Am J Transl Res 2022;14(2):1220-1233
www.ajtr.org /ISSN:1943-8141/AJTR0134187

Original Article

KLF8 promotes invasive outgrowth of breast cancer by inducing filopodium-like protrusions via CXCR4

Debarati Mukherjee[1,2], Jie Hao[1], Heng Lu[1,3], Satadru K Lahiri[1,4], Lin Yu[1], Jihe Zhao[1]

[1]Burnett School of Biomedical Sciences, University of Central Florida College of Medicine, 6900 Lake Nona Boulevard, Orlando, FL 32827, USA; [2]Duke University School of Medicine, Box 3813, C334 LSRC, 308 Research Drive, Durham, NC 27710, USA; [3]University of Miami Miller School of Medicine, 600 NW 10th Ave #1140, Miami, FL 33136, USA; [4]Cardiovascular Research Institute and Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030, USA

Received April 12, 2021; Accepted June 23, 2021; Epub February 15, 2022; Published February 28, 2022

Abstract: Post-therapeutic relapse remains the biggest challenge to breast cancer management. The re-initiation of proliferation of dormant tumor cells in either metastatic or primary tumor location marks the final rate-limiting step of malignancy and mortality. The underlying molecular mechanisms remain poorly understood. We have recently demonstrated that KLF8 promotes breast cancer metastasis via CXCR4 upregulation. Here we report a role and mechanisms for KLF8 in driving the recurrence-like tumor outgrowth in both secondary and primary sites in a CXCR4-dependent manner. Treatment of an MDA-MB-231 breast cancer cell variant with the CXCR4 ligand, CXCL12, induces formation of filopodia in monolayer culture and filopodium-like protrusions (FLPs) in 3D culture. The FLP^+ cells proliferate significantly faster than FLP^- cells in the 3D culture supplemented with CXCL12. Both the FLP formation and enhanced proliferation in the 3D culture can be prevented by silencing KLF8 expression in the cells. From this prevention, the cells can be rescued by overexpressing wild-type CXCR4 but not its inactive mutant form in the cells. Overexpression of KLF8 or CXCR4 in the cells dramatically enhances their invasive outgrowth and metastasis after being implanted into immunocompromised mice. Mechanistically, we found that the activated FAK was recruited to the nascent FLPs and that proliferation of the cells was completely prevented with a FAK-specific inhibitor. Taken together, these results shed new light on the role of KLF8 in promoting breast cancer recurrence, the fatal episode of the disease, by inducing CXCR4-dependent FLP formation.

Keywords: KLF8, CXCR4, filopodium-like protrusion, breast cancer relapse

Introduction

In the past few decades, adjuvant therapies and surgical resection have been proved to be effective for primary breast tumors that are well-confined. However, metastatic tumors remain very difficult to cure and post-therapeutic recurrent growth in either primary or secondary site is responsible for over 90% of the patient mortality [1, 2]. Understanding the underlying mechanisms is critical for developing effective therapies.

Recurrent tumor surge occurs after mainstay therapies including primary chemotherapy and surgical tumor removal and relies on gaining resistance to therapies, and formation of filopodium-like protrusions (FLPs) in the cancer cells at either secondary or primary tumor site. FLPs are actin-rich cellular structures and were originally identified in a very small fraction of extravasated breast cancer cells that can survive the colonization process, the final rate-limiting step of successful metastasis [3]. FLPs were subsequently proven to also play a critical role in a small fraction of orthotopically implanted experimental breast cancer cells that can survive and successfully form primary tumors [4]. These studies beautifully answered a long-lasting question that not all but only a fraction of micro-metastasized or orthotopically implanted cancer cells are capable of fully growing to form clinically significant metastatic or primary tumors. These studies also implicated the important signaling cascades including integrin β1 to Rif/mDia2, ILK/β-parvin/

cofilin and FAK/Erks in FLP formation and subsequent proliferation critical for the tumor outgrowth [3-6]. However, the molecular regulators of FLP formation, particularly those from the extracellular matrix side, remain understudied.

We have recently identified the G-protein coupled C-X-C motif chemokine receptor 4 (CXCR4) [7] as a novel target gene of transcriptional activation by Krüppel-like transcription factor 8 (KLF8) in human breast cancer cells [8]. CXCR4 interaction with and activation by its cytokine ligand CXCL12 has been strongly implicated in breast cancer metastasis. Physiologically, the CXCR4 receptor directs the trafficking or homing of $CXCR4^+$ leucocytes and hematopoietic stem cells towards CXCL12-rich tissues via CXCL12 gradient-dependent chemotaxis [7, 9, 10]. The CXCR4 is aberrantly overexpressed in malignant breast cancer cells and patient's tumors. These CXCR4-high cancer cells can "hijack" aforementioned cell homing mechanism to migrate along the CXCL12 gradient to establish metastasis in the same CXCL12-rich tissues such as the lungs, liver, bone marrow, brain and lymph nodes [7, 11].

Notably, another recent study from our lab has demonstrated a highly correlated, aberrant co-overexpression of CXCR4 and KLF8 in tumor tissue samples from large cohorts of breast cancer patients [8, 12, 13]. It is well documented that the aberrant overexpression of KLF8 [14-16] plays a critical role in breast cancer progression such as cell cycle progression [17], oncogenic transformation [18], epithelial to mesenchymal transition (EMT) [19], cancer stem cell induction [20], transendothelial migration [8] and DNA damage response [14]. However, a potential role of KLF8 for the very final step of breast cancer malignancy responsible for the initiation of the fatal tumor recurrence has rarely been investigated.

In this study, we report a novel finding that KLF8 at the aberrantly high levels in breast cancer cells promotes CXCR4-dependent induction of FLP formation. The formation of FLPs is responsible for the invasive proliferation and outgrowth of breast cancer cells both in 3D culture and in mice that recapitulate breast cancer relapse.

Materials and methods

Reagents and antibodies

The AMD3100 (a.k.a. octahydrochloride hydrate), a CXCR4-specific antagonist (A5602) and the PF573228, a FAK-specific pharmacological inhibitor (PZ0117) were purchased from Sigma (St. Louis, MO, USA). The recombinant CXCL12 (300-28A) of human source was purchased from Peprotech (Rocky Hill, CT, USA). Antibodies included GFP (B-2) polyclonal antibody (Santa Cruz Biotechnology Inc), Ki67 (8D5) antibody (Cell Signaling Technology Inc), and pY397-FAK antibody (Invitrogen, 44625G, Carlsbad, CA, USA). The Alexa Fluor® 568 Phalloidin (Thermo Fisher Scientific, A12380) was used for actin staining.

Generation of plasmids, cell lines, and cell culture

The doxycycline-inducible, KLF8-overexpressing MCF-10A cell line (10A-iK8) and the doxycycline-inducible shRNA-based KLF8 knockdown MDA-MB-231 cell line (231-K8ikd) were prepared as previously described [15]. The 231-K8ikd cell line that expresses the ectopic CXCR4 or its dN20 mutant was described in our previous report [8]. Generation of the MDA-MB-231 cell lines including 231-Vector, 231-KLF8 and 231-CXCR4 was described in our recent report [13]. Cell culture was described previously [8].

