Inhibition of signaling between human CXCR4 and zebrafish ligands by the small molecule IT1t impairs the formation of triple-negative breast cancer early metastases in a zebrafish xenograft model

Claudia Tulotta1, Cristina Stefanescu1, Elena Beletkaia2, Jeroen Bussmann1,3, Katsiaryna Tarbashevich4, Thomas Schmidt2 and B. Ewa Snaar-Jagalska1,*

ABSTRACT

Triple-negative breast cancer (TNBC) is a highly aggressive and recurrent type of breast carcinoma that is associated with poor patient prognosis. Because of the limited efficacy of current treatments, new therapeutic strategies need to be developed. The CXCR4-CXCL12 chemokine signaling axis guides cell migration in physiological and pathological processes, including breast cancer metastasis. Although targeted therapies to inhibit the CXCR4-CXCL12 axis are under clinical experimentation, still no effective therapeutic approaches have been established to block CXCR4 in TNBC. To unravel the role of the CXCR4-CXCL12 axis in the formation of TNBC early metastases, we used the zebrafish xenograft model. Importantly, we demonstrate that cross-communication between the zebrafish and human ligands and receptors takes place and human tumor cells expressing CXCR4 initiate early metastatic events by sensing zebrafish cognate ligands at the metastatic site. Taking advantage of the conserved intercommunication between human tumor cells and the zebrafish host, we blocked TNBC early metastatic events by chemical and genetic inhibition of CXCR4 signaling. We used IT1t, a potent CXCR4 antagonist, and show for the first time its promising anti-tumor effects. In conclusion, we confirm the validity of the zebrafish as a xenotransplantation model and propose a pharmacological approach to target CXCR4 in TNBC.

KEY WORDS: CXCR4, CXCL12, IT1t, Triple-negative breast cancer, Metastases, Inter-species crosstalk, Xenograft, Zebrafish

INTRODUCTION

CXCR4 is a chemokine receptor, first described in the early 1990s (Baggioolini et al., 1997; Herzog et al., 1993; Jazin et al., 1993; Nomura et al., 1993) and identified as a co-receptor for human immunodeficiency virus (HIV) entry (Feng et al., 1996). It is a seven-transmembrane G-protein-coupled receptor with a major role in physiological processes such as hematopoiesis (Nagasawa et al., 1996; Rosu-Myles et al., 2000), leukocyte trafficking (Day and Link, 2012; Sallusto and Bagnoli, 2008; Sallusto et al., 2000), cell migration and organogenesis during ontogeny (Bussmann and Raz, 2015), as well as pathological conditions like HIV pathogenesis (Vicenzi et al., 2013), WHIM syndrome (warts, hypogammaglobulinemia, infections and myelokathexis syndrome) (Gulino, 2003) and cancer (Balkwill, 2004; Chatterjee et al., 2014). Its cognate ligand is the homeostatic cytokine CXCL12 (Bleuel et al., 1996; Oberlin et al., 1996) [also known as stromal cell-derived factor 1 (SDF-1)]. However, recent reports indicate that ubiquitin and macrophase migration inhibitory factor (MIF) can also bind to and signal through CXCR4 (Saini et al., 2010; Bernhagen et al., 2007; Saini et al., 2011; Pawig et al., 2015). Upon CXCL12 binding, CXCR4 triggers cell migration, proliferation and transcriptional control of downstream targets via G-protein-dependent or -independent mechanisms (Pawig et al., 2015). Dissociation of Gαi and Giβγ subunits leads to Ca2+ release and activation of the PI3K/AKT and MAPK signaling pathways (Teicher and Fricker, 2010). CXCR4 dimerization occurs after ligand binding; subsequently, phosphorylation by JAK kinases takes place, followed by STAT signaling initiation in a G-protein-independent mechanism (Mellado et al., 2001; Vila-Coro et al., 1999). Moreover, initiation of β-arrestin signaling can take place, resulting in G-protein coupled receptor (GPCR) signaling blockade (Lefkowitz, 1998; Shukla et al., 2011) or ERK1/2 activation (Luttrel et al., 1999). CXCR4 activity is regulated by mechanisms of desensitization, through phosphorylation of the C-terminus and internalization, which is followed by degradation or recycling to the plasma membrane (Busillo and Benovic, 2007). Moreover, CXCL12 binds also to CXCR7 (Balabanian et al., 2005). However, differently from other chemokine receptors, CXCR7 does not signal through G proteins and acts as a ligand scavenger in a β-arrestin-mediated pathway (Rajagopal et al., 2010). Interestingly, a key role of the CXCL12-CXCR4-CXCR7 axis in collective tissue migration has been studied in zebrafish embryos. In the migration of the lateral line primordium, Cxc12 scavenging by Cxcr7 leads to the formation of a self-generated gradient and cell migration after Cxcr4 activation, along tissues where Cxc12 is homogeneously distributed (Boldajipour et al., 2008; Dona et al., 2013; Venkiteswaran et al., 2013).

A link between CXCR4 and cancer, in particular metastatic breast cancer, has been reported (Muller et al., 2001). CXCR4-expressing tumor cells preferentially colonize distant organs that secrete high levels of CXCL12, such as brain, lungs, lymph nodes, liver and bone marrow (Janowski, 2009). Among highly aggressive malignancies of the breast, triple-negative breast cancer (TNBC) is often associated with relapse and poor patient prognosis (Palma et al., 2013; Venkiteswaran et al., 2013).
Increased metastatic behavior in a zebrafish

We first characterized the expression profile of TNBC cells display high proliferation rate than the parental line (data not shown). To propose a potential treatment to impair CXCR4 signaling and effect inhibition of early metastatic events (He et al., 2012). Highly aggressive cancer cells, adhering to the intravascular endothelium, initiated early metastatic events in the tail, sustaining tumor progression until 4 days post-implantation (dpi). In our model, in which tumor cells are inoculated directly into the blood circulation to study the formation of experimental micrometastases, bypassing initial modifications in a primary tumor mass, early metastatic events coincided with tumor foci formation and expansion, tumor extravasation, with adherence to the extravascular endothelium, and invasion. In line with previous work from our group, the tail fin region, in proximity of the CHT, a temporary site of hematopoiesis analogous to the fetal liver in mammalian development, was a preferential early metastatic site (He et al., 2012).

