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Regulation of pluripotency genes by CX3CR1 in cancer cells

A Thesis Submitted to the Faculty of

Drexel University College of Medicine

by María Sofía Castelli

in partial fulfillment of the

requirements for the degree of

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ABSTRACT

Regulation of pluripotency genes by CX3CR1 in cancer cells
María Sofía Castelli
Alessandro Fatatis, MD, PhD

Prostate and breast cancers are among the most frequently diagnosed malignancies in the United States, leading to approximately 60,000 deaths every year. Although localized disease has a high survival rate, over 30% of the patients with either form of tumor develop metastatic recurrence over time. Metastatic disease remains incurable, accounting for most cancer-related deaths. Evidence suggests that while most cancer cells within a primary tumor lack the ability to initiate new tumors, a small group of cells known as cancer stem cells (CSCs) can initiate metastatic lesions and sustain their growth. CSCs are characterized by stemness features, which are mediated by the expression of pluripotency transcription factors, including OCT4a and NANOG. Recent evidence suggests that chemokine receptors may play an important role in the regulation of cell stemness. Our lab has previously demonstrated that the chemokine receptor CX3CR1 is implicated in tumor progression and metastasis in prostate and breast cancers. Furthermore, our studies suggest that prostate and breast cancer cells with high CX3CR1 expression ($CX3CR1^{High}$) display stem-like features and express pluripotency genes. In contrast, cancer cells with low CX3CR1 expression ($CX3CR1^{Low}$) do not display stemness features but undergo phenotypic plasticity, reacquiring the expression of CX3CR1 and pluripotency genes over a period of time. In this study, we set to determine if CX3CR1 is involved in regulating the expression of pluripotency genes during phenotypic plasticity. To this end, we used flow cytometry to sort prostate and breast cancer cells based on their CX3CR1 expression levels,

and then cultured CX3CR1^{Low} cells over a period of time to assess the timeframe for the re-expression of CX3CR1, OCT4a and NANOG. We also cultured these cells in the presence of a CX3CR1 small molecule inhibitor, FX-68, in order to assess the effect of blocking CX3CR1 in the re-expression of pluripotency genes. Our preliminary results show that both prostate and breast cancer CX3CR1^{Low} cells increase their expression of CX3CR1, OCT4a and NANOG following *in vitro* culture, and this re-expression appears to be impaired when CX3CR1 is blocked. These results suggest that CX3CR1 may be involved in regulating its own expression and the expression of pluripotency genes and may thus play a role in dictating stemness features and promoting metastasis initiation. Our study provides further evidence for the involvement of CX3CR1 in cancer progression and its potential as a therapeutic target to treat metastatic disease.

1. INTRODUCTION AND LITERATURE REVIEW

1.1. Prostate Cancer Overview

1.1.1. Prostate Cancer Statistics

Prostate cancer is the second most frequent malignancy in men and the fifth leading cause of death worldwide (Bray et al., 2018). In the United States, prostate cancer is the most commonly diagnosed cancer, accounting for more than 1 in 5 new diagnoses, and it is the second leading cause of cancer-related deaths in men, with over 30,000 deaths every year (Siegel, Miller, & Jemal, 2020). Prostate cancer prevalence increases with age, and about 60% of the cases are diagnosed in men over the age of 65 years (Howlader et al., 2020). In patients with localized prostate cancer, the 5-year survival is close to 100%; however, in patients with distant metastases, the 5-year survival drops to 31% (Siegel et al., 2020). Therefore, the main cause of death from prostate cancer is metastatic progression.

1.1.2. Prostate Cancer Diagnosis, Progression and Treatment Course

Prostate cancer is typically detected on the basis of elevated prostate specific antigen (PSA) levels (PSA > 4 ng/mL). The diagnosis is usually confirmed by a tissue biopsy (Rawla, 2019). The treatment options for clinically localized prostate cancer include radical prostatectomy, prostate brachytherapy, external beam radiation, and active surveillance (Brawley, Mohan, & Nein, 2018). Early-stage, localized prostate cancer treatments have high cure rates. However, approximately 35% percent of patients treated for localized disease will experience biochemical recurrence, i.e. an elevation of PSA levels, within 10 years of treatment. Among patients who develop biochemical recurrence,

one-third will present radiographic evidence of metastatic disease within 8 years (Bruce, Lang, McNeel, & Liu, 2012).

Following recurrence, the primary treatment is androgen-deprivation therapy (ADT) by chemical and surgical castration, since the growth of prostate cancer cells depends on androgen stimulation. Androgens bind to the androgen receptor (AR) forming a complex that translocates to the nucleus, where it binds to Androgen Responsive Elements (ARE), affecting the transcription of androgen-regulated genes, including PSA. The activation or repression of genes by this complex ultimately stimulates proliferation and inhibits apoptosis of prostate cancer cells (Dutt & Gao, 2009). Although more than 90% of the patients respond to ADT, all patients eventually progress to castration-resistant prostate cancer (CRPC) after a median of 24–36 months (Karantanos, Corn, & Thompson, 2013). CRPC patients no longer respond to ADT, because prostate cancer cells adapt to the androgen-deprived environment induced by this therapy. Some of the proposed mechanisms for this adaptation include increased local production of androgens by prostate cancer cells, AR amplification, AR gene mutations leading to promiscuous ligand interaction, enhanced AR signal transduction through alterations in coactivators/corepressors, and activation of the AR or downstream regulatory molecules by cross-talk with other signaling pathways (Mostaghel, Montgomery, & Nelson, 2009)

The treatment options for CRPC include hormonal therapy, chemotherapy, immunotherapy, and radionuclide therapy, as well as genetically targeted agents for certain subsets of biomarker-selected patients. However, despite recent progress in the development of new therapeutic agents, CRPC continues to be incurable. Therefore,

treatments at this stage aim to prolong patient survival, palliate symptoms, improve and maintain quality of life, and prevent complications (Nuhn et al., 2019).

1.1.3. Prostate Cancer Metastasis

Metastasis of solid tumors, including prostate cancer, involves multiple steps, including angiogenesis, local migration, invasion, intravasation, circulation, extravasation of tumor cells and then angiogenesis and colonization at the secondary organ (J. K. Jin, Dayyani, & Gallick, 2011).

The model of metastasis of solid tumors, including prostate cancer, is guided by the "seed and soil" hypothesis (Paget, 1889). In this model, tumor cells only "seed" or metastasize to specific organs or "soil" that are well suited for the tumor's growth. In other words, the tropism of tumor cells tends to be the result of a specific organ's microenvironment. Prostate cancer cells that leave the primary tumor show high tropism for the bone. Studies show that approximately 90% of the patients who succumb to metastatic prostate cancer were previously diagnosed with bone metastases (Wong et al., 2019). Treatment with recently developed drugs can provide an extension in life expectancy; however, the median survival of men with metastatic CRPC ranges from 15 to 36 months (Crawford, Petrylak, & Sartor, 2017). Thus, current therapies for this disease remain far from curative.

1.2. Breast Cancer Overview

1.2.1. Breast Cancer Statistics

Breast cancer represents an important health challenge globally, as it is the most commonly diagnosed cancer and the leading cause of cancer death in women worldwide, accounting for almost 1 in 4 cancer cases among women (Bray et al., 2018). In the United States, breast cancer alone accounts for 30% of female cancers and over 42,000 deaths each year (Siegel et al., 2020). Metastatic disease remains the underlying cause of death for most breast cancer patients. In patients with localized breast cancer, the 5-year survival is close to 100%; however, in patients with distant metastases, the 5-year survival drops to 27% (Siegel et al., 2020).

1.2.2. Breast Cancer Diagnosis, Progression and Treatment Course

Breast cancer is typically detected by a mammography. Diagnostic mammograms are performed either through screening or in women who have a symptom of breast disease, such as a palpable mass or pain. The diagnosis is then confirmed via histopathological assessment of a breast biopsy (McDonald, Clark, Tchou, Zhang, & Freedman, 2016).

Once breast cancer is diagnosed, the intervention method depends on the age of the patient, the extent of disease and the histological grade of the breast tumor (Nounou et al., 2015). Based on the American Joint Committee on Cancer (AJCC) guidelines, breast cancer staging is determined based on the extent of cancer as defined by tumor size (T), lymph node status (N), and distant metastasis (M), combined with the tumor grade and the status of the biomarkers human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR) (Koh & Kim, 2019). The main types of

treatment for breast cancer are surgery, radiation therapy, chemotherapy, endocrine (hormone) therapy, and targeted therapy (Nounou et al., 2015). Most patients are diagnosed at an early disease stage. For early, localized nonmetastatic breast cancer, the aim of therapy is to eradicate the tumor from the breast and regional lymph nodes and to prevent metastatic recurrence. The standard treatment for localized breast cancer consists of surgical resection and sampling or removal of axillary lymph nodes, sometimes with the addition of postoperative adjuvant therapy with radiation to reduce the risk of local recurrence (Nounou et al., 2015; Waks & Winer, 2019). Unfortunately, about 30% of patients with breast cancer who are free of disease after initial local and regional treatments experience metastatic recurrence (Colleoni et al., 2016). Following recurrence, the therapeutic goals are to prolong survival and palliate symptoms. Currently, metastatic breast cancer remains widely incurable (Waks & Winer, 2019).

1.2.3. Breast Cancer Metastasis

Metastasis in breast cancer, like in the rest of solid tumors, comprises a series of steps that cancer cells go through in order to depart from the primary tumor and colonize secondary organs. The tropism of breast tumors also depends on the microenvironment of specific organs. The most common target organs for breast cancer metastasis include the bone, lung, liver, brain, and distant lymph nodes. However, the tropism of cancer cells depends largely on the breast cancer subtype. For instance, for ER+ tumors, bone is the predominant metastatic site, whereas the brain is less affected, making this subtype have a better prognosis compared with others. One factor that plays a role in this preference is that the bone is rich in estrogen, which gives a particular advantage for the proliferation of ER+ cancer cells at this site. In contrast, triple negative breast tumors preferentially metastasize

to visceral organs, including brain and lung, leading to a worse prognosis (X. Jin & Mu, 2015). Despite recent advances in treatment, metastatic breast cancer still represents a major hurdle in the path to curing breast cancer.

1.3. Cancer Stem Cells

1.3.1. Tumor Recurrence

The risk of tumor recurrence remains a major concern among many cancer patients. Recurrence rates vary between cancer types; while approximately 17% of colorectal cancer patients develop recurrence, glioblastoma recurs in nearly all patients, despite treatment (Nabors et al., 2017; Pugh et al., 2016). The majority of cancer patients initially show signs of improvement when treated with standard therapies such as surgery and systemic adjuvant treatment, which eliminate most of the tumors at the primary site and throughout the body. However, in some patients, after a period with no clinical signs of cancer that could last for months to decades, clinically detectable metastatic lesions start to emerge. Following tumor recurrence, systemic treatment may induce a temporary decrease in tumor burden. Unfortunately, eventually treatment resistance ensues, ultimately leading to patient death (**Figure 1**) (Celia-Terrassa & Kang, 2016). As a result, most of the deaths that occur in cancer patients with solid tumors are not caused by the primary tumor, but are rather due to metastasis (Gupta & Massague, 2006).

1.3.2. Cancer Stem Cells (CSCs)

A number of different mechanisms have been proposed to explain tumor recurrence. One hypothesis that has recently been supported by increasing evidence is based on the existence of cancer stem cells (CSCs). CSCs represent a small population of cells within tumors with stem-like capabilities that allow them to initiate tumor growth. These cells have been identified and characterized in many cancer types, including prostate, breast, colon, ovarian, and melanoma, among others (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Maitland & Collins, 2008; O'Brien, Pollett, Gallinger, & Dick,

2007; Schatton et al., 2008; Zhang et al., 2008). CSCs that are able to initiate the growth of primary tumors are known as tumor-initiating cells (TICs) while cancer cells that seed clinically significant metastatic colonies in secondary organs are known as metastasis-initiating cells (MICs) (Celia-Terrassa & Kang, 2016). CSCs have been associated with drug resistance, tumor recurrence, and metastasis, thus playing an important role in tumor initiation and expansion. In addition, gene expression studies from clinical data show that the expression of genes associated with stem cell pathways identify aggressive cancers and are associated with poor outcomes to therapy in a wide range of cancers (Ben-Porath et al., 2008). The hypothesis underlying the association between CSCs and tumor recurrence and metastasis is based on the fact that CSCs are resistant to chemotherapy (**Figure 2**). Solid tumors are heterogeneous, with a small subpopulation of CSCs and a large subpopulation of non-stem bulk tumor cells. When a cancer patient is treated with chemotherapy, most fast dividing non-stem bulk tumor cells die, leaving resistant CSCs behind, which may later expand and differentiate to give rise to recurrence. Recurrent tumors metastasize widely and continue to evolve, developing resistance to first line therapy and eventually leading to patient death (Roberts, Cardenas, & Tedja, 2019).

1.3.3. Tumor-Initiating Cells (TICs)

TICs have features that distinguish them from differentiated, non-stem-like cancer cells. They can divide in an asymmetric fashion, giving rise to one stem cell and one differentiated cell. This allows for self-renewal, because more stem-like cells are generated, maintaining a steady state population of TICs within the bulk tumor population (Bajaj, Zimdahl, & Reya, 2015). TICs also have slow proliferation rates, which partially explains their resistance to anti-proliferative therapies, including chemotherapy and

radiation treatments (Moore & Lyle, 2011). Other mechanisms of resistance include the expression of high levels of multidrug resistance proteins and the enhancement of mechanisms of protection against DNA damage-induced cell death (Phi et al., 2018). The ability of TICs to resist conventional cancer therapy underscores their involvement in tumor relapse. While treatment with first-line chemotherapy will eliminate the majority of the susceptible non-stem-like cancer cells, TICs will remain. These cells can then undergo genetic alterations, expand and differentiate, leading to the development of metastatic lesions and patients' demise (Roberts et al., 2019).

