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TGFβ-dependent mechanism of the bone marrow-mediated resistance against PARP inhibitors in leukemias

PhD thesis

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ABBREVIATIONS

Abbreviation	Description
ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
ATM	Ataxia-Telangiectasia mutated
ATR	Ataxia Telangiectasia and Rad3-related
BCR-ABL1	Fusion protein consisting of: Breakpoint Cluster Region protein and Abelson murine Leukemia viral oncogene homolog 1
BER	Base excision repair
BMC	Bone marrow cell
BMM	Bone marrow microenvironment
BMM-cm	Bone marrow microenvironment-conditioned medium
BMM-dc	Bone marrow microenvironment-direct contact
BMM-idc	Bone marrow microenvironment-indirect contact
B-NHEJ Alt-NHEJ	Alternative non-homologous end joining
BRCA	Breast cancer susceptibility protein
BSA	Bovine serum albumin
CLL	Chronic lymphoid leukemia
CML	Chronic myeloid leukemia
CML-BC	Chronic myeloid leukemia-blast crisis
CML-CP	Chronic myeloid leukemia-chronic phase
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR Associated protein 9
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	C-X-C chemokine receptor 4
CXCR4i	C-X-C chemokine receptor 4 inhibitor
DNA-PKcs	DNA-dependent protein kinase catalyzed subunit
D-NHEJ	Canonical non-homologous end joining
DSB	Double-strand break
ECs	Endothelial cells
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FLT3	FMS-like tyrosine kinase 3
FLT3(ITD)	FMS-like tyrosine kinase 3 (internal tandem duplication)
FLT3(TKD)	FMS-like tyrosine kinase 3 (tyrosine kinase domain)
FLT3(WT)	FMS-like tyrosine kinase 3 (wild-type)
FLT3i	FMS-like tyrosine kinase 3 inhibitor
G-CSF	Granulocyte-colony stimulated factor
GFP	Green fluorescent protein
HIF	Hypoxia-inducible factor
HPC	Hematopoietic progenitor cell
HPV	Human papilloma virus

HR	Homologous recombination
HSC	Hematopoietic stem cell
IL	Interleukin
LPC	Leukemia progenitor cell
LSC	Leukemia stem cell
MDS	Myelodysfuntion syndrome
miRNA	MicroRNA
MPN	Myeloproliferative neoplasm
MSCs	Mesenchymal stromal cells
NHEJ	Non-homologous end joining
OTK	Oncogenic tyrosine kinase
PARP	Poly ADP-ribose polymerase
PARPi	Poly ADP-ribose polymerase inhibitor
PBL	Peripheral blood leukocyte
PBM	Peripheral blood microenvironment
PBS	Phosphate buffer saline
RF	Replication fork
RFP	Red fluorescent protein
RT	Room temperature
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulfate-Poly acrylamide gel electrophoresis
SPL	Splenocyte
SSB	Single-strand break
TAE	Tris-Acetic acid-EDTA
TET2	Ten-eleven translocation 2
TGF-β	Transforming Growth Factor-beta
TGFβR	Transforming Growth Factor-beta receptor
TGFβRi	Transforming Growth Factor-beta receptor inhibitor
TKD	Tyrosine kinase domain
TKi	Tyrosine kinase inhibitor
TME	Tumor microenvironment
TPO	Thrombopoietin

ABSTRACT

Leukemia is a group of blood cancer deriving from bone marrow, in which hematopoietic stem/progenitor cells are malignantly transformed into leukemia stem/progenitor cells by activation of oncogenes or inactivation of tumor suppressor genes. Typically, activation of oncogenic tyrosine kinase (TK) receptor FLT3(ITD) occupies approximately 23% cases of acute myeloid leukemia (AML) and oncogenic tyrosine kinase BCR-ABL1 in Philadelphia fusion chromosome is observed in around 95% of chronic myeloid leukemia (CML). Treatment of these leukemias by TK inhibitor (TKi) individually or in combination with standard chemotherapies has obtained initial remission. However the disease relapse has been acquired rapidly after delaying this strategic therapy, leading to urgent requirement for more efficient therapeutic approach.

Poly ADP-ribose polymerase inhibitor (PARPi)–induced synthetic lethality is one of the current promising anticancer strategies, which has been successfully used in *BRCA1/2*–mutated breast/ovarian cancer cells. This also resulted in a promising effects in leukemia despite of lacking *BRCA1/2* mutations in the hematopoietic malignancy, which has been shown by different groups including ours. We found that several oncogene-positive leukemias (eg. AML1-ETO, BCR-ABL1, IDH1/2) led to deficiencies in BRCA1/2, resulting in sensitivity to PARPi. Moreover, specific tyrosine kinase inhibitor such as FLT3(ITD) inhibitor (AC220) induced "BRCAness" phenotype, enhancing efficacy of PARPi against FLT3(ITD)–positive AML both *in vitro* and *in vivo*. However, in spite of effective potency of PARPi in BRCA1/2–deficient cancers, resistance to PARPi–mediated synthetic lethality has been reported in both preclinical research and clinical trials.

In this study, we discovered a novel mechanism of resistance against PARPi in BRCA–deficient leukemias mediated by the stromal cells-dependent bone marrow microenvironment (BMM), mediated by the transforming growth factor-beta 1 (TGF-β1) – TGFβ receptor (TGFβR) signaling pathway. Genetic/pharmacological targeting of TGF-β signaling pathway restored sensitivity of leukemia cells to PARPi in BMM *in vitro*. Remarkably, TGFβR serine/threonine kinase inhibitor (SB435142) enhanced anti-leukemia effect of [PARPi + TKi] in the leukemia-engrafted mice *in vivo*, therefore, prolonging the survival of leukemia-bearing mice. In conclusion, as the strategic therapy did not caused cytotoxic effect on the bone marrow healthy cells, we propose that the FDA–approved TGFβR kinase inhibitors are good candidates for clinical trials and treatment of leukemia patients currently receiving [PARPi +/- TKi] to enhance drug response as well as improve therapeutic efficiency.

STRESZCZENIE

Białaczki to nowotwory krwi wywodzące się ze szpiku kostnego, w których dochodzi do przekształcenia hematopoetycznej komórki macierzystej / progenitorowej w białaczkowe komórki macierzyste / progenitorowe, porzez aktywację onkogenów lub inaktywację genów supresorowych nowotworu. Przykładowo, aktywacja onkogennego receptora kinazy tyrozynowej (TK) FLT3 (ITD) wystepuje w około 23% przypadków ostrej białaczki szpikowej (AML), a onkogennej kinazy tyrozynowej BCR-ABL1 na skutek powstania fuzyjnego chromosomu Philadelphia wystepuje w około 95% przypadków przewlekłej białaczki szpikowej (CML). Leczenie tych białaczek inhibitorami kinaz tyrozynowych (TKi), zarówno w terapii pojedynczej jak i skojarzonej, w połączeniu ze standardowymi chemioterapiami daje korzystne efekty terapeutyczne i prowadzi do poczatkowej remisji. Jednak nawroty choroby i pojawienie się oporności jest częste, co prowadzi do konieczności poszukiwania nowych strategii leczenia i pilnego zapotrzebowania na bardziej skuteczne podejście terapeutyczne.