In vitro matrigel-on-top assay

The Matrigel-on-Top (MoT) three dimensional (3D) culture was performed as described [3, 13]. Matrigel Basement Membrane Matrix (Product #356234, Corning) was stored at 4°C overnight prior to the experiment. Precooled 8-well chamber slides (#C7057, Nunc® Lab-Tek® II Chamber Slide™ system) were coated with 40 µl of the Matrigel per well and incubated at 37°C for at least 30-40 minutes so as to allow the gel to solidify. Subsequently, a single suspension of the cells was generated, whereby 400 cells were suspended in 200 µl of culture media containing 2% Matrigel and seeded into the well of the chamber slide. Then, 10 ng/ml of CXCL12 in PBS or PBS alone was added directly into the well. The slides were then incubated at 37°C for the times indicated with the

media being changed every fourth day as applicable. For inhibitor treatment, the cells were pretreated with the AMD3100 (35 ng/ml) or the PF573228 (1 μM) for 3 hours prior to culturing cells under the MoT 3D conditions.

Immunofluorescent staining and confocal analysis of the 3D cell colonies

These assays were performed as previously described [3, 13]. Briefly, after fixation permeabilized and blocking, the cell colonies were stained with Alexa Fluor 568 phalloidin for visualizing FLPs, or stained with the antibody for Ki67 or active FAK followed by mounting with ProLong Gold antifade (#P36931, Invitrogen) containing DAPI. The cell imaging was done with the Ultraview Confocal Microscope (Perkin Elmer). 3D images of cell colonies were captured and quantified using the Volocity software as per the manufacturer's instruction.

Bioluminescent imaging (BLI), immunohistochemical (IHC) staining and statistical analysis

The animal experiments were conducted by strictly abiding by our institutional animal care and use committee-approved protocol (PROTO-202000093). Monitoring of the tumor growth by BLI and subsequent IHC staining of the tumor tissue samples for the expression of GFP and Ki67 as well as data analysis for statistical significance were carried out as previously described [8, 13, 15, 21-24].

Results

KLF8 promoted CXCR4-dependent filopodial formation in monolayer culture

We have recently demonstrated that overexpression of KLF8 upregulates CXCR4 expression in the non-tumorigenic human mammary epithelial cell line MCF10A with conditionally inducible KLF8 ectopic expression (10A-iK8) and that CXCR4 is a critical signaling player downstream of KLF8 in promoting breast cancer metastasis in the experimental metastatic models using the human breast cancer cell line variant MDA-MB-231 [25, 26] with conditionally inducible KLF8 knockdown (231-K8ikd) and immunocompromised mice [8]. To understand the cellular mechanism underlying the KLF8-CXCR4 signaling axis in the human breast cells, we first treated the 231-K8ikd cells with the CXCR4 ligand CXCL12 in the monolayer culture (**Figure 1**). We found that significantly larger number of these CXCL12-treated cells, compared to those cells mock-treated with PBS, developed many more filopodia. However, this filopodial development is almost completely prevented when KLF8 expression is silenced or CXCR4 is inactivated with its specific antagonist AMD3100 [27] regardless of the CXCL12 stimulation (**Figure 1A-C**). Conversely, the 10A-iK8 cells do not develop filopodia in response to CXCL12 when expression of the ectopic KLF8 is not induced. This is not surprising given that MCF10A expresses neither KLF8 nor CXCR4 to a detectable level [8, 18, 19]. Once the ectopic KLF8 expression is induced, however, treatment with CXCL12 induces abundant filopodia in many folds. And again, this CXCL12-stimulated filopodial formation is prevented by inactivating CXCR4 (**Figure 1D-F**). These results indicate that KLF8 plays an important role in promoting the remodeling of the actin cytoskeleton to form filopodia that are a critical cellular structure contributing to migration and invasion, and that this role of KLF8 depends upon the downstream CXCR4 engagement with CXCL12.

KLF8 expression was required for CXCR4-dependent FLP formation in breast cancer cells in 3D culture

Filopodia are thin actin-cytoskeletal protrusions like antenna for cells to probe their environment. They have been implicated in several physiological processes such as cell adhesion and migration. Recent reports suggest that the filopodia extended by monolayer cells appear to be very similar to FLPs extended by extravasated cancer cells that play a critical role in enabling cellular connection to extracellular matrix in the tissues of metastatic sites [3]. It is known that metastatic cancer cells proliferate only if they can interact productively with the extracellular matrix. Since we have already observed filopodial formation in the CXCL12-treated 231-K8iKd cells in monolayer culture, we tested whether or not these cells can extend FLPs under the MoT 3D culture conditions [3]. Our result showed (**Figure 2**) that the 231-K8ikd/U cells formed FLPs as early as 24 hours after CXCL12 exposure but failed to form FLPs upon mock treatment with PBS. Both the

