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Targeted therapy for triple-negative breast cancer

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Chapter 1

Introduction, Aim and Outline of this Thesis

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1. Triple-negative breast cancer

Triple-negative breast cancer continues to represent a significant problem for modern oncology practice, the disease being associated with a particularly poor prognosis in comparison to other subtypes of breast cancer. This review attempts to comprehensively summarise potential molecular targets for this intractable illness, paying particular attention to the plethora of drug resistance mechanisms employed by breast cancer cells to evade eradication and how these may be overcome. Additionally, the molecular classification of breast cancer and its relation to the triple-negative subtype is explained in detail. Particular emphasis is also placed on the utility of targeting the basal transcriptional apparatus as a means of combating triple-negative breast cancers as well as a method for subverting their resistance to other targeted therapies.

1. Aetiology, Pathophysiology & Cancer Stem Cells

Breast cancer is both the most commonly diagnosed cancer and the leading cause of cancer-related death amongst women worldwide[1]. Breast cancer has a diverse aetiology: exposure to tobacco or ionising radiation, consumption of alcohol, high BMI, nulliparity or Cowden syndrome, all increase the risk of breast cancer development[2–6]. Approximately 5-10% of breast tumours arise due to autosomal-dominant mutations in BRCA1 and BRCA2 genes, genes which play a crucial role in DNA repair by promoting checkpoint activation and homologous recombination, respectively[7]. Histologically, breast tumours arise from the glandular, terminal duct lobular units which contain branched, tubuloaveolar milk-producing glands centred around a lumen. Tumours arise from either the basal, structurally-supportive, contractile myoepithelial cells or the luminal epithelial cells responsible for milk production, both of which are believed to be derived from a breast stem cell

population[8]. This breast stem cell population is thought to have the potential to differentiate into either luminal-restricted progenitors, myoepithelial-restricted progenitors or bipotent progenitor cells, the latter retaining developmental plasticity, being capable of producing both luminal-restricted and myoepethelial-restricted progenitor populations responsible for the generation of fully differentiated luminal and myoepethelial cells, respectively[9,10]. Moreover, evidence suggests that this intricate hierarchy is also prone to oncogenic disruption at various levels, with transformation of the breast stem and progenitor populations increasingly thought to be responsible for the generation of breast cancer stem cells (BCSCs): self-renewing cancer cells capable of maintaining tumours via differentiation into tumour bulk, from which all tumour cells are, in theory, derived[11]. This cancer-driving subpopulation of cells is believed to be responsible for disease recurrence in later life and is notoriously refractory to therapy in various tumour types[12–19]. Various markers specific for BCSCs have been identified, including a CD44+/CD24- phenotype and a high ALDH1 activity[20,21]. However, the precise hierarchical origins of the CSC population in breast cancer and other tumours remain somewhat elusive, due to inconsistencies in marker expression and utility, discrepancies between mouse and human tissue and the lack of validated *in vivo* assays to accurately determine their abundance in human tumours[22]. Furthermore, given the striking inter-and intratumoural heterogeneity of breast cancers it is likely that the BCSC model may be too simplistic to fully explain the pathophysiology of the disease. This intracellular genetic variation may instead be explained by a “clonal evolution” model in which all tumour cells retain a certain degree of developmental plasticity, with clonal differences being attributed to epigenetic and genetic aberrations acquired during tumourigenesis[11]. In essence, understanding the factors governing this plasticity, be

they epigenetic or microenvironmental, will be crucial in unravelling the complexities of breast cancer tumourigenesis and heterogeneity. Typically, the most aggressive forms of breast cancer are enriched in these CSC populations, with standard cytotoxic chemotherapy against tumour bulk known to enhance this enrichment via the induction of hypoxia, highlighting the therapeutic utility of specifically targeting these tumourigenic cell populations and the influence of microenvironmental factors on CSC behaviour[27,28].

1.2 Histological Classification

The high degree of cellular heterogeneity in breast cancer translates to an equally varied histological presentation. Two broad histological classifications exist for terminal duct lobe-derived breast cancers: *in situ* breast carcinoma and invasive breast carcinoma[30–32]. *In situ* breast carcinoma can be classified as either Ductal Carcinoma In Situ (DCIS) or Lobular Carcinoma In Situ (LCIS), depending on its anatomical origins[30–32]. DCIS can be further sub-divided, based on morphological features, into Comedo, Cribiform, Micropapillary, Papillary and Solid forms[30–32]. Invasive breast carcinomas on the other hand comprise a more heterogeneous group of tumours similarly classified on the basis of histology. These include Invasive Ductal Carcinoma (IDC), Invasive Lobular Carcinoma (ILC), Tubular Carcinoma, Mucinous Carcinoma and Medullary Carcinoma, with IDC being the most common form of breast cancer, comprising around 80% of all cases[32]. IDC and ILC are tumours which have invaded the surrounding ductal or lobular tissue and have subsequently penetrated the ductal or lobular walls, permitting further invasion and destruction of stromal breast tissue and paving the way for metastasis to distant organs[33].

1.3 Molecular Classification

Besides being categorised based on histological characteristics, breast cancer has traditionally been broadly classified as either hormone receptor positive (HR+) or hormone receptor negative (HR-), referring to the expression of oestrogen receptor-α (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2; ERBB2), with this classification influencing breast cancer management[34]. Influential studies which profiled the gene expression patterns of multiple breast tumours using DNA microarrays unveiled a greater degree of complexity to this complicated disease[35,36]. Based on these gene expression profiles, five main transcriptional subtypes of breast cancer were identified: claudin-low, basal-like, luminal A, luminal B and HER2-enriched (***Figure 1***)[35,36]. Luminal-derived breast tumours are more frequently HR+ than those derived from the basal myoepithelial cells[35]. HR+ breast cancers are often dependent upon deranged hormone-mediated signalling for their proliferation and progression, rendering these tumours amenable to treatment with oestrogen antagonists such as tamoxifen[34]. Moreover, HR+ tumours frequently display gene amplification of HER2, resulting in these cancers being dependent upon aberrant pro-proliferative signalling through constitutive activation of the HER2 pathway. ATP-competitive inhibitors of HER2-tyrosine kinase activity (e.g. lapatinib) and anti-HER2 monoclonal antibodies (e.g. trastuzumab) are therefore appropriate for the treatment of these tumours[37,38]. In contrast to HR+ breast cancers, triple-negative breast cancer (TNBC) is characterised as lacking gene expression or amplification of ERα, PR and HER2, respectively[34]. Consequently, TNBC is not responsive to treatment with anti-hormonal therapeutics nor to anti-HER2 agents. Compared to HR+ breast cancer, TNBC has a far poorer prognosis, characterised by a lower 5-year survival rate post-diagnosis and although