Syntetyczna letalność indukowana przez inhibitory polimerazy ADP-rybozy (PARPi) jest jedną z obiecujących i aktualnie intensywnie badanych nowych strategii przeciwnowotworowych. Dotychczas stosowano ją z powodzeniem w komórkach raka piersi/jajnika, niosących mutacje genów BRCA1/2. Nasze wczesniejsze badania oraz badania innych grup wykazały, że zastosowanie inhibitorów PARP wykazuje również obiecujący wpływ terapeutyczny w przypadku białaczek, mimo iż nie należą one do nowotworów związanych z mutacjami BRCA1/2. Stwierdziliśmy, że ekspresja białaczkowych onkogenów (np. AML1-ETO, BCR-ABL1, IDH1/2) prowadziła do niedoborów BRCA1/2, co skutkowało wrażliwością na inhibitory PARP. Ponadto, specyficzny inhibitor kinazy tyrozynowej - FLT3(ITD) indukował fenotyp “BRCAness”, zwiększając skuteczność inhibitorów PARPi w komórkach białaczki, zarówno *in vitro*, jak i *in vivo*. Jednak pomimo skutecznego zastosowania PARPi w nowotworach z niedoborem BRCA1/2, zarówno w badaniach przedklinicznych, jak i klinicznych zaobserwowano pojawiającą się oporność. W naszych badaniach odkryliśmy nowy mechanizm oporności na PARPi w białaczkach z niedoborem BRCA1/2, związany z mikrośrodowiskiem szpiku kostnego (BMM) oraz regulowany przez szlak sygnałowy: transformujący czynnik wzrostu beta 1 (TGF-β1) - receptor TGFβ (TGFβR). Genetyczne/farmakologiczne zahamowanie tego szlaku sygnałowego przywracało wrażliwość komórek na PARPi w mikrośrodowisku szpiku in vitro. Co ważne, w badaniach in vivo wykazaliśmy, że inhibitor receptora TGFβR (SB435142) nasilał działanie przeciwbiałaczkowe [PARPi + TKi] i przedłużał przeżycie myszy z bialaczką. Ponieważ badana przez nas strategia terapeutyczna nie powodowała działania cytotoksycznego na zdrowe komórki szpiku kostnego, proponujemy, że inhibitory kinazy receptora TGFβR, zatwierdzone przez FDA, mogą być kandydatami do badań klinicznych nad łączoną terapią pacjentów z białaczką, obecnie otrzymujących [PARPi +/- TKi], w celu zahamowanie oporności i poprawy odpowiedzi na lek, a także poprawy skuteczności terapii.

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1. INTRODUCTION

This doctoral dissertation investigates the potential efficacy of Poly-ADP-Ribose Polymerase (PARP) inhibitors (PARPi) and PARPi–induced synthetic lethality as an anti-leukemic treatment, despite rare BRCA1/2 mutations in leukemic cells. Several oncogene-positive leukemias (eg. AML1-ETO, BCR-ABL1, MLL-AF9), that carry deficiencies in BRCA1/2 proteins, have been eliminated by PARPi (as a single treatment) [1-3]. However, synergistic therapies of PARPi with other targeted inhibitors could likely enhance efficacy of PARPi in BRCA1/2-proficient leukemias. Thus, in the first part of presented studies, we hypothesized that inhibition of FLT3(ITD) oncogenic tyrosine kinase (OTK) receptor by FLT3 inhibitor AC220 (also known as quizartinib) would sensitize FLT3(ITD)–positive leukemia cells to PARP inhibitors. On the other hand, resistance to common anti-leukemic therapies has been often described as connected to the leukemic bone marrow microenvironment. Hence, the next parts of the studies were aimed at verifying a novel and constitutive mechanism that induces resistance against PARPi in leukemia cells, activated by the stromal component of the bone marrow microenvironment. Conversely, targeting the discovered mechanism restored efficacy of PARPi against leukemic cells, indicating clinical potential of obtained results. Introduction of this thesis contains brief description of typical OTKs leading to leukemic transformation of hematopoietic progenitor cells, alongside with descriptions of OTK inhibitors, concept of synthetic lethality strategy, DNA double-strand break (DSB), DSB repair, PARP1 and PARPi –mediated synthetic lethality, published mechanisms of PARPi resistance and another possible mechanism of this resistance in leukemias, mediated by the bone marrow microenvironment.

1.1 Leukemia – a hematopoietic cancer

Leukemias are defined as a group of clonal blood cancers originating from hematopoietic stem cells (HSCs), which normally give rise to every cellular component of blood. In the hierarchy of hematopoiesis, HSCs divide, self-renew and also further differentiate into two common progenitors - myeloid and lymphoid progenitors (Figure 1.1). At this stage of hematopoietic differentiation, activation of oncogenes and/or loss of tumor suppressor genes (due to DNA mutations) can result in malignant transformation of HSCs or myeloid/lymphoid progenitors, into leukemia stem/progenitor cells (LSCs/LPCs). Following

this transformation, malignant cells are capable of proliferating uncontrollably and without cell death induced by apoptosis. This also results in impaired cell differentiation in the bone marrow – disrupting formation of red blood cells (erythrocytes), platelets and white blood cells, including functional immune cells (T, B cell precursors, NK cells etc.). Furthermore, after initial development in the bone marrow, LPCs continue expansion and eventually spread into peripheral blood as leukemic blasts. Based on the type of progenitors and the growth rate of malignant cells in the bone marrow, leukemias are categorized into four major groups:

- Acute myeloid leukemia (AML)
- Chronic myeloid leukemia (CML)
- Acute lymphoblastic leukemia (ALL)
- Chronic lymphocytic leukemia (CLL)

To diagnose and screen leukemia, the most common methods are total blood cell counts, bone marrow biopsy and monitoring the symptoms. Signs and symptoms of leukemia in affected individuals vary, depending on transformed progenitors in the hematopoietic hierarchy.

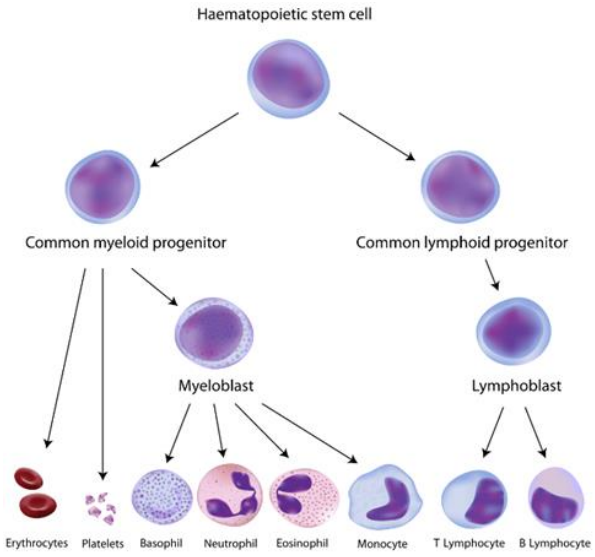

Figure 1.1. Hematopoietic hierarchy, in which HSCs initiate myeloid and lymphoid progenitors. Differentiation of myeloid progenitor drives the formation of red blood cells (erythrocytes), platelets and myeloid white blood cells (basophil, neutrophil, eosinophil, monocyte). Meanwhile, lymphoid progenitor differentiates into lymphoid immune cells (T, B lymphocytes, NK cells). Source (unprotected by lawful enforcement): https://vector.childrenshospital.org/2014/04/can-blood-cells-be-rebooted-into-blood-stem-cells.

In myeloid leukemias, malignant transformation of the myeloid progenitor abrogates formation of red blood cells (erythrocytes) and platelets production in the bloodstream, leading to bruising, excessive bleeding, pale skin and even fever in patients. Similarly to myeloid leukemias, in lymphoid leukemias loss of functional immune cells results in frequent infections in affected individuals, ranging from infection in tonsils, sores in mouth, diarrhea, to life-threatening pneumonia. Anatomically, progression of leukemia is associated with enlargement of spleen and liver [4]. In Poland, according to the most recent report from the World Health Organization (WHO), fatal cases of leukemia in 2017 reached 2,655, 0.77% of the affected population. Moreover, in terms of leukemia–related death rate, Poland has been standing at the 79th in over the world, with approximately 4.15 in 100,000 people. In the United States of America (USA), where cancer statistics are updated more frequently, total new incidences of leukemia in 2020 are predicted to reach 60,530, while the incidence rate in the period 2012 – 2016 was 14.2 in 100,000 people and death rate in the period 2013 - 2017 was 6.4 per 100,000 individuals. Moreover, in the USA, amongst all types of cancer, leukemias take up 2.5% of incidences and 3.5% of the total death rate.

Amongst the four major groups of leukemia, AML has been diagnosed in approximately 90% of cases of leukemia in adults [5]. Meanwhile, CML has represented 15% of leukemia incidences and 15-25% of all adult leukemias in Western countries [6]. A type of lymphoid leukemia – ALL - has been the most common hematopoietic malignancy in children from two to five years old and accounts for 25% of all cancers in this range of age [7]. Conversely, CLL has been only observed in adults over 50, establishing just 1% of all cancers [8]. Besides the popular environmental risk factors potentially leading to leukemias (such as ionizing/non-ionizing radiation, chemicals and smoking), the genetic alterations such as chromosomal translocations, deletions and insertions (deriving from acquired somatic mutations) are greatly implicated in leukemia development [9]. For example, translocation between chromosomes 8 and 21 [t(8;21) (q21;q22)] results in expression of the oncogene AML1-ETO (RUNX1;RUNX1T1) found in 5-12% of AML cases [10], t(9;11) (q23;p13.3) constitutes fusion oncogene TCF3-PBX1 accounting for 5% of ALL and 20% of pre B-ALL [11], whereas t(9;22) (q34;q11) leads to formation of the tyrosine kinase BCR-ABL1 responsible for CML [12]. In general, acquired somatic mutations mostly influence structure or

expression of the proteins performing key functions in normal hematopoiesis [13]. Hyperactive tyrosine kinases (TK) and receptor tyrosine kinases (RTK) are important oncoproteins, leading to cancers (including leukemias) [14, 15], whereas normally expressed and/or non-mutated TK/RTK perform significant roles in hematopoiesis [16-19]. Hence, number of studies on the TK/RTK associated with leukemia has been increasing year by year in the hematological area. In the following chapters, we describe the two most common oncogenic TK/RTK directly involved in the pathogenesis of AML and CML.

