7. EPSTI1 interaction with VCP is critical for the activation of NF- κ B.

(a) EPSTI1 expression decreases the expression of I κ B α depending upon its binding to VCP. The 10A-iE1 or MCF7-iE1 cells were cultured under U or I conditions for 24 h. The I-cells were then transfected with the VCP-N187 peptide (lanes 3 or 6) or the control vector (lanes 2 or 5) for another 24 h prior western blotting. (See Figure S2A for quantitative data). (b) EPSTI1 expression promotes the nuclear translocation of NF- κ B. The 10A-iE1 or MCF7-iE1 cells were cultured under U or I conditions for 24 h. Then the nuclear and cytoplasmic fractions were isolated for western blotting. SP1 and vinculin were used as a marker for nucleus and cytoplasm, respectively. (See Figure S2B and S2C for quantitative data). (c) EPSTI1 expression increases the transcriptional activity of NF- κ B. MCF7 cells were co-transfected with the NF- κ B reporter or control vector with EPSTI1 or control vector for 24 h before luciferase activity was determined. Data are representatives of at least three independent experiments in duplicate. *P < 0.05.

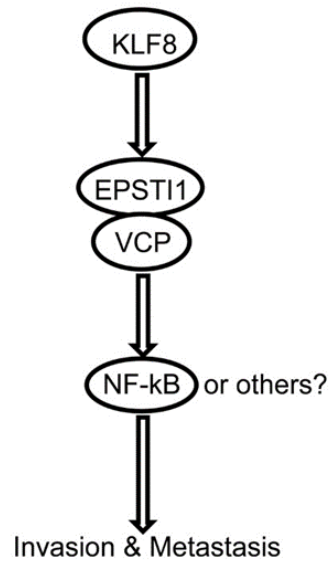


Figure 8. A Model of mechanism of action.

KLF8 upregulates EPSTI1 at the transcriptional level. EPSTI1 interacts with VCP to induce the degradation of $\text{I}\kappa\text{B}\alpha$, causing the nuclear translocation of NF- κB to activate certain target genes associated with tumor invasion and metastasis.

Supplemental Table 1. Positive correlation of co-expression of KLF8 and EPSTI1 with the invasiveness and aggressiveness of the patient tumors.

| Tissue | | Number of case | EPSTI1+ | KLF8+ | EPSTI1+ & KLF8+ |
|----------------------|---------------------------|----------------|---------|-------|-----------------|
| Normal Breast Tissue | | 3 | 0 | 0 | 0 |
| Benign tumor | Fibroadenoma | 3 | 0 | 0 | 0 |
| Malignant tumor | Cyctoscarcoma phtllodes | 2 | 1 | 0 | 0 |
| | Intraductal carcinoma | 7 | 2 | 0 | 0 |
| | Invasive ductal carcinoma | 60 | 46 | 28 | 21 |
| Total | | 75 | 49 | 28 | 21 |

Supplemental Table 2. Sequences of the primers and oligonucleotides used (5' to 3').

Cloning primers:

hEPSTI1-RV-F: AGATATCGAACACCCGC

hEPSTI1V2-RI-R :ACGGAATTCGGCCAGATAGG

Kozak-RFP-F: GCCATGGTGAGCAAGGGCGAGG

T2A-RFP-R: AGAAGACTACCTCTACCTTCCTTGTACAGCTCG

T2A-R: TCCTCCACGTCACCGCAAGTAAGAAGACTACCTCTACCTTC

T2A-HA-F: TGGAGGAAAACCCGGGGCCGACTTCTAGAATGTAC T2A-F:

TCTTCTTACTTGCGGTGACGTGGAGGAAAACCCGGGGCCG

pKH3-R primer: gga caa acc aca act aga atg cag

hEPSTI1p-F: ATGCGAAGCTTCCCCACTTTCTG

hEPSTI1p-R: ATGCGAAGCTTGCAGAGGCAG

hEPSTI1p-mGT1-F: TGTTTTAACGTCTCATCCTCCAAG

hEPSTI1p-mGT1-R: ACTTGGAGGATGAGACGTTAAAAC

hEPSTI1p-mGT2-F: TCTCCTGTCCTCTCATGGCCCACCC

hEPSTI1p-mGT2-R: AGGGTGGGCCATGAGAGGACAGGA

hEPSTI1p-mGT3-F: ACCCTCGCCTCTCATTCAATTTTCATTC

hEPSTI1p-mGT3-R: ATGAAATTGAATGAGAGGCCAGGGTG

hEPSTI1p-mGT4-F: ATGCCAGAGCTAAACACTGAACCTAC)

hEPSTI1p-mGT4-R: TGTAGGTTTCAGTGTTTAGCTCTGGCATTC

VCP-BHI-F: AAAAGGATCCAAGCTTGCGCG

VCP-BHI-R: AAATCGCCCTTGCTCACCATGG

VCP-BHI-187-R: ATATATGGATCCCTCCCCTTCG

VCP-BHI-188-F: ATAATGGGATCCCCTATCAAACGAGAGGATG

nGL-F: TGGCCACAACCATGGCTTC

nGL-R: AGACTAGTGCTTACACGGCGATC

dT-F: ACGCGTCTATGGTGAGCAAGGGCG

dT-R: TCACCATAGACGCGTGGGCCTC

qRT-PCR primers:

hEPSTI1-F, agcaggagctggccaacctgga, -R, ttgtgggccacaaacagcact.

GAPDH-F, tcgtacgtggaaggactca, -R, ccagtagaggcagggatgat.

ChIP primers for hEPSTI1 promoter:

F, tcctagaatgcaggtagc, R, tctccaggcagccttgc.

BOP oligos for hEPSTI1p GT-box 1:

WT-sense:

TGGGAGCTTTTCAAAAATATGAATGCCAGAGCTCACCCCTGAACCTACAGG

AACTGAGCCTTACACATC CATATTTTAAAATGTCC

WT-antisense:

GGACATTTTAAAATATGGATGTGTAAGGCTCAGTTCCTGTAGGTTTCAGGGG
TGAGCTCTGGCATTTCATATT TTTGAAAAGCTCCCA

mGT1-sense:

TGGGAGCTTTTCAAAAATATGAATGCCAGAGCTAAACACTGAACCTACAGG
AACTGAGCCTTACACATC CATATTTTAAAATGTCC

mGT1-antisense:

GGACATTTTAAAATATGGATGTGTAAGGCTCAGTTCCTGTAGGTTTCAGTGTT
TAGCTCTGGCATTTCATATT TTTGAAAAGCTCCCA

CHAPTER 3: IDENTIFICATION OF EPIDERMAL GROWTH FACTOR RECEPTOR AS A NOVEL KRÜPPEL-LIKE FACTOR 8 TARGET IN PROMOTING BREAST CANCER MALIGNANCY

Abstract

Krüppel-like factor 8 (KLF8) is a dual transcriptional factor critical for tumor progression in breast cancer. Epidermal growth factor receptor (EGFR), a family member of the epidermal growth factor receptor, is overexpressed in most types of breast cancer, but more frequently in triple-negative breast cancer (TNBC). Its overexpression is associated with larger tumor size, metastasis and poor clinical outcomes. Here we found out a high level of co-expression between KLF8 and EGFR in invasive breast cancer cells and patient tumor samples. Ectopic overexpression of KLF8 in the noninvasive, MCF-10A cells induced the expression of EGFR, whereas KLF8 knockdown from the invasive, MDA-MB-231 cells decreased EGFR expression. Promoter activation and binding assays indicated that KLF8 promoted EGFR expression by directly binding on the gene promoter of EGFR. Interestingly, we also revealed that miR141, a microRNA that could be repressed by KLF8, inhibited EGFR expression at its protein level. Using EGFR tyrosine kinase inhibitor AG1478, we found that KLF8 promoted invasion and proliferation partially through EGFR. We also revealed that overexpression of miR141 could inhibit KLF8's role in both invasion and proliferation. KLF8 performed its function through a KLF8-EGFR-Erk1/2 signal

pathway and this was proved by western blotting, a WST-1 assay and an invasion assay. Experiments using nude mice demonstrated that a knockdown of KLF8 inhibited both tumor growth and lung metastasis, whereas overexpression of miR141 performed similar functions. Taken together, these findings demonstrate a novel KLF8 to EGFR signaling pathway potentially crucial for breast cancer progression.

Introduction

Breast cancer is the leading cause of cancer death among women worldwide and both tumor growth and metastasis remain the major cause of death of a patient. Thorough understanding of the underlying mechanisms has been a main focus of breast cancer research for development of a more effective therapy to improve patient survival.

Krüppel-like factor 8 (KLF8) is a dual transcription factor [25, 27, 29, 30, 32, 35-37, 43, 85, 86] that is overexpressed in various human cancer including breast cancer [25, 29, 30, 32]. It was found to play a crucial role in breast cancer progression by promoting cell cycle progression [26, 34, 39], transformation [26, 27], epithelial-mesenchymal transition (EMT) [28, 29, 85, 87] and DNA damage repair [32]. Even though lots of progress has been made to demonstrate its transcriptional and post-translational levels as well as its nuclear localization [32, 36, 37, 85, 86, 88], the molecular and signaling mechanisms of how KLF8 promotes human breast cancer progression is still largely unknown.

Epidermal growth factor receptor (EGFR) is a family member of epidermal growth factor receptors whose members are among the most crucial cancer molecular targets. Epidermal growth factor receptor family includes EGFR (also known as HER1 and ErbB1), HER2 (also known as HER2/neu and ErbB2), ErbB3 (also known as HER3), and ErbB4 (also known as HER4) [77]. Among the family members, EGFR overexpression in breast cancer is associated with large tumor size, EMT, metastasis and poor clinical outcomes [75, 104]. EGFR is frequently overexpressed in triple-negative breast cancer (TNBC), which is especially aggressive and hard to cure [105, 106]. Because of the crucial role of EGFR in breast cancer, several therapies that target EGFR, including gefitinib, cetuximab, lapatinib, and others, have been developed. However, results of clinical studies of EGFR-targeted therapy in breast cancer have been disappointing [107]. Thus, better understanding of the molecular mechanisms of EGFR especially the upstream target of EGFR becomes more and more important for finding more effective therapeutic strategies and improving patient survival.

MicroRNAs are a class of small (~20-25 nucleotides), non-coding RNAs that bind to partially complementary target sites in messenger RNA (mRNA) 3' untranslated regions (UTRs), which results in degradation of the target mRNAs, or translational repression of the encoded proteins [108]. Over 650 human miRNAs have been described, and each of them is predicted to target tens to hundreds of different mRNAs [109]. Plenty of miRNAs have been reported to be involved in breast cancer and

repression of miR141, a microRNA-200 family member, strongly promoted EMT and invasion in breast cancer [110].

