Cell-autonomous and host-dependent CXCR4 signaling in cancer metastasis : insights from a zebrafish xenograft model

Tulotta, C.

Chapter 1

Introduction and thesis outline

The tumor microenvironment

Tumors are in constant interaction with the surrounding microenvironment. The tumor microenvironment consists of stromal cells such as cancer-associated fibroblasts (CAFs), endothelial cells, mesenchymal stem cells (MSCs), tumor-associated macrophages (TAMs) and neutrophils (TANs), adaptive immune cells and extracellular matrix (ECM) [1]. The interaction between cancer and stroma cells results in either tumor promoting or inhibiting effects and the tumor microenvironment differentially contributes to the efficacy of cancer therapies [2]. Tumor cells engage cells from the microenvironment, either educating resident stromal cells or inducing the recruitment of distal ones to further support malignant growth, motility and dissemination. Along with the angiogenic switch, where endothelial cells are educated by malignant cells to form new vasculature to provide oxygen and nutrients, the immunosuppressive switch phenomenon takes place: the polarization from pro-inflammatory to anti-inflammatory neutrophils and macrophages (N1 to N2 and M1 to M2), where the sub-type 2 associates with a tumor-promoting function, links to immunosuppression, characterized by reduced cytotoxic T cell (CTL) and enhanced T regulatory (Treg) and myeloid-derived suppressor (MDSCs) cell infiltration [3]. Interestingly, the cooperation between different subsets of leukocytes and its role in cancer metastases has been recently reported [4]. The plasticity phenomenon in the microenvironment has been described also for fibroblasts, which respond to a neoplastic lesion in a similar fashion as to a never healing wound [3]. The interaction between tumor and the microenvironment is controlled by a plethora of signaling molecules, such as chemokines, and their complex networking in cancer requires further understanding to inhibit tumor development.

CXCR4, cancer and the tumor microenvironment

Chemokines are chemotactic cytokines that guide directional cell migration in development and disease and more than 50 chemokine ligands and 18 chemokine receptors have been described in Homo sapiens [5]. Chemokines are classified into four classes, depending on the presence and position of the conserved cysteine residues (CXC, CC, (X)C and CX3C) at the N-terminus, involved in the formation of disulphide bonds between the first and third or second and fourth cysteines [6] . The chemokines belonging to the CXC subgroup are further classified into angiogenic ELR+ and angiostatic ELR-, whether they are positive or negative for the Glu-Leu-Arg (ELR) motif at the N-terminus [7, 8]. Chemokine ligands can bind multiple chemokine receptors, which possibly work in concert to control signaling activation and inhibition [7].

CXCR4 is a seven-transmembrane, chemokine, G-protein coupled receptor (GPCR). The chemokine CXCL12 binds both CXCR4 and CXCR7 receptors in order to guide a directional and collective migration of cell primordia, during the formation of sensory organs in zebrafish [9-11]. CXCL12 binding to CXCR4 induces the dissociation of the G protein

αβγ trimer and activation of PI3K/AKT/mTOR, MAPK, PKA and PLC/Ca2+ pathways. Moreover, MAPK cascade activation and CXCR4 internalization occur via β-Arrestin, independently from G-proteins (Fig.1A). In addition, CXCR4 can form homodimers, activating the JAK/STAT pathway and Ca2+ release from intracellular storage into the cytoplasm (Fig.1B). CXCR4 can also form heterodimers with CXCR7. Whereas CXCR4 is internalized and degraded after CXCL12 binding, CXCR7 is internalized and recycled to the plasma membrane. Via β-Arrestin, CXCR7 has either CXCL12 scavenging functions or triggers MAPK signaling activation (Fig.1C). CXCL12 signaling via CXCR4 and CXCR7 controls cell chemotaxis and migration as well as cell proliferation and survival [12, 13].

