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TGFβ signaling in cancer progression

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Chapter 7

General discussion

Cancer is the second leading cause of death. Globally, approximately 1 in 6 deaths are due to cancer (1). Patients with metastatic cancer are normally treated with systemic therapies, including chemotherapy, targeted therapy, hormonal therapy, and more recently immunotherapy (1). In breast cancer, HER2-positive patients can be treated with *trastuzumab* (*Herceptin*), a monoclonal antibody targeting the HER2 protein (2). Breast cancer patients who are carriers of germline BRCA mutations can be treated with poly(ADP-ribose) polymerase (PARP) inhibitors (3). However, for most TNBC patients, who have the most aggressive breast cancer phenotype and the worst poor prognosis, there is no clinically meaningful targeted therapy available. Thus, there is an urgent need to identify new therapeutic targets and develop novel treatment regimens (4). In this thesis, we focused on uncovering novel signaling mechanisms that promote TNBC metastasis and identifying new druggable targets for the treatment of TNBC patients. For metastatic melanoma, patients with the V600E BRAF mutation can benefit from BRAF inhibitors. However, approximately 40% of patients will develop resistance during chemotherapy, and melanoma recurs (5). Hence, in this thesis, I investigated the possibility of overcoming the drug resistance of metastatic melanoma by targeting TGFβ signaling with a small molecule TGFβ type I receptor kinase inhibitor.

Uncovering the DUB activity landscape in breast cancer

In the late stage of breast cancer, TGFβ-induced cytostatic effects are blunted, and instead, TGFβ promotes cancer progression by stimulating epithelial to mesenchymal transition (EMT), invasion and metastasis of cancer cells (6). The reversible ubiquitination of TGFβ signaling components is emerging as a key process regulating the intensity, duration and specificity of TGFβ intracellular signaling pathways. Deubiquitinating enzymes (DUBs) that are overly active (or amplified or mutated in cancer) in aggressive cancers and promote TGFβ-induced pro-oncogenic effects are considered potential targets for specific inhibitor development. In **Chapter 2**, we provide an overview of the DUBs that regulate TGFβ/BMP signaling and discuss the potential of several DUB inhibitors for cancer treatment. Breast cancer is a very heterogeneous disease, and it has been divided into multiple subclasses based upon histopathological characteristics and molecular/cellular features. To further classify breast cancer and develop prognostic and predictive biomarkers, large-scale conventional genomic, proteomic and metabolomic profiling studies have been performed (7). However, no large-scale DUB activity profiling has thus far been performed on breast cancer cell lines and clinical samples. Thus, in **Chapter 4**, we describe experiments in which we established two DUB activity profiling platforms to uncover the landscape of global DUBs in 52 human breast cancer cell lines and 52 patient tumor tissues. In our study, both profiling methods identified UCHL1 as a potential target in TNBC and aggressive tumors. These two DUB activity profiling methods can also be applied in the future study of other cancers or diseases.

Establishing animal xenograft models for studying cancer metastasis

To better understand the molecular mechanisms that underlie cancer metastasis *in vivo* and identify and validate new therapeutic targets, we need rapid, robust and clinically relevant animal models. We established an efficient, reliable and low-cost model in which human fluorescently labeled cancer cells are injected into early zebrafish embryos. In **Chapter 3**, a detailed protocol is provided on how to construct these zebrafish xenograft model

experiments by injecting human breast cancer cells into the perivitelline space or duct of Cuvier (Doc) to analyze the intravasation or extravasation of cancer cells at 6 days after injection. We took advantage of the transparency of transgenic (*fli:EGFP*) zebrafish embryos (8), which have enhanced green fluorescent protein-labeled vasculature, to quickly assess the invasive behavior of the injected mCherry fluorescently labeled cancer cells in the zebrafish embryos. Moreover, the pharmacological inhibition of druggable targets can be easily performed by adding small-molecule compounds to the water containing zebrafish eggs. We applied this model in experiments in **Chapter 4** to test UCHL1's function in TNBC metastasis by overexpressing/knocking down the gene encoding the UCHL1 protein or inhibiting UCHL1 activity with the specific covalent inhibitor 6RK73. In addition, we also describe experiments using this model to investigate the potential for genetic and pharmacological targeting of the TGFβ type I receptor in vemurafenib-resistant melanoma in **Chapter 6**. In addition to the zebrafish models, rodent xenograft cancer models were employed, as detailed in **Chapter 4**, to further validate and consolidate the metastatic ability of UCHL1 by intracardially injecting TNBC cells with misexpression of UCHL1 into female BALB/c athymic nude mice. Both the zebrafish and mouse xenograft TNBC models showed that high UCHL1 activity correlated with pronounced metastatic traits.