After reaching the vascular plexus that infiltrates the CHT, MDA-MB-231 and MDA-MB-231-B displayed divergent phenotypes. From 1 until 4 dpi, the parental line MDA-MB-231 showed a weakened behavior over time (Fig. 1K-M′), whereas no invading cells were found in the tail fin at 4 dpi (16%). Invasive events were detected at 2 dpi (4.5%), and at 4 dpi (47%). In conclusion, formation of a compact tumor structure, cancer cell extravasation, and invasion of the CHT and tail fin tissues increased over time in MDA-MB-231-B and decreased in MDA-MB-231. Taken together, our data show that the TNBC cell line MDA-MB-231-B displays high CXCR4 expression levels and CXCR4 antagonism has been introduced in clinical trials to treat different tumor types, mainly leukemia and lymphomas (Ramsey and McAlpine, 2013). However, it has been reported to cause cardiotoxicity (Hendrix et al., 2004). AMD3100 also functions as an agonist for CXCR7 (Kalatskaya et al., 2009), which has been linked to breast cancer cell proliferation (Salazar et al., 2014). In addition, an anti-CXCR4 antibody is currently in clinical trials (Kuhne et al., 2013; Vela et al., 2015). More CXCR4 antagonists have been developed and in vitro as well as animal models are required to further explore clinical applications in patients.

Zebrafish is increasingly being used as an animal model for translational research in oncology (Amatruda et al., 2002; Barriuso et al., 2015; Ghotra et al., 2015). In particular, transparent zebrafish embryos allow following the behavior of fluorescent tumor cells in a living organism. Human cancer cells engrafted in the blood circulation of 2-day-old transgenic embryos, with fluorescently traceable endothelial (Lawson and Weinstein, 2002) and immune cells (Ellett et al., 2011; Renshaw et al., 2006), have been described to induce angiogenesis and form micrometastases in concert with immune cell interaction (He et al., 2012). Tumor angiogenesis and colonization of secondary tissues can be visualized in a short time period (2-6 days) in the small and fast-developing larvae. Although numerous discoveries have been made using zebrafish embryos as a xenotransplantation model, lack of knowledge about the communication between human and zebrafish cells has questioned its validity and partially limited its use.

Here, we report that the CXCR4-CXCL12 axis acts across zebrafish and humans and drives the formation of tumor micrometastases of human TNBC cells in zebrafish. Cell treatment with IT1t, a potent CXCR4 antagonist, and genetic silencing of CXCR4 effectively inhibited early metastatic events in vivo. Therefore, using zebrafish as a xenotransplantation model, we proposed a potential treatment to impair CXCR4 signaling and reduce the metastatic onset of TNBC.

**RESULTS**

TNBC cells display high CXCR4 expression levels and increased metastatic behavior in a zebrafish xenotransplantation model

We first characterized the expression profile of CXCR4 and CXCR7, both chemokine receptors for CXCL12, in the TNBC line MDA-MB-231-B. Because this cell line derives from bone metastases of MDA-MB-231, after repeated engraftments into a murine host (Wetterwald et al., 2002), we used the parental line as a reference. We found that, compared to the original TNBC line, derived from human pleural metastases, the bone clone expressed higher CXCR4 and lower CXCR7 mRNA levels (Fig. 1A,B). Moreover, when compared in vitro, MDA-MB-231-B displayed a higher proliferation rate than the parental line (data not shown). To determine whether TNBC cells with increased CXCR4 expression displayed a different behavior, we engrafted both MDA-MB-231 and MDA-MB-231-B in zebrafish. As previously reported (He et al., 2012), tumor cells were inoculated in the blood circulation of 2-days-post-fertilization (dpf) embryos via the duct of Cuvier, a vein plexus that opens into the heart (Fig. 1C,C′). Fluorescent tumor cells derived from both cell lines entered the blood vessels and, at 5 hours post-injection (hpi), they were mainly found in the tail and trunk vessels of the Tg(kdr:EGFP)463 zebrafish reporter line with green fluorescent vasculature (Fig. 1D,E). Injected embryos were examined by microscopy and embryos with 25-50 tumor cells hematogenously disseminating into the dorsal aorta (DA), caudal vein (CV) and vessel branches of the caudal hematopoietic tissue (CHT), in the region between the urogenital opening and the end of the tail, were selected for the experiment. Tumor cells spread through the embryo via blood circulation of the head, trunk and tail. Intravascular and perivascular cancer cells were found in the basilar artery (BA), branchial arches (BAs) and optic vessels in the head region (Fig. 1F-H), and in intersegmental vessels (ISVs), dorsal longitudinal anastomotic vessels (DLAVs) and the DA and CV in both the trunk and tail areas (Fig. 1I,J). Moreover, tumor cells were often positioned near vessel branching points (Fig. 1I), as to follow a path in a similar fashion to nascent lymphatic vessels, known to express cxcr4a/b receptors (Cha et al., 2012). Interestingly, cxcl12a and cxcl12b are expressed at these sites in developing zebrafish embryos (Cha et al., 2012; Fujita et al., 2011; Hess and Boehm, 2012). Highly aggressive cancer cells, adhering to the intravascular endothelium, initiated early metastatic events in the tail, sustaining tumor progression until 4 days post-implantation (dpi). In our model, in which tumor cells are inoculated directly into the blood circulation to study the formation of experimental micrometastases, bypassing initial modifications in a primary tumor mass, early metastatic events coincided with tumor foci formation and expansion, tumor extravasation, with adherence to the extravascular endothelium, and invasion. In line with previous work from our group, the tail fin region, in proximity of the CHT, a temporary site of hematopoiesis analogous to the fetal liver in mammalian development, was a preferential early metastatic site (He et al., 2012).
Fig. 1. CXCR4 expression levels correlate with metastatic potential in a zebrafish xenotransplantation model. The bone clone (MDA-MB-231-B) expressed higher levels of CXCR4 mRNA (A) and lower levels of CXCR7 mRNA (B), compared to the parental cell line MDA-MB-231, originated from metastatic triple-negative breast cancer (TNBC) \( \text{unpaired } t \)-test, (A) \( \text{** } P=0.0016 \), (B) \( \text{** } P=0.0019 \). Upon engraftment into the duct of Cuvier (C, C’ red arrow) of 2-dpf zebrafish embryos (C’), MDA-MB-231 circulated in the vascular system (D), in a comparable manner to MDA-MB-231-B (E). Arrowhead in C represents the site of injection. CHT, caudal hematopoietic tissue. (F-J) Tumor cells disseminated throughout the embryo, in the head (F-H), the eye (H), the trunk and the tail (I,J), and extended filopodia at vessel branching points (I, arrowhead). BA, basilar artery; BAs, branchial arches; CV, caudal vein; DA, dorsal aorta; DLAV, dorsal longitudinal anastomotic vessel; ISV, intersegmental vessel. (K-P’) Over time, a weaker phenotype was detectable for the MDA-MB-231 cell line (K-M,K’-M’), whereas evident secondary tumor mass formation, extravasation and tail fin invasion persisted when MDA-MB-231-B cells were implanted (N-P,N’-P’). Arrows in O’ and P’ indicate invasive cancer cells that are not in contact with the endothelium and are found in the tail fin tissue, after extravasation. Images were acquired using a Leica TCS SPE confocal microscope with an HC PL FLUOTAR 10× DRY objective (0.30 N.A.) in panel C and with an HC APO 20× DRY objective (0.7 N.A.) in panel H. All other images were acquired using a Leica MZ16FA fluorescent microscope coupled to a DFC420C camera. Scale bars: 50 µm.