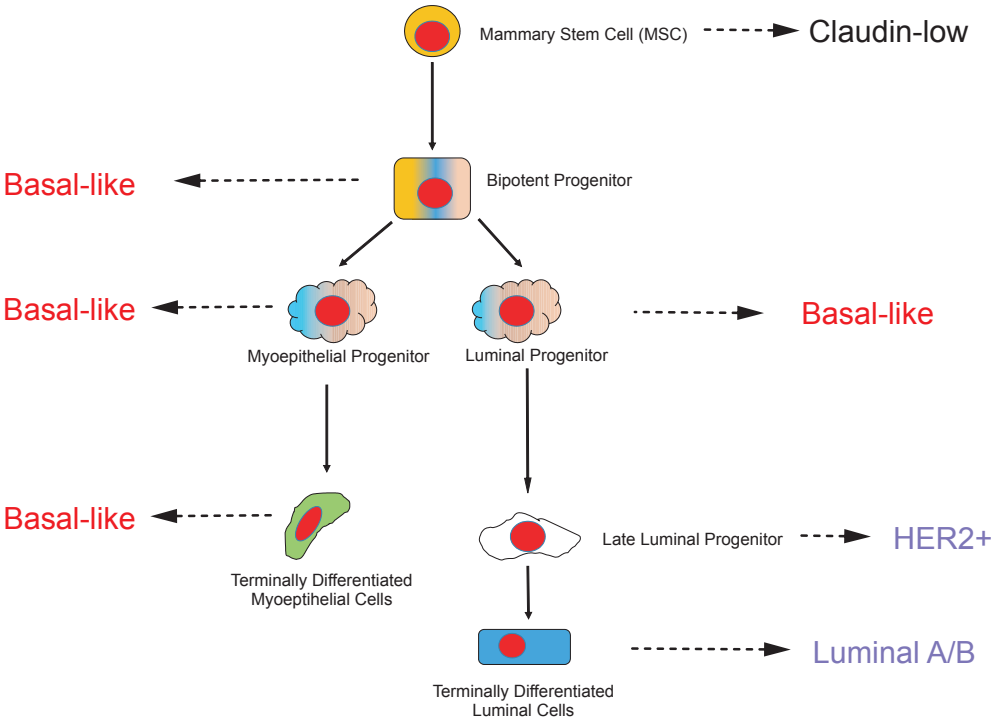

Figure 1. Molecular Classification of Breast Cancers. Diagram illustrating the association between the intrinsic subtypes of breast cancer and their putative cells-of-origin. The intrinsic subtypes of breast cancer are as follows: Claudin-low, basal-like, HER2-enriched, Luminal B and Luminal A. Each of these intrinsic subtypes is believed to originate from different cell populations during normal mammary gland development. Claudin-low tumours arise from mammary stem cells (MSCs) whilst basal-like tumours may arise from bipotent progenitor cells, myoepithelial progenitor cells, or terminally differentiated myoepithelial cells. As such, claudin-low tumours are frequently triple-negative, expressing high-levels of EMT markers and stem cell-related genes, whilst basal-like tumours exhibit high expression of basal cytokeratins (CK5, CK14, CK17) and EGFR, a minority of which may also show HER2 amplification[35,53–55]. HER2-enriched tumours may be derived from late luminal progenitor cells which acquire HER2 gene amplification. These tumours express high levels of HER2 and associated genes such as GRB7[36]. Luminal A and Luminal B tumour types may be a result of oncogenic transformation of differentiated myoepithelial or luminal cells. Luminal A tumours are frequently ER+ PR+ and HER2-, whilst Luminal B tumours are frequently "triple-positive", though these definitions are not mutually exclusive[56,57]. Luminal B tumours are often more highly enriched for pro-proliferative and cell cycle-related genes, compared with Luminal A tumours[58]. Adapted from *Prat & Perou (2009)*[59].

it accounts for approximately 15-20% of total breast cancer cases, it is disproportionately responsible for breast cancer-associated deaths[35,39–41]. Epidemiologically speaking, TNBC is more prevalent in pre-menopausal African-

American women younger than 40 years of age[42,43]. At diagnosis, TNBC often presents as a poorly differentiated, highly proliferative, high grade IDC with a necrotic core and varying levels of lymphocytic infiltration[42,44,45]. The pattern of metastasis is also markedly different for triple-negative tumours, with TNBC more likely to spread to the lungs and brain than HR+ tumours, which instead show greater propensity to spread to the bones[46,47]. *Lehmann et al* further sub-classified TNBC into six molecular subtypes (TNBCType) using k-means and consensus clustering: Basal-like 1 (BL1), Basal-like 2 (BL2), Mesenchymal (M), Mesenchymal stem-like (MSL), Immunomodulatory (IM) and Luminal Androgen Receptor (LAR)[48]. These subtypes displayed unique gene expression profiles with BL1 and BL2 subtypes enriched for expression of genes involved in cell cycle and growth factor signalling (specifically EGFR, IGF1R, Wnt/β-catenin and MET), respectively. Contrastingly, genes involved in regulation of cell motility and differentiation (e.g. Rho and TGF-β signalling pathways) are prevalent in the M subtype. Whilst the MSL subtype shares this enrichment, it is unique in also being enriched for genes linked to growth factor signalling pathways. Unsurprisingly, the IM subtype shows enrichment in genes linked to immune system regulation and cytokine signalling (e.g. IL-12, IL-7 and TH1/TH2 pathways). Interestingly, the LAR subtype is strongly enriched in pathways governing androgen/oestrogen metabolism and steroid synthesis. Moreover, the LAR subtype expresses greater levels of androgen receptor (AR) than the other subtypes, accompanied by up-regulation of signalling components or direct downstream targets of AR. These profiles have also been borne out in clinical practice, being highly relevant regarding drug sensitivity. *Masuda et al* analysed the response of 130 TNBC patients, classified into Lehmann et al's subtypes using gene expression microarrays, to neoadjuvant chemotherapy[51]. Basal-like TNBC is often the most sensitive to

standard chemotherapy, consistent with its enrichment in DNA-repair-related pathways, however, BL2-type tumours are considerably less sensitive to chemotherapeutic agents compared with BL1-type tumours, with pCR rates of 0% versus 52%, respectively, indicating that *Lehmann et al's* classifications have important implications for the clinical management of TNBC[51]. Nonetheless, this study was confounded by small sample sizes for certain tumour groups (particularly for BL2), and the lack of perfect overlap between gene expression profiles obtained from microarrays and those identified by *Lehmann et al*, precluding absolute translation of clinical relevance. Further research conducted by *Lehmann et al* reduced the original six subtypes to four (BL1, BL2, M, LAR), after refinement which took the low cellularity of IM and MSL tumours into account[52]. A retrospective analysis of 306 TNBC patients treated with neoadjuvant chemotherapy revealed substantial differences between these four classifications with BL2-type and LAR-type tumours again showing the lowest pCR rates, 18% and 23% respectively[52], signifying the need for targeted therapeutics in these instances, most likely receptor tyrosine kinase (RTK) inhibitors or androgen antagonists to counteract dependence on growth factor signalling and hormonally-regulated pathways[51].

2. Current Therapeutic Strategies and Mechanisms of Resistance in TNBC

2.1. Current management of TNBC

First-line treatment for TNBC is usually tumour-reductive surgery which consists of either simple or radical mastectomy or, if possible, breast-conserving surgery (BCS) in the form of lumpectomy or quadrenectomy[60,61]. Post-operative administration of radiotherapy is invariably given to patients receiving BCS and evidence suggests it is also beneficial to those who have undergone mastectomy by reducing 15-year breast

cancer mortality rates[61]. Given that TNBC is refractory to hormone antagonists such as tamoxifen, the primary systemic treatment is adjuvant or neoadjuvant chemotherapy. This typically consists of a combination of anthracyclines (e.g. doxorubicin), taxanes (e.g. paclitaxel) and platinum-based agents (e.g. carboplatin)[51,62]. Paradoxically, TNBC patients usually display higher rates of pCR in response to treatment with neoadjuvant chemotherapy compared to other BC subtypes[63–66]. However, only 30-45% of patients achieve pCR, with those who do not respond often relapsing far more rapidly with metastatic disease[41,67–70]. Upon progression into Stage III or IV TNBC, survival drops substantially, with a median survival of 2-3 years depending on location of the metastatic lesions[63,71]. The development of metastatic disease is therefore frequently met with fatal consequences and treatment is often palliative rather than curative[63,72]. Patients who present with inoperable metastatic disease are treated with neoadjuvant chemotherapy to render tumours amenable to resection[61]. Radiotherapy is used as a last resort for tumours which remain inoperable post-systemic chemotherapy[61]. The primary issue underlying the lack of response to therapeutic intervention, eventually leading to metastatic dissemination, is that of intrinsic or acquired drug resistance.

