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

Tulotta, C.

Chapter 7

Summary and Discussion

Cell-to-cell communication guarantees homeostasis in a multi-cellular organism. Cancer-to-microenvironment communication sustains malignant growth and dissemination [1]. Whereas the accumulation of mutations is at the origin of malignant cell transformation and neoplasia onset, the interaction between cancer and the surrounding stroma influences the balance between tumor regression and tumor progression [2]. To study how the interaction between cancer and stromal cells is disadvantageous or beneficial for tumor progression, the use of a transparent *in vivo* model, bears important research potentials. Zebrafish has been increasingly used as animal model to study tumor biology [3, 4]. The use of transparent zebrafish embryos, with fluorescent endothelial and immune cells [5-7], allows the visualization of cell-to-cell interaction, among host cells themselves and between zebrafish stroma and implanted human cancer cells (Figure 1A). In particular, tumor-induced angiogenesis, metastasis formation and relative chemical approaches to inhibit these processes have been studied using zebrafish as a xenotransplantation model, complementing current knowledge developed through the use of *in vitro* and other *in vivo* models (**Chapter 2**). Upon localized or hematogenous engraftment of cancer cells, zebrafish xenografts allow qualitative and quantitative assessment of tumor burden and tumor-microenvironment interaction (**Chapter 3**), representing a powerful pre-clinical model to unravel cancer mechanisms and to develop new therapeutic strategies.

Cell-autonomous CXCR4 signaling: the CXCR4 antagonist IT1t impairs early human metastatic events, in a zebrafish xenograft model where the interspecies cross-talk takes place

Chemokines direct tumor and stromal cell bidirectional migration [8]. CXCR4 is a seven-transmembrane G-protein coupled receptor. It plays a physiological role in hematopoiesis [9, 10], leukocyte trafficking [11-13], cell migration and embryo development [14], as well as a pathological function in HIV pathogenesis [15], WHIM syndrome [16] and cancer [17, 18]. Its cognate ligand is the chemokine CXCL12 (or stromal cell-derived factor-1, SDF-1) [19, 20]. Additionally, CXCR4 can bind ubiquitin [21], macrophage migration inhibitory factor (MIF) [22-24] and CXCL14 [25]. The CXCR4-CXCL12 signaling axis is known to play a critical function in cancer cell spreading, when tumor cells expressing high levels of CXCR4 communicate with CXCL12-secreting stromal cells of distant organs that function as metastatic and secondary growth “soils” [26]. In **Chapter 4**, we show that the impairment of the cell autonomous CXCR4 signaling blocks triple-negative breast cancer (TNBC) early metastatic events in the zebrafish xenograft model (Figure 1B). In our model, human triple-negative breast cancer cells, derived from bone metastases developed in a mouse model, were implanted directly into the blood circulation of zebrafish embryos. Using this model, the formation of the primary tumor and the initial steps of metastasis (local invasion and intravasation into the blood circulation) were by-passed. Tumor cells, inoculated into the blood circulation, were found to form early metastases, by adhering to the endothelial wall, forming aggregates and invading the local tail fin tissue. Experimental metastases occurred in proximity

of the caudal hematopoietic tissue, an intermediate site of hematopoiesis and a functional analogue of the fetal liver during mammalian development. This observation was in line with breast cancer metastasis formation in the bone [27] . Moreover, we demonstrated that the CXCR4 signaling functions across human and zebrafish systems, because CXCR4-expressing human cells respond to zebrafish Cxcl12 ligands and Cxcr4-expressing zebrafish cells migrate towards human CXCL12, showing that the zebrafish xenograft model is a valid approach to study human tumors. Taking advantage of the same *in vivo* model, where the interspecies crosstalk is validated, we propose a recently described CXCR4 antagonist, IT1t, as a possible therapeutic to inhibit early metastasis of TNBC.

