Oncogene
https://doi.org/10.1038/s41388-017-0036-8

ARTICLE

Breast cancer metastasis to liver and lung is facilitated by Pit-1-CXCL12-CXCR4 axis

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Received: 17 July 2017 / Revised: 13 October 2017 / Accepted: 31 October 2017

Abstract

Development of human tumors is driven by accumulation of alterations in tumor suppressor genes and oncogenes in cells. The POU1F1 transcription factor (also known Pit-1) is expressed in the mammary gland and its overexpression induces profound phenotypic changes in proteins involved in breast cancer progression. Patients with breast cancer and elevated expression of Pit-1 show a positive correlation with the occurrence of distant metastasis and poor overall survival. However, some mediators of Pit-1 actions are still unknown. Here, we show that CXCR4 chemokine receptor and its ligand CXCL12 play a critical role in the pro-tumoral process induced by Pit-1. We found that Pit-1 increases mRNA and protein in both CXCR4 and CXCL12. Knock-down of CXCR4 reduces tumor growth and spread of Pit-1 overexpressing cells in a zebrafish xenograft model. Furthermore, we described for the first time pro-angiogenic effects of Pit-1 through the CXCL12-CXCR4 axis, and that extravasation of Pit-1 overexpressing breast cancer cells is strongly reduced in CXCL12-deprived target tissues. Finally, in breast cancer patients, expression of Pit-1 in primary tumors was found to be positively correlated with CXCR4 and CXCL12, with specific metastasis in liver and lung, and with clinical outcome. Our results suggest that Pit-1-CXCL12-CXCR4 axis could be involved in chemotaxis guidance during the metastatic process, and may represent prognostic and/or therapeutic targets in breast tumors.

Introduction

Metastasis is the cause of almost all breast cancer deaths. The spread and growth of tumor cells to distant organs represents the most devastating hallmark of cancer [1]. A notable feature of this multistep and highly organized process is the variation in metastatic tissue tropism displayed by different types of cancer cells. Secondary growth in breast cancer commonly occurs in regional lymph nodes, bone marrow, lung, brain, and liver [2, 3]. One of the reasons for this non-random affinity is the chemokine expression pattern by target organs and their cognate receptor by breast cancer cells [4–6]. Chemokines are a group of 8–10 kDa chemotactic cytokines with the ability to bind G-protein-coupled receptors that play a pivotal role in a wide range of physiological and pathological processes [6]. CXCL12 (also known as SDF-1) belongs to the CXC chemokines family that signals through CXCR4 and CXCR7 receptors. Among other functions, this axis regulates cell migration during organogenesis, leukocyte trafficking, and mobilization of hemopoietic stem cells [7]. Müller et al. [4] have demonstrated that CXCR4 is highly

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Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41388-017-0036-8) contains supplementary material, which is available to authorized users.

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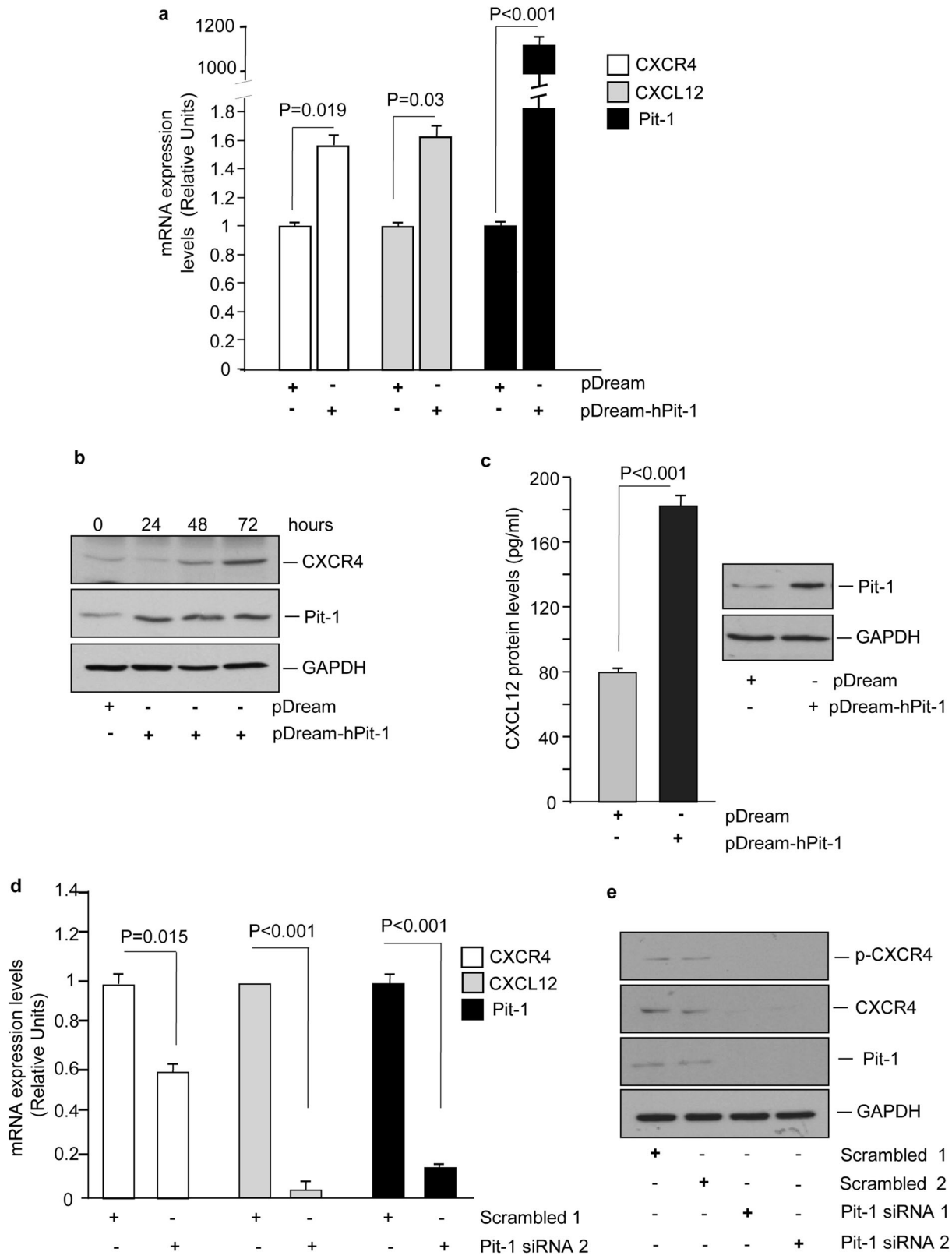
Published online: 11 January 2018



◀ **Fig. 1** Pit-1 regulates CXCR4 and CXCL12 expression. **a** MCF-7 cells were transfected with the pDream-hPit-1 overexpression vector or the pDream as control, and 48 h later a real-time PCR was carried out to evaluate CXCR4, CXCL12, and Pit-1 mRNA expression. **b** Western blot of CXCR4, Pit-1, and GAPDH (as loading control) in MCF-7 cells 24, 48, and 72 h after Pit-1 overexpression. **c** ELISA of CXCL12 in culture medium of MCF-7 cells 48 h after Pit-1 overexpression. Western blot of Pit-1 used as ELISA assay control. **d** Real-time PCR of MCF-7 cells 48 h after Pit-1 knock-down shows a decrease of CXCR4 and CXCL12 mRNA expression. **e** Western blot of phosphorylated (p)-CXCR4, CXCR4, Pit-1, and GAPDH in MCF-7 cells 48 h after Pit-1 knock-down

expressed in breast cancer compared to normal breast tissue. Moreover, breast cancer CXCR4 expressing cells are attracted by chemotaxis to tissues with high CXCL12 expression, which coincidentally are the most common organs of breast cancer metastasis [4, 5]. Recent studies suggest not only a critical role of CXCL12-CXCR4 in organ-specific metastases of breast cancer, but also in regulation of more steps of breast cancer progression, including proliferation, angiogenesis, and modulation of tumor microenvironment [8–10].