Phenotype assessment was carried out at 1, 2 and 4 dpi, evaluating the ability of both cell lines to form a secondary tumor mass, to extravasate and to invade the surrounding tail fin. Images are representative of embryos injected with MDA-MB-231 and number of individuals was 51 (5 hpi) (D), 45 (1 dpi) (K), 44 (2 dpi) (L) and 25 (4 dpi) (M) or with MDA-MB-231-B and number of individuals was 44 (5 hpi) (E), 42 (1 dpi) (N), 36 (2 dpi) (O) and 34 (4 dpi) (P). Percentages relative to tumor mass (TM), tumor extravasation (TE) and tumor invasion (TI) are reported for each stage, for both MDA-MB-231 and MDA-MB-231-B cell lines.
expression and enhanced metastatic behavior in the zebrafish embryo.

The CXCR4-CXCL12 signaling axis is cross-activated in zebrafish and humans

Cancer cells expressing CXCR4 form distant metastases in secondary organs that produce high levels of CXCL12, in human specimens and murine models (Muller et al., 2001). Our initial findings showed that TNBC cells initiating early metastatic events in the zebrafish xenotransplantation model express high levels of CXCR4. To establish whether CXCR4 sustained tumor metastatic properties in a Cxcl12-dependent manner, we first assessed whether the CXCR4-CXCL12 axis acts across zebrafish and human. Two cxcl12 genes, cxcl12a and cxcl12b, are found in zebrafish, following duplication events during teleost evolution. In a multiple alignment (Fig. 2A), human CXCL12 (α-isoform) shared 47.73% identical residues with both zebrafish Cxcl12a and Cxcl12b, whereas 75.26% was the percentage of identity between zebrafish Cxcl12 paralogs. Pair-wise sequence alignment showed 59% identity on residues involved in receptor binding, when the...
human ligand was compared to each zebrafish homolog, and 92.85% between Cxcl12a and Cxcl12b. Full identity in the motif involved in receptor activation was found (Fig. 2B). Therefore, considering the level of conservation, we verify that the CXCR4 signaling on human tumor cells is activated by both zebrafish Cxcl12 ligands. For this purpose, Ca\textsuperscript{2+} flux was measured. Whereas human CXCL12 (100 nM) failed to induce Ca\textsuperscript{2+} mobilization from intracellular storage into the cytoplasm in the parental line MDA-MB-231 (Fig. S1), time-lapse microscopy revealed that calcium sensor fluorescent signal intensity increased when MDA-MB-231-B cells were stimulated with either the human or the zebrafish ligands. In the bone clone, human CXCL12 elicited a response that increased and decreased rapidly. Zebrafish Cxcl12a and Cxcl12b triggered a slower but still significant response, in a non-synchronized fashion. Therefore, considering the level of conservation, we verify that the CXCR4 signaling on human tumor cells is activated by both zebrafish Cxcl12 ligands.

Next, we investigated whether human CXCL12 activates zebrafish Cxcr4. As for the Cxcl12 ligands, two Cxcr4 receptors have been described in zebrafish, Cxcr4a and Cxcr4b. ClustalW alignments of the human CXCR4 with the zebrafish Cxcr4a and Cxcr4b (Fig. 3A) showed a percentage of identity equal to 68 and 63.22 on whole sequence, respectively (Fig. 3B). When ligand-binding regions were considered, the pair-wise identity reached 48.6% (CXCR4-Cxcr4a and CXCR4-Cxcr4b), whereas, in the dimerization regions, 69.4% (CXCR4-Cxcr4a) and 55.5% (CXCR4-Cxcr4b) of the residues were identical. Moreover, the signaling motif was 100% conserved (Fig. 3B). In addition, the zebrafish Cxcr4 paralogs displayed 74.15%, 60%, 66.6% and 100% identity at the whole-sequence level, ligand-binding and receptor-dimerization regions, and signaling motif, respectively (Fig. 3B). Besides partial redundancy, Cxcl12 and Cxcr4 zebrafish paralogs seemed to play distinct functions and to have a different spatial expression during embryo development, as reviewed (Bussmann and Raz, 2015). In particular, ccr4b is found to be expressed by myeloid cells (Walters et al., 2010). To verify whether inter-species crosstalk exists between human ligands and zebrafish receptors, human recombinant CXCL12 (0.4 mg/ml) was injected in the hindbrain ventricle (HBV) of 30-32 hours post-fertilization

**Table 1.** Comparison of human and zebrafish CXCR4 sequences.

| Human CXCR4 | Zebrafish Cxcr4a | Zebrafish Cxcr4b |
|-------------|------------------|------------------|
| Percent identity | 68% | 63.22% |
| Ligand binding region | 48.6% | 48.8% |
| Dimerization region | 60% | 69.4% |
| Signaling motif | 66.6% | 55.5% |

**Fig. 3.** CXCR4 alignment shows similarity between human and zebrafish proteins. Human CXCR4 was aligned to the zebrafish homologs using ClustalW (A). Asterisks (*) represent fully conserved residues, colons (:) and periods (.) indicate positions at which residues share strong or weak similarity, respectively. The tyrosine (Y) in brown belongs to both the signaling motif and dimerization region. Amino acid residues were conserved in the ligand-binding region, and dimerization and signaling motifs in the CXCR4/Cxcr4 receptors (B).
Embryos, where macrophages are fluorescently labeled (Fig. 4A). A 57% increase in the number of cells that migrated to the site of injection was observed compared to the mock-injected group, and in a similar fashion to the zebrafish chemokine Cxcl11aa, previously shown to be a chemoattractant for this class of phagocytes (Torraca et al., 2015) (Fig. 4B,C). Furthermore, macrophage motility towards the human CXCL12 (α-isoform) was found to be Cxcr4-dependent. Macrophages did not respond to the human CXCL12 in the ody mutant line, with a non-functional Cxcr4b receptor. Injection of the human ligand in the HBV led to a 43% increase in macrophage number compared to the water-injected group, in the wild-type (wt) siblings, whereas no differences were detected in the CXCL12- and mock-injected groups when compared to the mock-injected group in wild-type (wt) siblings (D,E). Moreover, we excluded a possible Cxcr4b-dependent alteration of basal motility and total macrophage number in ody mutants compared to wild-type siblings (Fig. S2). In conclusion, zebrafish is a valuable in vivo model to study human cancers, particularly focusing on the interaction between cancer and host stromal cells, because human attractants trigger zebrafish cell migration and zebrafish ligands are sensed by human cells.

