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Zebrafish xenograft model: Identification of novel mechanisms driving prostate cancer metastasis

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Chapter 1

Introduction and thesis outline

Prostate cancer (PCa) is one of the most prevalent cancer in males and is the second leading-cause of death in cancer-related disease around the world [1]. Initial treatments such as prostatectomy, radiation and chemotherapy are employed to target cancer cells at the primary site [2]. Although the majority of the patients can benefit from the present clinical treatments, 20%-30% of the patients who originally respond to the therapy still develop incurable, castration-resistance bone metastases, which is a main cause of death in PCa [3]. Bone metastasis is initiated by a small group of cancer cells (cancer stem/progenitor-like cells (CSCs)) [4]. These cells harbor similar characteristics as normal prostate stem cells [5]. They indefinitely self-renew to maintain the CSC pool, and produce transient amplifying (TA) cells through asymmetric division. TA cells are able to rapidly proliferate and differentiate into committed basal (CB) cells, which subsequently form terminally differentiated luminal cell populations generating tumor architecture at both the primary and the metastatic site [4]. Notably, evidence shows that partially differentiated cancer cells can re-enter the CSC pool through dedifferentiation on response to the tumor microenvironment [6]. This “so called” cancer stem-plasticity is thought to induce cancer relapse and metastasis after clinical treatments. Uncovering cell non-autonomous cues regulating cell plasticity could therefore reveal important potential targets for clinical disease prediction and intervention.

In order to identify the molecular cues present in the cancer cell-microenvironment interaction, that might drive metastatic tumor initiation of PCa CSCs, throughout the course of my PhD, I have combined multiple research platforms including zebrafish and mice xenografts, patients’ dataset analysis, 2D/3D cellular assays and patient derived organoids. This multi-platform approach has allowed me to understand the interplay between general mechanisms of PCa metastasis development such as Cripto-dependent transient EMT, mechanosensing and mechanotransduction, NF-κB-Activin A axis activity, and AMPK-Autophagy-associated metabolic-stress coping machinery. Ultimately, the targeting of these signaling pathways may have significant therapeutic potential for disease intervention. In this introduction, I briefly introduce the hallmarks of cancer, metastasis development of PCa, and discuss how the combination of zebrafish and mice xenograft models can be utilized to study this disease.

The hallmarks of cancer

Cancer is a complex disease that involves genetic alterations and malignant transformation of normal cells into cancer cells. This malignant transformation is controlled by typical hallmarks of cancer including sustain proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, reprogramming of energy metabolism, evading immune destruction and activating invasion and metastasis [7]. Apart from cancer cells, tumor tissue also contain many other types of stromal cells such as cancer associated fibroblasts (CAFs), inflammatory cells, endothelial cells and mesenchymal stem cells. These stromal cells provide a wide range of essential signals to assist the development of cancer. Below, I will elaborate on the hallmarks of cancer in more details.

Cancer expansion is sustained by proliferative signaling. In normal tissues, the development, homeostasis and architecture are well-organized processes controlled by chemical and physical cues. Those cues control cell proliferation, differentiation and organization during the tissue generation/repairing [7]. In cancer, the genomic and epigenetic alteration in malignant cells can autonomously provide pro-proliferation factors in either autocrine or paracrine manner, leading to sustainable proliferation [8, 9]. Cancer cells also create a special tumor microenvironment through recruiting and modulating the neighboring stromal cells, which in turn constantly provides pro-proliferation signals to the cancer cells [10]. Additionally, mutations in oncogenic drivers trigger a hyper-activation of the proliferative signaling inducing uncontrolled growth [11, 12]. Notably, induction of proliferative signaling alone is often not sufficient to induce cell malignancy due to the existence of other natural tumor-suppressing mechanisms in the cells [7]. Hence, additional alterations are still required for a complete cancer transformation.

For example, cancer cells have a capacity of evading growth suppressing signals. The tumor suppressors like pRb, P53 and Pten present in normal tissues, determine whether cell proliferation should be stopped. In cancer, these genes are commonly mutated, that activate cells to overrule the growth-inhibitory signals, finally resulting in uncontrolled cell growth [13-15].

