Uncovering vulnerabilities in triple-negative breast cancer

He, J.

Chapter 6

Discussion and future perspectives

Worldwide, breast cancer remains the leading cause of female cancer-related death[1]. TNBC, characteristic of the absent expression of ER, PR and HER2 amplification, represents the most aggressive form of all breast cancer subtypes, is highly heterogeneous and associated with worse prognosis[186, 187]. Despite the initial increased response to the standard-of-care chemotherapy, TNBC often exhibits intrinsic or acquired drug resistance, and subsequently, recurs in local and distal organs. Targeted therapies have long been pursued for the treatment of TNBC, but rarely demonstrate satisfactory clinical outcomes. Therefore, improved understanding of the intricate biological basis underlying TNBC insensitivity to targeted agents and defining new therapeutic opportunities are of the upmost importance. The work presented in this thesis was aimed at understanding the molecular mechanisms of TNBC drug resistance, discovering TNBC-specific kinase signaling transduction dependencies and identifying novel drug targets and effective therapies for TNBC. The results from the chapters were summarized and discussed in a broader context and future perspectives were also provided.

1. FRET imaging in cancer drug discovery

With the advent of a vast number of fluorescent proteins and advanced microscopy facilities, fluorescence optical imaging has been widely employed in preclinical cancer drug discovery. Fluorescence-based functional imaging approaches have been extending our understanding of cancer biology and drug efficacy, by providing high-throughput phenotypic readouts, including changes in morphology, cell proliferation, invasion, migration and angiogenesis[99, 272]. In **Chapter 2** I exploited a FRET (fluorescence resonance energy transfer)-based high throughput imaging approach to investigate the dynamics of ERK and AKT kinase activity in response to a collection of kinase inhibitors (KIs) in TNBC cell lines. FRET, also known as Förster resonance energy transfer, refers to the nonradiative transfer of excited state energy between colocalized donor and acceptor fluorophores[273-275]. When combined with genetically encoded kinase reporters, FRET microscopy enables the direct visualization of kinase activity in highly spatiotemporal resolution in living cells[119].

ERK and AKT are two key signaling elements of the canonical Ras/MAPK and PI3K/AKT/mTOR pathways, respectively. Activation of PI3K/AKT/mTOR pathway is common in TNBC[41, 112, 134], due to the recurrent copy number loss of negative regulators (e.g., PTEN, INPP4B) and activating mutation of PIK3CA[19]. Analogous to PTEN loss, Ras/MAPK pathway is often activated by the loss of NF1 and DUSP4, despite the scarcity of activating mutations in TNBC. In **Chapter 2**, the MEKi-resistant and AKTi-resistant TNBC cell models were transfected to stably express FRET-based biosensors specifically for ERK and AKT activity, allowing for high-throughput FRET imaging. Upon exposure to KI library, real-time ERK and AKT activity dynamics were recorded and quantified, and subsequently, the key parameters were correlated with proliferative response. This study has associated

ERK and AKT kinase activity with the anti-tumor effects of kinase drugs and revealed differential kinase dependencies of treatment-refractory TNBC cells. The work has not only advanced the live cell high-content imaging-based quantification of kinase activity profiles in TNBC, but also discovered possible off-target effects of clinical kinase drugs.

Considering the heterogeneous nature of this disease and the intricate pathway interconnections, potential use of our FRET imaging-based approach has yet to be made by incorporating multiple kinase biosensors in various TNBC cell lines representative for different molecular subtypes. A simultaneous application in non-TNBC cells and normal mammary cells could also aid in finding TNBC-specific kinase signaling addictions and discovering undesired adverse effects. Besides ERK and AKT sensors, a large amount of other FRET probes (kinases, GTPases, phosphoinositides, Ca^{2+} and metabolites) have also been developed and used in multiple biological imaging studies[276-278]. Recently, Kuchenov and colleagues have described a high-content imaging platform using 40 FRET biosensors to profile cancer-relevant signaling networks[279]. Despite that the authors announced the potential of the platform to image up to 384 FRET sensors in a single experiment, how the complex signaling crosstalk is related to the various biological processes and eventually influence the cancer phenotypes is yet to be addressed. Our study in **Chapter 2** demonstrated the feasibility of quantifying real-time kinase activity in TNBC cells in response to clinical kinase drugs and correlating with cell proliferation, a hallmark of cancer. Our work has revealed the differential kinase dependencies in MEKi- and AKTi-resistant TNBC cells, thus providing new opportunities to explore effective therapeutic kinase targets in treatment-refractory cancer cells, as well as assess the drug efficacy and possible off-target effects of clinically used drugs.