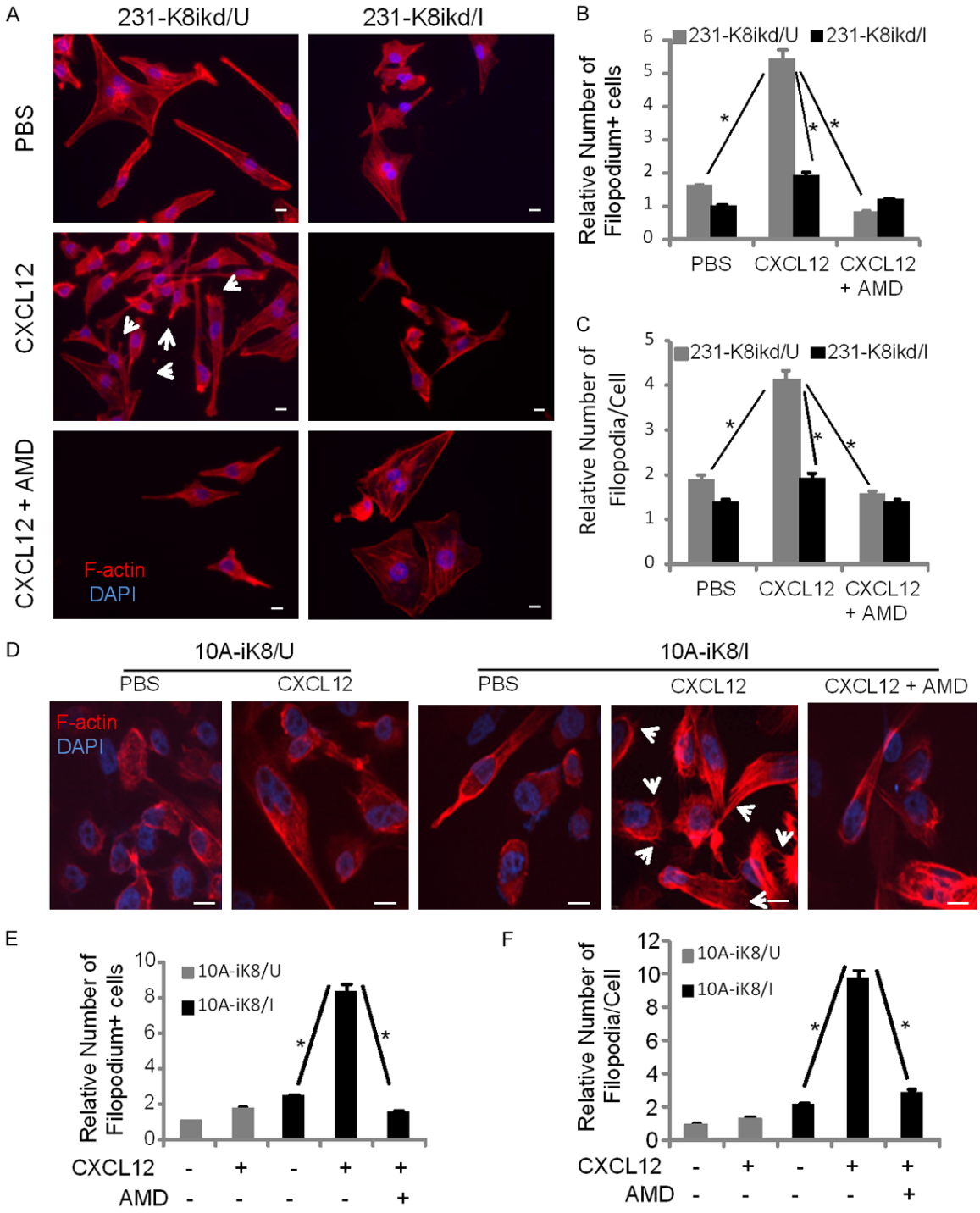

Figure 1. KLF8 promotes CXCR4-dependent filopodial formation in monolayer culture. (A-C) Silencing KLF8 expression or inactivating CXCR4 in breast cancer cells inhibits filopodial formation. The 231-K8ikd cells grown under the induced (I) or uninduced (U) conditions were exposed to PBS or CXCL12 alone, or CXCL12 supplemented with AMD3100 (AMD) for 24 h prior to phalloidin staining (Red. Nuclei in blue) for analysis of filopodia as described in Materials and methods. (D-F) Overexpressing KLF8 in breast epithelial cells induces CXCR4-dependent filopodial formation. The 10A-ik8 cells cultured under the U or I conditions were treated with PBS or CXCL12 alone, or CXCL12 supplemented with AMD for 24 h prior to filopodial staining and analysis. Representative confocal images and quantification are shown derived from three fields for each of the conditions in three separate experiments. Arrows are pointed to filopodia. The scale bars are 10 μm with a magnification factor of 400× in (A) or 630× in (D). *$P < 0.01$.

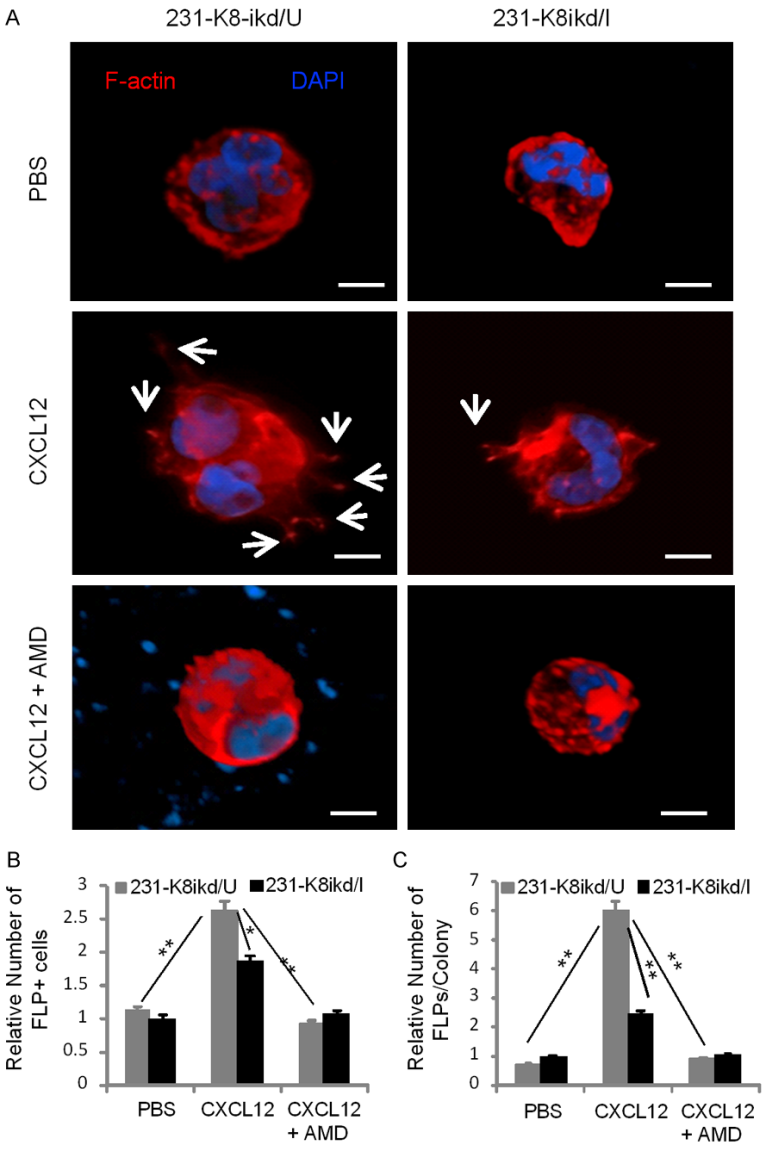

Figure 2. KLF8 expression is required for CXCR4-dependent FLP formation in breast cancer cells in 3D culture. A. Silencing KLF8 expression or inactivating CXCR4 in breast cancer cells inhibits FLP formation. The 231-K8ikd cells grown under the U or I conditions were exposed to PBS or CXCL12 alone, or CXCL12 supplemented with AMD in the MoT 3D culture for 48 h prior to analysis of FLPs. The scale bars are 10 μm with a magnification factor of 630×. B, C. Quantifications of FLP-positive cells and number of FLP-positive cell colonies were done. The 3D culture and quantification procedures were described in Materials and methods. Arrows are pointed to FLPs. $^{*}P < 0.05$; $^{**}P < 0.01$.

number of FLPs per cell colony and the overall FLP-positive colonies are significantly decreased by KLF8 knockdown (231-K8ikd/I) or by blocking the CXCR4 receptor activity (AMD) regardless of the CXCL12 presence. These results suggest that the KLF8-CXCR4 signaling axis plays an important role in FLP formation which could potentially contribute to the initiation of breast cancer recurrence.

KLF8 expression was required for CXCL12-dependent surge of proliferation in breast cancer cells in 3D culture

Loss of polarity of cell colonies in 3D culture has been associated with a concurrent failure to arrest proliferation [28, 29]. To assess the proliferative potential of the 231-K8ikd cells under the 3D culture conditions, we traced the number of cells in the colonies for a 10-day period of time (**Figure 3**). We found that the 231-K8ikd cells proliferated significantly faster to form much larger cell colonies in the presence of CXCL12, compared with the cells mock-treated with PBS. This enhancement in proliferation, however, was markedly diminished by KLF8 knockdown. These results indicate that the FLP formation promoted by KLF8 and stimulated by CXCL12 is critical for the cancer cell proliferation in a 3D environment that mimics the invasive tumor outgrowth particularly during colonization at the metastatic tumor sites.

In addition to CXCR4 receptor, CXCL12 can also interact with CXCR7 receptor [7, 9]. To determine if the long-term outgrowth of the 3D colonies stimulated by CXCL12 depends upon CXCR4 expression, rather than CXCR7, we cultured the 231-K8ikd cells under the 3D culture conditions for a 5-day period of time and examined the FLP formation and the colony expansion over the time course (**Figure 4**). Clearly, both the FLP formation and colony expansion were dramatically inhibited upon KLF8 silencing (I compared to