1.3.4. Metastasis-Initiating Cells (MICs)

Like TICs, MICs can also make use of normal stem cell pathways to acquire stemness features. Studies have shown that early stage metastatic cells possess a distinct stem-like gene expression signature and significant tumor initiation capacity, and support the existence of stem-like cells driving metastatic colonization (Lawson et al., 2015). However, the expression of stemness features appears to be insufficient for metastasis initiation, since MICs must also be able to survive the highly inefficient metastatic process (Celia-Terrassa & Kang, 2016). Metastasis requires that cells from a primary tumor detach, invade the vascular or lymphatic system, survive in circulation and migrate to distant sites, extravasate, and then colonize secondary organs (Nguyen, Bos, & Massague, 2009). During this process, cancer cells need to survive significant stress, which forces MICs to adopt additional protective mechanisms. Therefore, besides the maintenance of TIC ability, MICs require other capabilities to successfully initiate metastasis, such as the ability to undergo bidirectional transitions between the epithelial and mesenchymal states, resistance to apoptosis and anoikis (apoptosis induced by lack of correct cell-extracellular matrix

attachment), entry into and exit from dormancy, evasion of immune system attack, high metabolic adaptability and stress resistance, interclonal cooperation, and the ability to co-opt a supportive stromal niche (Celia-Terrassa & Kang, 2016).

The acquisition of MIC features seems to involve a combination of genetic and epigenetic events that occur during the metastatic process. Studies suggest that rather than acquiring additional driver mutations, MICs select pre-existing oncogenic mutations already present in the primary tumor site and undergo epigenetic regulation after escaping the primary site, both of which provide metastatic competence (Celia-Terrassa & Kang, 2016). Additionally, the loss of differentiation factors and increased activity of stem cell factors has been linked to metastasis. Furthermore, epithelial-mesenchymal transition (EMT), a process during which epithelial cells lose polarity and cell-cell adhesions to gain mesenchymal properties, is thought to be associated with the initiation of primary tumors and metastasis by promoting invasion and inducing stem cell-like properties (Celia-Terrassa & Kang, 2016). Cells in an epithelial state are in close contact with their neighbors through intercellular adhesion complexes, display apico-basal polarity and express epithelial markers. In contrast, cells in a mesenchymal state are nonpolarized, lack intercellular junctions, express mesenchymal markers and have migratory capabilities (Acloque, Adams, Fishwick, Bronner-Fraser, & Nieto, 2009). EMT promotes migration and invasion and is often used by cancer cells to escape from the primary tumor, whereas the opposite process, mesenchymal-to-epithelial transition (MET), seems to be required for metastatic outgrowth (Celia-Terrassa & Kang, 2016). Recently, it has been shown that MICs hijack wound healing mechanisms to regenerate tumors in different organs. For instance, the L1 cell adhesion molecule (L1CAM) was shown to play an essential role in

intestinal epithelial regeneration. In colorectal cancer, the expression of this molecule was shown to be required for orthotopic carcinoma propagation, liver metastatic colonization and chemoresistance (Ganesh, 2020). The expression of this molecule appears to be driven by loss of epithelial integrity, which leads to phenotypic plasticity and favors the selection of regenerative traits that support metastasis (Ganesh, 2020). Overall, the core characteristic of MICs is their high cellular plasticity, which appears to enable them to acquire many of the MIC properties (Celia-Terrassa & Kang, 2016). Although recent studies have unveiled some of the molecules and pathways driving the acquisition of metastatic traits, further research is needed to fully understand the mechanisms that lead to a metastasis-initiating phenotype.

1.3.5. Transcription factors that regulate stemness in CSCs

Since CSCs seem to play a major role in cancer recurrence and metastasis, it is essential to investigate which genes and pathways drive the stemness phenotype of these cells. Cancer cell stemness is known to be regulated by a number of pluripotency-associated transcription factors, including octamer binding transcription factor 4 (OCT4), sex determining region Y - related high mobility group box 2 (SOX2) and nanog homeobox (NANOG). It has been previously described that the upregulation of these genes promotes CSC-like properties, and they are considered critical regulators of self-renewal and pluripotency, mediating tumor proliferation and differentiation (Jeter, Yang, Wang, Chao, & Tang, 2015; Takeda et al., 2018; Y. J. Wang & Herlyn, 2015).

OCT4 (also known as OCT3) is a transcription factor encoded by the *Pou5f1* gene. In humans, this gene can generate three isoforms by alternative splicing, known as OCT4a, OCT4b and OCT4b1 (X. Wang & Dai, 2010). OCT4a is normally referred to as OCT4,

and it has been established as a marker for human pluripotent embryonic stem cells that is essential for the maintenance of the pluripotent state during embryonic development, while its loss leads to stem cell differentiation (Boiani & Scholer, 2005; X. Wang & Dai, 2010). OCT4b and OCT4b1 seem to play a role in the biologic response of cells to stress (Farashahi Yazd et al., 2011; X. Wang et al., 2009). Accumulating evidence suggests that OCT4 is involved in the maintenance of stemness features in cancer, playing a major role in self-renewal, cell survival, metastasis and drug resistance in CSCs through the regulation of its target genes (Y. J. Wang & Herlyn, 2015). OCT4 is overexpressed in CSCs in various cancers, and its high expression correlates with poor clinical outcome (Mohiuddin, Wei, & Kang, 2020). OCT4 forms heterodimers with other transcription factors, including SOX2. The POU domain within OCT4 interacts with the major groove of the DNA, whereas a high-mobility group (HMG) domain of SOX2 interacts with the minor groove of DNA (Tapia et al., 2015). This allows them to form a synergistic interaction to drive the transcription of many target genes, including *Sox2* and *Pou5f1* (the gene encoding OCT4) themselves, as well as *Nanog* (Rodda et al., 2005).

SOX2 is a transcription factor that belongs to the Sry-related HMG box (SOX) family of proteins, which bind to specific DNA sequences via a highly conserved HMG domain. SOX2 is involved in the regulation of pluripotency and self-renewal of stem cells, playing a role during embryogenic development and adult tissue regeneration (Novak et al., 2019). SOX2 has also been found to play a major role in tumorigenesis in a wide range of cancers, including breast, prostate, brain, lung, kidney and skin cancers. Some of the processes that SOX2 seems to promote through the regulation of a number of target genes include cancer cell growth, invasion, migration, metastasis and chemoresistance (K. Liu et al., 2013).

Overexpression of SOX2 correlates with a stem-like phenotype in numerous cancer studies and has been involved with poor survival rates in cancer patients (K. Liu et al., 2013).

NANOG is a transcription factor with a DNA-binding homeodomain that has been described as important for the maintenance of pluripotency in embryonic stem cells, and that is downregulated upon differentiation (Mitsui et al., 2003). As mentioned before, the OCT4/SOX2 complex is important for the regulation of NANOG expression; however, it has been observed that NANOG can also be maintained without OCT4 (Pan & Thomson, 2007). Like OCT4 and SOX2, NANOG has been found to play a role in many processes in cancer, including cell survival, anti-apoptotic signaling, migration, invasion and chemoresistance. Its overexpression has been observed in various cancers, including breast, prostate, ovarian, melanoma, and others (Gawlik-Rzemieniewska & Bednarek, 2016).

Together, these three transcription factors form a core that is crucial for the establishment of a pluripotent state in embryonic stem cells. This is achieved by activating the expression of other pluripotency factors, as well as their own expression, and by repressing genes encoding lineage-specific factors (Young, 2011). In cancer, co-expression of these transcription factors has been found in a wide range of cancer types, especially in poorly differentiated tumors, where they control the fate of stem-like cells during cancer development (Liu, Yu, & Liu, 2013). In addition, several studies have demonstrated that increasing the expression of these pluripotency transcription factors in non-stem-like cancer cells leads to the acquisition of a stemness phenotype (A. Liu et al., 2013). Furthermore, SOX2, NANOG, and OCT4 have been correlated with an increase in metastasis of numerous cancers, including prostate cancer, breast cancer, bladder cancer,

lung cancer, head and neck squamous cell carcinoma, and others (Celia-Terrassa & Kang, 2016; Hepburn et al., 2019). Although further studies are warranted to establish the link between these transcription factors and metastasis, the evidence so far suggests that these proteins may influence the metastatic behavior of cancer cells and the formation of MICs (Celia-Terrassa & Kang, 2016).

Given that OCT4, SOX2 and NANOG play crucial roles in the maintenance of stemness in CSCs, a greater knowledge of the mechanisms that regulate the expression of these factors is needed to design new therapeutic interventions that can eventually overcome issues like chemotherapy resistance, tumor recurrence and metastasis. The mechanisms that regulate the expression of pluripotency-associated genes in CSCs are not fully known. However, increasing evidence gathered lately suggests that their regulation is mediated by a complex network of biological pathways. Some of these include Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT), Hedgehog, Wnt, Notch, phosphatidylinositol 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN), and nuclear factor-κB (NFκB) signaling pathways (Matsui, 2016). Additionally, the relevance of each signaling pathway in the regulation of pluripotency genes seems to differ depending on the cancer type.

1.3.6. Plasticity of cancer cells

Cell plasticity shifts cancer cells between a differentiated state and a stem-like state and is responsible for long-term tumor growth. Previous studies have provided evidence to support cancer cell plasticity, by which cancer cells have the dynamic ability to shift between non-CSC and CSC states (da Silva-Diz, Lorenzo-Sanz, Bernat-Peguera, Lopez-Cerda, & Munoz, 2018). This process may be modulated by specific signals from the

microenvironment and cell interactions within the tumor niche (Cabrera, Hollingsworth, & Hurt, 2015). Evidence suggests that non-CSCs sorted from a tumor can convert to CSCs *in vivo* and re-establish the ratios of non-CSCs and CSCs from the original tumor, thereby recapitulating tumor heterogeneity (Quintana et al., 2008; Roesch et al., 2010). These studies support a model of 'dynamic stemness' by which cancer cells have the ability to interconvert between a CSC state and a non-CSC state in response to microenvironmental signals. The transformation of cancer cells driven by factors from the microenvironment can protect them from chemotherapeutic insults. Thus, phenotypic plasticity of cancer cells and the resulting tumor heterogeneity are believed to play a role in therapy resistance (Ahmed & Haass, 2018; Davies & Albeck, 2018). In addition, cancer cells plasticity allows for the maintenance of the CSCs pool and is thought to be involved in tumor recurrence, thus representing a major challenge to treatments (da Silva-Diz et al., 2018). Consequently, further studies are needed to provide insight into the mechanisms that regulate cancer cell plasticity, in order to design new therapeutic interventions to block this process.

1.4. Chemokines and Chemokine Receptors in Cancer Stem Cells

1.4.1. Chemokines and Chemokine Receptors

Chemokines are small cytokines that mediate the chemotaxis or migration of immune cells to specific organs, playing an essential role in inflammation and immunity. In addition to mediating chemotaxis, the most studied biological function of chemokines, these proteins are also involved in many other biological processes, including cell proliferation, survival and differentiation. Chemokines exert their effects by binding to chemokine receptors, and they are split into four subfamilies: CC, CXC, CX3C, and XC, based on the configuration of the two cysteines closest to the N terminus. Similarly, chemokine receptors are divided into four groups based on the subfamilies of chemokines they bind (Hughes & Nibbs, 2018). Conventional chemokine receptors are seven-transmembrane G protein-coupled receptors (GPCRs) and typically transduce signals through $G\alpha_i$ G-proteins, although some receptors couple to $G\alpha_q$ family members. The biological function of chemokines is mediated through various signal transduction pathways that are activated following their binding to chemokine receptors. G_i protein activation leads to the inhibition of PKA activity, the mobilization of Ca^{2+} from intracellular stores, and the activation of the PI3K pathway. The activation of chemokine receptors also leads to JAK/STAT signaling, which regulates gene expression and transduces cell adhesion and migration signals. Chemokines also trigger a signaling pathway mediated by G protein receptor kinase (GRK)/β-arrestin, which leads to receptor internalization, but also triggers other signaling pathways such as the p38 mitogen-activated protein kinase (MAPK) and the p44/p42 extracellular-regulated kinases (ERK1/2) cascades (Lacalle et al., 2017).

1.4.2. Chemokine/Chemokine Receptors in Cancer

The contribution of chemokines to tumor progression has been well documented and occurs not only by inducing the recruitment of leukocytes such as regulatory T cells (T_{regs}), but also by promoting other cancer-related processes, including tumor growth, proliferation and metastasis (Mollica Poeta, Massara, Capucetti, & Bonecchi, 2019). Chemokines contribute to the metastatic process in various ways, by promoting tumor cell proliferation and survival, supporting angiogenesis, and shaping the tumor microenvironment. Consequently, a strong correlation between chemokine receptors expression and the clinical outcome of cancer patients has been found in several studies. In many cases, the expression of a particular chemokine-receptor profile is associated with increased metastatic capacity (Marcuzzi, Angioni, Molon, & Cali, 2018). As a result of the association between chemokines and cancer progression, many inhibitors targeting chemokine receptors are being designed as therapeutics for cancer (Mollica Poeta et al., 2019). Furthermore, numerous studies have supported the involvement of chemokine/chemokine receptor pairs in the regulation of stemness features in CSC-like cells. This is consistent with the fact that chemokines signal through a complex network of pathways, many of which appear to play crucial roles in tumor initiation. In turn, several chemokine receptors are known to be upregulated during transformation to CSCs or during CSC sphere formation, and several of these molecules have confirmed roles in regulating stemness features (Choi et al., 2015). Furthermore, some chemokine receptors have been described as CSC markers in some cancer types, including CXCR1/2 and CXCR4 (Kim & Ryu, 2017).

