Uncovering vulnerabilities in triple-negative breast cancer

He, J.

Chapter 1

General introduction and scope of the thesis

1. Breast cancer

Worldwide, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer mortality among women. In 2018, about 2.1 million people were diagnosed, accounting for almost 1 in 4 cancer cases in females, and over half a million people died from this disease [1]. Early transcriptomic profiling studies have categorized breast cancer into at least four clinically relevant intrinsic subtypes: luminal-A, luminal-B, HER2-enriched and basal-like [2]. Breast cancer can also be classified into three major receptor subtypes, based on the presence of molecular markers estrogen (ER), progesterone (PR) receptors and human epidermal growth factor 2 (HER2), i.e. HR+/HER2- (70% of patients), HER2+ (15%-20%) and triple-negative (15%) [3]. While overlapping among the classifications, these subtypes have been characterized for distinct prevalence, prognoses and therapeutic strategies (Figure 1). HR+ tumors are more prevalent in older women, whereas triple-negative tumors are more likely to occur in women who are younger, African-American or Hispanic [4]. The prognoses of triple-negative tumors are worse than that of HR+ or HER2+, with approximately 1 year and 5 years median overall survival, respectively [5-7].

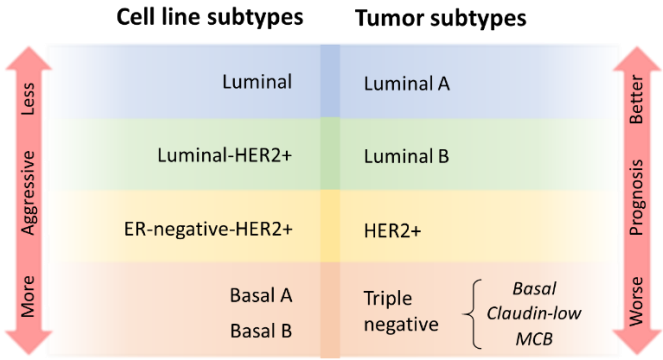

Figure 1. Breast cancer subtypes and prognosis. According to the status of ER, PR, HER2, breast cancer is classified as luminal A, luminal B, HER2 positive, and triple negative, where triple negative tumors can be further differentiated into at least basal, claudin-low, MBC (metaplastic breast cancer). The morphological features of the subtypes in tumors and cell lines accord well, with luminal tumors having better prognosis and luminal cell lines less aggressive than that in triple negative tumors and cell lines. (Adapted from Dai et al, 2017)

2. Triple-negative breast cancer

2.1 Molecular stratification

Triple-negative breast cancer (TNBC) represents about 15% of all breast cancers, but proves to be a highly malignant subtype, with earlier age of onset, high risk of metastasis and unfavorable clinical prognosis. Given the nature of heterogeneity, a collection of studies has profiled the distinct genetic landscape and therapeutic response of TNBC. By

analyzing transcriptomic profiles of 587 TNBC cases, Lehmann et al demonstrated that TNBC consists of seven subtypes (TNBCtype) and displays a heterogeneous biology with differential response to various therapies [8]. By the measurement of in total 2188 genes and consensus clustering, they recognized seven molecular subtypes, namely basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), luminal androgen receptor (LAR) and unstable (UNS). Each subtype represents a unique genetic background and driver signaling pathways. For example, BL1 subtype depicts increased cell cycle and DNA damage response gene signature, while BL2 involves high growth factor signaling. M illustrates gene enrichment in cell motility, growth and differentiation, which is partially resembled by MSL, but with low expression of proliferative genes. IM is associated with immune cell processes, whereas LAR characterized for elevated androgen signaling. To address the molecular heterogeneity of TNBC and the associated therapeutic responses, **Chapter 4** exploited a broad kinase inhibitor library screen across ~20 TNBC cell lines representative for the six main TNBC subtypes. Our research demonstrated a poor correlation of TNBC molecular subtypes with their proliferative responses to various kinase inhibitors. Of relevance, a retrospective study found the TNBCtype to be an independent predictor of pathological complete response (pCR) for patients receiving standard chemotherapy regimens [9]. Research by Ring et al revealed a small gene set algorithm (101 genes) showing the ability to recapitulate TNBCtype and predict therapeutic response, which might be more manageable in the clinic by focusing on the most relevant molecular alterations in the diverse TNBC categories [10]. Yet, limited by their small size of cohorts, these studies pinpoint the clinical precautions for the potential use of molecularly TNBC subtyping. Lehmann and colleagues recently refined their sub-classification into four (TNBCtype-4) tumor-specific subtypes (BL1, BL2, M and LAR), having recognized the influence of infiltrating lymphocytes and tumor-associated stromal cells on IM and MSL subtypes [11]. Other attempts to stratify TNBC using mRNA and DNA profiling include the four subtypes by Burstein et al: basal-like immune suppressed (BLIS), basal-like immune activated (BLIA), mesenchymal (MES) and LAR [12]. The diversity in TNBC classifications and their notable intersection not only confirm the great heterogeneity in this disease, but also accentuate a requirement for more comprehensive and optimized designation of TNBC molecular subtypes to eventually translate in the clinical settings.