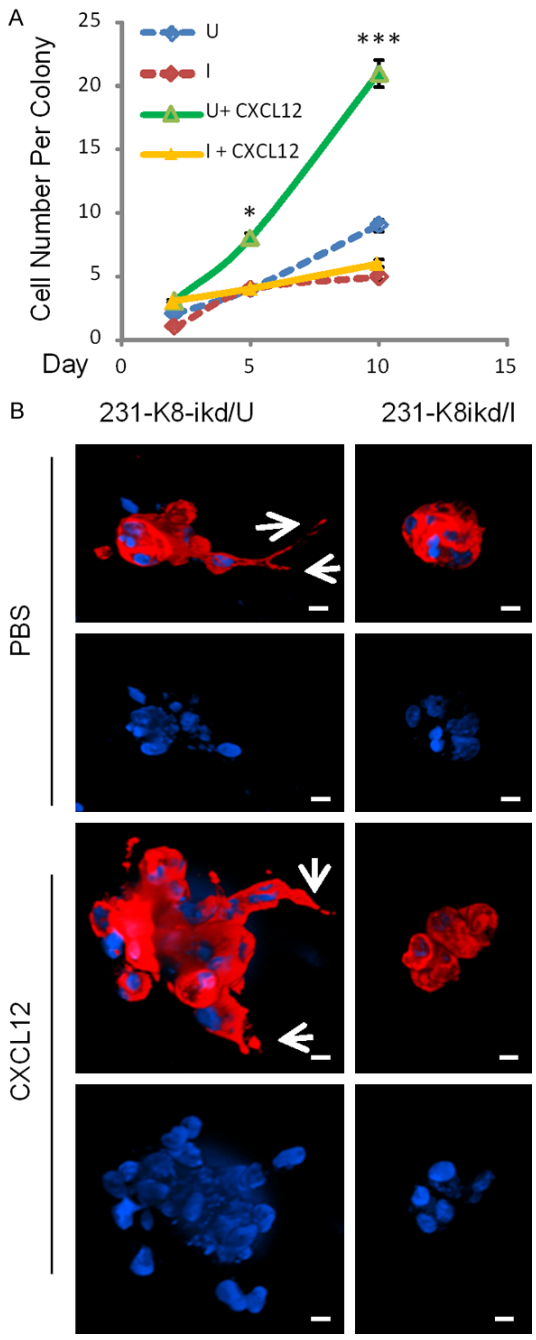

Figure 3. KLF8 expression is required for CXCL12-dependent proliferative enhancement in breast cancer cells in 3D culture. A. Silencing KLF8 expression in breast cancer cells inhibits FLP$^+$ colony expansion. The 231-K8ikd cells grown under the U or I conditions were exposed to PBS or CXCL12 in the MoT 3D culture for up to 10 days prior to quantification of the number of cells per colony. $^*P < 0.05$; $^{***}P < 0.001$. B. Representative confocal images of the colonies were taken after 10 days of culture. Arrows are pointed to FLPs. The scale bars are 10 μm with a magnification factor of 630×.

U). This inhibition was completely rescued by re-expression of the wild-type CXCR4 but not its inactive mutant form dN20. To determine the proliferative status of the cells, we examined the expression of the proliferation marker protein Ki67 after 48 hours (**Figure 5**). The results showed that knockdown of KLF8 expression significantly inhibited the cell proliferation (I compared to U), which was rescued upon re-expression of CXCR4 (I+CXCR4 compared to I+dN20). These results suggest that KLF8 is required for the outgrowth of the breast cancer cells in a 3D environment recapitulating invasive tumor locations *in vivo* and that CXCR4, rather than CXCR7, is a primary player with CXCL12 in mediating this function downstream of KLF8.

Overexpressing KLF8 or CXCR4 enhanced the invasive tumor outgrowth in vivo

We noticed that upon re-expression of CXCR4, the ability of the cells to form FLPs and proliferate in the 3D culture was not only recovered but also lifted to a level higher than that before KLF8 knockdown (**Figures 4** and **5**, compare I+CXCR4 with U groups). This suggests that the cellular response with FLP formation to CXCL12 stimulation echoes the levels of CXCR4, and thus possibly of KLF8, in the breast cancer cells. It also suggests that breast cancer cells already expressing high levels of KLF8 and/or CXCR4 could produce even higher levels of KLF8 and/or CXCR4 under certain pathological circumstances. Indeed, our recent study [13] has demonstrated that the KLF8-CXCR4 signaling axis can be over-induced in human metastatic breast cancer cells both in culture and in patient tumors in response to chemotherapeutic treatment. And their over-induction is highly correlated with the poor prognosis and responsible for the drastically extensive metastasis to multiple CXCL12-rich tissues of the cancer cells that otherwise metastasize to the lungs only [13, 25, 26]. These results strongly support a role of KLF8-CXCR4 signaling axis in enhancing metastasis, particularly after mainstay therapies, likely through inducing the FLP formation in the cancer cells.

FLP formation has also been implicated to be important for the invasive outgrowth of breast cancer cells in the primary tumor location [3, 5]. It is also known that CXCR4 signaling plays

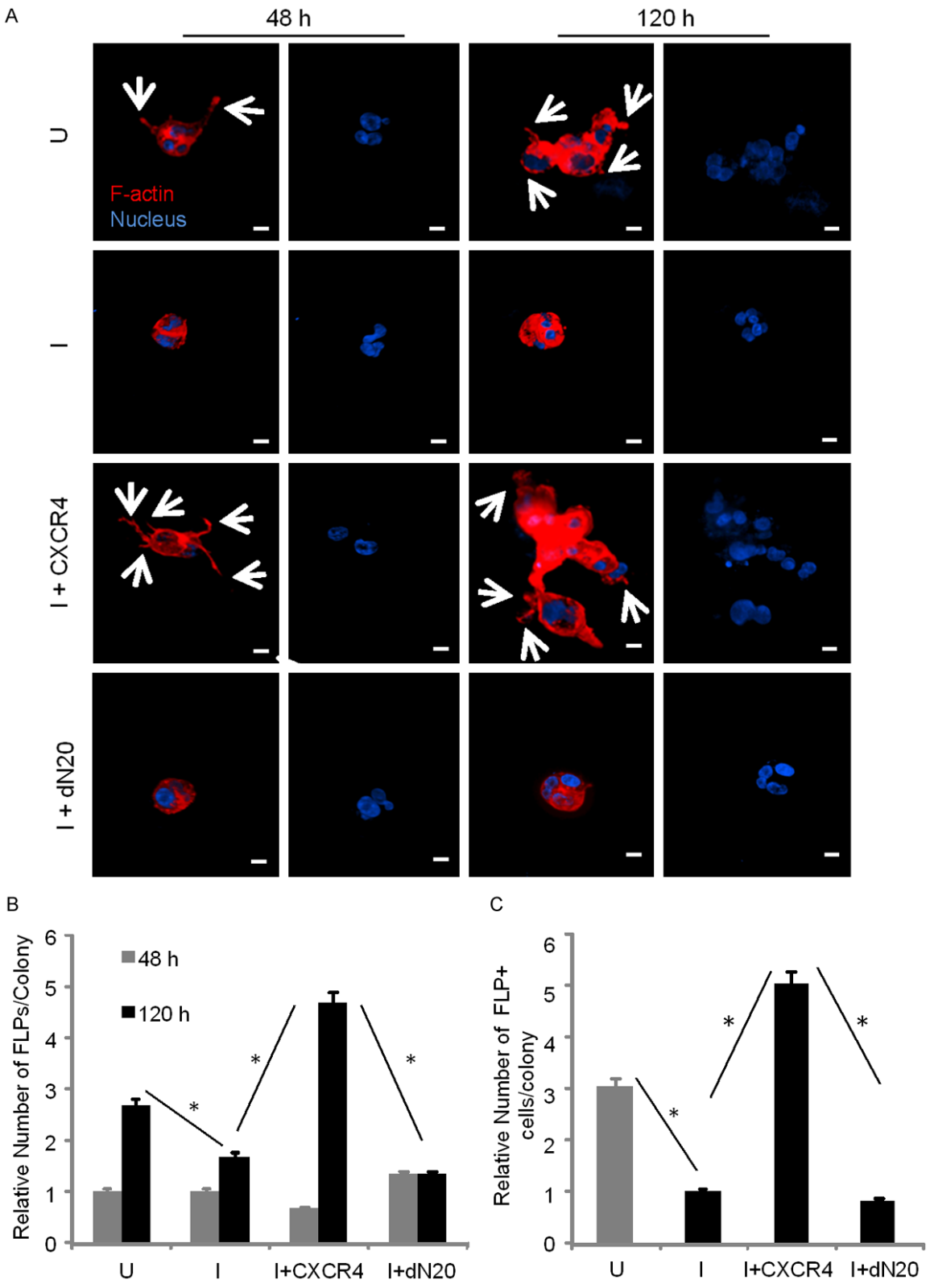

Figure 4. KLF8 expression is required for CXCR4-dependent FLP$^+$ cell colony growth in breast cancer cells in 3D culture. A. Overexpessing CXCR4 in breast cancer cells prevents FLP formation and proliferation from inhibition by silencing KLF8 expression. The 231-K8ikd cells overexpressing wild-type CXCR4 (CXCR4), its inactive mutant (dN20) or neither were grown under the U or I conditions in the MoT 3D culture supplemented with CXCL12. Representative