1.4.3. The CX3CR1/Fractalkine Axis in Cancer Progression and Metastasis

CX3CR1 is a G protein-coupled chemokine receptor for the chemokine CX3CL1, also known as fractalkine (FKN). In humans, CX3CR1 is expressed by several immune cells, including dendritic cells, natural killer cells and T cells. This receptor is also expressed by cells in the central nervous system, including neurons and microglial cells. Its ligand, FKN, is the only member of the CX3C chemokine family. This chemokine is expressed in several cell types, including endothelial cells, epithelial cells, dendritic cells, neurons, osteoblasts and keratinocytes (Tardaguila & Mañez, 2014). FKN is synthesized as a transmembrane protein with strong adhesive properties, and can be cleaved by the enzymes ADAM10 and ADAM17 into a soluble molecule with chemoattractant properties (Umehara et al., 2004). In addition to its role in chemotaxis and adhesion of leukocytes, studies have shown that FKN also supports the survival of several cell types during homeostasis and inflammation (White & Greaves, 2012). Membrane-bound FKN supports integrin-independent leukocyte adhesion when expressed on endothelial cells, whereas soluble FKN has chemoattractant activity for immune cells, including monocytes, natural killer cells and T cells (Umehara et al., 2004).

The role of this chemokine/chemokine receptor axis in cancer progression and metastasis has been described by the Fatatis lab and others. FKN has been shown to act as a tumor promoter by activation of pro-tumorigenic pathways in various cancer cells, including prostate, pancreas, breast, ovary and neouroblastoma (Tardaguila & Mañez, 2014). Furthermore, CX3CR1 expression is increased in a variety of clinical tumor samples, including breast and prostate cancer (Jamieson, Shimizu, D'Ambrosio, Meucci, & Fatatis, 2008; Shen et al., 2016; Tardaguila & Mañez, 2014). The expression of this

receptor by cancer cells has been implicated in metastasis to different organs, including the bone, lung and brain (Tardaguila & Mañez, 2014). Our lab previously showed that prostate cancer cells express CX3CR1, and that its activation by FKN promotes cell survival through PI3K/AKT signaling. This study also demonstrated that human osteoblasts express high FKN levels, facilitating the migration and adhesion of CX3CR1-expressing prostate cancer cells to the bone (Shulby, Dolloff, Stearns, Meucci, & Fatatis, 2004). Later studies from our lab showed that both normal and malignant breast tissues express CX3CR1, and that the ability of breast cancer cells to lodge in the skeleton of animal models is increased by the over-expression of this chemokine receptor. Furthermore, this study provided evidence that CX3CR1 regulates both adhesion and extravasation of breast cancer cells (Jamieson-Gladney, Zhang, Fong, Meucci, & Fatatis, 2011). In addition to facilitating metastasis to the bone in some cancer types, CX3CR1 has been implicated in the neurotropism of pancreatic ductal adenocarcinoma cells to local peripheral nerves by mediating adhesion to neural cells, chemotactic migration and survival by protecting cells from apoptosis (Marchesi et al., 2008).

The role of the CX3CR1/FKN receptor in metastasis can be explained by our model shown in Figure 3. During metastasis, cells depart from primary tumors and intravasate into blood or lymphatic vessels, at which point they become circulating tumor cells (CTCs). The extravasation of CTCs through the endothelium into secondary organs is facilitated by adhesive molecular interactions between CX3CR1 and the membrane-anchored form of FKN. Newly seeded cancer cells then migrate in response to the chemoattractant gradient established by the soluble form of FKN, which is released from cells of the surrounding

stroma. These cancer cells can then proliferate and establish metastases in the secondary organ (Worrede, Meucci, & Fatatis, 2019).

1.4.4. FX-68, a Novel CX3CR1 Antagonist

The important role of CX3CR1 and its ligand in the progression and metastasis of several cancer types makes it a valuable therapeutic target. Based on the model described in Figure 3, administering a CX3CR1 antagonist would block the initial CX3CR1-fractalkine interaction, preventing extravasation. This would cause CTCs to be retained in circulation, increasing their exposure to chemotherapeutic agents and thus improving clinical outcome (Worrede et al., 2019). Through collaborative efforts, our lab has generated a novel small molecule CX3CR1 antagonist. The synthesis of small-molecule inhibitors of CX3CR1 first led to the development of the small molecule compound JMS-17-2, followed by the improved compound FX-68. FX-68 showed high antagonistic potency (IC_{50} 15 nM) when evaluating the inhibition of MAPK activation in cancer cells stimulated with FKN. This compound proved to be highly selective when tested against 33 plasma membrane receptors and 364 different kinases (Qian et al., 2018). Compared to the previous compound JMS-17-2, FX-68 showed an improvement in water solubility, plasma stability, and hERG liability (IC_{50} 4.9 μM). Furthermore, FX-68 showed better mouse PK properties when given by IV, IP, and PO routes of administration (Qian et al., 2018).

1.5. Previous Studies from Our Lab

1.5.1. CX3CR1 plays a role in the seeding of prostate and breast cancer cells into bone.

Our lab previously described that CX3CR1 plays a major role in the dissemination of breast and prostate cancer cells to bone skeleton (Jamieson-Gladney et al., 2011) and that the inhibition of this receptor by the CX3CR1 antagonists JMS-17-2 or FX-68 reduces cancer cells dissemination in animal models. Our recent experiments comparing wild type and CX3CR1-overexpressing MDA-436 human breast cancer cells showed that not only CX3CR1 is important for seeding of cancer cells into secondary organs, but it also allows the cells to colonize and grow tumors more effectively (**Figure 4**). Similarly, blocking CX3CR1 impaired seeding and colonization of prostate cancer cells PC3-ML and breast cancer cells MDA-231 to the bone (**Figure 5**).

1.5.2. CX3CR1 expression and activity correlates with stemness features in prostate and breast cancer cells.

CSCs are characterized by a series of distinct features that distinguish them from more differentiated states. One of these defining features is the ability to form tumorspheres when single cells are cultured in serum-free, non-adherent conditions, in which only CSCs can survive and proliferate. A tumorsphere is a solid, spherical formation developed from the proliferation of one cancer stem/progenitor cell. The size and number of tumorspheres can be used to characterize the CSC population within the total population of cultured cancer cells *in vitro* (Johnson, Chen, & Lo, 2013). We recently showed that engineering human breast cancer cells MDA-436 to overexpress CX3CR1 leads to an increase in the diameter and number of tumorspheres compared with wild type cells (**Figure 6A**).

Additionally, experiments using MDA-436 cells that expressed a CX3CR1 inactive mutant (R128N) resulted in impaired formation of tumorspheres by these cells. The CX3CR1 R128 mutant contains an R-to-N mutation in the *DRY* sequence, which is required for G-protein activation, thus making the receptor incapable of intracellular signaling (Jamieson-Gladney et al., 2011). Therefore, our experiments suggested that functional CX3CR1 signaling is needed for the cells to display features similar to those found in CSCs (**Figure 6A**).

Similarly, when CX3CR1 was silenced in breast cancer cells, the number and diameter of tumorspheres was decreased (**Figure 6B**). Furthermore, the ability of cancer cells to form tumorspheres was reduced by FX-68 in both prostate cancer and breast cancer cells in a dose-dependent manner, further supporting the idea that CX3CR1 plays a major role in tumor initiation (**Figure 7**). To investigate if the tumor initiating potential deducted by tumorsphere formation is unique to CX3CR1, we evaluated two other chemokine receptors, CXCR4 and CCR5. Our results from the tumorsphere assay using prostate cancer cells showed that blocking these two receptors using the antagonists AMD-3100 and Maraviroc, respectively, did not alter tumorsphere formation as compared to the control (**Figure 8**). These data further suggest that among chemokine receptors, CX3CR1 has a preferential role in regulating tumorsphere formation *in vitro.*

We also analyzed the expression of the pluripotency-associated transcription factors OCT4a and NANOG in two distinct subpopulations of cells expressing high or low levels of CX3CR1 ($CX3CR1^{High}$ and $CX3CR1^{Low}$, respectively). These subpopulations were obtained by cell sorting using flow cytometry (**Figure 9 A, B**). Only a small proportion of the entire cell population was shown to express high levels of CX3CR1, which correlates

with the fact that CSCs are a minority of the cells within the tumor (**Figure 9B**). The results of qPCR analysis of the sorted subpopulations showed that CX3CR1^{High} cells had higher expression of the pluripotency genes OCT4a and NANOG compared to CX3CR1^{Low} cells, once again indicating that CX3CR1 expression is associated with stemness features (**Figure 10**). Another recognized stemness property is a low proliferation rate. Our experiments showed that CX3CR1^{High} cells displayed low proliferation rates compared to CX3CR1^{Low} cells (**Figure 11**), further supporting the association between CX3CR1 expression and a stemness phenotype.

We also tested another stem cell-like feature, which is the ability to undergo asymmetric division. From these experiments, we found that over a period of time (15 days), cells with high CX3CR1 expression (CX3CR1^{High}) gave rise to a heterogeneous population of cells with mixed CX3CR1 expression, as seen by a dilution in the expression of CX3CR1 (**Figure 12**). Thus, our results suggested that CX3CR1^{High} cells divide asymmetrically, supporting our hypothesis that CX3CR1 expression is associated with stemness properties. On the other hand, cells with low CX3CR1 expression (CX3CR1^{Low}) tested at 21 days after sorting transitioned into a mixed high and low CX3CR1 population, showing evidence of phenotypic plasticity (**Figure 13**). The same expression patterns were observed for the pluripotency-associated genes OCT4a and NANOG. Although plasticity of cancer cells has been described before (Meacham & Morrison, 2013), the exact mechanisms for this process are unknown, and they are thought to depend on signals derived from the tumor microenvironment *in vivo*, which would not explain the occurrence of this process *in vitro.* These striking results led us to the definition of the research aims for the project presented in this thesis.

1.6. Figures and Figure Legends

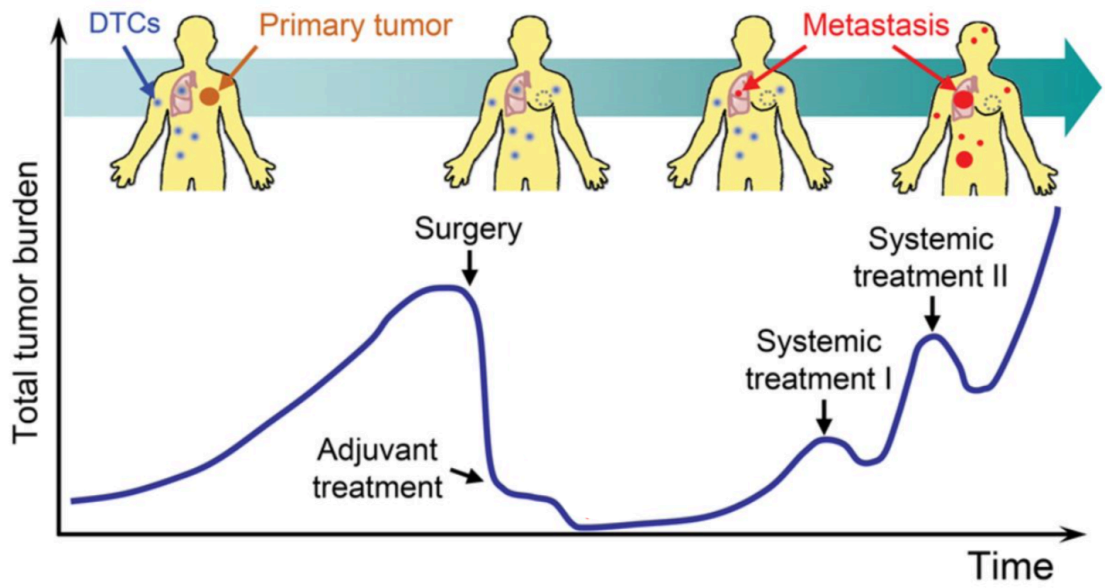

Figure 1. Typical course of metastatic progression from an early-stage cancer.

Surgery and systemic adjuvant treatment initially decrease tumor burden, but a few disseminated tumor cells (DTCs) survive, leading to metastasis after a period of dormancy. Subsequent systemic treatment often only temporarily reduces tumor burden before metastatic lesions develop resistance and eventually lead to patient lethality.

Modified from "Distinctive properties of metastasis-initiating cells" by Celià-Terrasa, T. & Kang, Y., 2016, *Genes and Development, 30*, p. 892–908.