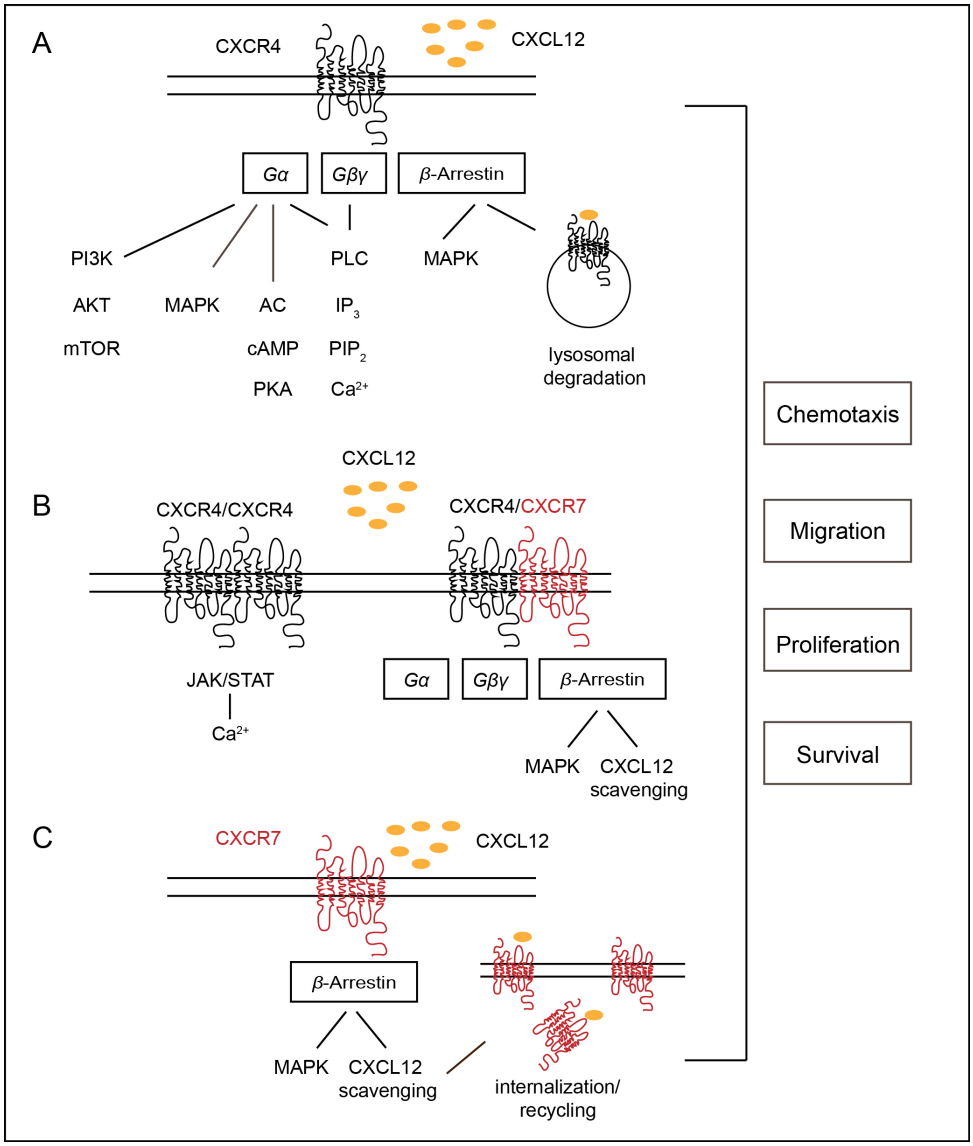

Figure 1. CXCL12-induced signaling via CXCR4 and CXCR7. (A) CXCL12 binds to CXCR4, inducing Gα and Gβγ dissociation and activation of PI3K, MAPK, AC, and PLC signaling pathways. CXCL12 binding to CXCR4 activates β-Arrestin, leading to MAPK signaling pathway activation or receptor internalization. (B) CXCR4 can form homo- and hetero-dimers with CXCR7. (C) CXCL12 binding to CXCR7 induces, via β-Arrestin, MAPK signaling activation, or CXCL12 scavenging functions, through receptor internalization and recycling to the plasma membrane. CXCL12-mediated signaling plays a role in cell chemotaxis, migration, proliferation and survival. PI3K, Phosphatidylinositide 3-kinase; MAPK, mitogen-activated protein kinases; AC, Adenyly cyclase; PLC, Phospholipase C. This scheme is an adaptation from [12, 13].