2.2. Mechanisms of drug resistance

Given the extensive molecular heterogeneity of TNBC, it is unsurprising that many of these tumours are often recalcitrant to standard chemotherapeutic regimens[69,73,74]. Drug resistance is pandemic amongst TNBC patients and proceeds via two broadly defined mechanisms: intrinsic or acquired resistance[75,76]. Intrinsic resistance denotes the outright insensitivity of tumour cells to anti-cancer agents, this being mediated by

pre-existing factors which render the tumour bulk impervious to treatment with chemotherapy or targeted agents[75–77]. Overexpression of transmembrane ATP-binding cassette (ABC) transporters, particularly BCRP and MDR1, promotes the efflux of anti-cancer drugs, and has been described as a mechanism of intrinsic resistance in breast cancer[78]. Moreover, CSCs often express higher levels of these drug pumps, including BCRP, thereby contributing to the insensitivity of this aggressive tumour cell population to therapy[79–81]. The quiescent state of CSCs also renders these cells refractory to treatment with DNA-damaging chemotherapeutics, since these agents preferentially target actively cycling cells[82,83]. Additionally, the elevated level of DNA repair systems in CSC populations confers resistance to DNA-damaging radiotherapy[84,85]. As such, the quality and efficiency of DNA damage repair in target cells is pivotal in determining the efficacy of DNA damaging agents[76,86]. Acquired resistance refers to an adaptive response to previously effective chemotherapeutics or targeted agents, which results in tumour cell survival in the presence of these drugs and develops over the course of treatment[76]. One such mechanism of acquired resistance comprises alterations in drug targets, which occurs in both solid and haematological malignancies alike[76]. BRCA1/2-mutant, homologous recombination-deficient breast cancers are sensitive to treatment with poly (ADP-ribose) polymerase (PARP) inhibitors[87,88]. The success of this synthetic lethal interaction is due to the inability of BRCA1/2-mutant cells to repair double-stranded DNA breaks (DSBs) in the absence of PARP, since intact HR pathways can overcome PARP inhibition[89–91]. However, in-frame deletions of BRCA2 can render cells insensitive to PARP inhibition by partially restoring BRCA2's HR capability[92,93]. Various cancers frequently become addicted to particular oncogenic kinases, as is the case in EGFR-mutant lung cancer, HER2+ BC and chronic myeloid leukaemia

(CML) driven by the BCR-ABL chromosomal translocation, in so-called "oncogene addiction[94–97]". EGFR-mutant lung cancer cells are therefore sensitive to EGFR-tyrosine kinase inhibitors (EGFR-TKI) such as lapatinib, erlotinib or gefitinib, by virtue of a constitutively active EGFR pathway[98,99]. Initially, patients respond to EGFR-TKI therapy but resistance to such agents is almost invariably acquired, with additional mutations in EGFR often being responsible for the development of resistance[100,101]. Similar responses are seen in CML in response to BCR-ABL inhibitor imatinib as well as in HER2+ BC in response to anti-HER2 therapies such as trastuzumab or lapatinib[102,103]. Besides alterations in the drug targets themselves, mutations acquired in pathway components downstream of receptor tyrosine kinases (RTKs) such as EGFR also contribute to acquisition of resistant phenotypes in multiple cancers, as is the case in BRAF-mutant melanoma where mutations in KRAS or MEK confer resistance to BRAF inhibitors[104,105]. In response to targeted agents which inhibit the activation of oncogenic RTKs or other kinases, an adaptive transcriptional response can be initiated which results in the up-regulation of other RTKs capable of funnelling growth-factor mediated signal transduction through commonly regulated downstream pathways. This aberrant reprogramming of cellular circuitry which exploits pathway redundancy is known as "oncogenic bypass" since the drug target remains inhibited whilst treatment promotes activation of alternative pathways through which signalling may still occur, thereby circumventing the effects of inhibiting the original oncogenic kinase or RTK (see ***Figure 2***)[75,76]. For example, in HER2+ BC, up-regulation of HER3 (ERBB3) is seen in response to HER2-targeted therapy cetuximab[106–108]. In TNBC, inhibition of MEK1/2 leads to a rapid kinome rewiring, resulting in the increased expression of platelet-derived growth factor receptors (PDGFRs) and other RTKs, the crux of which involved the proteolytic

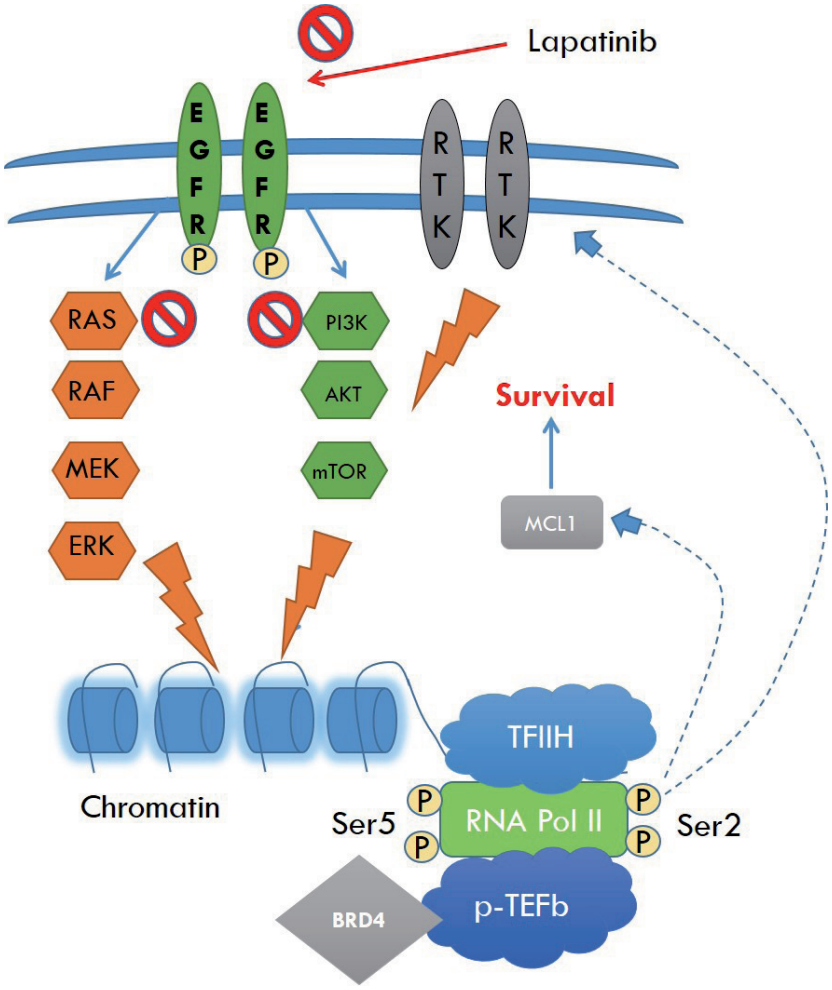

Figure 2. Oncogenic Bypass. Diagram illustrating oncogenic bypass-mediated mechanisms of resistance to RTK-targeted tyrosine kinase inhibitors in cancer. Inhibition of phosphorylation of the intracellular domains of RTKs by tyrosine kinase inhibitors such as lapatinib, prevents the downstream activation of signal transduction cascades responsible for controlling cell proliferation and survival. Amongst the most prominent of these pathways are the RAS-RAF-MEK-ERK and PI3K-AKT-mTOR pathways, which are frequently dysfunctional in multiple cancers. In cells with *de novo* or acquired resistance to such targeted therapies, an adaptive transcriptional response elicited by target kinase inhibition can promote the up-regulation of alternative RTKs which are capable of activating similar pro-proliferative downstream signal transduction cascades in the presence of inhibitor, despite complete inhibition of the originally targeted RTK, ultimately resulting in evasion of cell death. Mutations in downstream components of the MAPK or PI3K pathways which render certain kinases constitutively active, such as KRAS, BRAF or PIK3CA mutations as well as deletions in AKT's negative regulator PTEN can also contribute to oncogenic bypass since their presence renders the inhibition of upstream RTKs futile. Adapted from *Arteaga & Engelman*[140].

