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BREAST CANCER: ROLE OF PIT-1 AND CXCR4/CXCL12 IN THE METASTATIC PROCESS

Anxo Martínez Ordóñez

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Breast cancer: role of Pit-1 and CXCR4/CXCL12 in the metastatic process

D. Anxo Martínez Ordóñez

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BREAST CANCER: ROLE OF PIT-1 AND CXCR4/CXCL12 IN THE METASTATIC PROCESS

D. Román Pérez Fernández

D. Samuel Seoane Ruzo

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18s: 18S (subunit) ribosomal RNA

AKT/PKB: Protein kinase B

AMD3100: CXCR4 antagonist, plerixafor

ATP: Adenosine triphosphate

BCL2*:* B-cell lymphoma 2 gene

BRCA1: Breast cancer type 1 susceptibility protein gene

BRCA2: Breast cancer type 2 susceptibility protein gene

CAF: Carcinoma-Associated Fibroblasts

CPHD: Combinated Pituitary Hormone Deficiency

CM: Conditioned Medium

CSC: Cancer Stem Cell

CTC: Circulating Tumor Cell

DMEM: Dulbecco's Modified Eagle Medium

EMT: Epithelial-Mesenchymal Transition

ER: Estrogen Receptor

erbB2**:** Human Epidermal Growth Factor (ERBB2 o HER2)

FBS: Fetal Bovine Serum

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GFP: Green Fluorescence Protein

GH: Growth Hormone

GSEA: Gene Set Enrichment Analysis

HIF-1: Hypoxia Inducible Factor 1

HIF-2: Hypoxia Inducible Factor 2

IHC: Immunohistochemistry

Abbreviations

IL6: Interleukin 6

IP: Immunoprecipitation

JNK: c-Jun N-terminal kinase

kDa: Kilodaltons

MAPK: Mitogen-Activated Protein Kinase (MAPK or MAP kinase)

MET: Mesenchymal-Epithelial Transition

NF-ƙB: Nuclear Factor Kappa-light-chain-enhancer of activated B cells

PBS: Phosphate-Buffered Saline

PGK1: Phosphoglycerate Kinase 1

PI3K: Phosphoinositide 3-Kinases

Pit-1: Pituitary-1 transcription factor or POU1F1 or "POU class 1 homeobox 1"

POU1F1: POU class 1 homeobox 1 or POU domain, class 1, transcription factor 1

PPS: Post Progression Survival

PR: Progesterone receptor

PRL: Prolactin

RFS: Relapse Free Survival

RTK: Tyrosine Kinase Receptors

RU: Relative Units

shRNA: Short Hairpin RNA

siRNA: Small Interfering RNA

TAD: N-terminal Transactivating Domain

TAM: Tumor-Associated Macrophages

TGFβ: Transforming Growth Factor beta

TNBC: Triple Negative Breast Cancer

TNFα: Tumor Necrosis Factor alpha

TP53: Tumor Protein p53

TSHβ: Thyroid Stimulating Hormone, beta

VDR: Vitamin D Receptor

VEGFA: Vascular Endothelial Growth Factor A

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ABSTRACT

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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.

Keywords: Pit-1, CXCR4, CXCL12, breast cancer, metastasis.

Abstract

O desenrolo de tumores humanos é o resultado da acumulación nas células de alteracións nos xenes supresores de tumores e nos oncoxenes. O factor de transcrición POU1F1 (tamén coñecido como Pit-1) expresase na glándula mamaria e a súa sobreexpresión induce profundos cambios fenotípicos en proteínas involucradas na progresión do cancro de mama. As pacientes con cancro de mama e elevada expresión de Pit-1 amosan unha correlación positiva coa aparición de metástase a distancia e cunha deficiente supervivencia global. Porén, moitos dos mediadores das accións de Pit-1 permanecen descoñecidos.

Neste estudo, amosamos que o receptor de quimiocinas CXCR4 e o seu ligando CXCL12 teñen un papel crítico no proceso tumoral inducido por Pit-1. Achamos que Pit-1 aumenta os niveis de ARNm e proteína tanto en CXCR4 como en CXCL12. *In vivo*, nun modelo de xenoinxerto de peixe cebra, o bloqueo de CXCR4 reduce o crecemento tumoral e a diseminación das células que sobreexpresan Pit-1. Ademais, describimos por primeira vez os efectos proanxioxénicos de Pit-1 a través do eixo CXCL12-CXCR4, e que a extravasación de células de cancro de mama que sobreexpresan Pit-1 ata os tecidos diana redúcese fortemente se se reducía a expresión de CXCL12. Finalmente, en pacientes con cancro de mama, atopouse que a expresión de Pit-1 en tumores primarios correlacionábase positivamente con CXCR4 e CXCL12, con metástases específicas en fígado e pulmón e con resultado clínico. Os nosos resultados suxiren que o eixo Pit-1-CXCL12-CXCR4 podería participar nunha quimiotase dirixida durante o proceso de metástase e poderían representar dianas terapéuticas ou factores prognóstico.

Termos chave: Pit-1, CXCR4, CXCL12, cancro de mama, metástase.

El desarrollo de tumores humanos es resultado de la acumulación en las células de alteraciones en los genes supresores de tumores y en los oncogenes. El factor de transcripción POU1F1 (también conocido como Pit-1) se expresa en la glándula mamaria y su sobreexpresión induce profundos cambios fenotípicos en proteínas involucradas en la progresión del cáncer de mama. Los pacientes con cáncer de mama y elevada expresión de Pit-1 muestran una correlación positiva con la aparición de metástasis a distancia y una deficiente supervivencia global. Sin embargo, muchos de los mediadores de las acciones del Pit-1 permanecen desconocidos.

En este estudio, mostramos que el receptor de quimiocinas CXCR4 y su ligando CXCL12 desempeñan un papel crítico en el proceso tumoral inducido por Pit-1. Encontramos que Pit-1 aumenta los niveles de ARNm y proteína tanto en CXCR4 como en CXCL12. In vivo, en un modelo de xenoinjerto de pez cebra, el bloqueo de CXCR4 reduce el crecimiento tumoral y la diseminación de las células que sobreexpresan Pit-1. Además, describimos por primera vez efectos proangiogénicos de Pit-1 a través del eje CXCL12-CXCR4, y que la extravasación de células de cáncer de mama que sobreexpresan Pit-1 hacia los tejidos diana se reduce fuertemente si se reducía la expresión de CXCL12. Finalmente, en pacientes con cáncer de mama, se encontró que la expresión de Pit-1 en tumores primarios se correlacionaba positivamente con CXCR4 y CXCL12, con metástasis específicas en hígado y pulmón, y con resultado clínico. Nuestros resultados sugieren que el eje Pit-1-CXCL12-CXCR4 podría participar en un guiado quimiotáctico durante el proceso metastásico y pueden representar dianas terapéuticas o factores pronóstico.

Palabras clave: Pit-1, CXCR4, CXCL12, cáncer de mama, metástasis.

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INTRODUCTION

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1. CANCER

1.1. Definition of cancer

Cancer is a complex disease produced by alterations in DNA of cells, caused by multiple genetic and environmental factors. Genomic alterations, either hereditary or acquired, are the result of a balance between the capability of cells to maintain the integrity of genetic information and the modifying effects of the environment. The acquisition and accumulation of mutations in DNA and hereditary predisposition can transform the vital cycle of the cell, creating a new and abnormal context of chronic growth (new cells are formed when they are not needed), immortality (old or damaged cells survive when they should die) and invasion. Cancer cells form highly organized and complex new tissues called tumors and constitute a systemic disease (Figure 1).

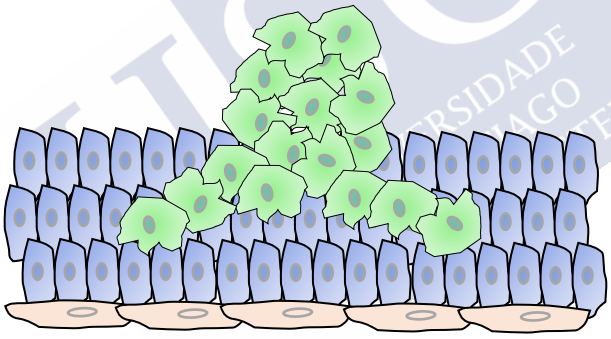

Figure 1. Cancer cells (in green) growing in epithelial tissue to form a tumor

1.2. Types of cancer

Cancers are named for the area in which they begin and the type of cell they are made of:

- **Carcinoma:** is the most common type of cancer. It is formed of epithelial cells. Carcinomas that begin in different epithelial cell types have specific names: for example; adenocarcinoma is a cancer that forms in epithelial cells that produce fluids or mucus. Tissues with this type of epithelial cell are sometimes called

glandular tissues. Most cancers of the breast, colon, and prostate are adenocarcinomas.

- **Sarcomas:** are cancers that form in bone and soft tissues, including muscle, fat, blood vessels, lymph vessels, and fibrous tissue (such as tendons and ligaments). They are much less common than carcinomas.
- **Lymphoma and Leukemia**: cancers that begin in the blood-forming tissue of the bone marrow. These cancers do not form solid tumors. Instead, large numbers of abnormal white blood cells (leukemia cells and leukemic blast cells) build up in the blood and bone marrow, crowding out normal blood cells.
- **Germ cell tumor**: cancers derived from pluripotent cells, most often present in in the testicles or ovaries (seminoma and dysgerminoma, respectively)
- **Brain and spinal cord tumors**: cancers that begin in the cells of the central nervous system. Most common types of brain tumor develops from glial cells and are called glioma (https://www.cancerreseachuk.org).

1.3. Cancer trends and statistics

Cancer is a major public health problem worldwide and is the fourth leading cause of death in developed countries. The world health organization estimates 18,078,957 million people (of both sexes and all ages) had at least one cancer type in 2018. The estimated number of deaths caused by cancer in 2018 is 9,555,027 million people. The number of prevalent cases (5 year) is 43,841,302 million people.

Among different types of cancer, lung cancer is the most frequent type, followed by breast, colorectum, prostate and stomach (Figure 2). Looking to the future, the forecast for incidence in 2040 is an increase to 29,532,994 million cases. Similarly, the forecast for cancer mortality will be 16,388,459 million people. In both cases this increase is due to demographic changes in human population (Siegel RL, 2018).

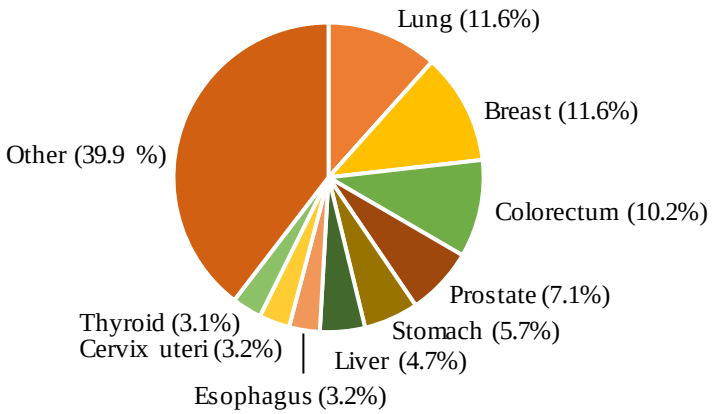

Figure 2. Pie chart of 2018 cancer statistics. Distribution of cases for the most common cancers in 2018 for both sexes.

1.4. Cancer hallmarks

Manifestations of cancers are notably diverse and complex, reflecting differences in the origin of normal cell types, acquisition of mutations, variably altered transcriptional networks, activation of normal tissue microenvironments and response to therapy. In order to simplify the study of cancer, the scientific community accept a common set of capabilities proposed by Hanahan and Weinberg (Hanahan D and Weinberg RA, 2011) that enable aberrant cells to progress and form a terrible disease. The functional capabilities of human cancer cells are called "hallmarks of cancer".

- **Sustaining proliferative signaling**: cancer cells have a complex set of inductive signals in order to begin cell cycle division to produce more and more cells. In the cancer contexts, these signals are chronic and involve the deregulation (by mutation or epigenic modification) of genes that normally act as transitory inductors of proliferation and growth such as tyrosine kinase receptors (RTKs), which include the ErbB2 gene, overexpressed in almost 30% of breast cancer patients (Lemmon MA and Schlessinger J, 2010). These activated genes are called oncogenes.
- **Evading growth suppressors**: in normal cells there are proteins that regulate negatively the activation and progression in the cell cycle. These genes are termed tumor suppressor genes. The most well-known gene suppressor involved in cancer is p53, which is mutated in almost 40% of cancers, predominating in ovarian and endometrial carcinomas (Kandoth C et al., 2013).

- **Resisting cell death**: aberrant cells can be destroyed by intrinsic signals of quality control that induce a cell death program such as apoptosis, necroptosis or autophagy. Cancer cells can attenuate or evade these mechanisms of death. For instance, it is known that many oncogenes such as Myc can force the expression of the anti-apoptotic Bcl-2 protein and evade apoptosis (Fernald K and Kurokawa M, 2013)
- **Replicative immortality**: cancer cells have the capability to circumvent the proliferative barrier presented by the shortening and dysfunction of telomeres in aging. Cancer cells adapt similar strategies to embryonic and stem cells enhancing the expression of the telomerase enzyme and promoting the elongation of telomeres (Shay JW, 2016).
- **Angiogenesis:** as commented above, tumors are complex tissues and need a nutrient and oxygen supply, as well as a method to eliminate metabolic waste. Tumor vasculature supports this function and the formation of new vessels is commonly activated in many tumors. However, tumor associated vasculature is usually aberrant. Blood vessels on tumors are dilated, leaky and not functional (Schaaf MB, 2018).
- **Metabolic reprogramming:** cancer cells have more enhancement uptake of glucose than normal cells, which is metabolized and fermented to lactate, even in the presence of oxygen, which should favor oxidative phosphorylation. As result of this process (known as Warburg effect) tumors obtain less ATP but get more macromolecules required for cell growth and high proliferation rates (Pavlova NN and Thompson CB, 2016). Furthermore, lactate produced by cancer cells is secreted and has tumor promoting capabilities (Dhup S et al., 2012)
- **Avoiding immune destruction:** it has been demonstrated that the immune system reacts against aberrant cells or incipient neoplasia. However, tumors have developed ways to evade this barrier (Gonzalez H et al., 2018). Cancer cells can circumvent immune recognition, losing expression of tumor antigens or promoting immunosuppression by the secretion of certain factors that induce immune tolerance. In this way, reactivation of the immune system is one of the great hopes for the future of cancer treatments.
- **Invasion and metastasis:** dissemination of cancer cells from primary tumors and their subsequent seeding of new tumor colonies in distant tissues is responsible

for almost 90% of cancer deaths (Lambert AW et al., 2017). Tumor plasticity and the capability of adaptions to invade and grow in different tissue environments is the least understood aspect of cancer biology. Figuring out the mechanisms of cancer cells to form metastasis is the biggest challenge of oncology.

Taken together, cancer cells are characterized by the acquisition and accumulation of these functional capabilities and features, which can be facilitated by: a) genome instability, which is the result of the DNA balance between damage and repair or/and b) inflammation, which was first proposed by Rudolf Virchow in 1863 after the observation that infiltrating leukocytes were a hallmark of tumors. Inflammation triggers activation of programs related with wound healing such as migration, proliferation and growth (Colotta F et al., 2009). Today, it is accepted that chronic inflammation is a critical promoter of cancer, with at least 20% of cancers associated with it (Grivennikov SI et al., 2010).

1.5. Tumor microenvironment: ecosystem of cancer cells

In addition to tumor-intrinsic genetic features, tumor growth is dependent on tumor-extrinsic factors. Solid tumors incorporate not only transformed cells but also a variety of normal tissue cells and bone marrow-derivations that are re-educated by tumor cells to support and collaborate with cancer progression (Figure 3) (Hanahan D and Coussens LM, 2012). It is now clear that tumors are also diverse by nature and the activation state of their non-epithelial compartment or stroma plays a critical role in the metastasis process (Quail DF and Joyce JA, 2014). Stromal cell compartment of tumors can be classified into three classes of cells that include a lot of phenotypes:

- **Angiogenic vascular cells (AVC)**: consisting of endothelial cells and supporting pericytes. Endothelial cells play an evident role during angiogenesis but now it is known that this also contributes to other tumor capabilities like inhibition of apoptosis or promoting tumor growth and metastasis through the release of growth factors and cytokines such as VEGF, CXCL12 or CCL2 (Butler JM et al., 2010)
- **Cancer associated fibroblasts (CAF):** encompasses heterogeneous populations of cells with fibroblast-like characteristics derived from mesenchymal stem cells,

resident fibroblasts, myofibroblasts or even adipocytes (Bochet L et al., 2013). Carcinoma associated fibroblasts are mainly a state rather than a cell (Madar S et al 2013). In many cancers, like pancreatic or breast carcinoma, CAF can compose up to 80% of the total tumor tissue (Gascard P et al., 2016). Signals from tumors induce a CAF state by epigenetic reprogramming (Kang N et al., 2015). At the same time CAFs trigger multiple malignant phenotypes in neighboring tumor epithelial cells as well as other stromal cell types by secretion of proteases, growth factors and/or cytokines that favor cancer progression (Lazennec G and Richmond A, 2010).

- **Infiltrating immune cells (ICC):** immune cells are recruited to the vicinity of tumors as the result of unresolved inflammation and cytokines released by tumors. Once immune cells infiltrate tumors, they undergo a local education to support tumor growth, angiogenesis and metastasis (Wellestein MD and de Visser KE, 2018). The tumors promoting ICC includes neutrophils, myeloid progenitors, subtypes of B and T lymphocyte and macrophages which represent the largest population of infiltrating inflammatory cells (Lewis CE and Pollard JW 2006).

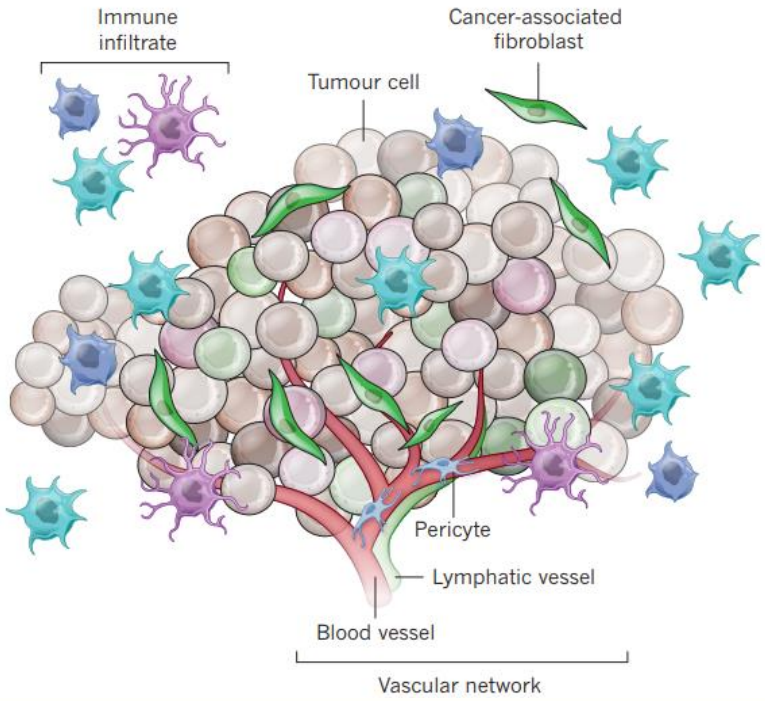

Figure 3. Tumor microenvironment collaborates with tumor cells to induce cancer progression. Reprinted by permission from Springer Nature.

To add more complexity, the tumor microenvironment contributes to tumor heterogeneity with intricate communications and relations between different stromal cell types. Furthermore, genome profiling techniques (especially single cell DNA genome sequencing) shows that tumors are mosaics composed of cancer cell subpopulations with different phenotypes, epigenetic profiles and genetic solutions to adapt and survive in the hostile environment of tumors (Gawad G, 2016). One of the most enigmatic cell subpopulations in tumors are cells showing similarities with normal tissue stem cells. These cancer stem cells (CSC) have enhanced ability to initiate new tumors despite the fact that they proliferate more slowly than abundant cancer cells in tumors (Nguyen LV, 2012). The source of CSC is still not clear. One origin of CSC could be a normal stem cell that was transformed and derives in differentiated cancer cells to form a tumor. In other cases, the CSC phenotype appears from a dynamic interconversion by cancer cells that adopt this function (Polyak K and Weinberg RA, 2009). Notably, epithelial-to-mesenchymal transition (EMT) during metastasis process has been related with CSC switch in tumors (Mani SA et al., 2008).

2. BREAST CANCER

2.1. Breast function and anatomy

Mammary glands are a distinguishing feature of mammals. The function of the breast is to provide nourishment to the offspring by secretion of milk. The organ develops from the primordially derived breast tissue, which anatomically matures as a modified sweat gland. Hormonal changes during the life of the female gender such as puberty, pregnancy, and menopause regulate an extensive postnatal development and functional activity of the mammary glands (Bland KI, 2018).

Breasts consist of 15 to 20 lobes of glandular tissue that surround the nipple in a radial manner. Longitudinal fibrous connective stroma support the lobes and adipose tissue fills the space between the lobes (https://www.mskcc.org/cancer-care/types/breast/anatomy-breast). Inside these lobes are smaller sections, called lobules. At the end of each lobule there are tiny sockets that produce milk. These structures are linked together by small tubes called ducts or lactiferous ducts, which carry milk to the

nipples. The nipple is in the center of a dark area of skin called the areola. The areola contains small glands that lubricate the nipple during breastfeeding (Figure 4). There are no muscles in the breasts, but pectoralis major and intercostal muscles lie under each breast to cover the ribs. Each breast also contains blood vessels, nodes and lymph vessels, nerves and Cooper ligaments that provide support to the breast parenchyma (https://www.nationalbreastcancer.org/breast-anatomy and Bland KI, 2018).

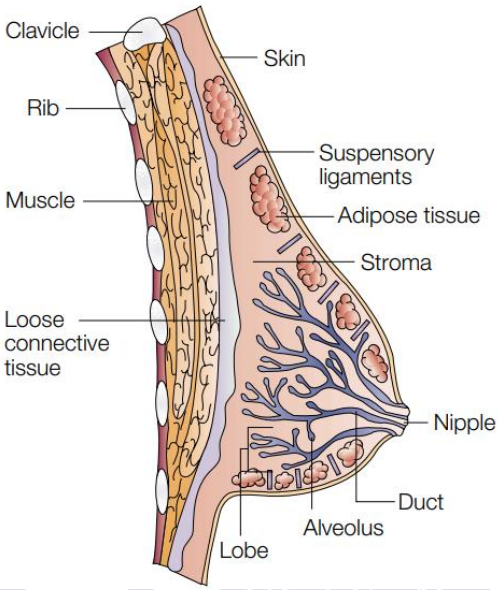

Figure 4. Schematic representation of the mammary gland anatomy. Reprinted by permission from Springer Nature.

2.2. Definition and classification

Breast cancer is cancer that begins in cells of the mammary glands. Worldwide, breast cancer is the most common invasive cancer in women (Siegel RL 2018). This disease can occur in both men and women, but it is far more common in women. The vast majority of breast tumors (approximately 70-80%) begin in epithelial cells in the ducts (ductal carcinoma) or also may begin in epithelial cells in the lobules, (lobular carcinoma). In both cases, cancer cells can proliferate confined in the lumen of the duct or in the lobule (carcinoma in situ). In contrast, if these aberrant cells spread and infiltrate into the stroma, blood and lymph vessels, it is called invasive carcinoma. Furthermore, there are other less frequent subtypes of invasive breast carcinoma based on

histopathological classification: medullary, tubular and metaplastic carcinomas (Weigelt B et al., 2008 and Lakhani S et al., 2014).

In conjunction with histopathological assessment, tumor grade or stage, the standard evaluation of breast cancer for clinical purposes involves immunohistochemical (IHC) characterization of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Around 70-80% of all breast cancer patients are hormone receptor-positive (ER+ and PR+). In this case, it is expected that these patients respond to hormone-based therapies such as tamoxifen or aromatase inhibitors. Generally, this type of breast cancer has a good prognosis. HER2+ represents another additional predictive marker used by clinicians. Approximately 10-15% of breast cancers display HER2 overexpression and/or amplification with around half of these being positive for ER or PR (Konecny G et al., 2003). The prognosis of these patients has been improved in recent years, by the development of anti-HER2 based therapies, including the monoclonal HER2 antibody, trastuzumab, which targets the extracellular domain of the HER2 receptor. The remaining 10-15% of breast cancers are defined by hormone receptor and HER2 negativity (i.e., triple negative cancers), which represent a big challenge for oncologists due to the lack of targeted therapies. Therapeutic advances have eluded this group of patients, making it the breast cancer subtype with poorest prognosis (Dawson SJ et al., 2009).

Although the histological classification of breast cancer subtypes has been helpful for clinicians, this classification has significant limitations. One of them is the fact that response to therapies and prognosis is variable among patients with the same breast cancer subtype. Furthermore, this classification is too simple for explaining the different molecular complexity and activated pathways in breast cancer. For that reason, the guide for modern cancer management and treatment is the molecular characterization of breast tumors.