Host-dependent Cxcr4 signaling: Cxcr4 controls the tumor metastatic niche preparation, by regulating intrinsic myeloid cell functions and responses to cancer cells

Immune cells are programmed to recognize and eliminate transformed cells. However, cancer cells have evolved mechanisms that reprogram the immune defense and make the foe-to-friend switch an important support for survival and progression. The combination of chemotherapy and immunotherapy is a current strategy in the clinic [28]. Galluzzi *et al.* have recently reviewed anti-cancer therapies that re-activate the immune system, such as tumor-targeting antibodies, adoptive cell transfer and oncolytic viruses (all classified as passive immunotherapy), dendritic cell-based immunotherapies, anti-cancer vaccines, immune-stimulatory cytokines, immunomodulatory antibodies, inhibitors of immunosuppressive metabolism, Pattern Recognition Receptor (PPRs) agonist, and immunogenic cell death inducers (all classified as active immunotherapy). Antibodies against CXCR4 are included in immunotherapeutic agents that skew the balance between M2/M1 tumor associated macrophages (TAMs) toward the pro-inflammatory and anti-tumor M1 phenotype [29]. In **Chapter 5**, the role of the host dependent CXCR4 signaling in supporting early metastatic events is described in the zebrafish xenograft model. Previous work from our group has shown that neutrophils are involved in the metastatic niche preparation by conditioning the ECM during their apparent random walk in the transmigration from the CHT (caudal hematopoietic tissue, transient hematopoietic site) to the tail tissue of zebrafish embryos [30]. Because CXCR4 is known to regulate the retention of hematopoietic stem progenitor cells (HSPCs) and differentiated leukocytes in the bone marrow in mammals [31], and is highly expressed in zebrafish myeloid cells [32], we hypothesized that CXCR4 signaling plays a role in controlling intrinsic neutrophil motility in physiological conditions (Chapter 5). We found that neutrophils display altered motility and their number fluctuates during embryo development, leading to the conclusion that CXCR4 regulates neutrophil development in zebrafish. The neutrophilic response towards cancer cells was also altered in zebrafish mutants with a non-functional Cxcr4 (Cxcr4b). We identified a population of neutrophils that was mainly retained in the CHT and a population of neutrophils that even if moving in the tissue, displayed the inability to infiltrate tumor cell aggregates in the tail fin of

Cxcr4b-null mutants. In the surrounding of cancer cells, *cxcr4b*-expressing neutrophils reduced their speed in motility, while Cxcr4b-null neutrophils maintained similar speeds as in neutrophils that had not been challenged by cancer cells. Interestingly, CXCR4-espressing macrophages, generally localized in the surrounding of invading cancer cells in the tail fin, were less recruited in a *cxcr4b* homozygote mutant. Therefore, we propose that Cxcr4 controls neutrophil development as well as both neutrophil and macrophage responses to tumor cells, initiating early metastatic events (Figure 1C). RNA sequencing performed on sorted neutrophils and macrophages from wild-type or *cxcr4b*$^{-/-}$ zebrafish larvae supported our conclusion that motility and adhesion are altered in myeloid cells with a non-functional Cxcr4 signaling.

CXCR4 and MDMX: a potential duo to inhibit tumor proliferation and metastatic onset of Ewing sarcoma

P53, known as "the guardian of the genome", is a transcription factor with key regulatory functions of cell survival. It is found mutated in almost all tumors, ranging between 10% (in hematopoietic cancers) and 96% (high grade ovarian serous carcinoma) of tumors [33]. Ewing sarcoma is the second most common bone tumor in children and young adults and in ~90% of the cases P53 is wild-type [34]. Mutations in P53 regulators (such as MDM2 and MDMX) are often found when P53 is wild-type [35]. Developing pharmacological approaches that target P53 regulators to induce P53 reactivation is a promising strategy in Ewing sarcoma [36]. In **Chapter 6**, we propose that P53 reactivation by *MDMX* genetic interference inhibits Ewing sarcoma burden *in vitro* and in a zebrafish xenograft model, suggesting that MDMX is a candidate target for therapies. Importantly, MDMX inhibition resulted in increased *CXCR4* expression levels. Because CXCR4 expression correlates with metastatic disease in Ewing sarcoma [37], we chemically and genetically inhibited CXCR4 in tumor cells engrafted in a zebrafish embryo model of experimental micrometastasis. CXCR4 inhibition resulted in reduced early metastatic events in Ewing sarcoma, in line with findings for TNBC described in **Chapter 4**. In conclusion, we propose that the dual inhibition of MDMX and CXCR4 represents a possible effective treatment against metastatic Ewing sarcoma and further research is needed to validate this hypothesis *in vitro* and *in vivo*.