degradation of c-MYC, allowing de-repression of PDGFR, AXL and VEGFR expression, ultimately rescuing TNBC cells from MEK1/2 inhibitor-induced death[109]. Up-regulation of MET is also known to promote resistance to EGFR-TKIs in a subset

of EGFR-mutant lung cancer patients[110]. Downstream of RTK-controlled signal transduction pathways, acquired resistance to targeted therapies can also be attributed to dysfunctional regulation of the equilibrium between pro- and anti-apoptotic factors. The resistance of tumour cells to apoptosis has previously been described as one of the hallmarks of cancer, the interplay between BCL-2 family proteins controlling whether a tumour cell undergoes apoptosis or not[111–114]. This family can be roughly sub-divided into two groups: anti-apoptotic members (e.g. BCL-2, BCL-XL and MCL-1) and pro-apoptotic members (e.g. BAX, BAK, BIM, BAD, BID and NOXA). Pro-survival BCL-2 family members such as MCL-1, BCL-2 and BCL-xL bind to and antagonise the oligomerisation of pro-apoptotic BH3-only proteins, thereby preventing loss of mitochondrial membrane integrity, leakage of cytochrome c and subsequent cell death[112,114–116]. Inhibitor of apoptosis proteins (IAPs) (e.g. XIAP and Survivin) act in tandem with anti-apoptotic BCL-2 family members by preventing the cleavage of caspases 3, 7, 8 and 9, hindering the caspase-mediated induction of apoptosis[117–119]. Dependency upon multiple pro-survival BCL-2 family member proteins, including MCL-1, BCL-2 and BCL-xL is frequent in haematological malignancies where the equilibrium between pro- and anti-apoptotic factors is skewed in favour of cell survival[120,121]. Expression of pro-apoptotic BIM is also essential in regulating EGFR-TKI-induced apoptosis in NSCLC patients, with deletions of this gene conferring resistance to such inhibitors[122–124]. Consequently, multiple BH3-mimetics which interfere with the function of pro-survival, BCL-2 family member proteins have been developed as potential cancer therapies which have proven valuable as combination therapies. High levels of BCL-2 expression are also seen across breast cancer subtypes, with the highest levels seen primarily in ER+ breast cancer, though a proportion of TNBCs also exhibit similar

BCL-2 levels[125,126]. Additionally, MCL-1 is a key survival factor in TNBC cells, MCL-1 silencing proving more lethal than sole knockdown of BCL-xL alone with dual silencing of MCL-1 and BCL-xL incompatible with TNBC cell survival[127]. In TNBC tumour xenografts, treatment with BH3-mimetic ABT-737 sensitised tumours expressing high levels of BCL-2 to docetaxel, suggesting BCL-2 proteins contribute to docetaxel insensitivity illustrating the utility of manipulating BCL-2 and other similar proteins in overcoming resistance to chemotherapy[128]. Interestingly, MCL-1 and oncogene MYC have also been shown to cooperate in maintaining a BCSC population in chemotherapy-resistant TNBC by promoting mitochondrial oxidative phosphorylation-induced HIF-1α expression, a process upon which BCSCs are dependent[129]. Combining HIF-1α inhibition with chemotherapy dramatically reduced expansion of the BCSC population, indicating that the oncogenic interaction between BCL-2 family members, transcription factors and altered metabolism is amenable to therapeutic intervention[130]. Evidence increasingly suggests that the tumour microenvironment is also critically involved in conferring resistance to anticancer therapies in both solid and haematological tumours[131,132]. Tumours interact with their surroundings via integrin-mediated adhesion; integrins are large, cell surface proteins which interact with tumour extracellular matrix (ECM) components and in turn control a multitude of pro-proliferative pathways (e.g. PI3K-AKT and NF-κB) as well as intrinsic and extrinsic apoptosis[131]. This is strictly controlled by integrin ligation status and cooperation with RTKs. For example, αvβ3 integrin interacts with fibroblast growth factor receptor (FGFR) to promote signal transduction via RAS-RAF pathway, thereby exerting adhesion-dependent control over tumour cell proliferation[133,134]. In HER2+ breast cancer, enhanced β1-integrin-mediated adhesion to extracellular matrix (ECM) components causes resistance to anti-HER2 monoclonal antibody

trastuzumab[135]. The tumour stroma itself is composed of cancer-associated fibroblasts (CAFs; fibroblasts which have been activated by tumour-derived growth factors) which can communicate with tumour cells via paracrine signalling and the secretion of cytokines and/or growth factors such as EGF, HGF or FGF[136]. Stromal-derived cytokines and growth factors have clear roles in anticancer drug resistance across a vast number of tumour types; CAF-regulated secretion of HGF confers resistance to vemurafenib in melanoma, to lapatinib in HER2+ breast cancer and to EGFR-TKIs in non-small cell lung cancer (NSCLC)[137]. Immune evasion is another property conferred to tumours by the microenvironment; CAFs recruit macrophages and monocytes which can shift the immune microenvironment of the tumour towards tolerance and immune suppression, thus concealing the tumour from cytotoxic T-cell responses[138,139].

3. Possible therapeutic targets in TNBC

Despite the heterogeneous nature of TNBC tumours and the plethora of resistance mechanisms they adopt in response to treatment, promising drug targets amenable to pharmacological manipulation in TNBC do exist. Here, a number of these are described, particular emphasis being placed on RTK inhibitors and cyclin-dependent kinase (CDK) inhibitors as prospective therapies.

3.1. Epidermal Growth Factor Receptor (EGFR)

Epidermal Growth Factor Receptor (EGFR; also known as ERBB1/HER1) is a transmembrane receptor tyrosine kinase (RTK) consisting of an extracellular ligand-binding domain and an intracellular tyrosine kinase domain[141]. Epidermal growth factor (EGF) binds to the extracellular domain, triggering homodimerisation of the

receptor, followed by auto-phosphorylation of the intracellular tyrosine kinase domain, resulting in the activation of many signalling cascades, most notably the PI3K-AKT and RAS-RAF-MEK-ERK axes, which promote cell proliferation, migration, differentiation and survival[142–146]. EGFR expression varies considerably amongst TNBC tumours, though it is frequently overexpressed in basal-like tumours where high rates of EGFR gene amplification have also been reported[147–150]. This overexpression is also linked to a significantly poorer overall survival compared to TNBC patients lacking EGFR overexpression[147,148]. Contrastingly, EGFR is rarely expressed in luminal, ER+ tumours[151]. Constitutive activation of pathways downstream of EGFR is also common in TNBC tumours, particularly constitutive activation of BRAF, suggesting some TNBC tumours depend upon EGFR-mediated signalling pathways for their growth[120,152,153]. Furthermore, *Lehmann et al*[48] also demonstrated that EGF signalling is highly enriched in the BL2 and mesenchymal TNBC subtypes identified in their expression analyses. However, the use of EGFR-TKIs or anti-EGFR monoclonal antibodies in a monotherapeutic context has yet to deliver satisfactory results against TNBC in clinical trials. A Phase II clinical trial for gefitinib monotherapy demonstrated that disease progression occurred in 61.3% of metastatic breast cancer patients with no complete or partial tumour responses detected[154]. Despite clear inhibition of EGFR and downstream MAPK phosphorylation, AKT phosphorylation was unaffected by treatment and no impact on tumour proliferation markers (Ki67) was noted. Other Phase II trials have demonstrated similarly disappointing results, with *Dickler et al*[155] reporting a disease progression rate of 87% amongst patients after Erlotinib treatment, with only two confirmed partial responses identified. These trials did not, however, select for tumours displaying EGFR overexpression or gene amplification, or which were

dependent upon active EGFR signalling. Nor did they assess potential genetic aberrations present in the tumours which may allow constituent cells to bypass inhibition of EGFR-related signalling, such as oncogenic RAS mutations or PTEN deletions[156,157]. Nuclear translocation of EGFR is also associated with resistance to radiotherapy, chemotherapy and EGFR-targeted agents, since this protects the receptor from tyrosine kinase inhibition occurring at the cell membrane[158,159]. Nuclear EGFR acts as a transcription factor which can up-regulate the expression of cell cycle-related genes such as Cyclin D1, thereby promoting proliferation and cell cycle progression even in the presence of EGFR-TKIs or anti-EGFR monoclonal antibodies[160–162]. Moreover, these trials were not stratified according to breast cancer subtype, with both HR+ and HR- breast cancer patients included. However, Phase II trials of second generation EGFR-TKI afatinib have proven equally unsuccessful in patients stratified as TN or HER2-HR+ or in inflammatory breast cancer (IBC) treated with EGFR-TKI lapatinib[163,164]. Despite these disappointing results, combination therapies including EGFR inhibitors have been moderately more successful. A Phase II trial evaluating neoadjuvant chemotherapy combined with panitumumab (EGFR-mAb) showed pCR rates of 47% in cases of operable TNBC, whilst combining cetuximab and docetaxel in another cohort of operable TNBC tumours resulted in pCR rates of 24%[165,166]. Overall response rates (ORR) after co-treatment of metastatic TNBC tumours with irinotecan and cetuximab were approximately 11%, indicating modest benefit of combining EGFR-targeted therapies with standard chemotherapeutic regimens[167]. The use of EGFR-targeted radioimmunotherapy in the form of a radiolabelled anti-EGFR monoclonal antibody in combination with PARP inhibitors and chemotherapy (docetaxel and doxorubicin) has also shown pre-clinical benefit; this triple combination eliminated established metastases in xenograft

models, induced tumour apoptosis *in vivo* and significantly reduced the amount of CD44+ CD24- BCSCs present[168]. Evidently, the future of EGFR-targeted therapies in TNBC is dependent upon their efficacy when combined with other well-tolerated agents.