2.2 Chemotherapy

Notwithstanding the extensive efforts in discerning the molecular landscape and complexity of tumor biology, no targeted therapies have been approved for TNBC. To date, cytotoxic chemotherapy remains the standard of care in the management of TNBC. Commonly used chemotherapeutic agents include alkylating agents, anti-tubulins,

anthracyclines, platinums and antimetabolites. Typical adjuvant and neoadjuvant therapy consists of an anthracycline (Adriamycin) plus an alkylating agent (Cyclophosphamide). Patients with TNBC have higher neoadjuvant response rate than those with other breast cancer subtypes [13, 14]. The phenomenon of high likelihood of pCR but with worse prognosis is referred to as triple-negative paradox [15], which might be attributed to its highly proliferating property and high risk of recurrence.

Despite progress in optimizing systemic therapy, very few patients with metastatic breast cancer (including TNBC) have benefited from the treatment [16]. Efforts in exploring combination chemotherapy have been attempted to improve the clinical outcomes. Although heightening response rates in comparison with single agents, combination therapies have to be compromised with increased adverse effects and no significant survival benefits [17]. With the exception of poly ADP-ribose polymerase (PARP) inhibitors for the treatment of germline BRCA-mutated (gBRCA) HER2- disease [18], there are currently no targeted options beyond chemotherapy in the TNBC settings.

Considering that optimal systemic chemotherapy has yet to be established, and that molecular research has been assisting in the discovery of driver mutations in TNBCs, novel alternate therapeutic strategies are underway and more targeted treatments could become accessible.

2.3 Targeted therapy

Triple-negative tumors are likely to relapse after chemotherapy despite initial response. Patients with TNBC who do not respond to neoadjuvant and adjuvant regimens, in a large proportion, represent intrinsic or acquired drug resistance. Large-scale genomic profiling of TNBC tumors has identified major mutations such as TP53 loss (84%), c-MYC amplification (40%), PTEN loss (35%) and PIK3CA mutation (7%) [19, 20]. Yet, these frequent mutations have not been druggable, pressing a necessity for exploring actionable targeted therapeutic options.

2.3.1 Poly ADP-ribose polymerase

PARP is a constitutively expressed nuclear enzyme essential for DNA repair in response to DNA single-strand and double-strand breaks, therefore facilitating genomic stability and cell survival. PARP deactivation leads to DNA double-strand breaks during replication. Tumor cells with wild-type BRCA1/2 rely much on homologous recombination for DNA repair. In BRCA1/2-deficient cells, double-strand breaks are repaired via PARP-mediated DNA metabolic processes, independently of homologous recombination. Thus, genetically or pharmacologically targeting PARP causes severe cell death in tumors representing BRCA1/2 deficiency, a classical example of synthetic lethality [21].

Critical findings in both preclinical and clinical studies have led to the approval of PARP inhibitor talazoparib by Food and Drug Administration (FDA) of the United States, for the treatment of germline BRCA-mutated HER2- breast cancer [18, 22]. In addition, alternate mechanisms underlying BRCA1/2 dysfunction have been recognized in different cancer types, including somatic mutations and epigenetic alterations, so-called "BRCAness" [23]. As such, PARP inhibition has gained much attention as a promising synthetic lethality therapeutic strategy for treating cancer with BRCA deficiency. Importantly, the anti-tumor activity of PARP inhibitors in combination with chemotherapy or targeted therapy has also been investigated in several clinical trials [20]. Nevertheless, it has to be noted that both gBRCA and "BRCAness" occurs merely in sporadic breast cancers.