**Zebrafish Cxcl12-sensing by human CXCR4 receptor sustains TNBC cancer burden in zebrafish larvae**

The tumor microenvironment plays a crucial role in the establishment of a favorable niche for the onset of cancer metastasis, and the CXCR4-CXCL12 axis, among other signaling pathways, guides the communication between tumor and microenvironment. We showed that crosstalk between human...
and zebrafish receptors and ligands (CXCR4/Cxcl12 and Cxcr4/CXCL12) occurs in vitro and in the zebrafish embryo model. Hence, we investigated whether CXCR4-expressing TNBC cells initiated early metastatic events in vivo after sensing zebrafish Cxcl12 ligands. MDA-MB-231-B cells were engrafted in the blood circulation of zebrafish embryos carrying null mutations in cxcl12a or cxcl12b. MDA-MB-231-B showed reduced localization in the head (BA and BAs) and trunk (ISVs) in larvae with a non-functional Cxcl12a (Fig. S3). However, tumor invasion developed similarly in cxcl12a−/−/cxcl12b−/− larvae and wt siblings at 4 dpi (Fig. 5A). The same effect was observed in the cxcl12a−/−/cxcl12b+/− and cxcl12a+/−/cxcl12b−/− siblings at 4 dpi (Fig. S4A). Like tumor invasion, also the overall micrometastasis burden in the tail fin was the same in cxcl12a−/−/cxcl12b+/− and cxcl12a+/−/cxcl12b−/− siblings at 2 dpi (Fig. S4B,C). However, the response of tumor cells in the Ca2+ assay to both zebrafish Cxcl12a and Cxcl12b supports the hypothesis that human tumor cells sense the Cxcl12a ligand in a cxcl12b mutant and the Cxcl12b ligand in a cxcl12a mutant. Therefore, in this scenario, tumor invasion and tumor burden could still occur in each single-ligand mutant line. Hence, the xenogeneic implantation was performed in the cxcl12a−/−/cxcl12b−/−/Tg(kdrl:EGFP)s843 double-mutant embryos. For this purpose, the cxcl12a−/−/cxcl12b+/−/Tg(kdrl:EGFP)s843 family was in-crossed and tumor engraftments were performed in the siblings of the F1 generation (experimental groups were blinded). Tumor burden and tumor invasion were significantly decreased in the double mutants compared to cxcl12a−/−/cxcl12b+/− siblings at 4 dpi (Fig. 5B,C and D,E, top panels). Mutant larvae for both ligands were distinguished from the siblings by screening for abnormal formation of the hypobranchial arteries at 6 dpf (Fig. 5D,E, bottom panels). Therefore, we suggest that the CXCR4-CXCL12 axis functions in a paracrine fashion across species and is responsible for driving the formation of TNBC micrometastases in zebrafish. Importantly, a potential role of the human CXCL12

Fig. 5. CXCR4-expressing TNBC cells fail to initiate metastatic events in cxcl12a- and cxcl12b-null zebrafish mutants. (A) No differences in tumor cell invasion were found in the medusa (cxcl12a−/−) mutants, compared to wild-type (wt) siblings. (B,C) Breast cancer cells failed to form micrometastases in 4-dpi zebrafish embryos deficient for both cxcl12a and cxcl12b ligands, whereas tumor invasion (B) and tumor burden (C) occurred in the cxcl12a−/−/cxcl12b−/− and cxcl12a−/−/cxcl12b−/− siblings. Unpaired t-test: (A) ns, P>0.05 (wt: n=73; medusa: n=66), (B) **P=0.0012 and (C) **P=0.0033 (n=11 in each group). Graphs in A-C are cumulative of two independent experiments. (D,E) Top panels: MDA-MB-231-B breast cancer cells were highly invasive in the siblings, whereas few tumor cells remained in the metastatic region in zebrafish that were mutant for both ligands. Images were acquired using a Leica MZ16FA fluorescent microscope coupled to a DFC420C camera. Bottom panels: vessel connections are compared to distinguish siblings and double mutants: the hypobranchial arteries (HA), indicated by asterisks, failed to connect to the mandibular arch (AA1) in the cxcl12a−/−/cxcl12b−/− larvae. Images were acquired using a Leica TCS SPE confocal microscope with an HC PL FLUOTAR 10× DRY objective (0.30 N.A.). Scale bars: 50 µm. (F) CXCL12 expression is lower in the MDA-MB-231-B compared to MCF-7 breast cancer cell line. Unpaired t-test, with Welch's correction. **P=0.006. qPCR was performed on two biological replicates.
autocrine loop in driving the formation of TNBC micrometastases in vivo is unlikely. CXCL12 expression levels were undetectable in the parental line MDA-MB-231 (data not shown) and significantly lower in MDA-MB-231-B compared to the Luminal A (ER+, PR+/−, Her2−) MCF-7 breast cancer cell line (Fig. 5F). Accordingly, a very low expression of CXCL12 in MDA-MB-231-B argued against the generation of a potential autocrine loop to activate the receptor CXCR4. Taken together,
the onset of early metastatic events in this experimental system is enhanced by the CXCR4-CXCL12 axis in a zebrafish xenotransplantation model in which human tumor cells respond to zebrafish ligands.

The CXCR4 antagonist IT1t reduces the formation of TNBC early metastases in vivo

The use of CXCR4 antagonists as a therapeutic targeted approach to inhibit tumor spreading and the formation of metastases has been introduced in clinical trials for different cancer types. Despite the fact that CXCR4 is highly involved in the establishment of secondary neoplasias, there are no approved FDA drugs to block CXCR4 in TNBC. In the zebrafish xenograft model in which CXCR4-Cxcl12 inter-species communication supports TNBC early metastasis onset, we test whether the isothiourea derivative IT1t, a recently described CXCR4 antagonist (Thoma et al., 2008), displays anti-neoplastic functions. This small molecule is an orthosteric competitor of the CXCL12 N-terminal signaling peptide and it impairs signaling activation by interfering with the docking of the ligand domain to the receptor (Wu et al., 2010).