Gene expression analysis using microarray-based technology has provided researchers with a fantastic tool to change to a comprehensive molecular profiling of breast cancer. As a result, five subtypes have been proposed based on gene expression: luminal A (ER+ and low grade), luminal B (ER+ but often high grade), HER2 overexpressing, basal-like (triple negative breast cancer, and many of BRCA1 gene mutated) and normal breast tissue-like (Perou CM et al., 2000; Sorlie T et al., 2003). This number of subtypes is a good example of the complexity in the biology of cancer: different mutations in different sets of genes trigger a common disease. More recently,

the intrinsic classification has been refined in a PAM50 assay based on the expression of 50 genes to classify patients into each of the five intrinsic subtypes (Nielsen TO et al., 2010).

Following the initial identification of the intrinsic molecular subtypes, gene expression studies have improved and a further sub-classification of breast cancers into new molecular entities was made. For example, a detailed analysis of genes differentially expressed in ER-negative tumors has demonstrated that basal breast cancers are a heterogeneous group with at least four main subtypes (Dawson SJ et al., 2013). Other studies have also identified a new breast cancer intrinsic subtype known as Claudin-low (Prat A et al., 2010). This subtype is characteristically triple negative but has low expression of cell-cell adhesion claudin proteins including E-cadherin, and frequently present lymphocytes infiltration. Importantly, Claudin-low tumors appear to be enriched with cells showing characteristics associated with mammary stem cells, that is tumor initiating potential and chemoresistance (Bruna A et al., 2012).

Finally, in 2012, Curtis et al published a seminal study of a large cohort of patients classifying breast tumors by genomic aberrations and transcriptome architecture. In this analysis 10 novel molecular subgroups with distinct clinical features and outcomes were revealed. The 10 integrative clusters (IntClust 1-10) were each associated with distinct copy number aberrations and gene expression changes (Curtis C et al., 2012). These clusters clearly showed the heterogeneity lost in tumors classified according to ER, PR and HER2 expression.

2.3. Risk factors

As commented above, cancer is the result of several genetics and/or environmental factors. For that reason, the well-known established risk factors that can cause breast cancer can be classified as:

- **Genetic factors:** those factors intrinsic to each person. Breast cancer occurs around 100 times more often in women than in men. However, epidemiological data has shown a clear increase in the incidence in breast cancer in men in recent years (Speirs V and Shaaban AM, 2009). Another intrinsic factor conditioning the occurrence of breast cancer is age. Cancer is the result of an accumulation of mutations, for that reason living longer increases the risk of breast cancer.

The vast majority (90-95%) of breast cancers are sporadic, meaning that they are the result of the acquisition of mutations during the life of the patients. Nevertheless, the remaining percentage of breast cancer cases is due to a hereditary or familiar susceptibility. Consequently, women who have a first degree relative with breast cancer have a double risk of disease, including early onset of disease and bilateral disease, compared with the general population (Key TJ et al., 2001). In this regard, in the last few decades, many inherited mutations in genes have been identified. The most well-known genetic link to breast cancer occurrence is a mutation in the tumor suppressor genes BRCA1 and BRCA2, involved in the repair of DNA double-strand breaks. Although the prevalence of the mutation in BRCA1/2 in the general population is low, the penetrance of these genes is high and increases 10-fold the risk of developing breast cancer. Curiously, BRCA1 mutated cancers often have a triple-negative phenotype, while BRCA2 mutated breast cancers tend to be luminal B subtype (Bai F et al., 2013; Larsen MJ et al., 2013). Other genes with low frequency and high penetrance are P53, which is associated with Li-Fraumeni syndrome, and PTEN, which results in Cowde-syndrome and has an estimated lifetime risk of breast cancer of 85% (Garcia-Closas M and Chanock S, 2009). Numerous other genes have been linked to a susceptibility to breast cancer but tend to be a low penetrance. Mutations in genes related with genome integrity and cell cycle check points such as ATM, BRIP1, PALB2, CHEK2 and RAD50 are associated with a 2-fold to 4-fold increased risk of breast cancer (Mavaddat N et al., 2010). In recent years, based on GWAS (genome wide association studies) in breast cancer, studies have discovered seven novel genetic susceptibility loci with low penetrance and low frequency: FGFR2, TNRC9, MAP3K1, LSP1, 8q24, 2q35, and 5p12 (Garcia-Closas M and Chanock S, 2009; Fanale D et al., 2012).

Finally, it is well demonstrated that there are reproductive factors which modulate the risk of breast cancer, more specifically in receptor-positive breast cancer. For example, early age-menopause has been found to increase the risk of breast cancer, whereas early menopause or having a child at a younger age reduces the risk of breast cancer (Vogel VG, 2018).

- **Environmental risk factors**: those factors that can be avoided or modulated. Exposure to radiation or some chemical compounds known as carcinogenic increases the risk of breast cancer. Other factors are related with the life style of

each person, for example a lack of physical activity, high fat diet, or alcohol consumption are closely related with a high risk of breast cancer (Vogel VG, 2018).

In recent years other risk factors (controversial in many cases) have been associated with breast cancer incidence such as dense breast tissue, abortion or breast-feeding.

3. CHEMOKINES AND CHEMOKINE RECEPTORS

3.1. Definition and function

Chemokines are chemotactic cytokines (approximately 8-17 kDa) with the ability to bind G-protein-coupled receptors in the cell membrane (Lazennec G and Richmond A, 2010). These small cytokines have a common structural feature: cysteine residues at the N-terminus. Based on the number and relative spacing of the N-terminal cysteine residues, chemokines are divided into CXC, CX3C, CC, and C subfamilies. X represents any amino acid between the two N-terminal cysteine residues (C) (Figure 5) (Karnoub AE and Weinberg RA, 2007). As commented above, chemokines exert their biological function through interaction with chemokine receptors, seven transmembrane G-protein-coupled receptors, present on the cell surface. They are also grouped into four subfamilies according to the subfamily of their major chemokine ligands (Zlotnik A and Yoshie O, 2012).

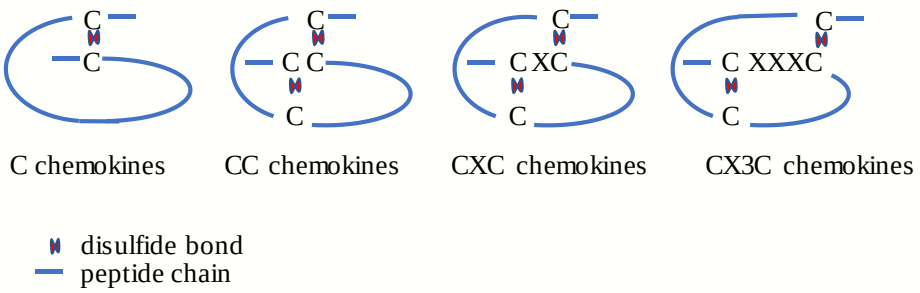

Figure 5. Structure of the chemokine classes depending on the spacing of their first two cysteine residues.

Chemokines were originally described as potent attractants for leukocytes, meaning that a gradient of chemokines can induce directional migration of the cell, a process known as chemotaxis (Lazennec G and Richmond A, 2010). In the immune system, chemokines play a critical role in cell to cell communications and tropism in situations associated with inflammation such as modulating the organization of lymphoid organs, lymphocyte differentiation and homing. The expression of these chemokines is induced by an inflammation process; thus, they are called inflammatory chemokines. However, some chemokines are constitutively expressed in specific tissues or cells and are important for other physiological processes related to directional chemotaxis such as hematopoiesis, angiogenesis, organogenesis and embryogenesis. These chemokines are called homeostatic chemokines (Zlotnik A et al., 2011). Throughout the last decade, the importance of deregulation of chemokines and chemokines receptors in cancer has been highlighted (Muller A et al., 2001). Chemokines are involved in critical steps of cancer progression such as proliferation, tumor microenvironment modulation, angiogenesis and site direct metastasis guide by chemotaxis. The human system of chemokine and chemokine receptors includes more than 40 chemokines and 18 chemokine receptors with the standard Gαi-dependent chemotactic activity have been identified in both humans and mice (Zlotnik A and Yoshie O, 2012). Notably, among different types of cancer there exists a different pattern of chemokine receptors. However, there is one chemokine receptor that seems to be overexpressed by most cancer types: the CXCR4 chemokine receptor (Balkwill F, 2004).

3.2. CXCR4 and its ligand CXCL12

CXCR4 receptor is a seven transmembrane G-protein-coupled receptor encoded on chromosome 2 (2q21). It is composed of 352 amino acids and is expressed by multiple cell types including lymphocytes, hematopoietic stem cells, endothelial cell, epithelial cells and fibroblasts (Guo F et al., 2016). CXCR4 signal is mainly activated by the endogenous chemokine CXCL12. However, a possible activation with CXCL11 and MIF has been also discovered (Bernhagen J et al., 2007; Singh AK et al., 2013)

CXCL12 chemokine (also known as SDF-1) belongs to the CXC chemokines family that signals through CXCR7 and CXCR4 receptors. This gene is located at 10q11.1

and has 6 exons. CXCL12 is expressed widely in multiple normal tissues but its expression can also be induced during pathological inflammation (Guo F et al., 2016). Like many other cytokines, there are multiple isoforms of CXCL12 in humans: α, β, γ, δ, ε, and φ. CXCL12 isoforms share the same initial three exons and differ from exon 4 onwards. All isoforms share the N-terminal 68 amino acids, which comprise the entirety of CXCL12-α isoform (Yu L et al., 2006; Ray P et al., 2015) Among these splicing variants, CXCL12-α is the most common and well-studied.

In the late 1990s, CXCR4 was discovered as a co-entry receptor for human immunodeficiency virus (HIV-1). This fact prompted the study of CXCR4 functions. The first approaches in CXCR4/CXCL12 deficient mice showed a lethal phenotype, confirming a critical role of this axis during embryonic development caused by their key role in cell motility of progenitor cells from their place of origin to their destination (Burger JA and Kipps TJ, 2006). In adult life, this motility system is used in the homing of hematopoietic stem cells in the bone marrow, which express high levels of CXCL12. In addition, CXCR4 expressed by cells of the immune system allows them to migrate along gradients of CXCL12 present in chronic or acute inflammatory processes such as wound healing or cancer progression (Lazennec G and Richmond A, 2010). In this regard, a seminal study published by Muller et al. in 2001 reported for the first time the involvement of CXCR4-CXCL12 in breast cancer metastasis (Muller A et al., 2001).

3.3. CXCR4-CXCL12 in breast cancer biology

CXCR4-CXCL12 expression is deregulated in many types of cancer, including breast cancer (Liu F et al., 2009). In fact, CXCR4 and CXCL12 expression are used as prognostic biomarkers or diagnostic markers for breast and ovarian cancer (Su YC et al., 2006; Popple A et al., 2012). CXCR4/CXCL12 expression can be regulated by epigenetic, post-transcriptional mechanisms such as microRNAs or at transcriptional level. In this way, many factors related with cancer progression such as transforming growth factor beta (TGFβ), tumor necrosis factor alpha (TNFα), SNAI2, c-Myc or estradiol can induce a positive transcriptional regulation of CXCR4-CXCL12 (Guo F et al., 2016). Interestingly, CXCR4 expression has been studied in the context of CSC. Many studies in breast cancer showed that CXCR4 is a key regulator of the stem activity and is overexpressed in breast cancer stem cell subpopulations (Ablett MP e al., 2014).

CXCL12 produced by cancer cells, can bind to CXCR4 receptor in an autocrine manner and stimulate signal transduction pathways that regulate critical steps of breast cancer progression:

- **Cell proliferation and tumor growth:** CXCR4 receptor activation induces tumor growth in primary breast tumors. The role of CXCR4-CXCL12 as tumor promoting growth was first demonstrated using a CXCR4 antagonist in mice models of breast cancer (Smith MC et al., 2004; Hassan S et al., 2011). It is well known that CXCL12 activates downstream pathways related with proliferation such as epidermal growth factor receptor (EGFR), MAPK, PI3K/Akt or ERK1/2 (Figure 6) (Barbero S et al., 2003; Porcile C et al., 2005). Interestingly, activation of MAPK by CXCL12 induces a c-Myc activation, which can upregulate the expression of CXCR4 constituting a positive feedback loop that promotes cancer proliferation (Thomas RM et al., 2008). HER2, described above as a critical receptor in breast cancer disease, is regulated by the activation of CXCR4 and constitutes another important positive feedback loop in breast cancer as HER2 can regulate positivity CXCR4 receptor (Li YM et al., 2004). In addition to an autocrine stimulation by tumor cells, there is another paracrine source of CXCL12 in tumors: the carcinoma associated fibroblasts (CAFs). Orimo et al. demonstrated that CXCL12 release by CAFs can induce tumor growth in breast cancer cells (Orimo A et al., 2005).
- **Angiogenesis:** increasing evidence suggests that CXCR4-CXCL12 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, downstream activation of PI3K/AKT proteins, and induction of VEGF release (Figure 6) (Liang Z et al., 2007); and (b) a paracrine mechanism through binding of CXCL12 released by cancer cells to endothelial CXCR4 and then increasing angiogenesis. In fact, a positive feedback between CXCR4-CXCL12 and VEGF has been demonstrated, and CXCR4 expression has been positively correlated with micro vessel density in many cancer types, including breast cancer (Bachelder RE et al., 2002; Liang Z et al., 2007). Furthermore, CXCL12 significantly upregulates several angiogenesis-related genes, such as IL-6, SOCS2, and cyclooxygenase-2 (Chu CY et al., 2009). In contrast, CXCL12 can downregulate a well-known anti-angiogenic factor PGK1 (Wang J et al., 2007). Notably, it has been revealed that the administration of CXCR4 inhibitors

decrease VEGF protein levels and disrupt tumor vasculature in a breast cancer mouse model (Hassan S et al., 2011).

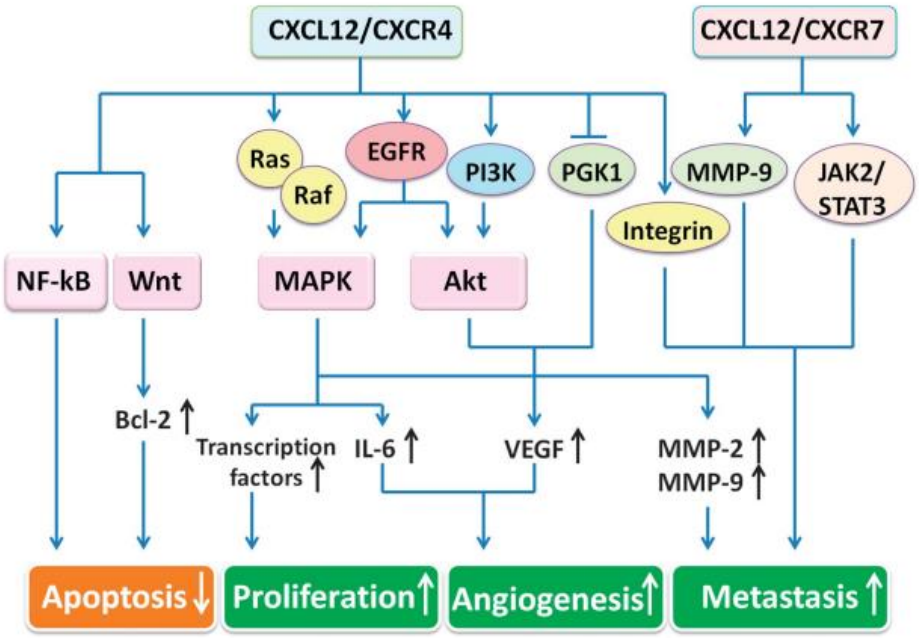

Figure 6. CXCL12-CXCR4 signaling pathway. CXCL12 binds to the CXCR4 receptor in the cell membrane and triggers an increase of proliferation, angiogenesis and metastatic behavior by activating or inhibiting multiple pathways and transcription factors. Reprinted by permission from Springer Nature.

- **CXCR4-CXCL12 in tumor micro-environment communication:** it is now clear that chemokines influence the nature and activation state of cellular components of the tumor micro-environment. CXCL12 released by tumor cells can trigger a recruitment of CXCR4 positive cells to the vicinity of tumors to assist and support tumor growth and progression. In fact, it has been demonstrated that CXCL12 can recruit monocytes/macrophages, fibroblasts and endothelial cell progenitors into the tumor mass (Orimo A et al., 2005; Ping YF et al., 2011; Domanska UM et al., 2013). Together, these cells increase tumor progression releasing growth factors, cytokines, chemokines and pro-angiogenic factors which amplify the signals secreted by tumor cells (Lazennec G and Richmond A, 2010). In this way, CXCL12 secretion is a bona fide CAF marker and constitutes an important secondary source of this chemokine (Orimo A et al., 2005). Furthermore, it is well known that CXCL12 released by tumor cells can switch

the phenotype of stromal cells. A recent study reported that CXCL12 released by myeloma cells can differentiate monocytes from macrophages and be reeducated to establish a tumor promoting micro-environment (Beider K et al., 2014). In addition, CXCL12 secreted by tumor cells can induce a phenotypic conversion from normal fibroblasts to myofibroblasts or activated CAFs in the tumor micro-environment (Toullec A et al., 2010). Preclinical studies support the use of CXCR4 antagonist plerixafor as a promising cancer treatment (AMD3100) by disrupting tumor-stroma interactions (Nervi B et al., 2009; Chen IX et al., 2019)

- **Invasion and site-directed metastasis:** a distinguishing feature of this multistep and highly organized process is the divergence in metastatic tissue tropism presented by different types of cancer cells. Secondary growth in breast cancer commonly happens in regional lymph nodes, bone marrow, lung, brain, and liver (Valastyan S and Weinberg RA, 2011). One of the explanations for this non-random affinity is the chemokine expression pattern by target organs and their cognate receptor by breast cancer cells. Muller et al. showed that CXCR4 receptor was overexpressed in breast cancer cells compared with normal breast epithelial cells and more importantly, these cells go on to metastasize organs with high CXCL12 expression such as bone, lung and liver, demonstrating a clear chemokine-dependency in the metastatic pattern (Muller A et al., 2001). This fact was confirmed in vivo by Kang et al. using MDAMB-231 breast cancer cell line. They recovered cells from bone metastasis and showed that CXCR4 was overexpressed in this subpopulation of MDAMB-231 as compared with MDAMB-231 previously injected (Kang Y et al., 2003). Furthermore, in the following years, more studies in mice xenografts showed that treatment with CXCR4 inhibitors was able to reduce the number and size of liver and lung metastasis (Liang Z et al., 2004; Gelmini S et al., 2009).

As commented above, CXCL12 binds to CXCR4 in the cell membrane and activates downstream signals such as PI3K/AKT, ERK and NF-KB, which are well studied and accepted migration pathways. This circumstance explains the role of CXCL12-CXCR4 in other capabilities of breast cancer metastasis such as invasion, wound healing or intra-extravasation (Kang H et al., 2005). CXCL12-CXCR4 is frequently used during immune and stem cell recruitment in the tissue repair process. CXCL12 is expressed in the basal lamina of endothelial cells and can activate integrins

that modulate cellular adhesion in the injured tissue. This physiological mechanism was adapted by cancer cells to intravasate to blood vessels and thus support hematogenous dissemination (Yang BG et al., 2007). More recently, it has been demonstrated that CXCL12 secreted by CAFs can facilitate tumor cell intravasation (Ahirwar DK et al., 2018).

Taken together, these data highlight the pivotal role of CXCR4-CXCL12 in breast cancer metastasis and suggest that the finding of new molecular components and regulators of this pathway will provide important therapeutic opportunities for the treatment of breast cancer.

4. PIT-1 TRANSCRIPTION FACTOR

Pit-1 protein (also known as POU1F1) is a member of the transcription factor family with a highly conserved POU domain. Pit-1 is composed of an N-terminal transactivating domain (TAD) involved in protein-protein interactions and a C-terminal domain characterized by two highly conserved protein domains, a POU-specific and Pou-homeo domain both involved in DNA binding and in interactions with transcriptional cofactors (Figure 7) (Ingraham HA et al., 1990; Andersen B and Rosenfeld MG, 2001).

Figure 7. Pit-1 transcription factor structure composed of an N-terminal TAD domain and a C-terminal domain which includes a POU-specific (POU-s) and POU-homeo (POU-h) domains.

The Pit-1 gene located in the chromosome 3, encodes a protein of 291 amino acids and was described for the first time in 1988 (Bodner M et al., 1988; Ingraham HA et al., 1988). This gene has two splicing variants resulting in an isoform alpha variant that uses an alternate in-frame splice site in the 5´coding region, compared to variant beta (also known Pit-2) that represents the longer transcript with an insertion of 78 bp resulting in 317 amino acids. However, Pit-2 is less abundant and has different target genes (Delhase M et al., 1995).

Little is known about the regulation of Pit-1 expression in humans. Analysis of Pit-1 regulatory regions has shown that its early activation involves different enhancers, one of them bound by the giant zinc finger protein ATBF1 (Qi Y et al., 2008). Pit-1 promoter has CRE domains to response AMPc. For this reason, Pit-1 expression seems to be regulated positivity by AMPc, through activation and nuclear translocation of PKA (Soto JL et al., 1995). Furthermore, it has been demonstrated that AMPc increase the levels of GH, PRL and TSH, which represent the most well-known targets of Pit-1 (Muller EE et al., 1999). Like many others transcription factors, Pit-1 has the capability of auto-regulation. Pit-1 can bind to its promoter and regulate the transcription (Ho Y et al., 2015). It has been also demonstrated that the administration of vitamin D can modulate the RNA and protein expression of Pit-1 through direct binding of vitamin receptors (VDR) to Pit-1 promoter to repress it (Seoane S and Perez-Fernandez R, 2006). Curiously, Pit-1 in cooperation with another transcription factor can increase the expression of VDR suggesting a negative feed-back regulation between these two factors (Seoane S et al., 2007).

4.1. Pit-1 function in anterior pituitary

Pit-1 expression in mammals is essential for cell differentiation process during the development of adenohypophysis (also known as anterior pituitary) as well as the regulation of growth hormone (GH) and prolactin (PRL) genes in the anterior lobe (Kelberman D et al., 2009). The Pit-1 transcription factor is involved in the specification and survival of lactotrophs, somatotrophs and thyrotrophs cells during pituitary organogenesis. In this way, a time-dependent relationship exists between the expression of Pit-1 and the expression of GH and PRL during the development (Andersen B and Rosenfeld MG, 2001). In fact, it has been demonstrated that Pit-1 can regulate GH, GHRH receptors, PRL and TSHβ subunit. In mouse models, the lack of Pit-1 shows anterior pituitary hypoplasia and dwarfism, as well as a clear reduction in levels of GH, PRL and TSH hormones (Li S et al., 1990)

More than 30 mutations of Pit-1 gene have been identified in humans, resulting in the well-known CPHD (combinated pituitary hormone deficiency) (Sobrier ML et al., 2016). Most mutations are in the POU domain, involved in DNA binding. However, only two mutations have been identified in the TAD domain of the gene. Recently, a novel

mutation has been identified in the intron 1 of the gene (Takagi M et al., 2017). All patients with CPHD show decreased levels of GH, PRL and TSH.

Pit-1 acts not only in the differentiation of lactotrophs and somatotrophs cells but also in the promotion of survival and cell proliferation through activation of cyclins. This data suggests that Pit-1 can regulate the entry in the cell cycle and DNA replication (Castrillo JL et al., 1991; Gaiddon C et al., 1999). In this sense, it is tempting to speculate that Pit-1 could be involved in anterior pituitary adenomas. In fact, high Pit-1 expression has been found in adenohypophysis tumors compared to normal tissue, suggesting a role for this factor (Sakagami Y et al., 2003) However, the meaning of Pit-1 overexpression in the anterior pituitary is still confusing. On the one hand, Pit-1 overexpression induces apoptosis and cell death through p53, a mechanism that could prevent diseases related with excess of GH secretion (Cañibano C et al., 2007; Diaz-Rodriguez E et al., 2012). On the other hand, other studies have demonstrated that the blockade of endogenous Pit-1 with short hairpin RNA or using a dominant negative mutant can drive a remarkable decrease in the proliferation in somatotroph cells (Roche C et al., 2012; Jullien N et al., 2015)

In summary, under normal conditions Pit-1 is important for the maintenance of cell survival and proliferation, but when it is overexpressed can induce cell death due to its function in the expansion/regression cycles of pituitary cell populations, suggesting a dose-dependent dual role of Pit-1.

4.2. Pit-1 in breast cancer

Pit-1 is expressed not only in the anterior pituitary. Pit-1 is also expressed in several extra pituitary tissues, including the mammary gland (Gil-Puig C et al., 2002). In breast cancer cell lines, Pit-1 can regulate both mRNA and protein GH and PRL levels, increasing cell proliferation by PRL dependent or independent mechanisms (Gil-Puig C et al., 2005; Ben-Batalla I et al., 2010b).

The regulation of these important hormones in breasts drove an intensive study of Pit-1 expression in normal and breast cancer tissues in order to figure out a possible role of Pit-1 in breast cancer. In this way, a clear increase of Pit-1 expression has been detected in breast cancer tissue compared with normal breast tissue (Gil-Puig C et al., 2005; Gao Z et al., 2016). Notably, a higher Pit-1 expression has been shown in the most aggressive

breast cancer cells lines of TNBC subtype compared to breast cancer cell lines belonging to luminal or HER-2 breast cancer subtypes (Seoane S et al., 2015). Moreover, it has been demonstrated that Pit-1 inhibits apoptosis and promotes migration and invasion in breast cancer cell lines. Notably, overexpression of Pit-1 regulates the epithelial-mesenchymal transition (EMT), a key event during metastasis process through regulation of Snail, E-cadherin and metalloproteases 1 and 13 (Ben-Batalla I et al., 2010; Sendon-Lago J et al., 2014). More recently, another group identified a hypomethylation profile in binding sites of Pit-1 transcription factor (among other transcription factors associated with stemness and proliferation such as NANOG, OCT4, SOX2 or SIN3A) in clusters of circulating tumor cells of breast cancer (Gkountela S et al., 2019).