2.3.2 Epidermal growth factor receptor and angiogenesis

Epidermal growth factor receptor (EGFR) is a transmembrane protein that, upon ligand binding, transduces extracellular signals (e.g. EGF, transforming growth factor-alpha, betacellulin) to intracellular signaling molecules, thereby triggering multiple signaling cascades regulating cell growth, migration, proliferation and apoptosis [24-26]. Overexpression of EGFR is commonly observed in several human cancers. EGFR is amplified in 2% of breast tumors, but is more frequently overexpressed in basal-like subtype than non-basal-like ones [19, 27]. Several agents targeting EGFR have been approved for clinical use, including small-molecule kinase inhibitors (KIs) and monoclonal antibodies (mAbs). However, no statistically significant prognostic improvements have been achieved in patients with TNBC in comparison to platinum-based therapy [28, 29]. Two independent studies reported that compensatory feedback loop via AKT and HER3 conferred acquired resistance against EGFR-directed treatments [26, 30]. In line with these findings, kinome-wide siRNA and lapatinib combination screen in **Chapter 3** has demonstrated that, a Src family member FYN, conferred TNBC resistance against EGFR kinase-targeted inhibition via negatively regulating EGFR/PI3K/AKT signaling. A multi-centric neoadjuvant Phase II study of cetuximab plus docetaxel demonstrated modest activity in operable TNBC, despite acceptable toxicity [31]. In another Phase II trial, there was no increased efficacy with the combination of panitumumab over that expected from chemotherapy alone in metastatic TNBC (mTNBC) [32].

Angiogenesis plays a central role in breast cancer metastasis and survival. Vascular endothelial growth factor (VEGF) is the most important angiogenic factor with proven significance in metastatic breast cancer. Given the high metastatic potential of TNBC, the development of VEGFR inhibitors is of great interest in combating this incurable disease. Bevacizumab is a mAb targeting angiogenesis by slowing the growth of new blood vessels, and approved for the treatment of a series of diseases, including colon cancer, lung cancer and glioblastoma. Several clinical studies have documented that treatment

with Bevacizumab improved pCR rates of patients with TNBC [33-35]. However, the survival benefits of Bevacizumab were marginal in most neoadjuvant trials.

2.3.3 PI3K/AKT/mTOR pathway

Activation of PI3K/AKT/mTOR pathway is repeatedly observed in TNBC, which could be attributed to loss of negative regulators such as PTEN (35%) and INPP4B (30%), as well as activating mutation of PIK3CA (7%) [19]. Provided the essential role in modulating tumor cell metabolism and proliferation, targeting this signaling axis represents a promising therapeutic avenue.

The anti-tumor activity of PARP inhibitors is limited to a small portion of TNBCs with gBRCA. A preclinical study showed that, in BCRA-proficient TNBC, PI3K blockade resulted in homologous recombination impairment and sensitization to PARP inhibition, and effectively suppressed tumor growth in patient-derived xenografts (PDXs) [36]. In BELLE-4 Phase II/III study, the addition of a pan-PI3K inhibitor buparlisib failed to show improvement in progression-free survival (PFS) in the full and PI3K pathway-activated populations with HER2- breast cancer [37].

Ipatasertib is an oral and highly selective AKT inhibitor and has been evaluated in several clinical trials. Results from a randomized Phase II study, LOTUS, demonstrated improved PFS of patients with advanced TNBC for ipatasertib plus paclitaxel group compared to chemotherapy alone (6.2 versus 4.9 months, respectively) [38]. Notably, the median PFS was 9.0 months with ipatasertib versus 4.9 months with placebo in the predefined cohorts with PIK3CA/AKT1/PTEN alteration. This has provided a rationale for the ongoing randomized phase III IPATunity130 trial testing the combination in patients with activated PI3K signaling (NCT03337724).

Phosphorylated mTOR, the active form, is present in the majority of TNBC populations [39]. **Chapter 4** also showed that rapalog-resistant TNBC cells presented a high phosphorylation level of mTOR in response to mTOR inhibition. A Phase II trial showed 36% clinical benefit rate from combination of everolimus and carboplatin in patients with mTNBC [40]. Another Phase I study on 52 females with mTNBC indicated that treatment with liposomal doxorubicin, bevacizumab, and temsirolimus or everolimus achieved improved responses, but the benefits were restricted to patients with aberrations in PIK3CA, AKT or PTEN [41]. In neoadjuvant setting, the addition of everolimus increased adverse events without additional benefits in patients with stage II/III TNBC [42].

Given the high prevalence of activation in TNBC, effectively targeting PI3K pathway warrants the development of more specific inhibitors and a better pathway aberration-based preselection of patients, as well as more clinical investigation.

2.3.4 Androgen receptor

The LAR subtype of TNBC is characteristic of AR signaling and demonstrates sensitivity to anti-androgen agents both in vitro and in vivo [43]. In a Phase II trial of 424 patients with HR- metastatic breast cancer, 12% of the cohort were tested to be AR+ [44]. Treatment with AR antagonist bicalutamide exerted 19% clinical benefit rate (defined as complete response, partial response, or stable disease) for more than 6 months in AR+ patients, with a median PFS of 12 weeks. Another clinical trial reported that AR-driven gene signature was associated with overall survival treated with enzalutamide, a highly potent anti-AR agent [45].