The POU class 1 homeobox 1 transcription factor (POU1F1, also known as Pit-1) was originally described in the pituitary gland, where it regulates cell differentiation during organogenesis and acts as an activator for pituitary gene transcription [11, 12]. Pit-1 is also expressed in human breast cell lines and tissue, including normal and tumor cells. However, Pit-1 expression is higher in human breast tumors compared to normal breast and its overexpression promotes tumor growth and metastasis [13–16]. In addition, elevated Pit-1 expression in patients with breast cancer positively correlated with the occurrence of distant metastasis and poor overall survival [16, 17].

Using human breast cancer cell lines for in vitro assays and a zebrafish model for in vivo studies, the present study evaluates CXCR4 and CXCL12 regulation by Pit-1, and the role of Pit-1-CXCL12-CXCR4 axis in cancer progression. In addition, human breast carcinomas samples were analyzed for Pit-1 and CXCR4 mRNA and protein expression, and these data were correlated with organ-specific breast cancer metastases and clinical outcome.

Results

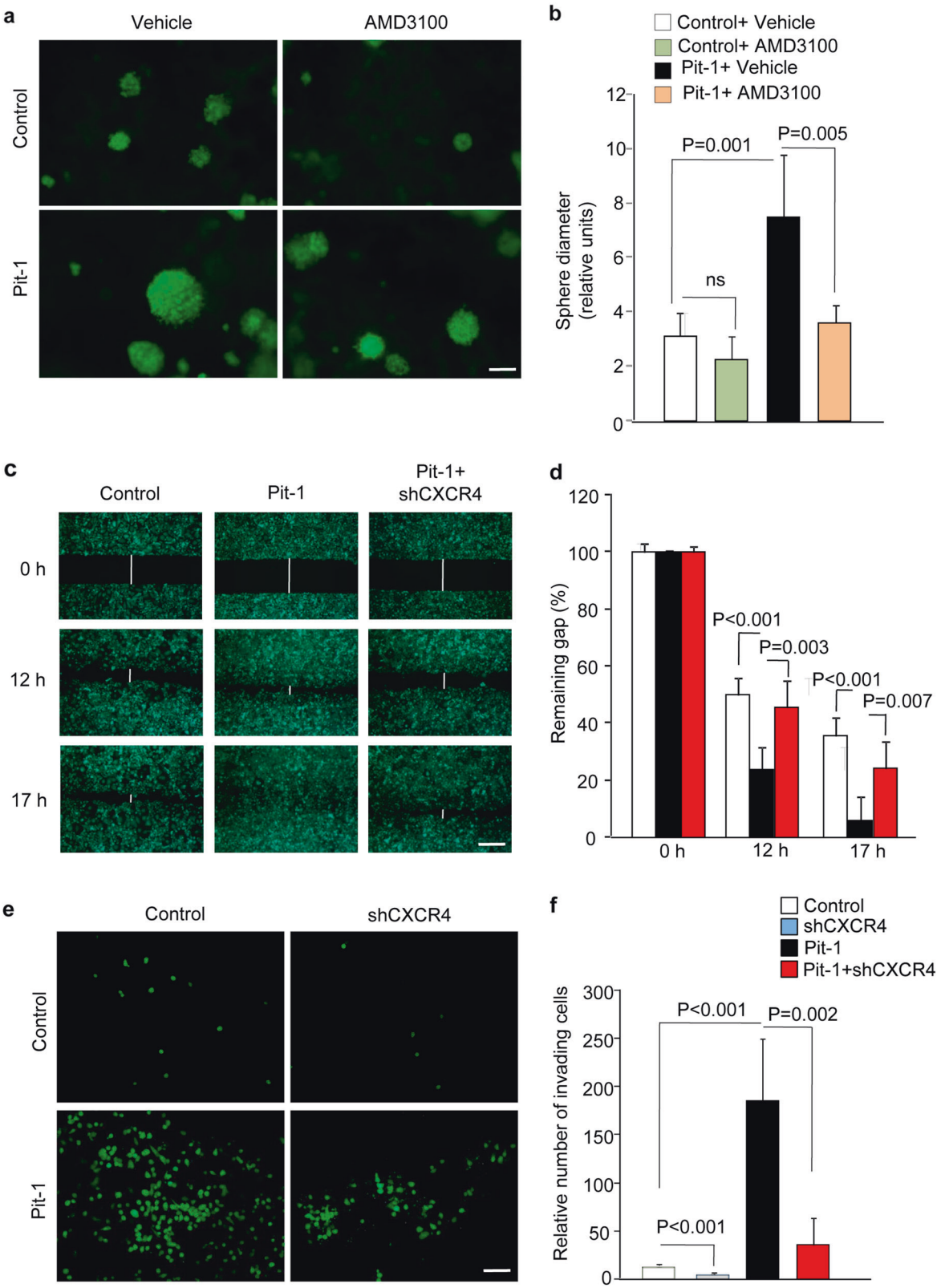
Pit-1 regulates the CXCL12 chemokine and its CXCR4 receptor and mediates tumor growth, migration, and invasion

To evaluate the effect of Pit-1 on CXCR4 and CXCL12, MCF-7 cells were transiently transfected with a Pit-1 overexpression vector. CXCR4 and CXCL12 mRNA and protein levels were determined by quantitative PCR (qPCR), western blot, and enzyme-linked immunosorbent assay (ELISA). Our data show that 48 h after Pit-1 overexpression both CXCR4 and CXCL12 significantly increase their levels as compared to baseline (Fig. 1a–c). Similar data were obtained in the human breast adenocarcinoma SKBR-3 (HER2 +) and MDA-MB-231 (basal-like) cell lines (Supplementary Fig. 1a–c). On the other hand, Pit-1 knock-down significantly reduces CXCR4 and CXCL12 mRNA, and CXCR4 and phosphorylated CXCR4 (p-CXCR4) protein expression in MCF-7 cells (Fig. 1d–e). To further evaluate the role of Pit-1-CXCL12-CXCR4 axis in cancer progression, we carried out a three-dimensional (3D) assay to evaluate tumor growth, as well as a migration assay and an invasion assay. MCF-7-GFP cells were infected with lentiviral activation particles to induce high Pit-1 expression (MCF-7-GFP-Pit-1) and then loaded into matrigel. After 7 days, cells were treated every 48 h with either AMD3100 (a known CXCR4 antagonist) or a vehicle for 5 days. Our results show that CXCR4 blockade significantly ($P = 0.005$) reduces sphere diameter (Fig. 2a, b, and Supplementary Fig. 2a). Wound-healing assay demonstrates that Pit-1 significantly ($P < 0.001$) increases migration of MCF-7-GFP cells at 12 and 17 h as compared to control cells, but CXCR4 knock-down significantly ($P = 0.007$) reduces cell migration with respect to Pit-1 overexpression alone (Fig. 2c, d and Supplementary Fig. 2b). Similarly, transwell invasion assays show a significant ($P = 0.002$) decrease of invading tumor cells after CXCR4 knock-down in MCF-7-GFP-Pit-1 cells (Fig. 2e, f and Supplementary Fig. 2c). In order to corroborate the in vitro effects in vivo, a zebrafish xenograft model was carried out to evaluate tumor growth and spread. MCF-7-GFP cells either (a) infected with control lentiviral activation particles + transfected with the pLKO control vector (MCF-7-GFP-control), (b) infected with Pit-1 lentiviral activation particles + transfected with the pLKO control vector, (MCF-7-GFP-Pit-1), or (c) infected with Pit-1 lentiviral activation particles + transfected with the pLKO-CXCR4 shRNA vector that knocked-down endogenous CXCR4 (MCF-7-GFP-Pit-1-shCXCR4) were injected into the yolk sac of zebrafish embryos and fluorescence intensity and area were measured 48 h later. Our data indicate a significant ($P = 0.021$) increase in tumor growth as well as in cell spread ($P = 0.003$) after Pit-1 overexpression with respect to MCF-7-GFP control cells. However, CXCR4 knock-down significantly reduces tumor growth ($P = 0.002$) and cell spread ($P < 0.001$) in relation to MCF-7-GFP-Pit-1 cells (Fig. 3a–c).