In cancer, malignant cells acquire higher CXCR4 levels, compared to normal tissues, and are found to preferentially metastasize in organs where CXCL12 is secreted, in line with the "seed and soil" theory [14]. Enhanced CXCR4 signaling has been identified in several malignancies such as gastrointestinal tumors, melanoma, basal cell carcinoma (BCC), head and neck squamous cell carcinoma, lung cancer, breast and ovarian tumors, renal cell carcinoma, prostate cancer, glioblastoma multiforme (GBM), Ewing sarcoma and leukemia. Elevated CXCR4 levels result in increased cell proliferation, dedifferentiation, migration and metastatic spreading of tumor cells, cancer stem cell (CSC) maintenance and it has been associated with the development of tumor resistance towards conventional therapies, leading to poor patient prognosis [15].

CXCR4 is expressed by both cancer cells and surrounding stromal cells (Figure 2). The recruitment of stromal cells expressing CXCR4 can be guided by the secretion of CXCL12 by cancer cells themselves or other stromal cells, such as MSCs and CAFs [16]. Moreover, CXCL12 secreted by CAFs displays effects on tumor cells, enhancing invasive potential [17] and functioning as a protective shield against T cells, boosting immune escaping mechanisms [18]. CXCR4 is involved in leukocyte trafficking, hematopoietic stem progenitor cells homing and neutrophil retention in the bone marrow during homeostasis, inflammation, infection and cancer [12, 19-22]. Infiltration of CXCR4hi neutrophils associates with faster tumor growth and angiogenesis in IFNβ deficient mice, injected with melanoma and fibrosarcoma [23]. CXCR4hi macrophages have been identified in CXCL12-enriched tumor areas after chemotherapies and are suggested to display pro-angiogenic functions that drive tumor-relapse [24]. Moreover, CXCL12 expressing glioblastoma cells induce VEGF production and angiogenesis in microvessel enriched areas with high CXCR4 levels [25]. In addition, CXCR4-expressing peripheral blood monocytes respond to CXCL12-secreting multiple myeloma (MM) tumor cells and acquire M2 associated properties [26]. Finally, the inhibition of CXCR4 signaling by oncolytic virotherapy limits the infiltration of Treg, decreasing immunosuppression [27].

Considering the major and intricate role of this chemokine receptor in cancer, its targeting represents an important pharmacological approach that is currently under development, through the use of CXCR4 antagonists, antibodies and CXCL12 binding agents. Importantly, the role of the stromal CXCR4 signaling needs to be considered in drug treatments that target CXCR4 to inhibit cancer spreading. Limiting cancer spreading by targeting the CXCR4 signaling in the tumor microenvironment is a promising