2.3.5 Cyclin-dependent kinases (CDKs)

Various cyclin-CDK complexes are responsible for the regulation of cell cycle progression both in normal and malignant cells. CDKs are naturally inhibited by CDK inhibitors. Loss-of-function mutations of these inhibitors or overexpressed cyclins lead to uncontrolled proliferation during tumorigenesis. mTOR inhibition could elevate cyclin D1 expression level in TNBC cells, therefore allowing for continuous proliferation of the rapalog-refractory cells, as shown in **Chapter 4**. Several cyclins are amplified in TNBCs [19]. Three CDK4/6 inhibitors, palbociclib, ribociclib and abemaciclib, have been approved by FDA for the treatment of HR+/HER2- breast cancer. Targeting CDK4 by palbociclib efficiently eliminated chemo-refractory cells and breast cancer stem cells in TNBC [46]. Amplification of MYC is frequently observed in TNBC (40%) [19]. Selective inhibition of CDK1 and CDK2 resulted in TNBC tumor regression in mouse xenografts harboring MYC amplification, highlighting the potential of targeting CDK1 and CDK2 in MYC-driven TNBC [47].

2.4 Emerging novel therapy

2.4.1 Immune checkpoint inhibitors

Among all breast cancer subtypes, TNBC has the highest mutational frequency, with an increased likelihood of generating neoantigens by immunogenic mutations [48, 49]. Gene expression profiling analysis has identified the IM subtype of TNBC, characteristic of elevated expression of genes modulating antigen production and T cell function [8], providing a strong rationale for testing immunotherapy. High PD-L1 expression was reported in 20% of patients with TNBC, associated with enriched tumor-infiltrating lymphocytes (TILs) [50]. Consistently, results from other studies indicated that elevated PD-L1 level strongly correlated with high TIL number and improved prognosis in neoadjuvant settings [51, 52]. Recently, while the manuscript being written, atezolizumab, a mAb against PD-L1, has received the approval for individuals with mTNBC based on the Phase III trial IMpassion130 [53]. Compared to nab-paclitaxel treated group, combination with

atezolizumab prolonged PFS from 5.5 to 7.2 months in the intention-to-treat population, and from 5.0 to 7.5 months in the PD-L1-positive subgroup, respectively. No new adverse effects were identified with the combination. Several other clinical trials evaluating the efficacy of immunotherapy in TNBC are still ongoing.

2.4.2 Antibody-drug conjugate (ADC)

Differential glycoprotein expression between malignant and normal cells has sparked the design and development of ADCs. Trop-2 is a commonly expressed glycoprotein in TNBC, making it an attractive therapeutic target [54]. Sacituzumab govitecan (IMMU-132), an ADC targeting Trop-2 for selective delivery of SN-38, has demonstrated 30% overall response rate with mild toxicity in heavily pretreated patients with mTNBC [54]. These findings have led to the breakthrough therapy designation by FDA and a confirmatory Phase III study is currently recruiting.

3. Drug resistance

Tumor heterogeneity is dominantly responsible for both intrinsic and acquired resistance and represents a major hurdle for established therapy. For the intrinsic resistance, sensitive tumor cells are eliminated, subsequently resulting in an accumulated population of residual tumor cells which are genetically and histologically distinct from the sensitive ones. Contrarily, acquired resistance occurs when initially susceptible tumor cells obtain the ability to resist the activity of the therapy despite continued drug administration.

3.1 Resistance to chemotherapy

TNBC is a highly heterogeneous disease with an unfavorable prognosis. Paradoxically, the initial higher pCR rate to chemotherapy fails to correlate with better overall survival. TNBC is much aggressive with high frequency of developing resistance to chemotherapy. Tumor recurrence and resistance can be due in part to intratumoral heterogeneity of TNBC, which allows selective enrichment for cancer stem cell-like subpopulation. Single-cell sequencing of TNBC patients has also demonstrated that resistance occurred through adaptive selection of pre-existing genotypes by neoadjuvant chemotherapy, with associated transcriptional reprogramming of the resistant signatures [55]. Some chemotherapeutic agents, such as doxorubicin and paclitaxel, are substrates of ATP-binding cassette (ABC) transporters. The efflux of drug by these transporters results in decreased drug concentration in tumor cells, hence weakening the efficacy [56, 57].