Unraveling the mechanism by which UCHL1 promotes TNBC metastasis

During breast cancer metastasis, EMT plays an important role by mediating breast cancer cell invasion. During EMT, the cobble stone-appearing and highly polarized epithelial cancer cell phenotype switches to a highly motile mesenchymal cell phenotype with a fibroblastic-like appearance. In **Chapter 4**, we first investigated the effect of UCHL1 knockdown in TNBC cells on several mesenchymal markers and found that UCHL1 depletion decreased mesenchymal markers at both the RNA and protein levels. Since TGFβ signaling is a key driver of the EMT process, we next examined the correlation between UCHL1 expression and TGFβ/SMAD signaling. We observed that UCHL1 can promote the levels of carboxy-tail phosphorylated and activated SMAD2/SMAD3 by protecting TβRI and SMAD2 from ubiquitination and subsequent degradation (Figure 1). The interaction of UCHL1 and TβRI occurred in early endosomes and was triggered by TGFβ ligand stimulation. Importantly, the promoting function of UCHL1 in metastasis could be blocked by a selective TβRI chemical inhibitor in TNBC cells. Although our results indicated a key role for UCHL1 in stimulating breast cancer metastasis by regulating TGFβ signaling, we do not exclude the possibility that UCHL1 may also promote metastasis by targeting other signaling pathways. Previous studies have shown that UCHL1 can also regulate AKT signaling (9) and hypoxia-inducible factor (HIF)1α signaling (10).

Discovery of potential blood-based biomarkers for metastatic TNBC

As UCHL1 is abundantly present in all neurons (accounting for 1-2% of total brain protein) (11), UCHL1 has been developed as a blood-based biomarker for the clinical diagnosis of traumatic brain injury (12). To investigate the possibility of using UCHL1 as a potential biomarker for the clinical diagnosis of metastatic TNBC, we tested UCHL1 levels by ELISA and observed that TNBC patient sera contained higher UCHL1 levels than sera from normal individuals (**Chapter 4**). Furthermore, we found that UCHL1 was highly enriched in the exosome fraction of ER- patient sera and TNBC cell conditioned media (Figure 1). Moreover,

exosomes isolated from TNBC cells were found to promote TGFβ/SMAD signaling and promote the migration and extravasation of recipient TNBC cells. Recently, two groups also detected UCHL1 in exosomes. One group found higher UCHL1 protein levels in patient serum exosomes than in serum exosomes from normal individuals, which could be correlated with chemotherapy resistance in breast cancer (13). Another group detected higher levels of UCHL1 mRNA in exosome preparations from serum samples from patients with early-stage high-grade neuroendocrine lung cancer than in exosomes derived from patients with early-stage non-small-cell lung cancer and healthy donors (14). Taken together, these studies suggest that extracellular vesicle-derived UCHL1 levels play an important role and that UCHL1 may act in cancer cells in both autonomous and paracrine manners to stimulate tumorigenesis.