Conclusions

Cancer is a complex, multi-step disease and a leading cause of death worldwide (14 million new cases and 8.2 million cancer-related deaths in 2012 (www.who.int, update February 2015, World cancer report 2014). Patients diagnosed with primary tumors are treated, when possible, with surgery. However, metastasis can occur years after surgical intervention [38]. Metastatic cancer associates with poor patient prognosis and represent a major challenge for clinical research. Chemotherapy is often the pharmacological choice to treat cancer, although side effects alter normal cell physiology and affect patient life quality. Moreover, cancer relapse and therapy resistance associate with poor prognosis. Progress in biomedical research has shown

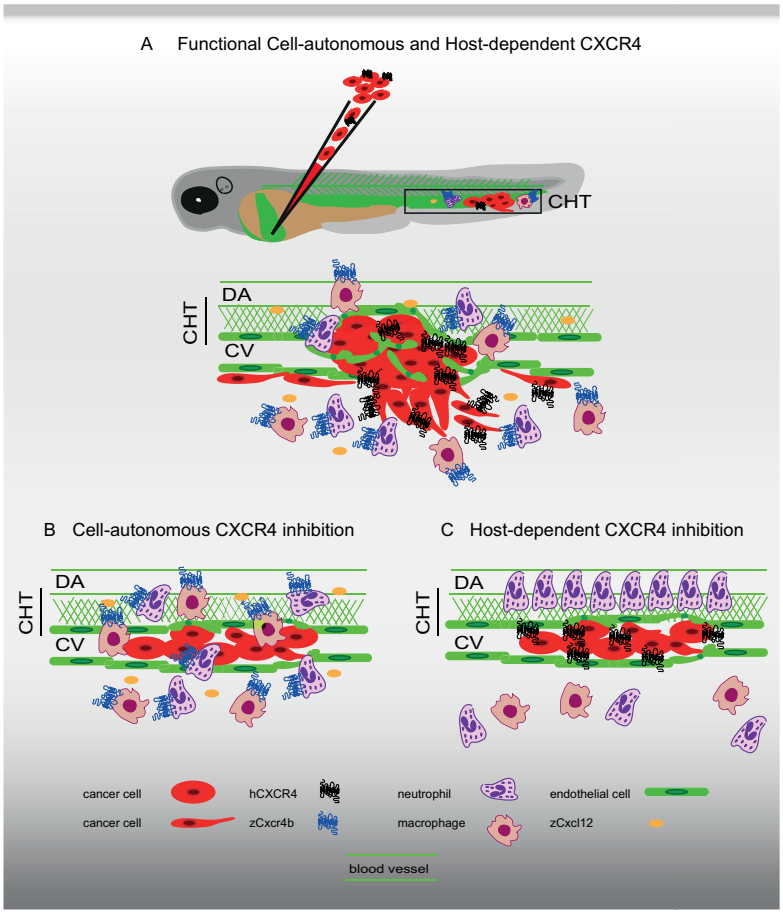

Figure 1. Role of cell-autonomous and host-dependent CXCR4 signaling in experimental metastasis formation in the zebrafish xenograft model. (A) Inoculation of human tumor cells into the blood circulation of zebrafish embryos results in experimental metastasis formation, characterized by tumor cell aggregates in the blood vessels, and extravasation and invasion in the surrounding tissue, in the region of the caudal hematopoietic tissue (CHT). During early metastatic events, endothelium alteration takes place and neutrophils and macrophages localize in the surrounding of the tumor. The CHT is a vascular plexus in the tail fin between the DA and the CV and is a hematopoietic site. (B) Upon disruption of the tumor cell-autonomous CXCR4 signaling, cancer cells are unable to initiate early metastatic events, while surrounded by myeloid cells. (C) The same inhibition of experimental metastasis formation occurs upon disruption of the host-dependent CXCR4 (Cxcr4b) signaling. Neutrophils are preferentially retained in the CHT, whereas neutrophil and macrophage recruitment at the metastatic site is impaired.