1.2 Leukemia–related oncogenic tyrosine kinases

1.2.1 FLT3(ITD) oncogenic receptor tyrosine kinase in AML

AML is a heterogeneous disease in which leukemic cells derive from myeloid progenitors and exhibit rapid, unlimited proliferation and lack of capacity to differentiate [5, 20]. Naive AML is coming from malignantly transformed progenitors, without effect of a previous disease or treatment. Meanwhile, secondary AML is usually associated with evolution of other hematopoietic malignancies, such as myelodysfunction syndrome (MDS) and myeloproliferative neoplasm (MPN), or due to an undesired consequence of initial chemotherapies with genotoxic/cytotoxic agents, as well as ionizing irradiation. Generally, secondary AML cases have been reported to account for 20-35% of overall AML cases, leading to more severe disease in affected individuals than the naive AML [21, 22]. In terms of genetic background, AML is caused by mutations in a wide range of genes encoding proteins performing functions in (ordered according to frequency in AML): receptor signaling (FLT3, KIT), DNA methylation and hydroxymethylation (DNMT3A, TET2, IDH1/2), chromatin remodeling (ASXL1, EZH2, MLL), myeloid transcription process (CEBPA, RUNX1), chromosomal translocation oncoproteins (RUNX1-RUNX1T1, PML-RARA, CBFB-MYH11), tumor suppression (TP53, WT1) [23]. Among types of mutagenesis related to AML, mutations in FLT3 receptor tyrosine kinase have been reported as the most common genetic derivation, accounting for approximately 1/3 of AML cases. AML-related FLT3 mutations contain internal tandem duplication [FLT3(ITD)] of 3-400 bp fragment observed in juxtamembrane region (associated with 23% of AML cases) and missense mutations in tyrosine kinase domain [FLT3(TKD)], responsible for 8-12% of AML occurrences [24-27]. Between two kinds of FLT3 mutations, FLT3(ITD) not only results in higher proportion of

AML incidences, but also leads to worse prognosis with higher leukemia burden and negative impact on disease management in patients [20, 25, 28-31].

FMS-like tyrosine kinase 3 (FLT3) is a transmembrane RTK, member of a receptor family together with FMS, KIT and PDGFRα/β. In hematopoiesis, FLT3 is expressed on the surface of HSCs/HPCs and the expression is reduced upon hematopoietic differentiation. Although expression of FLT3 is attenuated during later stages of hematopoiesis, the receptor has been described to be involved in cell survival, proliferation and differentiation [32-35]. In functional studies of FLT3 as a proto-oncoprotein, mice with simultaneous conditional knock-out of FLT3 receptor and FLT3 ligand revealed normal growth and survival without hematopoietic malignancies [36]. On the other hand, genetic knock-out of FLT3 receptor has been associated with decreased differentiation of B cell progenitors, NK cells and dendritic cells in the bone marrow, suggesting that wild-type (WT) FLT3 receptor has an important function in hematopoietic differentiation [37].

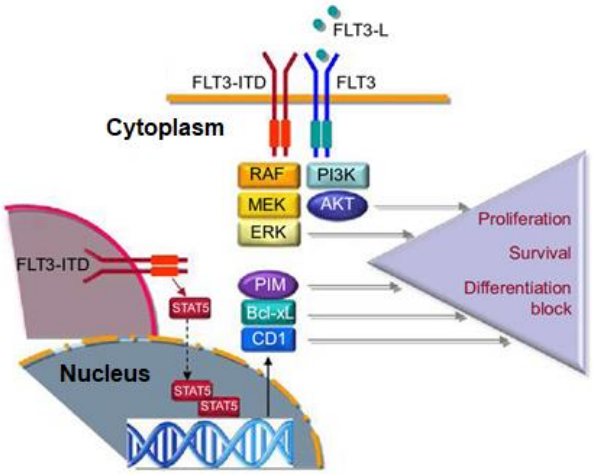

Figure 1.2 Molecular signaling pathways of FLT3(WT) receptor kinase stimulated by FLT3 ligand and FLT3(ITD) oncogenic receptor kinase with FLT3 ligand-independent manner [38-modified].

In terms of FLT3–mediated pathways, physiological stimulation of the receptor by FLT3 ligand leads to enhanced proliferation of HSCs/HPCs *in vitro*, when treated together with other growth factors, such as SCF, TPO and IL-3. This indicates that the activation of FLT3(WT) receptor in normal hematopoiesis requires interaction with FLT3 ligand. To further promote cell proliferation, survival and differentiation, activation of FLT3(WT) receptor by FLT3 ligand stimulation, triggers several downstream signals including PI3K/AKT,

RAS/RAF/MAPK, STAT5 [39]. However, when FLT3(ITD) mutation occurs in the juxtamembrane domain, FLT3 becomes an oncogenic receptor tyrosine kinase and is constitutively activated, without requirement of FLT3 ligand stimulation. This leads to constant proliferation of progenitor cells without differentiation (via RAS/RAF/MAPK), as well as their survival due to PI3K/AKT and STAT5–induced Bcl-xL expression and its anti-apoptotic function (Figure 1.2). Upon persistent activation of these signaling via MAPK, PI3K and STAT5, HSCs/HPCs are malignantly transformed into leukemic cells. Indeed, original studies verifying FLT3(ITD) association with AML have been conducted by diagnosis of the bone marrow samples induced by this mutation, and followed by disease relapse determination [40, 41]. Furthermore, convincing evidence confirming FLT3(ITD) as a leukemia–associated mutation comes from an observation that 75% of individuals with FLT3(ITD)–positive AML at initial diagnosis, still harbor this mutation at relapse [42]. Additionally, according to the Medical Research Council classification, FLT3(ITD) has been classified as one of the three most common mutations causing AML in adults, based on intermediate-risk karyotype analysis [43, 44]. Altogether, FLT3(ITD) can be clearly considered the most typical leukemia (especially in AML)–related OTK and a solid target for therapy by TK inhibitors (herein: FLT3 inhibitors). Thus, it should be thoroughly investigated to enable complete eradication of malignant cells.

1.2.2 Fusion oncogenic tyrosine kinase BCR-ABL1 in CML

CML is the second group of hematopoietic cancers deriving from myeloid progenitors. It is distinguished from AML by slower proliferation of malignant cells, as disease develops from chronic phase, via accelerated stage, and eventually to blast crisis. Genetic background of CML is related to generation of fusion OTK BCR-ABL1 in the Philadelphia chromosome, due to a translocation between chromosomes 9 and 22 [t(9;22) (q34;q11)] [45] (Figure 1.3) - responsible for more than 95% overall CML cases [46].

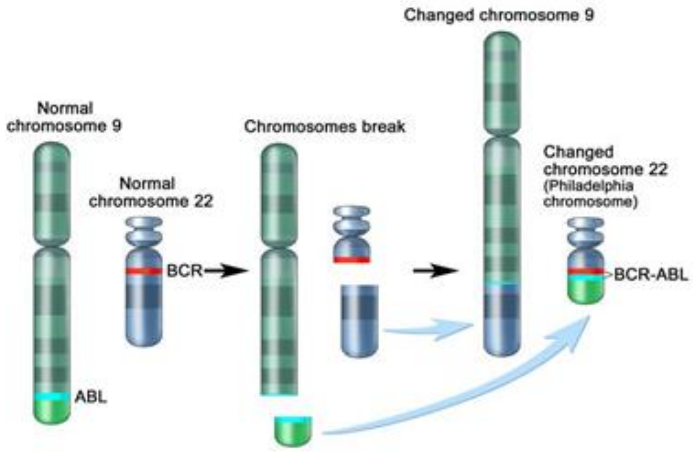

Figure 1.3. Translocation between chromosome 9 and 22 leads to constitution of fusion oncogenic tyrosine kinase BCR-ABL1 in Philadelphia chromosome. Source (unprotected by lawful enforcement): https://www.cancer.gov/publications/dictionaries/cancer-terms/def/bcr-abl-fusion-gene.