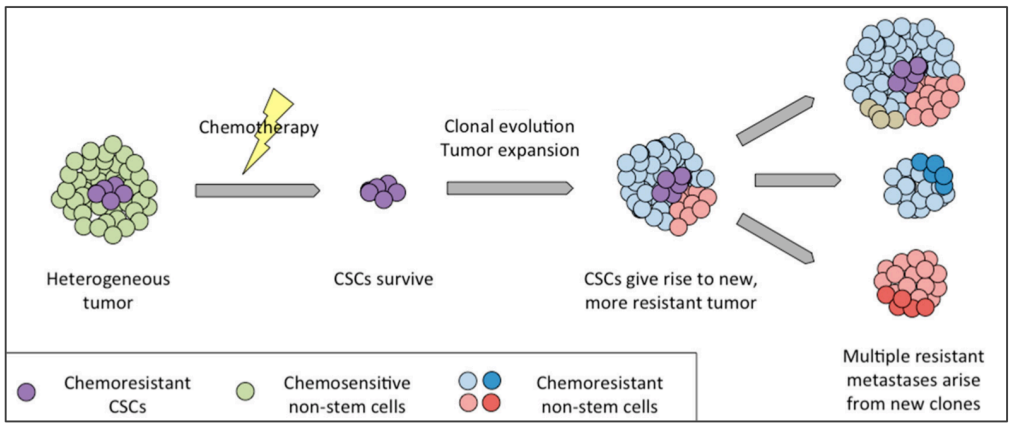

Figure 2. Contribution of cancer stem cells (CSCs) to recurrence and metastasis.

A solid tumor is heterogeneous, with a small subpopulation of CSCs (purple) and a large subpopulation of non-stem bulk tumor cells (green). When the tumor is treated with chemotherapy, this may eliminate all non-stem bulk tumor cells, leaving resistant CSCs behind. CSCs can then expand and differentiate, giving rise to recurrence. Recurrent tumors (red, blue) tend to be resistant to first line chemotherapy, metastasize widely, and continue to evolve (dark red, dark blue), ultimately leading to lethality to the patient.

Modified from "The Role of Intra-Tumoral Heterogeneity and Its Clinical Relevance in Epithelial Ovarian Cancer Recurrence and Metastasis" by Roberts, C.M., Cardenas, C., & Tedja, R., 2019, *Cancers, 11*, p. 1083.

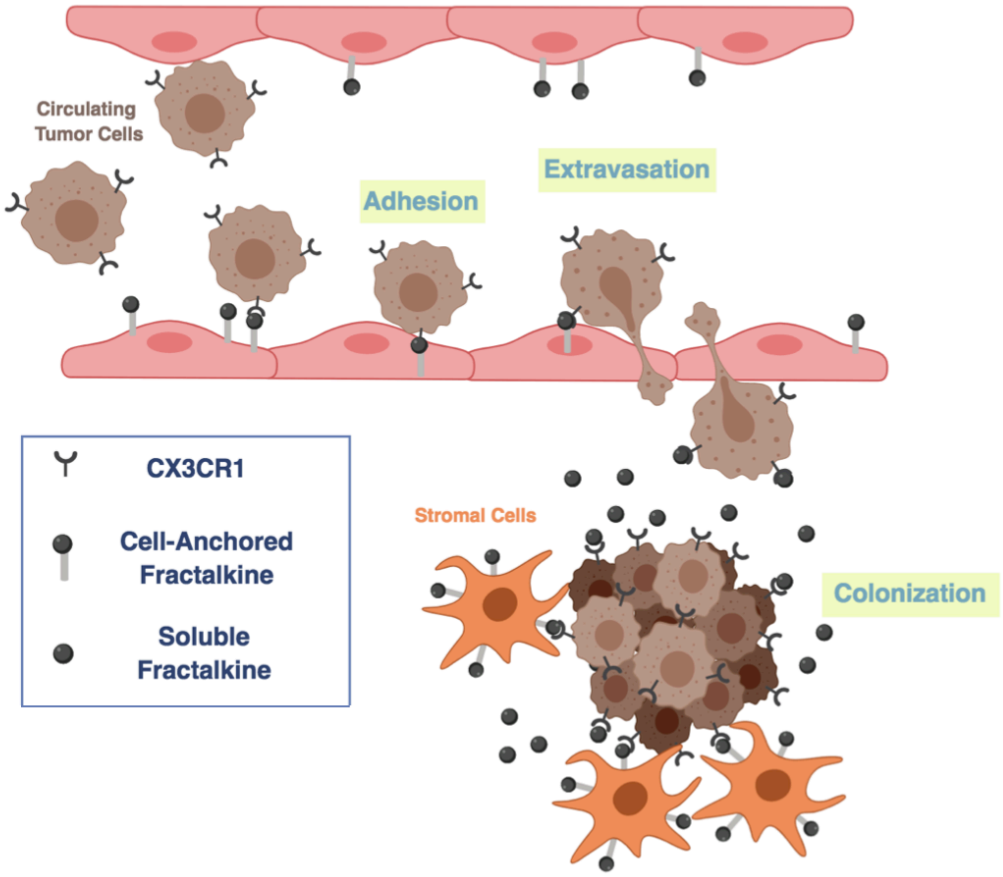

Figure 3. CX3CR1 facilitates metastatic seeding of cancer cells.

Circulating tumor cells (CTCs) expressing CX3CR1 adhere to cell-anchored fractalkine expressed by endothelial cells and extravasate to secondary organs, where they migrate towards soluble fractalkine secreted by stromal cells and eventually colonize, forming new metastatic lesions.

Modified from "Limiting tumor seeding as a therapeutic approach for metastatic disease" by Worrede, A., Meucci, O., & Fatatis, A., 2019, *Pharmacology & Therapeutics, 199*, p. 117-128.

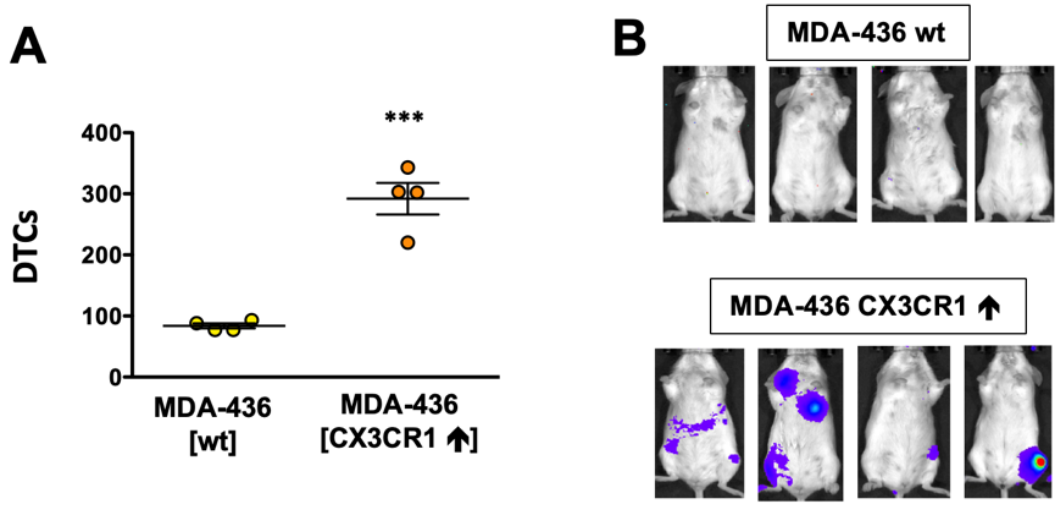

Figure 4. CX3CR1 expression drives breast cancer cells seeding to the bone.

A. CX3CR1 expression increases the number of disseminated tumor cells (DTCs) at the bone (***, *P = 0.0002).* **B.** Cells that do seed at the bone are unable to grow and colonize tumors effectively.

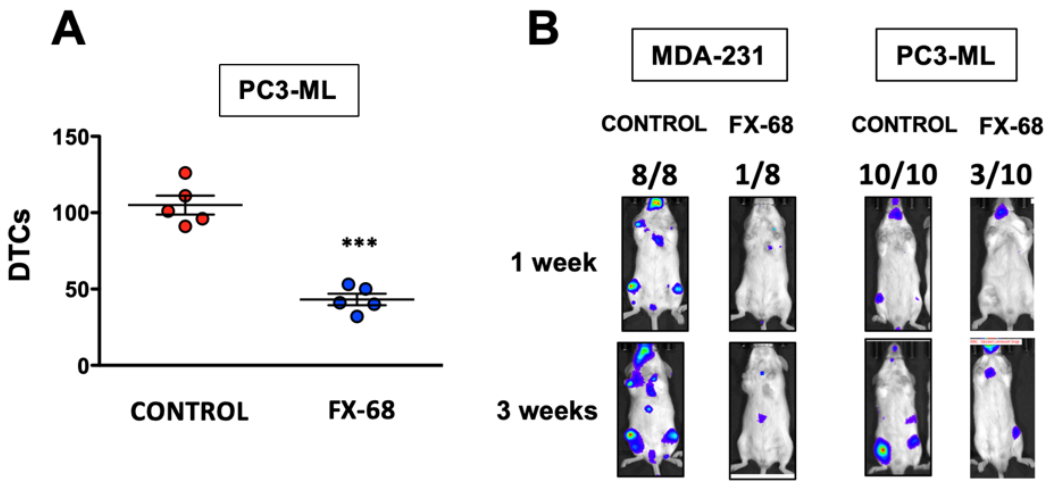

Figure 5. Blocking CX3CR1 with an antagonist (FX-68) impairs breast and prostate cancer cells seeding to the bone.

A. Blocking CX3CR1 decreases the number of prostate DTCs at the bone (***, P = 0.0007). **B.** Prostate and breast cancer cells that do arrive to the bone are unable to grow as effectively when CX3CR1 is blocked.

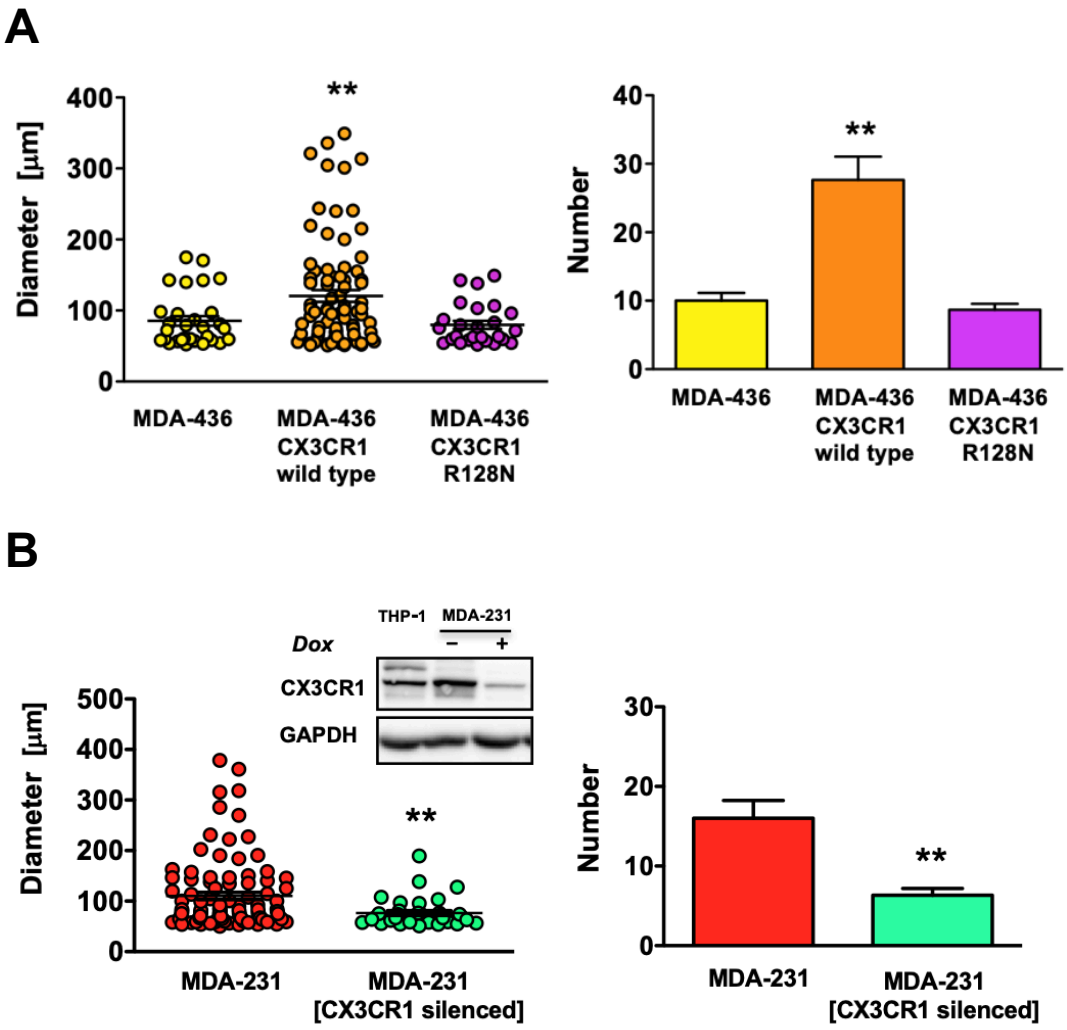

Figure 6. CX3CR1 expression is associated with tumorspheres formation.

A. MDA-436 cells overexpressing CX3CR1 generate tumorspheres in greater number and of higher diameter compared to the wild type cell line. This increase is not observed in cells engineered to express an inactive mutant of CX3CR1 (R128N) *(Diameter, **, P = 0.003; Number, **, P = 0.0012).* **B.** Silencing CX3CR1 in MDA-231 cells leads to a decrease in tumorsphere number and diameter *(Diameter, **, P = 0.006; Number, **, P = 0.002)*.