It is obvious that a lacking of tumor microenvironment has limited the translation of the findings derived from 2D cell culture into the clinic. This disadvantage can be compensated by utilizing the advanced and clinically relevant organoids and PDXs systems. As fluorescence imaging profoundly relies on the detection of emitted light, the absorbance and scattering by various tissues can constrain the depth in which the light penetrates. Another downside of fluorescence imaging is that the intrinsic fluorescence of biomolecules, and in some cases the kinase drugs, increases background signals. Another major application of FRET is fluorescence lifetime imaging microscopy (FLIM), which is suitable for the analysis of intracellular environment, independent of intensity-based ratiometric FRET calculation. In contrast to bulk FRET analyses where signals from FRET molecules in varied conformational states are captures and averaged, single-molecule FRET (smFRET) measurement allows for elucidating the structural heterogeneity of FRET in individual signaling molecules[280]. Nonetheless, along with the advances in optical microscopy, well-designed FRET probes and improved image analysis algorithms, FRET imaging has proved imperative and valuable in studying cancer biology and drug efficacy.

2. **Exploring novel therapies for TNBC via high-throughput screen**

High-throughput compound or gene screening plays an essential role in identifying novel therapeutic targets and combination treatment in cancer therapy. Protein kinases represent attractive drug targets due to their involvement in various cellular functions. **Chapter 2** utilized FRET imaging technology to screen a well-established inhibitor library containing about 400 kinase drugs in TNBC cells. The library spans 14 signaling pathways by targeting 118 kinases. Integrated proliferative response profiling and ERK- and AKT-based kinase activity dynamics analysis uncovered the differential kinase signaling dependencies of treatment-resistant TNBC cells. Specifically, MEKi-resistant cells were responsive to inhibitors against PI3K pathway but refractory to EGFR-targeted inhibitors, whereas AKTi-resistant cells were sensitive to EGFR/MAPK pathway blockade but showed resistance against mTOR inhibitors. These findings suggest that the molecularly heterogeneous TNBC may leverage alternate surviving pathways to by-pass kinase-targeted inhibition, thereby conferring resistance. However, the screening only partially explained the regulatory effects of the kinome on TNBC cell proliferation as the KIs target less than one third protein kinases (118/518). Moreover, less selective KIs could increase the likelihood of false discovery.

In contrast, genetic perturbation permits high-throughput screening in the desired set of genes, such as the entire genome or specifically the kinome. Suppression of gene expression can be achieved either by RNAi (siRNA and shRNA), or by CRISPR/Cas9 technology. Recently, an in vivo study has reported 40 novel modulators of breast cancer response to paclitaxel by performing genome-wide RNAi[281]. A gene signature was derived from the screen hits with the potential to predict patient outcomes. By carrying out kinome-scale siRNA screen, **Chapter 3** identified specific vulnerable kinase targets in EGFRi- and mTORi-resistant TNBC cells. Pharmacological inhibition of these targets greatly suppressed TNBC cell proliferation in different resistant scenarios, highlighting the potential of targeting these kinase vulnerabilities to combat the hard-to-treat disease. Moreover, a kinome-wide siRNA screen has been performed in combination with lapatinib in **Chapter 3** to study the synthetic lethality interactions with EGFR-targeted inhibition and discover potential therapies to overcome EGFRi-resistance. Unlike compound screen, siRNA screening provides a high specificity for targeting the protein kinases in question, with the potential to look into the kinase signaling network in the kinome scale. This work has led to the identification of FYN kinase as a negative regulator of EGFR/PI3K/AKT pathway in EGFRi-resistant TNBC cells. Thus, dual targeting of EGFR and FYN could lead to cell death in TNBC cells representing the intact pathway. In addition, targeting FYN also enhanced the anti-proliferative effects of PI3Ki and AKTi. This is in line with the finding in **Chapter 2** that EGFRi/MEKi-resistant cells were responsive to PI3K/AKT inhibition as the resistant cells were more likely to depend on elevated PI3K pathway for proliferation in

the presence of FYN. However, whether pharmacological inhibition of FYN could synergize with EGFR inhibitors has yet to be confirmed. Supportively, several FYN inhibitors have been developed, albeit less selective.

Using high-throughput KI screening, **Chapter 4** has discovered a novel combination therapy (i.e., AEE788 + rapamycin) to overcome mTORi-resistance in TNBCs. Various types of cancer arise owing to dysfunction of mTOR signaling, and can confer higher susceptibility to mTOR inhibitors. Despite rapalogs (rapamycin and its analogs) have proven effective in a range of preclinical studies, clinical success is restricted to only a few rare cancers. It was reported that rapalogs are cytostatic, thus unlikely to cause tumor regression. When used as monotherapy, rapalogs exhibited modest anti-tumor activity due in part to incomplete mTOR inhibition. Other mechanisms underlying this insensitivity involve redundant signal transduction and feedback loops. Rapalog-mediated mTORC1 inhibition is thereby not sufficient to induce comprehensive pathway inactivation, necessitating the development of combination therapy in this scenario. The AEE788 + rapamycin combination identified in **Chapter 4** represents a novel therapeutic strategy to combat TNBC. In this study, cheminformatics-based target prediction and validation using siRNA have revealed putative targets of AEE788, which play key roles in determining rapalog efficacy. The combination, by targeting multiple kinases, not only sustains inhibited MAPK activity, but also effectively suppresses mTOR signaling, thereby eliciting synergistic anti-proliferative effects in TNBC. In addition, the findings are complementary to the presently published target spectrum of the kinase drug AEE788. The synergistic effects of AEE788-everolimus combination have been reported in prostate, germ and renal tumor cell lines[198, 200, 201]. Specifically, by exploiting the integrated compound screen, target prediction and validation approach, I discovered the potential combination therapy for TNBC, and studied the mechanisms of action of the synergy in the highly heterogeneous TNBC. Provided the reported toxicity of AEE788 in clinical trials[282, 283], reduced doses of both drugs in the combination might decrease adverse effects while generating synergistic anti-tumor effect. Nevertheless, future studies assessing the effects in in vivo models are warranted for potential translation of the results in the clinic.