confocal images of the stained colonies were taken after 48 h and 120 h of culture. Arrows are pointed to FLPs. The scale bars are 10 μm with a magnification factor of 630×. B, C. Quantification and analysis of FLPs and Cells in the colonies are shown. *P < 0.01.

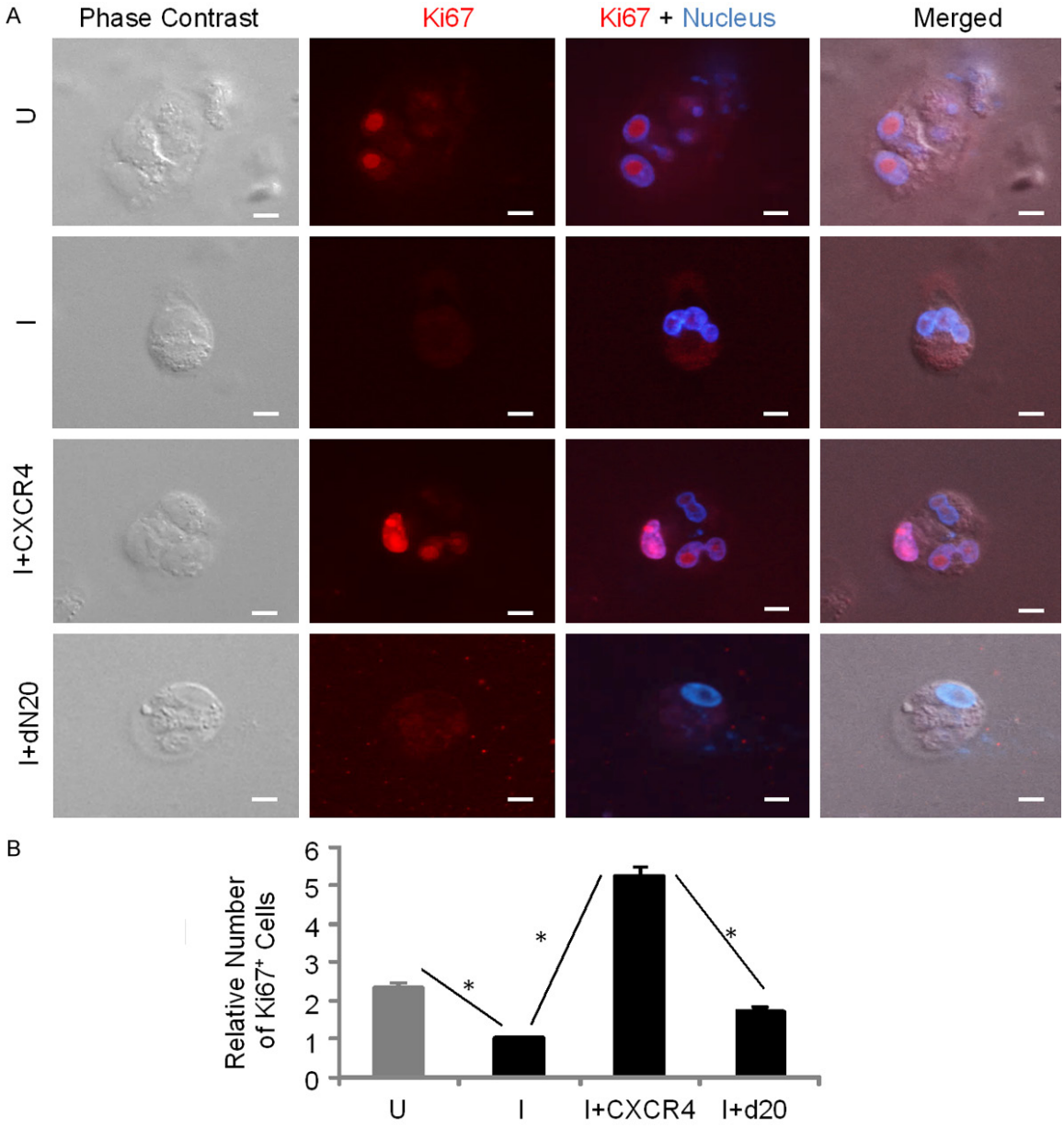

Figure 5. KLF8 expression is required for CXCR4-dependent proliferation in breast cancer cells in 3D culture. A. Overexpressing CXCR4 in breast cancer cells prevents cell proliferation from inhibition by silencing KLF8 expression. The 231-K8ikd cells overexpressing wild-type CXCR4 (CXCR4), its inactive mutant (dN20) or neither were grown under the U or I conditions in the MoT 3D culture supplemented with CXCL12. Representative phase contrast and confocal microscopic images of the stained colonies were taken after 48 h of culture. The scale bars are 10 μm with a magnification factor of 630×. B. Quantification and analysis of the proliferation marker protein Ki67$^+$ cells in the colonies are shown. $^*P < 0.01$.

an important role for invasive breast cancer tumor progression in the primary location [30]. Indeed, the KLF8-CXCR4 signaling axis is required for the primary tumor formation of the 231-K8ikd cells [8]. To mimic chemotherapeutic over-induction of KLF8-CXCR4 signaling, we

stably overexpressed KLF8 or CXCR4 in the cells. These cell lines do form more extensive FLPs in the MoT 3D culture [13]. Both CXCR4 and CXCL12 are extremely well conserved between human and mouse which allows the CXCR4-CXCL12 signaling to function across the species [13, 30, 31]. Indeed, these cell lines expressing human CXCR4 or KLF8 metastasize preferentially into the murine CXCL12-rich tissues [13]. To test if these cell lines outgrow more malignantly in the primary tumor location, we implanted the cells orthotopically, monitored the tumor growth rate in the mammary gland and analyzed the invasiveness and proliferation rate of the cells (**Figure 6**). We found that the KLF8 or CXCR4 overexpressing cells expanded to form tumors even faster (**Figure 6A-C**) with significantly enhanced invasiveness, as indicated by the severely lost basement membranes in the tumors labeled with GFP expression, and proliferation rate as reflected by the Ki67 staining results (**Figure 6D**). Consistently, previous reports have shown that the engagement of CXCR4 by CXCL12 *in vivo* is critical for breast tumor growth [30] and tissue-specific metastasis [7] in general and is a key contributor downstream of KLF8 to the aggressive breast tumor growth and metastasis in particular [8, 13]. These results strongly support a likely role of KLF8-CXCR4 signaling axis for boosting FLP formation and potentially subsequent invasive outgrowth of recurrent tumors following chemotherapeutic treatment [13].