that targeting cancer cells is not the only therapeutic option. The interaction between tumor and surrounding stroma supports cancer survival and spreading, representing therefore a possible new treatment strategy [39]. In this thesis, we used the zebrafish xenograft model to study early stages of experimental micrometastasis formation, engrafting fluorescent tumor cells in transparent zebrafish embryos with fluorescent endothelial and immune cells (**Chapter 2 and 3**). We propose that targeting CXCR4 signaling on cancer cells (**Chapter 4**) or in the tumor microenvironment (**Chapter 5**) is a valid approach to inhibit metastatic cancer and suggest that anti-CXCR4 therapy might have double treatment benefits. In addition, therapeutic modulation of the immune system might result in the reinforcement of the immune defense against cancer. However, we suggest that treatments designed to target malignant cells might affect tumor microenvironment intrinsic functions. Specifically, the intrinsic physiological role of myeloid cells can be affected by cancer treatment, resulting in an inability to mount a functional anti-cancer response or, on the other hand, in the ability to mount a tumor-supportive response. Moreover, as tumor proliferation inhibition by P53 reactivation through *MDMX* interference results in increased *CXCR4* expression, which associates with metastatic disease in Ewing sarcoma (**Chapter 6**), we propose that combinatorial treatments are a promising approach to effectively limit cancer progression and ameliorate patient prognosis.

References

1. Quail, D.F. and J.A. Joyce, Microenvironmental regulation of tumor progression and metastasis. Nat Med, 2013. 19(11): p. 1423-37.

2. Hanahan, D. and R.A. Weinberg, Hallmarks of cancer: the next generation. Cell, 2011. 144(5): p. 646-74.

3. Amatruda, J.F., et al., Zebrafish as a cancer model system. Cancer Cell, 2002. 1(3): p. 229-231.

4. Barriuso, J., R. Nagaraju, and A. Hurlstone, Zebrafish: a new companion for translational research in oncology. Clin Cancer Res, 2015. 21(5): p. 969-75.

5. Ellett, F., et al., mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. Blood, 2011. 117(4): p. e49-56.

6. Lawson, N.D. and B.M. Weinstein, In vivo imaging of embryonic vascular development using transgenic zebrafish. Developmental Biology, 2002. 248(2): p. 307-318.

7. Renshaw, S.A., et al., A transgenic zebrafish model of neutrophilic inflammation. Blood, 2006. 108(13): p. 3976-8.

8. Vandercappellen, J., J. Van Damme, and S. Struyf, The role of CXC chemokines and their receptors in cancer. Cancer Lett, 2008. 267(2): p. 226-44.

9. Nagasawa, T., et al., Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature, 1996. 382(6592): p. 635-638.

10. Rosu-Myles, M., et al., The human hematopoietic stem cell compartment is heterogeneous for CXCR4 expression. Proceedings of the National Academy of Sciences of the United States of America, 2000. 97(26): p. 14626-14631.

11. Day, R.B. and D.C. Link, Regulation of neutrophil trafficking from the bone marrow. Cellular and Molecular Life Sciences, 2012. 69(9): p. 1415-1423.

12. Sallusto, F. and M. Baggiolini, Chemokines and leukocyte traffic. Nature Immunology, 2008. 9(9): p. 949-952.

13. Sallusto, F., C.R. Mackay, and A. Lanzavecchia, The role of chemokine receptors in primary, effector, and memory immune responses. Annual Review of Immunology, 2000. 18: p. 593-+.

14. Bussmann, J. and E. Raz, Chemokine-guided cell migration and motility in zebrafish development. Embo Journal, 2015. 34(10): p. 1309-1318.

15. Vicenzi, E., P. Lio, and G. Poli, The Puzzling Role of CXCR4 in Human Immunodeficiency Virus Infection. Theranostics, 2013. 3(1): p. 18-25.

16. Gulino, A.V., WHIM syndrome: a genetic disorder of leukocyte trafficking. Curr Opin Allergy Clin Immunol, 2003. 3(6): p. 443-50.