3.2 Resistance to targeted therapy

Advancements in high-throughput next-generation sequencing technologies and massive parallel sequencing studies, as well as integrated bioinformatics-based tumor biology investigation have expanded our knowledge on the genomic complexity and intratumoral heterogeneity of breast cancer. Consequently, several targetable vulnerabilities have been identified in predefined patient subgroups to tailor treatment for improved therapeutic benefits. However, the activity of targeted therapy in the management of TNBC remains modest, due in a large part to drug resistance. Resistance involves reactivation of signaling pathways targeted by the drug and activation of compensatory signaling pathways, which can be resulted from dysregulated feedback loops and pathway crosstalk [58-60]. Evidences have shown that, in response to PI3K/mTOR inhibition, activated β-catenin served as an alternate survival pathway conferring glioblastoma and colorectal cancer resistance both in vitro and in vivo [61, 62]. Concordantly, a study employing colorectal cancer patient-derived sphere cultures and mouse tumor xenografts showed that blocking Wnt/β-catenin pathway by tankyrase inhibition reverted resistance to PI3K and AKT Inhibitors [63]. In TNBC, MEK-targeted inhibition triggered dynamic reprogramming of the kinome, thereby limiting its anti-cancer effects [58]. **Chapter 2** elucidated that drug resistant TNBC cells remained active ERK activity when treated with EGFR inhibitors. Proteolytic shedding and inactivated negative regulators of receptor tyrosine kinases (RTKs) have been shown to elevate surface RTK levels and enhance mitogenic signaling, resulting in kinase inhibitor resistance [64-66]. Contrarily, targeting FYN, a negative regulator of EGFR signaling identified in **Chapter 3**, released the activity of downstream PI3K and AKT signaling, rationalizing the co-targeting strategy to subvert resistance against inhibitors targeting EGFR/PI3K/AKT signaling axis. Recently, results from kinome dynamics mapping have concluded that maintenance of AURKA after drug treatment conferred therapy failure in breast cancer treated with inhibitors targeting PI3K/AKT/mTOR pathway [67]. Our research in **Chapter 4** found that elevated cyclin D1 expression contributed in part to mTORi resistance. It has also been reported that FAK/IGF1R dependent PI3K pathway activation drives tumor resistance against mTOR inhibitors in various cancer cell lines and mouse models, including TNBC [68].

Altogether, drug resistance remains one of the major determinants limiting drug efficacy in TNBC therapy. With the various resistance mechanisms being well studied, the discovery of new therapeutic strategies and novel attainable targets are still of high demand.

4. Novel therapeutic strategies and target identification approaches

4.1 Role of gene expression profiling in patient stratification

TNBC is a highly aggressive disease of a great histological and biological heterogeneity, which has a notorious impact on the primary end points in clinical trials. Gene expression profiling plays a central role in dissecting this complexity and generating clinical benefits. A comparative study on TNBC clinical outcomes has noted that genomic signatures strongly correlate with response and survival after polychemotherapy typically in the basal-like subgroup of triple-negative tumors [69]. These high-risk basal-like tumors with high proliferation scores are very sensitive to chemotherapy, whilst the lower-proliferating ones are less responsive with a worse prognosis. Thus, in the latter scenario, novel therapies are warranted. These findings highlight the importance of using gene expression data in patient stratification to predefine homogenous tumor groups and provide clinically relevant information. Supportively, encouraging results presented at ASCO 2018 showed that the biomarker selected group (i.e. with alterations in PIK3CA, AKT or PTEN genes) greatly contributed to the prolonged PFS by AKT-targeted therapy (AZD5363) plus first-line paclitaxel in the metastatic setting of TNBCs [69].

4.2 Exploiting combinatorial strategies to subvert resistance

Therapeutic inactivation of an essential protein, in some cases the protein complex, produces selective pressure, allowing tumor cells to evolve mechanisms of resistance. Provided the molecular complexity and interplay between signaling pathways within tumor cells, single agents are insufficient to block driving survival pathways to tackle TNBC, rationalizing multi-targeted remedy to neutralize intrinsic and/or acquired resistance. Multi-targeted therapy can be achieved by either combination of highly selective drugs or multiple-targeting single agents based on polypharmacology.

Inhibitors targeting different protein kinases have been tested in combinations, as well as with other therapy modalities. Activation of PI3K/AKT/mTOR pathway is common in TNBC. A randomized Phase II study has assessed the combination efficacy of AKT inhibitor AZD5363 with paclitaxel as first-line treatment for mTNBC [70]. The patients with alterations in PI3K pathway demonstrated remarkably improved median PFS of 9.3 months in the combination group, whereas the median PFS was 3.7 months for the paclitaxel alone group. Co-inhibition of MEK and BRAF has been attempted to target separate components in the same pathway to restrain downstream signaling reactivation. Indeed, a Phase III trial reported that the combination reduced the risk of disease progression compared to BRAF-targeted monotherapy [71]. In **Chapter 2**, FRET imaging-based high throughput kinase activity screen for ERK and AKT revealed that sustained ERK activity conferred EGFRi-targeted inhibition resistance in TNBC cells, presenting an excellent possibility to simultaneously target EGFR and downstream MEK/ERK signaling. Another approach for combination therapy is to targeting compensatory pathways in parallel. MEK inhibition was shown to reactivate ERK via upregulating multiple RTKs. The