Activation of Focal adhesion kinase (FAK) at FLPs is critical for cancer cell proliferation in 3D culture

Recently, FAK signaling has been suggested to play a role in FLP-dependent outgrowth of human breast cancer cells [3, 4]. In addition, several reports have indicated that engagement of CXCR4 by CXCL12 leads to FAK activation including our recently reported feed-forward signaling loop of KLF8-CXCR4/CXCL12-FAK-KLF8 in the human breast cancer cells used in this study [8]. To determine if FAK plays any part in the FLP formation and proliferation in the breast cancer cells under the MoT 3D culture conditions, we first stained the 231-K8ikd cell colonies using an antibody specific for the active form of FAK. We found that the active FAK proteins were highly enriched at the FLPs as early as 48 hours of culture which could be prevented by silencing KLF8 and protected by overexpressing the wild-type CXCR4 but not its inactive mutant dN20 (**Figure 7A**). The cells in the colonies stopped proliferating as indicated by the lost expression of the proliferation marker protein Ki67 upon treatment with the FAK-specific inhibitor PF228 (**Figure 7B** and **7C**). These results suggest that the active FAK serves as a crucial link FLPs to the cell proliferation and outgrowth promoted by KLF8-CXCR4 signaling axis.

Discussion

Our study demonstrates that KLF8-CXCR4 signaling axis is a critical player for FLP formation and proliferation in breast cancer cells. The KLF8-CXCR4 signaling axis seems to be indispensable for the FLP formation in the human breast cancer cells tested given that silencing KLF8 expression or blocking CXCR4 from binding to its ligand CXCL12 can almost completely prevent the FLP formation and proliferation. Our findings strongly suggest that the formation of FLPs is likely one of the important factors that mediate the function of the signaling cascade in the malignant proliferation of breast cancer cells and invasive tumor outgrowth.

Our results also indicate that it is the CXCR4 receptor that mediates the CXCL12-stimuated FLP formation downstream of KLF8. It is known that CXCL12 serves as a cytokine ligand not only for CXCR4 but also for CXCR7 [7, 9]. Yes, both the receptors are expressed in the breast cancer cells tested in this study. However, our results showed that the CXCR4-specific antagonist AMD3100 can completely inhibit the FLP formation in the cells. In addition, overexpression of CXCR4 not only can recapitulate the role of KLF8 in promoting FLP formation, proliferation and the invasive outgrowth under the 3D culture conditions as well as in the mice, but also can totally safeguard these behaviors of the breast cancer cells regardless of KLF8 knockdown. Consistently, CXCR4 is one of the primary target downstream genes of KLF8 as demonstrated in our previous report [8] and one of the most highly regulated genes by KLF8 as shown in our gene profiling studies that did not indicate any regulation of CXCR7 by KLF8 [32]. Therefore, we believe that the role of KLF8 in promoting the CXCR12-dependet FLP formation is CXCR4-dependent, although we do not

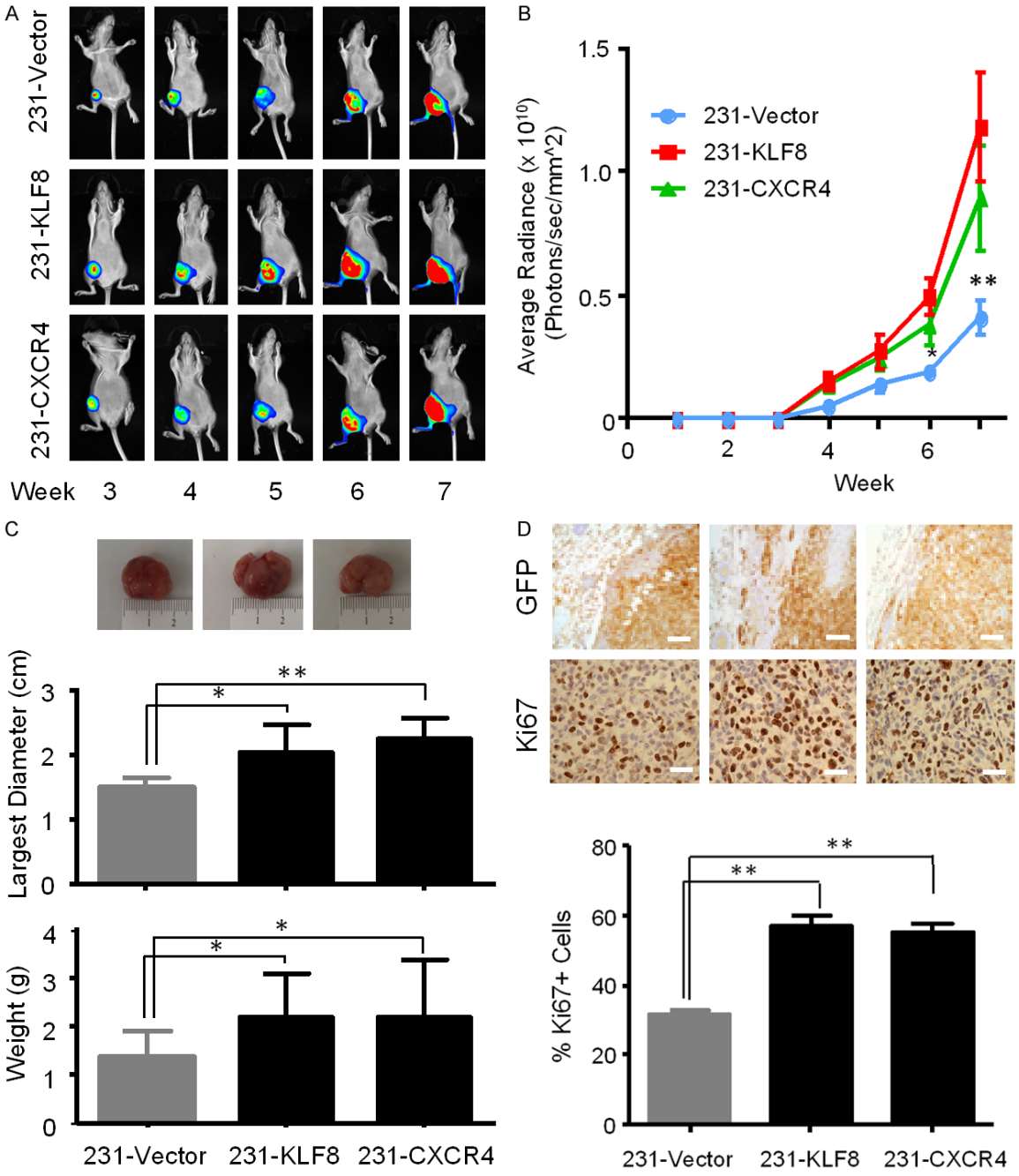

Figure 6. Overexpressing KLF8 or CXCR4 enhances the invasive outgrowth at the primary tumor location. A, B. The MDA-MB-231 cells overexpressing KLF8, CXCR4 or the empty vector were implanted into the mammary fat pad of immunocompromised mice. Representative weekly BLI images of whole mice and bioluminescent intensities in the tumors were shown. C. Tumor sizes after 7 weeks were measured as largest diameter and weight. D. The tumors after 7 weeks were processed for IHC staining for green fluorescent protein (GFP) harbored in the cancer cells and the proliferation marker protein Ki67. Representative IHC images as well as statistical analysis results for cell proliferation are shown. The scale bars are 100 μm with a magnification factor of 100× (GFP) or 25 μm with a magnification factor of 400× (Ki67). $^{*}P < 0.05$; $^{**}P < 0.01$.

rule out a possibility that CXCR7 may also play a role in the regulation of FLP formation in a signaling pathway other than KLF8.