In this study, we provide strong evidence to show that EGFR is a novel transcriptional target of KLF8. And miR141, which can be repressed by KLF8, represses EGFR. The KLF8-miR141-EGFR axis plays an important role in breast cancer progression.

Results:

KLF8 and EGFR are highly co-overexpressed in human metastatic breast cancer patient tumors.

Our cDNA microarray data indicated EGFR is one of the highly unregulated genes in 10A-iK8 cells [30] when KLF8 expression is induced compared with uninduced cells. Also, it is known that EGFR is overexpressed in TNBC cell line, MDA-MB-231 cells and these cells express high level of KLF8 [29, 111]. Because it is well known that both KLF8 and EGFR express aberrantly high levels in breast cancer cells and tissues [29, 30, 105], we want to know whether high expression of EGFR is due to high levels of KLF8. We have recently performed a tissue array analysis of KLF8 expression in breast cancer patient tumors and have shown a positive correlation with invasive ability of the tumors [30]. To see whether EGFR expression is correlated with KLF8 protein and/or the tumor invasion ability in the same tissue array, we did an IHC staining for EGFR expression. Interestingly, we did see high EGFR expression in KLF8 positive tumors

when compared with KLF8 negative tumor tissues and EGFR expression is also associated with the invasion potential of the tumors (Figure 9A-9D). These results highly demonstrated that EGFR expression is positively regulated by KLF8.

KLF8 upregulates EGFR expression at the transcriptional level.

We further verified high expression of EGFR in the 10A-iK8 cells when KLF8 is induced using real time PCR and western blotting (Figure 10a, compare lanes 2 with 1). Contrarily, EGFR expression was greatly decreased when KLF8 was knocked down in 231-K8ikd cells [30] (Figure 10a, compare lanes 4 with 3).

To further investigate if EGFR is a KLF8 transcriptional target, we first searched the human EGFR promoter sequence (EGFRp) and found 7 GT boxes (potential KLF8 binding sites) in a 2000bp region. We then cloned the EGFRp into a PGL3b luciferase reporter vector. Wild type KLF8 caused a more than 6 times increase in EGFR promoter activity whereas mutant KLF8 [36] didn't have this function (Figure 10b). These data suggested that EGFR is trans-activated by KLF8.

To further test the binding site of KLF8 at the EGFRp, we mutated the promoter and tested which region responds to KLF8. We found out that only when we mutated GT-box 1, KLF8's activation on EGFRp was abandoned. This result suggested GT box 1 as a strong candidate for KLF8 binding. Indeed, biotinylated oligonucleotide precipitation (BOP) (Figure 10d) and chromatin immunoprecipitation (ChIP) assays (Figure 10e, 10f)

indicated that KLF8 could interact with the EGFR promoter at the GT-box 1 site because mutation of the GT-box 1 (Figure 10d, lane 1) or lack of KLF8 expression (Figure 10e, lane 3, Figure 10f, lane 4) abolished this interaction.

All together, these results suggested that KLF8 binding to EGFR promoter through GT box 1 to activate the expression of EGFR.

KLF8 unregulated EGFR through inhibition of miR141.

We see only 6 times difference in EGFR promoter when KLF8 was transfected. But the EGFR protein level increase is over 3 times. This indicated some other mechanism might exist to help KLF8 promoting EGFR expression. MicroRNA is a recently identified small RNA that binding to the 3' UTR of the mRNA and play important role in the regulation of gene expression. Our lab has done a microRNA array [31] using 10A-iK8 cells compared between uninduced and induced cells. By searching candidate microRNAs that binding to EGFR 3' UTR using TargetScan 5.1, miRanda, miRbase and PITA, we found 2 microRNAs, miR7 and miR141, were predicted to binding to EGFR 3'UTR and downregulated when KLF8 was induced. MiR7 has been reported to repress EGFR in Glioblastoma [112]. MiR141 belongs to micorRNA 200 family and it is known that microRNA 200 family members regulate EMT by targeting ZEB1 and SIP1 [110, 113] and promote metastasis [114]. No one has reported that miR141 can repressed EGFR yet. To test the hypothesis that miR141 repressed EGFR, we first

validated the microRNA array data by real time PCR. And results showed that KLF8 induction decreased miR7 and miR141 expression (Figure 11a). We then cloned the 3'UTR of EGFR into a luciferase reporter plasmid [29]. Co-transfection of miR141 and reporter plasmid suggested a dramatically repression in the 3'UTR when miR141 was overexpressed (Figure 11c, lane 2). While this phenomenon was not seen when we used EGFR 3'UTR with miR141 binding site mutated (Figure 11c, lane 3). Overexpression of miR141 could not affect KLF8 induced EGFR promoter activity (Figure 11d) and mRNA expression (Figure 11e). But miR141 abolished KLF8 induced EGFR expression in the protein level (Figure 11f) suggested that miR141 repressed EGFR by inhibiting the translation of the gene [108]. As KLF8 repressing miR141, we then want to test if miR141 is a direct target of KLF8. We cloned the promoter of miR141 into the luciferase reporter plasmid and the promoter reporter assay was performed. KLF8 repressed the promoter activity whereas KLF8 repression domain mutant [36] could not. These data suggested that miR141 is a KLF8 direct repression target.

Taken together, our results strongly suggested that KLF8 represses miR141 and prevents miR141 to inhibit translation of EGFR and overall upregulates EGFR. This is an alternative mechanism of how KLF8 induces EGFR expression besides directly activation of the EGFR promoter.

KLF8 promoted invasion and proliferation through EGFR.

We have proved that KLF8 promoted EMT, migration, invasion and metastasis through repression of E-cadherin, activating MMP2, MMP9, and MMP14 [29, 30, 38]. Interestingly, EGFR is a well-known oncogene that promotes breast cancer progression [38, 77, 104, 106, 107, 111]. We then wanted to test whether KLF8 performed its function through EGFR. We found out that inhibition of EGFR activity by AG1478 could not only reverse the EMT process induced by KLF8 (Figure 12a, compare panels 3 and 2) but also the process induced by EGF (Figure 12a, compare panels 5 and 4) in 10A-iK8 cells. Inhibition of EGFR also inhibited 10A-iK8 cells invasion (Figure 12b) and proliferation (Figure 12c) induced by KLF8 or EGF. Western blotting results showed that EGFR inhibitor decreased EGFR phosphorylation (Y992, Y1068) and this explained why the inhibitor abolished KLF8 and EGF's function.

MiR141 partially abolished KLF8's role on invasion and proliferation.

Since miR141 can repress EGFR and KLF8 can repress miR141, we then want to know if miR141 can abolish KLF8's function. In order to test that, we made two stable cell lines from 10A-iK8, one with miR141 overexpression (10A-iK8-miR141), and another with miR141 sponge [31] overexpression (10A-iK8-miR141 sponge). We found out that miR141 abandoned the KLF8 induced invasion (Figure 13a, compare lanes 2 and 4)

in a matrigel invasion assay. Inhibition of miR141 by the sponge dramatically increased cell invasion ability under both KLF8 uninduced and induced condition (Figure 13a, Lanes 5 and 6). Similar phenomenon was seen in a WST-1 assay when we tried to test proliferation ability of the cells (Figure 13b) and cell survival when we performed a clonogenic assay (Figure 13c, 13d). Western blotting (Figure 13d) results showed that miR141 repressed EGFR expression induced by KLF8 and inhibition of miR141 unregulated EGFR even without KLF8.

KLF8 promoted proliferation through EGFR-ERK axis.

EGFR-ERK signaling and EGFR-AKT signaling pathways are two major pathways that regulated by EGFR [115, 116]. Our results showed that AKT activation could not be affected by KLF8. But KLF8 induction increased phosphorylation of EGFR and ERK1/2 (p-EGFR, p-ERK) (Figure 14a. compare lanes 2 and 1) and this could be inhibited by EGFR inhibitor (Figure 14a. compare lanes 4 and 3). Overexpression of miR141 decreased p-EGFR and p-ERK induced by KLF8 (Figure 14a. compare lanes 6 and 2). Also inhibition of miR141 increased EGFR and ERK activation even without KLF8 (Figure 14a. compare lanes 9 and 1). We also made two 231-K8ikd cell lines with overexpression of miR141 (231-K8ikd-miR141) or overexpression of miR141 sponge (231-K8ikd-miR141 sponge) respectively. In these cells, knocking-down of KLF8 decreased EGFR and ERK activation (Figure 14b. compare lanes 2 and 1).

Overexpression of miR141 decreased p-EGFR and p-ERK (Figure 14b. compare lanes 5 and 1) and inhibition of miR141 by the sponge increased EGFR and ERK activation (Figure 14b. compare lanes 2 and 10). We further used MEK inhibitor U0126 [117] to inhibit even downstream of EGFR. The results showed that the MEK inhibitor dramatically decreased cell proliferation induced by KLF8 or miR141 sponge (Figure 14c, 14d). In the protein level, western blotting results suggested that MEK inhibitor totally blocked ERK activation but not EGFR activation.

Taken together, all these data suggest that KLF8 performed the function through KLF8-miR141-EGFR-ERK axis.

Knocking-down of KLF8 or overexpression of miR141 inhibited tumor growth and invasion into the surrounding tissue.

To determine how KLF8-miR141-EGFR axis affects tumor progression in the breast, we injected 231-K8ikd cells or the 231-K8ikd-miR141 cells orthotopically into the mammary fat pad and then measured the tumor growth and invasion (Figure 7). We found out that tumor growth was inhibited when KLF8 was knocked down (I) or miR141 was overexpressed (U+miR141) (Figure 15a-15c). Interestingly, we also found out that tumor cells invasion to surrounding tissue was also being inhibited in I and U+miR141 condition by analyzing the H&E staining pictures (Figure 15d). Also IHC

staining indicated that KLF8 and EGFR expression was correlated and overexpression miR141 decreased EGFR expression without affecting KLF8 expression (Figure 15d).

Knocking-down of KLF8 or overexpression of miR141 inhibited tumor cells metastasis to the lung.