combination with multi-RTK targeted inhibitor sorafenib could sensitize TNBC cells to MEK inhibition and greatly suppressed tumor regression [58]. PI3K pathway is known to be activated by MEK inhibition, thus combining MEK-targeting drugs with inhibitors against PI3K/AKT/mTOR has been applicable. Significant tumor shrinkage by MEK inhibition was found in TNBC mouse model when combined with PI3K inhibitor [72, 73], as well as for the combination with AKT inhibitor in TNBC patients with both MEK and PI3K pathway activation [74]. Co-targeting of MEK and mTOR or upstream EGFR/VEGFR has also been tested in clinical trials (NCT02583542, NCT01586624 and NCT00600496). In our study, **Chapter 3** has identified FYN as a negative regulator of EGFR signaling, and proposed co-targeting FYN to reverse drug resistance in cancer cells with elevated EGFR expression, including TNBC. Recently, the emerging combinations of targeted therapy for the treatment of TNBC have been summarized in a review [75]. Alternatively, polypharmacology with multi-target KIs might be exploited to circumvent drug resistance. Targeting multiple signaling elements by single agents could resist pathway reactivation and reprogramming, thus ultimately delay the onset of resistance. An important example is the utilization of ponatinib, a dual PDGFRA/FGFR1 inhibitor, which overcomes PDGFRA inhibitor resistance by disrupting the driver signaling event (PDGFRA) and the adaptive FGFR1 pathway [76]. By utilizing an integrated systematic screening and cheminformatics approach, **Chapter 4** revealed the synergistic effects of multi-kinase targeted inhibitor AEE788 on rapalogs treatment in TNBCs and its underlying polypharmacology.

The discovery of immune checkpoints and recent clinical success of their blockade have led to a surge in cancer immunotherapy. It has been reported that lymphocytic infiltration correlates improved response to chemotherapy and clinical outcomes in TNBC, pressing the potential of combining immune checkpoint inhibition and chemotherapy for the treatment of this disease [50-52, 77]. Recently, results from Phase II TONIC trial have been presented at ASCO 2018, indicating that an upregulation of pro-immunogenic signatures and an increase in T-cells and T-cell clonality were observed after cisplatin or doxorubicin treatment, in which their combination with nivolumab (an anti-PD-1 mAb) enhanced the overall response rate in mTNBC [70]. And these higher-responsive cohorts will be expanded in the next phase of the trial owing to the encouraging results. More recently, FDA has approved the combination of PD-L1 inhibitor atezolizumab with nab-paclitaxel for PD-L1-positive, advanced TNBC [53]. Other clinical trials testing the efficacy of immunotherapy in combination with neoadjuvant or adjuvant chemotherapy in early-stage TNBC are currently on going (NCT03036488, NCT02954874, NCT03197935, NCT03281954 and NCT02926196) [78]. In addition to combination with chemotherapy, immune checkpoint inhibitors are also under clinical investigation along with molecularly targeted agents. The KEYNOTE-162 trial testing the combination of niraparib and pembrolizumab in mTNBC reported remarkably higher objective responses (67%) in

patients with gBRCA [78]. It was reported that Abemaciclib monotherapy increased T cell inflammatory signature in murine cancer model, whereas combination with anti-PD-L1 drugs resulted in complete tumor regression [79].

Dosing regimens of combined drugs may also have impact on therapeutic potency. For example, studies using TNBC xenografts argued that sequential administration of combined drugs effectively prevented oncogenic pathway rewiring and elevated apoptotic response, as illustrated by the sensitized response to doxorubicin by the pretreatment of EGFR-targeted inhibitor erlotinib [80]. In another preclinical study involving distinct sequential regimens of CDK inhibitor flavopiridol and topoisomerase inhibitor irinotecan, researchers found that the combination effectively induced apoptosis (43%) of colon cancer cells when administrated in a specific order (irinotecan followed by flavopiridol), whereas both the reverse order (15%) and simultaneous therapy (30%) were less effective [81].