CXCL12/CXCR4 are mediators of Pit-1-induced angiogenesis

To study the role of Pit-1-CXCL12-CXCR4 axis in cancer progression, we focus on angiogenesis. First, we carried out a western blot of MCF-7 cell extract before and after Pit-1 overexpression to evaluate phosphorylation and expression of proteins involved in multiple physiological and pathological processes, including angiogenesis. As shown in Fig. 4a, clear activation of phosphorylated (p)-CXCR4, p-AKT, p-JNK, p-ERK1/2, p-P38, and total VEGF-A protein expression were observed. Then, and given the well-known effects of the vascular endothelial growth factor A (VEGF-A) on endothelial cell growth and angiogenesis, VEGF-A mRNA levels were evaluated by qPCR in MCF-7 cells after Pit-1 overexpression and/or CXCR4 knock-down. Pit-1 significantly ($P = 0.001$) raises CXCR4 and VEGF-A

mRNA, while CXCR4 knock-down significantly ($P < 0.001$) reduces VEGF-A mRNA levels (Fig. 4b). After Pit-1 overexpression and CXCR4 knock-down, a significant ($P = 0.007$) decrease in VEGF-A mRNA was seen with respect to Pit-1 overexpression alone (Fig. 4b). To clarify the functional role of Pit-1-CXCL12-CXCR4 axis in tube



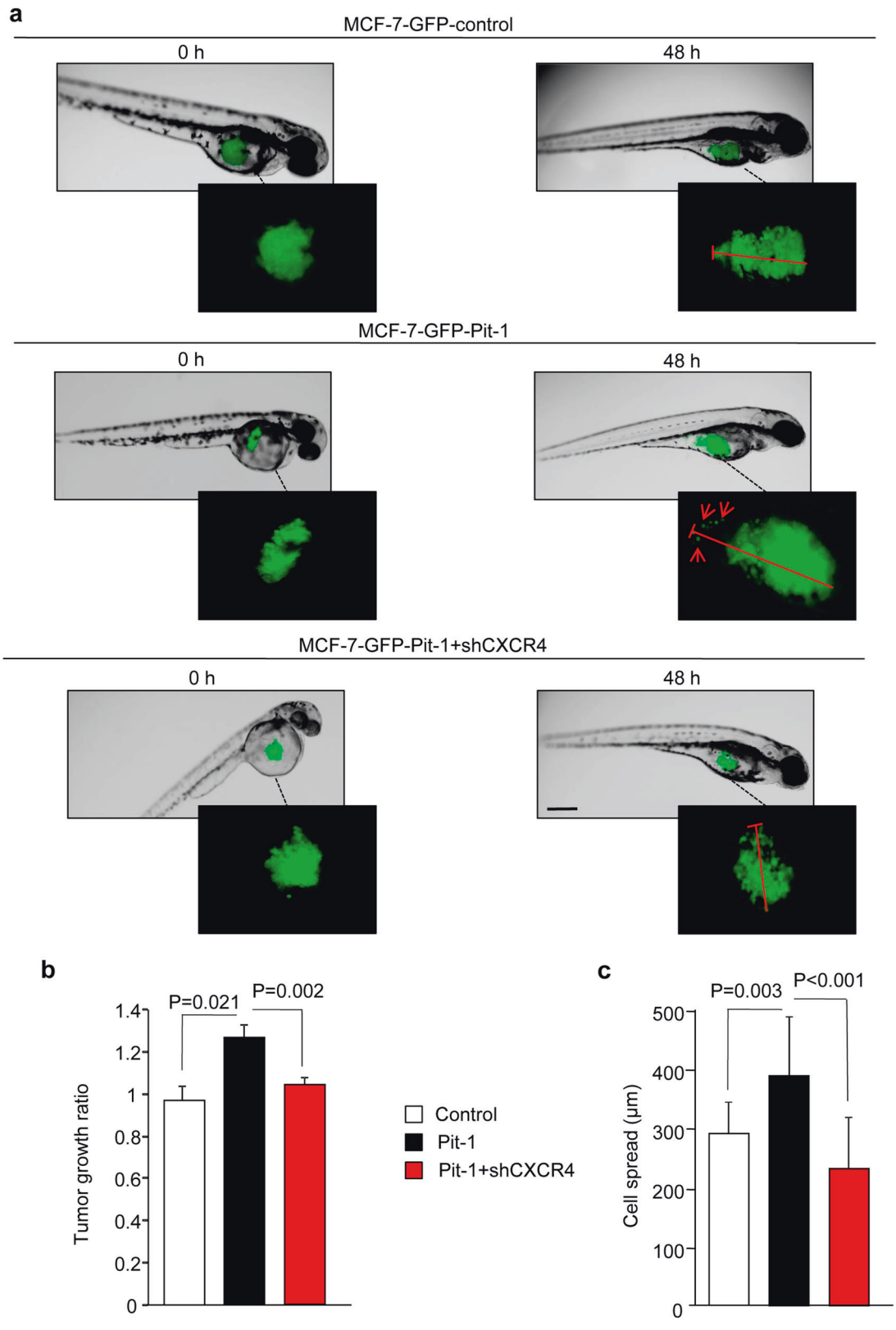
◀ **Fig. 2** CXCR4 blockade reduces pro-oncogenic features induced by Pit-1 overexpression in MCF-7 cells. **a** Representative figure of three-dimensional (3D) cultures of control cells (MCF-7-GFP-control, upper panel) and Pit-1 overexpressed cells (MCF-7-GFP-Pit-1, lower panel) treated every 48 h for 5 days with vehicle (left) or AMD3100 (right). Scale bar: 100 µm. **b** Quantitation of three independent experiments as described in **a**. Numbers represent mean±SD. **c** Representative figure of wound healing assay of control cells (MCF-7-GFP-control), Pit-1 overexpressed cells (MCF-7-GFP-Pit-1), and MCF-7-GFP-Pit-1 overexpressed and CXCR4 knocked-down cells (Pit-1 + shCXCR4). Distance between the wound edges was measured at 0, 12, and 17 h. Scale bar: 150 µm. **d** Quantitative analyses of three experiments as described in **c**. Numbers represent mean±SD. **e** Representative figure of cell invasion in MCF-7-GFP-control cells, Pit-1 overexpressed cells (Pit-1), CXCR4 knocked-down cells (shCXCR4), and Pit-1 overexpressed and CXCR4 knocked-down cells (Pit-1 + shCXCR4) Scale bar: 75 µm. **f** Quantitative analyses of three experiments as described in **e**. Numbers represent mean±SD

formation, HUVEC cells were cultured for 6 h with conditioned medium (CM) obtained from: (a) control MCF-7-GFP cells (CM-control), (b) MCF-7-GFP cells after knock-down of CXCR4 (CM-shCXCR4), (c) MCF-7-GFP cells overexpressing Pit-1 (CM-Pit-1), (d) MCF-7-GFP cells overexpressing Pit-1 and immunoprecipitation (IP) of CXCL12 (CM-Pit-1-IP-CXCL12), (e) MCF-7-GFP cells overexpressing Pit-1 and CXCR4 knock-down (CM-Pit-1-shCXCR4), and (f) MCF-7-GFP cells overexpressing Pit-1, CXCR4 knock-down, and IP of CXCL12 (CM-Pit-1-shCXCR4-IP-CXCL12). Our data show a significant ($P < 0.001$) increase in total number of junctions and vessels percentage area in cells cultured with CM-Pit-1 (Fig. 4c–d). However, CM-Pit-1-IP-CXCL12, CM-Pit-1-shCXCR4, and CM-Pit-1-shCXCR4-IP-CXCL12 significantly decreased total number of junctions ($P < 0.001$, $P = 0.001$, and $P < 0.001$, respectively) and vessels percentage area ($P < 0.001$, $P = 0.007$, and $P < 0.001$, respectively) as compared to HUVEC cells cultured with CM-Pit-1 (Fig. 4c–d). In fact, proliferation of HUVEC cells significantly ($P = 0.008$) increase after culture with CM-Pit-1 in relation to CM-control cells, but significantly ($P = 0.045$) decreases after culture with CM-Pit-1 + IP-CXCL12 cells (Fig. 4e). Western blot of HUVEC cells show a clear increase in CXCR4, P38, and AKT phosphorylation after treatment for 30 min with CXCL12 and with CM-MCF-7-Pit-1, which diminishes after IP-CXCL12 (Fig. 4f). All data suggest that the effects of Pit-1 in angiogenesis could be mediated by this chemokine.

