Exploring the mechanisms of metastatic onset for novel treatment strategies

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Chapter 6

Summary and discussion

Cancer, one of the most lethal diseases [1], has witnessed a concerning increase in diagnosis rates over the past few decades, affecting even young individuals [2]. With a multitude of cancer types, organs, tissues, and cells resulting in diverse cancer phenotypes [3, 4], the treatment of tumors requires the development of tailored therapeutic approaches based on their specific characteristics [4]. Despite substantial progress in cancer research, aggressive metastasis, significant heterogeneity, and drug resistance pose significant challenges in eradicating tumor cells through conventional treatments, often leading to secondary or multiple recurrences and eventual patient mortality [5-8]. Hence, it is crucial to prioritize the development of more targeted, efficient, and safe treatment options.

As outlined in **Chapter 1**, tumor metastasis is a highly intricate process involving the alteration of cell adhesion to neighboring cells and the extracellular matrix within the original tumor site. This transformation triggers the activation of crucial cellular pathways, including integrin, adhesion proteins, and CD44, resulting in the remodeling of the cytoskeleton, cell morphology, and a transition from an epithelial-like to mesenchymal-like phenotypes known as epithelial-to-mesenchymal transition (EMT) [9]. Subsequently, a subset of these cells invade the vasculature, disseminating throughout the body via the circulatory system, where they face significant environmental stresses [10, 11]. While the majority of these cells succumb to harsh conditions, a small fraction, named circulating tumor cells (CTCs), can survive and eventually exit the vasculature through a process called extravasation [12]. Following extravasation, CTCs revert back their transformation, transitioning from a mesenchymal-like to an epithelial-like state through a process known as mesenchymal-to-epithelial transition (MET), and commence rapid growth, generating new tumors [10]. Increasing evidence underscores the significance of a distinct subset of tumor cells possessing stem cell-like properties characterized by CD44+/CD24-, known as cancer stem cells (CSCs), in executing this cascade of events and driving tumor metastasis [13, 14]. Given the pivotal roles played by CTCs and CSCs in cancer metastasis, we aimed to elucidate the intricate intracellular signaling pathways associated with these cell types using zebrafish xenograft models and patient-derived organoids.

In **Chapter 2,** we focused on CSC-like cells derived from prostate cancer (PCa) patients. Through comprehensive analysis of clinical datasets, we observed high expression of cell-matrix interaction genes, cell adhesion proteins, and the putative mechanosensor TAZ within this population. We generated patient-derived xenograft (PDX) zebrafish and organoid models to assess the mechanical response of cells to matrix stiffness, including cell-generated forces. Our findings demonstrate that mechanical transduction plays a key role in shaping the PCa CSC-like population during metastasis. This mechanical signaling axis operates through the involvement of β1-integrin, ILK, CDC42, N-Wasp-dependent cytoskeletal tension, and TAZ nuclear translocation. Activation of this pathway induces the expression of stemness genes NANOG and OCT4, ultimately initiating the formation of metastatic tumors. As a proof of concept, we used pharmacological inhibition of TAZ to successfully abolish PCa metastasis in zebrafish and the growth of PDX-derived organoids (**Table 1**). These results underscore the role of mechanotransduction in driving the aggressiveness of prostate cancer, thereby establishing this pathway as a promising therapeutic target for future research.

Table 1. Novel cancer treatment strategies explored in this thesis.