Furthermore, *in vivo*, mice xenografts of MCF7 breast cancer cell line with Pit-1 overexpression show an increase in tumor growth and lung metastasis (Ben-Batalla I et al., 2010; Sendon-Lago J et al., 2014). These data in mice seems to be in accordance with data in humans: elevated Pit-1 expression in patients with breast cancer positively correlated with the occurrence of distant metastasis and poor overall survival (Ben-Batalla I et al., 2010; Gao Z et al., 2016). Although some mediators of Pit-1 actions are still unknown, these data suggest that Pit-1 could be a prognostic factor in breast cancer and may represent a novel therapeutic target.

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OBJECTIVES

1. To study the regulation of CXCL12 and CXCR4 expression by Pit-1 in human breast adenocarcinoma cell lines.

2. To evaluate cell proliferation, migration, invasion and angiogenesis in breast cancer cell lines after Pit-1 overexpression and CXCL12-CXCR4 blockade.

3. To evaluate cell spread and tumor growth of breast cancer cells with Pit-1 overexpression and CXCR4 blockade in a zebrafish xenograft model *in vivo*.

4. To analyze the relation between Pit-1, CXCL12 and CXCR4 and clinical outcome in breast cancer patients.

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MATERIAL AND METHODS

1. PATIENTS AND BREAST CANCER SAMPLES

Patient sample characteristics for IHC analysis are described in Table 1. Women were treated according to the guidelines used by Fundación Hospital de Jove. The study adhered to Spanish regulations and was approved by the Fundación Hospital de Jove Ethics and Investigation Committee (Code Number 80/2013). Inclusion criteria were as follows: invasive ductal carcinoma, at least 10 histopathologically assessed axillary lymph nodes, a minimum of 10 years follow-up in women without tumor recurrence. Exclusion criteria were as follow: metastatic disease at presentation, prior history of any type of malignant tumor, bilateral breast cancer presentation, having received any type of neoadjuvant therapy, development of loco-regional recurrence during the follow-up period, development of a second primary cancer, and absence of enough tissue in the paraffin blocks for analysis. Of the patients fulfilling these criteria, 48 were randomly selected and divided into two groups with similar size and stratified with regard to development of visceral metastatic disease. The median follow-up period was 130.5 months in patients without metastases and 51 months in patients with metastases. Breast carcinoma tissue samples were obtained at the time of surgery.

2. CELL LINES AND CULTURES

The human breast adenocarcinoma cell lines MCF-7, MDA-MB-231, SKBR-3, and the 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 cell passaging after they reach confluence, the cells are washed with PBS and then are detached from the surface of the culture vessel by enzymatic digestion with Trypsin-EDTA (Sigma). The cells are seeded again 1:3 in fresh medium.

3. PLASMIDS, VIRUSES AND SMALL INTERFERING RNA (siRNA)

For transient Pit-1 overexpression, the pDream2.1/MCS plasmid (obtained from GenScript, Piscataway, USA) containing an ORF clone of the human Pit-1 gene (pDream-hPit-1) was used. The empty vector (pDream) was used as control. Silencing of CXCR4 was achieved by transfecting CXCR4 shRNA expressing plasmids (Sigma) in the pLKO plasmid. The empty vector was used as control. The shRNA targeting sequences were the following: CXCR4 shRNA1 (TRCN0000256866), CXCR4 shRNA2 (TRCN0000256864), CXCR4 shRNA3 (TRCN0000256865), CXCR4 shRNA4 (TRCN0000355689), and CXCR4 shRNA5 (TRCN0000004056).

Pit-1 or control lentiviral activation particles (Santacruz biotechnology, sc-401671-LAC and sc-437282, respectively) were transduced in MCF-7-GFP cells to permanent activate endogenous Pit-1 promoter. Each lentiviral particle contains a synergistic activation mediator (SAM) transcription activation system based on CRISPR technology. SAM activation elements are composed for: a deactivated Cas9 (dCas9) nuclease (D10A and N863A) fused to the transactivation domain VP64, an MS2-p65-HSF1 fusion protein, and a target-specific 20 nt guide RNA to Pit-1 gene, resulting in their transcriptional activation.

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 described in Silencer^{TM} siRNA Contruction Kit (Ambion, Austin, USA). Two silencer negatives (Scrambled 1 and Scrambled 2) (Ambion) were employed as control. Sequences of siRNAs were as follow:

Pit-1 siRNA-1 site 1, 5'-AACCCCTTGTCTTTACAAGTTCCTGTCTC-3' (antisense) site-2, 5'-AATTAAGTTAGGATACACCCACCTGTCTC-3' (antisense) site-3, 5'-AATTGAATCTCGAGAAAGAAGCCTGTCTC-3' (antisense).

Pit-1 siRNA-2 site-1, 5'-AATGTTGCTGTAGACATCACACCTGTCTC-3´ (antisense) site-2, 5´-AAGCTTTCAGTTTGCATGCATCCTGTCTC- 3´ (antisense) site-3, 5´ -AACTTTCTCCAGATTCAGTTCCCTGTCTC- 3´ (antisense).

4. TRANSIENT TRANSFECTIONS AND INFECTIONS

Transfections with plasmids were performed with jetPEI (Polyplus transfection, Illkirch, France). 3 x 10^6 MCF7 or MCF7-GFP were harvest and seeded in a 10 cm culture

plate. The next day the transfection was performed using 10 µg of plasmid and 30 µl of jetPEI for each cell plate. Plasmid expression was tested 24 h to 72 h after transfection.

Transfections with small interfering RNA (siRNA) were performed with jetPRIME (Polyplus transfection). MCF7 cells were seeded at 40-50% of confluency to improve efficiency of siRNA Pit-1 transfection. 24 h later, siRNA was diluted for a final concentration of 40 nM per well.

For establishment of MCF-GFP cell with Pit-1 overexpression; 2 x 10^5 cells were seeded in a 6-well tissue culture plate 24 h prior to transduction. The next day, cells were treated with Polybrene (Santacruz biotechnology; sc-134220) at a final concentration of 5 µg/ml and then were infected with the lentiviral particles encapsulated with the SAM system. After 96 h, cells expressing Pit-1 were selected with puromycin dihydrochloride (2 µg/ml), hygromycin B (500 µg/ml) and blasticidin S HCl (10 µg/ml). Resulting selective antibiotic-resistant clones were analyzed for Pit-1 gene activation by Western blot and real time PCR.

5. mRNA ANALYSIS

5.1. RNA isolation

RNA from breast cancer cell lines was obtained using the TRIzol reagent (Invitrogen, Paisley, UK). Resulting solution was precipitated by centrifugation with isopropanol, washed with etanol 75% concentrated and resuspended in DEPC-treated water (with Diethyl pyrocarbonate 0.1%) nuclease free. Their purity and concentration were tested in a NanoDrop 2000 (Thermo Fischer Scientific, Rockford, USA), a UV-Vis spectrophotometer. RNA is kept in -80ºC until their use.

5.2. cDNA synthesis

cDNA was synthesized with 400 units of M-MLV-RT (Invitrogen) from 1 µg of total RNA in a final solution of 30 µl composed of 6 µl of tampon solution, 2 mM of each dNTP (Invitrogen), 500 ng of random primers (Invitrogen), 20 units of RNase inhibitor

(RNase Out, Invitrogen). The solution is incubated as follows: 50 min at 37ºC, 15 min at 42ºC and 5 min at 95ºC.

5.3. Quantitative PCR (q-PCR)

Reactions of quantitative real time PCR were done using 2 µl of cDNA as template for SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, USA) on StepOnePlus Real time PCR System (Applied Biosystems). The Pit-1, CXCR4, CXCL12, VEGFA and 18S samples were denatured at 95°C for 5 sec, annealed at 55°C for 10 sec and extended at 72°C for 40 sec, for a total of 40 cycles. The samples were quantified using Sequence Detection Software 1.4 (Applied Biosystems), with 18S as normalization control. The oligonucleotide sequences used in real-time PCR were as follows:

gene	**forward**	**reverse**
Pit-1	attcttgacgcctctgcaact	ccataggttgatggctggtt
CXCR4	gaaccctgtttccgtgaaga	cttgtccgtcatgcttctca
CXCL12	cgattcttcgaaagccatgt	ctttagcttcgggtcaatgc
VEGF-A	cgcaagaaatcccggtataa	aaatgctttctccgctctga
18S	gtaacccgttgaaccccatt	ccatccaatcgctagtagcg

6. PROTEIN ANALYSIS

6.1. Western Blot

Breast cancer cells and HUVEC were lysated in RIPA Lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EGTA, 1.5 mM ClMg2, 1% SDS, 10% glycerol, 1% Triton X-100) with protease inhibitor cocktail (Sigma). Protein extract was kept in ice for 30 min and then was centrifugated at 14000g for 10 min at 4ºC. After discard the pellet, protein extract was quantified by Bradford method. Load buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 2% 2-Mercaptoethanol and bromophenol blue) was added to the protein extract and the solution was boiled for 5 min. The electrophoresis was performed in poly-acrilamide gel (SDS-PAGE) at 120V for 2-3 h. Proteins were transferred to a nitrocellulose membrane at 100V for 1 h and then the membrane was blocked for 1 h at

room temperature in blocking solution (0.2% I-Block in PBS-Tween-20). After that, the primary antibody was added and was incubated overnight at 4ºC. The next day, the membrane was washed three times with PBS-Tween-20 and incubated with the appropriate secondary antibody for 1 h. The signal was detected with the Pierce enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Fisher Scientific, Rockford, USA), and visualized by placing the blot in contact with standard X-ray film. CXCL12 recombinant human protein (R&D Systems, Minneapolis, USA) was used as positive control for studying the CXCR4 activation pathway. The antibodies used were as follow:

Antigen	**Source**	**Application**
CXCL12	Santa Cruz Biotechnology (FL-93)	IP
Pit-1	Santa Cruz Biotechnology (X-7) Abmart	WB, IP IHC
p-CXCR4	Abcam (ab74012)	WB
CXCR4	Abcam (ab124824)	WB, IHC
p-ERK1/2	Cell Signalling (D13.14.4E)	WB
p-AKT	Cell Signalling (D9E)	WB
AKT	Santa Cruz Biotechnology (H-136)	WB
p-JNK	Santa Cruz Biotechnology (G-7)	WB
p-P38	Cell Signalling (D3F9)	WB
VEGF-A	Santa Cruz Biotechnology (A-20)	WB
GAPDH	Santa Cruz Biotechnology (6C5)	WB

6.2. ELISA

CXCL12 was measured by ELISA (RayBiotech, Norcross, USA) in culture media after 48 h of transient Pit-1 overexpression in MCF7 cells. For ELISA assay, culture media was centrifuged to avoid cell debris and then was diluted in assay diluent 2-fold. 100 µl of each standard or sample was added into appropriate wells and incubated at room temperature for 2.5 h with gentle shaking. The wells were washed 4 times with 1X wash

solution and then 100 µl of 1X prepared biotinylated antibody to each well was added. After 1 h of incubation at room temperature the washed step was repeated. The next step was to add 100 µl of streptavidin solution to each well and incubate for 45 min at room temperature. After washing, 100 µl of TMB One-Step Substrate Reagent was added and incubated for 30 min at room temperature in darkness, then 50 µl of Stop solution to each well was added and absorbance was measured at 450 nm.

7. IMMUNOPRECIPITATION OF CULTURE MEDIUM

For HUVEC treatments, conditioned medium (CM) from control MCF-7-GFP cells, from CXCR4 knock-down, from Pit-1 overexpression, and/or from CXCL12 immunoprecipitation (IP) was obtained by culturing cells in the VascuLife basal medium for 48 h. IP was performed with 70 µl of Protein A/Sepharose and 5 µl of CXCL12 antibody (Santa Cruz Biotechnology). Conditioned medium was collected and centrifuged at 300 g for 5 min to avoid cell debris and the solution of Protein/A-CXCL12 antibody was added and incubated overnight at 4ºC. The next day, after a brief centrifugation of 3000 g for 10 min, the supernatant was collected. Non-specific IgG (Santa Cruz Biotechnology) was used as control.

8. CHROMATIN IMMUNOPRECIPITATION ASSAY

ChIP (Chromatin Immunoprecipitation) assay was performed using a ChIP assay Kit (Millipore, Temecula, USA). MCF-7 cells were transfected with 15 ug of pDream or pDream-hPit-1 for 48 h, fixed with 1% formaldehyde for 10 min at 37ºC and washed with PBS containing a protease inhibitor cocktail (Sigma) and centrifuged at 600 g for 4 min at 4ºC. The pellet was collected and resuspended in 200 µl of lysis buffer for 10 min on ice. The next step was a sonication of 16 cycles at 15W for 5 seconds/ each. After centrifugation the supernatant was diluted in a 10X buffer. As positive control, 20 µl were taken and the crosslinking was reverted for 4 h at 65ºC. The remaining volumen of sample was splitted in two tubes: one of them was immunoprecipitated overnight at 4ºC with a Pit-1 antibody (Santacruz Biotechnology) and the other was immunoprecipitated with a IgG (Sigma) as negative control. The next day, the samples were washed and the crosslinking was reverted (4 h, 65ºC). DNA was extracted using phenol-chloroform and was resuspended in 30 µl of nuclease-free water. PCR conditions were as follows: 1

minute at 95ºC, 1 minute at 56ºC and 1 minute at 72ºC, with a final step of extension at 72ºC for 10 min. The number of cycles was 35. PCR product was loaded in agarose gel (1%) and amplicons were visualized in a Digital system of UV light (Molecular Analyst, BioRAD, Irvine, USA) by tinction with RedSafe nucleic acid staining solution (iNtRON Biotechnology, South Korea). Oligonucleotides used of analyze the CXCR4 promoter were as follow:

gene	**Sequence**
Promoter CXCR4 (A)	Fwd: 5´-TGGATCCCCAACGCCTAGAA-3´ Rev: 5´-TCACTAGGGTCAGGTGCAGA-3´
Promoter CXCR4 (B)	Fwd: 5´-CCGCTATTATGAAATTACTGAGCA-3´ Rev: 5´-TCCAGACCTGGGAATGCTAC-3´
Promoter CXCR4 (C)	Fwd: 5´-CCTGGGCCTCAGTGTCTCTA-3´ Rev: 5´-GCAGACGCGAGGAAGGA-3´
Promoter CXCR4 (D)	Fwd: 5´-CCCTCCTTCCTCGCGTCT-3´ Rev: 5´-TAACCGCTGGTTCTCCAGAT-3´

9. THREE DIMENSIONAL (3D) CULTURE

For 3D cell culture, 12 mm coverslips were coated with 60 µL of ice-cold Matrigel (BD Biosciences) and incubated at 37°C for 20 min to allow Matrigel to solidify. A single cell suspension containing 5×10^3 MCF-7-GFP-control or MCF-7-GFP-Pit-1 cells per 100 µL of culture medium, supplemented with 2% (vol/vol) of Matrigel, was carefully placed on the coverslips on top of the solidified Matrigel and incubated at 37°C for 30 min. Coverslips were then placed in six-well plates with 500 µL of culture medium per well. After 7 days, cells were treated every 48 h with 1 µM of the CXCR4 receptor antagonist AMD3100 (Sigma) or vehicle for 5 days. Photographs of the 3D cultures were taken with a Nikon Eclipse Ti-S inverted microscope (Izasa, Barcelona, Spain) equipped with a ProgRes C3 camera and the ProgRess Capture Pro 2.7 software. Quantification of the sphere diameters was performed manually by tracing a straight line across the diameter of the sphere and scoring its value as relative units.

10. WOUND-HEALING

To test the ability of breast cancer cells to close the gap made in a monolayer of cell culture, 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 2 culture-inserts (ibidi, Munich, Germany) on 35-mm dish. After cell attachment, culture inserts were removed, and the dishes were refilled with the appropriated culture medium. A cell-free gap is created in which the cell migration can be visualized. Remaining gap between cells after wounding was measured under a fluorescence microscope at 0, 12, and 17 h.

11. CELL INVASION ASSAY

Cell invasion assay was performed in 24-well tissue culture plates with invasion chambers inserts of 8-µm pore size (Corning, New York, USA). Invasion chambers were coated with 100 µl of growth factor-reduced Matrigel (BD Biosciences) diluted in deionized water (1:4). In the lower chamber, 500 µl of growth medium with 20% FBS was added. In the upper chamber, 5 x 10^4 MCF-7-GFP or MCF-7-GFP-Pit-1 or MCF-7-GFP-shCXCR4 or MCF-7-GFP-Pit-1-shCXCR4 cells were seeded in culture growth medium without FBS. The cells are incubated 24 h at 37ºC in 5% CO_2. Then, the cells were fixed with cold ethanol (75%) and observed under a fluorescence microscope. Values for cell invasion were expressed as the mean number of cells per field over four fields per filter.

12. CELL EXTRAVASATION ASSAY

For extravasation assays, each invasion chamber (Corning) was coated with 100 µl of growth factor-reduced Matrigel (BD Biosciences) diluted in deionized water (1:5). HUVEC cells (7 x 10^4) were seeded in 100 µl Vasculife basal medium supplemented with LifeFactorsVasculifeEnGS and incubated at 37ºC for 24 h to reach a monolayer. Culture medium of HUVEC cells was then removed and 8 x 10^4 tumor cells (MCF-7-GFP or MCF-7-GFP-Pit-1) were added to the upper chamber in 200 µl of DMEN medium without FBS. In the lower chamber, 400 µg of liver, lung, brain or muscle of female mice (C57BL/6) protein extracts in 1 ml of DMEM were added before and after immunoprecipitation with an CXCL12 antibody coated with sepharose beads protein A (Santacruz biotechnology) or IgG as control. After 48 h, cells that migrated to the lower surface of the filters were fixed in ethanol and counted under fluorescence microscopy. Values of extravasated cells were expressed as the mean number of cells per field over four fields from triplicate in at least two experiments.

13. HUVEC TUBE FORMATION ASSAY

For HUVEC tube formation assay, 100 µl of growth factor-reduced Matrigel (BD Biosciences), was loaded into each well of a 96-well plate and allowed to polymerize for 30 min at 37ºC. HUVEC cells (4 x 10^5) were added to each well and incubated at 37ºC in 5% CO_2 for 6 h with different conditioned mediums from tumor cells. Formation of capillary-like structures was observed under a fluorescence microscope after cell staining with 0.5 µM CellTracker Red CMTPX Dye (Thermo Fisher Scientific, Waltham, USA) for 15 min. Tube formation was evaluated using the AngioTool software.

14. CELL PROLIFERATION (MTT) ASSAY

Cell proliferation experiments were carried out using MTT assay. HUVEC (2.5×10^4 cells/ml) were seeded in a volume of 0.5 ml in 24-well tissue culture plates. MTT assay is an indirect method of live cell estimation. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan in the mitochondria of living cells and therefore conversion can be directly related to the number of viable (living) cells. The absorbance of this colored solution can be quantified by measuring at 590 nm.

After 48 h of HUVEC seeding, 55 µl of MTT (Millipore) in a final concentration of 500 µg/ml were added to the well. After 1 h of incubation, the culture medium was discarded and 500 µl of DMSO were added in order to solubilize the amount of formazan produced. The absorbance of the samples at 590 nm in a multiwell plate reader (LB 940 Mithras, Berthold Technologies, Oak Ridge, USA). Results were plotted as the mean ± SD values of quadruplicates from at least two independent experiments.

15. IMMUNOHISTOCHEMISTRY (IHC)

Routinely fixed (overnight in 10% buffered formalin), paraffin-embedded tumor samples stored at the Fundación hospital de Jove were used after approval by the ethical and scientific committee. Histopathologically representative tumor areas without necrosis were defined on H&E-stained sections. Serial 5-µm sections were consecutively cut with a microtome (Leica HM335S, Leica Microsystems, Wetzlar, Germany) and transferred to adhesive-coated slides. The Pit-1 antibody was obtained from Abmart (Shangai, China)

and the CXCR4 antibody from Abcam (Cambridge, UK). The monoclonal Pit-1antibody (Mab Pit-1) recognizes the amino acids sequence –GEALAAVHGS- corresponding to positions 154-164 in Pit-1 sequence. Antibody validation was carried out using human breast tissue as positive control and recombinant human Pit-1 protein (sc-4014, Santa Cruz biotechnology) for Pit-1 antibody preadrsortion as negative control. Paraffin-embedded biopsies from human breast tumors were immunostained with the Mab Pit-1 using TechMAte TM50 autostainer (Dako, Glostrup, Denmark). Consecutive sections were also analyzed with the anti-CXCR4 antibody. To enhance antigen retrieval, tissue sections were treated in a PT-Link (Dako) at 97ºC for 20 min, in citrate buffer pH 6.1 for Pit-1, or in EDTA buffer pH 9 for CXCR4. Endogenous peroxidase activity was blocked by incubating the slides in peroxidase-blocking solution (Dako) for 5 min, sections were treated with anti-CXCR4 (1/500 dilution) for 60 min and Mab Pit-1 (1/200 dilution) for 25 min, at room temperature. Bound antibody was detected using EnVision Detection kit (Dako). Sections were counterstained with hematoxylin. Immunostaining levels were scored by an expert pathologist blinded to clinical outcome of patients, evaluated the percentage of positive cancer cells for Pit-1 or CXCR4, and intensity using a numeric score ranging from 0 to 3 as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. Using a Excel spreadsheet, the score was obtained by multiplying the percentage of positive cells by intensity level.

This study was performed in collaboration with Dr. Francisco Vizoso laboratory.

16. IN VIVO ZEBRAFISH TUMOR XENOGRAFT ASSAYS AND IMAGE ANALYSIS

Adult zebrafish (Danio rerio, wild-type) were maintained in 30 L aquaria at 28.5ºC at a rate of 1 fish per liter of water, with a light-dark cycle of 14:10. Zebrafish embryos were obtained from mating adults. Care use and treatment of zebrafish were performed in agreement with the Animal Care and Use Committee of the University of Santiago de Compostela. Experiments were performed under the experimental project permission MR110250 in the center authorized with REGA code ES270280346401. At the end of the experiments, embryos were euthanized by tricaine overdose. In order to perform the xenografts, 48 h post fecundation zebrafish embryos were anesthetized with 0.003% tricaine (Sigma). MCF-7-GFP, MCF-7-GFP-Pit-1 and MCF-7-GFP-Pit-1-shCXCR4 cells were incubated at 37ºC and 5%CO_2 before injection until they reach >80%

confluence. Afterwards, cells were collected and resuspended at 10,000-20,000 cells/µl of complete medium and maintained at room temperature for no longer than 2 h before they were injected. Borosilicate glass capillary needles (1 mm O.D. x 0.78 mm ID, Harvard Apparatus, Holliston, USA) were used in order to inject cells into the yolk manually using IM-31 Electric Microinjector (Narishige, East Meadow, USA) with an output pressure of 34 kPa and 30 m/s injection time. Poorly or non-injected embryos were discarded.

After injection, zebrafish embryos were incubated for 48 h post injection (hpi) at 36ºC in 24-well plates with salt de-chlorinated tap water and a pcr-tape to avoid evaporation. Analysis of tumor growth was performed with self-made software based on ImageJ yielding the number of GFP pixel in each image, which represents the area of the cells inside the yolk sac of the embryo and the GFP Intensity Medium Value, which represents the medium intensity of the GFP inside the embryo. The results were processed to obtain a tumor growth ratio (1: the number at which the cells are maintained during incubation, >1: tumor cell proliferation during incubation, and <1: tumor cell death during incubation. Spread of tumor cells was examined 48 hpi under fluorescent microscopy. To quantitatively analyze cell spread, cells lying furthest away from the primary tumor mass were chosen, and the distance between these cells and the furthest border in primary tumor was measured using the NIS-Elements software (Nikon). Three experiments with at least 40 embryos in each group were used to obtain statistically significant values.

This study was performed in collaboration with Dr. Laura Sánchez Piñón laboratory.

17. TUMOR SAMPLES FOR mRNA AND CLINICAL OUTCOME ANALYSIS

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, USA) and qPCR was performed as described above. 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 (Code Number: 2014/177). For outcome analyses, the KM Plotter Online Tool (http://www.kmplot.com) was used to evaluate the relationship between Pit-1 and CXCR4 mRNA expression and patient clinical outcome.

18. MICROARRAY AND GSEA ENRICHMENT ANALYSIS

MCF-7 cells were transfected with the pcDNA3 (control, two samples/condition) or the pcDNA3-Pit-1 overexpression vector (two samples/condition) for 48 h. After that, RNA extraction was performed. RNA was purified with PureLinkTM Micro-to-midi kit (Invitrogen). The cDNA and then the cRNA were synthesized using an oligo T7-polyT and the BioArray RNA label kit (Enzo, USA), respectively.

Microarray assay of mRNA was analysed using an Affymetrix Human Gene 1.0 ST Array (GEO database access no. GSE64101). Gene expression (.gct) and phenotype labels (.cls) were used as input for Gene set enrichment analysis generated according to GSEA v2.0.14 software instructions (http://www.broadinstitute.org/gsea/index.jsp). GSEA was run under 1000 gene-set permutations using the gene-ranking metric Signal2Noise with C5 MSigDb collection.