3.2. Fibroblast growth factor receptors (FGFRs)

Fibroblast growth factor receptors (FGFRs), upon binding FGFs 1-10, transduce pro-proliferative and pro-survival signals via downstream activation of PI3K-AKT and MAPK pathways[169]. Overexpression of FGFR1 and FGFR2 is less frequent than EGFR amplification, with FGFR2 amplification occurring in approximately 4% of TNBC cases[170]. The expression of FGFR ligand FGF2 is significantly higher in basal-like breast tumours, whilst RNAi-mediated silencing of FGF2 impedes the growth of multiple TNBC cell lines, which are incidentally also sensitive to selective FGFR1 inhibitor PD173074[171]. Inhibition of FGFR-controlled signal transduction prevents phosphorylation of both ERK1/2 and AKT, induces G1 cell cycle arrest and induces apoptosis whilst also leading to a significant reduction in the growth of FGFR1-expressing TNBC xenografts[171]. Furthermore, analysis of 782 TNBC tumours revealed high-level FGFR1 expression is more frequent in basal-like tumours and is associated with a significantly poorer overall survival (OS)[172]. Dovitinib, an FGFR-TKI with activity against FGFR1-3, has shown moderate efficacy against FGFR-amplified, metastatic breast tumours in a Phase II clinical trial, resulting in stable disease in a subset of HR+ patients, though no complete responses (CRs) and few pCRs were seen[173]. It therefore remains to be seen whether anti-FGFR therapies are of benefit to TNBC patients.

3.3. Platelet-derived growth factor receptors (PDGFRs)

Platelet-derived growth factors (PDGFs) PDGF-A, PDGF-B, PDGF-C, PDGF-D bind to two either PDGFR-α or PDGFR-β as hetero- or homodimers[174]. Dimerisation of PDGFR-α and/or PDGFR-β subsequently promotes autophosphorylation of their intracellular tyrosine kinase domains, thereby triggering downstream signal cascades[174]. PDGFR-α is expressed at higher levels in TNBC compared with HR+ breast cancer[175]. Activation of PDGFR-α in inflammatory breast cancer (IBC) patients is linked to a significantly poorer metastasis-free survival, suggesting PFGFR-α activity is linked to metastasis formation and disease progression[176]. The recruitment of bone marrow-derived mesenchymal stem cells (BM-MSCs) to the TNBC tumour microenvironment is a pivotal step in the metastatic cascade. Secretion of inflammatory cytokines (e.g. CCL5) by BM-MSCs activates AKT via binding to CCR5, thereby promoting tumour cell proliferation, subsequent extravasation into the vasculature and distant organ colonisation[177]. Additionally, BM-MSCs exhibit remarkable plasticity and can reversibly differentiate into CAFs which secrete pro-tumourigenic factors capable of inducing epithelial-to-mesenchymal transitions (EMTs), facilitating a pro-metastatic environment[178]. Tumour-secreted factors also enhance the BM-MSC/CAF-mediated secretion of these factors, indicating the presence of a signalling loop between the constituent cells of the primary tumour and its microenvironment[178]. PDGFR-β plays a fundamental role in regulating BM-MSC recruitment to primary and metastatic tumour sites[179]. PDGFR-β inhibition prevents intravasation of BM-MSCs into primary TNBC xenografted tumours, and reduces the number of lung metastases[179]. However, clinical trial data for PDGFR-specific inhibitors is scarce. Unfortunately, non-specific inhibitors of PDGFRs such as sunitinib and sorafenib, which also inhibit vascular endothelial growth factor

receptors, performed poorly in clinical trials either as single agents or in combination with chemotherapy[180,181]. In summary, targeting PDGFRs in TNBC may therefore be of use in preventing metastasis of TNBC cells, though the utility of PDGFR-specific inhibitors in a clinical context remains unknown.

3.4. PI3K-AKT-mTOR and MAPK Pathways

Downstream of most RTK-mediated signal transduction pathways, both the PI3K-AKT-mTOR and mitogen-activated protein kinase (MAPK) axes constitute vital pro-proliferative, pro-survival cascades for tumour cells. Heterodimeric phosphoinositide-3 kinases (PI3K) consist of regulatory (p85) and catalytic (p110) subunits; activation of a multitude of RTKs (including EGFR) and downstream adaptors induces recruitment of the regulatory p85 subunit, resulting in a conformational change permitting p110-mediated phosphorylation of PIP2 to PIP3, which subsequently activates AKT[182–184]. Negative feedback in the form of PTEN counteracts activation of the pathway by dephosphorylating PIP3[185]. PI3K-AKT-mediated regulation of mTOR activity controls cellular metabolism and protein translation[186,187]. mTOR forms complexes with RAPTOR and RICTOR proteins to form mTORC1 and mTORC2, respectively[182]. mTORC1 phosphorylates 4E-BP1 and S6K to enhance ribosomal biogenesis and protein translation whereas mTORC2 re-inforces AKT-mediated signalling[187–190]. Enhanced activation of the PI3K-AKT-mTOR axis is frequent in TNBC, as are oncogenic PIK3CA (p110) mutations and PTEN deletions which lead to constitutive activation of AKT[191–194]. PI3K inhibitors and mTOR inhibitors have been extensively tested both pre-clinically and clinically in TNBC with varying degrees of success[70,182]. As single agents, mimimal efficacy has been observed, whilst combination therapies appear to be of particular benefit. The PI3K pathway is critical in maintaining stability of the HR complex during DNA

repair[195,196]. Silencing PIK3CA in TNBC cells reduces BRCA1 levels which can be exploited by combining PI3K (p110)-selective inhibitor BKM120 with PARP inhibitor olaparib[197]. This combination sensitised BRCA1/2-proficient TNBC cells to olaparib *in vitro* and in TNBC patient-derived xenograft models[197]. Enhanced PFS has been observed in TNBC patients with PI3K-AKT-mTOR pathway alterations treated with combinations of PI3K-AKT-mTOR inhibitors and chemotherapy[198]. Nonetheless, adaptive resistance mechanisms due to the extensive functional overlap and cross-inhibition observed between the PI3K-AKT-mTOR and MAPK axes, invariably arise. MAPK signalling itself proceeds via activation of the RAS-RAF-MEK-ERK cascade; growth factor-induced activation of RAS GTPase phosphorylates effector kinase RAF which subsequently phosphorylates effector kinase MEK and finally ERK1/2, which promotes the function of terminal transcription factors (e.g. c-MYC and c-FOS) eventually leading to increased expression of pro-survival transcripts[199–201]. High expression of ERK1/2 correlates with poorer survival in TNBC whilst increased copy number of RAS or RAF leads to pathway overactivation, with mutations in RAS or RAF uncommon events in TNBC[69,152]. MEK inhibitors induce up-regulation of the PI3K-AKT-mTOR axis in TNBC xenografts and cell lines due to de-repression of MEK-induced inhibition of this pathway, likely via prevention of ERK-mediated GAB1 phosphorylation[202–204]. The apoptosis-inducing effect of PI3K inhibitors has also been attributed to their transient effects on the adjacent RAS-RAF-MEK-ERK pathway[202]. Intriguingly, pulsatile PI3K inhibition or combined MEK or AKT inhibition led to enhanced apoptosis in multiple cancer cell lines, highlighting important implications for the dosage of such targeted therapies[202].