Pit-1 induces extravasation of tumor cells toward tissues with elevated levels of CXCL12 and is correlated with metastasis in liver and lung in human breast tumors

Given that our data seems to indicate that the CXCL12-CXCR4 pathway mediated the Pit-1 effects in angiogenesis and breast cancer progression, we explore if mammary tumor cells with Pit-1 overexpression (MCF-7-GFP-Pit-1) have preference to metastasize in specific tissues. HUVEC cells were seeded in matrigel in the upper chamber of the transwell system for 24 h, and then MCF-7-GFP or MCF-7-GFP-Pit-1 cells were seeded on HUVEC cells. Mouse tissue extracts from liver, lung, brain, or muscle were added in the bottom chamber of wells before and after IP-CXCL12 (Fig. 5a). After 48 h, tumor cells that broke through HUVEC-cell/matrigel layer were counted. We found a significant increase of MCF-7-GFP-Pit-1 cells in liver ($P = 0.001$), lung ($P = 0.035$), and brain ($P = 0.04$) tissue extracts with respect to control MCF-7-GFP cells (Fig. 5b, c). However, IP-CXCL12 in liver and lung tissues (but not in brain or muscle) dramatically decreases the presence of MCF-7 Pit-1 cells ($P < 0.001$, $P = 0.004$, respectively, Fig. 5b, c).

To study the relationships between Pit-1, CXCL12, and CXCR4 in human tumors, 37 human breast tumors obtained from the frozen tissue of patients with breast cancer but without clinical follow-up were analyzed by qPCR to evaluate Pit-1, CXCR4, and CXCL12 mRNA. As shown in Fig. 6a–c, Pit-1 mRNA expression significantly ($P < 0.001$) correlated with CXCR4 and CXCL12 mRNA expression. Given the preference of tumor cells with high Pit-1 expression to in vitro target lung and liver tissues with high CXCL12 levels, primary breast tumor samples from patients with clinical follow-up either developing metastasis in lung and liver ($n = 24$) or without metastasis ($n = 24$) (Table 1) were analyzed by IHC to evaluate Pit-1 and CXCR4 protein expression (Fig. 6d, and Supplementary Fig. 3). Out of the total, 45 tumors (93.8%) were stained positively for Pit-1 and 33 (68.8%) were stained positively for CXCR4, showing clear differences with respect to intensity and percentage of cells stained. The mean score value for Pit-1 was 20 (range 0–140) and for CXCR4 was 20 (range 0–225). Distribution of Pit-1 and CXCR4 score values is shown in Supplementary Fig. 4a, b. We examined all possible score values obtained by IHC staining for Pit-1 and CXCR4 as cutoff points for predicting relapse-free survival (Supplementary Fig. 4a, b). For Pit-1, we found the optimal cutoff value to be 30 ($\chi^2 = 12.3$; $P < 0.001$). A subgroup of 13 patients (27.1%) presented scores above this threshold, and were at high risk for visceral metastasis. For CXCR4, we found the optimal cutoff point to be 40 ($\chi^2 = 4.2$; $P = 0.041$). A subgroup of 12 patients (25.0%) presented scores above this threshold and were at high risk of visceral metastasis. Figure 6e, f shows relapse-free survival curves for Pit-1 and CXCR4. In addition, using the online KM plotter (http://www.kmplot.com) [18] a significant relation was found between Pit-1 and CXCR4 mRNA expression with recurrence free survival (RFS, $P = 0.0015$, $n = 3951$) and PPS ($P = 0.0015$, $n = 414$) in breast cancer patients (Supplementary Fig. 5).


a
MCF-7-GFP-control
0 h
48 h
MCF-7-GFP-Pit-1
0 h
48 h
MCF-7-GFP-Pit-1+shCXCR4
0 h
48 h
b
P=0.021
P=0.002
Tumor growth ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Pit-1
Pit-1+shCXCR4
c
P=0.003
P<0.001
Cell spread (µm)
500
400
300
200
100
0

◀ **Fig. 3** CXCR4 knock-down in Pit-1 overexpressed MCF-7 cells reduces tumor growth and spreading in zebrafish embryos. **a** Representative images of tumor growth and cell spreading in zebrafish embryos ($n = 40$ per group) injected in yolk sac with control cells (MCF-7-GFP-control), Pit-1 overexpressing cells (MCF-7-GFP-Pit-1), and Pit-1 overexpressing + CXCR4 knocked-down cells (MCF-7-GFP-Pit-1 + shCXCR4) at 0 h post injection (hpi) and 48 hpi. Red line indicates cell spread, and red arrows in MCF-7-GFP-Pit-1 cells indicate early metastasis. Fish images are a superposition of a fluorescence field image over a bright field image. Scale bar: 250 µm **b** Tumor growth ratio in zebrafish embryos injected with the cells described in **a**. Data were obtained using the ZF tool software. **c** Cell spread (in µm) in zebrafish embryos injected with the cells described in **a**

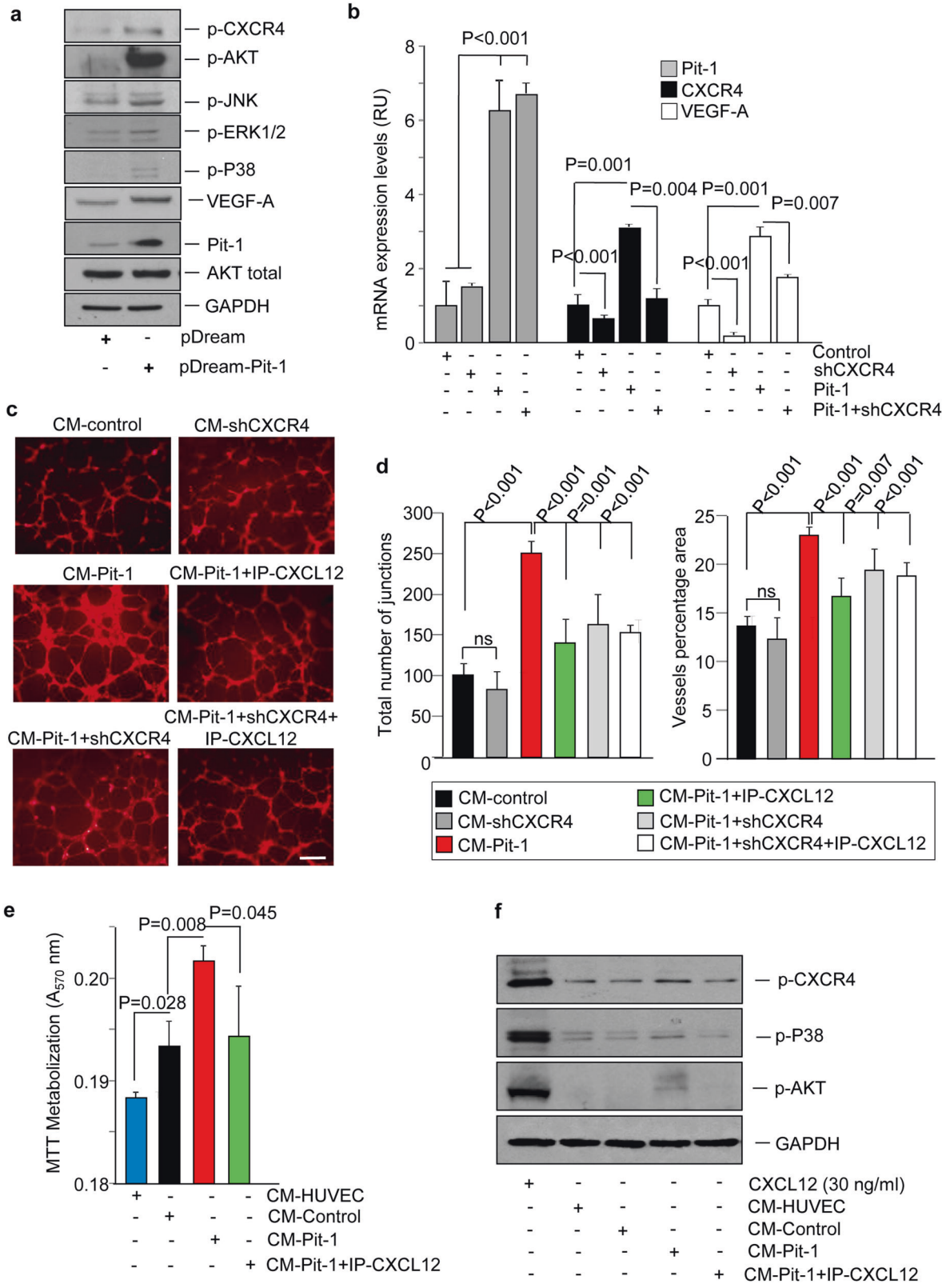
Discussion

In this study we found that Pit-1 regulates CXCL12 and CXCR4 in breast cancer cells. Our data indicated that CXCR4 knock-down in Pit-1 overexpressing breast cancer cells reduces tumor growth and cell spread. In addition, both CXCL12 and CXCR4 are involved in Pit-1-induced angiogenesis. Finally, in human breast tumor samples CXCL12 and CXCR4 positively correlated with Pit-1, and high Pit-1 expression in human breast tumors is related with metastasis in liver and lung as well as poor clinical outcome.