We have recently showed that KLF8 promotes breast cancer lung metastasis [30, 38]. To demonstrate whether the miR141-EGFR axis performed similar role, we injected 231-K8ikd cells or the 231-K8ikd-miR141 cells into the tail vein and then measured the lung metastasis by bioluminescent imaging (BLI) (Figure 8a and 8b). Lung metastasis began to be detected by BLI at week 6-7 and became able to be detected by eye at week 9 (Figure 16a-16c). Interestingly, the KLF8 knocking-down induced group (I) and miR141 overexpression group (U+miR141) showed significantly slower metastasis rate (Figure 16a and 16b) and less metastasis nodules (Figure 16c) compared with uninduced group (U). The human origin of tumor was verified by human vimentin IHC staining (Figure 16d). IHC staining using KLF8 and EGFR antibodies suggested the co-expression of two protein and this correlation was been abolished by miR141 expression (Figure 16d).

Discussion

This report identified a novel KLF8 and EGFR signaling mechanism for the tumor growth and lung metastasis of human breast cancer (Figure 17). In this signaling model of tumor progression, the abnormally high expression of KLF8 in the cancer cells ensures the high level of EGFR. On the one hand, KLF8 activates EGFR at the transcriptional level by directly binding to the gene promoter, On the other hand, KLF8 represses miR141 expression to release EGFR from translation repression. High level of KLF8 and low level of miR141 result in further activation of the EGFR gene. Once the high level of EGFR is produced in the cells, it will further activate the downstream ERK pathway and promote tumor growth and metastasis.

EGFR plays a crucial role in the breast tumor growth and metastasis [107]. Our cDNA microarray identified EGFR as one of the most unregulated genes by KLF8 and we had validated this result at both mRNA and protein levels (Figure 2). Furthermore, we have correlated the co-expression of KLF8 and EGFR with the metastatic potential of the patient tumors (Figure 1). In addition, KLF8 and EGFR share some common target genes, including cyclin D1 [25, 76], MMP9 [30, 74, 118], and cellular functions such as EMT, proliferation, and metastasis [30, 38, 39, 119, 120]. Altogether, these results strongly suggest that EGFR is a potential KLF8 target and KLF8 to EGFR signaling is potentially correlated with breast cancer progression.

MiR-141 is known to work as tumor suppressor by repression of ZEB1 [110, 113, 114]. It belongs to the miR-200 family which is a microRNA family that play crucial role in EMT, invasion and metastasis [113, 114]. In our lab's microRNA microarray data [31], KLF8 repressed miR7, miR141 and miR200c and this has been validated by real time PCR (Figure 3a). MiR141 and miR200c belong to the same family and share same promoter. Both of them can be repressed by KLF8 suggested that KLF8 may directly repress their promoter activity and this was proved by a promoter reporter assay (Figure 3g). MiR7 has been reported to inhibit EGFR [112] and we have confirmed KLF8's role on repression miR7 (Figure 3a). This might be the 3rd mechanism of how KLF8 upregulated EGFR. Since KLF8 can repress miR141 and miR200c, it can work upstream of ZEB1, which is a strong EMT inducer. This actually makes KLF8's role to be more important during the EMT process. MiR141 has been shown to have reversed expression pattern when compared with EGFR [121]. But no one has reported that miR141 repressed EGFR. Our data indicated that miR141 repressed EGFR and this is the 2nd mechanism of how KLF8 promoting EGFR expression.

Since EGFR is crucial in breast cancer, several therapies has been developed to target EGFR but the results in clinical studies in breast cancer have not been convincing. We have established a KLF8-miR141-EGFR axis and we could use KLF8 as a novel target for new therapies because KLF8 is strong oncogene and upstream of EGFR.

In summary, this work provides a novel insight into the mechanisms of breast cancer progression and opens a new gate in the study of both KLF8 and EGFR. The KLF8 to miR141 to EGFR to ERK signaling promotes breast tumor growth and metastasis to the lung. More studies can be done to pursue the details in the signaling.

Materials and Methods

Cell culture and reagents.

Antibodies. Antibodies used for western blotting were EGF Receptor rabbit polyclonal antibody (#4267), Phospho-EGF Receptor (Tyr992) rabbit polyclonal Ab (#2235), Phospho-EGF Receptor (Tyr1068) rabbit polyclonal Ab(#3777) (Cell Signaling Technology, Inc., Danvers, MA, USA), β -actin (C4) mouse monoclonal Ab (sc-47778), p-ERK (E-4) mouse monoclonal Ab (sc-7383), ERK 1 (C-16) rabbit polyclonal Ab (sc-93), HA-probe (F-7) mouse monoclonal Ab (sc-7392), (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), human vimentin mouse monoclonal Ab (550513) (BD Biosciences Inc, San Jose, CA, USA), HRP-conjugated donkey anti-mouse IgG (715-035-150) or anti-rabbit IgG (711-035-152), Peroxidase substrate kit (DAB) (SK-4100) (Vector laboratories Inc., Burlingame, CA, USA). Antibodies used for BOP and ChIP assays were HA probe (F-7) and KLF8 rabbit polyclonal Ab (8477), respectively as previously described [25, 29, 30].

Plasmids. pLenti 4.1 Ex miR200c-141 (#35534) was purchased from Addgene (Cambridge, MA, USA). The human miR141 cDNA was cloned and inserted into pLenti 4.1 through EcoRI and XhoI sites to generate pLenti 4.1 –miR141 plasmid. The human hEGFR-3'UTR was cloned and inserted into pIS0 vector through SacI and XbaI sites to generate pIS0-hEGFR3'UTR plasmids. The human EGFRp was cloned and inserted into PGL3b vector through KpnI and NarI sites to generate PGL3-EGFRp plasmid. MiR141 sponge was inserted into the pBABE-puro-mcherry vector through EcoRI and AgeI sites to generate pBABE-puro-mcherry-miR141 sponge plasmid. The human miR141 promoter was cloned and inserted into PGL3b through NheI and HindIII sites to generate PGL3-miR141p plasmid.

Cell lines. The HEK293 and NIH3T3 [101-103], MCF-10A, and MDA-MB-231 [26, 29], 10A-iK8 and the 231-K8ikd [30, 32] cell lines were described previously. These cells were maintained in DMEM/F-12 or DMEM with 10% fetal bovine serum or calf serum. 10A-iK8-miR141 and 231-K8iKD were generated by infected 10A-iK8 and 231-K8iKD cells by pLenti 4.1 miR141 plasmid followed by puromycin selection. 10A-iK8-miR141 sponge and 231-K8iKD-miR141 sponge cell lines were generated by pBABE-puro-mcherry-miR141 sponge infection followed by puromycin selection. The cell lines were maintained under U (in the absence of doxycycline or Dox) or I (in the presence of Dox) conditions depending on the experimental requirement. Doxycycline hydrochloride was purchased from Sigma-Aldrich Corp. (D3072, St. Louis, MO, USA).

Quantitative real-time PCR and western blotting.

These assays were done as described previously [29]. Cells and antibodies used were described above.

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

These assays were performed as described previously [29, 30, 38]. The plasmids containing the wild-type or mutant human hEGFRp, wild-type or mutant human hmiR141p the wild-type or mutant human EGFR 3'UTR reporter and the control vector, HEK293, MEF, 10A-iK8 and 231-K8ikd cell lines were described above.

Bioluminescence imaging (BLI) analysis of tumor growth and metastasis.

All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Female athymic Nude-Foxn1nu nude mice (the Harlan laboratory) 4–6 weeks old were used for the tail vein and mammary fat pad injection. 10^6 viable 231-K8iKD and 231-K8-iKD-miR141 cells were washed and harvested in 0.1 ml phosphate-buffered saline and subsequently injected into the tail vein or mammary fat pad of the mice. The mice were fed with food (Bioserve,

Frenchtown, NJ, USA) supplemented with doxycycline containing Diet (S3888) to induce the knockdown of KLF8 expression in the cells in vivo or with the Control Diet containing no doxycycline (S4207). After injection, tumor growth or lung metastasis was monitored by BLI. For BLI, mice were anaesthetized and injected with 150 mg/kg of D-luciferin (15 mg/ml in PBS) (LUCK-1, Gold Biotechnology, Inc., St. Louis, MO, USA) by i.p injection. Imaging was completed in 3 min after injection using the Kodak image system together with analysis software.

Matrigel invasion assays.

Matrigel invasion assays were done using BD BioCoat invasion chambers. Serum in the complete medium was used as the chemoattractant. The invasion chambers were incubated with the culture medium for 2 hours at 37°C for hydration. Then 5×10^4 cells were loaded into the upper chamber. 16-20 hours after incubation at 37°C, the upper chamber was scratched and washed following by crystal violet staining.

WST-1 assay.

2000 cells were seeded into each well of 96 wells plates. The second day, WST-1 substrate was added into the medium and the cells were then cultured for two hours and then measure the absorbance of each sample using a microplate reader at a wavelength of 450 nm.

Clonogenic assay.

2000 cells were seeded into each well of 6 wells plates. The cells were cultured in the plates for 14 days then stained with crystal violet. Picture was taken and then the cells were washed by methanol and the measured the colorimetric under OD 540.

Hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining.

The mammary tissues and the lungs were collected, fixed in formaldehyde solution (F8775, Sigma-Aldrich Co., St. Louis, MO, USA), embedded in EM-400 Embedding Medium Paraffin (3801320, Leica Microsystems Inc., Buffalo Grove, IL, USA), and sectioned with the Manual Rotary Microtome for Routine Sectioning (Leica RM2235, Leica Microsystems Inc., Buffalo Grove, IL, USA). H&E and IHC staining was performed as described previously [27]. Human breast cancer tissue array (BR1503, US Biomax, Rockville, MD, USA) was analyzed as described previously [27, 30]. KLF8, EGFR, vimentin antibodies were described above. Leica DFC295 microscope was used for the microscopy of sectioned tissues (Leica Microsystems Inc., Buffalo Grove, IL, USA).

Statistical analysis.

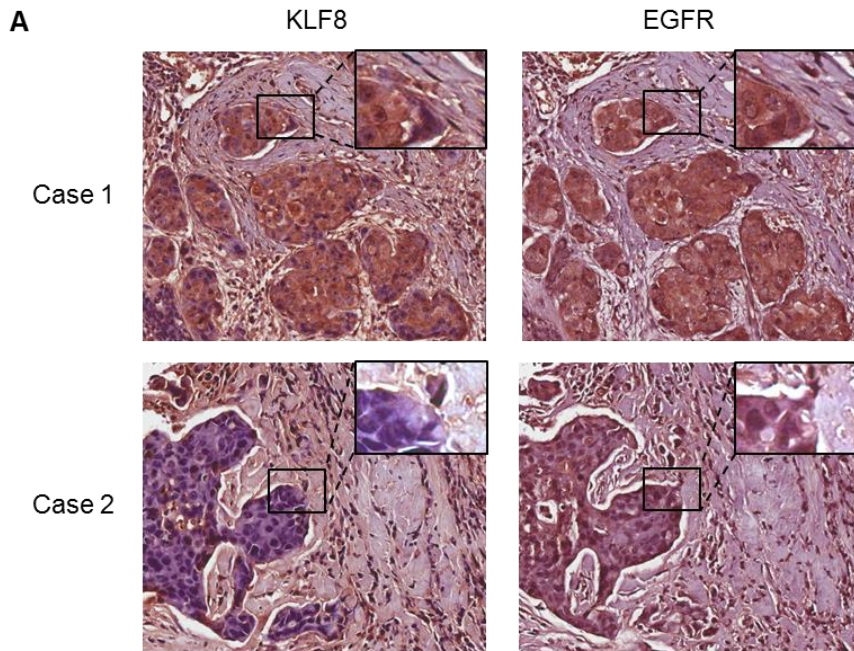
All data are presented as mean \pm s.d. with a minimum of three observations per group.