MDA-MB-231-B cells were treated in vitro for 24 h and subsequently engrafted in zebrafish embryos. Cells proliferated in treated (20 μM) and untreated conditions (Fig. 6A). Cell survival was not significantly changed: the percentage of live cells was found to be comparable in both groups (97% DMSO, 92% IT1t) with a 39.5% and 60% reduction at 2 and 4 dpi, respectively (Fig. 6D-G). An increase in tumor burden was found from 2 to 4 dpi with a 39.5% and 60% reduction at 2 and 4 dpi, respectively (Fig. 6D-G). CXCR4 chemical inhibition affected tumor burden, and tumor burden assessed at the metastatic site at 2 and 4 dpi decreased in sh#1 and sh#2 compared to scrambled control shRNA, showing a knockdown efficiency of 66% and 80%, respectively (Fig. 7A). Subsequently, xenograft experiments were performed. Tumor cell invasion at the metastatic site was effectively reduced upon CXCR4 silencing (Fig. 7B), similar to the antagonist IT1t (Fig. 7C). Therefore, using chemical and genetic approaches, we demonstrate that CXCR4 silencing inhibition reduces the formation of TNBC early metastases in vivo and describe IT1t as a potential therapeutic for metastatic TNBC.

DISCUSSION

Metastatic TNBC is a major challenge for biopharmaceutical and clinical research because tumor relapse and cell spreading represent the main cause of death for patients. The development of targeted therapies, in combination with conventional chemotherapy, is an important approach to prolong patient lifetime. Although steps forward in elucidating cancer dissemination have been made, generally the pathogenesis of metastases is not fully understood. Monitoring single tumor cells while crossing the blood vessel boundaries in vivo is an optimal scenario to unravel early metastatic events. For this purpose, zebrafish is an advantageous model. The transparency of the embryos and the use of reporter lines make the zebrafish an excellent host to study human tumor cell growth and invasion at early stages (Movie 4). In the last decade the zebrafish xenotransplantation model has been used to study human tumor progression (Konantz et al., 2012) and to discover potential treatments (van der Ent et al., 2014a,b; Veinotte et al., 2014; Zoni et al., 2015). However, the translational validity of a zebrafish xenotransplantation approach has been questioned. Concerns have emerged on possible inter-species crosstalk and lack of species-specific environmental cues. Here, we show that the cross communication between human tumor cells and zebrafish ligands is maintained, because zebrafish Cxcl12 activates human CXCR4 signaling in vitro and supports the formation of TNBC early metastases in vivo. We found that TNBC cells with high CXCR4 expression levels exhibit aggressive features in zebrafish, in agreement with findings in patients and other models. In addition to CXCR4 and CXCR7 mRNA levels, other differences in gene expression between MDA-MB-231-B and MDA-MB-231 might be present and influence tumor cell behavior. Notably, we proved that CXCR4-linked tumor burden occurred in a zebrafish Cxcl12-dependent manner. The involvement of the human CXCL12 ligand...
is questioned owing to very low expression levels, in line with the evidence that CXCL12 expression is higher in non-metastatic breast tumors, compared to metastatic ones (Zhao et al., 2014). However, CXCR4 activation by autocrine mechanisms cannot be fully excluded and CXCL12 knockdown is required to completely rule out this possibility. Moreover, Cxcr4-expressing macrophages migrate towards human CXCL12, demonstrating that the intercommunication takes place in both directions and confirming the validity of the zebrafish embryo model to study human tumors.

Using the zebrafish xenograft model, we observed that TNBC cells make contact with the endothelium, after inoculation in the blood circulation. Then, tumor-endothelium interaction favors tumor mass formation and, consequently, tumor extravasation and invasion. Interestingly, the invasive process frequently recurs when tumor mass formation and growth are observed at earlier stages, whereas a minimized or absent invasion takes place if no tumor mass is present. This scenario is in accordance with the hypothesis, proposed by Ewing in 1929 (Ewing, 1929), that there is a link between metastasis and organization of the vascular system, stressing the mechanical nature of cancer homing to different sites in the body. At the same time, we could not observe tumor aggregates and invasion phenomena in other tissues along the trunk that are also perfused by the DA and the CV in the zebrafish larvae. The aggressive tumor phenotype occurred mainly in the CHT, a site of hematopoiesis in zebrafish larvae. This is in line with the frequent presence of TNBC metastases in the bone marrow of adult mammals (Shi et al., 2014) and correlates with Cxcl12 ligand expression in zebrafish. In the zebrafish tail region, ccl12a is generally expressed in the CHT (Walters et al., 2010), and expression of ccl12a and ccl12b is normally found in the endothelium of the CV and DA, respectively (Cha et al., 2012). Perhaps both Cxcl12a and Cxcl12b work in concert in sustaining tumor adhesion to the endothelium and consequent tumor burden. Using the zebrafish model, we propose that receptor activation via ligand stimulation and not necessarily in response to a Cxcl12 gradient enhances tumor burden and subsequent invasion. This observation is in line with the ‘seed and soil’ theory proposed by Paget in 1889 (Paget, 1989): tumor cells, the ‘seeds’, form a secondary mass in a growth-supportive microenvironment or ‘fertile soil’. Moreover, the preferential growth and invasion in the CHT partially explains why the bone clone and not the parental line MDA-MB-231, more commonly used in other animal models, showed aggressive features in the zebrafish embryo. In conclusion, our data are in agreement with previous theories (Fidler, 2003) that both mechanical and microenvironment-related factors contribute to tumor mass formation and cell invasion to ultimately initiate the metastatic process.

Attempts in the clinic have been made to pharmacologically interfere with CXCR4-CXCL12 signaling. AMD3100 (plerixafor), the most commonly used drug to inhibit the receptor CXCR4, is currently in clinical trials for glioma, leukemia, Ewing sarcoma, neuroblastoma and brain tumors and is already FDA-approved for currently in clinical trials for glioma, leukemia, Ewing sarcoma, the most commonly used drug to inhibit the receptor CXCR4, is used to interfere with CXCR4-CXCL12 signaling. AMD3100 (plerixafor), (medusa) (Valentin et al., 2007), ccl12b (Bussmann et al., 2011) and ccr4b (Bussmann et al., 2011) mutants were identified, before raising, for incomplete migration of the lateral line primordium at the larval stage. Adult fins were clipped and DNA extraction for genotyping was performed. Genotype identification was carried out using a KASP assay. The following primers were used: A1 (reverse) 5'-TCGCTGGTTAC- TGGGAAGGCAC-3', A2 (reverse) 5'-CTGTTGGTACTGTGAGAC- GCCAT-3' and C1 (forward) 5'-AGCAAAGGCCATACGCTGTA-3' for ccl12a; A1 (forward) 5'-GTGCTGGTTCGCTCCACG-3' and C2 (reverse) 5'-AAC- TTGATCGATGTTCCGTTT-3' for ccl12b; and A1 (reverse) 5'- TACGTTGTCTCATTGCGTCTT-3', A2 (reverse) 5'-TGACGGTGGTT- CTTCACTGCTCA-3' and C1 (forward) 5'-CAAGAATCCTGAGAC- TGACTCTA-3' for ccr4b. KASP assay results were confirmed by sequencing, using the following primers: 5'-AGGAGTGTGCTCGGTTT- TAC-3' (forward) and 5'-TGTTGTTGTCTGACTAAAGC-3' (reverse) for ccl12a; and 5'-AGAACCCATACGTGCTGGGAGAGG-3' (forward) and 5'-TGCGCCCTTGGTTGCTGTAACCTG-3' (reverse) for ccl12b. Primers for ccl12b (Bussmann et al., 2011) and ccr4b (Miyasaka et al., 2007) identification were previously described and used for sequencing. For experiments, ccl12a(+/+)/ccl12a(−/−)/tg(kdrl:EGFP)and ccr4b(+/+)/ccr4b(−/−) were considered as control groups.