Name	*Mechanism*	*Drug type*	*Application*
K975	K975 inhibits the interaction between YAP1/TAZ and TEAD, thereby reducing stemness of cancer cells	Chemical molecule	Prostate Cancer: PC-3, PC-3M-Pro4, C4-2B/LAPC9 Organoids
Rapalink-1 and SBI-0206965	Synergistic inhibition of mTOR and AMPK pathways to prevent survival of CTCs	Chemical molecule	Prostate Cancer: PC-3M-Pro4, LAPC9 Organoids
Ru-p(HH)	Local photoactivation releases metallic ruthenium ions inducing toxicity	Chemical molecule	Glioblastoma: U87MG
Ru-p(MH)	Local photoactivation releases metallic ruthenium ions inducing toxicity	Chemical molecule	Glioblastoma: U87MG
Ru-p(MM)	Local photoactivation releases metallic ruthenium ions inducing toxicity	Chemical molecule	Glioblastoma: U87MG
AKPC-siYAP/TAZ	CD44-specific targeting LNPs deliver YAP1/TAZ siRNA to tumor cells to inhibit tumor growth	siRNA	Breast Cancer: MDA-MB-231, HCC38 Prostate Cancer: LAPC9 Organoids
APC-sgPLK1	CD44-specific targeting LNPs deliver Cas9 mRNA and PLK1 sgRNA to tumor cells to inhibit tumor growth	Cas9 mRNA + sgRNA	Melanoma: SK-MEL-28/A375

In **Chapter 3**, our research focused in the transformation of prostate cancer cells into CTCs, which requires metabolic rewiring to adapt to the diverse stresses encountered within the circulatory system. While previous studies highlighted the contribution of metabolic remodeling of the AMPK and mTOR pathways to promote tumor cell growth, it remained unclear whether similar processes occur in previous steps, such as CTC survival. To address this knowledge gap, we investigated the dynamic activation of AMPK and mTOR in circulation. Through a series of *in vitro* and *in vivo* experiments, we discovered that AMPK can partially compensate for the metabolic deficiencies resulting from impaired mTOR function during cellular stress, thereby promoting the survival of tumor cells. Furthermore, we identified that administration of an mTOR inhibitor, as it is currently used in chemotherapeutic treatments, induced the activation of AMPK. Based on this information, we devised a new therapeutic approach utilizing both mTOR and AMPK inhibition to target metastatic onset. This combined treatment strategy exhibits synergistic efficacy in eliminating tumors in both *in vitro* in LAPC9 organoids derived from prostate cancer patients and *in vivo* in zebrafish models. These findings underscore the potential of therapeutic strategies that concurrently target both the mTOR and AMPK pathways for inhibiting prostate cancer and shed light on the intricate interplay between metabolic rewiring and tumor cell survival during metastasis.

In Chapter 4, we evaluated the therapeutic efficacy of integrin-specific targeted photoactivated drugs. To enhance selectivity and biocompatibility, peptide conjugation has emerged as a promising approach for anticancer drug development. In this study, we designed

cyclic, photoactivated ruthenium peptide prodrugs specifically tailored for active targeting of glioblastoma cells. Our novel compounds employed the Ac-X_1RGDX_2-NH_2 pentapeptide, which targets integrin and binds to the ruthenium center through two light-cleavable coordination bonds involving X1 and X2 amino acid residues (X1 or X2 = His/H or Met/M). We synthesized and characterized three compounds with the general formula [Ru(Ph_2phen)^2(Ac-X_1RGDX_2-NH_2)]Cl_2 (Ph_2phen = 4,7-diphenyl-1,10-phenanthroline). We conducted comprehensive *in vitro* and *in vivo* investigations to reveal that the mechanism of photoactivated cell killing varied depending on the specific metal-bound amino acid residues. Interestingly, despite the distinct mechanisms, all three Ru-peptide conjugates, namely Ru-p(HH), Ru-p(MH), and Ru-p(MM), exhibited comparable antitumor effects against glioblastoma in the zebrafish xenograft model. These compounds demonstrated effective targeting of brain tumors, which can be attributed to their ability to traverse the brain-blood barrier (BBB), which is a significant hurdle in the delivery of anticancer drugs to glioblastoma patients. These findings highlight the potential of integrin-specific targeted photoactivated drugs in glioblastoma treatment.