Functional studies have confirmed the association between CML and expression of BCR-ABL1 in normal $CD34^+$ HSCs *in vitro*, as well as knock-in of BCR-ABL1 into mice *in vivo* (by retroviral transduction of HSCs), led to rise of CML hallmarks in BCR-ABL1–expressing cells [47, 48], as well as in the bone marrow and peripheral blood of oncogenic knock-in mice [49, 50]. In another *in vivo* study, in mice harboring BCR-ABL1 in Philadelphia chromosome together with existence of a missense mutation in *ABL1* gene (K1176R) (which results in completely impaired kinase activity of the BCR-ABL1), no symptoms of CML in either both bone marrow and peripheral blood were found [51]. The leukemia–initiating role of BCR-ABL1 has been further validated in mice with expression the OTK inducible by tetracycline. The animals, in which tetracycline-induced OTK expression occurred, developed CML, while the counterparts without the induction by tetracycline were capable to survive without signs of malignant transformation in myeloid progenitors [52].

To induce molecular signaling of BCR-ABL1 protein kinase, auto-phosphorylation of Tyr117 on BCR triggers the entire signaling cascade, that further results in malignant transformation of myeloid progenitors into leukemic cells [53] (Figure 1.4).

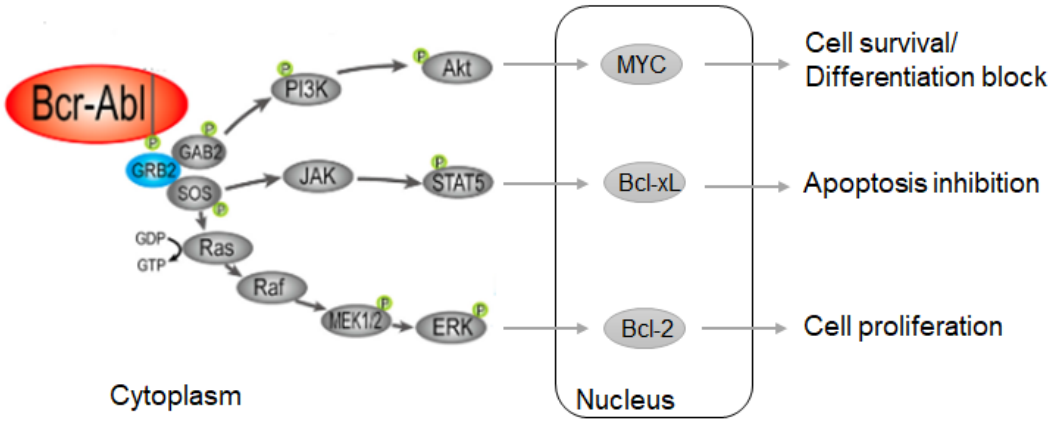

Figure 1.4. The major molecular signaling pathways of fusion oncogenic tyrosine kinase BCR-ABL1 [54-modified].

In more detail, phosphorylation of Tyr117 recruits GRB2 (growth factor receptor-bound protein 2) co-binding with GAB2 (GRB2 –associated binding protein 2) and SOS (son of sevenless, a guanine-nucleotide exchanger of RAS). The complex of GRB2-SOS activates RAS/RAF/MAPK pathways to enhance cell proliferation in CML-chronic phase [55, 56], and prevent apoptosis, due to RAS–mediated increased expression of Bcl-2 in the nucleus [57]. To verify if the signal from phospho-Tyr117 leads to activation of RAS/MAPK, a mutation in *BCR,* substituting Tyr117 to phenylalanine (Tyr117Phe) was introduced and has indeed resulted in inhibition of the binding of GRB2 to SOS, leading to inhibition of BCR-ABL1–induced RAS/MAPK activation. Consequently, due to inactivated RAS/MAPK pathway, this mutation of *BCR* abrogated malignant transformation of primary bone marrow cells, despite of presence of completely functional protein kinase ABL1 [58]. Moreover, in the BCR-ABL1 signaling pathway, GRB2-GAB2 binding leads to constitution of active PI3K/AKT signaling, to enhance survival of myeloid progenitors [59]. Additionally, the activation of phospho-AKT increases expression of *MYC* gene, subsequently resulting in stabilization of MYC and causing differentiation arrest in myeloid progenitors [60]. The final major molecular pathway induced by BCR-ABL1 (involved in CML transformation) is induction of phosphorylated STAT5 (pSTAT5) directly, or indirectly through Janus kinase 2 (JAK2). This is followed by translocation of pSTAT5 from cytoplasm into the nucleus, to up-regulate transcription of *Bcl-xL*. In co-operation with RAS-induced Bcl-2, Bcl-xL prevents apoptotic cell death [61]. Altogether, it can be clearly stated that BCR-ABL1–mediated molecular signals represent co-operative involvement in CML transformation, that has been confirmed by expression of

dominant negative mutant of each pathway including: RAS/MAPK, PI3K/AKT and JAK2/STAT5. Consequently, complete impairment of two out of these three signaling pathways by the dominant negative mutants led to apoptosis of BCR-ABL1–positive K562 cells [62]. Taken together, undoubtedly, strong kinase activity exerted by BCR-ABL1 is well established in promotion of malignant transformation of myeloid progenitors to CML cells.

The progression of CML includes two main stages, as disease develops from chronic phase (CP) to blast crisis (BC). The expression of BCR-ABL1, as well as its constitutive kinase activity and activation of the related signaling pathways are responsible for the expansion of malignant cells. Indeed, CML-BC cells exhibit increased expression of BCR-ABL1 on both mRNA and protein levels [63, 64]. Moreover, the elevated transcript level of *BCR-ABL1* has been found in $CD34^+$ CML and granulocyte-macrophage progenitors analyzed during expansion of CML-BC [65, 66]. Moreover, BCR-ABL1 has been reported to modulate transcription of several genes encoding proteins involved in hematological differentiation [67, 68]. The most canonical example is of transcription factor CCAAT/enhancer-binding protein-α (CEBPα), which controls normal granulocyte differentiation and is expressed in both normal bone marrow cells and CML-CP counterparts. However, in CML-BC cells, the expression of CEBPα was not detected and the elevated activation of BCR-ABL1 in CML-BC has been revealed to drive this phenomenon by stabilization of poly(rC)-binding protein heterogeneous nuclear ribonucleoprotein E2 (hnRNPE2) [69]. Besides participation in progression from CML-CP to CML-BC through increased expression level and inhibition of progenitor cell differentiation, BCR-ABL1 has been demonstrated to trigger reactive oxygen species (ROS) release. This leads to sporadic oxidative DNA damage, including DNA double-strand breaks (DSB) in S and G_2/M phases of cell cycle, as well as mutagenesis [70]. Furthermore, BCR-ABL1 induces impairment of DNA repair activity by down-regulating ataxia telangiectasia and RAD3-related protein (ATR) expression which is responsible for DNA damage response prior to cell cycle arrest [71]. More importantly, deficiencies in DSB repair pathways (comprising homologous recombination, non-homologous end joining, base excision repair) are derived from elevated expression of BCR-ABL1 [70]. Altogether, ROS induction (mediated by BCR-ABL1) leads to accumulation of oxidative DNA damage and insufficient DNA repair, enhancing genomic instability in malignant cells. Such mechanism enables rapid progression from CML-CP into CML-BC. With crucial role of BCR-ABL1 protein

kinase in CML initiation and progression, analogically to FLT3(ITD)–positive AML, it is important to develop and investigate OTK inhibitors against BCR-ABL1 to eliminate leukemia cells.