Cell culture

The breast cancer cell line MDA-MB-231 [American Type Culture Collection] derived bone clone MDA-MB-231-B and MCF-7 were cultured in DMEM complemented with 10% fetal calf serum (FCS) and grown at 37°C and 5% CO2. pLenti-tdtomato plasmid was introduced via lentiviral transduction in MDA-MB-231, whereas MDA-MB-231-B cells stably expressed dsRed fluorescent protein. Blasticidin and G418 were used to select cell clones that were tdtomato- or dsRed-positive, respectively.

**MATERIALS AND METHODS**

**Zebrafish husbandry**

Zebrafish lines were maintained according to standard protocols, described in zfin.org, and handled in accordance with the Dutch animal welfare regulations and the EU Animal Protection Directive 2010/63/EU.

**Zebrafish lines**

In the present study, the reporter lines Tg(kdr1:EGFP) and Tg(mpeg1-mCherry) were used to monitor tumor cell behavior, macrophage recruitment and motility, respectively. Mutant lines used were ccl12a(+/+) (medusa) (Valentin et al., 2007), ccl12b(+/+) (Bussmann et al., 2011) and ccr4b(+/+) (Bussmann et al., 2011) and ccr4b(−/−) (Bussmann et al., 2011) and ccr4b(−/−) (Bussmann et al., 2011) and ccr4b(−/−) (Bussmann et al., 2011) and ccr4b(−/−) (Bussmann et al., 2011) and ccr4b(−/−) (Bussmann et al., 2011) and ccr4b(−/−) (Bussmann et al., 2011) and ccr4b(−/−) (Bussmann et al., 2011).
Cell lines were regularly tested for mycoplasma with the Universal Mycoplasma Detection Kit (30-1012k, ATCC).

**Proliferation assay**
MDA-231-B tumor cells were treated with increasing concentrations (5, 10 and 20 μM) of CXCR4 antagonist ITT1 (239821, Calbiochem) to assess cell growth. A total of 30,000 cells were seeded in a single well of a 96-well plate. The following day, inhibitor treatment was carried on for 24 h. WST-1 tetrazolium salt (05015944001, Roche) was added into the cell medium and absorbance of the reduced by-product was measured to quantify cell viability.

**RNA interference**
CXCR4 stable knockdown in MDA-MB-231-B was obtained using shRNA containing constructs derived from Sigma MISSION library (TRCN0000004054 or #1): 5'-CCTTGTGATATCAGGTTTCC-3' and TRCN0000004056 or #2: 5'-GAAATACGTAAGAGTCTGAA-3'. Lentivirus virions were produced by transfecting HEK293T cells with pKLO1-puro plasmid (containing the CXCR4-targeting shRNA or a non-mammalian shRNA control), pCMV- VSV-G (envelope plasmid), pMDLG- RRE (gag and pol elements) and pRSV-REV (rev or HIV1gp6) as packaging vectors. Plasmid mix was added to cell medium together with CaCl₂ and incubated for 18-20 h. Virus-containing supernatant was collected and used to infect the target cells.

**Chemokine injection, L-P staining and in silico analysis**
Human CXCL12-α (0.4 mg/ml) (300-28A, Peprotech) or zebrafish Cxxcl11a (1.5 mg/ml) (Torraca et al., 2015) chemokines, or water control (1 ml), were injected in the hindbrain ventricle (HBV) of 30-32 hpf embryos. Sample fixation was done at 3-3.5 hpi with 4% paraformaldehyde (PFA) (O.N. at 4°C or for 3 h at room temperature). Macrophages were counted as mpeg+ cells in the Tg(mpeg1:mCherry) line and L-plastin (L-P)+/− mice. Images were acquired using a Leica TCS SPE confocal microscope with an HC PL FLUOTAR 10×/0.40 DRY objective (0.30 N.A.). In each micrograph, embryos are shown in lateral view and oriented head (left)-to-tail (right), as shown by representative cartoons.

**Statistical analysis**
Unpaired, two-tailed t-test was used to compare the means of two groups and Welch’s correction applied when variances were significantly different (P<0.05). For datasets of three or more groups, one-way ANOVA with Bonferroni post-hoc test was performed. Raw or normalized data are mean±s.e.m. of pooled data points from at least two independent experiments. Statistics were performed with GraphPad Prism 6.

**Acknowledgements**
The authors would like to thank A. H. Meijer for critical reading of the manuscript and V. Torraca, A. Groenewoud (Leiden University), M. J. Smit (VU, Amsterdam) and E. Raz (University of Münster) for scientific discussion. The authors are grateful to M. Gómovski (IIMB, Warsaw) for providing MDA-MB-231 transduced with the pLenti-tdtomato plasmid, and P. ten Dijke and Y. Drabsch for MDA-MB-231-B dsRed cells (LUMC, Leiden). CXCR4-targeting shRNAs were provided by R. Hoeben and
Bussmann, J. and Raz, E. (2015). Chemokine-guided cell migration and motility in zebrafish development. EMBO J. 34, 1309-1318.

Bussmann, J., Wolfe, S. A. and Siekmann, A. F. (2011). Arterio-venous network formation during brain vascularization involves hemodynamic regulation of chemokine signaling. Development 138, 1717-1726.

Ch, Y. R., Fujita, M., Butler, M., Isogai, S., Kochhan, E., Siekmann, A. F. and Weinstein, B. M. (2012). Chemokine signaling directs trunk lymphatic network formation along the preexisting blood vascular. Dev. Cell 22, 834-836.

Chatterjee, S., Azad, B. B. and Nimmagadda, S. (2014). The intricate role of CXCR4 in cancer. Adv. Cancer Res. 124, 31-82.