In Chapter 5, our focus shifted to the development and assessment of the therapeutic efficacy of siRNA-based anticancer agents delivered by lipid nanoparticles (LNPs). Similar to photoactivated therapy, RNA therapy has emerged as a promising modality for cancer treatment, offering precise regulation of cancer-related genes. While LNPs currently represent the most advanced clinically approved non-viral vectors for RNA therapy, their antitumor effectiveness is limited by their tendency to accumulate primarily in the liver following systemic administration. Consequently, there is a need to enhance the delivery efficiency of LNPs to tumor cells. To address this, we introduced a set of lipopeptides (AKPC) into LNPs, incorporating a CD44-specific targeting peptide A6 (KPSSPPEE). Through this modification, we achieved a tumor-specific LNP delivery system capable of co-delivering siRNAs targeting the transcriptional co-activators YAP and TAZ. We observed significant improvement in the tumor targeting of LNPs to breast cancer both *in vitro* and *in vivo* after the CD44-specific lipopeptide modifications. Upon encapsulation of siYAP/TAZ, CD44 peptide-modified LNPs effectively mediated gene silencing, apoptosis, and cell death in 2D cell cultures and 3D spheroids. In a zebrafish breast tumor cell xenograft model, systemic administration of CD44-targeted LNPs induced robust silencing of YAP/TAZ and downstream genes, leading to significantly enhanced tumor suppression compared to bare LNPs. In summary, our study presented an efficient lipid nanoparticle platform for targeted cancer therapy.

Throughout the research presented in this thesis, we explored the molecular mechanisms that govern tumor cell behavior during metastasis. We employed a combination of *in vitro* experimental validations, zebrafish models, and patient-derived organoids to investigate the impact of key pathways involved in CSCs and CTCs regulations during tumor metastasis. Based on these findings, we successfully developed and validated a diverse range of novel therapeutic strategies targeting tumors, including the utilization of chemical inhibitors targeting of the YAP/TAZ pathway, combination therapies involving dual inhibitors for mTOR/AMPK, light-activated chemotherapy facilitated by ruthenium-peptide conjugates, and systemic gene therapy employing peptide targeting, nanoparticle delivery, and RNA interference.

At present, although mTOR inhibitors are used as tumor suppressor drugs [15-17], there are also reports that tumor cells develop resistance to mTOR inhibitors [18, 19]. Considering the cross-talk between AMPK and mTOR, we believe that the drug combination targeting both of them can increase treatment effectiveness and overcame resistance. We are hopeful that continued research on the molecular mechanisms and pathways that cancer cells use to survive will new options and possibility of other drug combinations. Extending beyond conventional small molecule inhibitors, our investigation into targeted light-activated chemotherapy has exhibited promising outcomes for brain cancer therapy, which displayed favorable results in parallel with already established chemotherapy approaches used in cancer treatments. Particularly noteworthy is the higher efficacy of ruthenium couplers that have been fine-tuned with targeting peptides in treating brain cancer. Importantly, we provide compelling evidence that these compounds can successfully trespass the blood-brain barrier, one of the major obstacles for those treatments. This breakthrough underscores the potential clinical utility and application of these compounds as therapeutic agents.

As we have mentioned, a significant challenge associated with chemical drugs lies the appearance of treatment resistance [20, 21]. To overcome that challenge, the current focus has shifted to the rapid development of gene-based anticancer drugs. However, these therapeutic agents are usually unstable. In consequence, it is of critical importance to devise delivery system for effective cellular uptake in a short time window. Leveraging our peptide modification approach in tandem with an FDA-approved nanoparticle system, we achieved a marked enhancement in both the delivery efficiency of nucleic acid therapeutics and in precisely targeting tumor cells. Looking ahead, this nanoparticle modification methodology holds promise for integration with proteomic techniques, facilitating the identification of even more precise tumor cell-specific targeting sites. Such advancements will pave the way for the development of increasingly tailored and refined nanoparticle delivery systems.

Although we acknowledge that the results achieved thus far are limited in their preclinical application, we believe that they represent a robust proof of concept for highlighting their significant potential.

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