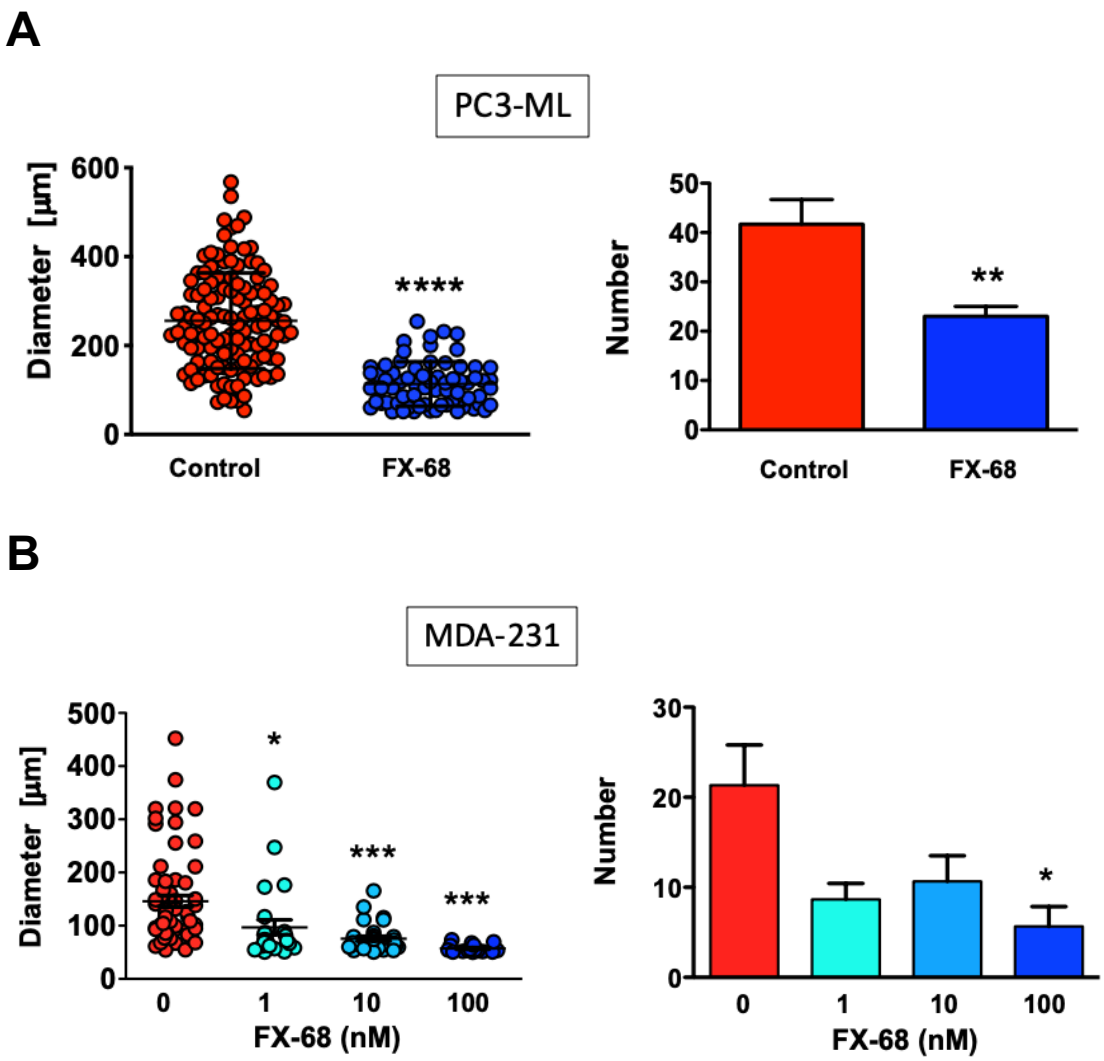

Figure 7. Blocking CX3CR1 with FX-68 impairs tumorsphere formation.

A. FX-68 treatment decreases the number and diameter of tumorspheres (Diameter, ****, $P < 0.0001$, Number, **, $P = 0.004$). **B.** FX-68 impairs tumorsphere formation in a dose-dependent manner (Diameter, *, $P = 0.01$, ***, $P < 0.0001$; Number, *, $P = 0.03$).

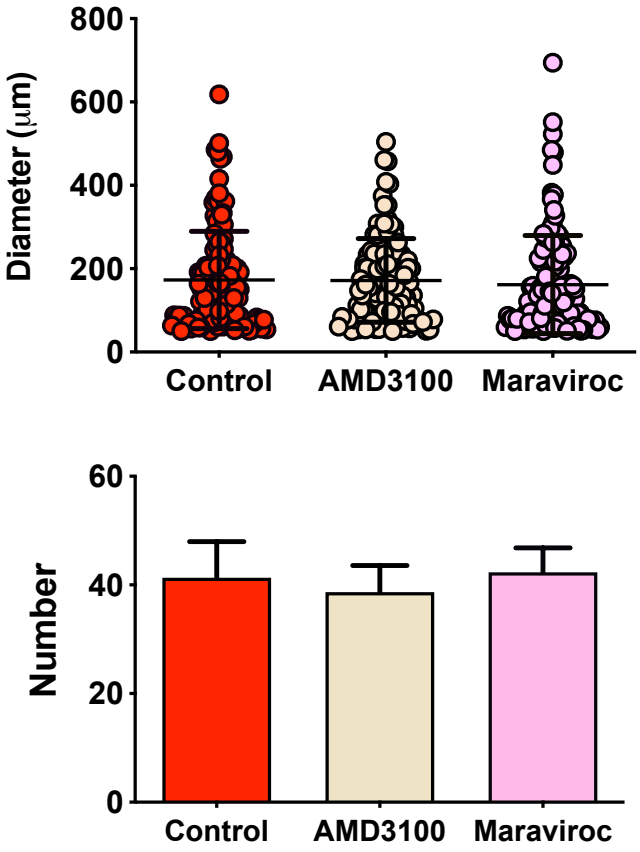

Figure 8. CXCR4 and CCR5 antagonism do not alter tumorsphere formation.

AMD-3100, a CXCR4 antagonist, and Maraviroc, a CCR5 antagonist, have no effect on tumorsphere formation.

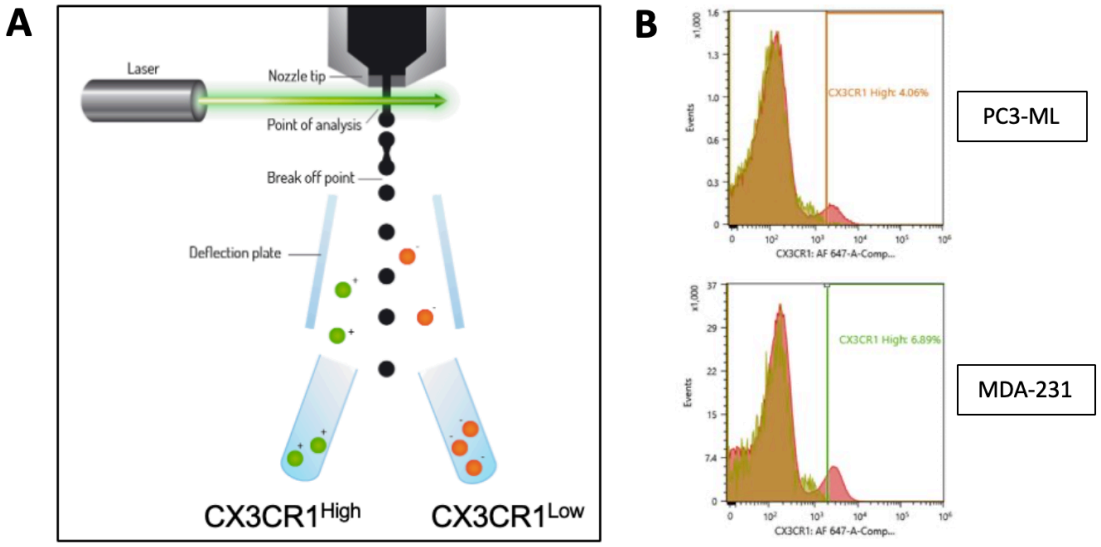

Figure 9. Breast and prostate cancer cell lines can be sorted into CX3CR1^{High} and CX3CR1^{Low} subpopulations.

A. Staining of prostate cancer cells (PC3-ML) or breast cancer cells (MDA-231) followed by flow cytometry can select subpopulations of cells that express high and low levels of CX3CR1. **B.** Flow cytometry histogram following staining of PC3-ML (top) or MDA-231 (bottom) cells using an anti-CX3CR1 antibody.

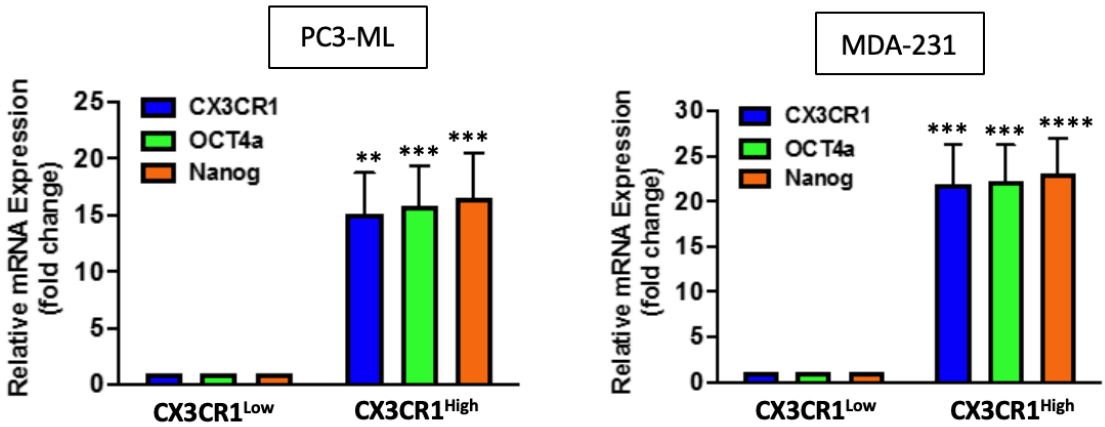

Figure 10. CX3CR1 expression is correlated with pluripotency genes.

$CX3CR1^{High}$ and $CX3CR1^{Low}$ cells were stained and sorted by flow cytometry and subjected to qPCR analysis. $CX3CR1^{High}$ cells express significantly higher levels of the pluripotency genes OCT4a and Nanog *(PC3-ML **, $P = 0.0016$, ***, $P = 0.009$, ***, $P = 0.007$; MDA-231 ***, $P = 0.0002$, ***, $P = 0.0001$, ****, $P<0.0001$).*

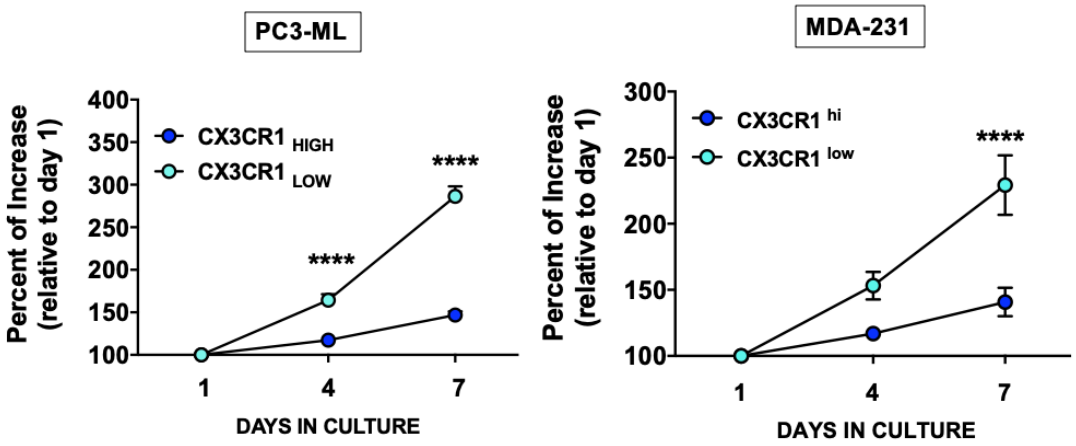

Figure 11. CX3CR1^{High} cells display lower proliferation rates compared with CX3CR1^{Low} cells.

The results of XTT assays of CX3CR1^{High} and CX3CR1^{Low} proliferation studies show that cells with high CX3CR1 expression proliferate at slower rates compared with CX3CR1^{Low} cells *(****, $P < 0.0001$).*

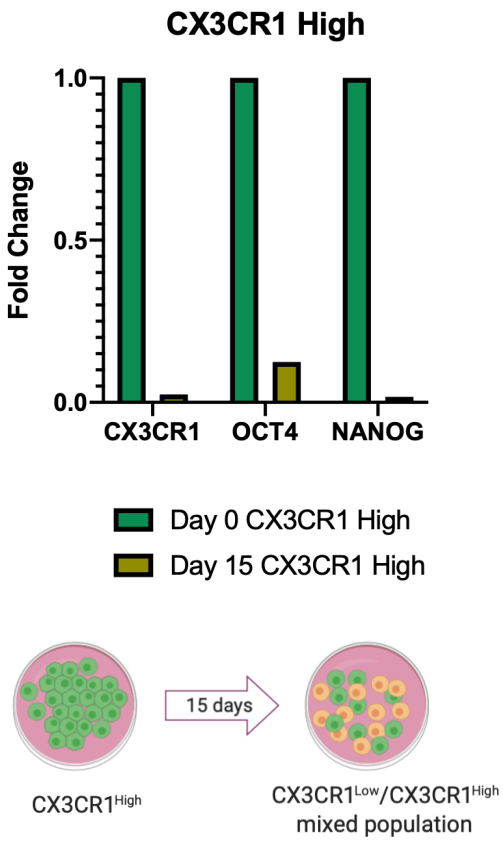

Figure 12. CX3CR1^{High} cells show evidence of asymmetric division.