Student's t-test was used to prepare the significance between two groups of data. The

two by two tables for human data were analyzed by Fisher's exact test. Significance

was determined by the p value.

Figures and Table



B

Total

| | EGFR+ | EGFR- | Total | P-Value |
|-------|-------|-------|-------|---------|
| KLF8+ | 21 | 7 | 28 | |
| KLF8- | 14 | 33 | 47 | |
| Total | 35 | 40 | 75 | 0.000 |

C

IDC

| | EGFR+ | EGFR- | Total | P-Value |
|-------|-------|-------|-------|---------|
| KLF8+ | 21 | 7 | 28 | |
| KLF8- | 13 | 19 | 32 | |
| Total | 34 | 22 | 60 | 0.00 |

D

| | EGFR+ | EGFR- | Total | P-value |
|----------|-------|-------|-------|---------|
| None IDC | 1 | 14 | 15 | |
| IDC | 34 | 26 | 60 | |
| total | 35 | 40 | 75 | 0.000 |

Figure 9. Correlated upregulation of KLF8 and EGFR expression in human breast cancer patient tumors.

Figure 9. Correlated upregulation of KLF8 and EGFR expression in human breast cancer patient tumors.

(a-d) Positive correlation of protein expression between EGFR and KLF8 in metastatic breast cancer patient tumors. IHC staining of EGFR or KLF8 (brown) in the human breast cancer tissue array was performed. Images representing specimens in duplicate from 75 patient tumors or normal tissues were shown in (a). Correlation of EGFR and KLF8 expression was shown for all the samples (b) or in the invasive ductal carcinoma (IDC) specimens only (c). Positive correlation of EGFR expression with the invasive potential was outlined in (d). Statistical significance was determined by χ^2 -test.

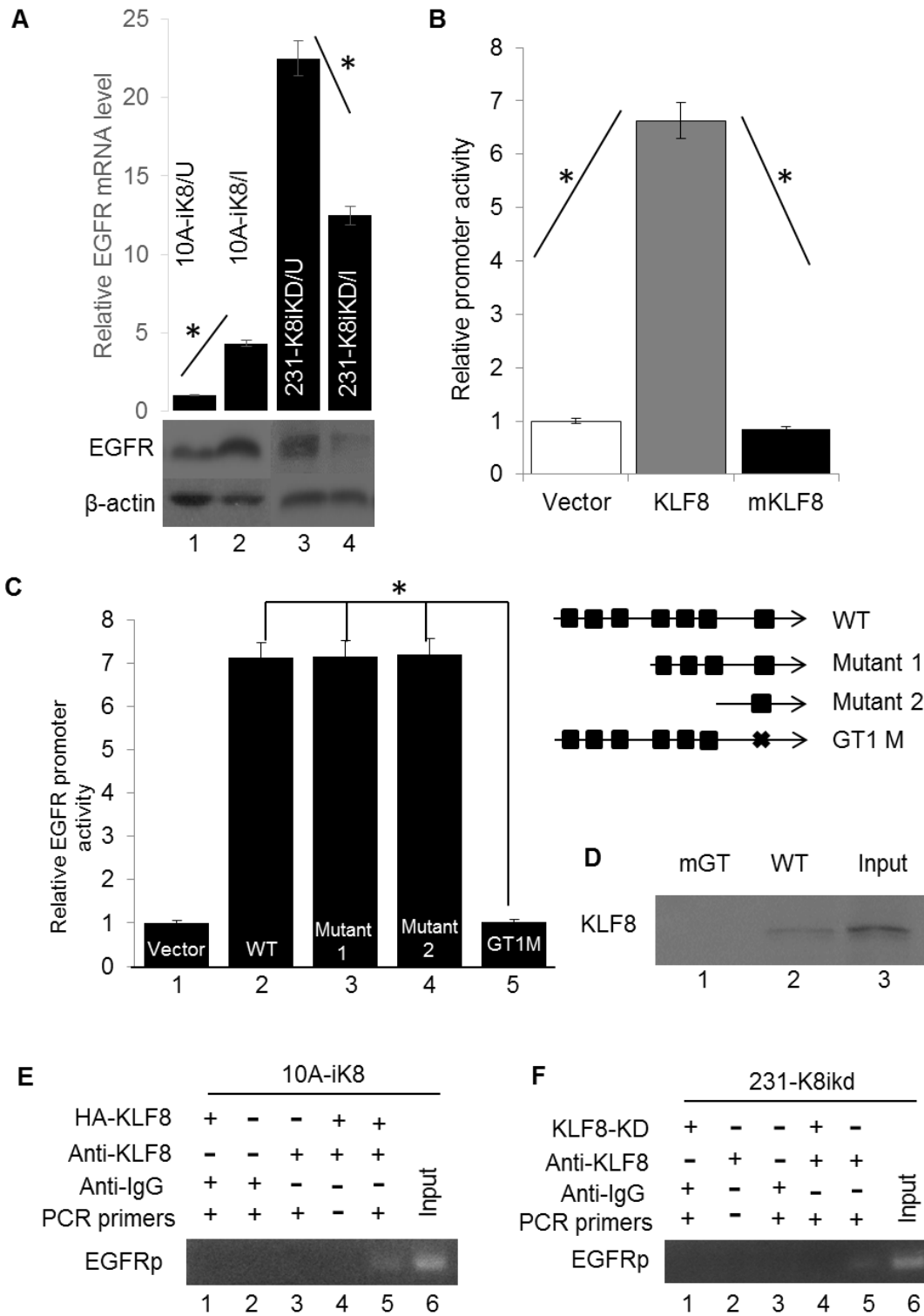


Figure 10. KLF8 upregulates EGFR expression at the transcriptional level.

Figure 10. KLF8 upregulates EGFR expression at the transcriptional level.

KLF8 expression is both sufficient and necessary for EGFR expression. The 10A-ik8 and 231-k8ikd cells were grown under induced (I) or uninduced (U) conditions for 48h. (a) Real time PCR and western blotting were performed to test the expression of EGFR. (b) KLF8 activates the EGFR gene promoter (EGFRp). The human EGFRp was cloned into a luciferase reporter plasmid and co-transfected into the NIH3T3 cells with wild-type KLF8, its transactivation-defective mutant (mKLF8) or the control vector plasmid for 16 h. The reporter activity was determined as described in the Material and Methods (*P < 0.01). (c) The GT-box 1 (GT1) is required for the activation of EGFRp by KLF8. The GT-boxes were individually mutated. The wild-type (WT) or mutant (mGT) reporters were used for similar reporter assay. *P < 0.01. (d) KLF8 directly binds EGFRp at the GT-box 1 site. HEK293 cell lysate containing HA-KLF8 and oligos spanning the wild-type GT1 (WT) or its mutant (mC+GT1) were used for BOP assay as described in the Material and Methods. (e and f) KLF8 binds EPSTI1p in vivo. The 10A-iK8 or 231-K8ikd cells were cultured under the uninduced (U) or induced (I) conditions for 72 h. ChIP assay was the done as described in the Material and Methods.

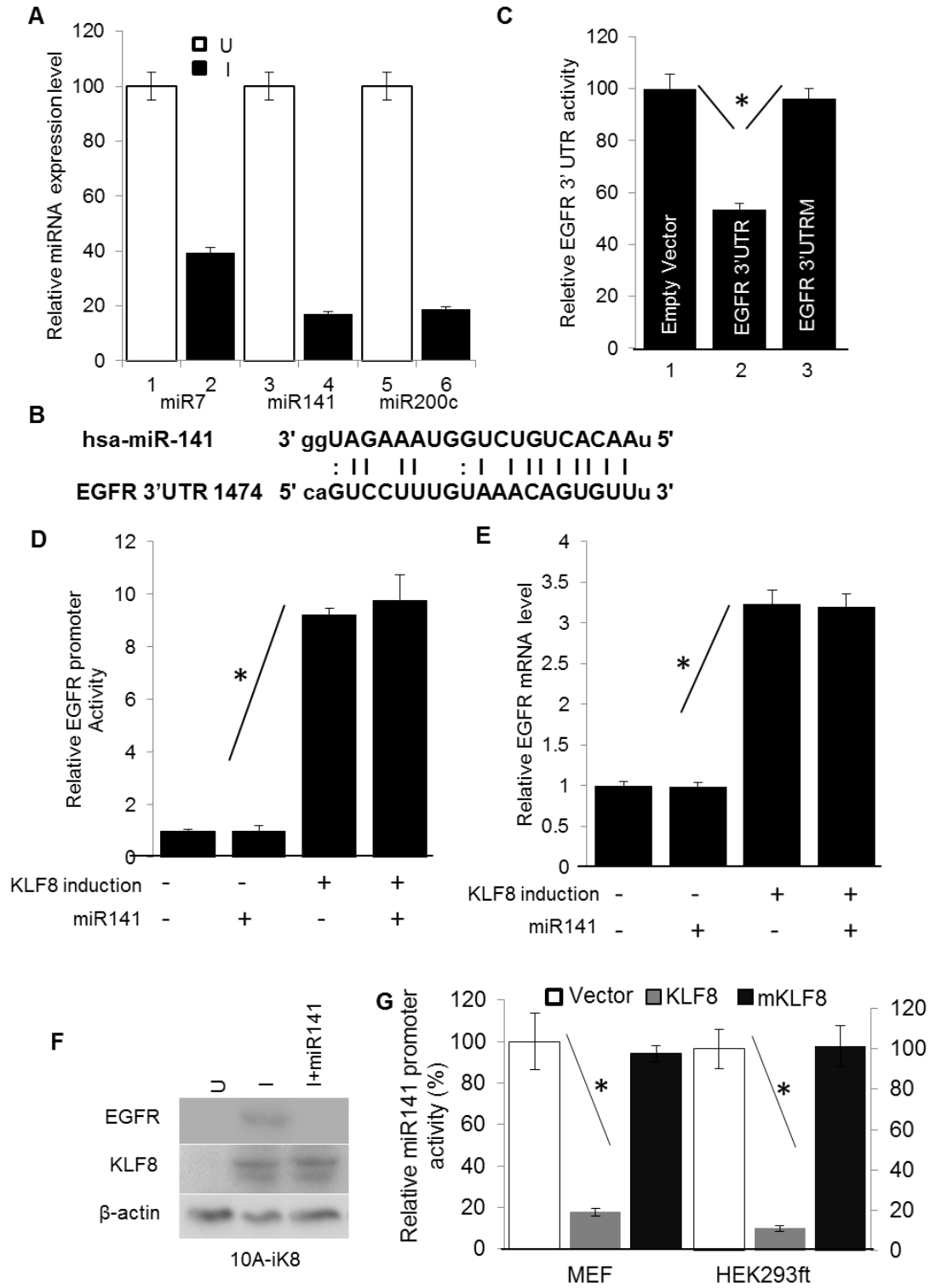


Figure 11. KLF8 unregulated EGFR through inhibition of miR141.