19. 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. Correlation between mRNA expression levels was calculated by using the Pearson correlation coefficient. P values of less than 0.05 were considered statistically significant. 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."

RESULTS

1. Pit-1 regulates CXCR4 and CXCL12

1.1. Pit-1 overexpression in MCF-7 breast cancer cell line induces chemokine regulation

To evaluate Pit-1 mediators that could activate their tumoral-promoting actions, we carried out a microarray before and 48 h after transient transfection of MCF7 cells with a Pit-1 overexpression vector (pcDNA3-hPit-1) or the empty vector as control. In order to analyze this data, a heatmap and a Gene set enrichment analysis were performed.

We found that Pit-1 overexpression induced a clear enrichment of cytokine-mediated signaling pathway and chemokine activity signatures (NES=3.05, NES=2.53, respectively) (Figures 8-9), including among them, CXCR4 and CXCL12.

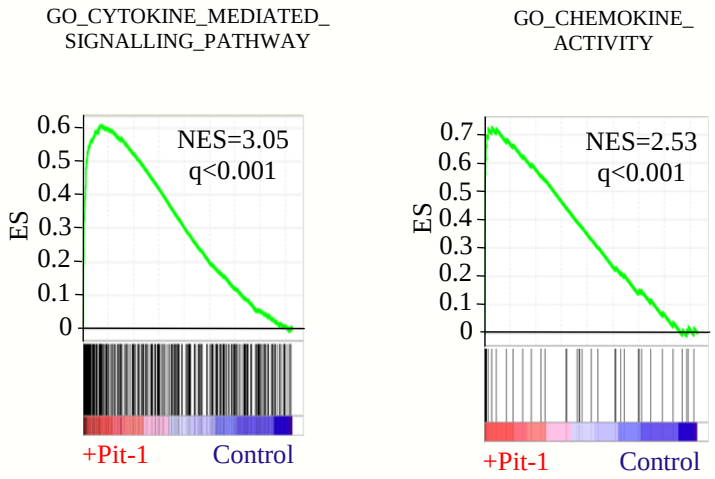

Figure 8. GSEA of C5 signatures (Cytokine mediated signaling pathway and Chemokine activity) in microarray data of +Pit-1 and control.

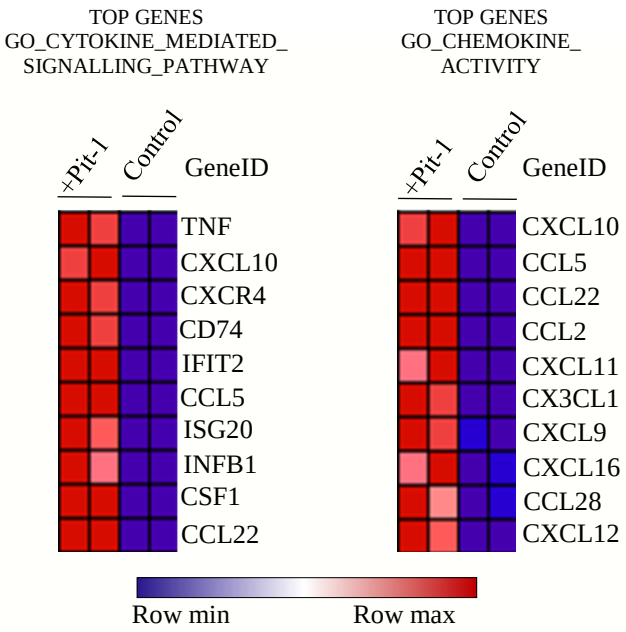

Figure 9. Heatmap of top 10 differentially expressed genes in Pit-1 overexpressing cells vs control microarray GSE64101 from the C5 signatures in GSEA.

1.2. Effect of Pit-1 overexpression or knock-down in CXCR4 and CXCL12 mRNA expression levels

Pit-1 is expressed in all breast cancer cell lines, being more elevated in high aggressive breast cancer cell lines. To evaluate the effect of Pit-1 on CXCR4 and CXCL12, MCF-7 (luminal A subtype), SKBR-3 (HER2 +) and MDA-MB-231 (basal-like) cells were transiently transfected with a Pit-1 overexpression vector (pDream-hPit-1). CXCR4 and CXCL12 mRNA levels were determined by quantitative PCR (qPCR), using 18S as load control. Our data shows that 48 h after Pit-1 overexpression both CXCR4 and CXCL12 significantly increase their levels in MCF-7 cells as compared to controls (Figure 10A). Similar data were obtained in the human breast adenocarcinoma SKBR-3 and MDA-MB-231 cell lines (Figure 10B-C).

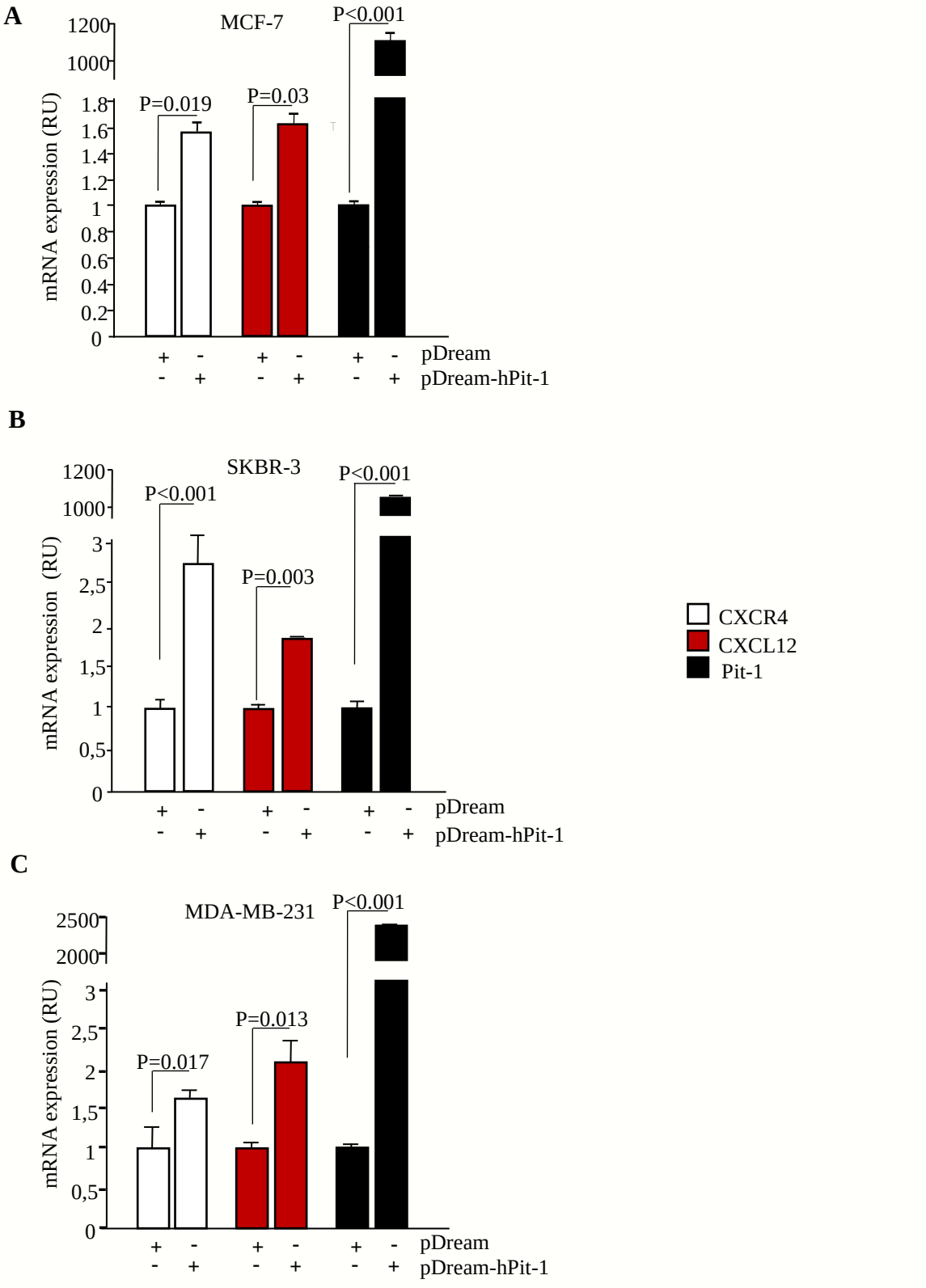

Figure 10. Pit-1 overexpression increases CXCR4 and CXCL12 mRNA. **(A)** MCF-7, **(B)** SKBR-3 and **(C)** MDA-MB-231 cells were transfected with the pDream-hPit-1 overexpression vector or the pDream vector as control, and 48 h later a real-time PCR was carried out to evaluate CXCR4, CXCL12, and Pit-1 mRNA expression. Quantitation of three independent experiments. Means were compared using 2-tailed Student's t-test.RU indicates relative units.

MCF-7 cells were also transfected using a small interfering RNA (siRNA) (40 nM) of Pit-1 to knock-down the Pit-1 expression. 48 h later, a quantitative PCR was performed to test the expression of CXCR4 and CXCL12. Figure 11 shows a significant reduction of CXCR4 and CXCL12 mRNA in MCF-7 cells after Pit-1 knock-down.

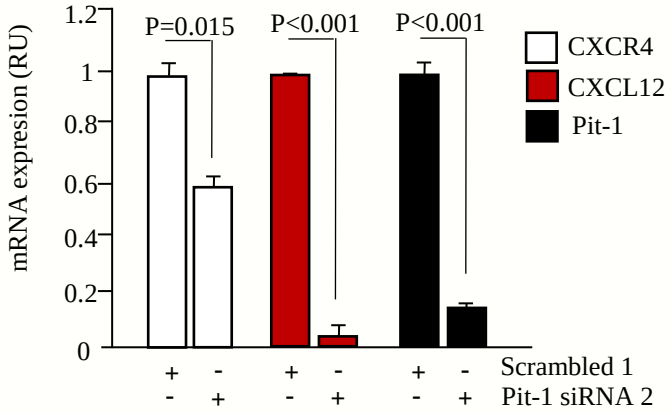

Figure 11. qPCR of MCF-7 cells 48 h after Pit-1 knock-down shows a decrease of CXCR4 and CXCL12 mRNA expression. Quantitation of three independent experiments. Means were compared using 2-tailed Student's t-test.RU indicates relative units.

1.3. Effect of Pit-1 overexpression or knock-down in CXCR4 and CXCL12 protein expression levels

To study the effect of Pit-1 expression on CXCR4 protein expression, a Western blot at 24, 48 and 72 h after transient overexpression of pDream-hPit-1 (and pDream as vector control) in MCF-7 cells was performed. GAPDH was used as load control. Figure 12 shows a clear increase of CXCR4 expression 48 and 72 h after Pit-1overexpression.

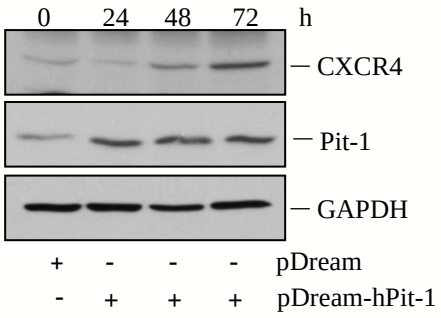

Figure 12. Western blot of CXCR4, Pit-1 and GAPDH (as loading control) in MCF-7 cells 24, 48, and 72 h after Pit-1 overexpression.

Given that CXCL12 is a cytokine released to the culture medium, the medium was collected 48 h after transient transfection with pDream-hPit-1 or the empty vector in MCF-7 cells and then, an ELISA was performed. At the same time, a protein extract of these cells was loaded in a Western blot to verify Pit-1 overexpression. The overexpression of Pit-1 in MCF-7 cells significantly increased the protein levels of CXCL12 (Figure 13).

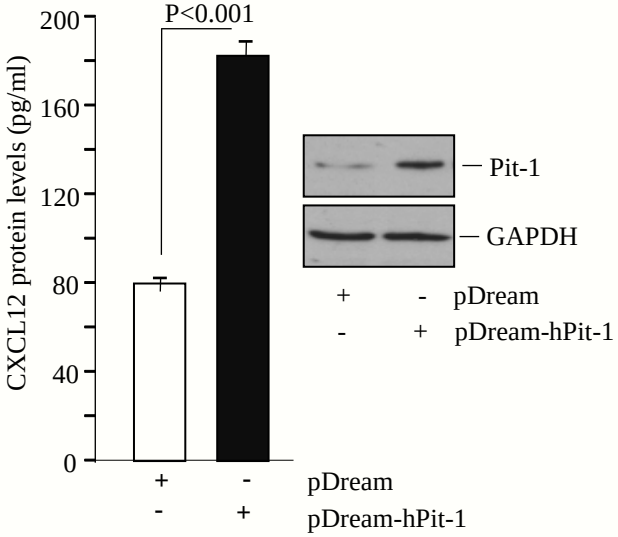

Figure 13. ELISA of CXCL12 in culture medium of MCF-7 cells 48 h after Pit-1 overexpression. Western blot of Pit-1 was used as transfection control. Quantitation of three independent experiments. Means were compared using 2-tailed Student's t-test.

Similar results were found in SKBR-3 and MDA-MB-231 cells. A Western blot was carried out 48 h after Pit-1 overexpression to test CXCR4, phosphorylated CXCR4 (p-CXCR4) (as indirect indicator of CXCL12 levels), Pit-1 and GAPDH levels. Figure 14 shows an increase in the signal of CXCR4, p-CXCR4 and Pit-1 protein expression in both cell lines.

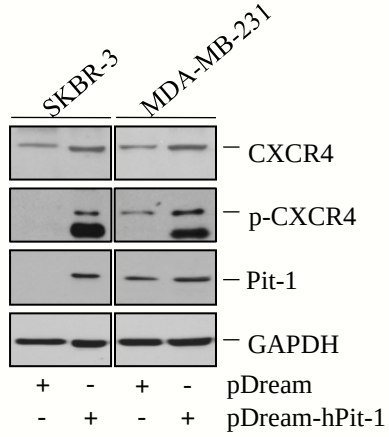

Figure 14. Western blot of CXCR4, phosphorylated (p)-CXCR4, Pit-1 and GAPDH in SKBR-3 and MDA-MB-231 cells 48 h after Pit-1 overexpression.

On the contrary, using two siRNAs to knock-down Pit-1 expression in MCF-7 cells and two missense RNA as controls (40 nM), a decrease in CXCR4 and pCXCR4 protein levels was found (Figure 15).

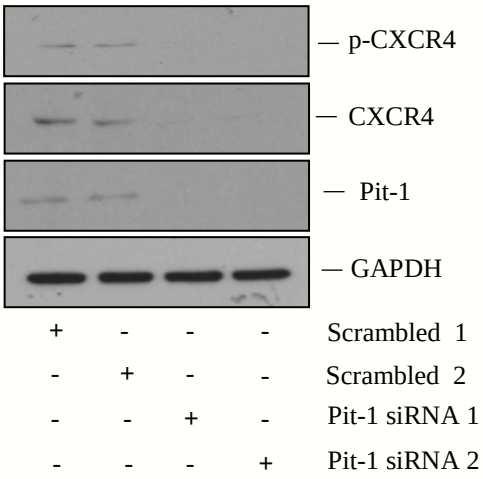

Figure 15. Western blot of phosphorylated (p)-CXCR4, CXCR4, Pit-1 and GAPDH in MCF-7 cells 48 h after Pit-1 knock-down.

2. Transcriptional regulation of CXCR4 by Pit-1

Given that Pit-1 modifies both CXCR4 mRNA and protein expression levels in breast cancer cell lines, next we tested a possible transcriptional regulation of CXCR4 by Pit-1 in a Chromatin Immunoprecipitation assay (ChIP).

Using an online software, we found two putative binding sites for Pit-1 in the CXCR4 promoter. Therefore, four pairs of primers were designed to amplify these regions of the promoter (Figure 16). MCF-7 were transfected with Pit-1 overexpressing vector (pDream-hPit-1) or with the empty vector (pDream) as control. After 48 h, the cells were fixed with formaldehyde and the lysate was immunoprecipitated with a Pit-1 antibody. The immunoprecipitated DNA fragments were amplified by PCR.

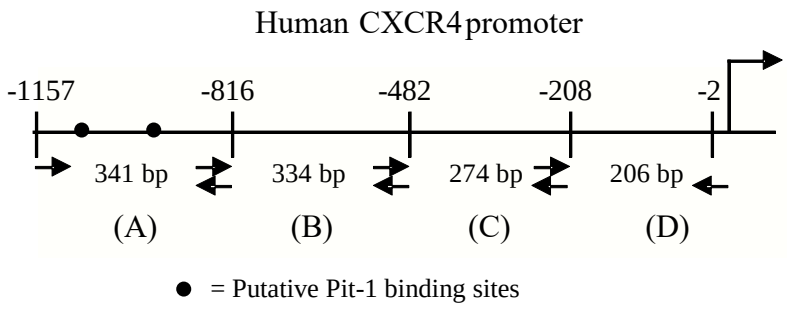

Figure 16. Schematic representation of CXCR4 promoter. Regions selected for PCR amplification are represented by A, B, C and D in the ChIP assay.

Figure 17 shows that Pit-1 bound to the region A in the CXCR4 promoter in both control and Pit-1 overexpressing cells, indicating a Pit-1 binding-site in a region between -1157/-816 bp upstream to the transcription starting site.

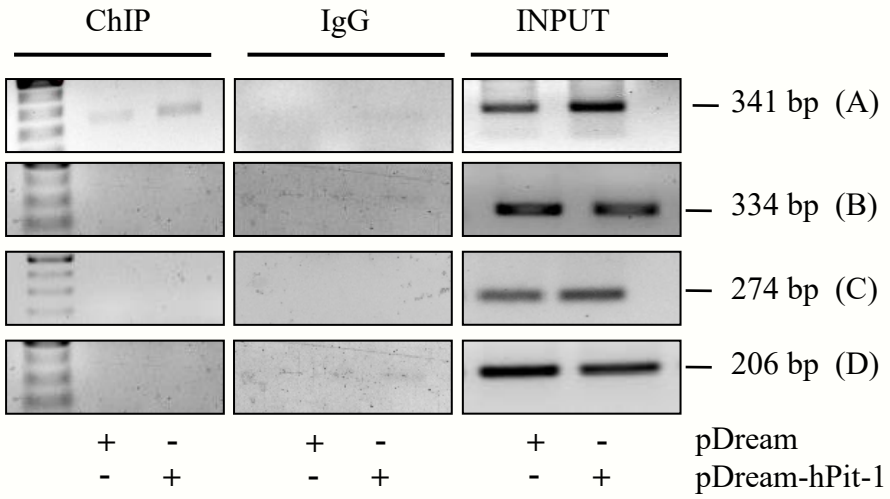

Figure 17. Chromatin immunoprecipitation assay (ChIP). Chromatin of MCF-7 cells was immunoprecipitated with a Pit-1 antibody (or IgG as negative control) and the DNA was amplified by PCR. The input was amplified with total DNA.

3. Three-dimensional (3D) cell growth

To further examine the function of Pit-1-CXCL12-CXCR4 axis in cancer progression, we used a well-known CXCR4 antagonist (AMD3100, also called plerixafor) to test the role of CXCR4 in Pit-1 overexpressing cells.

CXCR4 inhibition in MCF-7 cells was tested by Western blot after AMD3100 (10 μM) administration at 1, 2 and 4 h. The decrease of p-CXCR4 signal in MCF-7 cells, even in presence of CXCL12 (30 ng/ml), indicates a successful activity of AMD3100 (Figure 18).

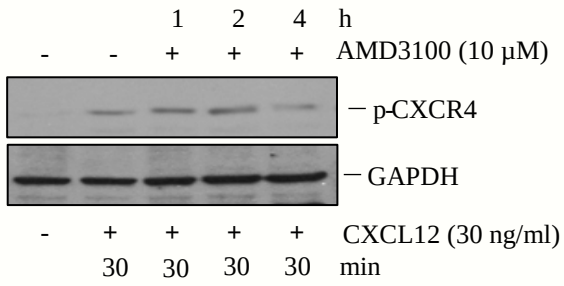

Figure 18. Western blot of p-CXCR4 and GAPDH in MCF-7 cells after treatment with AMD3100 (a CXCR4 inhibitor) for 1, 2, and 4 h and CXCL12 for 30 min.

Next, we carried out a three-dimensional (3D) assay to evaluate tumor growth *in vitro*. MCF-7-GFP cells were infected with lentiviral activation particles to induce high Pit-1 endogenous expression (MCF-7-GFP-Pit-1) and then loaded into matrigel. After 7 days, cells were treated every 48 h with either AMD3100 or a vehicle for 5 days. Our results show that CXCR4 blockade significantly (P=0.005) reduces sphere diameter (Figure 19A-B).

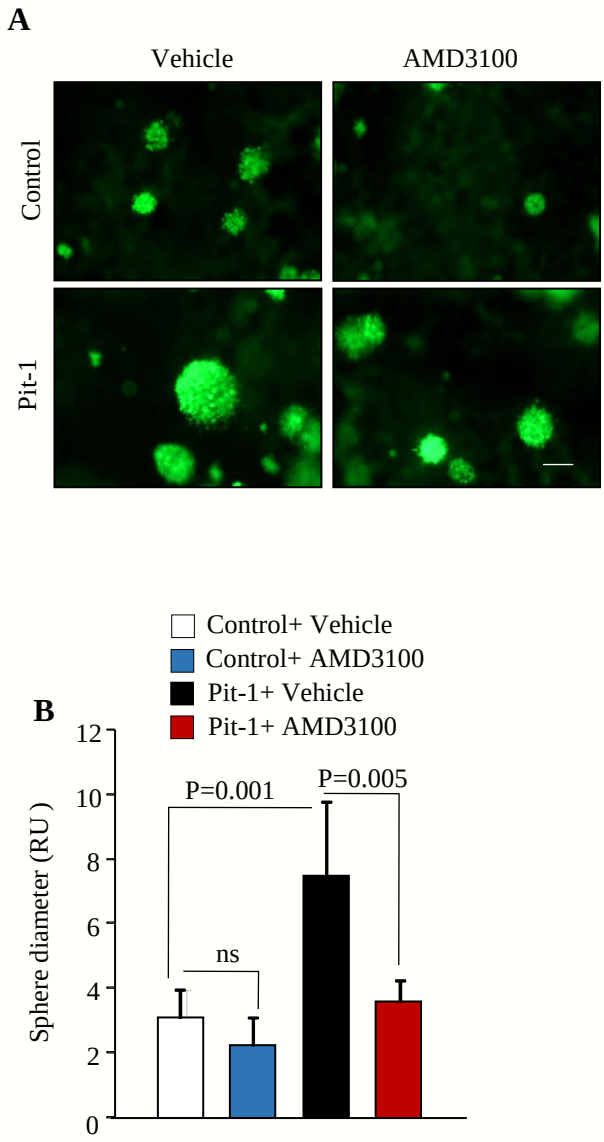

Figure 19. (A) Representative figure of three-dimensional (3D) cultures of control cells (MCF-7-GFP-control, upper panel) and Pit-1 overexpressed cells (MCF-7-GFP-Pit-1, lower panel) treated every 48 h for 5 days with vehicle (left) or AMD3100 (right). Scale bar: 100 µm. **(B)** Quantitation of three independent experiments as described in A. Quantitation of three independent experiments. Means were compared using 2-tailed Student's t-test. Numbers represent mean ± SD.

4. Cell migration and invasion

To evaluate whether CXCR4-CXCL12 modulates migration of Pit-1 overexpressing cells, a wound- healing assay was performed. First, by Western blot we demonstrated a significant reduction of CXCR4 expression after CXCR4 knock-down using five shRNA (Figure 20). The first (shCXCR4-1) was chosen to perform the assays.

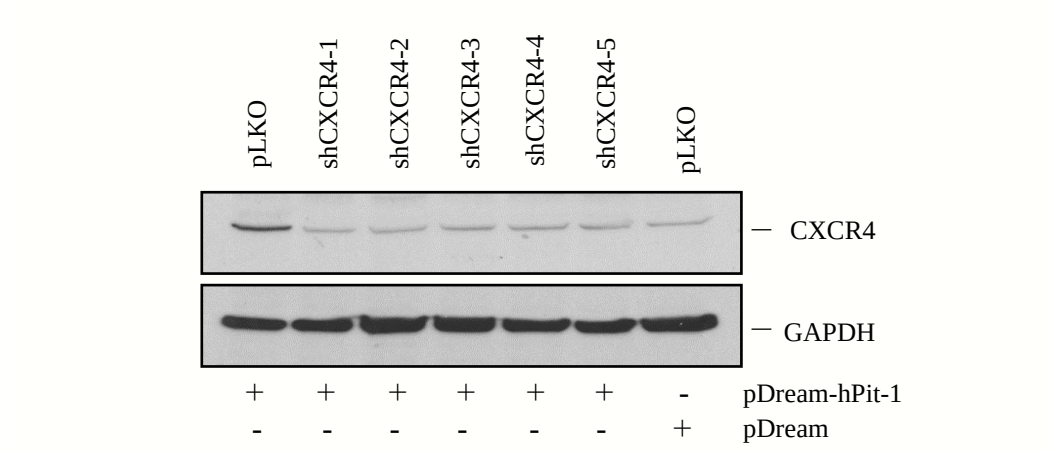

Figure 20. Western blot of CXCR4 and GAPDH in MCF-7 cells 48 h after Pit-1 overexpression (pDream-hPit-1) and CXCR4 knock-down with five different short hairpin pLKO-shCXCR4 vectors. The pLKO and the pDream vectors were used as controls.