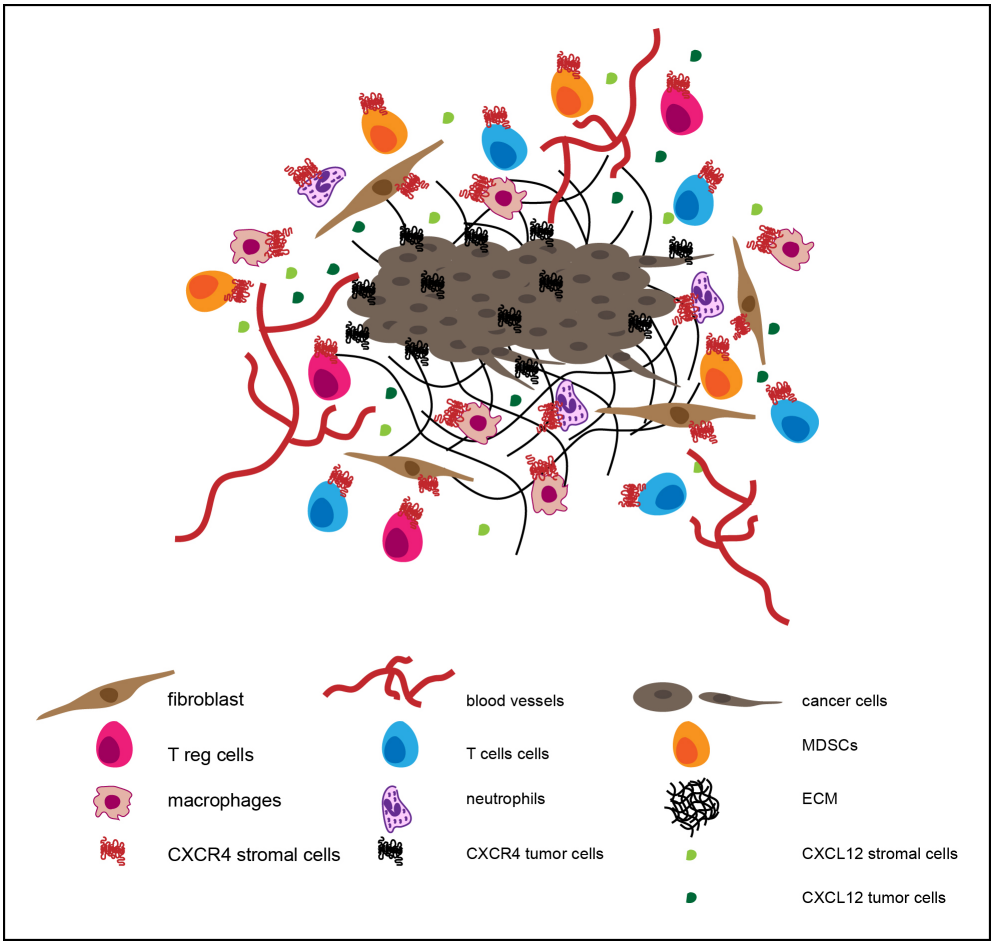

Figure 2. CXCR4 drives the interaction between cancer and stromal cells. The CXCR4-CXCL12 axis signals in a bi-directional fashion. CXCR4 is expressed by both tumor cells and cells that form the surrounding stroma, (fibroblast, T cells, T reg cells, myeloid derived suppressors cells (MDSCs), macrophages and neutrophils), embedded in the extracellular matrix (ECM). The CXCR4 cognate ligand CXCL12 is secreted by both cancer cells and cells in the microenvironment.

approach that requires further investigations to become an alternative therapeutic form of intervention.

Zebrafish xenograft as a model to study cancer

Research performed in pre-clinical *in vivo* models is constantly under development to provide further insights into the communication between tumor and the surrounding microenvironment. Zebrafish is a tropical freshwater teleost, increasingly used to study a range of disease processes [28] as well as being an excellent tool for the study of development. Several important advances in understanding of cancer and inflammation

have arisen from studies in zebrafish [29-31]. The rapid and external development of transparent embryos [32], availability of reporter lines with traceable fluorescent cells [33-35], ease of genetic manipulation [36, 37] and pharmacological approaches [38] make the zebrafish an excellent *in vivo* model to visualize single cell interactions in real time and to uncover the signaling mechanisms involved, on a whole organism level. Human tumor cells engrafted into the blood circulation of 2-day-old zebrafish embryos induce angiogenesis and form micrometastasis sustained by neutrophils and macrophages, nearby hematopoietic sites [39]. Therefore, the zebrafish xenograft model bears the potential to elucidate crucial kinetics and key mechanisms that regulate tumor-microenvironment interaction and ultimately support tumor spreading.