In addition, cancer cells are resistant to apoptosis, the programmed cell death that serves as a natural barrier for oncogenesis [14]. Apoptosis of a cell is triggered by extracellular or

intracellular signals [16]. It occurs when cells are exposed to physiological stresses, DNA damages, and/or immune challenges. These stress signals lead to the activation of a proteolytic caspase pathway, resulting in cell disassembly and cell death. Bcl-2 and its closest relatives Bcl-XL and Mcl1 are the major inhibitors of apoptosis [17]. Some cancer cells have gained the ability to escape apoptosis by overexpression of Bcl-2 (or its relatives). In addition, cancer cells can also harbor loss-of-function mutations in tumor-suppressors like TP-53, an apoptosis inducer [13]. In both cases cancer cells are resistant to the cell-death signals. Targeting of Bcl-2 to trigger apoptosis has been therefore considered as an efficient clinical approach to kill cancer cells.

Cancer cells sustain replicative immortality. In contrast to normal cells, which only divide a few times followed by cell senescence and crisis/apoptosis, cancer cells have the potential for unlimited duplication [18]. This replicative immortality is related to the elongation and/or maintenance of telomeres, a repetitive DNA sequence capping the terminals of chromosomes. Telomeres control chromosome stability by preventing deterioration or fusion between chromosomes [19]. After each time of division, in normal cells telomeres are shorted, which induces chromosome instability, resulting in cell senescence and cell death. Telomeres can be prolonged/fixed by telomerases, ribonucleoproteins which prevent shortening of telomere during chromosome duplication by editing the DNA sequence to the 3' end of telomeres. Telomerases are low expressed in most cells, but become highly expressed in cancer, which endows the malignant cells with the potential of indefinite division [20].

Cancer cells have the ability to induce angiogenesis, which leads to formation of neovascularization in the tumors, to provide nutrition and oxygen for their rapid growth [21]. These neovessels are excessively branched, leaky and microhemorrhaging due to the abnormal angiogenetic signaling produced by tumors [22]. Tumor angiogenesis is induced by a series of ligands including VEGF and FGF, which drive sprouting and expansion of the existing vessels towards the tumor [23]. These pro-angiogenetic ligands are controlled by the hypoxic microenvironment, and by activation of oncogenic pathways like RAS-MAPK signaling [24, 25]. In addition, stromal cells such as macrophages and cancer-associated fibroblasts further promote tumor angiogenesis by secreting analogous angiogenetic ligands [26].

Following the malignant progression, some of the cancer cells invade and metastasize to distant organs. Metastasis is a multiple-step process starting with primary cancer cell invasion into the surrounding tissues, followed by intravasation into vessels, where they have to cope with the mechanical stresses in the blood flow, extravasation at distant organs, and initiation of a metastatic tumor outgrowth [27]. The metastatic cascade is mediated by a developmental program, called the epithelial-to-mesenchymal transition (EMT) [28]. When EMT is induced, cancer cells acquire a series of mesenchymal characteristics such as a spindle-like morphology, loss of cell-cell junctions, enhanced cell-ECM adhesion, gain in motility, and expression of matrix metalloproteases (MMPs). EMT patterns promote cell invasion, survival, and extravasation and is also associated with CSC induction in multiple type of cancers. It is therefore thought to be crucial driver of tumorigenicity at both the primary and the metastatic site [29]. Following the metastatic process, an EMT-reversed program, the mesenchymal-to-epithelial transition (MET) is induced, which endows cancer cells with the capacity of rapid proliferation at the metastatic site [30]. Stromal cells also contribute to the metastatic process [31]. Cancer associated fibroblasts (CAFs) are capable of driving cancer cell invasion and metastasis [32] through reorganizing the composition of the extracellular matrix (ECM) by changing ECM stiffness, which seems to guide cancer cell invasion. In addition, CAFs produce serial critical cytokines such as Wnt, TGF-β, IL-1 β and FGF, leading to EMT induction and further CSCs enrichment [33].

Tumor tissues are generally under severe metabolic challenge due to cancer cell hyper-proliferation and inefficient vascularization. To survive and rapidly grow in a low-energy and hypoxic microenvironment, cancer cells have reprogrammed their energy metabolism into the so-called “aerobic glycolysis” where they largely depend on glycolysis (Warburg-effect) for energy consumption [34, 35].

Cancer is capable of evading immune destruction. Most of pre-neoplastic cells harboring oncogenetic mutation are recognized and removed by adaptive immunity. However, malignant neoplastic cells can escape immune destruction [36]. Due to accumulation of mutations in the growing tumor, the presentation of neo-antigen by human leukocyte antigen class I (HLA-1) may be lost, causing a failure of the body’s immunosurveillance. In addition, cancer cells are able to express different types of immune checkpoints to suppress eradication of tumors by immune system [37]. The tumor stromal cells also play a role in promoting

immune evasion. Tumor associated neutrophils and macrophages, for example, have been indicated to suppress CD-8^+ cytotoxic T lymphocytes (CTLs) via activation of the T-reg pathway [38].