1.3 Oncogenic tyrosine kinase inhibitors

1.3.1 FLT3 inhibitors in treatment of FLT3-mutant AML

As described above, OTKs are crucial for malignant transformation of myeloid progenitors into AML and CML cells. Therefore, development of TK inhibitors (TKi) to abrogate activation of OTKs is necessary as a targeted therapy in leukemias. To target FLT3(ITD) or FLT3(TKD)-positive AML, several first generation FLT3 inhibitors (including lestaurtinib, sunitinib, sorafenib, and midostaurin) have been developed and efficacy was tested in affected leukemia patients [72-76]. Despite possibility to target activated FLT3-mutant receptor, mono-therapy with all first generation FLT3 inhibitors has shown limited efficacy in FLT3(ITD) –positive AML patients, indicating requirement of combinational strategy of FLT3 inhibitors with e.g. chemotherapeutic agents [76, 77]. However, even in combination with extensive chemotherapy, lestaurtinib and sunitinib did not significantly improve treatment outcome of AML patients carrying FLT3 mutations [78, 79]. On the other hand, combination of sunitinib with cytarabine/doxorubicin yielded complete remission (CR) in 50% of FLT3(ITD) –positive AML cases and 38% of FLT3(TKD) –positive AML cases [76]. Efficacy of sorafenib and midostaurin against FLT3–mutant AML was insufficient [80, 81], further demonstrating limited anti-leukemic effect of the FLT3 inhibitors. Altogether, development of next generation of FLT3 inhibitors that more precisely and potently target the activated FLT3–mutant receptor has been advised.

Therefore, the next generation FLT3 inhibitors, including gilteritinib, crenolanib and quizartinib, have been verified in clinical trials. These FLT3 inhibitors manage to more potently and selectively inhibit FLT3-mutant receptor than the first generation inhibitors, which also happen to target multiple other pathways besides FLT3 [73]. Among the three next generation FLT3 inhibitors, gilteritinib and crenolanib belong to type I inhibitors which inhibit FLT3 receptor activated by both FLT3 ligand and FLT3 mutations, whereas quizartinib belongs to type II, which selectively targets FLT3(ITD) oncogenic receptor [82]. Gilteritinib or crenolanib used as a single agent have been validated in phase two of clinical trials, and

overall response rate (ORR) (including CR and partial remission (PR)) did not exceed 50% [83, 84]. However, because these two inhibitors also target FLT3 receptor activated by FLT3 ligand, such treatment has led to unexpected effect on hematopoiesis. Indeed, gilteritinib and crenolanib elicited CR with incomplete blood count recovery in 30% and 23% in AML patients, respectively [83, 84]. To deal with these side effects of type I FLT3 inhibitors, type II FLT3 inhibitor (quizartinib), has been developed as an orally-delivered, selective and potent compound [85, 86]. In clinical trials, monotherapy with quizartinib of naïve FLT3(ITD) – positive leukemia patients has yielded 46-56% CR with ORR reaching 74-77%, leading to enhanced overall survival (OS) in affected individuals [87]. Moreover, in that study, in relapsed FLT3(ITD)–positive AML patients over 60 years old, single treatment with quizartinib provided 56% CR and 77% ORR. Importantly, in individuals with relapsed FLT3(ITD)–positive AML over 18 years old, after failure of initial chemotherapy or relapse after allogeneic HSCs transplantation, monotherapy with quizartinib delivered 46% CR and 74% ORR. Moreover, combined treatment with quizartinib and standard chemotherapeutic agents (such as cytarabine or doxorubicin) has also been reported to deliver remarkable remission in both younger and older AML patients harboring FLT3(ITD) [88, 89]. Altogether, next generation type II FLT3 inhibitor, quizartinib (also known as AC220), can be considered as the most promising FLT3-targeting therapy, to eradicate FLT3(ITD)–positive acute myeloid leukemia.

1.3.2 Imatinib mesylate in treatment of BCR-ABL1–positive CML

Since BCR-ABL1 is responsible for CML development and progression, in 2003 a massive study conducted by IRIS (International Randomized Study of Interferon and STI571) resulted in introduction of imatinib mesylate - a TKi targeting the BCR-ABL1 fusion oncoprotein. Imatinib treatment induced an anti-leukemic effect in CML-CP, in comparison with previously used treatments - low dose cytarabine and INF-α [90]. The inhibitory activity of imatinib occurs due to the binding of imatinib to the BCR-ABL1 kinase domain, which blocks its kinase activity and transfer of phosphate group into tyrosine residue of substrates, resulting in deactivation of BCR-ABL1-mediated downstream signaling pathways. Importantly, imatinib has been the first specifically targeting drug, designed based on the *in silico* modeling, which has become a revolution in the drug development field (comprehensively reviewed in [91]).

The first IRIS study, an 18 month-long clinical trial, revealed that in comparison with combined chemotherapy (cytarabine) and INF-α, imatinib treatment resulted in increase of major cytogenetic responses and complete cytogenetic responses (CCgR) from 34.7% to 87.1% and from 14.5% to 76.2%, respectively [90]. Moreover, follow-up study has shown that 5-year CCgR in CML patients (N = 359) receiving imatinib was recorded at 87% with approximately 89% of overall survival [92]. In the second study of IRIS, including 8-year monitoring of imatinib efficacy in CML patients, the OS achieved 85%. Meanwhile, the annual rate of patients progressing from CML-CP to CML-BC between the 4^{th} and 8^{th} year of imatinib treatment ranged from 0.9%, 0.5%, 0%, 0%, to 0.4%, respectively each year. Moreover, in patients obtaining CCgR upon imatinib, the rate of CML-CP progression to CML-BC was only 3% and the percentage even reduced to 0% in patients who achieved major molecular response [93]. Thanks to remarkable potency against BCR-ABL1 in CML, imatinib has been accepted by the US Food and Drug Administration (FDA) as the first TKi in cancer therapy, as a first line treatment for CML. In overall, imatinib has been reported to be beneficial for 60% of CML patients [94].

1.3.3 Resistance against OTK inhibitors in leukemias

In FLT3(ITD)–positive AML, though quizartinib induces a potent and selective anti-leukemic effect, resistance against this inhibitor can occur due to various mechanisms. Firstly, in a case when FLT3 receptors [both FLT3(WT) and FLT3(ITD)] interact with FLT3 ligand. Notably, the overproduction of FLT3 ligand by stromal cells in the bone marrow microenvironment (BMM) stabilizes the FLT3/MAPK pathway. This provides the pro-survival signal protecting FLT3(ITD)-positive leukemia cells against apoptotic effect of FLT3 inhibitors [95, 96]. Additionally, resistance to FLT3 inhibitors in the BMM can also be associated with activated FLT3/STAT5 signaling and high secretion of fibroblast growth factor 2 (FGF2) [97, 98]. Secondly, the resistance against FLT3 inhibitors can occur due to novel missense mutations in FLT3 receptor, especially mutations in TKD [99, 100]. Another mechanism inducing resistance to FLT3 inhibitors comes from hyper-activation of alternative pathways, such as PI3K/AKT/mTOR, RAS/MEK/MAPK in AML cells, besides activation of the oncogenic FLT3–mutant oncogenic receptor [97].

In targeted therapy of CML with imatinib, though the inhibitor has been effective in 60% of CML patients, the resistance against imatinib is unavoidable and can be divided according to two major mechanisms, i.e. BCR-ABL1–dependent and BCR-ABL1–independent resistance. In the BCR-ABL1-dependent mechanism, 50-90% of CML patients, who did not respond to imatinib treatment, have been reported to carry point mutations in TKD of the fusion *BCR-ABL1* oncogene [101-104]. All TKD missense mutations contributing to the resistance have been recorded in 12 amino acid residues comprising M244, G250, Q252, Y253, E255, V299, F311, T315, F317, M351, F359 and H396, of which, T315I is the most common one associated with attenuation of imatinib effects [105]. Moreover, in another BCR-ABL1–dependent mechanism, increased expression of the oncoprotein can provide persistent survival signal to CML cells, possibly inducing acquired resistance to imatinib without point mutations in TKD [106]. As for the BCR-ABL1-independent mechanism, in spite of lacking BCR-ABL1 TKD mutations or increased expression of the oncoprotein, failure of imatinib treatment has been observed in 40% of CML patients harboring normal/unmutated *BCR-ABL1* in a clinical study [107]. Similarly to FLT3 inhibitor resistance, high levels of several stroma-derived growth factors in the BMM have been implicated in imatinib resistance. In this instance, diminished anti-leukemic effect of imatinib occurs due to elevated phospho-STAT3, ERK and BMP that provide pro-survival and proliferative signals for leukemia progenitor cells or promote leukemia migration and homing in the bone marrow by CXCL12-CXCR4 axis [108-110]. Secondly, besides the involvement of BMM–dependent growth factors, several signaling pathways can be upregulated in response to imatinib treatment. These include the PI3K/AKT/mTOR and RAS/RAF/MAPK pathways in CML cells, which after being up-regulated, become alternative drivers of CML transformation alongside with BCR-ABL1, simultaneously inducing resistance to imatinib [111, 112].