Costantini, S., Di Bernardo, G., Cammarota, M., Castello, G. and Colonna, G. (2013). Gene expression signature of human HepG2 cell line. Gene 518, 335-345.

Cui, C., Benard, E. L., Kanwal, Z., Stockhammer, O. W., van der Vaart, M., Zakrzewski, A., Spaenk, H. P. and Meijer, A. H. (2011). Infectious disease models and innate immune function in zebrafish embryos. Zebrafish 10, 273-308.

Day, R. B. and Link, D. C. (2012). Regulation of neutrophil trafficking from the bone marrow. Cell. Mol. Life Sciences 69, 1415-1423.

Dono, E., Barry, J. D., Valentin, G., Quirin, C., Khemlinskii, A., Kunze, A., Durdu, S., Kostin, L. R., Fernandez-Minan, A., Huber, W. et al. (2013). Directional tissue migration through a self-generated chemokine gradient. Nature 503, 285-289.

Elliott, F., Pase, L., Hayman, J. W., Andrianopoulos, A. and Lieschke, G. J. (2011). mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. Blood 117, e49-e66.

Ewing, J. (1929). Neoplastic Diseases, 6th edn. Philadelphia: W. B. Saunders.

Fry, E., Broder, C. C., Kennedy, P. E. and Berger, E. A. (1996). HIV-1 entry cofactor: functional CD4NA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272, 872-877.

Fidler, I. J. (2003). Timeline: the pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited. Nat. Rev. Cancer 3, 453-458.

Fujita, M., Cho, Y. A., Pham, V. N., Sakurai, A., Roman, B. L., Gutkind, J. S. and Weinstein, B. M. (2011). Assembly and patterning of the vascular network of the vertebrate hindbrain. Development 138, 1705-1715.

Ghotta, V. P. S., He, S., de Bont, H., van der Ent, W., Spaenk, H. P., van der Water, E. E., Snaar-Jagalska, B. E. and Danen, E. H. J. (2012). Automated whole animal bio-imaging assay for human cancer dissemination. PLoS ONE 7, e31281.

Ghotta, V. P. S., He, S., van der Horst, G., Nijhoff, J. S., de Bont, H., Lekerkerker, A., Janssen, R., Jenster, G., van Leenders, G. J. L. H., Hoogland, A. M. M. et al. (2015). SYK is a candidate kinase target for the treatment of advanced prostate cancer. Cancer Res. 75, 230-240.

Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paer, J. and Lopez, R. (2010). A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res. 38, W695-W699.

Guilino, A. V. (2003). WHIM syndrome: a genetic disorder of leucocyte trafficking. Curr. Opin. Allergy Clin. Immunol. 3, 443-450.

Hanahan, D. and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell 144, 646-674.

He, S., Lamers, G. M. E., Beenakker, J.-W. M., Cui, C., Ghotta, V. P. S., Danen, E. H. J., Meijer, A. H., Spaenk, H. P. and Snaar-Jagalska, B. E. (2012). Neutrophil-mediated experimental metastasis is enhanced by VEGF inhibition in a zebrafish xenograft model. J. Pathol. 227, 431-445.

Hendrix, C. W., Collier, A. C., Lederman, M. M., Scholls, D., Pollard, R. B., Brown, S., Jackson, J. B., Coombs, R. W., Glesby, M. J., Flexner, C. W. et al. (2004). Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. J. Acquir. Immune Defic. Syndr. 37, 1253-1262.

Herzog, H., Hort, Y. J., Shine, J. and Selbie, L. A. (1993). Molecular cloning, characterization, and localization of the human homolog to the reported bovine NPY Y3 receptor: lack of NPY binding and activation. DNA Cell Biol. 12, 465-471.

Hess, I. and Boehm, T. (2012). Intravital imaging of thymopoiesis reveals dynamic lymphocyte-epithelial interactions. Immunity 36, 298-309.

Janowsky, M. (2009). Functional diversity of SDF-1 splicing variants. Cell Adh. Migr. 3, 243-249.

Jazin, E. E., Yoo, H., Blomqvist, A. G., Yee, F., Weng, G., Walker, M. W., Salon, J., Larhammar, D. and Wahlestedt, C. (1993). A proposed bovine neuropeptide Y (NPY) receptor cDNA clone, or its human homologue, confers neither NPY binding sites nor NPY responsiveness on transfected cells. Regul. Pept. 47, 247-258.

Jin, S.-W., Beis, D., Mitchell, T., Chen, J.-N. and Stainier, D. Y. R. (2005). Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development 132, 5199-5209.

Kalatskaya, I., Berichiche, Y. A., Gravel, S., Limberg, B. J., Rosenbaum, J. S. and Hevezker, N. (2009). AMD3100 is a CXCR7 ligand with allosteric agonist activity. Mol. Pharmacol. 75, 1240-1247.

Knaut, H., Werz, C., Geisler, R., The Tübingen 2000 Screen Consortium Robert and Nusslein-Volhard, C. (2003). A zebrafish homologue of the chemokine receptor Cxcr1 is a germ-cell guidance receptor. Nature 421, 279-282.

Konantz, M., Balci, T. B., Hartwig, U. F., Delaire, G., Andre, M. C., Berman, J. N. and Lengerke, G. (2012). Zebrafish xenografts as a tool for in vivo studies on human cancer. Ann. N. Y. Acad. Sci. 1266, 124-137.

Kuhne, M. R., Mulvey, T., Belanger, B., Chen, S., Pan, C., Chong, C., Gao, F., Nieberg, W. K. A. et al. (2013). BMS-936554/Mdx-139, a fully human anti-CXCR4 antibody induces apoptosis in vitro and shows antitumor activity in vivo in hematologic malignancies. Clin. Cancer Res. 19, 357-366.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilim, A., Lopez, R. et al. (2007). Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948.

Lawson, N. D. and Weinstein, B. M. (2002). In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev. Biol. 248, 307-318.
Shi, J., Wei, Y., Xia, J., Wang, S., Wu, J., Chen, F., Huang, G. and Chen, J. (2014). CXCL12-CXCR4 contributes to the implication of bone marrow in cancer metastasis. Future Oncol. 10, 749-759.

Shukla, A. K., Xiao, K. and Lefkowitz, R. J. (2011). Emerging paradigms of beta-arrestin-independent seven transmembrane receptor signaling. Trends Biochem. Sci. 36, 457-469.

Stoletov, K., Kato, H., Zardouzian, E., Kelber, J., Yang, J., Shattil, S. and Klemke, R. (2010). Visualizing extravasation dynamics of metastatic tumor cells. J. Cell Sci. 123, 2332-2341.

Teicher, B. A. and Fricker, S. P. (2010). CXCL12 (SFDF-1) CXCR4 pathway in cancer. Clin. Cancer Res. 16, 2927-2931.