3.5. DNA-Damaging Agents & Checkpoint Inhibitors

By their very nature, cancers display inherently high levels of genomic instability, a known hallmark of cancer which underlies their proclivity for the accumulation of DNA damage[205]. Oncogene activation causes replication-linked DNA lesions as a result of disproportionate replication origin firing due to excessive pro-proliferative signals, the implication of this being that malignant cells possess more endogenous replication errors than non-malignant cells[206–208]. Moreover, proteins involved in the DNA damage response (DDR) and associated cell cycle arrest are often mutated or dysfunctional in multiple cancers; tumour suppressor p53 being a prominent example[209,210]. Radiotherapy and chemotherapy take advantage of these properties by inducing exogenous DNA damage such that tumour cells can no longer repair the damage, leading to cell cycle arrest and ultimately apoptosis[208]. DNA-damaging agents induce this irreparable damage by various mechanisms: topoisomerase poisons (e.g. etoposide and doxorubicin) act by preventing topoisomerase II-mediated cutting of DNA double-helices and release of torsional strain, thereby trapping obstructing DNA replication resulting in double-stranded breaks (DSBs)[211–213]. Alkylating agents (e.g. cisplatin) bind to guanine residues in DNA, forming damaging adducts and intra-strand crosslinks[214]. Antimetabolites (e.g. 5-fluorouracil and gemcitabine) interfere with nucleotide metabolism by competing with other nucleotides during DNA synthesis, thus preventing incorporation of the correct nucleotides and inducing termination of chain elongation[215]. However, DNA-damaging agents are associated with considerable dose-limiting, off-target toxicity given their effects on non-malignant, rapidly dividing cells in the gastrointestinal system and bone marrow[216–218]. The redundancy between various DNA repair pathways is amenable to targeted therapy since cancer cells deficient for a particular

form of repair often become addicted to another, as is the case with the use of PARP inhibitors against BRCA1/2-deficient breast cancer and ovarian cancer[87,88]. Interfering with DNA damage checkpoints in the cell cycle is another strategy whereby aberrant cell cycle progression can lead to apoptosis due to accumulation of DNA lesions. These checkpoints are strictly controlled by ataxia telangiectasia mutated (ATM) and ataxia telangiectasia Rad3-related (ATR) proteins which are activated by DSBs and single-stranded breaks (SSBs), respectively[219,220]. In response to DSB detection, ATM phosphorylates histone H2AX which induces a change in chromatin architecture permitting the recruitment of other repair factors and ubiquitin ligases (e.g. MDC1, MRN and RNF8), ultimately leading to localisation of BRCA1, p53 and other vital repair factors to the site of damage. ATM-mediated phosphorylation of SMC1, MRN or KAP1 induces lesion repair, dissemination of damage signals or chromatin relaxation, respectively[221–223]. Checkpoint kinases 1 and 2 (CHK1 and CHK2) are serine-threonine kinases downstream of ATM and ATR, which act to halt cell cycle progression in the presence of DNA damage[224]. Phosphorylation of CHK2 (Thr68) and CHK1 (Ser345/Ser317) by ATM and ATR, respectively, halts cell cycle progression via CHK2- or CHK1-mediated phosphorylation of cdc25A or cdc25C, priming them for ubiquitination and proteasome-mediated degradation[225,226]. Degradation of cdc25A inhibits cyclin-dependent kinase 2 (CDK2) activity, thus arresting cell cycle progression in G1/S-phase. Whilst cdc25C degradation obstructs CDK1-CyclinB function, leading to G2/M arrest[225,226]. Activated CHK2 also phosphorylates substrates which amplify the DDR, including BRCA1, BRCA2 and XRCC1[227–229]. BRCA1 and BRCA2 are also substrates of ATM, indicating that CHK2 may also act to augment ATM function in DNA repair[227,228]. With respect to targeting CHK1 and CHK2 function in TNBC,

CHK1 inhibitor MK-8776 improves the radiosensitivity of multiple TNBC cell lines and xenografts by preventing CHK1-mediated activation of DNA repair and abrogating damage-induced G2/M arrest[230]. P53-deficient TNBC cell lines and xenografts are exquisitely sensitive to CHK1 inhibition, while p53 inhibition sensitises p53-proficient TNBC cells to CHK1 inhibitors UCN-01 and AZD7762, suggesting that stratifying patients based on p53 status may identify patients eligible for anti-CHK1/2 therapy[231]. Extensive pre-clinical evaluation of combining CHK1/2 inhibitors with chemotherapeutics has not necessarily been translated into successful clinical application, however[232]. Co-treatment of metastatic TNBC tumours with UCN-01 and irinotecan in a Phase I clinical trial was not associated with significant clinical benefit due to pharmacodynamic issues with UCN-01 and tumour heterogeneity. Induction of apoptosis markers in patients' tumours was minimal, alluding to the existence of possible bypass mechanisms[233]. The results of an ongoing clinical trial investigating the effect of LY2606368, a relatively specific CHK1 inhibitor with weaker effects on CHK2, in BRCA1/2-associated BC and TNBC on induction of complete and partial responses are yet to be published but will be made available in 2019 (NCT02203513). Considering that repair of DNA damage requires the function of multiple, often parallel, signalling pathways, the identification of novel synthetic lethal combinations using DNA damage checkpoint inhibitors is likely to be invaluable in designing efficacious, multi-faceted therapies which prevent tumour cell escape facilitated by pathway redundancy in TNBC and other malignancies.

3.6. Cyclin-dependent kinases

3.6.1. The role of cyclin-dependent kinases in controlling cell cycle progression

Cyclin-dependent kinases (CDKs) are serine/threonine protein kinases involved in control of cell proliferation and transcription, the expression of which is strictly controlled throughout the cell cycle and whose activity is dependent upon their binding to specific cyclin molecules[236–240]. Progression through the various phases of the cell cycle is governed by multiple CDKs and their corresponding cyclins with specific CDK-Cyclin complexes regulating the passage of the cell from one specific cell cycle phase to another[241]. During G1 phase, the transcriptional repressor Rb binds chromatin-modifying enzymes (HDAC1) and transcription factors (E2F1) which normally promote expression of genes necessary for transition into S phase[242–244]. The CDK4/6-Cyclin D1 complex phosphorylates Rb, leading to dissociation of both HDAC1 and E2F1 from Rb, thereby de-repressing E2F1's transcriptional activity and promoting S phase entry[242–244]. Rising levels of Cyclin E1/E2 expression as a result of enhanced E2F1-mediated transcription result in the formation of the CDK2-Cyclin E complex which subsequently hyperphosphorylates Rb, leading to increased expression of genes critical for DNA synthesis during S-phase[245–247]. The binding of Cyclin A, the transcription of which is dependent upon E2F1 activity, to CDK2 then terminates S phase by phosphorylating E2F1, thus abrogating its DNA-binding ability and forming a negative feedback loop[248–250]. During late S phase, Cyclin A forms a complex with CDK1 and is subsequently degraded upon entry into G2/M, resulting in formation of CDK1-Cyclin B complexes which bind to, phosphorylate and enhance the activity of FOXM1 transcription factors, thereby upregulating the expression of genes indispensable for mitosis[251,252]. Phosphorylation of the cytoplasmic-retention sequence on Cyclin B during late prophase is a critical step in mitotic progression,

resulting in rapid nuclear translocation[253]. The CDK1-Cyclin B complex subsequently phosphorylates multiple nuclear-localised substrates resulting in reorganisation of the nuclear architecture and eventual degradation of the nuclear membrane in preparation for mitosis[253–257]. CDK1-Cyclin B1-mediated activation of APC eventually promotes its own destruction, allowing chromosomal separation and cytokinesis[252]. Protein phosphatase 1 (PP1) then dephosphorylates Rb, allowing it to once more repress and bind E2F1 and HDAC1, thus preventing repetition of the cell cycle until appropriate mitogenic stimulation is received[258,259].