RT-qPCR was performed in CX3CR1^{High} cells pre- and post-culture for 15 days. The dilution in CX3CR1, OCT4a and NANOG mRNA expression suggests that CX3CR1^{High} cells generated a mixed population of CX3CR1^{High} and CX3CR1^{Low} cells by asymmetric division.

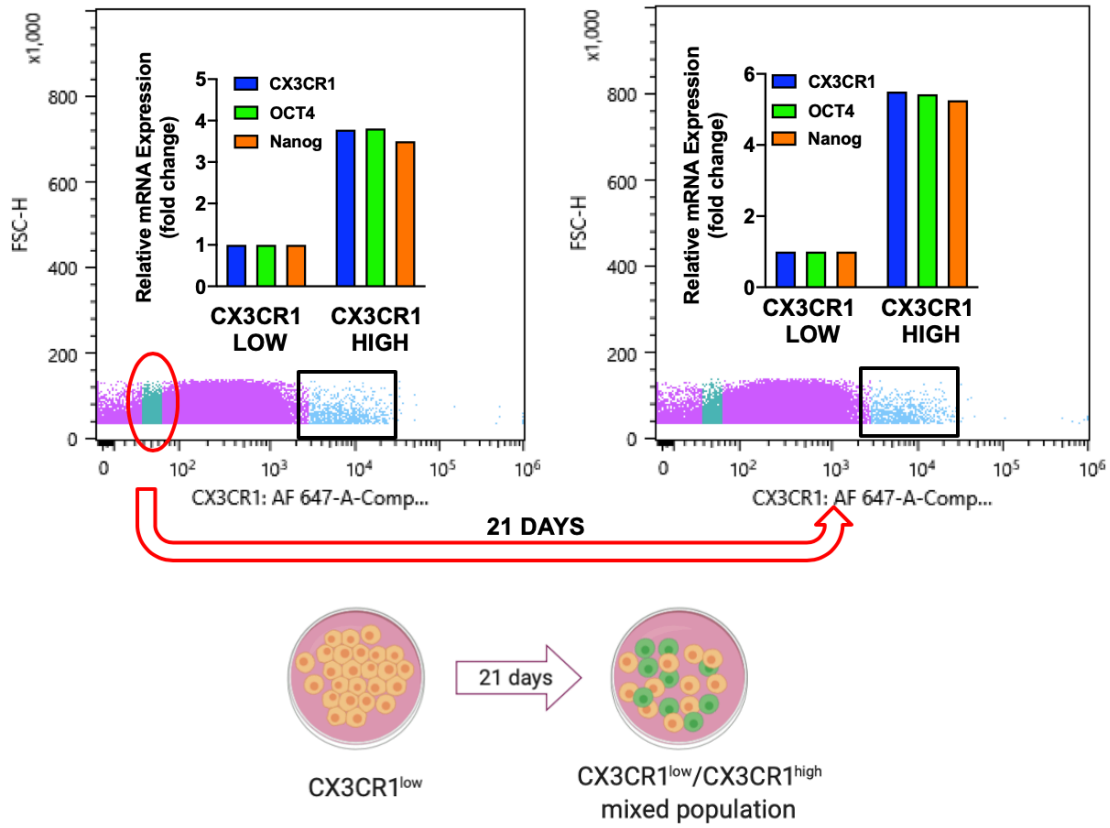

Figure 13. CX3CR1^{Low} cells display evidence of phenotypic plasticity following prolonged incubation.

CX3CR1^{High} and CX3CR1^{Low} cells were stained and sorted by flow cytometry and subjected to qPCR analysis. CX3CR1^{High} cells express significantly higher levels of the pluripotency genes OCT4a and Nanog (Left). CX3CR1^{Low} cells were incubated *in vitro* and after 21 days they were stained for flow cytometry. The resulting cell population consisted of mixed CX3CR1^{High} and CX3CR1^{Low} cells, which were also subjected to qPCR analysis. Again, CX3CR1^{High} cells express significantly higher levels of the pluripotency genes OCT4a and Nanog (Right).

2. RESEARCH OBJECTIVES

The primary objective of this project is to elucidate the role of the chemokine receptor CX3CR1 in the regulation of stemness and the phenotypic plasticity of cancer cells.

2.1. Rationale and Strategy

Metastasis remains the main cause for cancer-related deaths. In order to develop novel therapies to target metastasis, it is essential to understand the molecular mechanisms driving this process. Mounting evidence has shown that stem-like cells are responsible for the initiation of both primary tumors and metastatic lesions. Our previous studies implicated the CX3CR1-FKN axis in tumor initiation and revealed that the expression of this chemokine receptor in prostate and cancer cells is associated with a stem-like phenotype. Furthermore, our results suggested that CX3CR1 may be involved in the phenotypic plasticity of cancer cells converting from a non-stem-like phenotype to stem-like cells.

Based on our preliminary studies, we hypothesize that CX3CR1 is involved in the regulation of the expression of the pluripotency genes OCT4a and NANOG, and that this regulation is performed through the activation of intracellular pathways. To test this hypothesis, we will use molecular and pharmacological approaches to delineate the timeframe for the conversion of $CX3CR1^{Low}$ to $CX3CR1^{High}$ cells, and to establish whether there is a correlation between the expression of CX3CR1 and the master regulator genes of pluripotency.

2.2. Innovation

Our experiments will test our hypothesis that the chemokine receptor CX3CR1 drives the plasticity of cancer cells towards a stem-like state by regulating the expression of pluripotency genes. In order to do this, we will use various strategies to define the role of CX3CR1 in the expression of the aforementioned genes. Our studies will determine the timeframe by which the plasticity of cancer cells occurs. Additionally, we will assess if blocking this chemokine receptor with FX-68, a novel small-molecule CX3CR1 antagonist, is enough to counteract the re-expression of pluripotency genes by CX3CR1.

The innovation of our studies lies in the fact that the mechanisms of cell plasticity are not yet fully comprehended, and the expression of CX3CR1 has not been previously associated with this phenomenon. A better understanding of the process of cell plasticity would have important implications for addressing major mechanisms of resistance to chemotherapeutic and radiation therapies in the clinic.

2.3. Specific Aim

The aim of the work presented here was to establish a correlation between CX3CR1 and the master regulator genes of pluripotency OCT4a and NANOG. The goal of our studies is to investigate the changes in the pattern of expression of CX3CR1 and the transcription factors encoded by the master regulator genes in $CX3CR1^{Low}$ cells, as well as the timeframe for these changes.

3. EXPERIMENTAL METHODS

3.1. Cell Lines and Cell Culture

Human prostate cancer (PC3-ML) and breast cancer (MDA-231) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (Hyclone) and 0.1% gentamicin (Invitrogen). Cells were cultured at 37° C and 5% CO_2. Control for Mycoplasma contamination and cell authentication by Single Tandem Repeat were performed by IDEXX Radil.

3.2. Flow Cytometry Analyses

Prostate and breast cancer cell lines were sorted into $CX3CR1^{High}$ and $CX3CR1^{Low}$ subpopulations using flow cytometry. Cells were detached from dishes with cell stripper and centrifuged at 1000 rpm for 5 min. The cells were then resuspended in a staining buffer (1% FBS in ice-cold PBS) and incubated with a Brilliant Violet conjugated CX3CR1 antibody (Biolegend, 341619) for 30 mins at 4°. Following incubation, cells were washed 3 times with staining buffer and then sorted with a SH800S cell sorter (Sony), using a Brilliant Violet 421 isotype matched immunoglobulin as control.

3.3. SDS-PAGE And Western Blotting

Protein levels for CX3CR1 and NANOG were assessed by Western blotting. After incubation, cells were lysed in RIPA lysis and extraction buffer (#89900 Thermo Fisher) containing a phosphatase inhibitor cocktail (Calbiochem), a protease inhibitor cocktail (Calbiochem), 10% glycerol, and 0.5 M EDTA (Thermo Fisher). Protein concentrations were determined with the BCA™ protein assay kit (Pierce). Proteins were separated via 10% SDS-PAGE and afterwards transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation). Membranes were blocked for one hour at room

temperature with either 5% non-fat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for CX3CR1 detection or 5% bovine serum albumin (BSA) in TBST for OCT4a and NANOG detection. After blocking, membranes were washed with TBST and incubated with antibodies against CX3CR1 (ab8021, Abcam, 1:1000), OCT4a (C30A3, Cell Signaling, 1:1000), NANOG (3580S, Cell Signaling, 1:1000) or Actin (A2066, Sigma Aldrich, 1:1000) at 4°C overnight. A secondary, HRP-conjugated antibody (Pierce) was used at 10 ng/ml. Blotted membranes were processed with SuperSignal Femto chemiluminescence substrates (Pierce) and visualized using a FluorChem imaging system (ProteinSimple).

3.4. Statistics

Results are reported as mean ± SEM. When comparing two experimental groups, statistical significance was determined by Student's t-test with Welch's correction (GraphPad Prism 5.0). When comparing multiple experimental groups, statistical significance was determined by one-way ANOVA with Dunnett's post-test (GraphPad Prism, version 8.4.2) In both cases, statistical significance was achieved by $p<0.05$.

4. RESULTS

4.1. CX3CR1^{Low} cells display evidence of phenotypic plasticity *in vitro*, which is at least partially impaired by pharmacological targeting of CX3CR1

To investigate the timeframe for the cell plasticity observed in our preliminary studies, we set to establish a timeframe for the re-expression of CX3CR1 and the pluripotency transcription factors OCT4a and NANOG in CX3CR1^{Low} prostate (PC3-ML) and breast (MDA-231) cancer cells. To this end, we sought to detect the expression of CX3CR1, OCT4a and NANOG at different timepoints during the course of 21 days by using a Western blot assay. Any changes in the expression of these proteins during the time course of the experiment were then registered and analyzed.

After the CX3CR1^{Low} subpopulation for each cell line was obtained by flow cytometry, about 350,000 cells were seeded onto a cell culture flask, and about 350,000 cells were seeded onto a cell culture dish. The cells contained within the cell culture flask were cultured in DMEM at 37° C and 5% CO_2. Upon confluency (approximately every 3-5 days), these cells were split again, and an appropriate number was seeded onto a new cell culture flask and a new cell culture dish. This procedure was repeated over a period of 21 days. At each of the different timepoints, the cells in each cell culture dish were lysed and collected to perform a Western blot assay, probing for the proteins CX3CR1, OCT4a and NANOG (**Figure 14**). To investigate the implication of CX3CR1 signaling in the regulation of pluripotency genes, we also cultured CX3CR1^{Low} cells in the presence of FX-68 (100 nM) in order to block CX3CR1. Before proceeding with the main experiments, we performed validation of the antibodies to be utilized in the Western blot assays (**Figure 15**).

Based on the preliminary results from our qPCR studies, CX3CR1^{Low} cells grown for 21 days in vitro showed re-expression of CX3CR1, OCT4a and NANOG at the transcriptional level. Here we found that these changes also translated at the protein level, since CX3CR1^{Low} cells progressively re-expressed CX3CR1 when cultured for 21 days. This increase in CX3CR1 expression started being detected after just 4-5 days of culture. At the latest timepoint, the increase was up to 3.3-fold in PC3-ML prostate cancer cells (**Figure 16, A**) and up to 7.5-fold in MDA-231 breast cancer cells (**Figure 16, B**). These results show evidence of phenotypic plasticity in cancer cells *in vitro*. We also assessed the expression of CX3CR1 protein levels over time following the incubation of CX3CR1^{Low} cells in the presence of FX-68. Our results show that the increase in CX3CR1 protein expression was, at least in part, impaired when this receptor was pharmacologically blocked (**Figure 16, C and D**).

In line with our hypothesis that CX3CR1 expression is associated with stemness features, we also tested the expression of the pluripotency-associated transcription factors OCT4a and NANOG. When we performed Western blot assays probing for OCT4a, we encountered a challenge in the detection of this protein in CX3CR1^{Low} cells, although this protein was easily detected in the positive control, i.e. the embryonic cell line NTERA-2, which express high levels of pluripotency transcription factors. The reason for this issue was most likely the low amount of protein available for detection in our samples. Despite trying different approaches for troubleshooting (loading higher protein levels, using higher primary antibody concentration, performing cell lysis with nuclear extraction) we were not able to increase the sensitivity of the assay to obtain bands of higher intensity. For this reason, we decided to focus on the expression of NANOG. However, we were able to detect

OCT4a in one replicate of the experiment in PC3-ML cells in the absence of FX-68. Although we would need further replicates to draw any conclusion, the result we obtained seems to be in line with our hypothesis, since OCT4a expression appears to increase overtime (**Figure 17**).

When we performed Western blot assays probing for NANOG, we observed that the levels of this protein also increased over time following incubation of $CX3CR1^{Low}$ cells, although to a lesser extent compared with the increase in CX3CR1 levels (**Figure 18, A and C**). These results again suggest the existence of phenotypic plasticity in cancer cells. We then assessed the expression of NANOG protein levels over time following the incubation of $CX3CR1^{Low}$ cells in the presence of FX-68. Our results show that the increase in NANOG protein expression in PC3-ML cells was, at least in part, impaired when CX3CR1 was blocked (**Figure 18C**). This suggests that CX3CR1 is involved in the regulation of NANOG, since pharmacologically targeting this receptor partially halts the increase in NANOG expression. This effect was not observed in MDA-231 cells, since the levels of NANOG were not affected by the presence of FX-68 (**Figure 18D**).