The most important mechanism leading to resistance against both quizartinib and imatinib, as well as generally to TK inhibitors, is existence of the minimal residual disease (MRD) in the form of quiescent leukemia stem cells (LSCs). In FLT3(ITD)–positive AML and BCR-ABL1–positive CML, the quiescent $Lin^{-}CD34^{+}CD38^{-}$ cells establish approximately only 0.5% of total leukemia population [113, 114]. Moreover, TKi are only capable of targeting proliferating leukemia cells due to OTK–induced activation of RAS/MAPK pathway. Therefore, although accounting for a quite small proportion of leukemic cell population, quiescent LSCs are most

refractory to TKi as well as chemotherapeutic agents, resulting in ineffectiveness of TKi and, eventually, disease relapse [115, 116]. Furthermore, especially high expression of BCR-ABL1 was found in $Lin^{-}CD34^{+}CD38^{-}$ CML cells and imatinib–induced CML quiescent LSCs and quizartinib–induced AML quiescent cells have been reported to induce resistance to TKi [66, 117, 118]. Taken together, to combat all mechanisms of TKi resistance, it is necessary to introduce novel therapies for use in combination with TKi, to possibly eliminate LSCs and other TKi-refractory cells.

1.4 Synthetic lethality

Genomic instability is one of the hallmarks of cancer [119]. It occurs when activation of oncogenes and/or inactivation of tumor suppressor genes induce production of endogenous reactive oxygen species (ROS). This leads to accumulation of oxidative DNA damage in cancer cells, together with secondary DNA mutations (e.g. derived from initial treatment with chemotherapeutic agents), causing inhibition of apoptosis/senescence/DNA checkpoint pathways.

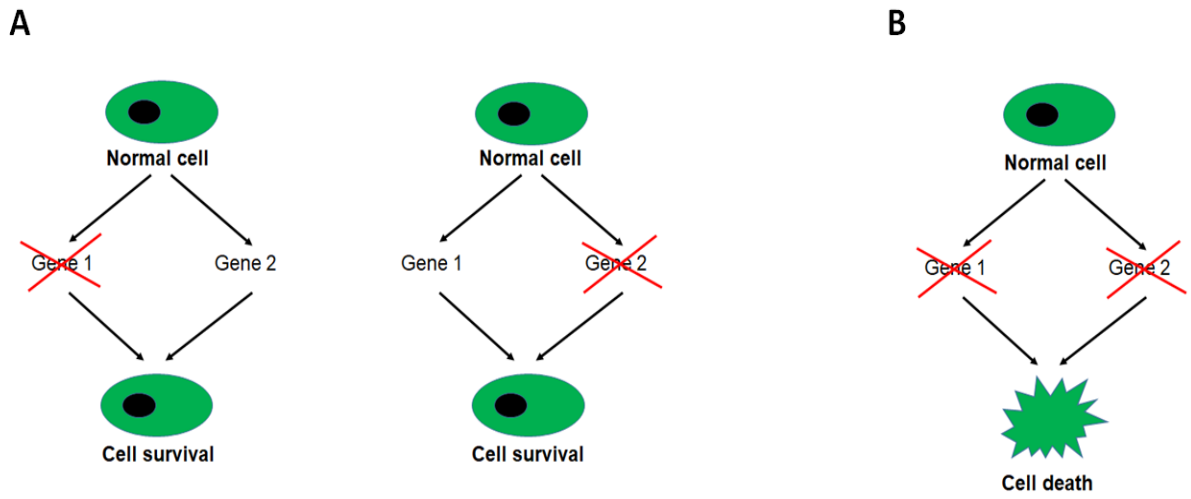

Figure 1.5. General concept of the synthetic lethality phenomenon. **A**. Cells survive under inactivation of only one gene. **B**. Simultaneous inactivation of at least two genes in the same survival mechanism leads to cell death.

To survive in the state of elevated DNA damage, cancer cells are protected by enhanced/altered DNA repair pathways [120]. Although enhancement of DNA repair provides a pro-survival effect in cancer cells, it also reveals an “Achilles heel” of cancer cells that can be therapeutically targeted by an approach called synthetic lethality. In theory, cell

death caused by synthetic lethality is based on inactivation of two genes encoding two parallel proteins/pathways that function in a complementary mechanism required for cell survival (Figure 1.5B). In such case, maintenance of one functional gene enables a compensatory response to prevent cell death when the other parallel gene is inactivated (Figure 1.5A) [121]. Historically, the first study describing the interplay between two parallel genes has been conducted in *Drosophila melanogaster* in 1922 [122] and the term "synthetic lethality" was introduced in 1946 when the same result was found in *Drosophila Pseudoobscura* [123].

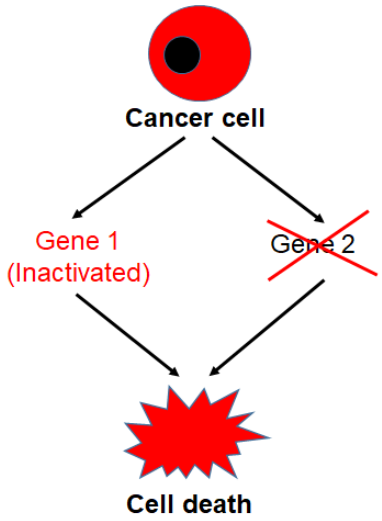

Figure 1.6. Concept of synthetic lethality in cancer cells in which one gene is inactivated due to DNA mutation and targeting of the parallel gene causes cell death.

In cancer cells, accumulation of DNA damage can cause inactivation of one gene due to mutations, resulting in dependence of cells on the parallel gene to survive. In such instance, the remaining active gene becomes a weakness of cancer cells that can be therapeutically exploited in the synthetic lethality mechanism, to eliminate malignant cells without unwanted effect on normal cells (Figure 1.6). As DNA repair pathways are altered and induced to support survival of cancers cell under high levels of oxidative DNA damage, the synthetic lethality-based targeting of DNA repair pathways is a promising approach to develop a novel therapeutic strategy for cancer treatment.

1.5 DNA double-strand break repair pathways

Among all types of DNA damage, the DNA double-strand break (DSB) is the most lethal type of DNA lesion to cells. The DNA break derives from risk factors of DNA damage such as γ-irradiation, ROS, DNA–damaging chemical agents or intracellular processes, leading to DSBs as a consequence of stalled replication forks [124]. As such DNA lesions are lethal, if DSBs are not effectively repaired, chromosomal inactivation and/or cell death will occur [125]. On the other hand, unsuccessful repair of DSBs may result in additional DNA mutations and chromosomal translocations, which lead to constitution of oncogenes or inactivation of tumor suppressor genes, thereby triggering tumorigenesis. Altogether, DSB repair is the perfect target for application of the concept of synthetic lethality in cancer cells.

1.5.1 Homologous recombination pathway

In general, DSB repair consists of two pathways, including homologous recombination (HR) and non-homologous end joining (NHEJ). Out of those, HR is considered an accurate DSB repair pathway, because it depends on a sister chromatid of a cell in mitotic phase, as a template for DNA synthesis and to repair a DSB [126]. Therefore, HR is capable of repairing DSBs only in proliferating cells, due to the existence of HR at replication fork [127].

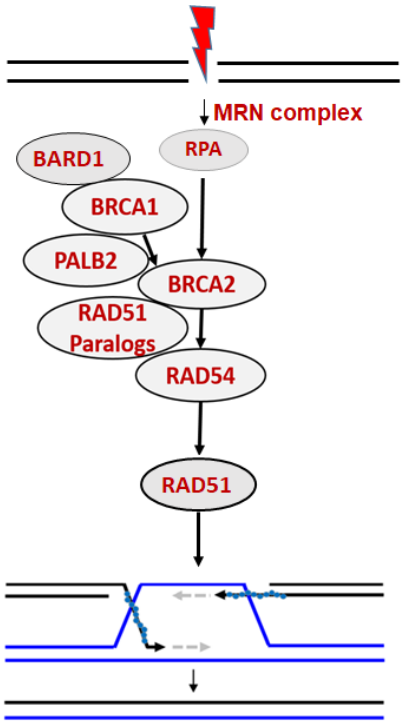

Figure 1.7. Procedure of DSB repaired by BRCA1/2–mediated homologous recombination pathway. **MRN**: MRE11/RAD50/NBS1, **RPA**: Replication Protein A.