Our first aim was to evaluate the correlation between Pit-1 and CXCL12-CXCR4 expression in breast cancer cell lines. We demonstrated that Pit-1 overexpression or knock-down is accompanied by CXCL12 and CXCR4 increase or decrease, respectively, in breast cancer cell lines, suggesting that CXCL12 and CXCR4 regulation by Pit-1 is independent of breast cancer subtype. In fact, although it has been demonstrated that CXCR4 and CXCL12 expression seems to be higher in triple negative and HER-2 breast cancer subtypes compared with luminal subtype, this ligand and its receptor have not yet been associated with a specific breast cancer subtype [19, 20].

Our group previously showed that Pit-1 induced tumor growth and invasion in breast cancer [15, 16]. Therefore, we sought to determine whether these actions could be partially mediated by CXCL12-CXCR4 activation. 3D cultures of Pit-1 overexpressing cells show significant cell growth, which decreases after treatment with a CXCR4 inhibitor. Furthermore, CXCR4 knock-down in Pit-1 overexpressing cells reduces cell migration and invasion, suggesting an involvement of the CXCL12-CXCR4 axis in these actions. The CXCL12 chemokine and its CXCR4 receptor have remained well conserved throughout evolution [21]. As in humans, both are expressed in zebrafish during development and adult period, playing a key role in certain physiological processes such as primordial germ cell migration and brain development [22–24]. Pit-1 starts to be expressed in zebrafish at a much earlier stage of adenohypophysis development, indicating that both conserved and class-specific aspects of Pit-1 function during pituitary development in a variety of vertebrate species [25]. Given that endogenous CXCL12 in zebrafish activates CXCR4 signaling in human cells [26], we evaluated these effects in a zebrafish xenograft model in vivo. Our results demonstrate a significant decrease of tumor growth and spreading after CXCR4 knock-down in Pit-1-overexpressing cells. This is in line with recent studies demonstrating the role of CXCL12-CXCR4 in induction of cell proliferation, migration, and invasion in other animal models [6, 27, 28].

Increasing evidence suggests that CXCL12-CXCR4 axis plays a key role in angiogenesis and that two mechanisms could be involved: (a) an autocrine mechanism implying binding of CXCL12 released by cancer cells to its CXCR4 receptor, phosphorylation of the PI3K/AKT proteins, and induction of VEGF release; [29, 30] and (b) a paracrine mechanism through binding of CXCL12 released by cancer cells to endothelial CXCR4 and then increasing angiogenesis [31–33]. In fact, a positive feedback between CXCR4 and VEGF has been demonstrated [9, 30], and CXCR4 expression has been positively correlated with microvessel density in many cancer subtypes, including breast cancer [10]. Furthermore, CXCR4 inhibitors decrease VEGF protein levels and disrupt tumor vasculature [28, 34]. Our data seems to indicate that Pit-1 induces angiogenesis through both mechanisms. Firstly, Pit-1 overexpression in tumor cells increases phosphorylation of CXCR4, AKT, JNK, ERK1/2, and P38 proteins, all of which are involved in the angiogenesis pathway. Pit-1 also induces VEGF-A expression in tumor cells, but CXCR4 knock-down (either alone or after Pit-1 overexpression) significantly reduces VEGF-A mRNA expression. And secondly, HUVEC cells treated with conditioned medium (CM) from Pit-1-overexpressing cancer cells increases both the number of junctions and percentage of vessels. However, reduced levels of CXCL12 (after IP) and/or CXCR4 knock-down significantly decrease total number of junctions and percentage of vessels. In fact, an increased proliferation of HUVEC cells cultured with CM-Pit-1 but not after CM-Pit-1-IP-CXCL12 was observed. In addition, HUVEC cells cultured with CM-Pit-1 showed increased CXCR4, AKT, and P38 phosphorylation. In summary, our results strongly suggest a critical function of CXCL12-CXCR4 axis on Pit-1-induced angiogenesis, both through autocrine and paracrine pathways. Other studies have demonstrated an indirect paracrine effect of CXCL12 released from cancer-associated fibroblasts (CAF) through chemotaxis recruitment of endothelial progenitor cells to the vicinity of tumor mass and induction of neovascularization [35]. In spite of the fact that our study has not evaluated this mechanism, Pit-1 is expressed in fibroblasts (data not shown) and, therefore, CXCL12 regulation by Pit-1 in CAF could be possible.


a
p-CXCR4
p-AKT
p-JNK
p-ERK1/2
p-P38
VEGF-A
Pit-1
AKT total
GAPDH
pDream
pDream-Pit-1
b
mRNA expression levels (RU)
Pit-1
CXCR4
VEGF-A
P<0.001
P=0.001
P=0.004
P=0.001
P=0.007
P<0.001
P<0.001
Control
shCXCR4
Pit-1
Pit-1+shCXCR4
c
CM-control
CM-shCXCR4
CM-Pit-1
CM-Pit-1+IP-CXCL12
CM-Pit-1+shCXCR4
CM-Pit-1+shCXCR4+ IP-CXCL12
d
Total number of junctions
Vessels percentage area
P<0.001
P<0.001
P=0.001
P<0.001
ns
P<0.001
P<0.001
P=0.007
P<0.001
ns
CM-control
CM-shCXCR4
CM-Pit-1
CM-Pit-1+IP-CXCL12
CM-Pit-1+shCXCR4
CM-Pit-1+shCXCR4+IP-CXCL12
e
MTT Metabolization (A570 nm)
P=0.008
P=0.045
P=0.028
CM-HUVEC
CM-Control
CM-Pit-1
CM-Pit-1+IP-CXCL12
f
p-CXCR4
p-P38
p-AKT
GAPDH
CXCL12 (30 ng/ml)
CM-HUVEC
CM-Control
CM-Pit-1
CM-Pit-1+IP-CXCL12

◀ **Fig. 4** Pit-1 induces angiogenesis through CXCL12-CXCR4. **a** Western blot of phosphorylated (p) CXCR4, AKT, CXCR4, JNK, ERK1/2 and P38, and VEGF-A, AKT, Pit-1 as transfection control, and GAPDH as loading control in control and Pit-1 transfected MCF-7 cells. **b** Real-time PCR of Pit-1, CXCR4, and VEGF-A in control MCF-7 cells and 48 h after knock-down of CXCR4 (shCXCR4), Pit-1 overexpression (Pit-1), and Pit-1 overexpression and CXCR4 knock-down (Pit-1 + shCXCR4). RU indicates relative units. **c** Representative image of tube formation assay in human umbilical vein endothelial (HUVEC) cells after 6 h of treatment with conditioned medium (CM) from control MCF-7 cells (CM-control), from MCF-7 cells after CXCR4 knock-down (control + shCXCR4), from MCF-7 cells after Pit-1 overexpression (CM-Pit-1), from MCF-7 cells after Pit-1 overexpression and CXCL12 immunoprecipitation (IP) (CM-Pit-1 + IP-CXCL12), from MCF-7 cells after Pit-1 overexpression and CXCR4 knock-down (CM-Pit-1 + shCXCR4), and from MCF-7 cells after Pit-1 overexpression + CXCR4 knock-down + IP-CXCL12 (CM-Pit-1 + shCXCR4 + IP-CXCL12). Scale bar: 300 µm. **d** Quantitative analyses of HUVEC tube formation assay after treatments described in **c**. Angiogenic parameters were calculated using the Angiotool software. Numbers represent mean±SD. **e** Proliferation (MTT) assay of HUVEC cells after 48 h of treatment with CM from their own cells (CM-HUVEC), from control MCF-7 cells (CM-Control), from Pit-1 overexpressing MCF-7 cells (CM-Pit-1), and from Pit-1 overexpressing and CXCL12 immunoprecipitated MCF-7 cells (CM-Pit-1 + IP-CXCL12). **f** Western blot of phosphorylated (p) CXCR4, P38, and AKT, and GAPDH in HUVEC cells stimulated for 30 min with CXCL12 ligand (30 ng/ml) and CM as described in **e**