However, it is worth noting that only two replicates could be obtained for these experiments due to lack of time. Thus, it would be necessary to repeat the experiments in order to make any conclusions with statistical confidence.

4.2. Figures and Figure Legends

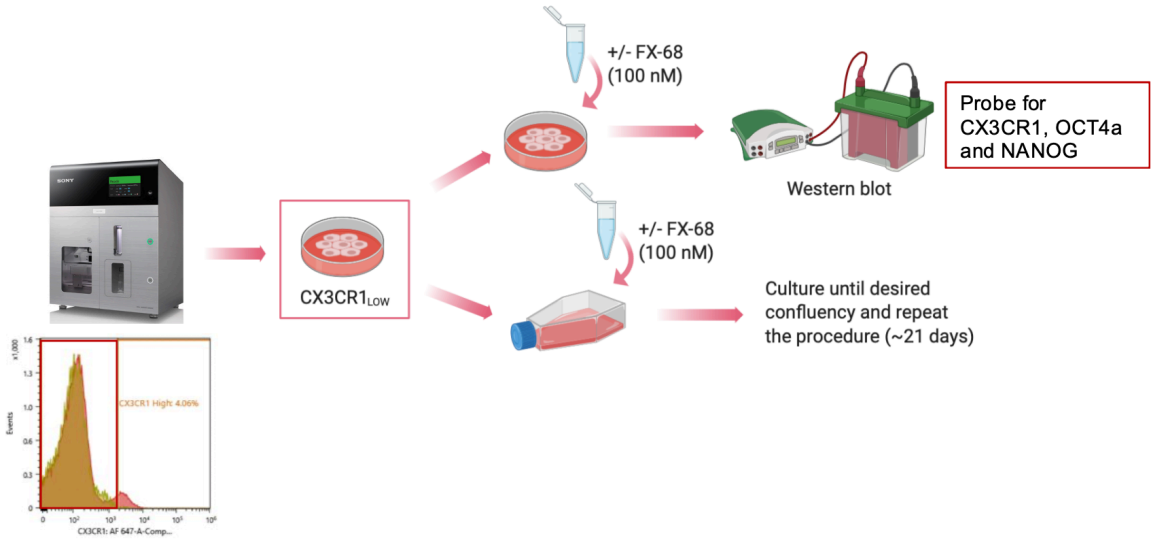

Figure 14. Schematic for the investigation of the timeframe of phenotypic plasticity in CX3CR1^{Low} cells.

Prostate cancer cells (PC3-ML) or breast cancer cells (MDA-231) were stained and sorted by flow cytometry, obtaining two subpopulations with high and low levels of CX3CR1 expression (CX3CR1^{High} and CX3CR1^{Low}, respectively) After the CX3CR1^{low} subpopulation for each cell line was obtained, about 350,000 cells were seeded onto a cell culture flask, and about 350,000 cells were seeded onto a cell culture dish. The cells contained within the cell culture flask were cultured in DMEM at 37° C and 5% CO_2, either in the absence or in the presence of the CX3CR1 antagonist FX-68 (100 nM). Upon confluency (approximately every 3-6 days), these cells were split again, and an appropriate number was seeded onto a new cell culture flask and a new cell culture dish. This procedure was be repeated over a period of a 21 days. At each of the timepoints obtained, the cells will be lysed and collected to perform a Western blot assay, probing for the proteins CX3CR1, OCT4a and NANOG.

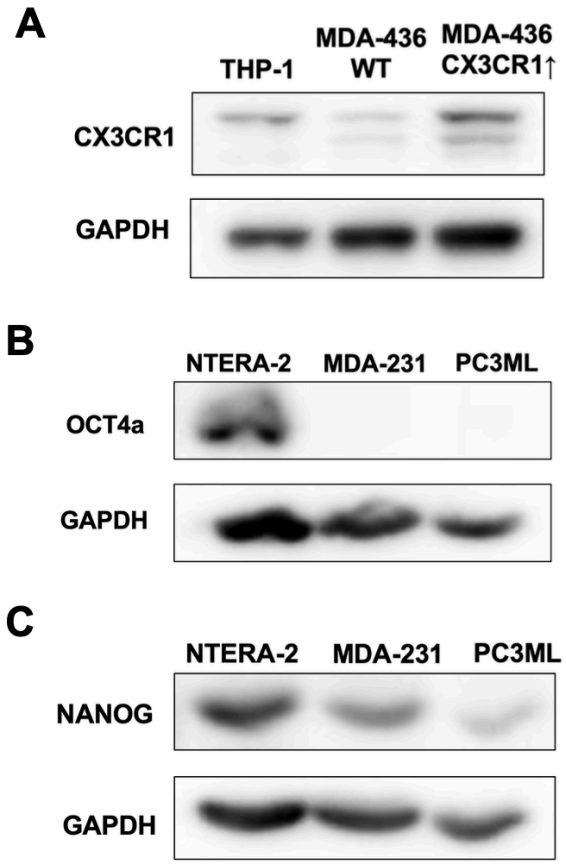

Figure 15. Validation of antibodies utilized for Western blot analysis to detect the proteins CX3CR1, OCT4a and NANOG.

A. For the detection of CX3CR1, THP-1 human monocytic cells, which constitutively express CX3CR1, were used as positive control, together with MDA-436 human breast cancer cells engineered to exogenously overexpress CX3CR1. Wild type MDA-436, which express minimal levels of CX3CR1, were used as a negative control. The three bands observed represent the three isoforms of the receptor produced by alternative splicing. **B, C.** For the detection of the pluripotency genes OCT4a and NANOG, the pluripotent NTERA-2 human embryoid carcinoma cells were used as a positive control. Human prostate cancer cells PC-3ML and breast cancer cells MDA-231 showed lack of Oct4a expression and reduced levels of Nanog, as compared to NTERA-2 cells.

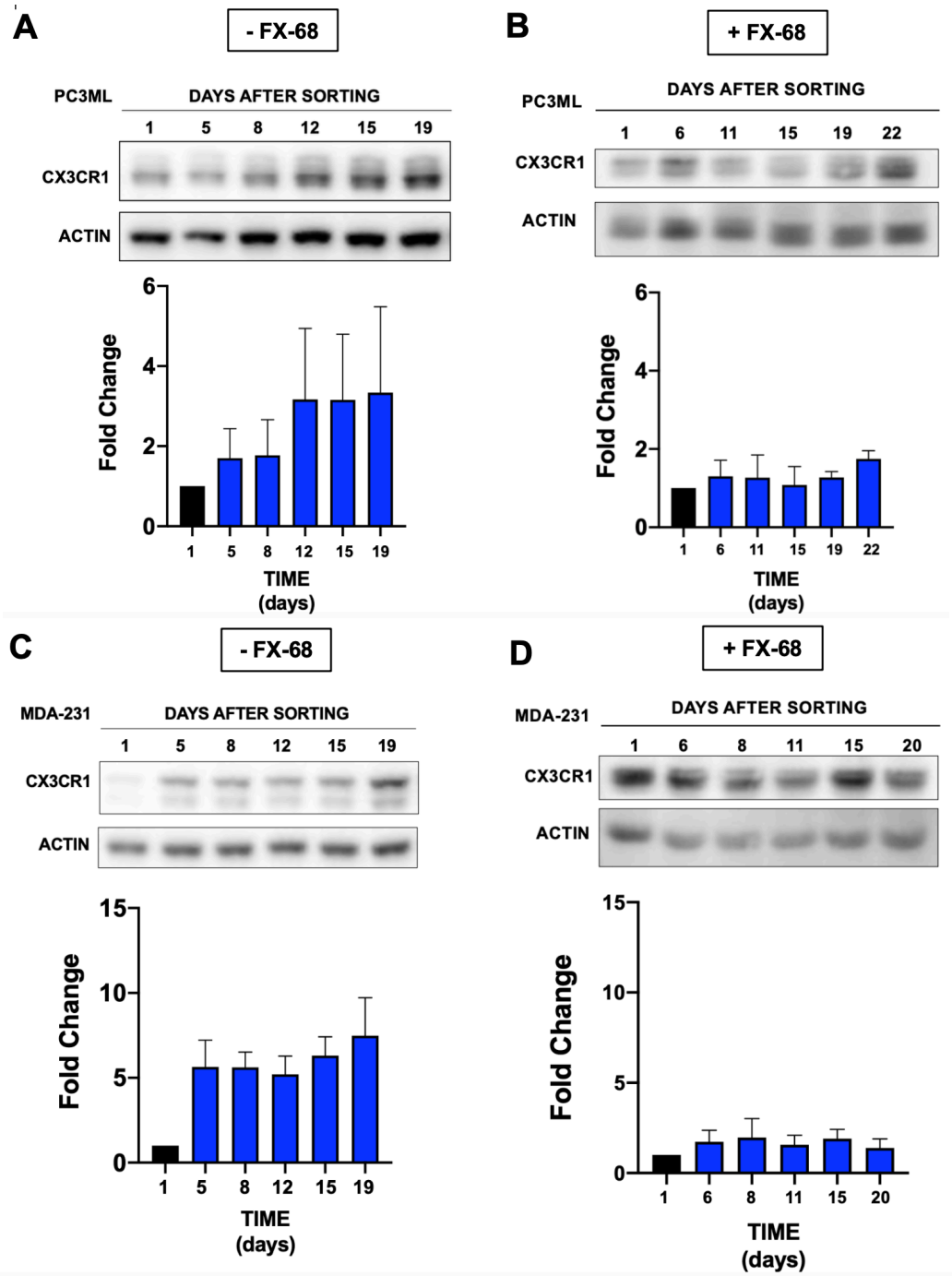

Figure 16. Western blot analysis and quantification of CX3CR1 protein expression in CX3CR1^{Low} cells cultured in the absence and presence of FX-68.

CX3CR1^{Low} cells were sorted from the total population of PC3-ML **(A, B)** or MDA-231 **(C, D)** cells and cultured in DMEM at 37 °C and 5% CO_2, either in the absence **(A, C)** or presence **(B, D)** of the CX3CR1 antagonist FX-68. At 6 different timepoints, cells were lysed, and western blot analysis was performed (two biological replicates/time point).

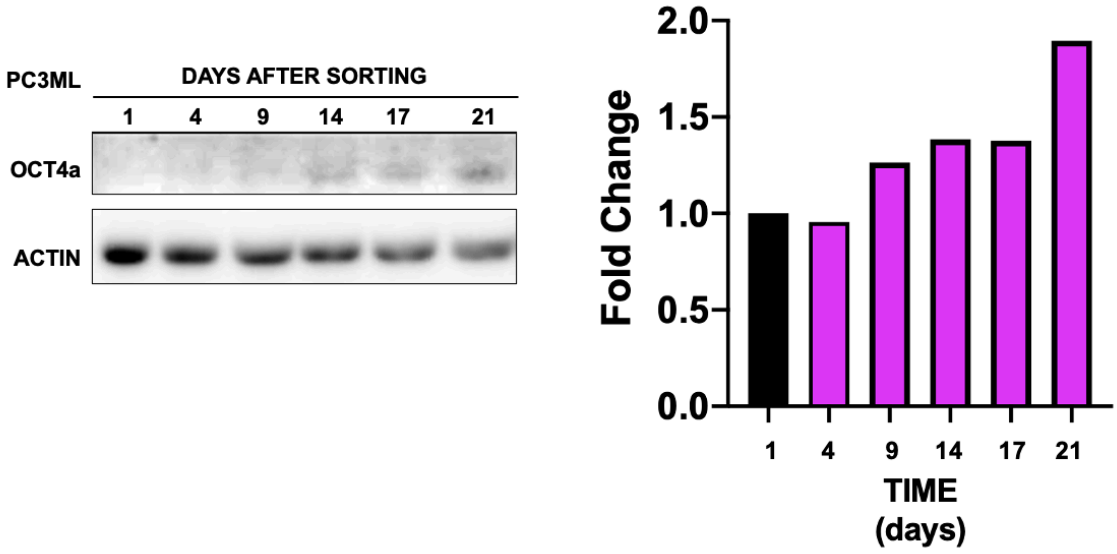

Figure 17. Western blot analysis and quantification of OCT4a protein expression in CX3CR1^{Low} cells over time.

CX3CR1^{Low} cells were obtained by flow cytometry from the total population of PC3-ML cells and cultured in DMEM at 37 °C and 5% CO_2. At 6 different timepoints, cells were lysed, and western blot analysis was performed (results from a single experiment).