Figure 11. KLF8 unregulated EGFR through inhibition of miR141.

(a) KLF8 induction inhibited the expression of miR7, miR141 and miR200c. 10A-iK8 cells were cultured under uninduced (U) or induced (I) conditions for 2 days. Then RNA was extracted and miRNA was isolated. The expression of miRNAs was then detected by real time PCR. (b) The miR-141 binding site in EGFR 3'UTR was aligned with the miR-141 sequence. (c) The wild type (WT) EGFR 3'UTR or its mutant defective in miR-141 binding (EGFR 3'UTRM) was cloned into a luciferase reporter vector. The reporter plasmids were transfected into the HEK293ft cells with overexpressing of miR141. (d) MiR141 expression could not affect EGFR promoter activity. 10A-iK8 with stable overexpression of miR141 or the normal 10A-iK8 cell lines were used to perform the luciferase assay. The cells were cultured with or without KLF8 induction for 2 days, then luciferase assay was performed. (e) MiR141 expression could not affect EGFR expression in mRNA level. The cells were treated as same as figure 3c, then real-time PCR was performed. (f) MiR141 expression repressed EGFR expression in protein level. The cells were treated as figure 3c, then cell lysate was collected and western blotting was done. (g) KLF8 repressed miR141 promoter. MiR141 promoter was cloned in to luciferase reporter plasmid and then co-transfection of miR141p reporter and KLF8, or mutant KLF8, or empty vector.

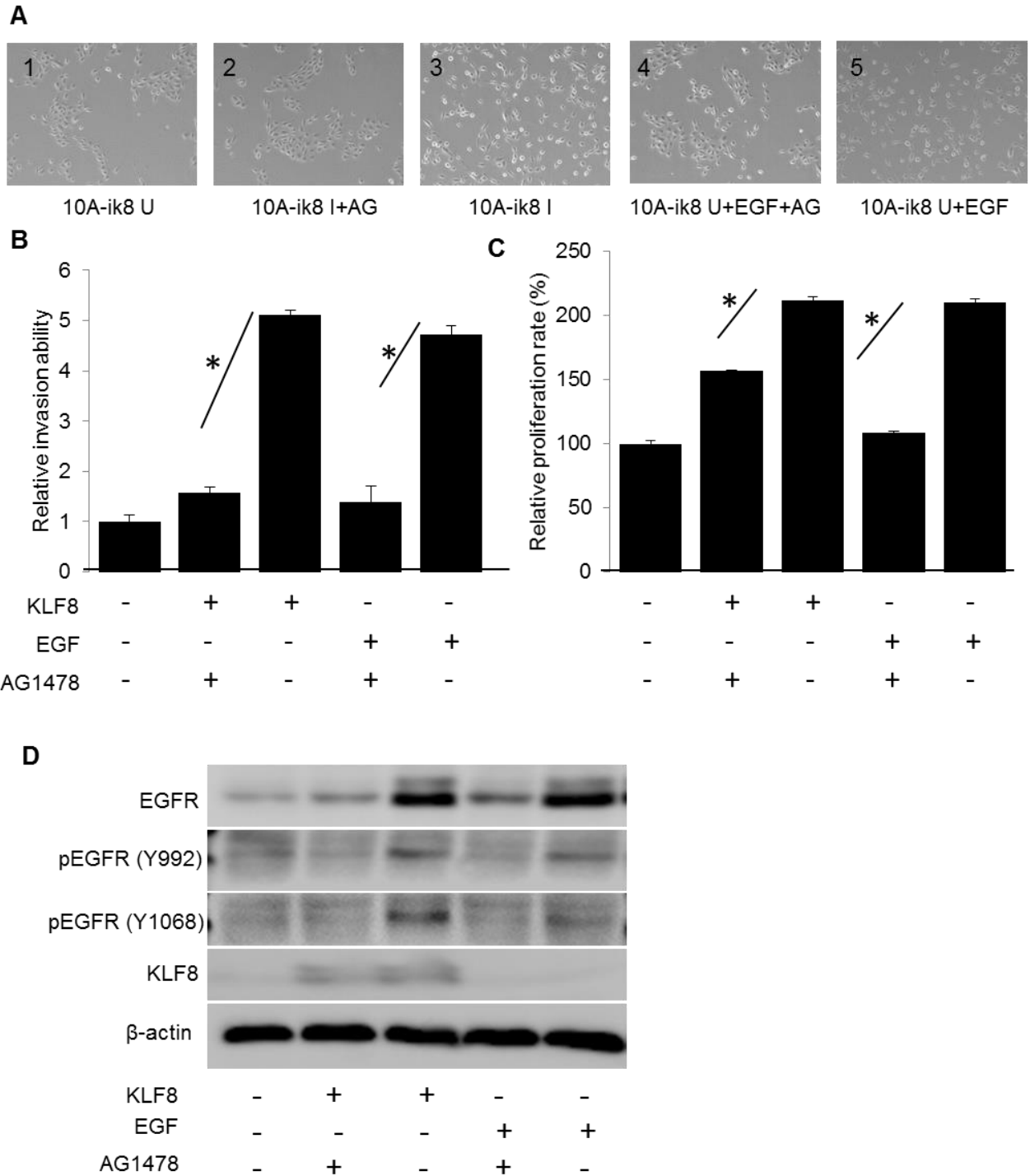


Figure 12. KLF8 promoted invasion and proliferation through EGFR.

Figure 12. KLF8 promoted invasion and proliferation through EGFR.

(a) Inhibition of EGFR activation reversed KLF8 induced EMT. 10A-iK8 cells were cultured under uninduced (U) or induced (I) conditions for 5 days. Then the cells were treated with DMSO or AG1478 for 30 min. For EGF treatment, the cells were treated with high dose of EGF for 5 days, and then DMSO or AG1478 were added for 30 min. The cells were reseeded. (b and c). Inhibition of EGFR activation reversed both KLF8 and EGF's function on promoting Invasion and proliferation. The cells were treated as figure 4a, and then matrigel invasion assay (b) or WST-1 assay (c) was performed. (d) Inhibition of EGFR activation reversed both KLF8 and EGF's function on activating EGFR. The cells were treated as figure 4a, then cell lysate was harvested and western blotting was performed.

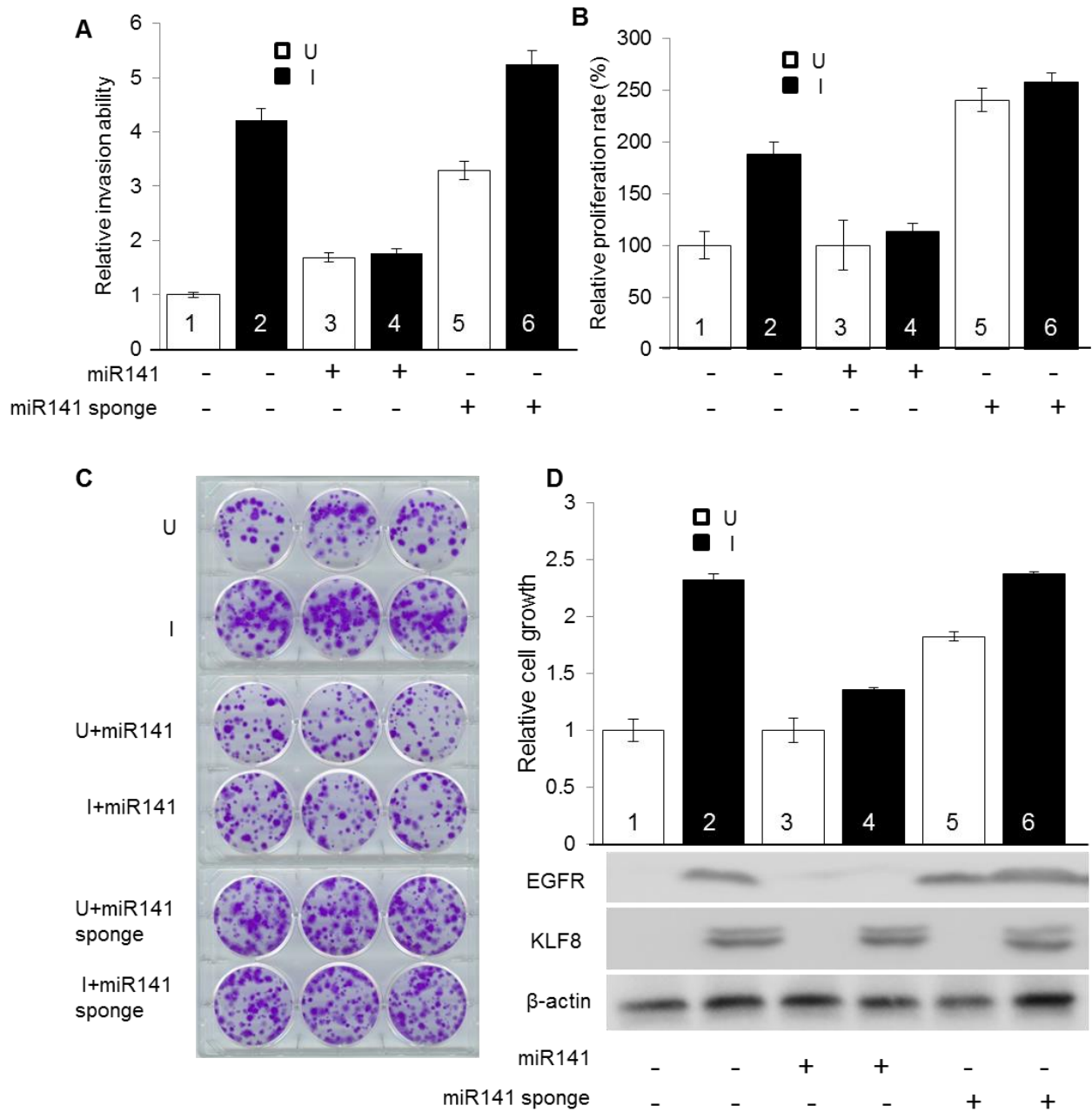


Figure 13. miR141 partially abolished KLF8's role on invasion and proliferation.