A critical point in the process of metastasis is the choice of new "soil" by cancer cells [3]. Primary tumor cells enter into systemic circulation (intravasation), and exit by breaking the junctions of endothelial cells (extravasation), and finally reach the target organ. In order to test in vitro if the Pit-1-CXCL12-CXCR4 axis could be involved in this process, different mouse tissues, including lung, liver, and brain were studied in a trans-endothelial invasion assay. Muscle tissue was used as a non-metastatic control [4]. Lung, liver, and brain were target tissues of Pit-1 overexpressing breast cancer cells, but, surprisingly, CXCL12 immunoprecipitation in these tissues dramatically decreased trans-endothelial migration of breast cancer cells to liver and lung, but not to brain tissue. These data suggest a critical role of both CXCL12 in target tissues and CXCR4 in primary tumor cells, at least in Pit-1-induced breast cancer metastasis. In fact, it has been shown that high levels of CXCL12 in metastatic target tissues acts as chemoattractant for primary tumor cells with high CXCR4 expression, and that CXCR4 blockade significantly reduces metastasis to these tissues [4, 8, 27, 28].

Finally, to test if our in vitro and in vivo data were transferable to human breast tumors, we correlated Pit-1 with CXCR4 and CXCL12 mRNA expression in a set of human breast tumor samples. Our results show a significant correlation between Pit-1 and CXCL12 and CXCR4. In addition, to study clinical relationships between Pit-1 and CXCR4, human primary breast tumors from patients with metastasis in lung and liver and from patients without metastasis were analyzed by immunochemistry. We found a significant correlation between Pit-1 and CXCR4 protein levels in primary breast tumors and high risk of visceral metastasis. A significant correlation has previously been demonstrated between high CXCR4 expression and visceral metastasis in triple-negative breast cancer [20], and colon and melanoma tumors [36]. In addition, data obtained from public databases reflected significant relationships between Pit-1 and CXCR4 mRNA expression levels and RFS as well as post-progression survival (PPS) in breast cancer patients [18].

Based on our data, targeting the Pit-1-CXCL12-CXCR4 axis in breast cancer could be carried out by inhibition of the DNA-binding activity of Pit-1, which has been demonstrated in vitro using a small molecule (a phenyl-furan-benzimidazole dication, DB293) [37], and by transcriptional repression of Pit-1 through 125-dihydroxyvitamin D3 and its analogs [38, 39]. Several CXCR4 inhibitors have been developed, and some of them currently are under clinical trials, including solid tumors [10, 40]. In fact, studies involving patient-derived xenografts have recently reported that CXCR4 inhibitors improve response to Trastuzumab and taxanes in chemoresistant HER2 breast cancer [19].

CXCL12/CXCR4 can be regulated by many factors, such as the hypoxia-inducible factor, and the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) [10, 41]. In the present study, we also show that CXCL12 and CXCR4 are positively regulated by Pit-1, suggesting an important role of the Pit-1-CXCL12-CXCR4 axis in breast cancer progression, including cell proliferation, migration, tumor growth, invasion, spreading, angiogenesis, and extravasation of cancer cells to form metastasis in lung and liver (Fig. 6g). Thus, these factors could be used as prognostic as well as therapeutic targets in breast tumors.

Material and methods

Patients, breast cancer samples, and immunohistochemistry (IHC)

Samples from 48 patients with invasive breast cancer, half with metastasis in liver and/or lung and the other half without metastasis were obtained from archived, formalin fixed, paraffin embedded, surgically resected mammary specimens from patients with breast cancer in accordance with the Fundación Hospital de Jove Ethics and Investigation Committee approved protocols and were used for IHC. Patient sample characteristics are described in Supplemental methods and Table 1. IHC was performed using a Pit-1 (Abmart, Shangai, China) and a CXCR4 (Abcam, Cambridge, UK) antibodies as previously described [16, 42] and detailed in Supplemental methods.