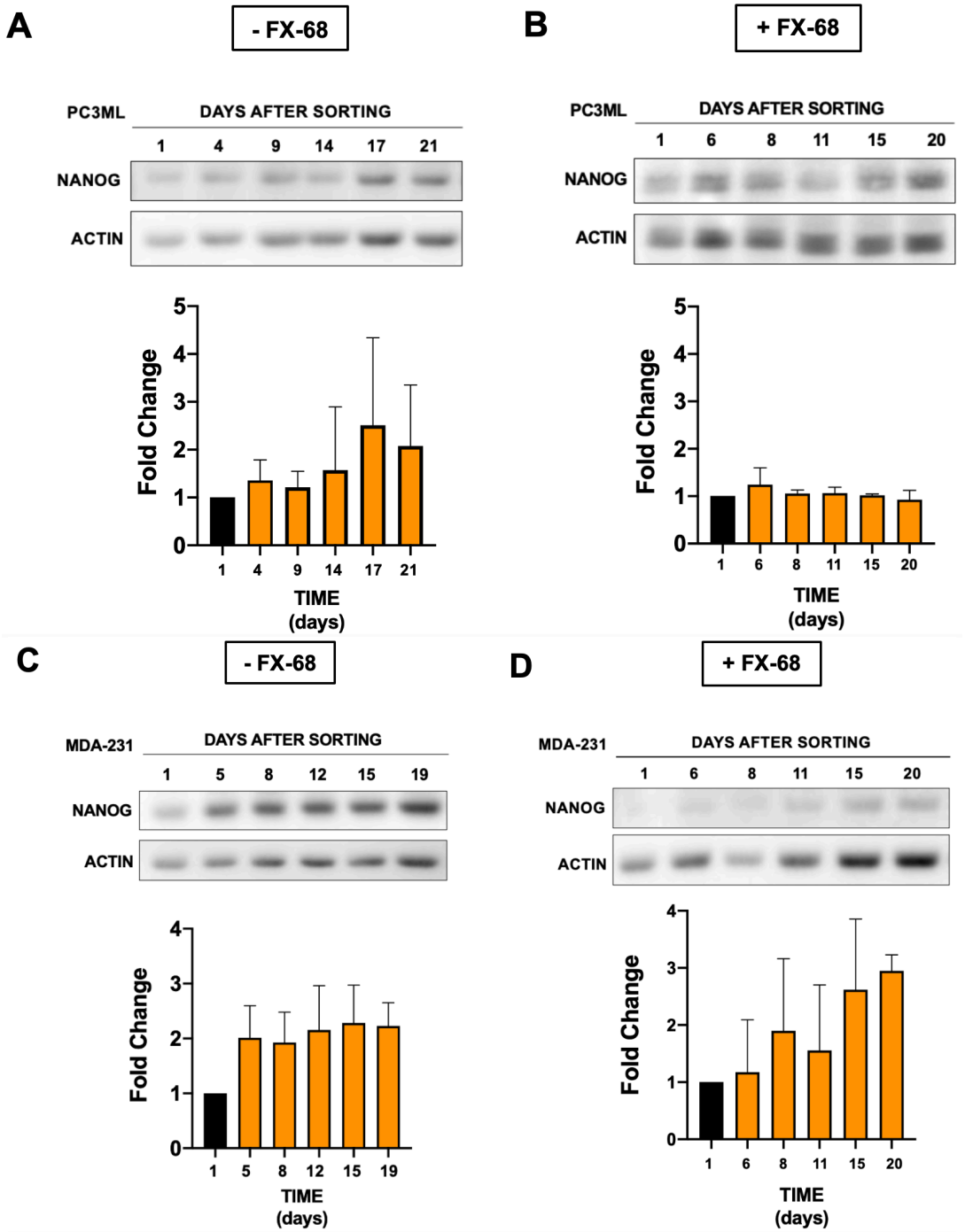

Figure 18. Western blot analysis and quantification of NANOG protein expression in CX3CR1^{Low} cells cultured in the absence and presence of FX-68.

CX3CR1^{Low} cells were obtained by flow cytometry from the total population of PC3-ML **(A, B)** or MDA-231 **(C, D)** cells and cultured in DMEM at 37 °C and 5% CO_2, either in the absence **(A, C)** or presence **(B, D)** of the CX3CR1 antagonist FX-68. At 6 different timepoints, cells were lysed, and western blot analysis was performed (two biological replicates/time point).

5. DISCUSSION

Our studies show that CX3CR1^{Low} cells reacquire CX3CR1 expression over time, a process that seems to be impaired by pharmacological targeting of this receptor. These results suggest that CX3CR1 may be involved in its own regulation through a positive feedback mechanism; however, more mechanistic studies are warranted in order to support this finding. Our results also show that CX3CR1^{Low} cells reacquire NANOG and potentially OCT4a expression over time. This re-expression of pluripotency genes may be accompanied by an increase in stemness features such as the ability to form tumorspheres, slower proliferation rates and drug resistance, although this remains to be tested with further experiments. Furthermore, the reacquisition of NANOG expression seemed to be impaired by blocking CX3CR1 in prostate cancer cells, suggesting that CX3CR1 signaling may play a role in the regulation of NANOG expression. However, additional replicates for this experiment, as well as further mechanistic studies, are needed to unveil the role of CX3CR1 in regulating pluripotency.

In order to understand the mechanisms by which CX3CR1 may regulate its own expression, it is crucial to elucidate the pathways that regulate the expression of this receptor. In monocytes, the regulation of this receptor depends on different cytokines, including CCL2, which increases CX3CR1 expression through the p38 MAPK pathway, and IL-15 and IL-2, which oppositely regulate CX3CR1 expression by differentially recruiting NFAT1 and NFAT2 to the *CX3CR1* promoter (Barlic et al., 2004; Green et al., 2006). In prostate cancer cells, hypoxia increases CX3CR1 expression by HIF-1 and NF-κB activation (Xiao et al., 2012). Further studies are needed to provide a better insight into the molecular mechanisms that regulate CX3CR1 expression in cancer cells.

Since our studies show that CX3CR1 may be involved in the regulation of pluripotency genes, it is of relevance to investigate the signaling pathways triggered by CX3CR1 activation in cancer. CX3CR1 has been shown to induce activation of several pathways in different cancer types, including PI3K/AKT, AKT/NF-kB, JAK/STAT, and ERK1/2 signaling cascades (Shulby et al., 2004; Tardaguila et al., 2013; Wada et al., 2015; H. Wang et al., 2017; Wei, Cao, Yu, Liu, & Wang, 2015). These pathways activated by CX3CR1 in cancer have also been involved in the regulation of pluripotency genes in cancer stem cells (Matsui, 2016). Thus, our results provide a starting point to investigate the molecular mechanisms by which CX3CR1 expression is associated with a stemness phenotype in cancer.

The preliminary results obtained in this study suggest that CX3CR1 may play a role in the phenotypic plasticity of cancer cells. These results could have important implications if the cell plasticity observed *in vitro* should also be observed *in vivo*. A direct consequence of cellular plasticity and the conversion of non-stem-like cells into stem-like cells would be the rise of chemotherapy resistant cells, which are ultimately responsible for the initiation of metastasis and, thus, patient lethality. Cell plasticity involving the acquisition of stem-like properties has been described in several cancers (Chen, Dong, Haiech, Kilhoffer, & Zeniou, 2016).

Studies have shown that the conversion from a non-CSC state to a CSC state seems to be induced either by microenvironmental cues, such as the infiltration of inflammatory cells, cytokines, hypoxia, low pH, nutrition deprivation, and oxidative stress, or as a consequence of treatment. Thus, depending on the physiological conditions, cancer cells may modify their gene expression pattern to change their phenotypes accordingly (Agliano,

Calvo, & Box, 2017; Chen et al., 2016). Strikingly, our experiments showed that prostate and breast cancer cells experience plasticity *in vitro*, which shows that cancer cells can modify their phenotype in a cell-autonomous way, even in the absence of signals from the tumor microenvironment. Importantly, the risk of cell plasticity must be considered when designing new therapies to treat metastasis.

6. SUMMARY AND FUTURE DIRECTIONS

6.1. Summary

Prostate cancer and breast cancer represent a major health issue in the United States and worldwide. Both cancer types are among the most frequently diagnosed malignancies, leading to thousands of deaths every year. Although there are many treatment options for early/localized disease, which has high survival rate, over 30% of the patients develop metastatic recurrence over time. Unfortunately, metastasis remains incurable, accounting for most cancer-related deaths. Therefore, it is imperative to elucidate the molecular mechanisms underlying metastasis initiation in order to develop novel therapeutic approaches to target this process.

Mounting evidence suggests that a small group of cells within primary tumors have the ability to initiate metastatic lesions. These cells display stemness features, including self-renewal, asymmetric division, slow proliferation rates and chemotherapy and radiotherapy resistance. These characteristics are mediated by the expression of pluripotency transcription factors, including OCT4a and NANOG. Recent studies have demonstrated that certain chemokine receptors are overexpressed in stem-like cancer cells, and that signaling through these receptors leads to the regulation of stemness by activation of different pathways. Together, these studies have revealed a potential for the therapeutic targeting of chemokine receptors and the pathways they activate in order to block the self-renewal capabilities of stem-like cells.

The chemokine receptor CX3CR1 has been previously implicated in tumor progression and metastasis in prostate and breast cancer, as well as in other cancer types. Furthermore, pharmacological targeting of this receptor with our novel small molecule antagonist FX-

68 was able to impair seeding and growth of cancer cells in an animal model of metastasis. Recent studies conducted in our lab suggested for the first time that CX3CR1 is a marker for tumor initiation and its expression is associated with stem-like features in prostate and breast cancer cells *in vitro*. Through the use of flow cytometry, we were able to separate two different subpopulations in prostate and breast cancer cell lines based on the expression levels of CX3CR1, namely $CX3CR1^{High}$ and $CX3CR1^{Low}$. The results of the experiments conducted on these subpopulations showed that $CX3CR1^{High}$ displayed features associated with stem-like cells, as opposed with $CX3CR1^{Low}$ cells, which appeared to lack stemness features. Strikingly, our studies also showed that $CX3CR1^{Low}$ cells reacquire the expression of CX3CR1 and stemness markers at the transcript level when cultured over a period of time. This interesting observation led us to define the aim for this thesis.

The main objectives of this project were to elucidate the role of the chemokine receptor CX3CR1 in the regulation of pluripotency genes and to investigate the phenomenon of phenotypic plasticity in prostate and breast cancer $CX3CR1^{Low}$ cells.

Our studies support the hypothesis that $CX3CR1^{Low}$ cells reacquire CX3CR1 expression over time. This increase in CX3CR1 expression was at least partially blocked by FX-68, suggesting the existence of a positive feedback loop in the regulation of this chemokine receptor. Our results also support the hypothesis that $CX3CR1^{Low}$ cells reacquire NANOG expression over time, a process that seems to be impaired by blocking CX3CR1 in prostate cancer cells, suggesting that CX3CR1 signaling may play a role in the regulation of NANOG expression. Together, our studies suggest the existence of phenotypic plasticity *in vitro* in prostate and breast $CX3CR1^{Low}$ cancer cells and suggest the involvement of CX3CR1 activation in this process.

It is worth noting that the work presented here could not be completed due to unforeseen circumstances caused by the COVID-19 global pandemic, which led to the interruption of research activities at Drexel University for several months.

The results presented in this thesis laid the foundation for future studies that will allow us to better understand the role of CX3CR1 in the regulation of stemness and phenotypic plasticity. Further elucidation of the mechanisms underlying this process should be helpful to develop novel therapies to target metastasis.

6.2. Future Directions

6.2.1. Expanding upon our experiments with CX3CR1^{Low} cells

To expand upon our previous results, we need to obtain more information on the phenotypic plasticity observed in cancer cells. One potential experiment is to assess if CX3CR1^{Low} cells also reacquire stemness features following prolonged culture, such as tumorsphere formation, slow proliferation rates, and drug resistance.

We will also use gene reporter experiments in order to complement our Western blot results and follow the re-expression of CX3CR1 and NANOG using time-lapse video microscopy (Etaluma LS720 Microscope). Using a construct that includes the GFP gene downstream from the promoter for CX3CR1, we can identify the cells with high CX3CR1 expression as the cells with green fluorescence. With this technique, we can follow the re-expression of CX3CR1 more closely in each individual cell. We will also use a construct for NANOG using an m-cherry reporter to follow the re-expression of this protein. This way, we can double-transfect cells and see the expression of both proteins at the same time, and therefore observe the sequence for the re-expression. This experiment can be done in the presence and absence of FX-68, to observe the re-expression pattern when CX3CR1 is blocked.

6.2.2. Investigating if CX3CR1 is responsible for regulating the expression of pluripotency genes and the acquisition of stemness features in cancer cells

In order to definitively conclude whether CX3CR1 participates in the regulation of the pluripotency genes OCT4a and NANOG, we need to conduct further experiments. For instance, CX3CR1^{Low} cancer cells could be exposed to fractalkine in culture to determine whether phenotypic plasticity can be forced to occur more rapidly or to a greater extent.

6.2.3. Elucidating the molecular events that modulate the re-expression of CX3CR1 and pluripotency genes in cancer cells during phenotypic plasticity

To reveal the molecular mechanisms underlying the phenotypic plasticity observed in our experiments, we need to investigate the signaling pathways involved in this process. This can be achieved by incubating CX3CR1^{Low} cells with inhibitors of different oncogenic signaling pathways to reveal if one or more of these pathways regulates CX3CR1 expression. One approach to narrow down the potential pathways involved is to use RNA-seq for transcriptome profiling of both CX3CR1^{High} and CX3CR1^{Low} subpopulations, in order to determine the genes that are upregulated and downregulated in each of these groups. Following this analysis, cell signaling experiments can be performed based on the main pathways affected by CX3CR1 expression.

6.2.4. Determining if the phenotypic plasticity observed *in vitro* also occurs *in vivo*

In addition to mechanistic studies *in vitro*, the completion of an *in vivo* experiment would enable us to observe if the phenotypic plasticity of cancer cells is also observed in these conditions. This can be performed by injecting CX3CR1^{Low} cells into mice, both in the presence and absence of FX-68, and then verifying if these cells are able to initiate tumors.

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