Prostate cancer development and androgen independence

The majority of prostate cancers are adenocarcinoma that develop within the prostatic gland. In very rare cases (less than 1%), other types of prostatic carcinoma are developed such as transitional cell carcinoma, small cell carcinoma and neuroendocrine tumors [39]. PCa is developed from CSCs which are produced due to genetic alterations in prostate stem cells, housed in basal ($CK5^+$) and/or luminal ($CK8^+$) cell layers of prostate gland [4, 40, 41].

The initial development of PCa relies on androgen, a steroid hormone that regulates development of male sex organs including prostate [42]. Although androgen is not required for the maintenance of the PCa CSC pool [43], like normal prostatic luminal cells, survival and growth of the luminal PCa cells crucially depend on androgen signaling [42]. The circulating androgen, testosterone, is mainly secreted by the testes but can also be formed by peripheral conversion of adrenal steroids. After been taken up by PCa cells, testosterone is converted by the enzyme 5α-reductase (SRD5A2) to dihydrotestosterone (DHT). DHT has 5 times higher affinity than testosterone to the steroid–thyroid–retinoid nuclear receptor, androgen receptor (AR) in PCa cells. After binding to DHT, ARs are dissociated from heat-shock proteins (HSP), phosphorylated by protein kinase A, and subsequently form homodimers. The dimerized AR further translocate into the nucleus and bind to target gene promotors, leading to cell survival, growth and production of prostate-specific antigen [44, 45].

Considering the importance of androgen signaling in PCa progression, androgen depletion is the most primary clinical approach to treat PCa at the early stage of the disease. However, after androgen depletion, castration-resistant prostate cancer (CRPC) can still develop, which grow and metastasize independently of androgen [46, 47]. Up to now, five possible mechanisms of androgen independency have been proposed that include the hyposensitive pathway, the promiscuous pathway, the outlaw pathway, the bypass pathway and lurker cell pathway [47].

In **the hyposensitive pathway**, PCa cells circumvent the ablation of androgen by increase of their sensitivity to the low level of blood androgen. This increased cell sensitivity can be caused by amplification of AR expression or increase of AR sensitivity to androgen, allowing sufficient activation of AR signaling by low level of androgen to promote PCa cell survival and growth [48, 49].

In **the promiscuous pathway**, mutations in ARs decrease AR specificity to androgen and allow activation by other non-androgen steroids and androgen antagonists, leading to androgen-independent tumor growth [50-52]. Alternatively, mutations occurring in AR co-activators or co-suppressors can enhance expression of AR targeting genes [53, 54].

In **the outlaw pathway**, growth factors like insulin-like growth-factor-1 (IGF1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF) activate ARs without androgen [55]. This outlaw pathway is induced by the elevated expression of growth factors, mutations in ARs, and hypo-activation of Receptor-tyrosine-kinase pathways (RTKs). The RTK hypo-activation can be induced by constant overexpression of HER-2/neu and/or loss of Pten, which induces phosphorylation and activation of ARs through MAPK and/or PI3K-AKT cascades in the absence of androgen, rendering androgen independent cell growth [56]. In all of the androgen-independent pathways mentioned above, ARs are indispensable.

In **the bypass pathway**, ARs are not necessary for cell survival and growth due to genetic alteration in other signaling [47]. Overexpression of BCL-2 is one of the causes of the bypass pathway [57]. In androgen-dependent PCa where BCL-2 is rarely expressed, androgen ablation induces cell apoptosis. This androgen depletion-induced apoptosis is blocked by BCL-2 overexpression [58]. The bypass pathway can also be induced by other proteins in addition to BCL-2 but more studies are still required.

In **the lurker cell pathway**, CRPC is generated by CSCs after differentiation in a distinct cell lineage when facing the stress from androgen ablation [59]. As discussed above, the basal CSCs are androgen independent and can differentiate into androgen-dependent PCa in the presence of androgen [43, 59]. In case of androgen ablation, although the majority of the PCa cells are suppressed, the androgen-independent CSCs survive and subsequently generate alternated androgen-independent cell populations. Apart from these five pathways, the presence of other androgen-independent pathways cannot be excluded [47]. Understanding

of the underlying mechanisms of androgen independency in each patient is therefore essential for the selection of clinical therapies.