4.3 Identification of novel therapeutic targets

High-throughput genetic perturbation drug screens have led to the discovery of gene essentialities, synthetic lethality interactions and drugs with the potential to improve the treatment of cancer, including TNBC [82-84]. For example, a genome-wide siRNA screen has identified proteasome addiction in basal-like TNBC cells [83]. Pharmacologically targeting proteasome inhibited TNBC tumor growth in mice. Another siRNA-based screen across 117 cell lines spanning 10 cancer types revealed cancer driver gene dependencies and enabled the prediction of cancer cell drug responsiveness [84]. Provided the off-target limitation of RNA interference (RNAi), a genome-scale shRNA screen was performed across 501 cancer cell lines, accompanied by DEMETER computational algorithm to remove false positives [85]. Novel kinase dependencies in TNBC have been discovered through a FRET biosensor-based kinase inhibitor screen in **Chapter 2** with a possibility to prioritize therapeutic targets. Comprehensive drug combination screens also reported pivotal findings, resulting in 44 effective combinations evaluated in mouse models [86]. These evaluations have led to the clinical testing of two promising combinations bortezomib + clofarabine and paclitaxel + nilotinib in Phase I trials (NCT02211755 and NCT02379416, respectively).

Gene expression-based approaches have been employed to identify therapeutic targets in TNBC. The integration of genomic and proteomic platform on breast cancer cell lines and tumors revealed five potential candidate genes specifically enriched in TNBC, including STAT5A, POSTN, MYLK, HLA-A and EPHA2 [87]. Recently, an integrative analysis of mutations, copy-number changes, mRNA expression, gene fusions and DNA methylation profiles has identified co-occurring actionable alterations, suggesting opportunities for

combination therapies [88]. Additionally, computational algorithm-based aggregation in recurrently altered genomic regions has also assisted in the identification of novel cancer drivers/therapeutic targets [89-91]. **Chapter 5** has exploited the robust ADMIRE algorithm to prioritize 148 candidate genes driving TNBC cell growth and proliferation. siRNA-based functional screen further validated, besides known EGFR and MYC oncogenes, novel driver genes including ASAP1 which showed high amplification frequency and gene expression in TNBC cohorts, and associated with poor prognosis in patients. A pooled shRNA breast cancer cell line screen, integrated with siMEM algorithm and omics data, has identified BRD4 as a potential target in luminal breast cancer. In addition to known drivers, this approach also found two potential new amplified drivers ZNF652 and YEATS4, depletion of which significantly inhibited cell proliferation in breast cancer cell lines with corresponding amplification [91]. Another integrative study assessing frequent copy number alterations (CNAs) in TNBCs identified and functionally validated 13 TNBC addiction genes. The role of one potential drug target KIFC1 was mechanistically studied and, accordingly, a selection biomarker was developed to identify patients with tumor exhibiting centrosome amplification [89].

5. Recent advances and new opportunities

5.1 Tackling cancer heterogeneity

Cancer cell lines (CCLs) represent easy-to-manipulate systems for high-throughput drug and genetic screens. Equipped with automated liquid handling techniques, large-scale high-throughput screens are now much attainable and less time-consuming. However, faithfully recapitulating cancer heterogeneity requires large panels of available CCLs. With the goal of capturing this diversity, two large projects have been initiated, namely the Cancer Cell Line Factory and the Human Cancer Model Initiative. The former one aims to create over 10,000 CCLs for research use [92], while the latter one aims to generate about 1000 new in vitro cancer models [93]. Recently, organoids have emerged as novel in vitro 3D cancer models with the capability of self-organizing and phenocopying essential facets of organs where they derive [93]. Multiple living organoid biobanks have been established for a collection of cancer types, including breast cancer. Mouse models are highly important in vivo systems in preclinical cancer research, ranging from cell line-derived xenografts (CDXs) to PDXs, to genetically engineered mouse models (GEMMs), and to the recently revisited syngeneic mouse models owing to the breakthrough of immunotherapy.

5.2 Improved gene manipulation

RNA interference (RNAi) allows broad gene inactivation and functional analysis of suppressed genes, which can be achieved transiently by siRNA, or stably by shRNA. Multiple RNAi libraries have been established targeting almost the entire genome or subsets, such as the druggable genome, the adhesome or the kinome. RNAi-based screens have been used to identify addiction genes and drug response-modulating genes. However, this approach is limited by its off-target effects and incomplete gene inactivation. CRISPR/Cas9 is a rapidly developing gene-editing tool with high efficiency and specificity. It has been substantially used in cancer research for generation of cancer models, synergistic gene study and target identification and validation [94, 95]. In a comparative study of genome-wide CRISPR/Cas9 dropout screen and shRNA screen, researchers found that CRISPR/Cas9 screen was more capable of detecting cancer essential genes than shRNA screen [96]. Though, false-positive hits were discovered in highly amplified regions owing to the induction of DNA damage response. It was reported that sgRNA-associated target mismatches caused cell lethality, necessitating the design of more specific sgRNAs. Notably, for both RNAi screen and CRISPR/Cas9 screen, improved algorithms have been developed to examine on- and off- target effects, holding the promise to remove false-positives [85, 94]. In context of CRISPR/Cas9 screens, modified sgRNA design algorithms have also been developed to improve screen sensitivity [97].