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 (Figure 21A-B).

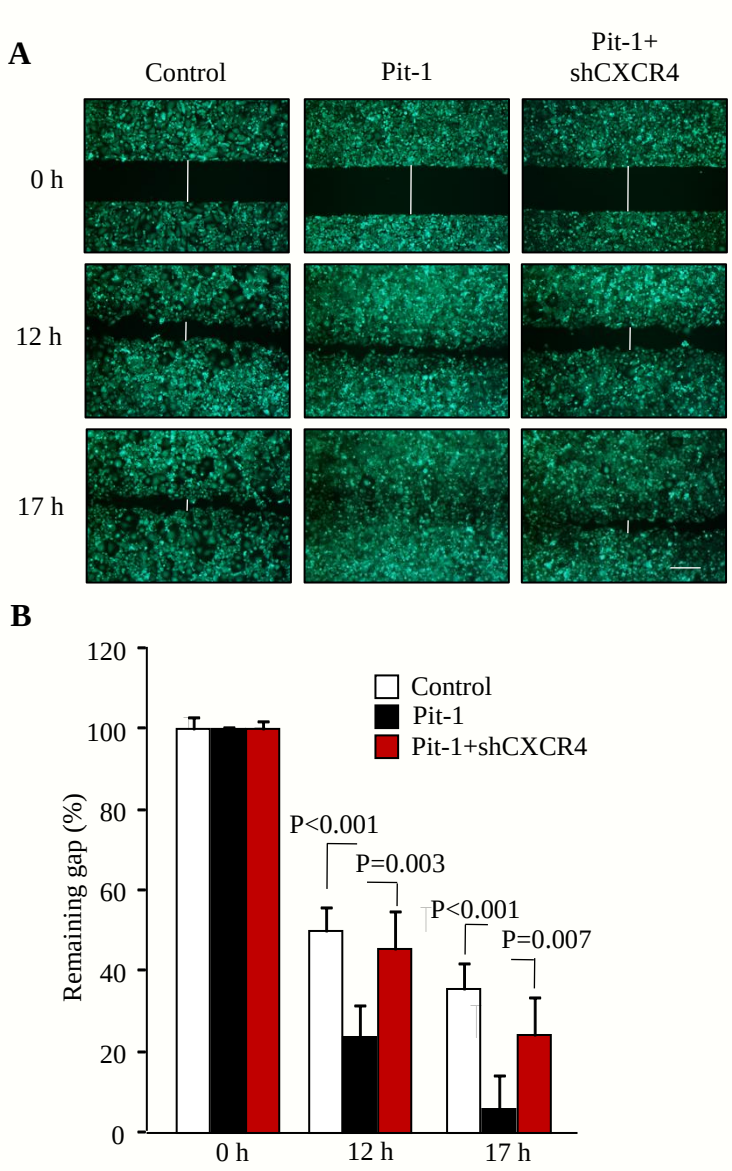

Figure 21. (**A**) Representative figure of wound healing assay of control cells (MCF-7-GFP), Pit-1 overexpressed cells (MCF-7-GFP-Pit-1), and Pit-1 overexpressed and CXCR4 knocked-down cells (Pit-1+shCXCR4). Distance between the wound edges was measured at 0, 12 and 17 h. Scale bar: 150 µm. (**B**) Quantitative analyses of three experiments as described in A. Quantitation of three independent experiments. Means were compared using 2-tailed Student's t-test.Numbers represent mean ± SD.

Another key step in the metastatic process is the invading capability of tumor cells. To test this competence, an invasion assay was performed using trans-well chambers coated with Matrigel. Pit-1 overexpressing cells show a significant increase of invading capability as regard to control cells (MCF-7-GFP) (Figure 22A-B). However, after CXCR4 knock-down in MCF-7-GFP-Pit-1 cells a significant (P=0.002) decrease in invading tumor cells was observed.

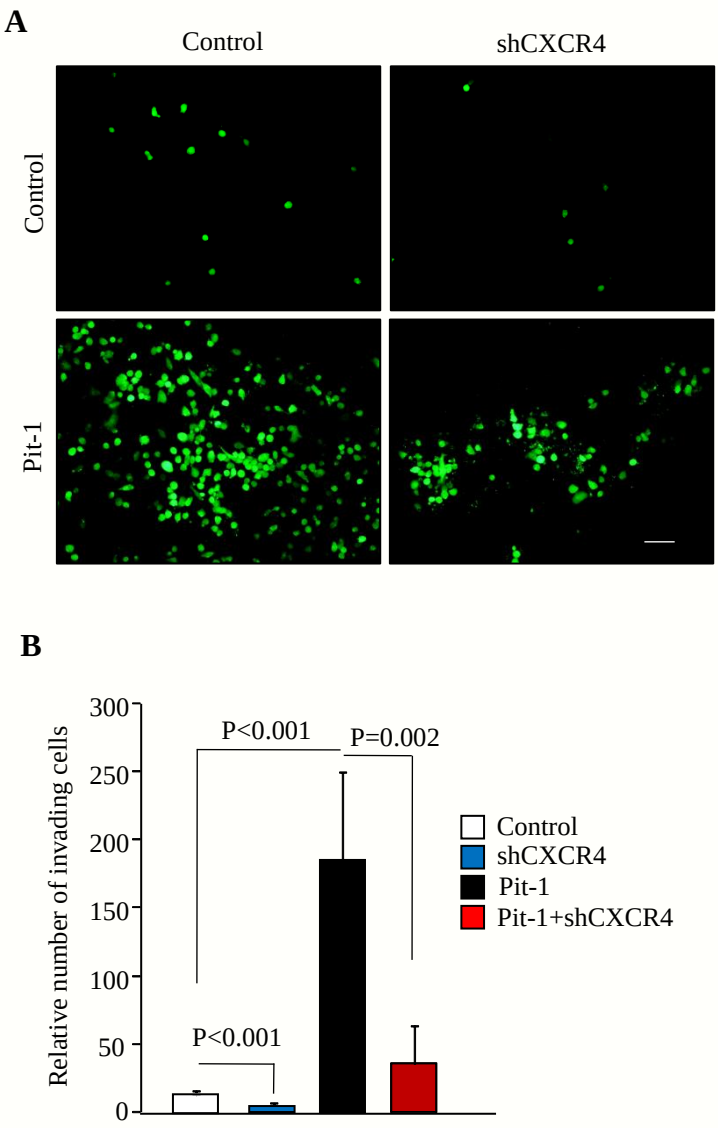

Figure 22. (A) Representative figure of invasion in MCF-7-GFP-control cells, Pit-1 overexpressed cells (Pit-1), CXCR4 knocked-down cells (shCXCR4), and Pit-1 overexpressed and CXCR4 knocked-down cells (Pit-1+shCXCR4) Scale bar: 75 μm. **(B)** Quantitation of three independent experiments. Means were compared using 2-tailed Student's t-test.Numbers represent mean ± SD.

5. Pit-1 overexpression and CXCR4 knock-down in tumor growth and cell spread in zebrafish xenografts

In order to corroborate the in vitro effects, a zebrafish xenograft model was carried out to evaluate tumor growth and spread in vivo. Either MCF-7-GFP, or MCF-7-GFP-Pit-1, or MCF-7-GFP-Pit-1-shCXCR4 cells were injected into the yolk sac of zebrafish embryos and 48 h later fluorescence intensity and area were measured. Previously, before injecting into the yolk sac, Pit-1 and CXCR4 expression were tested by Western blot (Figure 23).

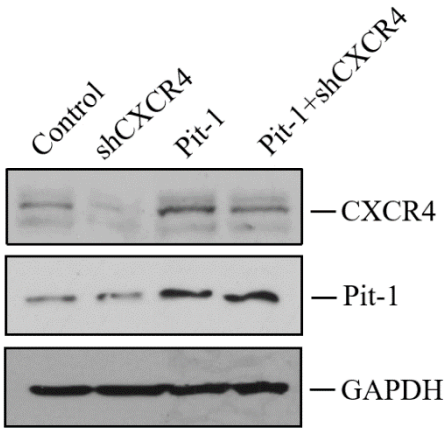

Figure 23. Western blot of Pit-1, CXCR4 and GAPDH in control cells (MCF-7-GFP+pLKO+pDream, control), CXCR4 knocked-down MCF-7 cells (pLKO-shCXCR4, shCXCR4), Pit-1 overexpressing MCF-7 cells (MCF-7-GFP+pDream-Pit-1, Pit-1), and Pit-1 overexpressing MCF-7 cells + CXCR4 knock-down (MCF-7-GFP+pDream-Pit-1+ pLKO-shCXCR4, Pit-1+shCXCR4).

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 spreading (P<0.001) in relation to MCF-7-GFP-Pit-1 cells (Figure 24A-C).

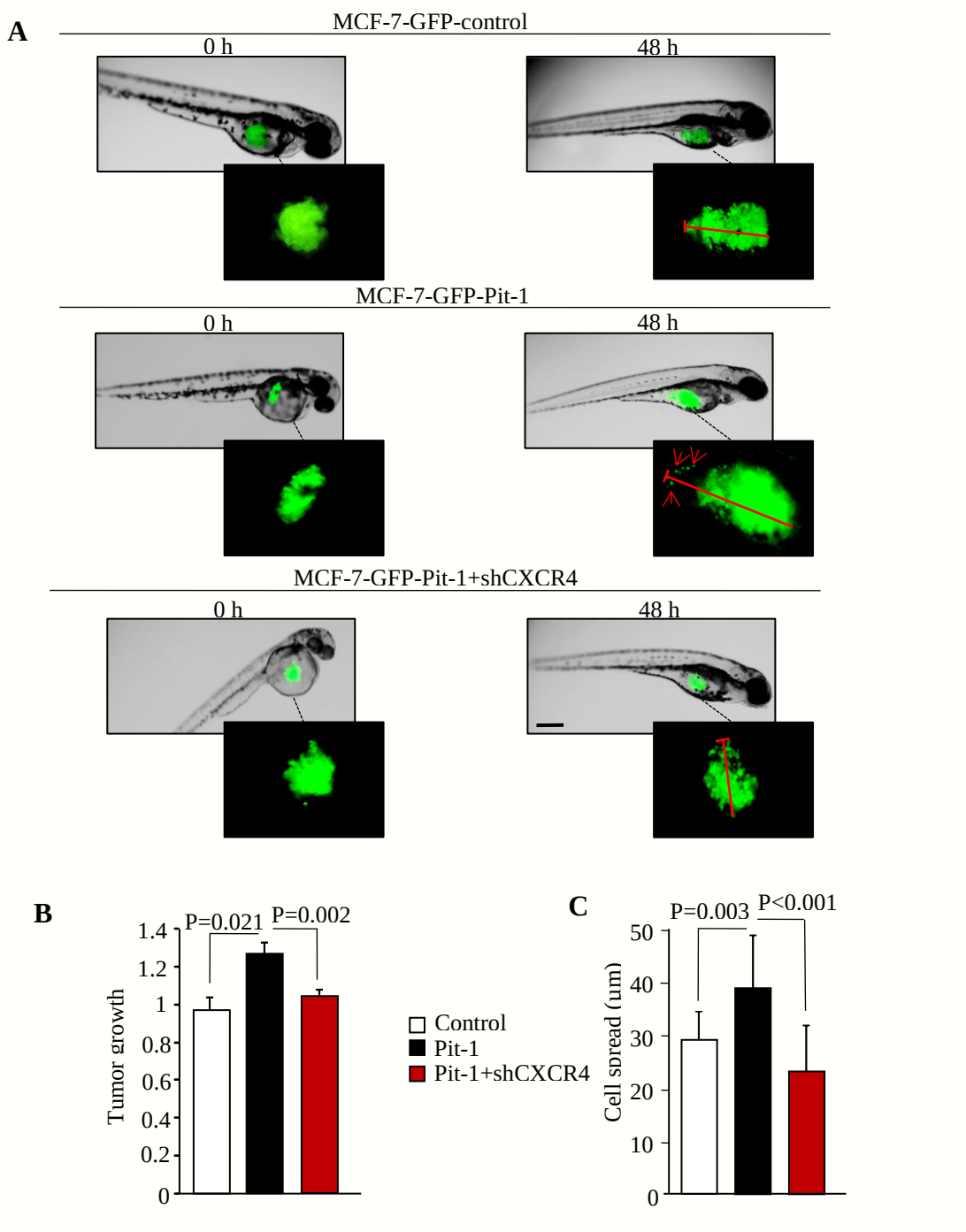

Figure 24. (**A**) Representative images of tumor growth and cell spreading in zebrafish embryos (n=40 per group) injected in yolk sac with control cells (MCF-7-GFP), Pit-1 overexpressing cells (MCF-7-GFP-Pit-1), and Pit-1 overexpressing+CXCR4 knocked-down cells (Pit-1+shCXCR4) at 0 h and 48 h post injection. 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. Means were compared using 2-tailed Student's t-test.Data are represented as mean ± SD.

6. Effect of Pit-1 overexpression and CXCR4 knock-down in angiogenesis

6.1. Effects of Pit-1 overexpression in proteins involved in the angiogenesis pathway

To study the role of Pit-1-CXCL12-CXCR4 axis in angiogenesis, we carried out a Western blot of phosphorylated proteins involved in the angiogenesis pathway in MCF-7 cells before and after transient overexpression of Pit-1. As shown in Figure 25, a clear activation of p-CXCR4, p-AKT, p-JNK, p-ERK1/2, p-P38 and total VEGF-A protein expression was observed.

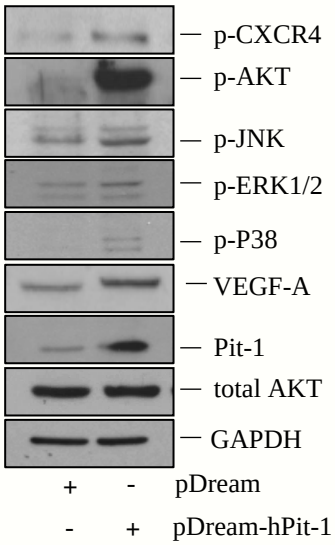

Figure 25. Western blot of p-CXCR4, p-AKT, p-JNK, p-ERK1/2 and p-P38, total AKT, Pit-1 (as transfection control), and GAPDH (as loading control) in MCF-7 cells and Pit-1 transfected MCF-7 cells.

6.2. Effect of overexpression of Pit-1 and CXCR4 knock-down in VEGF-A mRNA expression levels

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 (Figure 26). 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 (Figure 26).

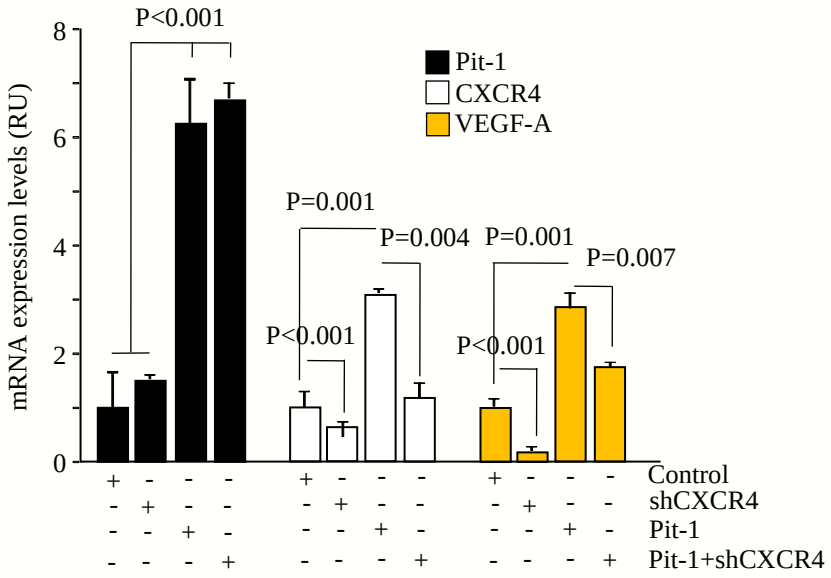

Figure 26. Real-time PCR of Pit-1, CXCR4 and VEGF-A in control MCF-7 cells and 48 h after knock-down of CXCR4 (shCXCR4), Pit-1 overexpression (Pit-1), and Pit-1 overexpression and CXCR4 knock-down (Pit-1+shCXCR4). Quantitation of three independent experiments. Means were compared using 2-tailed Student's t-test.

6.3. Effect of Pit-1 overexpression, CXCR4 knock-down and CXCL12 depletion in Human umbilical vein endothelial cells (HUVEC) tube formation assay

To clarify the functional role of Pit-1-CXCL12-CXCR4 axis in tube 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-Pit-1 cells (CM-Pit-1), d) MCF-7-GFP-Pit-1 cells and CXCL12 immunoprecipitation (IP) (CM-Pit-1-IP-CXCL12), e) MCF-7-GFP-Pit-1 and CXCR4 knock-down (CM-Pit-1-shCXCR4), and f) MCF-7-GFP-Pit-1 cells and CXCR4 knock-down, and CXCL12 IP (CM-Pit-1-shCXCR4-IP-CXCL12). Our data show a significant ($P<0.001$) increase in the total number of junctions and vessel percentage area in cells cultured with CM-Pit-1 (Figure 27A, B). However, CM-Pit-1-IP-CXCL12, CM-Pit-1-shCXCR4, and CM-Pit-1-shCXCR4-IP-CXCL12 significantly decreased the total number of junctions ($P<0.001$, $P=0.001$, and $P<0.001$, respectively) and vessel percentage area ($P<0.001$, $P=0.007$, and $P<0.001$, respectively) as compared to HUVEC cells cultured with CM-Pit-1 (Figure 27A-B).

In fact, proliferation of HUVEC cells significantly (P=0.008) increases after culture with CM-Pit-1 in relation to CM-control, but significantly (P=0.045) decreases after culture with CM-Pit-1+IP-CXCL12 (Figure 28)

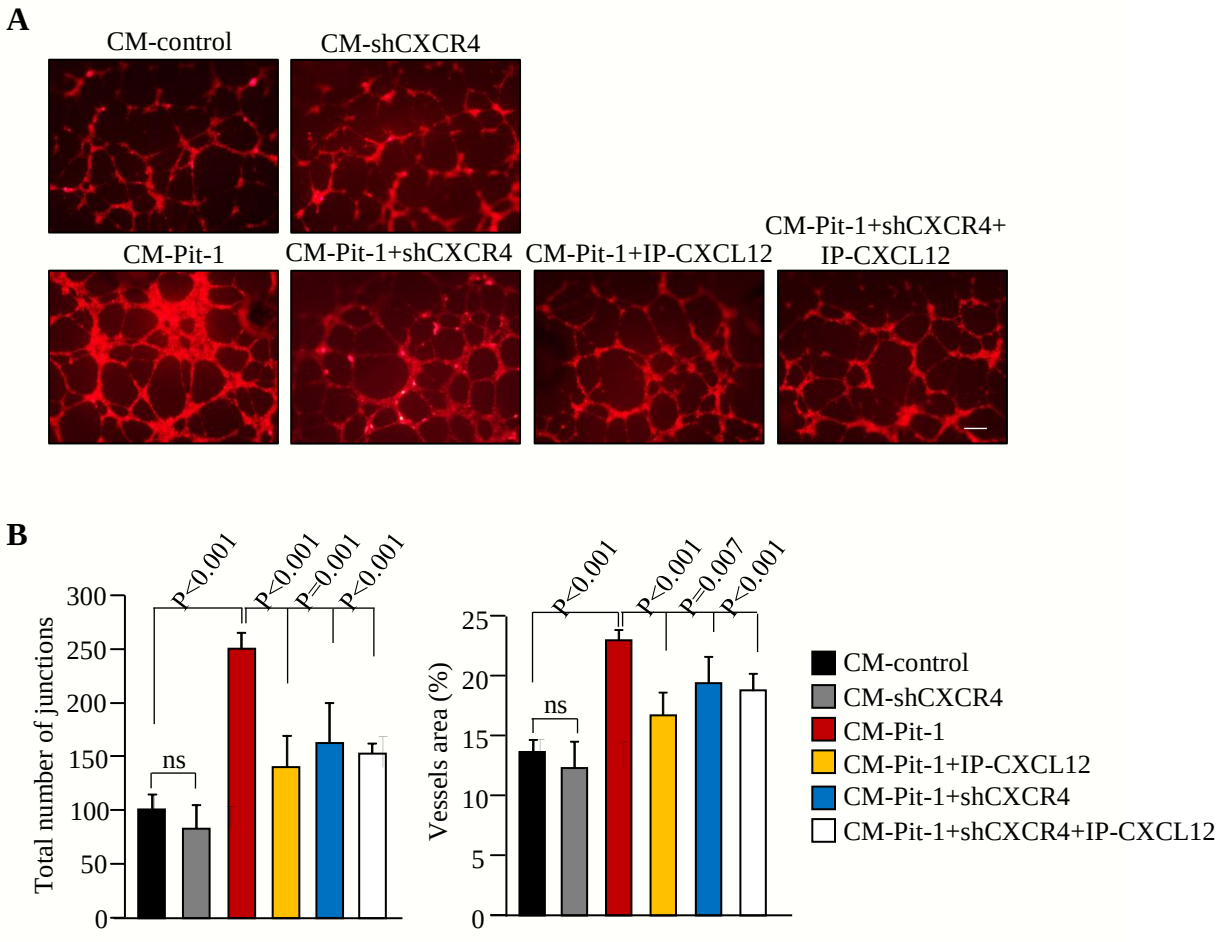

Figure 27. (**A**) Representative image of tube formation assay in human umbilical vein endothelial (HUVEC) cells after 6 h of treatment with conditioned medium (CM) from control MCF-7 cells (CM-control), from MCF-7 cells after CXCR4 knock-down (control+shCXCR4), from MCF-7 cells after Pit-1 overexpression (CM-Pit-1), from MCF-7 cells after Pit-1 overexpression and CXCL12immunoprecipitation (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. (**B**) Quantitative analyses of HUVEC tube formation assay after treatments described in A. Angiogenic parameters were calculated using the Angiotool software. Quantitation of three independent experiments. Means were compared using 2-tailed Student's t-test.Numbers represent mean ± SD.

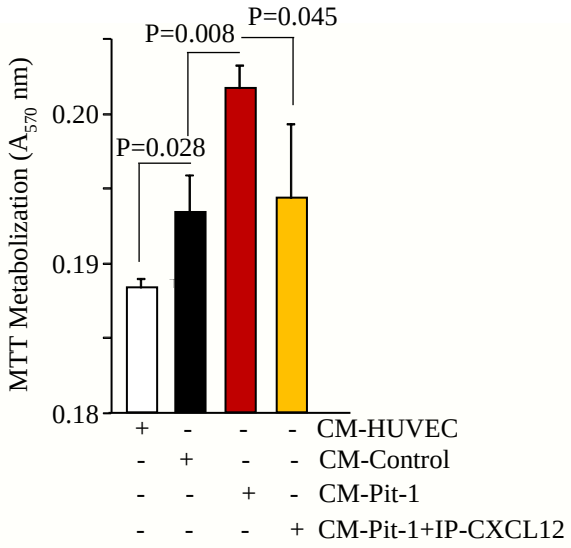

Figure 28. Proliferation (MTT) assay of HUVEC cells after 48 h of treatment with CM from their own cells (CM-HUVEC), from control MCF-7 cells (CM-Control), from Pit-1 overexpressing MCF-7 cells (CM-Pit-1), and from Pit-1 overexpressing and CXCL12 immunoprecipitated MCF-7 cells (CM-Pit-1+IP-CXCL12). Quantitation of three independent experiments. Means were compared using 2-tailed Student's t-test.

6.4. Effect of Pit-1-induced CXCL12 secretion on HUVEC activation pathways

Western blot of HUVEC cells shows a clear increase in p-CXCR4, p-P38, and p-AKT after treatment for 30 min with CXCL12 and with CM-MCF-7-Pit-1, which diminishes after IP-CXCL12 (Figure 29), demonstrating that CXCL12 activates angiogenesis-related pathways in HUVEC cells.

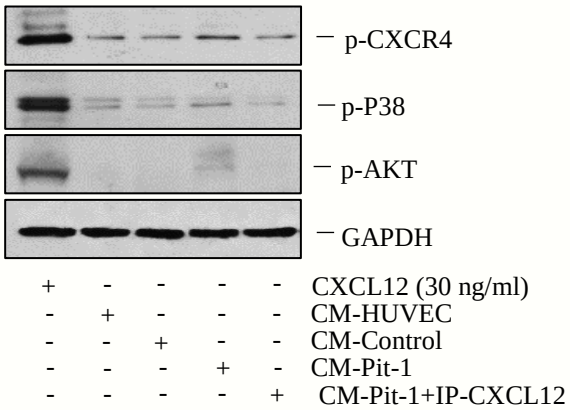

Figure 29. Western blot of p-CXCR4, p-P38, and p-AKT, and GAPDH in HUVEC cells stimulated for 30 min with CXCL12 ligand (30 ng/ml) and CM as described in Figure 28.

7. Extravasation of Pit-1 overexpressing cells toward tissues with elevated levels of CXCL12

Given that our data suggest the CXCL12-CXCR4 involvement of Pit-1-effects in angiogenesis and breast cancer progression, we explored the possibility that mammary tumor cells with Pit-1 overexpression (MCF-7-GFP-Pit-1) have a preference to metastasize in specific tissues. HUVEC cells were seeded in Matrigel in the upper chamber of a 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 (Figure 30). After 48 h, tumor cells that migrated 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 (Figure 31A-B). However, IP-CXCL12 dramatically decreases the presence of MCF-7 Pit-1 cells in liver and lung tissues (but not in brain or muscle) (P<0.001, P=0.004, respectively (Figure 31A-B).