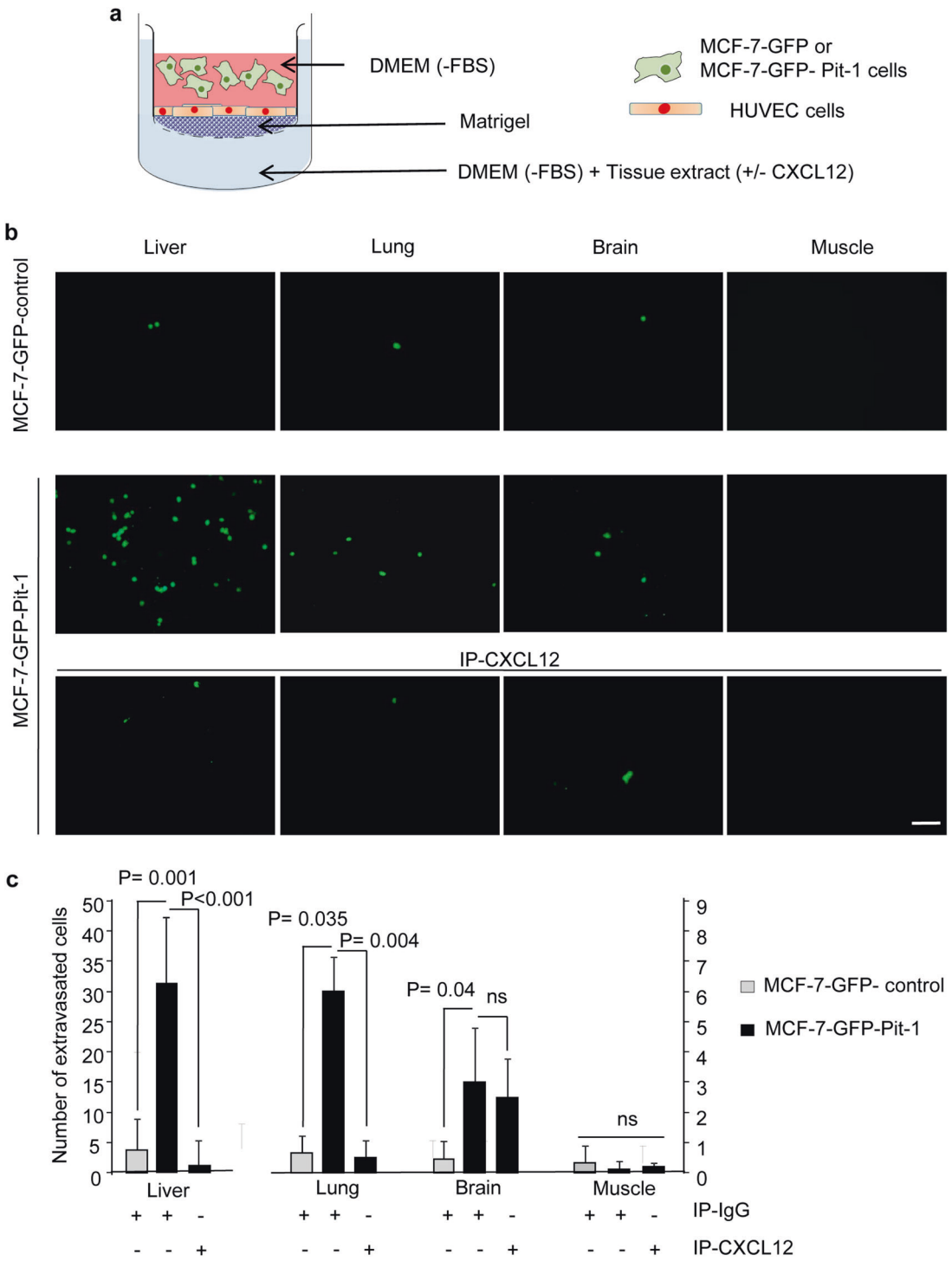
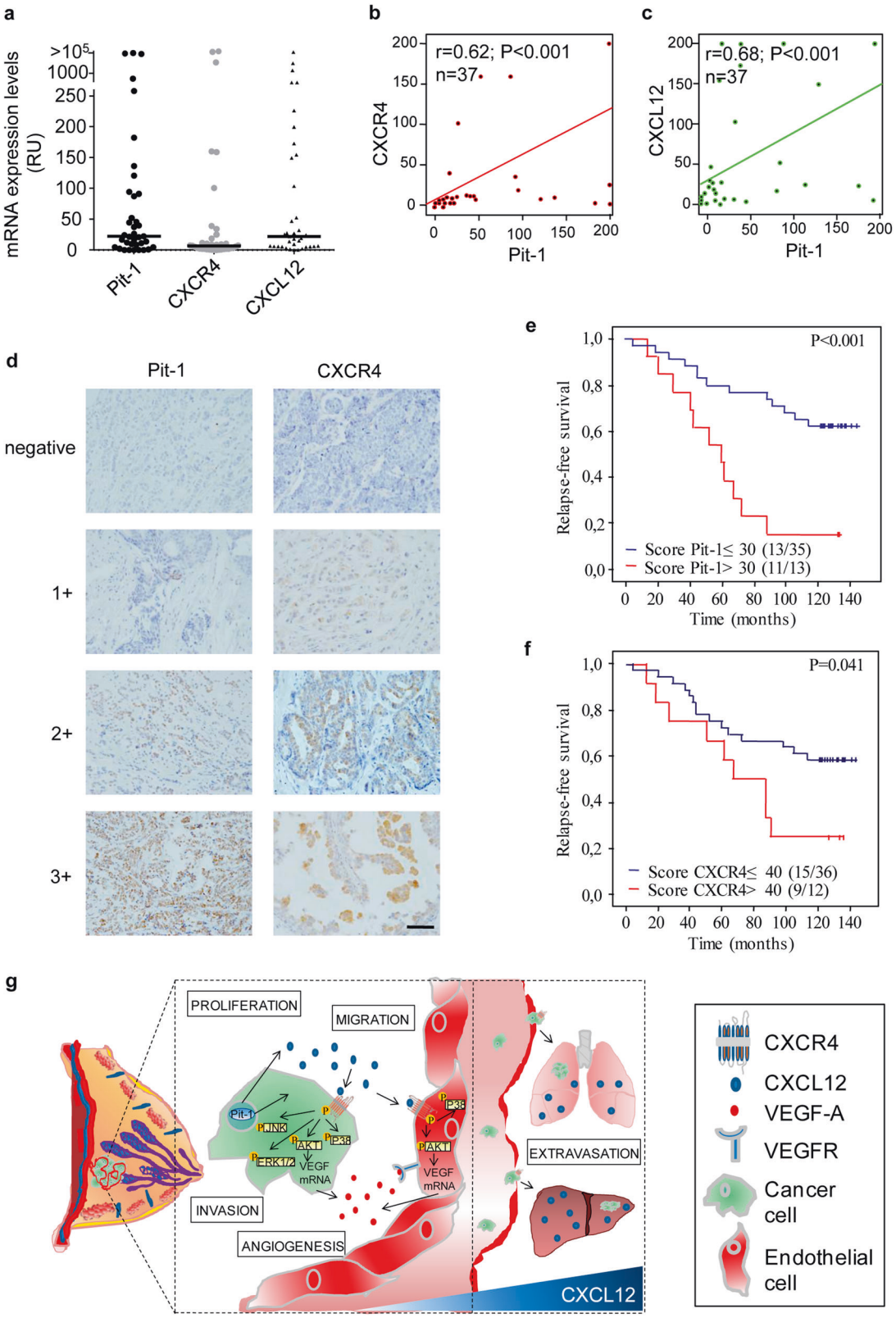


Fig. 5 CXCL12 levels determines extravasation of MCF-7-Pit-1 overexpressing cells to liver and lung tissue. **a** Schematic extravasation assay. Control (MCF-7-GFP-control) or Pit-1 overexpressing (MCF-7-GFP-Pit-1) cells are plated into the upper chamber of transwell and migrate across HUVEC cells, matrigel and pore membrane system to gain access to the lower chamber filled with tissue extract from mouse liver, lung, brain, or muscle (used as negative control). **b** Representative images of extravasated control cells (MCF-7-GFP-control), Pit-1 overexpressing MCF-7 cells (MCF-7-GFP-Pit-1), and Pit-1 overexpressing MCF-7 cells after CXCL12 immunoprecipitation in tissue extracts (IP-CXCL12). Scale bar: 100 µm. **c** Quantitative analyses of three experiments as described in **b**. Numbers represent mean±SD


a
mRNA expression levels (RU)
>10^5
1000
250
200
150
100
50
0
Pit-1
CXCR4
CXCL12
b
r=0.62; P<0.001
n=37
CXCR4
Pit-1
c
r=0.68; P<0.001
n=37
CXCL12
Pit-1
d
Pit-1
CXCR4
negative
1+
2+
3+
e
P<0.001
Relapse-free survival
Score Pit-1≤ 30 (13/35)
Score Pit-1> 30 (11/13)
Time (months)
f
P=0.041
Relapse-free survival
Score CXCR4≤ 40 (15/36)
Score CXCR4> 40 (9/12)
Time (months)
g
PROLIFERATION
MIGRATION
INVASION
ANGIOGENESIS
EXTRAVASATION
Pit-1
JNK
AKT
P38
ERK1/2
VEGF mRNA
CXCL12
CXCR4
CXCL12
VEGF-A
VEGFR
Cancer cell
Endothelial cell

◀ **Fig. 6** Pit-1 correlates with CXCR4 and CXCL12 expression in breast cancer tumor samples. **a–c** Pit-1, CXCR4, and CXCL12 mRNA expression in 37 breast tumor samples were evaluated by qPCR. Dispersion plot indicates a significant ($P < 0.001$) positive correlation between Pit-1 and CXCR4 and CXCL12 mRNA. **d** Representative set of negative and positive (1 +, 2 +, and 3 +) Pit-1 and CXCR4 immunodetection in human breast invasive ductal carcinomas. Scale bar: 100 μm. **e**, **f** Probability of relapse-free survival in 48 breast cancer patients with clinical follow-up as a function of the optimal cutoff point for Pit-1 and CXCR4 score values (30 and 40, respectively). Ratio of number of events/total cases is indicated in each graphic. **g** Schematic representation of Pit-1 overexpressing breast tumors and metastatic progression to liver and lung. To establish secondary growth, tumor cells must overcome sequential steps known as metastatic cascade. In breast tumors, deregulation of Pit-1 drives an increase of the chemokine CXCL12 and its receptor CXCR4. Activation of this pathway promotes proliferation, migration, and invasion. Furthermore, CXCL12 released by Pit-1 overexpressed cells affects to endothelial cells in tumor microenvironment increasing angiogenesis. Once Pit-1 and CXCR4 positive cells entry in blood vessels (intravasation) they circulate through bloodstream until to escape (extravasation) to target tissues with high CXCL12 expression, where they survive and grow to form metastases

Cell lines, cultures, and conditioned medium

The human breast adenocarcinoma cell lines MCF-7, MDA-MB-231, SKBR-3, and human umbilical vein endothelial cells (HUVEC) were obtained from ATCC-LGC (Barcelona, Spain). MCF-7-GFP cells were obtained from Cell Biolabs (San Diego, USA). Cell lines were tested and authenticated according to microscopic morphology, growth curve analysis, and mycoplasma detection according to the ECACC cell line verification test recommendations. Breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, ThermoFisher Scientific, Waltham, USA), and HUVEC cells were cultured in VascuLife basal medium supplemented with LifeFactors VascuLifeEnGS (Lifeline Cell Technology, Frederick, USA) at 37 °C in 5% CO_2. For HUVEC treatments, conditioned medium (CM) from control MCF-7-GFP cells, and after MCF-7-GFP knock-down of CXCR4, overexpression of Pit-1, and/or CXCL12 immunoprecipitation (IP) was obtained by culturing cells in the VascuLife basal medium for 48 h. IP-CXCL-12 (MCF-7-GFP-Pit-1-IP-CXCL12) was obtained after IP of CM-MCF-7-GFP-Pit-1 for 4 h with an anti-CXCL12 antibody (Santa Cruz Biotechnology, Dallas, USA). Non-specific IgG (Santa Cruz Biotechnology) was used as control.