Thesis outline

In this thesis we describe the role of CXCR4 signaling in tumor progression, considering its signature both on cell autonomous and host dependent mechanisms. Specifically, we unravel the role of the chemokine receptor during metastasis initiation *in vivo*, following extravasation events of circulating human cancer cells, engrafted in zebrafish embryos.

In **Chapter 2**, we report recent findings that have contributed to the understanding of cancer angiogenesis and metastasis through the use of the zebrafish embryo as a xenotransplantation model, in line with other *in vitro* and *in vivo* models. We highlight the transparency of the embryo as a clear advantage to image human tumor cells during early metastatic events after leaving the blood circulation and focus on the interaction between fluorescent cancer cells and the zebrafish microenvironment (endothelial and immune cells).

To study human cancer metastasis, our group generated a xenotransplantation model of experimental micrometastasis. In **Chapter 3**, we provide a detailed description of the engraftment procedure of human tumor cells directly into the blood circulation of zebrafish embryos and define tumor phenotype assessment *in vivo*, using imaging techniques at cellular resolution to study tumor burden at micrometastatic sites and interaction with the surrounding innate immune cells.

The characterization of human cancer cell behavior in the zebrafish host is followed by the identification of putative signaling pathways involved in the observed phenotypes. As previously mentioned, we hypothesize that the CXCR4 signaling, both on the tumor cells and the zebrafish host side, is responsible for the ability of cancer cells to initiate early metastatic events, mainly at hematopoietic sites.

In **Chapter 4**, the chemical and genetic inhibition of the cell autonomous CXCR4 signaling impairs triple negative breast cancer (TNBC) early metastatic events in a zebrafish xenograft model of experimental micrometastasis. IT1t, a recently described

CXCR4 antagonist, is used for the first time to inhibit cancer progression, via blocking the interspecies crosstalk between human cells and the zebrafish microenvironment. In this chapter we demonstrate that the xenograft approach in zebrafish is a valuable model to study human tumors as the CXCR4 signaling functions in human cells upon zebrafish CXCL12 stimulation and *vice versa* CXCR4-expressing zebrafish cells respond to the human cognate chemokine.

In **Chapter 5**, we focus on the role of neutrophils and macrophages, expressing high levels of CXCR4, in early metastatic events initiated by human cancer cells in a transient hematopoietic site in zebrafish larvae. Lacking of a functional CXCR4 receptor leads to altered neutrophil motility and development as well as an atypical response towards cancer cells by both innate immune cell populations. Transcriptomic profiles of neutrophils and macrophages lacking a functional CXCR4 receptor confirm basal alterations in cell motility and adhesive properties, independently of cancer cells. We propose that these alterations are responsible for the impaired tumor niche preparation and inhibition of early micrometastasis formation of TNBC and prostate human cancer cells.

In **Chapter 6** we identify MDMX as a negative regulator of CXCR4. In line with previous work performed by our group, P53 stabilization inhibits Ewing sarcoma proliferation *in vitro* and *in vivo*. To stabilize P53 in Ewing sarcoma cells with a wild type form of this tumor suppressor, we target MDMX, a negative regulator of P53, with inducible RNA interference. Whereas inhibiting tumor burden *in vivo*, MDMX interference increases *CXCR4* mRNA levels, which are linked to metastatic Ewing sarcoma. Chemical and genetic inhibition of the cell autonomous CXCR4 signaling impairs Ewing sarcoma early metastatic events *in vivo*. Moreover, the same inhibition of micrometastasis onset is observed in a zebrafish mutant host with a non-functional CXCR4. Therefore, we suggest that an MDMX/CXCR4 combined treatment is required to impair Ewing sarcoma proliferation, limiting at the same time metastatic onset.

In a conclusive **Chapter 7** we summarize our findings on the role of the cell autonomous and host dependent CXCR4 signaling on human tumor progression *in vivo*, using zebrafish xenotransplantation as a model. In the same chapter we frame our findings in the current scientific landscape and discuss future perspectives.

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