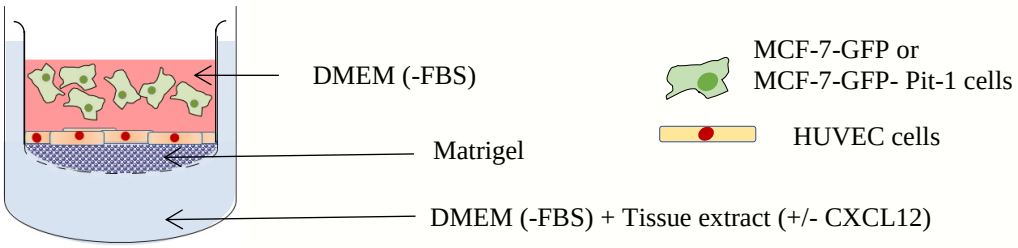

Figure 30. Schematic extravasation assay. Control (MCF-7-GFP-control) or Pit-1 overexpressing (MCF-7-GFP-Pit-1) cells are plated into the upper chamber of transwell and migrate across HUVEC cells, Matrigel and pore membrane system to gain access to the lower chamber filled with either liver, lung, brain or muscle (used as negative control) tissue extract from mouse.

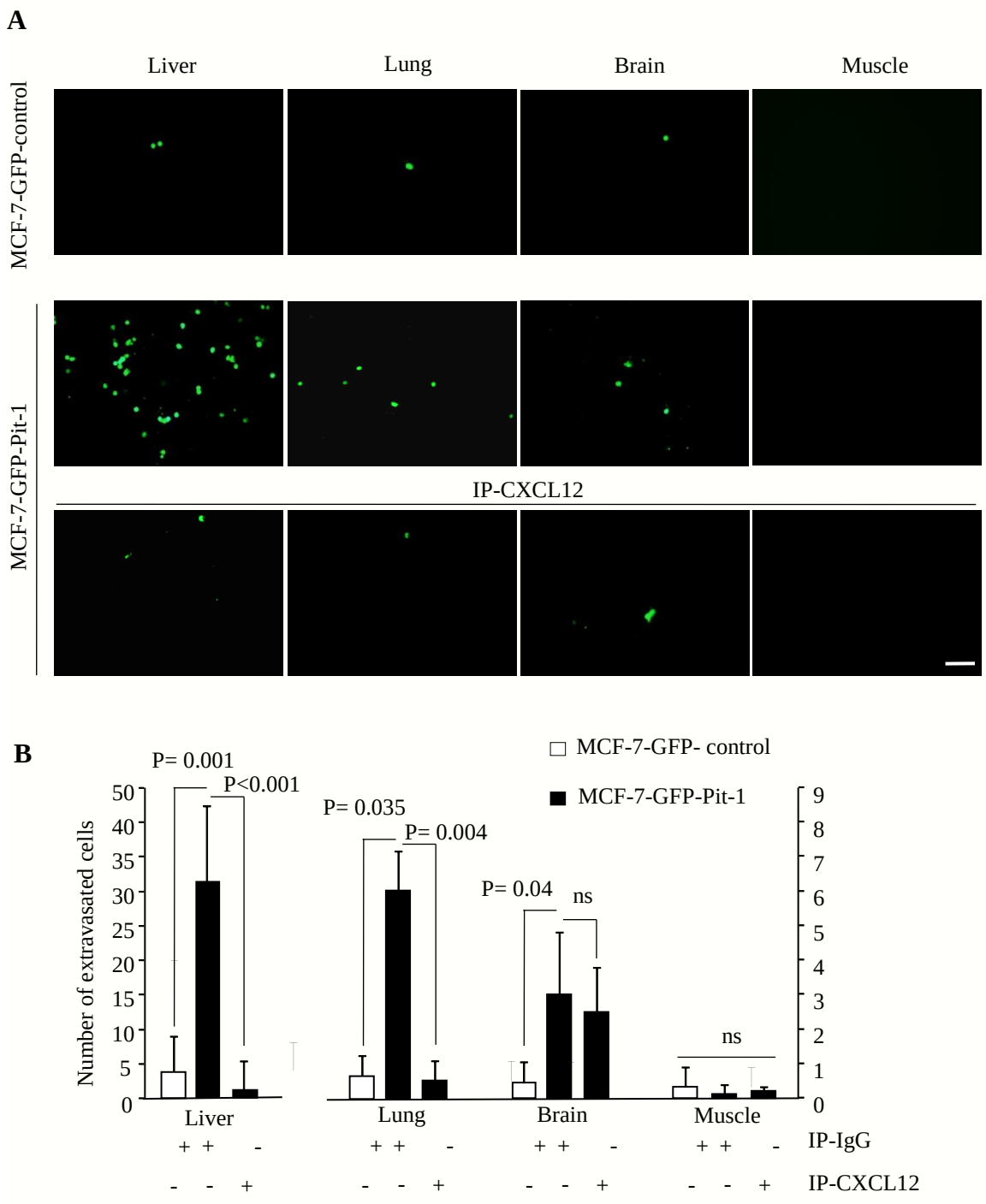

Figure 31. (**A**) Representative images of extravasated control cells (MCF-7-GFP-control), Pit-1 overexpressing MCF-7 cells (MCF-7-GFP-Pit-1), and Pit-1 overexpressing MCF-7 cells after CXCL12 immunoprecipitation in tissue extracts (IP-CXCL12). Scale bar: 100 µm. (**B**) Quantitative analyses of three experiments as described in b. Means were compared using 2-tailed Student's t-test.Numbers represent mean ± SD.

8. Pit-1, CXCR4 and CXCL12 expression in breast cancer patients and clinical outcome

8.1. Pit-1, CXCR4 and CXCL12 mRNA expression in breast cancer patients

To study the relationships between Pit-1, CXCL12, and CXCR4 in cancer patients, thirty-seven human breast tumors obtained from frozen tissue from patients with breast cancer but without clinical follow-up were analyzed by qPCR to evaluate Pit-1, CXCR4 and CXCL12 mRNA. As shown in Figure 32A-C, Pit-1 mRNA expression significantly (P<0.001) correlated with both CXCR4 and CXCL12 mRNA expression

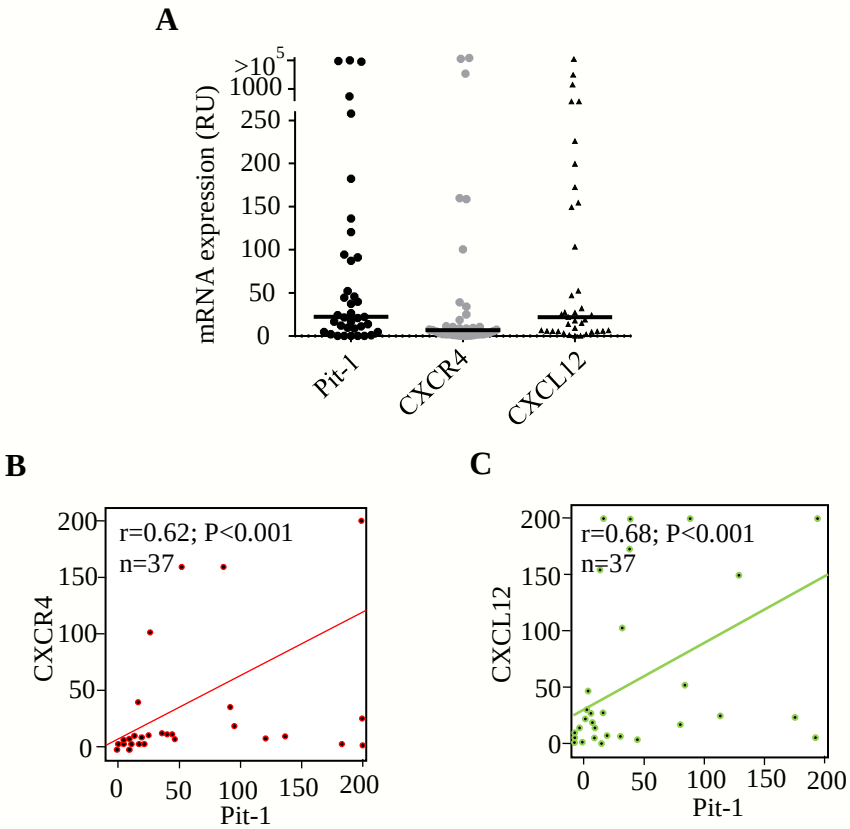

Figure 32. (A) Pit-1, CXCR4 and CXCL12 mRNA expression in 37 breast tumor samples were evaluated by qPCR. (**B**) Pearson correlation analysis of Pit-1 and CXCR4 expression in 37 breast cancer patients. **(C)** Pearson correlation analysis of Pit-1 and CXCL12 expression in 37 breast cancer patients. Dispersion plot indicates a significant (P<0.001) positive correlation between Pit-1 and CXCR4 and CXCL12 mRNA.

8.2. Effect of Pit-1 and CXCR4 mRNA expression in relapse free survival and post-progression survival

Using the online KM plotter (http://www. kmplot.com), a large database with mRNA gene expression of tumors and their clinical follow-up, a significant relation was found between Pit-1 and CXCR4 mRNA expression and the probability of relapse free survival (RFS, P=0.0015) in a cohort of 3951 breast cancer patients, and post-progression survival (PPS, P=0.0015) in 414 breast cancer patients (Figure 33A, B).

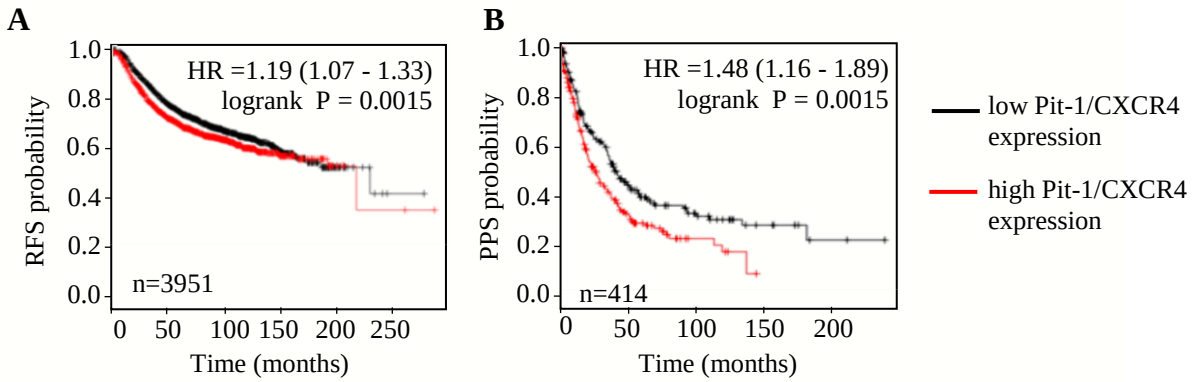

Figure 33. Association between Pit-1 and CXCR4 mRNA expression with relapse free survival (RFS) in A and post-progression survival (PPS) in B in human breast tumors using KM plotter online tool, as described in material and methods.

8.3. Protein expression of Pit-1 and CXCR4 in breast cancer patients with clinical follow-up

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 the clinical value of Pit-1 and CXCR4 expression as predictors of visceral metastasis in breast cancer (Figure 34A-C).

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 Figure 35A-B. We examined all possible score values obtained by IHC staining for Pit-1 and CXCR4 as cut-off points for predicting relapse-free survival.

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)
TMX + Trastuzumab	0 (0)	1 (4.2)
No treatment	1 (4.2)	0 (0)

TMX: Tamoxifen, CMT: Chemotherapy

For Pit-1, we found the optimal cut-off value to be 30 (χ^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 cut-off point to be 40 (χ^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 34B-C shows relapse-free survival curves for Pit-1 and CXCR4.

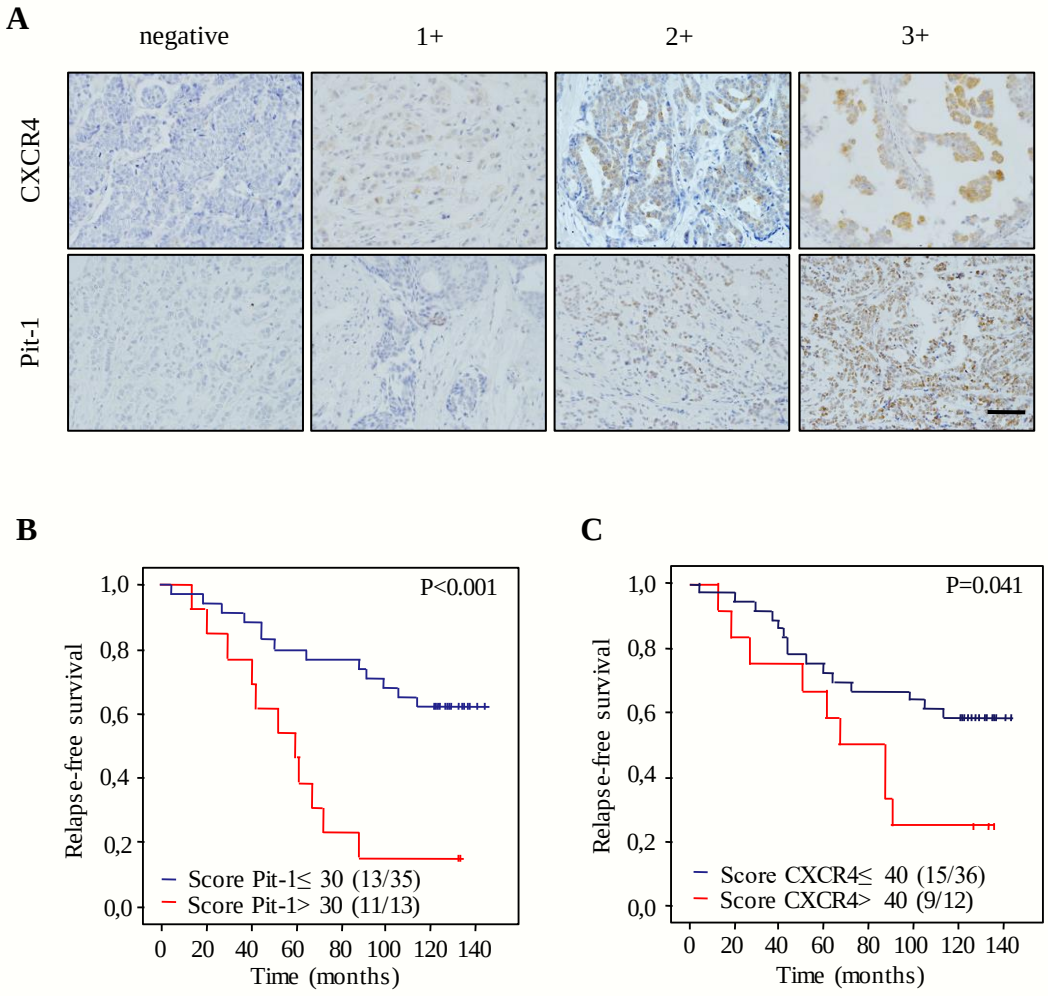

Figure 34. (**A**) Representative set of negative and positive (1+, 2+, and 3+) Pit-1 and CXCR4 immunodetection in human breast invasive ductal carcinomas. Scale bar: 100 µm. (**B-C**) Probability of relapse-free survival in 48 breast cancer patients with clinical follow-up as a function of the optimal 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.

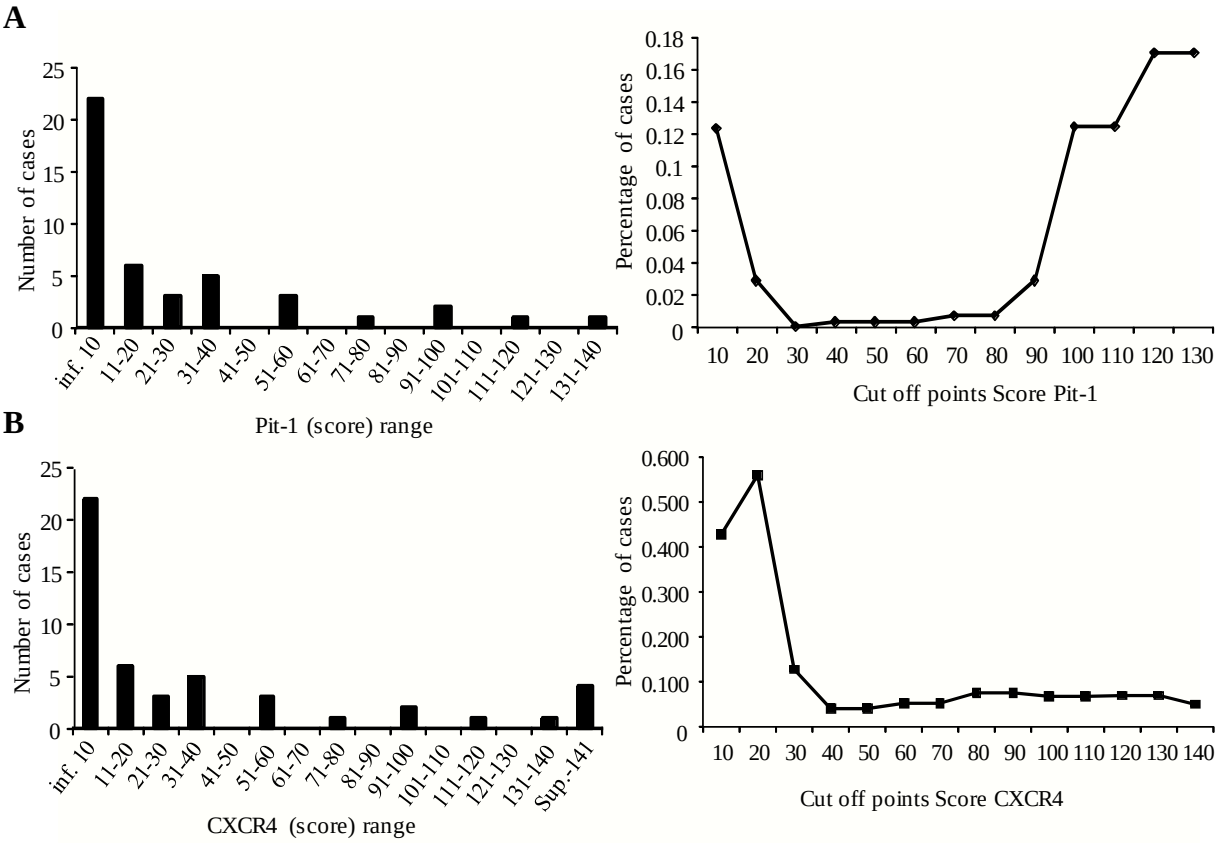

Figure 35. Distribution of score values obtained by IHC staining of Pit-1 (A) and CXCR4 (B) in 48 ductal invasive carcinomas of the breast.

DISCUSSION

Although in recent years knowledge and treatments against cancer and specifically in breast cancer have been improved, the appearance of metastasis remains the key problem and the main cause of death in patients with cancer. The survival and secondary growth of cancer cells in a foreign tissue, surrounded by unfamiliar cells, represents a significant example of evolution and adaptation to the environment; therefore, those cells that survive can generate metastatic tumors with a markedly different biology from that of the primary tumors (Anderson RL et al., 2019). Thus, the identification of early metastasis markers in tumors or in subpopulations of cancer cells in tumors can be more important than classic prognosis markers such as tumor size or tumor grade.

It has been known for well over a century that cancers show a tissue-specific pattern of metastases. For example, in breast cancer, secondary growth commonly occurs in regional lymph nodes, bone marrow, lung, brain, and liver. In contrast, prostate cancer metastasizes to the bone and only rarely to lungs or the liver. The idea that the pattern of metastatic dissemination of cancer cells can not only be explained by the anatomy of vascular and lymphatic drainage from the site of the primary tumor to the metastatic tissue dates to 1889 when Steven Paget published the "seed and soil" hypothesis in The Lancet journal (Langley RR and Fidler IJ, 2008). Paget proposed that the distribution of metastases cannot be due to chance: the"seed" refers to cancer cells in the blood or lymphatic system, and the "soil" is the organ or tissue providing an proper environment for the growth of cancer cells (Paget S, 1889). In summary, Paget suggested that the metastasis was a result of this match, the appropriate seed in a correct soil, and involves the interaction between the cancer cell and the host organ.

Given the well-defined role of chemokines and their receptors in site-directed leucocytic traffic and their crucial role during cell motility in organogenesis, these proteins have been proposed as master regulators of the metastatic spread of tumor cells to specific anatomical sites (Lazennec G and Richmond A, 2010). Muller et al. in 2001 were the first authors to place the focus on chemokines in terms of metastatic behavior. They have demonstrated that CXCR4 is highly expressed in breast cancer compared to normal breast tissue. In addition, 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. This receptor-ligand interaction clarifies perfectly the seed and soil hypothesis proposed by Paget: different pattern expression of chemokine receptors in the "seeds" can explain their different non-random affinity to

"soils" with high expression of their ligands. Recent studies suggest not only a critical role of CXCL12-CXCR4 in organ-specific metastases of breast cancer, but also in the regulation of more steps of breast cancer progression, including proliferation, angiogenesis, and modulation of tumor microenvironment (Smith MC et al., 2004; Chen IX et al., 2019).

The role of Pit-1 in breast carcinogenesis has been studied by our group and more recently by other groups (Gao Z et al., 2016). Unrestrained Pit-1 expression works as an oncogene in breasts. Pit-1 is overexpressed in breast cancer cells compared to normal cells and Pit-1 overexpression induces both *in vitro* and *in vivo* proliferation, EMT program induction, migration, invasion and lung metastasis in xenograft mice models (Ben-Batalla I et al., 2010; Sendon-Lago et al., 2014). Furthermore, a recent study showed a hypomethylation profile in the Pit-1 binding sites in breast circulating tumor cells (Gkountela S et al., 2019). Notably, high Pit-1 expression correlates with poor prognosis and clinical outcome in patients with breast cancer (Ben-Batalla I et al., 2010; Gao Z et al., 2016).

Given that many mediators of pro-tumoral actions driven by Pit-1 in breast cancer are unknown, a hypothesis that Pit-1 could regulate the CXCR4-CXCL12 was elaborated in this study for many reasons: a) Pit-1 and CXCR4 are key factors during organogenesis, b) Pit-1 and CXCR4 are overexpressed in breast cancer cells compared to normal breasts, c) it is known that there exists a direct link between the EMT program and the acquisition of epithelial stem cell properties. Pit-1 induces EMT program activation and CXCR4 is overexpressed in breast cancer stem cell subpopulations, and d) Pit-1 overexpressing cells have a predilection to metastasize into the lung, a tissue rich in CXCL12.

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 the liver and lungs as well as poor clinical outcome.

We started with an unbiased approach, a microarray after Pit-1 overexpression was performed in the MCF-7 cell line to investigate evidence of CXCR4 and CXCL12 regulation. A clear increase of both CXCR4 and CXCL12 was found in the microarray data. Interestingly, Pit-1 upregulates other important cytokines and chemokines related

with breast cancer metastasis. Several of them, for example CCL2 or CSF1, have been involved in the recruitment of macrophage into the tumor area and their polarization to TAM phenotype (Arendt LM et al., 2013; Noy R and Pollard JW, 2014), keeping the door open for future studies of TAM polarization mediated by Pit-1 in breast cancer.

Our next step was to specifically 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 followed by a 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 (Chen HW et al., 2013; Lefort S et al., 2017)

Based on *in vitro* data obtained in cell cultures, CXCR4 and CXCL12 could be regulated in multiple ways, including at epigenetic, transcriptional and post-transcriptional level. A hypomethylation profile in the CXCR4 promoter was found in numerous types of cancer, including breast cancer, promoting the upregulation of CXCR4 (Ramos EA et al., 2011). In terms of transcriptional factors, the hypoxia inducible factors 1 and 2 (HIF-1 and HIF-2) represent the most well-known regulators of CXCR4 and CXCL12 (Martin SK et al., 2010; Guo M et al., 2014). This mechanism allows the cells to adapt to a usual situation generated in the tumors by the aberrant and highly disorganized vasculature of tumors: hypoxia. The lack of oxygen triggers the expression of HIF-1 and HIF-2 in tumor cells, which upregulates the CXCR4-CXCL12 expression, and in turn induce angiogenesis by VEGFA secretion. In a similar way, TGF-β and NF-κβ transcriptions factors can induce an increase in mRNA and protein levels of CXCR4 in breast cancer cell lines (Helbig G et al., 2003; Zhao XP et al., 2010). Interestingly, Pit-1 can act similarly to these transcription factors, increasing both CXCR4 mRNA and protein levels in MCF-7 cells.

A negative regulator of CXCR4 was identified in the CXCR4 promoter, around -300 bp upstream to the transcription start site: the Ying-Yang 1 (YY1) transcription factor (Moriuchi M et al., 1999). More upstream than the YY1 binding site, Pit-1 binds to a region in the CXCR4 promoter between -1157 bp to -816 bp to the transcription start site, demonstrated by a ChIP assay in MCF-7 cells. Overall, our data indicate that Pit-1 regulates CXCR4 at transcriptional level by direct binding to the CXCR4 promoter.