Figure 13. miR141 partially abolished KLF8's role on invasion and proliferation.

(a) MiR141 abolished KLF8's function on promoting invasion. 10A-iK8, 10A-iK8-miR141, and 10A-iK8 miR141 sponge cell line were used. The cells were cultured under uninduced (U) or induced (I) conditions for 2 days. Then matrigel invasion assay was performed. Serum was used as the chemoattractant. (b) MiR141 abolished KLF8's function on increasing proliferation of the cells. The cells were treated same as figure 5a. Then WST-1 assay was performed. (c and d) miR141 partially abolished KLF8's role on cell survival. The cells were treated as figure 5a. Then 2000 cells were seeded into each well of 6 well plates. The cells were cultured in the plates for 14 days then stained with crystal violet. The picture was shown in c. The cells were washed by methanol and the measured the colorimetric under OD 540. Western blotting was also performed using cell lysate from parallel plates.

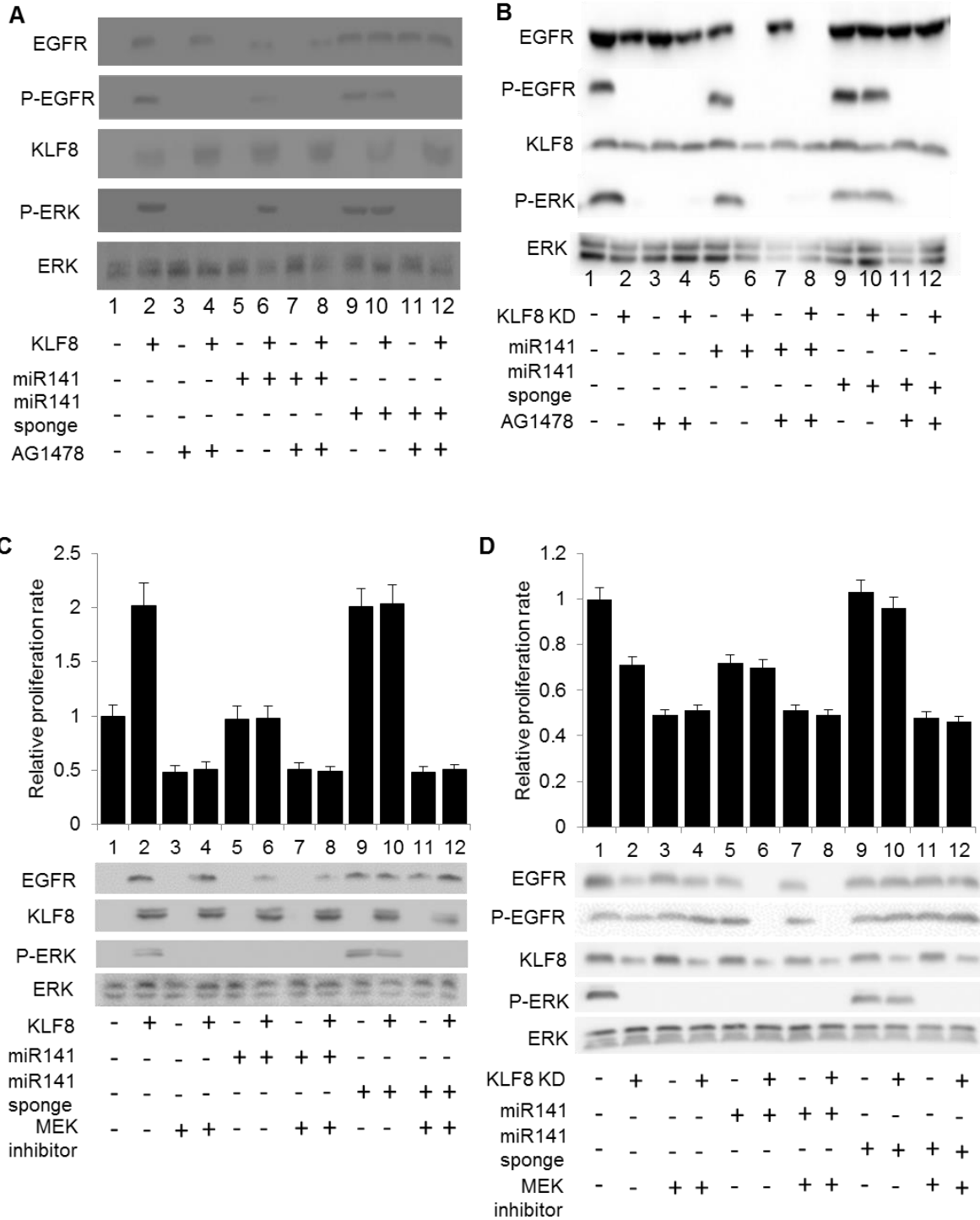


Figure 14. KLF8 promoted proliferation through EGFR-ERK axis.

Figure 14. KLF8 promoted proliferation through EGFR-ERK axis.

(a) Inhibition of EGFR activation abolished KLF8's role on activating Erk1/2 in 10A cells. 10A-iK8, 10A-iK8-miR141, and 10A-iK8 miR141 sponge cells were cultured under uninduced (U) or induced (I) conditions for 2 days. Then DMSO or AG1478 was added into the medium for 30 min. After changing the medium, the cells were cultured for 1 hour and then cell lysates were harvest for western blotting. (b) Inhibition of EGFR activation abolished KLF8's role on activating Erk1/2 in 231 cells. 231-K8iKD, 231-K8iKD-miR141, and 231-K8iKD- miR141 sponge cells were cultured under uninduced (U) or induced (I) conditions for 3 days. Then DMSO or AG1478 was added into the medium for 30 min. After changing the medium, the cells were cultured for 1 hour and then cell lysates were harvest for western blotting. (c) Inhibition of MEK activation abolished KLF8's role on promoting proliferation through activating Erk1/2 in 10A cells. 10A-iK8, 10A-iK8-miR141, and 10A-iK8 miR141 sponge cells were cultured under uninduced (U) or induced (I) conditions for 2 days. Then DMSO or MEK inhibitor U0126 was added into the medium for 15 min. The cells were then reseeded and WST-1 assay and western blotting were performed 1 day after re-plating. (d) Inhibition of MEK activation abolished KLF8's role on promoting proliferation through activating Erk1/2 in 231 cells. 231-K8iKD, 231-K8iKD-miR141, and 231-K8iKD- miR141 sponge cells were cultured under uninduced (U) or induced (I) conditions for 3 days. Then DMSO or MEK

inhibitor U0126 was added into the medium for 15 min. The cells were then reseeded and WST-1 assay and western blotting were performed 1 day after re-plating.

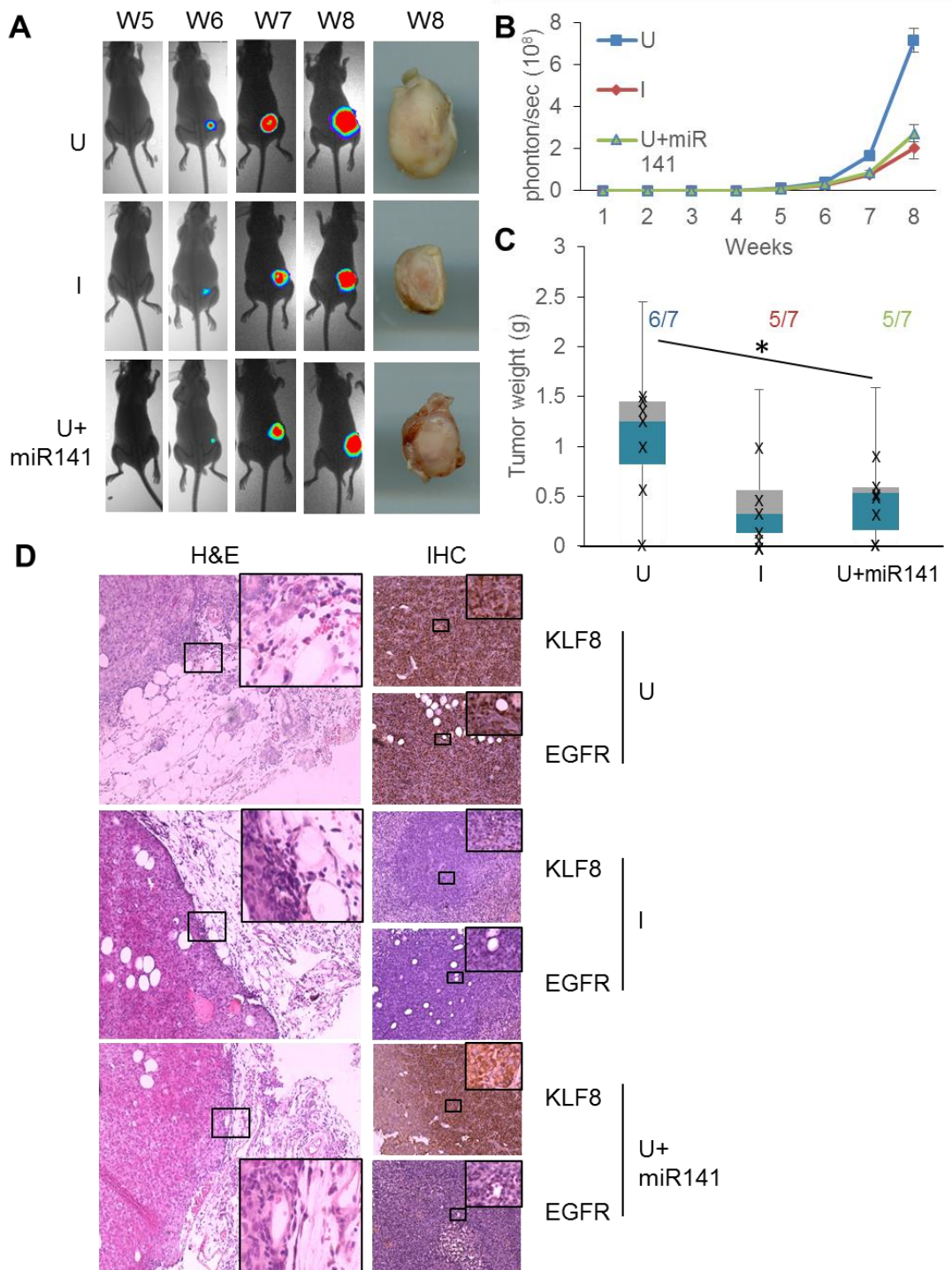


Figure 15. Knocking-down of KLF8 or overexpression of miR141 inhibited tumor growth and invasion into the surrounding tissue, after nude mice fatpad injection.