Prostate cancer stem cells, EMT and metastasis

As discussed above, the development, recurrence, therapeutic resistance and bone metastasis of PCa are facilitated by CSCs [4, 60]. These cells are androgen-(in)dependent and are able to indefinitely self-renew to maintain their CSC pool [61-63]. After asymmetrical division and differentiation, CSCs can develop tumors bearing whole cellular heterogeneity. Numerous proteins have been found as CSC markers, including CD133, CD44, integrin α2β1, EpCAM, CXCR4 and aldehyde dehydrogenases (ALDHs) [4, 64]. Similar to normal stem cells, CSCs display a higher expression of the pluripotency genes Nanog, Oct-3/4, Sox2, Klf-4 and c-Myc [5]. Among them, Nanog and Sox2 are particularly considered as CSC markers and shown to be functionally involved in cancer malignancy [65, 66]. CSCs identified by one or a combination of these markers show increased motility, clonogenicity and tumor spheroid formation *in vitro*. *In vivo*, low amount of CSCs are sufficient to develop primary tumor growth and metastases recapitulating original tumor heterogeneity.

CSCs can be enriched from non-CSCs. This CSC plasticity driven by microenvironmental factors plays a fundamental role in promoting PCa metastasis. It has been shown that the microenvironment of bone marrow can induce PCa CSC enrichment from non-CSCs leading to metastatic colonization [67, 68]. Shiozawa et al unveiled that PCa cells are able to compete with hematopoietic stem cells in their respective niche to establish footholds, which further support metastatic tumor growth through enlarging the size of the CSC subpopulation [68]. Different stromal cells are involved in this cancer cell-niche interaction including bone progenitor cells, osteoblasts, cancer associated fibroblasts and macrophages [27, 33, 69-71]. Those stromal cells provide a systemic signaling network to drive cancer stem cell plasticity and promoting bone metastasis. When co-cultured with osteoblasts, for instance, PCa cells acquired an enlarged CSC subpopulation accompanied by the occurrence of EMT, indicating a crucial role of osteoblasts in the regulation of CSC and EMT plasticity. The underlying mechanisms, however, remain unknown [71].

The role of EMT in the metastatic cascade of PCa is still under discussion. It is hypothesized that the non-proliferative, migratory EMT cells lead invasion, intravasation and extravasation

at invasive front closely followed by CSCs [72]. The latter subsequently develops metastases. Another hypothesis suggests that EMT plasticity occurs in the cancer cells during metastatic colonization. In this theory the individual non-proliferative EMT cells invade, extravasate and home to distant organs followed by occurrence of MET, which endows the cells with enhanced CSC traits and proliferative potential, inducing tumorigenesis [30, 72, 73]. Since EMT has been reported closely linked to CSC induction, it is also proposed that the insufficient, partial EMT not only endow the cells with enhanced motility but also lead to the generation of CSCs which initiate the growth of epithelial cancer cells [28, 29].] Several studies demonstrated the existence of two types of convertible CSCs: the epithelial CSCs ($ALDH^{hi}$) and the mesenchymal-CSCs ($CD44^{hi}$). The mesenchymal CSCs invade, intravasate, survive the stress in circulations and extravasate. After homing to the metastatic niche, these mesenchymal-CSCs can shift to epithelial-CSCs which subsequently facilitate metastatic tumor growth [74].

Zebrafish as a model to study cancer metastasis

Zebrafish has been widely employed as a vertebrate model to study human cancer disease due to its high genomic conservation with human [75]. Due to the absence of a matured adaptive immune system at the embryonic stage of zebrafish, human cancer cells can be engrafted into zebrafish embryos, resulting in tumor growth, inflammatory responses, angiogenesis, and metastasis [76]. Engrafted human cancer cells respond and modulate the host microenvironment, which in turn support the growth and metastasis of the engrafted cells [77, 78].

One of the main advantages of zebrafish in cancer research is their optical transparency. Transparent zebrafish can be easily generated by inhibition of melanocytes through treatment with 1-phenyl-2-thiourea (PTU) or double genetic mutations of *nacre* and *roy* allowing non-invasive imaging of the engrafted cancer at a single cell resolution and during the whole metastatic process [79].

Zebrafish is also a suitable model to study cancer-microenvironment interactions due to the presence of numerus transgenic reporter fish lines, allowing visualization of cancer cell-stromal cell interactions such as angiogenesis and inflammatory response [76]. Due to a high accessibility of genetic modifications using morpholino oligomer and/or Crispr-Cas9, it is

possible to specifically modify stromal cells in zebrafish and study how each types of host stromal cells contribute to cancer progression [80].