Despite our data showing that Pit-1 regulates positively the CXCL12 chemokine, it remains unknown if Pit-1 can bind directly to the CXCL12 promoter. Regarding this point, there are several studies demonstrating that CXCR4 and CXCL12 have numerous mutual regulators such as HIF-2, TGF-β or c-myb (Guo F et al., 2016).

Several studies have suggested that Pit-1 is directly involved in pituitary cell survival, determination of cell lineages and proliferation. Furthermore, in the mammary gland, Pit-1 can increase cell proliferation dependent or independent of GH and PRL regulation (Gil-Puig C et al., 2005; Ben-Batalla I et al., 2010b). Given that Pit-1 regulates CXCR4-CXCL12, and CXCR4 activation pathway and this has been associated with cell proliferation in numerous types of cancer, including breast cancer, we sought to determine whether Pit-1-induced proliferation could be partially modulated by CXCL12-CXCR4. In the present study, we demonstrate that Pit-1 induces proliferation via CXCR4 activation. Using 3D cultures, our data show that inhibiting CXCR4 with AMD3100, a well-known CXCR4 antagonist, significantly reduced Pit-1 overexpressing cells sphere diameter. In addition, we demonstrated Pit-1 activation of AKT and MAPK signaling pathways, which have been linked with high proliferation mediated by CXCL12-CXCR4. Interestingly, previous data from our group confirmed an increase of the Bcl-2 anti-apoptotic gene after Pit-1 overexpression. Bcl-2 is a well-known CXCL12 target, inhibiting tumor cell apoptosis (Guo F et al., 2016). Thus, it could be worthwhile to study in more depth Pit-1, CXCR4 and CXCL12 role in cell death.

The epithelial-mesenchymal transition (EMT) is a crucial event during organogenesis. Epithelial cells lose their cell polarity and cell-cell adhesion and acquire motility and invasive properties to become mesenchymal cells, which might once again back to an epithelial state by activation of the mesenchymal-epithelial transition (MET). EMT can occur during adult tissue repair or maintenance, and unfortunately has been adapted by tumor cells to invade surrounding tissues from primary tumors (Brabletz T et al., 2018). Activation of EMT is tightly regulated by transcription factors such as Slug, Twist or Snail which are expressed during organogenesis and they are closely related with the metastatic process (Hotz B et al., 2007). As described previously, Pit-1 is a key transcription factor during organogenesis and data from our group confirmed that it regulates Snail and activates the EMT program (Ben-Batalla et al., 2010). Given CXCR4-CXCL12 expression has been related with EMT in cancer and therefore with motility, we wanted to test if Pit-1-inducing migration and invasion could be, at least in part, mediated by CXCL12-CXCR4. The migratory capability of Pit-1 overexpressing cells was tested

using a wound healing assay, while invasion was tested using a trans-well coated with Matrigel. We observed that after knock-down of CXCR4, the remaining gap between wound edges of Pit-1 overexpressing cells was increased. Similarly, a decrease of invasion in Pit-1 overexpressing cells was found after CXCR4 knock-down. Our data seems to confirm and support the pro-invasive actions triggered by CXCL12-CXCR4 axis in primary tumors. Although further experiments are needed to link CXCR4-CXCL12 and EMT induced by Pit-1, it is very tempting to speculate about a dual role of Pit-1 on EMT- induction: a) by regulation of Snail and therefore activating EMT, and/or b) by activation of CXCR4 through autocrine CXCL12 release. Furthermore, we cannot discard other key mediators of Pit-1 in breast cancer. Both MMP-1 and MMP13, metalloproteases used by tumor cells to degrade stroma and connective tissue prior to invasion, are regulated by Pit-1, and both metalloproteases mediated Pit-1-induced migration and invasion (Sendon-Lago J et al., 2014).

The CXCL12 chemokine and its CXCR4 receptor have remained well conserved throughout evolution (DeVries ME et al., 2006). 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 (Doitsidou M et al., 2002; Tiveron MC et al., 2008; Palevitch O et al., 2010). 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 (Nica G et al., 2004). Zebrafish embryos have a number of advantages as an animal model in our study: a) endogenous CXCL12 in zebrafish activates CXCR4 signaling in human cells (Tulotta C et al., 2016), b) zebrafish embryos are transparent, and labelled tumor cells are highlighted, c) the immune system is not mature until 2-4 weeks after-fertilization which allows xenograft studies, d) a large number of animals per group can be used, and e) generate primary tumors quickly. 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 a recent study demonstrating inhibition of early metastatic events and decrease in tumor mass four days after i.v. inoculation of MDA-MB-231 cells with CXCR4 blockade in zebrafish embryos (Tulotta C et al., 2016). Furthermore, zebrafish data correlate with a number studies in rodents demonstrating the CXCL12-CXCR4 role in induction of cell proliferation, migration, and invasion in breast and ovarian cancers (Huang EH et al., 2009; Hassan S et al., 2011).

As commented in the introduction section, increasing evidence suggests a key role of CXCL12-CXCR4 axis in angiogenesis by an autocrine mechanism implying binding of CXCL12 to CXCR4 in cancer cells, and and induction of VEGF release; (Liang Z et al., 2007; Domanska UM et al., 2013), and by a paracrine mechanism through binding of CXCL12 released by cancer cells to endothelial CXCR4 (Salvucci O et al., 2002; Kryczek I et al., 2005) In fact, a positive feedback between CXCR4 and VEGF has been demonstrated (Bachelder RE, et al., 2002; Liang Z et al., 2007), and CXCR4 expression has been positively correlated with microvessel density in many cancer subtypes, including breast cancer. Furthermore, CXCR4 inhibitors decrease VEGF protein levels and disrupt tumor vasculature (Hassan S et al., 2011; Gil M et al., 2013). 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 mRNA 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 increase 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 the 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 (Orimo A et al., 2005). In spite that our study has not evaluated this mechanism, Pit-1 is expressed in mammary fibroblasts (data not shown) and, therefore, CXCL12 regulation by Pit-1 in CAF could be possible. VEGF blockade, using a monoclonal antibody, has contributed to the development of anti-angiogenesis therapies in cancer. However, most tumors became resistant to this treatment resulting in a limited benefit for patients (Bergers G and Hanahan D, 2008). Our data suggest that activation of Pit-1 and therefore CXCR4-CXCL12 induce angiogenesis by dependent and independent mechanisms of VEGF-A, and blockade of

Pit-1 in patients resistant to anti-angiogenesis therapies could open new treatment strategies.

A critical point in the metastasis process is the choice of a new "soil" by cancer cells (Nguyen DX et al., 2009). Primary tumor cells enter into systemic circulation (intravasation), exit by breaking the junctions of endothelial cells (extravasation), and finally reach the target organ. It has been recently demonstrated that extravasated breast cancer cells in bloodstream (circulating tumor cells, CTCs clusters) are hypomethylated at the Pit-1 binding sites, which occur with other transcription factors, such as NANOG or SOX2, suggesting an increase of Pit-1 activity and therefore a possible role of Pit-1 in intra/extravasation and metastatic potential (Gkountela S et al., 2019). Furthermore, the basal lamina of endothelial vessels is rich in CXCL12, which could attract breast cancer cells with high expression of CXCR4 and induce them to enter in blood (Yang BG et al., 2007). In order to test *in vitro* if the Pit-1-CXCL12-CXCR4 axis could be involved in this process, a trans-endothelial migration assay was performed using different mouse tissues to recreate the chemotactic guidance that could occurs during breast cancer dissemination. Lung, liver and brain protein extracts (as typical sites of breast cancer metastasis) were loaded in the trans-well bottom. Muscle tissue was used as a non-metastatic control (Muller A et al., 2001). 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 in zebrafish and mice (Muller A et al., 2001; Smith MC et al., 2004; Huang EH et al., 2009; Hassan S et al., 2011).

Finally, to test if our *in vitro* and *in vivo* data are 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, human primary breast tumors from both patients with metastasis in lung and liver and from patients without metastasis, were analyzed by immunochemistry to study clinical relationships between Pit-1 and CXCR4. We found a significant correlation between high Pit-1 and CXCR4 protein levels in primary breast tumors and high risk of visceral metastasis. Our data seems to confirm other studies,

which have demonstrated a significant correlation between high CXCR4 expression and visceral metastasis in triple-negative breast cancer, (Chen HW et al., 2013) and colon and melanoma tumors (Kim J et al., 2006).

CXCL12 is a chemokine expressed and released by cancer cells but also by other tumor microenvironment cell populations, such as carcinoma associated fibroblasts (CAFs) and tumor associated macrophages (TAMs). This could be why we did not find a significant correlation between Pit-1 and CXCL12 protein expression in tumor cells. As commented above, CAFs overexpress CXCL12, and this may suggest the question of whether Pit-1 regulates CXCL12 in fibroblasts and thus mediate the transformation of normal fibroblasts in CAF. In relation to this question, it has been shown that inhibition of CXCR4 decreases CAF recruitment in tumors and suggests a possible role of CXCL12 released by Pit-1 in this mechanism. Finally, data obtained from public databases indicate significant relationships between Pit-1 and CXCR4 mRNA expression levels and relapse free survival (RFS) as well as post-progression survival (PPS) in breast cancer patients (Györffy B et al., 2010).

Based on our data, targeting the Pit-1-CXCL12-CXCR4 axis in breast cancer could be carried out by the 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) (Peixoto P et al., 2008), and by transcriptional repression of Pit-1 through 1,25-dihydroxyvitamin D3 and its analogs (Seoane S et al., 2006; Seoane S et al., 2015). Notably, an advantage of vitamin D-based therapies is their dual action on tumor stroma and cancer cells. It has been demonstrated that vitamin D alleviates CAF transformation and desmoplasia in pancreatic, hepatic and colorectal cancer (Sherman MH et al., 2014; Duran A et al., 2016; Ferrer-Mayorga G et al., 2017). Several CXCR4 inhibitors have been developed, and some of them are currently under clinical trials, including solid tumors (Scala S, 2015). In fact, studies involving patient-derived xenografts have recently reported that CXCR4 inhibitors improve response to Trastuzumab and taxanes in chemoresistant HER2 breast cancer (Lefort S et al., 2017). Finally, it has been recently demonstrate that CXCR4 inhibition through AMD3100 treatment, reduces fibrosis, alleviates immunosuppression, and significantly enhances the efficacy of immunotherapy in metastatic breast cancer (Chen IX et al., 2019).

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-κβ) (Zhao XP et al., 2010; Helbig G et al., 2003). 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 36). Thus, these factors could be used as prognostic as well as therapeutic targets in breast tumors.

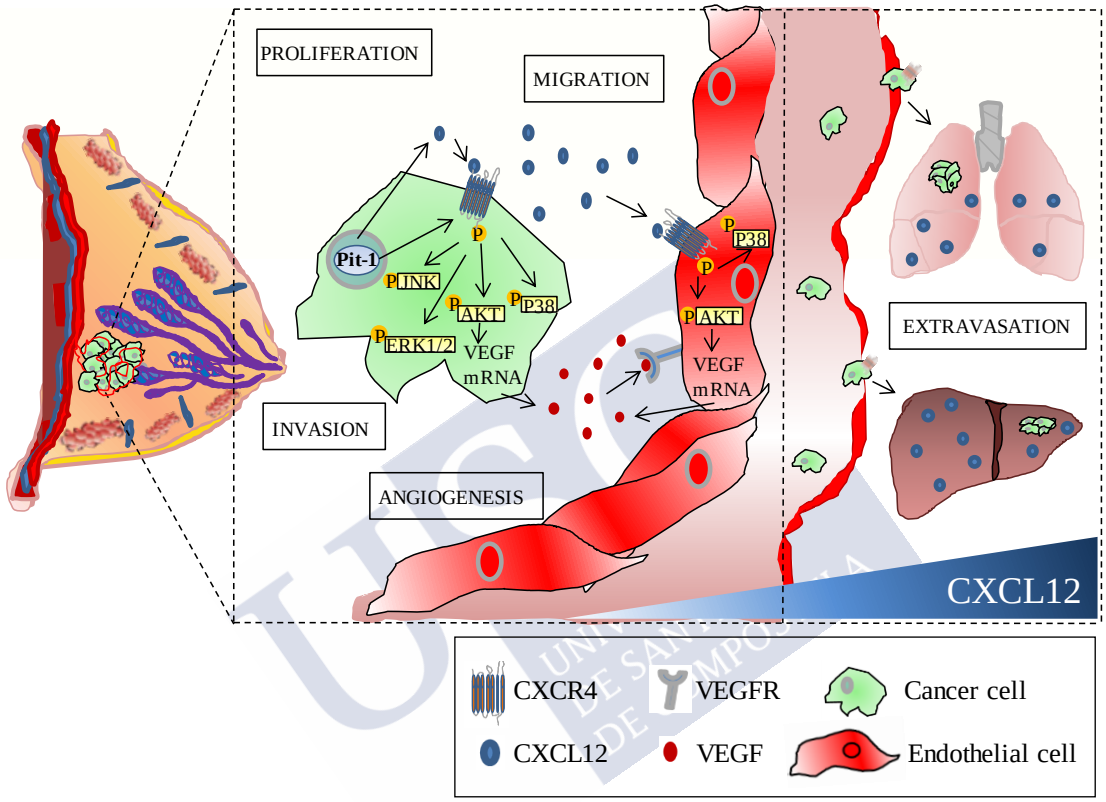

Figure 36. Schematic representation of Pit-1 overexpressing breast tumors and metastatic progression to liver and lung. To establish secondary growth, tumor cells must overcome sequential steps known as metastatic cascade. In breast tumors, deregulation of Pit-1 drives an increase of the chemokine CXCL12 and its receptor CXCR4. Activation of this pathway promotes proliferation, migration, and invasion. Furthermore, CXCL12 released by Pit-1 overexpressed cells affect to endothelial cells in tumor microenvironment increasing angiogenesis. Once Pit-1 and CXCR4 positive cells enter blood vessels (intravasation) they circulate through the bloodstream until escaping (extravasation) to target tissues with high CXCL12 expression, where they survive and grow to form metastases.

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CONCLUSIONS

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1. The Pit-1 transcription factor regulates the expression of CXCR4 and CXCL12 in MCF-7, SKBR-3 and MDAMB-231 breast cancer cell lines.

2. CXCR4 blockade reduces proliferation, migration, invasion and angiogenesis in breast cancer cell lines with Pit-1 overexpression.

3. In an in vivo zebrafish xenograft model, CXCR4 knock-down reduces tumor growth and cell spread in Pit-1 overexpressing breast cancer cells.

4. In breast cancer patients there is a significant positive correlation between Pit-1, CXCR4 and CXCL12 mRNA expression.

5. High Pit-1 and CXCR4 expression in tumors correlate with a high probability of developing visceral metastasis.

6. Our data suggest that Pit-1 and CXCR4-CXCL12 could be therapeutic targets in breast cancer patients with Pit-1 overexpression.

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ANNEX

USC
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1. SUMMARY (Galician)

O cancro de mama é a principal causa de morte por cancro en mulleres, sendo a metástase a etapa final na progresión destes tumores e a causa máis frecuente de morte. A metástase é un proceso con varias etapas: invasión polas células tumorais dos tecidos adxacentes, entrada das células tumorais na circulación sistémica (intravasación), circulación a través do torrente sanguíneo, extravasación en órganos distantes, e finalmente crecemento das células cancerígenas para producir tumores secundarios. A gran variabilidade no seu prognóstico, así como a ausencia dun tratamento universal e efectivo, obriga a realizar un enfoque terapéutico individualizado das pacientes. Por iso, cremos moi importante tratar de identificar un patrón de expresión de certos factores nas células do tumor primario que fosen preditivos de metástase. Iso tamén implicaría a localización de dianas terapéuticas. Neste proxecto estudouse a función do factor de transcrición Pit-1 na indución e desenvolvemento de metástase de cancro de mama, e en particular a regulación por Pit-1 do receptor de quimiocinas CXCR4 e o seu ligando CXCL12 (tamén coñecido como SDF1), os cales foron relacionados con metástase de cancro de mama.

O factor de transcrición Pou clase 1 homeobox 1 (POU1F1, tamén coñecido como Pit-1) pertence á familia de factores de transcrición Pit-Oct-Unc, que xogan un papel clave na inhibición e promoción da proliferación celular e na determinación de liñaxes celulares, así como na regulación da migración celular, supervivencia e diferenciación. Pit-1 é crítico para a diferenciación celular durante a organoxénese da hipófise anterior e como activador transcricional de xenes hipofisarios (transcrición de prolactina (PRL), hormona de crecemento (GH), e o propio Pit-1). Ratos con mutacións ou deleccións do xene de Pit-1 non xeran células somatotropas, lactotropas ou tirotropas, polo que presentan hipoplasia da hipófise anterior e ananismo, demostrando a importancia de Pit-1 na ontoxenia da glándula hipofisaria. Porén, Pit-1 exprésase tamén en liñas celulares e tecidos non hipofisarios, como a placenta, o tecido hematopoético ou a glándula mamaria. Nestes tecidos extra hipofisarios, suxeriuse que Pit-1 podería estar relacionado con proliferación celular e formación do tumor . Especificamente, en mama, o noso grupo demostrou unha maior expresión de Pit-1 en tumores que en tecido mamario normal, regulando tamén a expresión de dúas hormonas que foron relacionadas con cancro de mama: GH e PRL . Os nosos resultados indican que Pit-1 incrementa proliferación celular e diminúe apoptosis. Estes efectos son exercidos antes (a partir das 12 h de sobreexpresión

de Pit-1) que a indución de PRL ou GH (que se observa a partir das 36-48 h), o que parece indicar unha acción non mediada por GH/PRL. Recentemente, demostramos que a sobreexpresión e/ou inhibición de Pit-1 en liñas celulares de cancro de mama inducen cambios nos niveis de proteínas implicadas en proliferación, apoptose e invasión. Demostramos que Pit-1 dirixe ás células epiteliais a un fenotipo mesenquimal e xa que logo máis invasivo (inhibe e-cadherina e b-catenina, e incrementa vimentina). Observamos ademais, que algúns destes efectos pro-tumorais de Pit-1 son mediados pola metaloproteasa 1, 13 e Snai1 (un indutor clave na transición epitelio-mesénquima), os cales son regulados a nivel transcricional por Pit-1. En estudos *in vivo* con modelos de xenoinxertos en ratos demostramos que a sobreexpresión de Pit-1 incrementa crecemento/volume tumoral e induce metástase en pulmón. En mostras de pacientes con cancro de mama (carcinoma ductal infiltrante, n=110) e ganglios positivos, demostramos que elevados niveis de Pit-1 se relacionan significativamente cunha maior incidencia de metástase .

De forma conxunta, os nosos datos suxiren que a determinación de Pit-1 en biopsias de tumores de mama podería ser empregada como un factor prognóstico. Así, elevados niveis de Pit-1 indicarían un mal prognóstico (maior probabilidade de desenvolver metástase), sendo estas pacientes susceptibles dun tratamento máis agresivo e un seguimento estreito. Pola contra, pacientes cuxos niveis de expresión tumoral de Pit-1 fosen baixos, terían un prognóstico máis favorable, e nese caso poderían evitarse condutas terapéuticas máis agresivas . Cremos importante resaltar que Pit-1 presentou niveis de expresión proteica « moderados-intensos » no 36% das mostras analizadas (n=110), o cal parece indicar que serviría como posible factor prognóstico nun importante número de pacientes.

En relación co papel de Pit-1 como indutor de metástase, datos preliminares do noso grupo nos que cuantificamos expresión xénica tras sobreexpresión de Pit-1, indican que Pit-1 incrementa de forma significativa a expresión de varios factores relacionados coa inflamación. En particular, centrámonos en dous deles, o receptor de quimiocinas CXCR4 e o seu ligando CXCL12.

A función máis importante das quimiocinas é a de actuar como quimioatraentes para guiar a migración celular (quimiotase). Así, por exemplo, ocorre co recrutamento de leucocitos cara aos sitios de infección ou inflamación. Con todo, nos últimos anos demostrouse que as quimiocinas e os seus receptores están implicados no cancro, e en particular en procesos de metástase. É coñecido dende hai moito tempo que as células

cancerosas teñen tendencia a metastatizar en órganos específicos, o cal garda estreita relación coa migración celular normal. Foi demostrada unha elevada expresión do receptor de quimiocinas CXCR4 en células de cancro de mama humano, tumores de mama malignos e metástase. O seu ligando, CXCL12, tamén se expresa a maiores niveis en órganos que representan o primeiro destino das metástases de cancro de mama. A neutralización da interacción CXCL12/CXCR4, *in vivo*, impide de forma significativa metástase de células de cancro de mama a ganglios linfáticos rexionais e pulmón, o que parece indicar que este complexo (CXCL12-CXCR4) ten un papel crítico en determinar o destino metastático das células tumorais. Ademais de mediar no proceso de metástase, o grupo de Weinberg demostrou que fibroblastos recollidos de carcinomas de mama humana (fibroblastos asociados ao carcinoma, CAFs) inducen crecemento das células tumorais e anxioxénese debido á produción de CXCL12.

Os obxectivos deste estudo foron:

1. Estudar a regulación da expresión de CXCL12 e CXCR4 por Pit-1 en liñas celulares de adenocarcinoma de mama humano.
2. Avaliar a proliferación celular, a migración, a invasión e a anxioxénese en liñas celulares de cancro de mama despois da sobreexpresión de Pit-1 e o bloqueo de CXCL12-CXCR4.
3. Testar a diseminación celular e o crecemento tumoral de células de cancro de mama con sobreexpresión de Pit-1 e bloqueo de CXCR4 nun modelo de xenoinxerto de peixe cebra *in vivo*.
4. Analizar a relación entre Pit-1, CXCL12 e CXCR4 no resultado clínico en pacientes con cancro de mama.

Para acadar ditos obxectivos comezamos estudando o efecto da sobreexpresión de Pit-1 sobre os niveis de ARN mensaxeiro (ARNm) e de proteína de CXCR4 e CXCL12. Corenta e oito horas despois dunha transfección transitoria co plásmido de expresión pDream-hPit-1 na liña de cancro de mama MCF-7, fixemos unha extracción de ARN e observamos por PCR a tempo real un aumento significativo nos niveis de expresión de CXCR4 e CXCL12. Ademais, por Western Blot (WB) observamos un claro aumento da expresión de CXCR4 trala sobreexpresión de Pit-1. Do mesmo xeito, medindo a cantidade de CXCL12 liberado polas células de sobreexpresión de Pit-1 mediante un ELISA, observamos un aumento nos niveis proteicos de CXCL12.

De forma complementaria, observamos que tralo bloqueo de Pit-1 empregando dous *small interfering RNA* (siRNA), reduciamos de forma significativa os niveis de

CXCR4 e CXCL12, tanto de ARNm coma de proteína. Para comprobar que esta regulación tamén se podía estender a outras liñas celulares de cancro de mama, repetimos a transfección de Pit-1 na liña SKBR-3 (pertencente ao subtipo HER2+) e na liña MDA-MB-231 (pertencente ao subtipo triple negativo). Corenta e oito horas despois da transfección comprobamos que novamente incrementábanse os niveis de ARNm de CXCR4 e CXCL12, así como de proteína no referente a CXCR4.

Dado que Pit-1 modifica os niveis de expresión tanto do ARNm de CXCR4 como da proteína en liñas celulares de cancro de mama, a continuación probamos unha posible regulación transcricional de CXCR4 por Pit-1 nun ensaio de inmunoprecipitación de cromatina (ChIP).

Empregando un *software* en liña para predicir sitios de unión posibles, encontramos dous sitios de unión posibles para Pit-1 no promotor de CXCR4. Polo tanto, deseñáronse cebadores para amplificar estas rexións do promotor. Trala realización do ensaio observamos que,efectivamente, Pit-1 se unía a unha rexión do promotor comprendida entre -1157/-816 pares de bases *upstream* dende o inicio da transcrición do xene de CXCR4.

Para examinar máis a fondo o papel do eixo Pit-1-CXCL12-CXCR4 na progresión do cancro, empregamos un antagonista ben coñecido de CXCR4: o AMD3100, (tamén chamado plerixafor) para probar a dependencia da actividade de CXCR4 das células que sobreexpresan Pit-1. Nun ensaio de proliferación en tres dimensións observamos que tralo tratamento con este fármaco reduciamos de forma significativa o diámetro dos esferoides formados polas células tumorais que sobreexpresaban Pit-1. Ademais, nun ensaio de curación de ferida para valorar a migración celular tralo bloqueo de CXCR4 cun shRNA amosounos que Pit-1 aumenta significativamente a migración de células MCF-7 en comparación coas células control, pero o bloqueo de CXCR4 reduce significativamente a migración celular con respecto á sobreexpresión do Pit-1. De maneira similar, ensaios de invasión en *transwell* amosan unha diminución significativa das células tumorais invasoras despois da eliminación de CXCR4 en células MCF-7 con sobreexpresión de Pit-1.

Para corroborar os resultados obtidos *in vitro* levouse a cabo un modelo *in vivo* de xenoinxerto de peixe cebra para avaliar o crecemento e a diseminación do tumor. Células MCF-7 (a) infectadas con partículas de activación lentiviral control + transfectadas co vector control pLKO, (b) infectadas con partículas de activación lentiviral Pit-1 + transfectadas co vector control de pLKO , ou (c) infectadas con partículas de activación

lentiviral Pit-1 + transfectadas co vector de shRNA pLKO-CXCR4 que bloquea o CXCR4 endóxeno, inxectáronse no saco vitelino de embrións de peixe cebra e a intensidade de fluorescencia e a área medíronse corenta e oito horas máis tarde. Os nosos datos indican un aumento significativo no crecemento do tumor, así como na diseminación celular despois da sobreexpresión de Pit-1 con respecto ás células control MCF-7. Porén, o bloqueo de CXCR4 reduce significativamente o crecemento do tumor e a diseminación celular en relación coas células MCF-7 con sobreexpresión de Pit-1.