Figure 15. Knocking-down of KLF8 or overexpression of miR141 inhibited tumor growth and invasion into the surrounding tissue, after nude mice fatpad injection.

The 231-K8iKD cells or 231-K8-iKD with miR141 overexpression cells (U+miR141) were injected through the fatpad. The mice injected with 231-K8iKD cells were fed with food supplemented with (I, KLF8 knockdown) or without doxycycline (U, no KLF8 knockdown). The mice injected with the miR141 overexpressed 231-K8iKD cells were feed with normal food. The tumor growth was followed up for 8 weeks by BLI or stereomicroscopy at week 8 as described in Materials and Methods. (a) Representative images. (b) Quantitative results of the volumes of the tumor growth. (c) Tumor weight at week 8. *P < 0.05. (d) H&E and IHC staining with KLF8 or EGFR antibody confirmed the co-expression of KLF8 and EGFR.

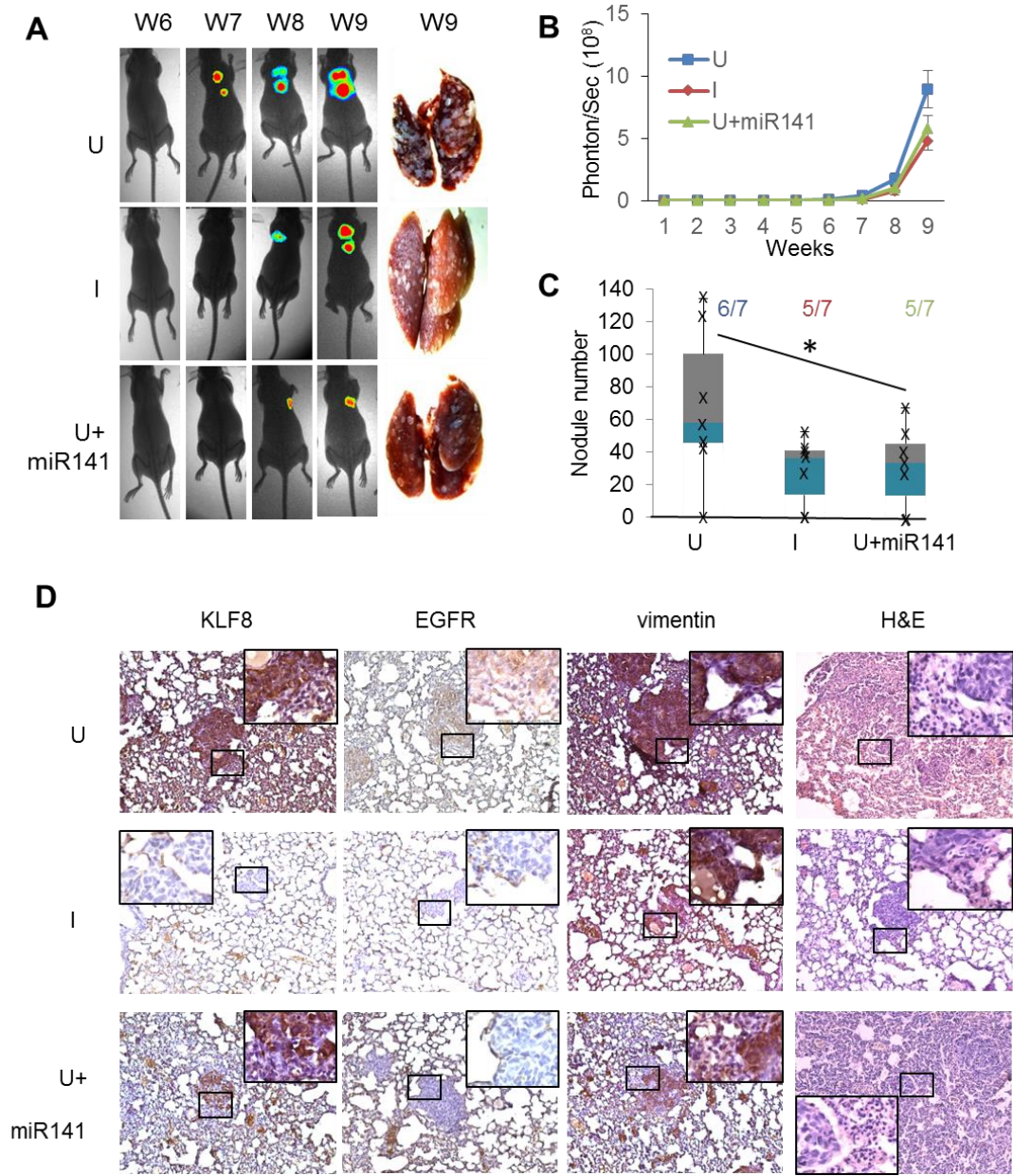


Figure 16. Knocking-down of KLF8 or overexpression of miR141 inhibited cancer cells metastasis to the lung.

Figure 16. Knocking-down of KLF8 or overexpression of miR141 inhibited cancer cells metastasis to the lung.

231-K8iKD-miR141 or 231-K8iKD was injected into the mice through tail vein. The mice were fed as described in Figure 7. BLI analysis and stereomicroscopy of the tumors (a-b) and H&E or IHC analyses (d) H&E and IHC staining with KLF8, EGFR, or vimentin antibody confirming the human origin of the lung metastasis. (c) Knockdown KLF8 or overexpression of miR141 could reduce the lung surface tumor nodules number.

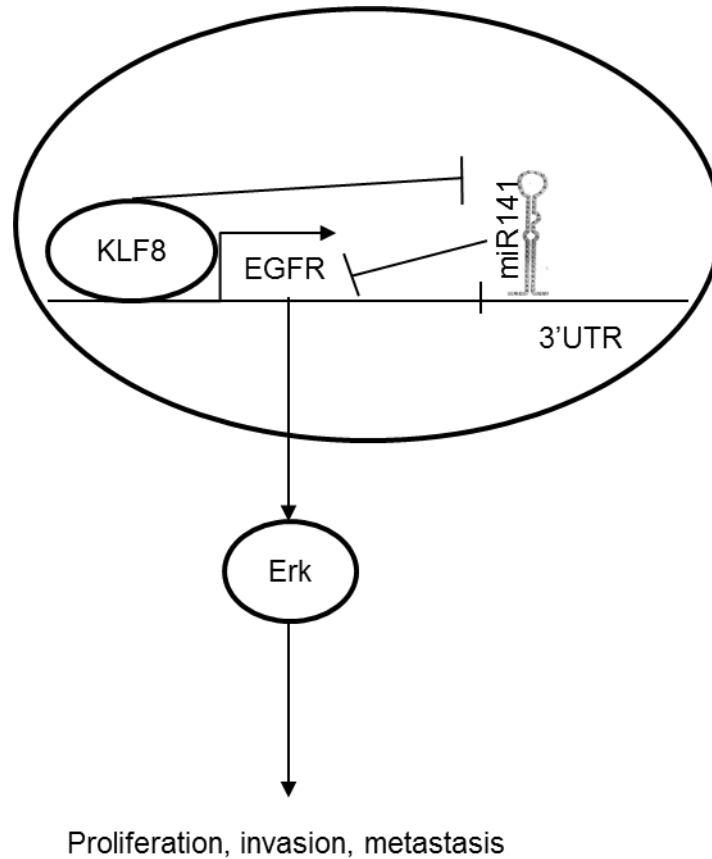


Figure 17. Model of mechanism.

KLF8 up-regulates EGFR at transcriptional level and miR141 can repress EGFR 3'UTR.

KLF8 can inhibit miR141 expression. Overexpression of EGFR then causes ERK1/2 activation and promotes breast cancer proliferation, invasion and metastasis.

CHAPTER 4: CONCLUSION

In summary, we have identified EPSTI1-to-NF κ B and EGFR-to-ERK signaling pathways as two novel signaling branches downstream of KLF8 that promote the invasion and metastasis of breast cancer. This work has opened a new avenue for breast cancer mechanistic studies and provided new foundation for developing novel targeted therapies against mortality associated with aggressive breast cancer. Specifically, we demonstrate for the first time that 1) KLF8 overexpression is well correlated with the overexpression of EPSTI1 and EGFR in the invasive patient breast cancer tumors including the TNBC tumors, 2) KLF8 upregulates expression of EPSTI1 and EGFR in breast cancer cells by direct transcriptional activation of their gene promoters, 3) KLF8 upregulates EGFR expression also by transcriptional repression of the expression of microRNA141 to release the inhibition of EGFR expression by microRNA141, and 4) EPSTI1 promotes breast cancer invasion and metastasis in vivo, 5) EPSTI1 interacts with VCP to activate NF κ B by promoting the degradation of I κ B α , EGFR primarily activates ERK downstream of KLF8, and 6) most importantly, the upregulation of EPSTI1 and EGFR and the downregulation of microRNA141 are required for KLF8 to promote the metastatic progression of breast cancer in vivo.

The interaction between carcinoma and stroma plays crucial roles in tumor progression. The most dramatic consequence of epithelial neoplasia and peritumoral interaction is the accumulation of myofibroblasts, or so-called cancer associated fibroblast (CAF) [122,

123]. CAF can be recruited to the tumor sites by TGF β secreted from tumor cells. Lots of works have suggested the positive roles of CAF in tumor formation and progression. It has been shown that CAF secretes various factors, such as stromal derived factor 1 (SDF-1) [124], hepatocyte growth factor (HGF) [125], and urokinase (uPA) [126] which function directly on tumor cells through specific receptors, CXCR4, cMET, and uPAR, respectively. EPSTI1 was originally identified in breast cancer cells when cocultured with myofibroblasts [49]. Recently, it has been reported that EPSTI1 is one of the most upregulated genes in response to interferon (IFN) in non-breast cancer cells [127]. It will be interesting and important to investigate what stromal factors are responsible for the induction of EPSTI1 in the tumor cells and to what degree KLF8 is involved in the induction process.