5.3 Advanced high-throughput screening

Nowadays, high-throughput screens have been advanced by exploitation of lentiviral barcoding and mixing genetically labeled cell lines. The novel platform PRISM enables simultaneous detection of a drug's activity on a mixture of CCLs in a single well, resulting in much higher throughput [98]. In addition, advanced high-throughput imaging allows for tempo-spatial detection of key cancer events in living cells and more complex intravital mouse model with improved fidelity and statistical robustness. Image-based screening approaches can provide high-throughput phenotypic readouts for cancer drug discovery, such as cell morphology, migration, programmed cell death and cell cycle progression [99]. Our approach described in **Chapter 2**, by leveraging FRET biosensor-based reporter cell systems, has also advanced the live cell high content imaging-based quantification of kinase activity profiles in TNBC cells. Multi-dimensional image analyses of kinase activity dynamics in high temporal resolution revealed differential kinase dependencies in various drug-resistant TNBC cells, as well as possible off-target effect of clinical kinase drugs.

5.4 Genomic sequencing and bioinformatics

Massive next-generation DNA/RNA sequencing studies represent an increasing reservoir of genetic aberration and gene expression information. Coupled with bioinformatics-based pathway analysis approaches, these databases allows for the exploration of

signaling pathway activation and active signaling components. TempO-Seq-based targeted whole genome RNA sequencing analysis performed in **Chapter 5** concluded that, the novel TNBC driver gene ASAP1 regulates various cytokine and apoptosis signaling components that are significantly associated with TNBC prognosis, supporting the potentiality of ASAP1 as a therapeutic target for the dismal disease. In addition, chemoinformatics helps to better understand the drug-target interactions. Chemoinformatics-guided target prediction and validation in **Chapter 4** has pronounced the polypharmacology mechanisms underlying the synergy of multi-kinase targeted inhibition on mTORi treatment in TNBC. An integrated application of these continuously advancing approaches/platforms will eventually lead to potential novel cancer therapy.

6. Aim and scope of this thesis

TNBC represents a highly aggressive disease, and incurable. Cytotoxic chemotherapy is the only option for systemic treatment. Prospective precision medicine in TNBC requires the development of new targeted therapeutic options. The main objectives of the studies described in this thesis are to i) understand the molecular basis of TNBC drug response/resistance to small-molecule KIs, ii) systematically identify vulnerabilities of refractory TNBC cells, and iii) eventually identify novel therapeutic targets and potential combination therapies. **Chapter 2** explored highly dynamic ERK and AKT kinase activity in TNBC cells in response to a well-established KI library. The integrated high-throughput FRET imaging and phenotypic readouts approach has revealed differential kinase dependencies for TNBC cell proliferation. The research also identified TNBC drug resistance against EGFR- and AKT/mTOR-targeted inhibitors, separately. These resistant cell lines were further used for synthetic lethality screens in **Chapter 3** and **Chapter 4**. EGFR inhibitors (EGFRi) have long been explored as targeted therapy for TNBC, but rarely beneficial in the clinic. In **Chapter 3**, a kinome-wide siRNA screen was performed in EGFRi-resistant TNBC cells in combination with lapatinib treatment. The combination screen aimed to identify key modulators of kinase signal transduction, conferring TNBC resistance against EGFR-targeted inhibition. Overactivation of PI3K/AKT/mTOR pathway is common in TNBC. Targeting mTOR, the convergent signaling element of multiple pathways, is of particular interest. A KI library screen was conducted in the absence or presence of rapamycin, as shown in **Chapter 4**. This study aimed to explore effective combined drug treatment to overcome TNBC resistance to mTOR inhibitors (mTORi). The research suggested using polypharmacology to circumvent mTORi resistance by combining multi-targeted kinase inhibition. The targets of the identified KI were predicted by cheminformatics-based survey and functionally validated by siRNA-mediated gene suppression. Over 80% of cases diagnosed with TNBC represent TP53 mutation, resulting

in high likelihood of gene instability. Thus, discovery of recurrent CNAs in the focal regions of TNBC genome provides an excellent possibility to identify therapeutic targets. Using a robust computational algorithm, **Chapter 5** identified not only known oncogenes but also novel putative cancer drivers. Following functional validation via gene inactivation, whole-genome RNA sequencing and pathway analysis were carried out to investigate the functionalities of the most clinically relevant drivers. Last of all, **Chapter 6** summarizes and discusses the findings of the work. Challenges and future perspectives are also provided.