Plasmids and transfections

The pDream (used as control) and the pDream-hPit-1 overexpression vectors were obtained from GenScript

Table 1 Basal characteristics of the 48 patients with breast cancer

Characteristics	Without recurrence *N* (%)	With recurrence *N* (%)
Total cases	24 (100)	24 (100)
Age median (years)		
≤58	10 (41.7)	14 (58.3)
>58	14 (58.3)	10 (41.7)
Menopausal status		
Premenopausal	5 (20.8)	4 (16.7)
Postmenopausal	19 (79.2)	20 (83.3)
Tumor size		
T1	14 (58.3)	9 (37.5)
T2	10 (41.7)	15 (62.5)
Nodal status		
N-	11 (45.8)	9 (37.5)
N+	13 (54.2)	15 (62.5)
Tumor stage		
I	7 (29.2)	4 (16.7)
II	17 (70.8)	17 (70.8)
III	0 (0)	3 (12.5)
Histological grade		
Well differentiated	8 (33.3)	2 (8.3)
Mod differentiated	5 (20.8)	13 (54.2)
Poorly differentiated	11 (45.8)	9 (37.5)
HER2 status		
Negative	16 (66.7)	15 (62.5)
Positive	8 (33.3)	9 (37.5)
Estrogen receptors		
Negative	7 (29.2)	8 (33.3)
Positive	17 (70.8)	16 (66.7)
Progesterone receptors		
Negative	9 (37.5)	9 (37.5)
Positive	15 (62.5)	15 (62.5)
Molecular subtypes		
Luminal A	10 (41.7)	8 (33.3)
Luminal B	8 (33.3)	7 (29.2)
Her2	3 (12.5)	3 (12.5)
Triple-negative	3 (12.5)	6 (25.0)
Metastasis site		
Lung	0 (0)	14 (58.3)
Liver	0 (0)	10 (41.7)
Adjuvant radiotherapy		
No	10 (41.7)	6 (25.0)
Yes	14 (58.3)	18 (75.0)
Adjuvant systemic therapy		
TMX	7 (29.2)	4 (16.7)
CMT	7 (29.2)	8 (33.3)
CMT + TMX	9 (37.5)	10 (41.7)
CMT + Trastuzumab	0 (0)	1 (4.2)

Table 1 (continued)

Characteristics	Without recurrence *N* (%)	With recurrence *N* (%)
TMX + Trastuzumab	0 (0)	1 (4.2)
No treatment	1 (4.2)	0 (0)

TMX tamoxifen, *CMT* chemotherapy

(Piscataway, USA), and the pLKO-CXCR4 shRNA and the pLKO (used as control) vectors were obtained from Sigma, as described in Supplemental methods. Transfections were performed with jetPEI (Polyplus transfection, Illkirch, France). Control or Pit-1 lentiviral activation particles (Santacruz biotechnology, sc-43728 and sc-401671-LAC, respectively) were transduced in MCF-7-GFP cells as described in Supplemental methods. Pit-1 knock-down was carried out using two different Pit-1 small-interfering RNA (siRNA) (Pit-1 siRNA-1 and Pit-1 siRNA-2), as previously described [16]. Sequences of Pit-1 siRNAs are described in Supplemental methods. Two silencer negatives (Scrambled 1 and Scrambled 2) (Ambion, Thermo Fisher Scientific) were employed as controls.

RNA isolation, qPCR, tumor samples for mRNA analysis, and clinical outcome analyses

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, USA) and qPCR was performed as previously described [15] and detailed in Supplemental methods. Thirty-seven breast tumor samples were provided by the BioBank Complejo Hospitalario Universitario de Santiago (CHUS), integrated in the Spanish National Biobanks Network and were processed following standard operating procedures. Approval by the Ethical and Scientific Committees was obtained. For outcome analyses, the KM Plotter Online Tool (http://www.kmplot.com) [18] was used to evaluate the relationship between Pit-1 and CXCR4 mRNA expression and patient clinical outcome.

Western blot and ELISA

Western blotting was carried out as described elsewhere [15]. Antibodies used are detailed in Supplemental methods. CXCL12 was measured in culture media after 48 h of transient Pit-1 overexpression by ELISA (RayBiotech, Norcross, USA) according to the manufacturer's instructions.

Three-dimensional (3D) culture, cell invasion, and extravasation assays

3D cultures were performed as previously described [39] and detailed in Supplemental methods. Cell invasion assays were performed as previously described [15]. Extravasation assays are detailed in Supplemental methods. Briefly, HUVEC cells were seeded in the upper chamber of transwells coated with growth factor-reduced Matrigel (BD Biosciences, Madrid, Spain), and cultured in Vasculife basal medium for 24 h. Then, tumor cells were added over HUVEC cells in DMEN without FBS. In the lower chamber, protein extracts in DMEM without FBS from liver, lung, brain or muscle of female mice were added after immunoprecipitation with either a CXCL12 antibody or an IgG (used as control). After 48 h, cells that migrated to the lower surface of the filters were counted under fluorescence microscopy.

HUVEC tube formation and wound healing assays

For HUVEC tube-formation assay, HUVEC cells were cultured in growth factor-reduced Matrigel (BD Biosciences) for 6 h with conditioned medium from tumor cells. Formation of capillary-like structures was observed under a fluorescence microscope after cell staining with CellTracker Red CMTPX Dye (Thermo Fisher Scientific) for 15 min. Tube formation was evaluated using the AngioTool software [43]. Detailed information is available in Supplemental methods. Wound healing assay was carried out after seeding 34×10^3 MCF-7-GFP, MCF-7-GFP-Pit-1, or MCF-7-GFP-Pit-1-shCXCR4 cells in ibidi culture-inserts (ibidi, Munich, Germany) according to the manufacturer's instructions. Remaining gap between cells after wounding was measured under a fluorescence microscope at 0, 12, and 17 h.

In vivo zebrafish tumor xenograft assays and image analysis

Procedure of zebrafish assays is detailed in Supplemental methods. Briefly, 48 h post fecundation, zebrafish embryos ($n = 40$ per group) were anesthetized and injected into the yolk with 15×10^3 cells/µl (MCF7-GFP, MCF7-GFP-Pit-1, or MCF7-GFP-Pit-1-shCXCR4 cells). Zebrafish embryos were incubated for 48 h post injection (hpi) at 36 °C in 24-well plates and photographed at 0 hpi and 48 hpi with AZ-100 Nikon fluorescence stereomicroscope in order to track tumor growth and cell spread of the injected GFP cells. The analysis was performed as detailed in Supplemental methods.

Statistical analysis

Each experiment was performed at least three times. Values are expressed as mean±SD. Means were compared using 2-tailed Student's *t*-test or 1-way ANOVA, with the Tukey-Kramer multiple comparison test for post-hoc comparisons.

SPSS 20.0 Software was used for statistical calculations. For in vivo experiments, homoscedasticity was tested for all the data and then an excel outlier analysis was carry out using interquartile range to discard possible outliers. U Mann–Whitney test for non-parametrical data was applied to non-homoscedastic data with confidence intervals of 95%. Probabilities of relapse-free survival were calculated with the Kaplan–Meier method and differences between curves were evaluated with the log rank test. To establish a cutoff point in order to convert score values obtained by IHC staining of Pit-1 and CXCR4 into a categorical variable by combining patients into two groups, we followed the "minimum *P*-value approach."

Acknowledgements This study was supported by Ministerio de Economía y Competividad (SAF2015-69221-R, MINECO/FEDER), and Conselleria de Cultura, Educación e Ordenacion Universitaria (GPC2014/001) to RP-F, and Ministerio de Educacion, FPU14/00548 to AM-O. We want to particularly acknowledge the patients and the BioBank Complejo Hospitalario Universitario de Santiago (CHUS) (PT17/0015/0002), integrated in the Spanish National Biobanks Network for its collaboration, to P. Peñas for providing biological samples, and to M. Fraile for help us with histological analyses.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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