The HR repair pathway is comprehensively modulated by proteins encoded by two classical tumor suppressor genes as BRCA1 and BRCA2 (Figure 1.7). In detail, upon the introduction of DSB, it is detected by MRE11/RAD50/NBS1 (MRN) complex to trigger DSB response mediated by ATM kinase [128, 129]. At this stage, ATM phosphorylates histone H2AX to generate γ-H2AX, which is considered an early intracellular DSB marker [130]. The formation of γ-H2AX foci results in recruitment of BRCA1-BARD1 complex to the DNA break, in which presence of BARD1 is required for active effector function of BRCA1 [131]. BRCA1-BARD1 complex plays two important roles in the HR pathway. The first one is stabilizing the CtIP–mediated end-resection of MRN complex, to generate 3' single-strand (ss) DNA ends. Meanwhile, the second function of BRCA1-BARD1 complex is initiation of the BRCA2 involvement at the break, via a binding interaction with PALB2. At this step, due to effector function of BRCA2, RAD51 is recruited into Replication Protein A (RPA)-coated ssDNA ends [132]. Based on the involvement of RAD51, to continue the pathway, BRCA2 performs loading of RAD51 paralogs (including RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3), leading to stabilization of RAD51 nucleoprotein filament. In the final step, together with the formation of RAD51 nucleoprotein filament, RAD54 generates the interaction between RAD51 and ssDNA, alongside with unwinding of dsDNA. This enables enhancement of RAD51–dependent strand invasion, in order to search for a homologous DNA template, to complete DSB repair in the HR pathway [133, 134]. Mutations in *BRCA1/2* genes have been found in a wide range of cancers, leading to inactivation of the HR pathway. In such case, cancer cells usually acquire an alternative DNA repair pathway to survive.

1.5.2 Non-homologous end joining pathway (NHEJ)

The second mechanism of DSB repair is NHEJ, which is an error-prone DSB repair mechanism, due to lack of sister chromatid as a DNA template. NHEJ is further divided into two pathways including canonical NHEJ (D-NHEJ) and alternative NHEJ (Alt-NHEJ). Both pathways allow for DSB repair in cells throughout all phases of the cell cycle. Therefore, unlike HR, DSBs in both quiescent and proliferating cells can canonically be repaired by D-NHEJ, with joint participation of Ku70/80, catalyzing kinase subunit of DNA-dependent protein kinase (DNA-PKcs), Artemis and the complex of XRCC4/Ligase IV/XLF [135] (Figure 1.8A). In details, when a DSB is introduced, Ku78/80 recognizes and binds to the DSB, immediately leading to activation of kinase activity of DNA-PKcs to generate a DNA-PK

holoenzyme. It further co-operates with Artemis nuclease and other enzymes to trigger DSB ends, followed by DNA ligation, catalyzed by complex of DNA ligase IV/XRCC4 [136, 137].

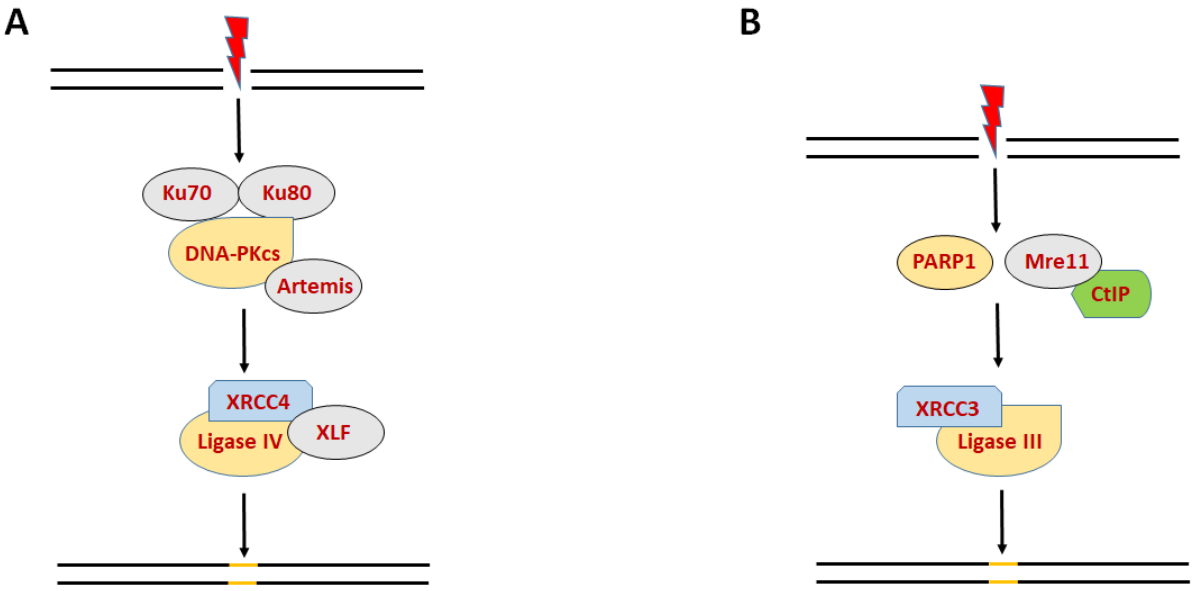

Figure 1.8. Process of DSB repair by non-homologous end joining (NHEJ) pathway. **A**. DNA-PKcs–mediated canonical NHEJ. **B**. PARP1–dependent alternative NHEJ.

Alt-NHEJ pathway is considered to be a "back-up" of D-NHEJ, in case when D-NHEJ is inactivated due to down-regulation of proteins engaged in this pathway. Although Alt-NHEJ also performs an error-prone DSB repair similar to D-NHEJ, it is more likely to result in alterations in DNA sequence than D-NHEJ, leading to an increased risk of chromosomal translocations [138]. The Alt-NHEJ pathway is fundamentally mediated by poly ADP-ribose polymerase 1 (PARP1), which has been reported to perform analogical function as DNA-PKcs in D-NHEJ [139]. In fact, PARP1 co-operates with MRE11 (of the MRN complex), DNA polymerase theta (Polθ), and WRN helicase to promote DSB end re-section processing, eventually leading to DNA ligation (which depends on catalytic activities of DNA ligase I and/or ligase III) [140, 141] (Figure 1.8B).

1.5.3 PARP1–dependent DNA repair - a backup of DSB repair pathways

1.5.3.1 The optional regulation between DSB repair pathways

The preferential repair of DSBs via either HR, D-NHEJ or Alt-NHEJ pathways is basically regulated by the cell cycle. For example, PARP1–dependent Alt-NHEJ repair activity is impaired by both Ku70/80 and DNA-PKcs. On the other hand, it is remarkably promoted in

D-NHEJ–defective cells, confirming previous reports describing an aggressive competition between Ku70/80 and PARP1 in DSB recognition [142, 143]. Moreover, Alt-NHEJ mediates DSB repair with slower frequency than D-NHEJ, highlighting an alternative role of Alt-NHEJ for both HR and D-NHEJ [144]. Indeed, Alt-NHEJ activity is significantly elevated in HR–deficient cells in S phase of cell cycle, validating the important backup role of PARP1 in proliferating BRCA1/2–mutated cancer cells, to prevent accumulation of unrepaired DSBs [145]. Although both D-NHEJ and Alt-NHEJ are considered as error-prone DSB repair pathways (especially in comparison with HR - as an accurate DSB repair mechanism), these pathways are required for sustainability of genomic integrity, thanks to function of different DSB repair mechanisms ,in both proliferative and quiescent cells. Moreover, D-NHEJ has been revealed to exhibit robust activity than the other two DSB repair pathways [146]. Therefore, D-NHEJ is a key element for prevention of genomic instability and, at the same time, prevention of development and progression of tumors. Altogether, this highlights the essential role of PARP1–dependent Alt-NHEJ in cells that are D-NHEJ–deficient.

1.5.3.1 PARP1–dependent single-strand break (SSB) repair

Together with being involved in the Alt-NHEJ pathway of DSB repair, PARP1 has been reported to mediate SSB repair by recruiting proteins of base excision repair (BER) pathway. Theoretically, BER pathway repairs base damage derived from intracellular metabolic processes, including oxidation, alkylation and deamination [147].