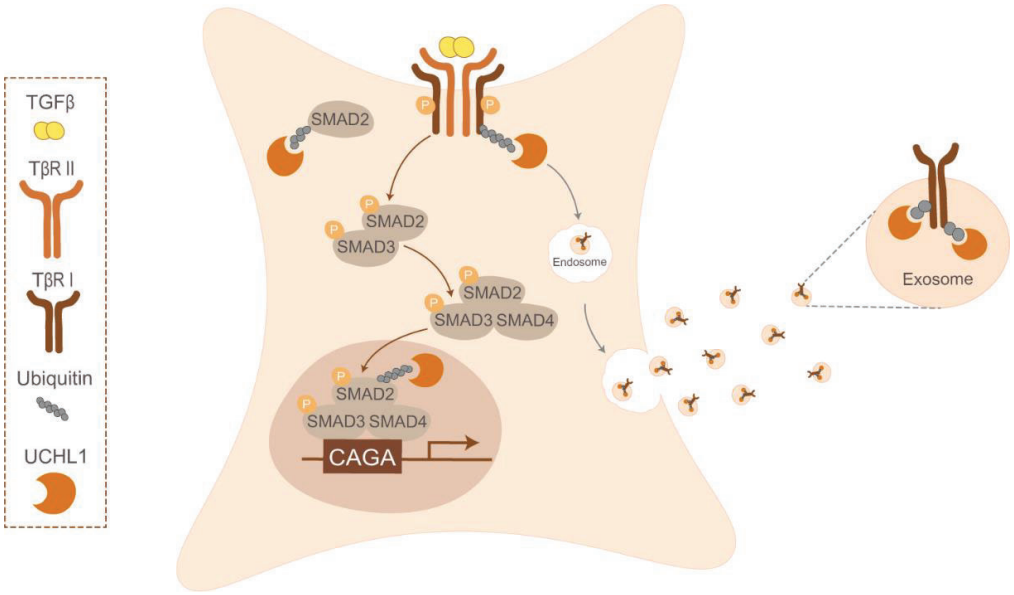

Figure 1. A working model for the role of UCHL1 in TGFβ signaling. UCHL1 promotes the phosphorylation of SMAD2 by protecting TβRI and SMAD2 from ubiquitination in TNBC cells. The interaction between UCHL1 and TβRI occurs in the early endosome. The interaction between UCHL1 and SMAD2 shifts from the cytoplasm to the nucleus after TGFβ treatment. UCHL1 can also be detected in TNBC cell exosomes.

Investigating a potential drug for targeting UCHL1 activity in TNBC

The ability of UCHL1 to promote TGFβ/SMAD signaling critically depends on its catalytic DUB activity. Mutation of the UCHL1 protein from WT to C90A (DUB activity-inactivating mutation) abolishes its stimulatory effect on TGFβ receptor signaling, migration and invasion in TNBC cells. We therefore attempted to find a specific inhibitor of UCHL1 activity. This inhibitor was identified in a patent application of Mission Therapeutics (15). This led to the chemical synthesis and characterization of highly selective and potent UCHL1 inhibitors, including 6RK73 (**Chapter 4**). The 6RK73 compound covalently binds to UCHL1 and blocks its activity both *in vitro* and *in vivo*. When we tested 6RK73 in TNBC cells and patient samples, it showed specific inhibition of UCHL1 among all the DUBs in the breast cancer cell and patient tissue lysates. Mechanistically, 6RK73 strongly inhibited pSMAD2, which functions in TGFβ signaling, by inducing the degradation of TβRI/SMAD2 in TNBC cells (Figure 2). Importantly, 6RK73 strongly inhibited TNBC cell migration and extravasation in

the scratch assay and zebrafish xenograft model. Thus, 6RK73 (or its analogues) has the potential to become a new drug for TNBC treatment. In addition, UCHL1 has been reported as a novel functional marker for liver fibroblasts and a therapeutic target in chronic liver disease (16). This study highlighted the opportunity for applying UCHL1 activity inhibitors in chronic liver disease treatment. However, many of the reported DUB inhibitors have off-target effects, which inhibits their clinical development (17). For 6RK73, we have not yet found such off-target effects, but future studies may reveal them. Another challenge is that DUBs are promiscuous and have multiple substrates. This may also lead to unwanted side effects of specific DUB inhibitors. A specific inhibitor that targets the DUB–substrate interaction may provide more accurate DUB-target interference (18). Therefore, in the future, we can target the UCHL1-TβRI interaction to more specifically interfere with the ability of UCHL1 to promote TGFβ receptor signaling.

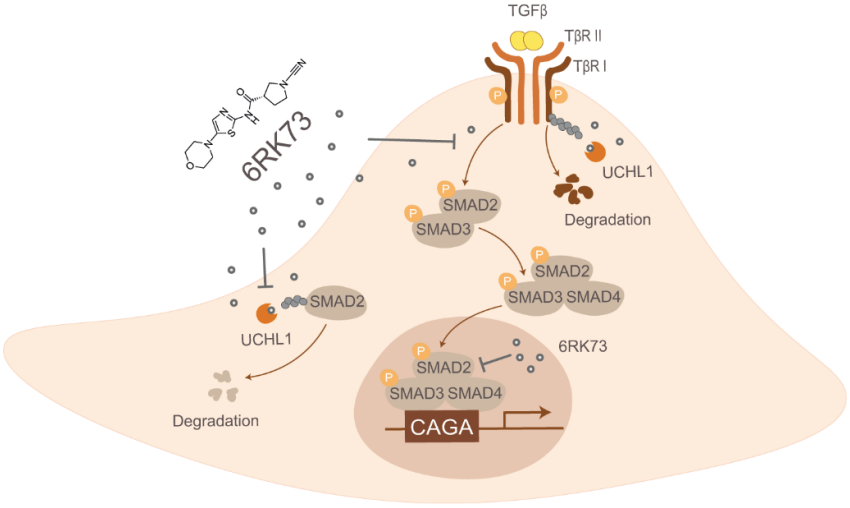

Figure 2. The role of 6RK73 in TGFβ signaling. 6RK73 blocks UCHL1 activity covalently and inhibits the phosphorylation of SMAD2 by decreasing the levels of TβRI and SMAD2 in TNBC cells.