The ATP-driven chaperone protein VCP plays crucial roles in ubiquitin-proteasome degradation [91]. We identified VCP as a novel EPSTI1 interaction protein. As the domain for co-factor binding, VCP's N 1-187 region is also the domain for EPSTI1 interaction, which suggests a potential role of EPSTI1 in VCP driven protein degradation. Besides the degradation of I κ B α , VCP has also been reported to degrade key apoptotic factors, such as Mcl1 or DIAP1 [128, 129] to regulate cell proliferation and survival. Future work will be performed to determine what other degrading target proteins such as Mcl1 and DIAP1 may play a role downstream of KLF8-to-EPSTI1 signaling pathway in

breast cancer. Nevertheless, the EPSTI1-VCP interaction could become a potential therapeutic target for the treatment of breast cancer metastasis.

NF- κ B is a transcription factor that is known to be involved in the inflammatory. Recent studies identified the crucial roles of NF- κ B in tumor progression. In this work, we found out NF- κ B accumulation and activation in the nucleus due to the degradation of I κ B α . The active NF- κ B then promoted invasion and metastasis in breast cancer. Because NF- κ B is downstream of KLF8 in our system, it will be very interesting to identify its potential targets downstream of KLF8-EPSTI1. Intriguingly, as KLF8 downstream target genes, MMP9 and cyclin D1 are also targets of NF- κ B. This further suggests that NF- κ B may serve as a second arm of KLF8 to ensure the activation of these critical target genes' expression in breast cancer.

Recent study has suggested that interaction between cancer cells and platelet induces EMT and promotes metastasis. Platelet-derived TGF β and direct platelet-tumor cells interactions synergistically activate the TGF β /Smad and NF- κ B pathways in cancer cells, causing an EMT like phenotype and promoted metastasis in vivo [130]. Given our findings that KLF8 promotes EMT process, upregulates EPSTI1 and further stimulates NF- κ B signaling and MMP9 that is known to play a role in the cleavage and releasing of the soluble and mature TGF β , and epithelial stromal interaction induces EPSTI1 expression, it will be very interesting to determine whether the direct physical contact between platelet or tumor stromal cells with the tumor epithelial cells and/or their

secreted TGF β plays a role in the induction of EPSTI1 expression through KLF8 in the tumor epithelial cells of breast cancer during invasion and metastasis.

EGFR is frequently overexpressed in TNBC that is especially aggressive and hard to cure. Because of the lack of therapeutic targets, TNBC has very low survival rate compared with other types of breast cancer. KLF8 promotes EGFR expression by directly activating EGFR promoter. Besides that, KLF8 can also repress miR7 and miR141 expression and this prevents the miRNA to repress the translation of EGFR. As a cell membrane receptor, EGFR activation will trigger the intracellular tyrosine kinase activity and promote survival, proliferation and invasiveness in cancer. Besides the function as a tyrosine kinase, recent studies have also identified EGFR's role in the nucleus as a transcription factor. By activation of cyclin D1, EGFR promotes proliferation [131]. More interestingly, cyclin D1 is also a KLF8 direct target gene. This suggests that KLF8-EGFR-cyclin D1 signaling is another potential pathway that promotes proliferation in cancer. Researchers also found out that combination of several EGFR antibodies significantly promoted the degradation of EGFR and inhibited the breast cancer progression in TNBC. As upstream regulator of EGFR that overexpressed in TNBC, KLF8 has great potential as a novel therapeutic target. Because KLF8 needs PARP-1 to be imported into the nucleus [88], inhibition of PARP-1 by the PARP-1 inhibitor can partially abolish KLF8's function. So besides discovering a direct KLF8 therapeutic drug,

combination of PARP-1 and EGFR inhibitors may become an effective way to treat the TNBC.

Researches are underway to determine role of other potential target genes as well as microRNAs downstream of KLF8. Similar to miR141, both miR7 and miR200c are upregulated by KLF8. Interestingly, miR7 has been reported to target EFGR in brain tumor. MiR200c is located at the same gene cluster of miR141 and is repressor of ZEB EMT inducer [110]. It has been reported that miR126 regulates tumor angiogenesis [132] and miR203 controls prostate cancer proliferation and invasion [133]. Indeed, among other top KLF8 target gene candidates identified by the microarray study described above, there are some genes that either are potential repression target of these microRNAs or known to play critical roles in tumor angiogenesis. Roles of KLF8 in tumor angiogenesis via these target genes or microRNAs are in progress.

It is important to mention that KLF8 is aberrantly overexpressed in many other types of human cancer in addition to breast cancer, and KLF8 expression is barely detectable in most of normal cells or tissue types. Therefore, while opening new avenues for KLF8 research in breast cancer, our work has provided significant new insights into potentially future KLF8-targeted therapy against KLF8-overexpressing breast cancer as well as other kinds of cancer, particularly those aggressive, metastatic types.

APPENDIX A: PUBLICATIONS

Li T, Lu H, Shen C, Lahiri S, Wason M, Mukherjee D, Yu L, Zhao J. Identification of epithelial stromal interaction 1 as a novel effector downstream of Krüppel-like factor 8 in breast cancer invasion and metastasis. *Oncogene*. Accepted

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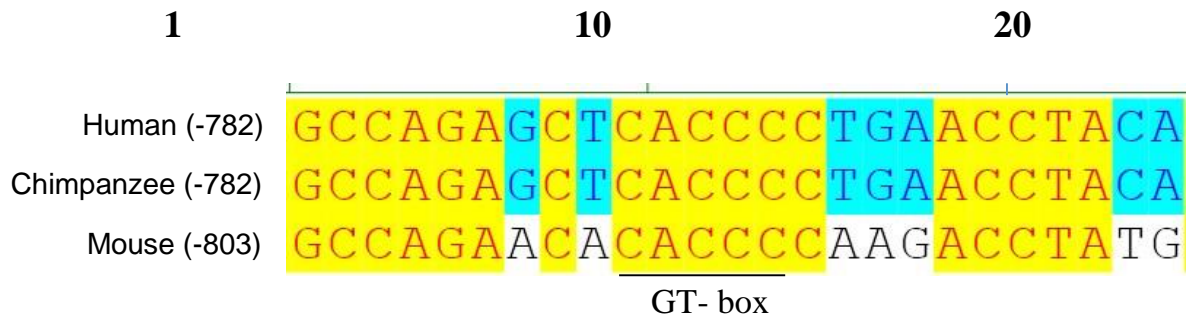
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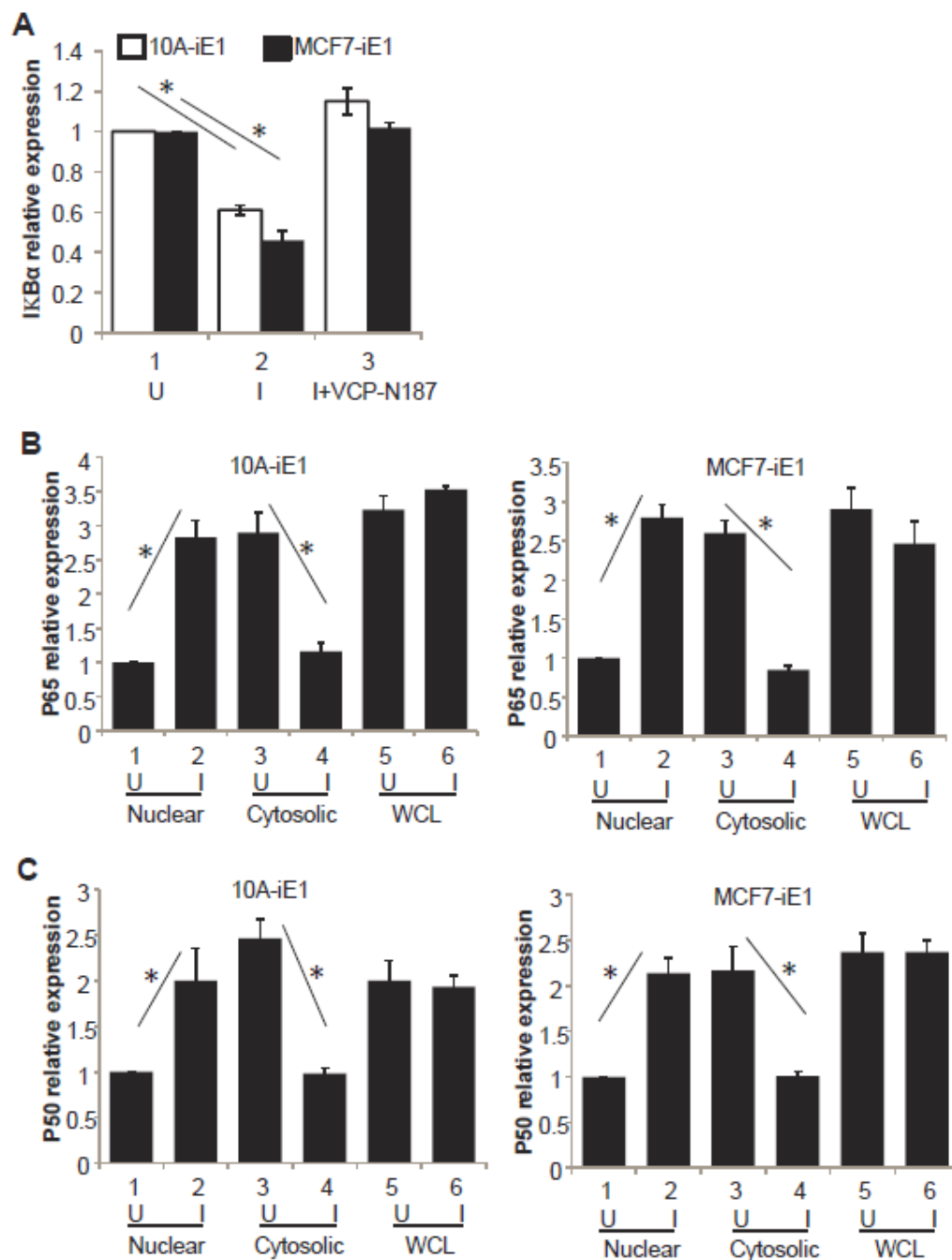
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APPENDIX C: SUPPLEMENTAL FIGURES



Supplemental Figure S1. The KLF8-binding GT-box at the EPSTI1 gene promoter is well conserved.



Supplemental figure S2. Quantitative data supplemental to Figure 7A (A) and Figure 7B (B, C). Western blots were carried out using Bio-Rad Gel Documentation System and quantified with Image Lab 3.0 software. *P<0.05

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