3.6.2. The role of CDKs in regulating transcriptional fidelity

Whilst CDKs are a vital mechanistic component in cell cycle regulation, they are equally as important for the correct transcription of protein-coding genes by RNA Polymerase II (RNA II). The C-terminal domain (CTD) of RNA II serves as a scaffold for the recruitment of key transcriptional regulators, including various CDKs, and the phosphorylation of numerous amino acid residues on this domain directly influences the fidelity of transcriptional initiation, elongation and termination[260,261]. For transcription to be initiated, RNA II must be recruited to the promoter of the gene in question and it must eventually vacate this position to permit transcriptional elongation[261]. The first step in transcriptional initiation involves the formation of the pre-initiation complex at the gene promoter. This process begins with the binding of the TATA-box binding protein (TBP) domain of general transcription factor TFIID to the core promoter and the subsequent association of RNA II with the Mediator complex by virtue of the affinity of its unmodified CTD for hydrophobic residues in the latter[262–264]. The Mediator complex is a crucial transcriptional co-activator which serves to recruit pro-elongation factors such as P-TEFb and to transduce signals from transcription factors bound at gene enhancer regions to the pre-initiation

complex machinery at gene promoters[265–268]. Mediator function is also governed by CDK8, which transiently associates with the Mediator complex and is therefore able to directly phosphorylate and thus modify the function of transcription factors, an event believed to be mutually exclusive with RNA II-Mediator binding and a putative transcriptional checkpoint[269,270]. The transition from initiation to elongation involves the recruitment of TFIIH to the pre-initiation complex, its helicase component inducing a conformational change in the DNA which facilitates the interaction of single-stranded DNA with the active site of RNA II[271,272]. CDK7 is a catalytic component of TFIIH and phosphorylates the CTD of RNA II at Ser5 and Ser7, thereby disrupting the interaction of RNA II with the pre-initiation complex and promoting the detachment of RNA II from the gene promoter[273,274]. CDK7 also possesses myriad functions other than transcriptional regulation, since it controls the functions of cell cycle-regulatory CDKs by phosphorylating CDK1 and CDK2, thus acting as a CDK-activating kinase (CAK) and linking transcription with cell cycle progression[275]. Elegant work conducted by Larochelle et al (2007) demonstrated that CDK7 inhibition during G1 phase impeded CDK2 activation and consequently hindered progression into S phase, whilst CDK7 inhibition during G2 phase and/or S phase prevented the onset of mitosis and activation of CDK1, with co-immunoprecipitation revealing disruption of the formation of CDK1-Cyclin B complexes[276]. Moreover, CDK7 has also been shown to regulate CDK4/6 function and is therefore essential for G1/S phase transition; CDK7 inhibition lead to the abrogation of Rb-kinase activity of both CDK4 and CDK6, due to impaired CDK4/6 activation and not due to inhibition of CDK4/6-Rb complex formation[277]. Furthermore, CDK7-mediated phosphorylation of the T-loops of CDK4 and CDK6 was required for maintenance of their activity, not just their activation, reinforcing the idea of CDK7 as

a master regulator of CDK function[277]. Intriguingly, apart from controlling transcriptional elongation and the activation of cell cycle-regulatory CDKs, CDK7 may also serve as a link between the transcriptional machinery and activation of the DNA damage response by p53. In response to DNA damage, CDK7 has been shown to phosphorylate and thereby activate p53, enhancing its binding with p53-responsive DNA elements[278,279]. In return, p53 binding reduces the activity of CDK7 at both the CTD of RNA II and CDK2, potentially leading to inhibition of transcription and subsequent cell cycle arrest[280]. CDK7-mediated CTD phosphorylation on Ser5 and Ser7 leads to “proximal promoter pausing” in which RNA II pauses approximately 20-100bps downstream of the transcriptional start site, thereby allowing mRNA modification and the binding of negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF) to RNA II, preventing productive elongation of mRNA transcripts[281,282]. For productive elongation to occur, positive transcription elongation factor B (P-TEFb) which is composed of CDK9 and Cyclin T1, must be recruited to promoters and the associated transcriptional machinery[283,284]. Bromodomain-containing protein 4 (BRD4) is a chromatin reader needed for the transmission of epigenetic signatures during cell division. BRD4 binds to acetylated histones in all cell cycle phases to promote the maintenance of acetylated chromatin and its high-order architecture[285–289]. Crucially, BRD4 is required for both the formation of P-TEFb and its recruitment to promoters; via its P-TEFb-interacting domain (PID), BRD4 associates with P-TEFb and facilitates the dissociation of negative regulators (e.g. 7SKsnRNA) from P-TEFb, thereby activating the complex. CDK9 then phosphorylates both NELF and DSIF, resulting in NELF dissociation and conversion of DSIF into a positive elongation factor[290–292]. Concurrently, CDK9 phosphorylates the CTD of RNA II at Ser2, allowing the recruitment of more

elongation factors and the initiation of productive mRNA elongation. Additionally, BRD4 has been shown to potentiate the kinase activity of P-TEFb at promoters located in regions of highly acetylated chromatin, and render it less susceptible to inhibition, despite lacking CTD kinase activity itself[293–295]. Notably, the recruitment of P-TEFb, and thus CDK9-mediated Ser2 phosphorylation, is dependent upon the presence of phosphorylated Ser5 and Ser7 residues in the CTD, also indicating cross-talk between CDK7 and CDK9[276,296]. The presence of phosphorylated Ser2 on the CTD of RNA II also recruits factors required for termination of transcription and polyadenylation of mRNA transcripts[297,298]; once a termination signal is recognised by RNA Pol II, the phosphatases CTDP1 and SSU72 remove CTD phosphorylation marks at Ser2 and Ser5, restoring CTD phosphorylation to pre-initiation levels, whilst SSU72 also promotes cleavage of pre-mRNA transcripts to complete the process, allowing detachment of RNA II from the promoter and termination of transcription[299,300].

3.6.3. Targeting transcriptional CDKs in oncology

Given their critical roles in the regulation of transcriptional elongation as well as the regulation of cell cycle components in the case of CDK7, the pharmacological inhibition of both CDK7 and CDK9 has been the subject of multiple investigations into their potential utility as anticancer drug targets. Various studies have shown that multiple cancers are indeed exquisitely sensitive to CDK7 inhibitors (e.g. THZ1) and/or CDK9-specific inhibitors, including ovarian carcinoma, neuroblastoma, glioma, non-small cell lung cancer (NSCLC) and T-cell lymphomas[302–309]. Common to the majority of these cases, CDK7/9 inhibition led to downregulation of MYC resulting in decreased levels of short-lived, pro-survival, anti-apoptotic proteins such as MCL-1, leading to reduced cell proliferation and apoptosis[302,303,306,307,310]. As a

consequence, cells were also rendered more sensitive to BH3-mimetics such as ABT-199, with strong synergy observed in both T-cell lymphomas and acute myeloid leukaemia[302,311]. Moreover, in MYC-addicted hepatocellular carcinoma, CDK9-mediated transcription of MYC-regulated genes was found to be indispensable for MYC-driven tumourigenesis[312]. Inhibition of transcriptional elongation, regulated by CDK9 via phosphorylation of RNA-Polymerase II (Ser2/5), using a pharmacological cdc7/CDK9 inhibitor resulted in tumour regressions, abolition of tumour growth and decreased proliferative markers[312]. Regarding TNBC specifically, Horiuchi et al demonstrated that TNBC cells with elevated levels of MYC were particularly sensitive to pan-CDK inhibitors such as Dinaciclib[313]. Taken together, the evidence suggests that targeting currently non-druggable, master transcriptional regulators such as MYC, which act as a downstream convergence point for signals transduced via RTK-mediated signalling, through CDK7 or p-TEFb inhibition may represent a potent new strategy to identify novel therapies for TNBC as well as other cancers, whilst also identifying novel synergistic combinations with enhanced efficacy. Elaborate studies conducted by *Wang et al* illustrated that TNBC cells are uniquely dependent on transcriptional regulation, particularly by CDK7 and CDK9[314]. Their research demonstrated that a cluster of genes (including EGFR) whose transcription is regulated by CDK7 and which are associated with super-enhancers (clusters of enhancers with high transcriptional activity and which are characterised by exceptionally high levels of master transcription factor and Mediator complex binding[315]) was critical for the growth of TNBC cells[314]. Using multiple EGFR-TKIs, it was shown that despite clear inhibition of phosphorylation of EGFR and of its downstream components, cell proliferation was unaffected. However, CRISPR/Cas9-mediated editing of EGFR was detrimental to cell proliferation, with similar results

obtained using the CDK7 inhibitor THZ1, suggesting that eliminating EGFR transcriptional activity represents a more effective approach[314]. Zawitowski et al have recently demonstrated that TNBC tumours treated with Trametinib, a MEK inhibitor, rapidly rewire their signalling circuitry in order to bypass the effects of drug treatment, upregulating the levels of various RTKs such as PDGFR-B and FGFR2[109,316]. Disrupting p-TEFb activation through BRD4 inhibition potently reversed this adaptive transcriptional response, precluding RTK-upregulation and leading to durable responses in xenograft models of TNBC[316]. Interestingly, combining lapatinib treatment with BRD4 inhibitor JQ1 in HER2+ BC similarly led to abolition of an