Development of an activity-based probe for monitoring UCHL1 activity *in vivo*

To further study UCHL1 activity *in vivo*, we attempted to make a UCHL1 activity-based probe. However, we were faced with many challenges. More than 100 DUBs have been identified, which can be grouped into different subfamilies. Within each subfamily, there is extensive sequence/structural similarity in the catalytic domains, making the design of selective inhibitors difficult. Additionally, creating inhibitors with the ability to penetrate the cell membrane and retain DUB inhibitor activity upon modification with fluorescent groups is difficult (19). In **Chapter 5**, we provided evidence for the first potential small-molecule UCHL1 activity-based probe (8RK59) that can specifically label an active version of UCHL1 *in vitro*. Moreover, this probe can efficiently pass through the cell membrane and monitor UCHL1 activity in (living) cells and in zebrafish embryos. In the follow-up study, we found that this probe could be used for tracking UCHL1 activity in TNBC cells during the metastatic process in a zebrafish xenograft model (Figure 3). Although 8RK59 only targets UCHL1 among all the DUBs, we also identified a non-DUB target, Parkinson's disease protein 7 (PARK7), using an unbiased mass spectrometry-based approach. UCHL1 is also called PARK5; both UCHL1 and PARK7 have been functionally linked to Parkinson's

disease (20). In addition, profiling Parkin-binding partners identified UCHL1 and PARK7 in the same protein-protein interaction network using tandem affinity purification (21), which indicated that these two proteins may interact with each other. However, further studies are needed to clarify the underlying correlation between UCHL1 (PARK5) and PARK7 and to further improve the selectivity of the UCHL1 activity probe.

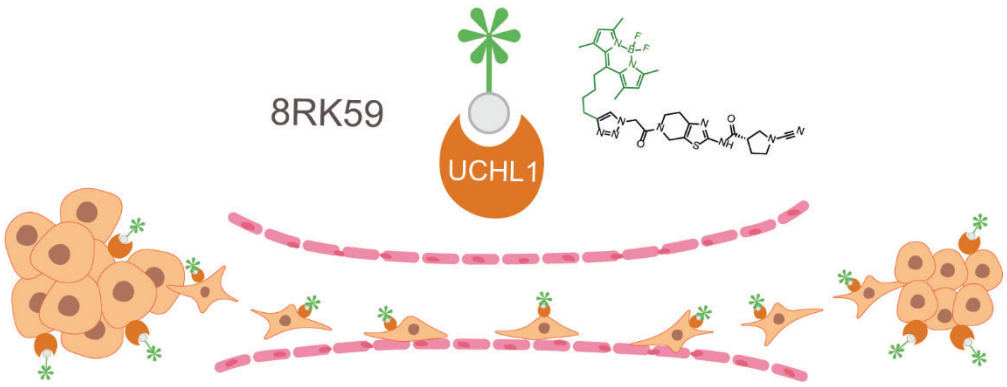

Figure 3. The application of 8RK59 in tracking UCHL1 activity during TNBC metastasis.

Targeting TGFβ signaling to improve drug-resistant melanoma treatment

Drug resistance is a major reason for the high mortality rate in late-stage melanoma. Recent studies have revealed elevated TGFβ signaling in BRAF inhibitor-resistant melanoma (22). However, the potential for targeting TGFβ signaling in cases of advanced melanoma has not been investigated. In **Chapter 6**, we provided evidence that the TβRI inhibitor SB-431542 blocked the proliferation and SMAD2 phosphorylation of vemurafenib-resistant patient-derived cells. Importantly, pharmacological and genetic inhibition of TβRI effectively blocked the clonogenicity of human BRAF-mutant melanoma cells and inhibited extravasation of melanoma in a zebrafish xenograft model. Although targeting of TGFβ signaling has been considered a potential therapy in several metastatic cancers, first-generation inhibitors targeting TβRI have failed because of overt cardiac toxicity (23). Hence, when applying TβRI inhibitors in clinical treatment, unwanted side effects need to be taken into consideration, and applying treatment with an intermittent dosing regimen may overcome cardiac toxicity (24-26).

Conclusion

Overall, this thesis uncovered the DUB activity landscape in breast cancer and identified UCHL1 as a potential tumor-promoting protein that facilitates TGFβ-induced TNBC metastasis. The UCHL1 activity inhibitor 6RK73 strongly mitigated TNBC invasion and metastasis. Moreover, the development of the UCHL1 activity-based probe 8RK59 has opened a new window for monitoring UCHL1 *in vivo*. Significantly, TNBC patient sera contain high UCHL1 levels, suggesting that UCHL1 may be a candidate blood-based biomarker. It will be interesting in future studies to test the value of measuring UCHL1 expression and/or activity in detecting disease early, selecting patients and/or monitoring therapy response. This thesis suggests the potential therapeutic value of targeting TGFβ signaling in the (pre)clinical setting for drug-resistant melanoma.

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