Para continuar estudando o papel do eixo Pit-1-CXCL12-CXCR4 na progresión do cancro, centrámonos na anxioxénese. Primeiro, realizamos un Western Blot dun extracto proteico de células MCF-7 antes e despois dunha sobreexpresión de Pit-1 para avaliar a fosforilación e a expresión de proteínas involucradas en múltiples procesos fisiolóxicos e patolóxicos, incluída a anxioxénese. Observouse unha clara activación na expresión de proteína fosforilada (p) de: CXCR4, p-AKT, p-JNK, p-ERK1 / 2, p-P38 e VEGF-A total. Despois, e dados os efectos ben coñecidos do factor de crecemento endotelial vascular A (VEGF-A) sobre o crecemento de células endoteliais e a anxioxénese, os niveis de ARNm de VEGF-A foron avaliados por PCR en tempo real en células MCF-7 despois da sobreexpresión de Pit-1 e/ou bloqueo de CXCR4. Pit-1 aumenta o ARNm de CXCR4 y VEGF-A de maneira significativa, mentres que o bloqueo de CXCR4 reduce os niveis de ARNm de VEGF-A, suxerindo que a elevación dos niveis VEGF-A inducida por Pit-1 estaría mediada pola activación e aumento de CXCR4. Para aclarar o papel funcional de Pit-1-CXCL12-CXCR4 na formación de vasos sanguíneos, realizouse un ensaio *in vitro* chamado HUVEC-*tube formation assay*. Brevemente, cultiváronse as células endoteliais (HUVEC) durante seis horas con medio acondicionado (MC) obtido de: (a) células MCF-7 control, (b) Células MCF-7 con bloqueo de CXCR4, (c) Células MCF-7 con sobreexpresión de Pit-1, (d) Células que sobreexpresan Pit-1 e bloqueo de CXCL12 despois dunha inmunoprecipitación (IP) de CXCL12 do medio condicionado, (e) Células MCF-7 que sobreexpresan Pit-1 e bloqueo de CXCR4 , e (f) Células MCF-7 que sobreexpresan Pit-1, bloqueo de CXCR4 e IP de CXCL12. Os datos obtidos amosan un aumento significativo no número total de unións e na porcentaxe de vasos formados en células cultivadas con MC de células con sobreexpresión de Pit-1. Porén, os MC das condicións (d), (e) e (f) diminuíron de xeito significativo o número total de unións e a porcentaxe de vasos en comparación co MC de células con sobreexpresión de Pit-1. Paralelamente, realizouse un ensaio para testar a proliferación das células endoteliais despois de seren tratadas cos medios descritos anteriormente. Os

resultados obtidos suxiren novamente que a activación de CXCR4 e CXCL12 xogan un papel clave na proliferación e función das células endoteliais.

Dado que os datos nos indicaban que o eixo CXCL12-CXCR4 mediaba os efectos de Pit-1 na anxioxénese e na progresión do cancro de mama, exploramos se as células tumorais mamarias con sobreexpresión de Pit-1, e polo tanto de CXCR4, tiñan preferencia por metastatizar en tecidos específicos. Para isto, comezamos cun experimento *in vitro* para avaliar a extravasión das células tumorais. Agregáronse como quimio-atraentes para as células tumorais distintos extractos de tecido de rato : de fígado, pulmón, cerebro ou músculo na cámara inferior dos pocillos tipo *transwell* antes e despois de facerlle unha IP de CXCL12. Despois de corenta e oito horas, contáronse as células tumorais que pasaron a través da monocapa de células HUVEC e matrixel. Encontramos un aumento significativo das células MCF-7 con sobreexpresión de Pit-1 no extracto proteico de fígado, pulmóns e no extracto de tecido de cerebro con respecto ao control das células MCF-7. Porén, trala inmunoprecipitación de CXCL12 en tecidos de fígado e pulmón (pero non en cerebro ou músculo) diminuía dramaticamente a presenza de células con sobreexpresión de Pit-1.

Para estudar as relacións entre Pit-1, CXCL12 e CXCR4 en tumores humanos, analizáronse trinta e sete tumores de mama obtidos de tecido conxelado de pacientes con cancro de mama pero sen seguimento clínico para avaliar os niveis de ARNm de Pit-1, CXCR4 e CXCL12. Observamos que a expresión de Pit-1 correlacionábase de xeito significativo coa expresión tanto de CXCR4 coma de CXCL12. Dada a preferencia *in vitro* das células tumorais con alta expresión de Pit-1 polos tecidos de pulmón e fígado, ambos con altos niveis de CXCL12, quixemos testar en pacientes con cancro de mama dos que dispoñiamos de seguimento clínico a posible relación entre a expresión de Pit-1 e CXCR4 coa aparación de metástase en fígado e/ou pulmón. Seleccionamos un grupo de vinte e catro pacientes con cancro de mama sen metástase e outro grupo de vinte e catro pacientes que desafortunadamente desenvolveu metástases viscerais. Tras avaliar nas respectivas mostras os niveis de Pit-1 e CXCR4 por inmunohistoquímica e realizar a análise estatística obtivemos que, como acontecía *in vitro,* aqueles pacientes con elevada expresión de Pit-1 e CXCR4 tiñan un maior risco de aparición de metástase en fígado e/ou pulmón. Ademais, utilizando a ferramenta *on line* KMplotter encontrouse unha relación significativa entre a expresión de ARNm de Pit-1 e CXCR4 coa supervivencia libre de recorrencia en pacientes con cancro de mama.

Polo tanto, resumindo, encontramos que Pit-1 regula CXCL12 e CXCR4 en células de cancro de mama. Os nosos datos indican que o bloqueo de CXCR4 nas células de cancro de mama que sobreexpresan Pit-1 reduce o crecemento do tumor e a diseminación celular. Ademais, tanto CXCL12 como CXCR4 están involucrados na anxioxénese inducida por Pit-1. Finalmente, nas mostras de tumores de mama humanos, CXCL12 y CXCR4 correlacionáronse positivamente con Pit-1, e a alta expresión de Pit-1 en tumores de mama humanos relaciónase coa aparición de metástase en fígado e pulmón, así como con resultados clínicos deficientes.

O noso primeiro obxectivo neste traballo foi avaliar a correlación entre a expresión de Pit-1 e CXCL12-CXCR4 en liñas celulares de cancro de mama. Demostramos que a sobreexpresión ou redución de Pit-1 está acompañada por un aumento ou diminución de CXCL12 e CXCR4, respectivamente, o que suxire que a regulación de CXCL12 e CXCR4 por Pit-1 é independente do subtipo de cancro de mama. De feito, aínda que se ten demostrado que a expresión de CXCR4 e CXCL12 parece ser maior nos subtipos de cancro de mama triple negativo e HER-2 en comparación co subtipo luminal, este ligando e receptor aínda non foron asociados con un subtipo específico de cancro de mama.

O noso grupo demostrou previamente que Pit-1 induce o crecemento de tumores e a invasión no cancro de mama. Polo tanto, intentamos determinar se estas accións poderían estar parcialmente mediadas pola activación de CXCL12-CXCR4. Os cultivos en 3D de células que sobreexpresan Pit-1 amosan un maior crecemento celular, que diminúe despois do tratamento cun inhibidor de CXCR4. Ademais, o bloqueo de CXCR4 en células que sobreexpresan Pit-1 reduce a invasión e a migración celular, o que suxire unha participación do eixo CXCL12-CXCR4 nestas accións. A quimiocina CXCL12 e o seu receptor CXCR4 mantivéronse ben conservados ao longo da evolución. Ao igual que nos seres humanos, ambos os dous exprésanse no peixe cebra durante o desenvolvemento e o período adulto, desempeñando un papel clave en certos procesos fisiolóxicos, como a migración de células xerminais primordiais e o desenvolvemento cerebral. Dado que o CXCL12 endóxeno no peixe cebra activa a sinalización de CXCR4 en células humanas, avaliamos estes efectos nun modelo de xenoinxerto de peixe cebra. Os resultados amosan unha diminución significativa do crecemento do tumor e a diseminación despois da eliminación de CXCR4 nas células que sobreexpresan Pit-1. Isto está en liña con estudos recentes que demostran o papel de CXCL12-CXCR4 na indución da proliferación celular, na migración e na invasión noutros modelos animais.

A crecente evidencia suxire que o eixo CXCL12-CXCR4 desempeña un papel clave na anxioxénese e que poderían estar implicados dous mecanismos: (a) un mecanismo autocrino que implica a unión de CXCL12 liberado polas células cancerosas ao seu receptor CXCR4, a fosforilación das proteínas PI3K / AKT e a indución da liberación de VEGF; e (b) un mecanismo paracrino a través da unión de CXCL12 liberado polas células cancerosas ao CXCR4 endotelial e logo aumentar a anxioxénese. De feito, demostrouse unha retro-alimentación positiva entre CXCR4 e VEGF, e a expresión de CXCR4 correlacionouse de xeito positivo coa densidade de microvasos en moitos tipos de cancro, incluído o cancro de mama. Ademais, os inhibidores de CXCR4 diminúen os niveis da proteína VEGF e interrompen a vasculatura tumoral. Os nosos datos xerados aquí, parecen indicar que Pit-1 induce a anxioxénese a través de ambos mecanismos. En primeiro lugar, a sobreexpresión de Pit-1 en células tumorais aumenta a fosforilación das proteínas CXCR4, AKT, JNK, ERK1/2 e P38, todas as cales están involucradas na vía de sinalización da anxioxénese. Pit-1 tamén induce a expresión de VEGF-A en células tumorais, pero a redución de CXCR4 (xa sexa só ou despois da sobreexpresión de Pit-1) reduce significativamente a expresión do ARNm de VEGF-A. E, en segundo lugar, as células HUVEC tratadas con medio condicionado (MC) das células cancerosas que sobreexpresan Pit-1 aumentan tanto o número de unións como a porcentaxe de vasos. Porén, os niveis reducidos de CXCL12 (despois da IP) e/ou CXCR4 diminúen significativamente o número total de unións e a porcentaxe de vasos. De feito, observouse unha proliferación aumentada de células HUVEC cultivadas con MC-Pit-1 pero non despois de facer a inmunoprecipitación de CXCL12. Ademais, as células HUVEC cultivadas con MC-Pit-1 amosaron unha maior fosforilación de CXCR4, AKT e P38. En resumo, estes resultados suxiren unha función crítica do eixo CXCL12-CXCR4 na anxioxénese inducida por Pit-1, tanto a través de vías autocrinas como paracrinas. Outros estudos teñen demostrado un efecto paracrino indirecto de CXCL12 liberado de fibroblastos asociados con cancro (CAF) a través do recrutamento por quimiotase de células proxenitoras endoteliais á proximidade da masa tumoral.

Un punto crítico no proceso de metástase é a elección dun novo "solo" por parte das células cancerosas. As células tumorais primarias entran na circulación sistémica (intravasación) e saen rompendo as unións das células endoteliais (extravasación), e finalmente chegan ao órgano diana. Para probar *in vitro* se o eixo Pit-1-CXCL12-CXCR4 podería estar involucrado neste proceso, estudáronse diferentes tecidos de rato, incluíndo pulmón, fígado e cerebro, nun ensaio de invasión trans-endotelial. Empregouse tecido

muscular como control non metastático. O pulmón, o fígado e o cerebro foron tecidos diana das células de cancro de mama que sobreexpresan Pit-1, pero, sorprendentemente, a inmunoprecipitación de CXCL12 nestes tecidos reduciu de forma drástica a migración trans-endotelial das células de cancro de mama ao fígado e ao pulmón, pero non ao tecido cerebral. Estes datos suxiren un papel crítico de CXCL12 en tecidos diana e de CXCR4 nas células tumorais primarias, polo menos en metástases de cancro de mama inducidas por Pit-1. De feito, ten sido demostrado que os altos niveles de CXCL12 en tecidos metastásicos diana actúan como quimioatraentes para células tumorais primarias con alta expresión de CXCR4, e que o bloqueo de CXCR4 reduce significativamente a metástase a estes tecidos.

Finalmente, correlacionamos Pit-1 coa expresión de ARNm de CXCR4 e CXCL12 nun conxunto de mostras de tumores de mama humana. Os nosos resultados amosan unha correlación significativa entre Pit-1 e CXCL12 e Pit-1 e CXCR4. Ademais, encontramos unha correlación significativa en tumores de mama primarios entre os niveis de proteína Pit-1 e CXCR4 e con alto risco de metástases viscerais. Neste senso, demostrouse previamente que existe unha correlación significativa entre a expresión alta de CXCR4 e a metástase visceral en cancro de mama triple negativo e nos tumores de colon e melanoma.

Segundo os nosos datos, o tratamento dirixido sobre o eixo Pit-1-CXCL12-CXCR4 en cancro de mama podería levarse a cabo mediante a inhibición da actividade de unión de Pit-1 no ADN, o cal foi demostrado *in vitro* utilizando unha pequena molécula (coñecida como DB293), e por represión transcricional de Pit-1 que pode ser exercida pola 1,25-dihidroxivitamina D3 e os seus análogos. Para CXCR4 desenvolvéronse varios inhibidores, e algúns deles atópanse arestora en ensaios clínicos, incluídos tumores sólidos. De feito, estudos con xenoinxertos derivados de pacientes puxeron de manifesto de forma recente que os inhibidores de CXCR4 melloran a resposta a Trastuzumab e taxanos no cancro de mama HER2 quimiorresistente.

CXCL12 e CXCR4 poden estar regulados por moitos factores, como o factor inducible por hipoxia e o factor nuclear kappa potenciador da cadea lixeira das células B activadas (NF-κB). No presente estudo, tamén mostramos que CXCL12 e CXCR4 están regulados positivamente por Pit-1, o que suxire un papel importante do eixo Pit-1-CXCL12-CXCR4 na progresión do cancro de mama, incluída a proliferación celular, a migración, o crecemento tumoral, a invasión, diseminación, anxioxénese e extravasación

de células cancerosas para formar metástases en pulmón e fígado. Polo tanto, estes factores poderían usarse como factores prognósticos e terapéuticos nos tumores de mama.

2. ETHICS APPROVAL AND COPYRIGHT PERMISSIONS

DICTAMEN DEL COMITÉ AUTONÓMICO DE ÉTICA DE LA INVESTIGACIÓN DE GALICIA

Paula M. López Vázquez, Secretaria del Comité Autonómico de Ética de la Investigación de Galicia

CERTIFICA:

Que este Comité evaluó en su reunión del día 26/06/2014 el estudio:

Título: Cáncer de mama: papel de PIT1 y CXCR4 en el proceso de metástasis
Promotor: Román Pérez Fernández
Código de Registro: 2014/177

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Posautorización de Tipo Observacional para medicamentos de uso humano, y la Circular nº 07 / 2004, investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del CEIC de Galicia

Emite un **INFORME FAVORABLE** para la realización del estudio por el/la investigador/a del centro:

Centros	Investigadores Principales
CIMUS (Universidade de Santiago de Compostela)	Román Pérez Fernández

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Teresa Vázquez Pumariño. Diplomada Universitaria de Enfermería.
Mª Asunción Verdejo González. Médico Especialista en Farmacología Clínica.

En Santiago de Compostela, a 01 de julio de 2014

Paula M. López Vázquez

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Comité Ético de Investigación Clínica
Regional del Principado de Asturias
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33006.-Oviedo
Tfno: 985.10.79.27/985.10.80.28
Fax: 985.10.87.11
e-mail: ceicr_asturias@hca.es

Área Sanitaria

Oviedo, 17 de Julio de 2013

El Comité Ético de Investigación Clínica Regional del Principado de Asturias, ha revisado el Proyecto de Investigación nº 80/2013 (FIS), titulado: "VALOR PRONÓSTICO DEL FENOTIPO DE LAS CÉLULAS MONONUCLEARES DE SANGRE PERIFÉRICA EN EL DESARROLLO DE METÁSTASIS Y MODULACIÓN DE SUS CARACTERÍSTICAS FUNCIONALES COMO NUEVO MECANISMO TERAPÉUTICO".Investigador Principal Dr. Francisco José Vizoso Piñeiro del Hospital Jove- Gijón

El Comité ha tomado el acuerdo de considerar que el citado proyecto reúne las condiciones éticas necesarias para poder realizarse y en consecuencia emite su autorización.

Le recuerdo que deberá guardarse la máxima confidencialidad de los datos utilizados en este proyecto.

Fdo: Eduardo Arnáez Moral
Secretario del Comité Ético de Investigación
Clínica Regional del Principado de Asturias

Annex

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MR110250

Laura Sánchez Piñón
Departamento de Xenética
Facultade de Veterinaria
Estrada da Granxa, s/n
27002 Lugo

Asunto: Comunicación de autorización de proxecto.

Remítolle Resolución de autorización do proxecto "El pez cebra y su aplicación en Biomedicina, acuicultura y medio ambiente" do que é responsable a investigadora Dona Laura Sánchez Piñón e que se vai levar a cabo no centro usuario de animais de experimentación da Fundación Rof Codina(AE-LU-003).

Lugo, 10 de xullo de 2014
O xefe do servizo provincial de Gandaría

PA
Belén Ferreiro Rodríguez

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RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTOS DE EXPERIMENTACIÓN ANIMAL

Expediente núm.:009/14
Interesado: Laura Sánchez Piñón
Procedemento: RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTOS DE EXPERIMENTACIÓN ANIMAL
Data de inicio: 1 de setembro de 2014
Forma de inicio: solicitude do interesado

ANTECEDENTES

O interesado, como responsable do proxecto **El pez cebra y su aplicación en Biomedicina, acuicultura y medio ambiente**, presentou con data 19 de xuño de 2014 e rexistro de entrada 72150/RX 660953 de 19 de xuño de 2014, solicitude para a realización do proxecto de experimentación animal cuxos datos detállanse a continuación:

Denominación do proxecto: **El pez cebra y su aplicación en Biomedicina, acuicultura y medio ambiente.**
Nome do centro usuario: Animalario da Facultade de Lugo (AE-LU-003).
Persoa responsable do proxecto: Laura Sánchez Piñón
Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo): Animalario da Facultade de Lugo (AE-LU-003).
Clasificación do proxecto : ☐ Tipo II **XTipo III** ☐

En oficio con data de 26 de xuño de 2014 remítese a interesada acuse de recibo da solicitude de autorización, no que se indican unha serie de deficiencias que hai que solucionar para poder continuar co procedemento de autorización do proxecto.

En escrito de 8 de xullo de 2014, rexistro de entrada 80469/RX 758185 de 8 de xullo de 2014 achégase documentación que arranxa as eivas sinaladas.

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1. O Real decreto 53/2013, de 1 de febreiro (B.O.E. nº 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.

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2. O artigo 89 da Lei 30/1992, de 26 de novembro, do réxime xurídico das Administracións Públicas e do procedemento administrativo común (B.O.E. núm. 285, 27 de novembro de 1992), modificada pola Lei 4/1999, de 14 de xaneiro, establece que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas do mesmo.

3. Esta xefatura territorial é competente para ditar resolución de conformidade co artigo 11 do Decreto 245/2009 de 3/ de abril, polo que se regulan as delegacións territoriais da Xunta de Galicia e o Decreto 46/2012, de 19 de xaneiro, polo que establece a estrutura orgánica da Consellería do Medio Rural e do Mar e do Fondo Galego de Garantía Agraria.

Revisada por parte do Servizo Provincial de Gandería de Lugo a documentación achegada na solicitude e o resultado **favorable** da avaliación do proxecto, realizada polo órgano habilitado Sección de Experimentación animal do Comité de Bioética da Universidade de Santiago de Compostela, este Departamento Territorial **RESOLVE,**

- AUTORIZAR o proxecto solicitado

A autorización deste proxecto terá unha **duración de 5 ANOS**, transcorridos os cales, deberá renovar a autorización do mesmo.

A autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandería.

Esta autorización poderá ser suspendida no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirala previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non pon fin á vía administrativa, poderá interpor **recurso de alzada** ante o conselleiro de Medio Rural da Xunta de Galicia no prazo **dun mes** contado a partir da recepción da notificación da presente resolución, conforme coa Lei 30/1992, do 26 de novembro, (B.O.E. núm. 285, 27 de novembro de 1992), de Réxime Xurídico das Administracións Públicas e do Procedemento Administrativo Común na súa redacción dada pola Lei 4/1999, do 13 de xaneiro.

Lugo,a 2 de xullo de 2014

O xefe do Departamento Territorial de Medio Rural e do Mar de Lugo

Asdo.: José Ramón Losada Fernández

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Remisión autorización

Cristina Castillo Rodriguez
Responsable benestar animal
Facultade de Veterinaria
Rúa Carballo Calero s/n
27002 Lugo

De acordo co establecido no RD 479/2004, de 26 e marzo, polo que se establece e regula o Rexistro xeral de explotacións gandeiras (REGA) e o disposto no Decreto 296/2008, do 30 de decembro, de protección dos animais utilizados para experimentación e outros fins científicos, incluída a docencia, e polo que se crea o Rexistro de centros de cría, de subministro e usuarios e a Comisión Galega de Benestar Animal dos Animais de Experimentación, comunícolle os códigos adxudicados ao centro de cría, subministrador e usuario de pez cebra(Danio rerio) situado no pavillón II da Facultade de Veterinaria.

- Nº do centro: AE-LU-003.
- Código REGA: ES270280346401.

Lugo, 2 de xullo de 2013
O xefe do servizo de Gandería

Manuel Corujo González

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Annex

ASUNTO:
INSCRICIÓN NO REXISTRO DE CENTROS DE CRÍA, SUBMINISTRADORES E USUARIOS DE ANIMAIS DE EXPERIMENTACIÓN
LEXISLACIÓN APLICABLE:
•**Real decreto 53/2013, de 1 de febreiro** (BOE nº 34 de 8 de febreiro), polo que se establecen as normas básicas aplicables para a produción dos animais utilizados en experimentación e outros fins docentes, incluíndo a docencia •**Decreto 296/2008, do 30 de decembro** (DOG nº11, do 16 de xaneiro), sobre protección dos animais utilizados para experimentación e outros fins científicos, incluída a docencia, e polo que se crea...

DATOS DO CENTRO:	
TITULAR **UNIVERSIDADE DE SANTIAGO DE COMPOSTELA-**	**N.I.F/C.I.F.** Q1518001A
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NOME DO REPRESENTANTE FRANCISCO GONZÁLEZ GARCÍA	**N.I.F./C.I.F.** 34908578Y

RESOLUCIÓN:

Visto:

O artigo 16 do Real Decreto 53/2013, de 1 de febreiro, polo que se establecen as normas básicas aplicables para a produción dos animais utilizados en experimentación e outros fins docentes, incluíndo a docencia

- que establece que os criadores, suministradores e usuarios e os seus establecementos deberán estar autorizados con carácter previo ao inicio das súas actividades
- O artigo 4º do Decreto 296/2008, do 30 de decembro, sobre protección dos animais utilizados para experimentación e outros fins científicos, que crea o Rexistro de centros de cría, subministradores e usuarios de animais de experimentación, que queda estruturado nas seccións de centros de cría, centros subministradores e centros usuarios.
- A solicitude e a documentación presentada por D.Francisco González García, en representación do centro USC-Facultade de Veterinaria de Lugo.
- O informe do Servizo Provincial de Gandería de Lugo.

En virtude da autorización outorgada, no uso das facultades conferidas na lexislación vixente,

R E S O L V O:

-Inscribir o centro, no Rexistro de centros de cría, subministradores e usuarios, có código **ES270280346401**

-Incluír o centro, dentro do citado Rexistro, na sección animais de experimentación

Esta resolución implica o cumprimento do especificado na normativa sobre protección dos animais utilizados para experimentación e outros fins científicos, e concretamente conleva as seguintes obrigas:

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-Deberá notificar, ao Servizo Provincial de Gandaría, no prazo dun mes desde que se produza, a modificación dos datos que figuren inscritos no rexistro, derivada de ampliacións, reducións, traslados, cambios de persoal ou outras circunstancias, así como suspensión, cesamento da actividade e cambio de titularidade.

-A cancelación da inscrición no rexistro terá lugar por solicitude do titular ou de oficio, cando se produzan incumprimentos do establecido na normativa ou cando se comprobe que o centro cesou a actividade.

-Deberá levar un rexistro de control dos animais, no que deberá anotar os datos que figuran no anexo VI do R.D. 53/2013, de 1 de febreiro.

-Deberá presentar, no Servizo Provincial de Gandaría, solicitude para a comunicación e autorización dos proxectos de acordo co determinado no artigo 33 do R.D. 53/2013, de 1 de febreiro. A documentación a presentar será a enumerada no anexo X do referido Real Decreto,

Contra e presente resolución, ao non ser firme en vía administrativa, poderá interpor recurso de alzada perante o conselleiro do Medio Rural, no prazo dun mes, a contar dende o día seguinte ao da súa notificación, consonte coa Lei 30/1992, do 26 de novembro, de Réxime Xurídico das Administracións Públicas e do Procedemento Administrativo Común na súa redacción dada pola Lei 4/1999, do 13 de xaneiro.

Lugo, 2 de xullo de 2013
O/A xefe/a territorial

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