FAK is known to be a critical cancer promoting protein when aberrantly overexpressed and/or activated [33]. The FAK activity has recently

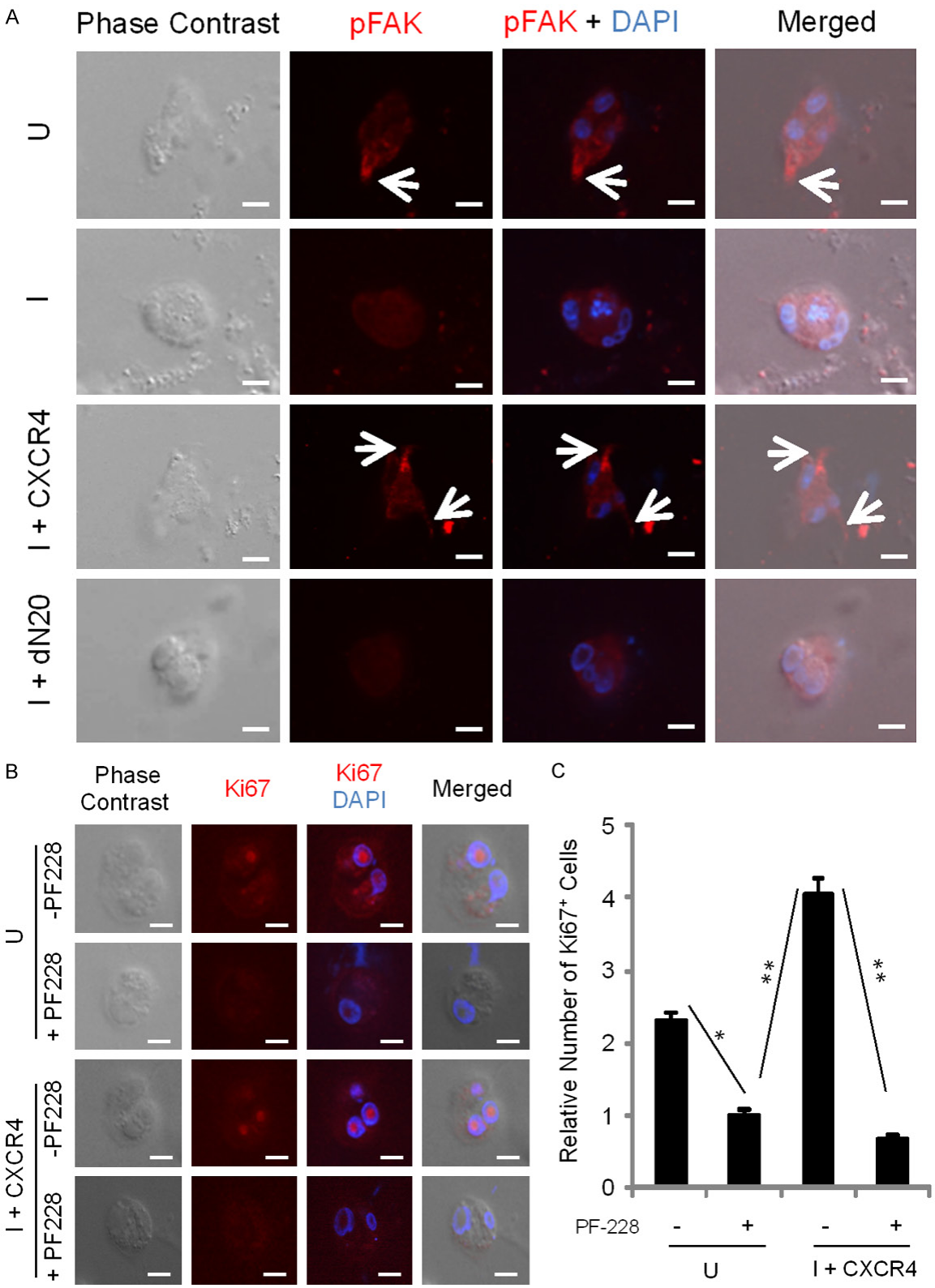

Figure 7. FAK activation at FLPs is critical for cancer cell proliferation in the MoT 3D culture. A. The 231-K8ikd cells overexpressing wild-type CXCR4 (CXCR4), its inactive mutant (dN20) or neither were grown under the U or I conditions in the MoT 3D culture supplemented with CXCL12. Representative phase contrast and confocal microscopic images of the colonies stained for active FAK (pFAK) and nuclei (DAPI) were taken after 48 h of culture. B, C. The 231-K8ikd cells overexpressing wild-type CXCR4 (CXCR4) or not were grown under the U or I conditions in the MoT

3D culture supplemented with CXCL12 along in the presence or absence of the FAK-specific inhibitor PF228. After 48 h of culture, phase contrast and confocal microscopic images of the colonies stained for proliferation (Ki67) and nuclei (DAPI) were taken. Representative images and statistical analysis of $Ki67^+$ cells are shown. The scale bars are 10 μm with a magnification factor of 630×. $^*P < 0.05$; $^{**}P < 0.01$.

been implicated also in promoting FLP-related outgrowth of cancer cells for colonization in the metastatic tumor site as well as invasive growth at the primary tumor site [4]. Intriguingly, forced or aberrant overexpression or overactivation of FAK as is in cancer cells leads to the upregulation of KLF8 [16, 17, 33]. Conversely, forced or aberrant overexpression of KLF8 in the cancer cells results in increased FAK activity when its downstream target CXCR4 meets the extracellular ligand CXCL12 [7]. Thus, it is not surprising that FAK plays a part in FLP associated cellular changes in the cancer cells that express aberrantly high levels of KLF8 and/or CXCR4 while located in a tissue rich for CXCL12. We are not sure exactly for the time being about the molecular and signaling changes that trigger the initiation of FLP formation in the KLF8 and CXCR4 overexpressing cancer cells in response to CXCL12 stimulation. Since the Rho family small GTPase proteins are among the most important regulators of actin cytoskeletal structures and functions in cancer cells [34], it will be interesting to look into a potential role of the small GTPase proteins such as RhoA and Cdc42, in addition to the RIF/mDia2 and ILK/β-Parvin/Cofilin signaling nodes, in initiating the FLP formation of the KLF8-CXCR4 signaling axis in the breast cancer cells.

Consistent with recent studies [3-5], this report along with our recent observation [13] indicates again that FLP formation is critical for invasive outgrowth of breast cancer cells in both primary and secondary tumor sites. This is particularly true when KLF8 or CXCR4 is forced to overexpress to the extent that recapitulates the patient situations when the expression of KLF8 and CXCR4 in the tumor tissues is over-induced by chemotherapeutic treatment [13]. These results provide a novel link between drug resistance and FLP formation both of which are potential driving forces of KLF8-CXCR4 signaling axis for the fatal recurrent outgrowth of breast cancer in multiple CXCR12-rich metastatic tumor sites as well as in the primary tumor location.

In summary, this study identifies KLF8 as a novel protein that promotes FLP formation in breast cancer cells and subsequent recurrence-like tumor outgrowth involving CXCR4 and FAK in the signaling cascade. Our results shed a new light on considering KLF8 as a potential therapeutic target during the course of chemotherapy to minimize the chance of breast cancer recurrence given that neither FAK-targeted strategy nor CXCR4-targeted strategy has been satisfactory due to the tumor specificity issue derived from their extensive expression in normal tissues.

Acknowledgements

This work was supported by an NIH grant (CA132977), a Florida Breast Cancer Foundation grant, the LIFE@UCF Richard Tucker Grant and an institutional grant to J.Z.

Disclosure of conflict of interest

None.