The UniProt Consortium (2015). UniProt: a hub for protein information. Nucleic Acids Res. 43, D204-D212.

Thoma, G., Streiff, M. B., Kovarik, J., Glickman, F., Wagner, T., Beeri, C. and Zenz, H.-G. (2006). Orally bioavailable isothiouronium block function of the chemokine receptor CXCR4 in vitro and in vivo. J. Med. Chem. 51, 7915-7920.

Torvaca, R., Cui, B., Boland, R., Bebelman, J.-P., van der Sar, A. M., Smit, M. J., Siderius, M., Spahn, H. P. and Meier, A. H. (2015). The CXCR3-CXCL11 signaling axis mediates macrophage recruitment and dissemination of mycobacterial infection. Dis. Model. Mech. 8, 253-269.

Valentin, G., Haas, P. and Gilmour, D. (2007). The chemokine SDF1alpha coordinates tissue migration through the spatially restricted activation of Cxcr7 and Cxcr4b. Curr. Biol. 17, 1026-1031.

van der Bent, W., Burrello, C., Teunisse, A. F. S., Ksander, B. R., van der Velden, P. A., Jager, M. J., Jochemsen, A. G. and Snaar-Jagalska, B. E. (2014a). Modeling of human uveal melanoma in zebrafish xenograft embryos. Invest. Ophthalmol. Vis. Sci. 55, 6612-6622.

van der Bent, W., Jochemsen, A. G., Teunisse, A. F. S., Krens, S. F. G., Szuhal, K., Spahn, H. P., Hodgendoorn, P. C. W. and Snaar-Jagalska, B. E. (2014b). Ewing sarcoma inhibition by disruption of EWSR1-FLIN transcriptional activity and reactivation of p53. J. Pathol. 233, 415-424.

van der Vaart, M., Korbee, C. J., Lamers, G. E. M., Tengeler, A. C., Hosseini, R., Haks, M. C., Ottenhoff, T. H. M., Spahn, H. P. and Meier, A. H. (2014). The DNA damage-regulated autophagy modulator DRAM1 links mycobacterial recognition via TLR-MYD88 to autophagic defense. Cell Host Microbe 15, 753-767.

Veinotte, C. J., Delaire, G. and Berman, J. N. (2014). Hooking the big one: the potential of zebrafish xenotransplantation to reform cancer drug screening in the genomic era. Dis. Model. Mech. 7, 745-754.

Vela, M., Aris, M., Llorente, M., Garcia-Sanz, J. A. and Kremer, L. (2015). Chemokine receptor-specific antibodies in cancer immunotherapy: achievements and challenges. Front. Immunol. 6, 12.

Venkiteswaran, G., Lewellis, S. W., Wang, J., Reynolds, E., Nicholson, C. and Knaut, H. (2013). Generation and dynamics of an endogenous, self-generated signaling gradient across a migrating tissue. Cell 155, 674-687.

Vicenzi, E., Lio, P. and Poli, G. (2013). The puzzling role of CXCR4 in human immunodeficiency virus infection. Therapostics 3, 18-25.

Vila-Coro, A. J., Rodríguez-Frade, J. M., Martín De Ana, A., Moreno-Ortiz, M. C., Martínez-A, C. and Mellado, M. (1999). The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. FASEB J. 13, 1699-1710.

Wahba, H. A. and El-Hadaad, H. A. (2015). Current approaches in treatment of triple-negative breast cancer. Cancer Biol. Med. 12, 106-116.

Walters, K. B., Green, J. M., Surfus, J. C., Yoo, S. K. and Huttenlocher, A. (2010). Live imaging of neutrophil motility in a zebrafish model of WHIM syndrome. Blood 116, 2803-2811.

Wetterwald, A., van der Plujum, G., Que, I., Sijmons, B., Bijls, K., Karperien, M., Lowik, C. W. G. M., Gaultushi, E., Thalmann, G. N. and Cecchini, M. G. (2002). Optical Imaging of cancer metastasis to bone marrow - a mouse model of minimal residual disease. Am. J. Pathol. 160, 1143-1153.

Wu, B., Chien, E. Y. T., Mol, C. D., Denali, G., Liu, W., Katritch, V., Abagyan, R., Walters, K. B., Green, J. M., Surfus, J. C., Yoo, S. K. and Huttenlocher, A. (2010). Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. Science 330, 1908-1912.

Yoshikawa, Y., Oishi, S., Kubo, T., Tanahara, N., Fujii, N. and Furuya, T. (2013). Optimization of G-protein-coupled receptor homology modeling: its application to the discovery of novel CXCR7 ligands. Curr. Top. Med. Chem. 13, 4236-4251.

Zhang, S., Chang, S. L., Linderman, J. J., Feng, F. Y. and Luker, G. D. (2014). A comprehensive analysis of CXCL12 isoforms in breast cancer. Transl. Oncol. 7, 429-438.

Zoni, E., van der Horst, G., van de Merbel, A. F., Chen, L., Rane, J. K., Pelger, R. C. M., Collins, A. T., Visakorpi, T., Snaar-Jagalska, B. E., Maitland, N. J. et al. (2015). miR-25 modulates invasiveness and dissemination of human prostate cancer cells via regulation of alpha(v)- and alpha(6)-integrin expression. Cancer Res. 75, 2326-2336.

Lefkowitz, R. J. (1998). G protein-coupled receptors: III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. J. Biol. Chem. 273, 18677-18680.

Loynes, C. A., Martin, J. S., Robertson, A., Trushell, D. M., Ingham, P. W., Whyte, M. K. B. and Renshaw, S. A. (2010). Pivotal advance: pharmacological manipulation of inflammation resolution during spontaneously resolving tissue neutrophilia in the zebrafish. J. Leukoc. Biol. 87, 203-212.

Luttrell, L. M., Ferguson, S. G. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F.-T., Kawakatsu, H., Owada, K., Luttrell, D. K. et al. (1999). Beta-arrestin dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 283, 655-661.

Mellado, M., Rodríguez-Frade, J. M., Manes, S. and Martínez-A, C. (2001). Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. Annu. Rev. Immunol. 19, 397-421.

Miyasaka, N., Knaut, H. and Yoshikawa, Y. (2007). Cxcr4 regulates chemokine signaling is required for placode assembly and sensory axon pathfinding in the zebrafish olfactory system. Development 134, 2459-2468.

Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, T., Range, U., Wagner, W., S. N. et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. Nature 410, 50-56.

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Takahashi, T., Takashima, S., Muller, A., Homey, B., Soto, H., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, T., Range, U., Wagner, W., S. N. et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. Nature 410, 50-56.