Moreover, the zebrafish is an ideal model for anti-cancer drug discovery. The high yield of zebrafish embryos enables high-throughput anti-cancer drug screening. Small chemical inhibitors are easily taken up by embryos from the water or can be intravascularly injected into zebrafish to test their toxicity, chemical kinetics and anti-cancer efficacy [81]. Importantly, even patient-derived cancer cells can be transplanted and do grow in zebrafish embryos, allowing high throughput personalized anti-cancer drug screening [82]. More recently, immunodeficient zebrafish bearing *prkdc* and *il2rga* mutation has been developed [83]. This fish lacks T, B and NK cells in its whole lifespan, permitting long-term (up to 1 month) growth of engrafted patient-derived cancer cells towards metastasis without immune rejection. This model permits the assessment of disease prognosis and therapy response of patient-cancer cells. Altogether, these advantages make zebrafish an excellent model to accelerate fundamental cancer research as well as personalized anti-cancer drug discovery.

Scope of the thesis

In this thesis, I combined an advanced zebrafish xenograft model with *in vitro* cellular approaches and mice xenografts to study the early stage of PCa metastasis. Using this comprehensive research platform, I identified multiple key signaling pathways that play essential roles in promoting the onset of PCa metastatis. The pathways I discovered include Cripto-associated EMT plasticity, CDC-42-N-Wasp-Cortactin associated mechanosensing and mechanotransduction, microenvironment dependent NF-κB-Activin A signaling pathway, and AMPK-Autophagy dependent metabolic stress coping pathway. In **chapter 2**, I discuss the advantages of using zebrafish for human cancer research. I introduce how zebrafish xenografts are useful to study metastasis and explain in detail how I utilize zebrafish xenografts to dissect the CSC-microenvironment interaction at the early stage of metastasis. In **chapter 3**, I specifically focus on the metastatic phenotype of PCa cells using the zebrafish model. I show that human PCa cells can respond to the zebrafish microenvironment resulting in the elevated expression of EMT and CSC markers in cancer cells at the early stage of metastasis, leading to metastatic tumor initiation. This cell plasticity is, at least partially, regulated by Cripto, a Nodal co-receptor. Targeting of either Cripto or EMT transcriptional

factors significantly suppressed metastatic tumorigenicity. In **Chapter 4**, I show how cell-autonomous factors contribute to cancer stemness and metastatic initiation. I reveal that the EMT marker integrin-linked kinase (ILK) promotes the stem-like phenotype of the PCa cells at metastatic onset through controlling cell adhesion, cytoskeleton re-organization and mechanotransduction. I show that the metastatic potential of the PCa CSCs is highly dependent on their capacity for mechanosensing and mechanotransduction. Enhanced mechanotransduction is mediated by integrin β1 and ILK, which leads to CDC42-N-Wasp-Cortactin associated actin reorganization, induction of TAZ activation, expression of pluripotency genes, all leading to metastasis initiation. Suppression of mechanotransduction by RNA interference or specific inhibitors sufficiently inhibited PCa metastasis in both zebrafish and mice xenografts, and suppressed growth of patient derived organoids, highlighting the therapeutic potential of targeting mechanical signaling for disease intervention. In **Chapter 5** I present how the molecular and cellular components of the microenvironment regulates cell plasticity and promotes metastatic onset. I performed a cross-species transcriptomic analysis of metastasized cancer cells in zebrafish and compared the outcome with data obtained from mice xenografts as well as from patient-derived clinical datasets. This approach allowed me to demonstrate that human cancer cells exhibit almost identical response to the microenvironmental cues in zebrafish, mice and human. The cancer cell-microenvironment interaction in all systems drives expression of Activin A through activation of NF-κB in the cancer cells/CSCs, which sustains the CSC-like phenotype and promotes metastasis. From this transcriptomic analysis, I also found that cancer cells are facing severe metabolic challenges during metastatic tumor growth. I therefore in **Chapter 6** investigated the role of the AMPK-Autophagy-dependent metabolic stress machinery in protecting cancer cells from the metabolic challenges during PCa metastasis. I show that the expression and activation of AMPK was closely associated with the metastatic phenotype of the PCa cells. Suppression of either AMPK or its downstream autophagy significantly inhibited cell invasion and metastasis, indicating a clinical value of targeting AMPK and/or autophagy in PCa treatment. Finally, in **Chapter 7**, I summarized my finding and discuss how our finding potentially contribute to future clinical application against PCa metastasis.

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