Address correspondence to: Jihe Zhao, Burnett School of Biomedical Sciences, University of Central Florida College of Medicine, 6900 Lake Nona Boulevard, Orlando, FL 32827, USA. Tel: 407-266-7099; Fax: 407-266-7002; E-mail: Jihe.Zhao@ucf.edu

References

[1] Barney LE, Jansen LE, Polio SR, Galarza S, Lynch ME and Peyton SR. The predictive link between matrix and metastasis. Curr Opin Chem Eng 2016; 11: 85-93.

[2] Gupta GP and Massague J. Cancer metastasis: building a framework. Cell 2006; 127: 679-695.

[3] Shibue T, Brooks MW, Inan MF, Reinhardt F and Weinberg RA. The outgrowth of micrometastases is enabled by the formation of filopodium-like protrusions. Cancer Discov 2012; 2: 706-721.

[4] Shibue T, Brooks MW and Weinberg RA. An integrin-linked machinery of cytoskeletal regulation that enables experimental tumor initiation and metastatic colonization. Cancer Cell 2013; 24: 481-498.

[5] Shibue T and Weinberg RA. Metastatic colonization: settlement, adaptation and propagation of tumor cells in a foreign tissue environment. Semin Cancer Biol 2011; 21: 99-106.

[6] Shibue T and Weinberg RA. Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. Proc Natl Acad Sci U S A 2009; 106: 10290-10295.
[7] Mukherjee D and Zhao J. The role of chemokine receptor CXCR4 in breast cancer metastasis. Am J Cancer Res 2013; 3: 46-57.
[8] Mukherjee D, Lu H, Yu L, He C, Lahiri SK, Li T and Zhao J. Kruppel-like factor 8 activates the transcription of C-X-C cytokine receptor type 4 to promote breast cancer cell invasion, transendothelial migration and metastasis. Oncotarget 2016; 7: 23552-23568.
[9] Cojoc M, Peitzsch C, Trautmann F, Polishchuk L, Telegeev GD and Dubrovska A. Emerging targets in cancer management: role of the CXCL12/CXCR4 axis. Onco Targets Ther 2013; 6: 1347-1361.
[10] Bleul CC, Farzan M, Choe H, Parolin C, Clark-Lewis I, Sodroski J and Springer TA. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature 1996; 382: 829-833.
[11] Balkwill F. Cancer and the chemokine network. Nat Rev Cancer 2004; 4: 540-550.
[12] Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q and Szallasi Z. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat 2010; 123: 725-731.
[13] Hao J, Lu H, Mukherjee D, Yu L and Zhao J. Role of kruppel-like factor 8 for therapeutic drug-resistant multi-organ metastasis of breast cancer. Am J Cancer Res 2021; 11: 2188-2201.
[14] Lu H, Hu L, Li T, Lahiri S, Shen C, Wason MS, Mukherjee D, Xie H, Yu L and Zhao J. A novel role of Kruppel-like factor 8 in DNA repair in breast cancer cells. J Biol Chem 2012; 287: 43720-43729.
[15] Wang X, Lu H, Urvalek AM, Li T, Yu L, Lamar J, DiPersio CM, Feustel PJ and Zhao J. KLF8 promotes human breast cancer cell invasion and metastasis by transcriptional activation of MMP9. Oncogene 2011; 30: 1901-1911.
[16] Lahiri SK and Zhao J. Kruppel-like factor 8 emerges as an important regulator of cancer. Am J Transl Res 2012; 4: 357-363.
[17] Zhao J, Bian ZC, Yee K, Chen BP, Chien S and Guan JL. Identification of transcription factor KLF8 as a downstream target of focal adhesion kinase in its regulation of cyclin D1 and cell cycle progression. Mol Cell 2003; 11: 1503-1515.
[18] Wang X and Zhao J. KLF8 transcription factor participates in oncogenic transformation. Oncogene 2007; 26: 456-461.
[19] Wang X, Zheng M, Liu G, Xia W, McKeown-Longo PJ, Hung MC and Zhao J. Kruppel-like factor 8 induces epithelial to mesenchymal transition and epithelial cell invasion. Cancer Res 2007; 67: 7184-7193.
[20] Wang X, Lu H, Li T, Yu L, Liu G, Peng X and Zhao J. Kruppel-like factor 8 promotes tumorigenic mammary stem cell induction by targeting miR-146a. Am J Cancer Res 2013; 3: 356-373.
[21] Lu H, Hu L, Yu L, Wang X, Urvalek AM, Li T, Shen C, Mukherjee D, Lahiri SK, Wason MS and Zhao J. KLF8 and FAK cooperatively enrich the active MMP14 on the cell surface required for the metastatic progression of breast cancer. Oncogene 2014; 33: 2909-2917.
[22] Li T, Lu H, Mukherjee D, Lahiri SK, Shen C, Yu L and Zhao J. Identification of epidermal growth factor receptor and its inhibitory microRNA141 as novel targets of Kruppel-like factor 8 in breast cancer. Oncotarget 2015; 6: 21428-42.
[23] Li T, Lu H, Shen C, Lahiri SK, Wason MS, Mukherjee D, Yu L and Zhao J. Identification of epithelial stromal interaction 1 as a novel effector downstream of Kruppel-like factor 8 in breast cancer invasion and metastasis. Oncogene 2014; 33: 4746-4755.
[24] Ganguly S, Basu B, Shome S, Jadhav T, Roy S, Majumdar J, Dasgupta PS and Basu S. Dopamine, by acting through its D2 receptor, inhibits insulin-like growth factor-I (IGF-I)-induced gastric cancer cell proliferation via up-regulation of Kruppel-like factor 4 through down-regulation of IGF-IR and AKT phosphorylation. Am J Pathol 2010; 177: 2701-2707.
[25] Minn AJ, Gupta GP, Padua D, Bos P, Nguyen DX, Nuyten D, Kreike B, Zhang Y, Wang Y, Ishwaran H, Foekens JA, van de Vijver M and Massague J. Lung metastasis genes couple breast tumor size and metastatic spread. Proc Natl Acad Sci U S A 2007; 104: 6740-6745.
[26] Minn AJ, Kang Y, Serganova I, Gupta GP, Giri DD, Doubrovin M, Ponomarev V, Gerald WL, Blasberg R and Massague J. Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. J Clin Invest 2005; 115: 44-55.
[27] Pusic I and DiPersio JF. Update on clinical experience with AMD3100, an SDF-1/CXCL12-CXCR4 inhibitor, in mobilization of hematopoietic stem and progenitor cells. Curr Opin Hematol 2010; 17: 319-326.
[28] Nelson CM and Bissell MJ. Modeling dynamic reciprocity: engineering three-dimensional culture models of breast architecture, function, and neoplastic transformation. Semin Cancer Biol 2005; 15: 342-352.
[29] Petersen OW, Ronnov-Jessen L, Howlett AR and Bissell MJ. Interaction with basement membrane serves to rapidly distinguish growth

and differentiation pattern of normal and malignant human breast epithelial cells. Proc Natl Acad Sci U S A 1992; 89: 9064-9068.
[30] Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL and Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 2005; 121: 335-348.
[31] Costa MJ, Kudaravalli J, Liu WH, Stock J, Kong S and Liu SH. A mouse model for evaluation of efficacy and concomitant toxicity of anti-human CXCR4 therapeutics. PLoS One 2018; 13: e0194688.
[32] Zhao J. KLF8: so different in ovarian and breast cancer. Oncoscience 2014; 1: 248-249.
[33] Zhao J and Guan JL. Signal transduction by focal adhesion kinase in cancer. Cancer Metastasis Rev 2009; 28: 35-49.
[34] Haga RB and Ridley AJ. Rho GTPases: regulation and roles in cancer cell biology. Small GTPases 2016; 7: 207-221.