3. Bioinformatics-based therapeutic target identification

Genetic alterations are thought to be favored during the initiation, development and progression of cancer in an evolutionary fashion[106, 284, 285]. Over 80% TNBCs exhibits TP53 mutation[19], being a major reason for causing gene instability in this disease. Insightful analysis of genomic sequencing data in breast cancer exploiting bioinformatics holds the promise for the identification of novel therapeutic targets[152, 286, 287]**.** For example, two independent bioinformatics-based studies have demonstrated that PIM kinase regulated chemotherapy response in TNBC and can be explored as novel targeted therapy[288, 289]. In

Chapter 5, ADMIRE analysis of copy number and gene expression profiles was performed across a set of triple-negative tumors. This work has led to the discovery of several novel TNBC driver genes, besides the known oncogene EGFR and MYC. Of the identified driver genes, I functionally validated the most promising driver gene ASAP1 (an Arf GTPase-activating protein) and revealed its biological role in TNBC progression through transcriptome-wide bioinformatics study. Of relevance, high level of ASAP1 expression correlates with poor prognosis in patients with TNBC. Several studies have also reported the role of ASAP1 in breast cancer invasion and metastasis[263, 290-292]. Studies by Onodera and colleagues showed that ASAP1 was localized at invadopodia together with cortactin and paxillin to form a trimeric protein complex, which accelerated extracellular matrix degradation and subsequently promoted tumor cell motility and invasion[263]. Consistently, among the most significantly enriched GO terms derived from DEGs upon ASAP1 depletion, as shown in **Chapter 5**, are extracellular matrix organization and regulation of protein complex assembly. With a couple of small molecule modulators of GTPase-activating proteins (including ASAP1) being developed[293, 294], the regulatory role of ASAP1 in tumor invasion and migration can be assessed in more relevant 3D systems (such as tumor organoids and PDXs), and thus assisting in the translation of the potential driver gene ASAP1 into a clinical therapeutic target.

4. Future perspectives

A major concern in this thesis is to which extent the findings can be translated into the clinic. Monolayer cell culture has been the main cancer model for conducting the research. Although several studies have argued that a sufficiently large panel of breast cancer cell lines represents the genomic and proteomic landscape of breast tumors and provides a reasonable model for breast cancer study[91, 284, 295], the results derived from 2D culture systems can be variable when tested in 3D and in vivo models, which more realistically mimic the cell-cell communication and microenvironment[93, 99, 296, 297]. By utilizing state-of-art CRISPR/Cas gene editing technology, genome-wide integration of fluorescent proteins into PDX models allows us to monitor the dynamics of gene expression in living TNBC cells affected by the identified novel therapies. The continuing efforts in enlarging the biobank of breast tumor organoid and well-established PDX mouse models both provide an excellent possibility to assess the findings of this thesis for potential clinical translation. Another concern is that the evaluation of the anti-cancer effect of the proposed therapeutic targeting strategies has been limited to proliferation assays. Multi-faceted functionality studies are merited in a context-dependent manner. Application of our established FRET imaging approach in 3D culture of PDX-derived TNBC cells could better dissect the tumor heterogeneity in both highly temporal and spatial resolution. In addition, single-cell sequencing has proved to be an imperative tool to study the transcriptional diversity and variability in therapeutic response within a range of cancer types, including

TNBC. More importantly, future assessment of the novel therapies discovered in this thesis using organoids and PDXs would prove invaluable in advancing precision medicine in TNBC.

5. Conclusions

Altogether, the aim of the studies presented in this thesis was to systematically identify gene/kinase susceptibilities of refractory TNBC cells, and reveal novel potent targeted therapies for TNBC as monotherapy or in combination with approved kinase drugs. The work has identified important kinase signaling dependencies of TNBC cell proliferation (**Chapter 2**) and discovered novel multi-kinase targeting strategy to overcome mTORi resistance (**Chapter 4**) using compound library screen, found key regulators of TNBC resistance against EGFR-targeted inhibitors (**Chapter 3**), and identified and validated novel driver genes via integrated computational algorithms and bioinformatics approach (**Chapter 5**). This work provides novel insights into the molecular basis of TNBC response to clinical kinase drugs and provokes potential therapeutic targeting strategies for the incurable TNBC.