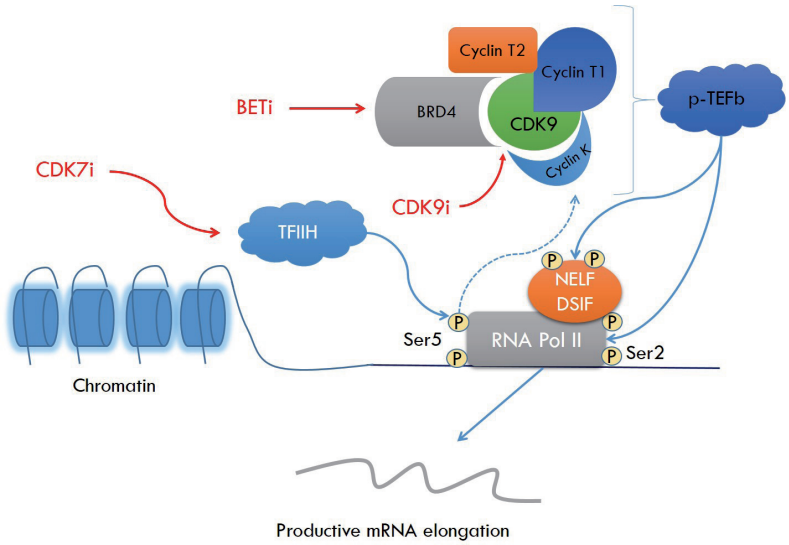

Figure 3: Drugging the transcriptional machinery in cancer. Possible ways of targeting the transcriptional machinery in malignant cells are represented here. Interfering with TFIIH function by inhibiting CDK7 would prevent phosphorylation of RNA Polymerase II's C-terminal domain (CTD) at Ser5, thereby precluding the initiation of transcription since TFIIH facilitates interaction of RNA Polymerase II with DNA. The TFIIH-dependent recruitment of the P-TEFb complex, a member of the super elongation complex (SEC) whose CDK9-mediated phosphorylation of RNA Polymerase II's CTD at Ser2 is vital for productive elongation of mRNA transcripts, would therefore also be averted. P-TEFb itself and its various components are also amenable to pharmacological manipulation. Kinase inhibitors which block the activity of CDK9 would obstruct CDK9-mediated phosphorylation of RNA Polymerase II's CTD at Ser2, as well as the dissociation of negative elongation factors NELF and DSIF, which induce proximal promoter pausing after CDK7-mediated phosphorylation of CTD residue Ser5, ultimately preventing resumption of productive mRNA transcription. Impeding BRD4 function by means of bromodomain inhibitors could also disrupt activation of the kinase component of P-TEFb, as well as its subsequent recruitment to the transcriptional machinery.

adaptive kinome response and reversal of resistance to lapatinib[317]. This evidence reiterates the utility of targeting the transcriptional program of cancer cells addicted to growth factor receptor signalling pathways by halting enhancer remodelling and transcriptional adaptation (see ***Figure 3***). However, there is a paucity of information regarding the possible use of EGFR-TKIs in combination inhibitors which interfere with P-TEFb function in TNBCs resistant to EGFR-TKI monotherapy. Since EGFR-TKIs are notoriously ineffective as monotherapies against TNBC, despite the dependence of these tumours on EGFR-mediated signalling, there is a clear rationale to inhibit both RTK-mediated signalling at the source and the P-TEFb complex which regulates the transcription of genes up-regulated via activation of these growth factor-mediated pathways. This two-pronged approach would seek to reverse the intrinsic resistance of TNBC cells to RTK-targeted therapies by eliminating their propensity for transcriptional plasticity in response to therapy, thereby sensitising them to EGFR-TKIs.

4. Conclusion

In summary, TNBC employs a vast arsenal of resistance mechanisms in order to evade elimination. This arsenal primarily consists of subversion of cellular signalling networks in response to kinase-targeted therapy, a testament to the plasticity of this disease. Evidently, given the critical role of transcription in the conduction and regulation of these adaptive responses, inhibition of such transcriptional machinery therefore represents an attractive strategy for the reversal of resistance to targeted therapies such as EGFR-tyrosine kinase inhibitors or PI3K-AKT-mTOR inhibitors. Notwithstanding the promising pre-clinical studies of transcriptional CDK inhibitors described here, the development of highly efficacious, selective agents remains an elusive prospect. This lack of specificity often results in undesirable or even life-

threatening side-effects, thereby limiting the anti-cancer potential of such agents. Future drug design programmes must therefore identify methods to circumvent the structural similarity between the various CDKs in order to address these issues. Moreover, whilst combining multiple kinase inhibitors as a means of depleting cancer cells of options to rewire their survival signalling appears sensible, the importance of practical considerations regarding dosage, toxicity and contraindications cannot be understated. Altogether, multiple options exist regarding the use of molecular targeted therapy against TNBC, including, but not limited to, DNA-damaging agents, EGFR-tyrosine kinase inhibitors, PI3K-Akt-mTOR inhibitors, FGFR inhibitors and CDK inhibitors. However, given the intricate and innumerable mechanisms of resistance, thinking one step ahead in order to predict and subsequently target possible pathways implicated in the development of resistance to such agents is essential if progress is to be made in improving the clinical management of TNBC.

5. Aims and Outline of this Thesis

The primary objective of the research described in this thesis was to identify novel therapeutic targets and synergistic combination treatments for TNBC. Primarily, this research focused on profiling the response of TNBC to multiple kinase inhibitors (e.g. MEK, AKT and EGFR inhibitors) and ascertaining which targeted agents were capable of subverting the refractory response of TNBC thereto, by means of high-throughput, compound library-based screening. Consequently, the potential of prospective, novel targeted agents, specifically CDK inhibitors, either as monotherapies or in combination with other kinase inhibitors was thoroughly evaluated. **Chapter 2** explores the differential sensitivity of TNBC cells to MEK and AKT inhibitors, illustrating the heterogeneity in response to such targeted agents. Here, the link between resistance to MEK/AKT inhibitors and cell cycle regulation is

emphasised, as well as how this cell cycle dependency can be exploited therapeutically in drug-resistant subtypes using potent CDK inhibitors. **Chapter 3** delineates the potential of combined cdc7/CDK9 inhibition as a means to overcome resistance to multiple EGFR-TKIs in TNBC. **Chapter 4** elaborates on these findings by describing the evaluation of a panel of novel CDK inhibitors as potential therapeutic agents in TNBC both *in vitro* and *in vivo*. Here, compound-based screening coupled with proliferation and apoptosis assays demonstrates that a selection of these novel CDK inhibitors are highly effective as anti-TNBC therapies. The effect of these drugs on relevant signal transduction pathways such as CDK-mediated transcription and the equilibrium between pro- and anti-apoptotic BCL-2 family members is extensively examined here. RNA Seq-based transcriptomic analysis was employed to further clarify the mechanisms of action of these compounds and define a set of transcription factors essential for TNBC proliferation, and which are exquisitely sensitive to CDK inhibition. The utility of combining EGFR-TKIs and BET inhibitor JQ1 with one of these novel CDK inhibitors is described in **Chapter 5**, in which it is shown that combining these agents is synergistic in inhibiting the proliferation of TNBC cells and augments the inhibitory effect of the CDK inhibitor on transcription and CDK-mediated signal transduction. Finally, **Chapter 6** provides a discussion and summary of the aforementioned results, focusing on the key conclusions derived from this research and possible avenues for future research.

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