17. Balkwill, F., The significance of cancer cell expression of the chemokine receptor CXCR4. Semin Cancer Biol, 2004. 14(3): p. 171-9.

18. Chatterjee, S., B.B. Azad, and S. Nimmagadda, The Intricate Role of CXCR4 in Cancer. Emerging Applications of Molecular Imaging to Oncology, 2014. 124: p. 31-82.

19. Bleul, C.C., et al., The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature, 1996. 382(6594): p. 829-833.

20. Oberlin, E., et al., The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1 (vol 382, pg 833, pg 1996). Nature, 1996. 384(6606): p. 288-288.

21. Saini, V., A. Marchese, and M. Majetschak, CXC Chemokine Receptor 4 Is a Cell Surface Receptor for Extracellular Ubiquitin. Journal of Biological Chemistry, 2010. 285(20): p. 15566-15576.

22. Bernhagen, J., et al., MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med, 2007. 13(5): p. 587-96.

23. Lourenco, S., et al., Macrophage migration inhibitory factor-CXCR4 is the dominant chemotactic axis in human mesenchymal stem cell recruitment to tumors. J Immunol, 2015. 194(7): p. 3463-74.

24. Shin, H.N., H.H. Moon, and J.L. Ku, Stromal cell-derived factor-1alpha and macrophage migration-inhibitory factor induce metastatic behavior in CXCR4-expressing colon cancer cells. Int J Mol Med, 2012. 30(6): p. 1537-43.

25. Lu, J., et al., CXCL14 as an emerging immune and inflammatory modulator. J Inflamm (Lond), 2016. 13: p. 1.

26. Muller, A., et al., Involvement of chemokine receptors in breast cancer metastasis. Nature, 2001. 410(6824): p. 50-6.

27. Weidle, U.H., et al., Molecular Mechanisms of Bone Metastasis. Cancer Genomics Proteomics, 2016. 13(1): p. 1-12.

28. Cook, A.M., et al., Chemotherapy and immunotherapy: mapping the road ahead. Curr Opin Immunol, 2015. 39: p. 23-29.

29. Galluzzi, L., et al., Classification of current anticancer immunotherapies. Oncotarget, 2014. 5(24): p. 12472-508.

30. He, S.N., et al., Neutrophil-mediated experimental metastasis is enhanced by VEGFR inhibition in a zebrafish xenograft model. Journal of Pathology, 2012. 227(4): p. 431-445.

31. Tchernychev, B., et al., Discovery of a CXCR4 agonist pepducin that mobilizes bone marrow hematopoietic cells. Proc Natl Acad Sci U S A, 2010. 107(51): p. 22255-9.

32. Walters, K.B., et al., Live imaging of neutrophil motility in a zebrafish model of WHIM syndrome. Blood, 2010. 116(15): p. 2803-11.

33. Walerych, D., K. Lisek, and G. Del Sal, Mutant p53: One, No One, and One Hundred Thousand. Front Oncol, 2015. 5: p. 289.

34. Neilsen, P.M., et al., Targeting the p53 Pathway in Ewing Sarcoma. Sarcoma, 2011. 2011: p. 746939.

35. Wade, M., Y.C. Li, and G.M. Wahl, MDM2, MDMX and p53 in oncogenesis and cancer therapy. Nat Rev Cancer, 2013. 13(2): p. 83-96.

36. van der Ent, W., et al., Ewing sarcoma inhibition by disruption of EWSR1-FLI1 transcriptional activity and reactivation of p53. J Pathol, 2014. 233(4): p. 415-24.

37. Bennani-Baiti, I.M., et al., Intercohort gene expression co-analysis reveals chemokine receptors as prognostic indicators in Ewing's sarcoma. Clin Cancer Res, 2010. 16(14): p. 3769-78.

38. Demicheli, R., et al., The effects of surgery on tumor growth: a century of investigations. Ann Oncol, 2008. 19(11): p. 1821-8.

39. Sounni, N.E. and A. Noel, Targeting the tumor microenvironment for cancer therapy. Clin Chem, 2013. 59(1): p. 85-93.