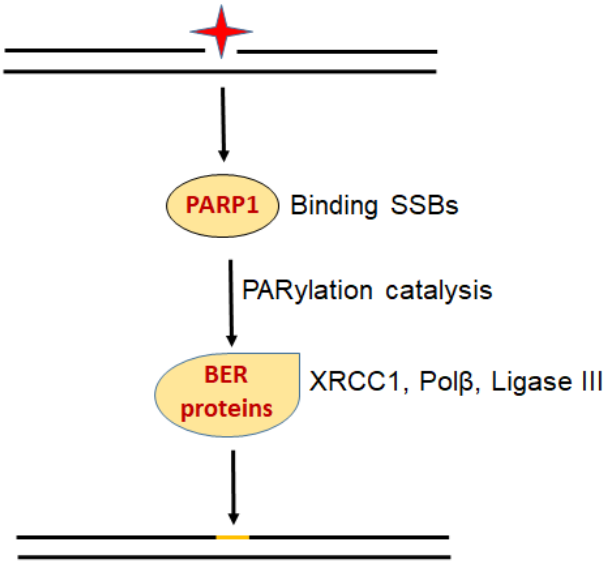

Figure 1.9. Scheme of PARP1–dependent DNA single-strand break repair. **BER**: Base excision repair.

In BER pathway, detection and excision processes of base damage are conducted by a wide range of glycosylases (based on the types of base damage), that form an apurinic/apyrimidinic (AP) site cleaved by an AP endonuclease, leading to intermediation of SSB. In this setting, PARP1 conducts a search throughout single DNA strand until it recognizes a SSB and binds to the break. At this point, elevated activity of a process named PARylation (catalyzed by PARP enzymes family) occurs. This includes covalent appending of long ADP-ribose forms poly ADP-ribose (PAR) on PARP1 and other targeted proteins [148], when essential proteins comprising XRCC1 complex, DNA polymerase β, and DNA Ligase III are recruited to repair SSB. In the final step, faulty nucleotide is quickly corrected by either sealing or ligation, depending on either a short path (to repair an individual nucleotide) or a long path (to replace at least two nucleotides) [149] (Figure 1.9). Notably, if SSB remains unrepaired during DNA replication, it will cause gene transcription arrest, leading to formation of a lethal DSB [150]. Therefore, PARP1–dependent SSB repair is considered very important for survival of HR–deficient proliferating cells, by preventing the conversion from SSB to DSB during DNA replication. This establishes a rationale for therapeutic use of PARP inhibitor (PARPi)–induced synthetic lethality in *BRCA*–mutated/deficient cancer cells.

1.6 PARP inhibitor (PARPi)–induced synthetic lethality

1.6.1 PARPi–induced synthetic lethality in *BRCA*–mutated cancers

Use of PARP inhibitors (which predominantly block activity of PARP1, together with dimmer effect against PARP2 and PARP3) in *BRCA*–mutated cancer cells has been the most typical and well-known example of synthetic lethality in tumor cells. Indeed, the development of different PARP inhibitors over the last decade has proven that PARPi-induced synthetic lethality is a significant breakthrough in therapy of *BRCA*–mutated cancers, with minimum cytotoxicity towards normal cells [151-153]. Moreover, the effectiveness of PARPi in eliminating *BRCA1/2*–mutated breast/ovarian tumors has initiated an era of personalized medicine with utilization of PARP inhibitors [154-156]. Mechanistically, mutations in *BRCA1/2* inactivate the HR pathway, and in order to survive, *BRCA1/2*–mutated cancer cells require function of PARP1 in SSB repair, to prevent formation of DSBs from unrepaired SSBs during DNA replication. Furthermore, the enhanced dependence of *BRCA1/2*–mutated cancer cells on PARP1 has been validated by overexpression studies of PARP1 in such types

of cancer cells [157]. Besides working effectively in *BRCA*–mutated cancers, PARPi–mediated synthetic lethality is capable of eliminating D-NHEJ–deficient cancer cells. For example, down-regulation of LIG4 (involved in D-NHEJ pathway, to perform DNA ligation) induced sensitivity of melanoma cells to PARPi (olaparib), without cytotoxic effect on normal melanocytes [158].

In terms of mechanism of action PARPi, it was initially thought that PARPi block the catalytic activities of PARP1 (to the strongest degree), PARP2 and PARP3 in the PARylation process, leading to inhibition of recruitment of BER proteins to repair SSBs. However, recent studies have shown that the inhibition of catalytic activity of PARPs is not the only mechanism triggering cytotoxicity [159]. Next generation PARP inhibitors are able to generate a trapping effect against PARP1 (and probably also PARP2) at a SSB site, which results in DNA replication and transcription arrest. Therefore, the PARP inhibitors perform remarkably elevated cytotoxicity and potency, while compared with inhibition of PARylation catalysis [160].

1.6.2 Development of PARP inhibitors in clinical trials

Until now, the major mechanism of action of currently developed PARPi has been connected to nicotinamide, interfering the accessibility of NAD^+ to PARP1 catalytic site, leading to inactivation of PARylation process [161]. Recently, PARPi, talazoparib (also known as BMN673), has been reported as approximately 20-200 times more efficient than earlier PARP inhibitors, validated via *in vitro* kinase activity assay [162]. The elevated efficacy of talazoparib results from its enhanced PARP1-trapping capacity, making talazoparib the best PARP trapping agent among all reported PARP inhibitors [163]. Thanks to reported significant potency, talazoparib has been clinically verified in breast cancer patients, with germline mutations of *BRCA1/2*, and other types of cancer that contain impaired DNA damage response. Phase III clinical trials of talazoparib demonstrated increased overall survival rate of metastatic breast cancer patients [164]. Besides talazoparib, another orally available PARPi (veliparib) is undergoing clinical trials. It shows the best selectivity against PARP1/2/3 catalysis, though this PARPi exhibits limited efficacy of PARP trapping [165]. This demonstrated that PARPi, which exerts a more potent and selective inhibitory effect on PARylation process, is also capable of entering clinical trials.

1.6.3 FDA–approved PARP inhibitors

Olaparib (commercial name - Lynpaza®) is the first orally distributed PARPi verified in clinical trials, and so far has been the most common PARPi used in studies on BRCA–deficient cancers. Historically, olaparib was the first PARPi approved by the FDA in December 2014, based on its significant efficacy in treatment of relapsed ovarian cancer individuals with *BRCA1/2* mutations [166]. In August 2017, olaparib obtained the second approval from FDA, as an extensive therapy for patients with recurrent fallopian tube, peritoneal, or epithelial ovarian cancer, who have achieved partial or complete remission after systematic chemotherapy [167]. Additionally, olaparib's application in cancer therapy has extended in January 2018, when FDA licensed the PARPi as a therapeutic strategy for germline *BRCA*-mutated metastatic breast cancer patients, who previously received chemotherapy. This marked olaparib as the first FDA–approved compound working effectively in individuals with hereditary breast cancer [168]. Back in 2016, another PARPi (introduced as an intravenous drug) named rucaparib (Rubraca®), became a FDA-approved therapy in women with *BRCA*-mutated advanced ovarian cancer, who previously received at least two systematic chemotherapies [169]. In the timeline of PARPi development, the last orally-delivered inhibitor, that obtained approval from FDA in March 2017, was niraparib (Zejula®), which has been approved as a therapy in individuals with recurrent ovarian, fallopian tube, or primary peritoneal cancer, who have already undergone platinum-based chemotherapy [170].

1.6.4 Demonstration of PARPi in BRCA–deficient leukemia and other hematopoietic malignancies

Although *BRCA1/2* mutations are predominantly detected in female breast and ovarian cancers or male prostate cancer, and are very rare in leukemia or other hematopoietic malignancies, PARPi–induced synthetic lethality can be exploited to eradicate hematological malignant cells as well. Indeed, expression of oncogenes related to myeloid and lymphoid malignancies, including AML1-ETO (also known as RUNX1-RUNX1T1), BCR-ABL1, MLL-AF9, TCF3-HLF and IDH1/2mut, IGH/MYC or inactivation tumor suppressor genes such as TET2, can cause down-regulation of BRCA1 and/or BRCA2, thus rendering malignant hematopoietic cells susceptible to synthetically lethal effect triggered by PARPi [1-3, 171-175]. Moreover, our group has conducted a comprehensive study on personalized precision medicine using PARPi. In our study, myeloid malignant cells derived from AML/ALL patients

with down-regulation of BRCA1/2 (as validated by Gene Expression and Mutation Analysis, based on RNA-seq or flow cytometry), were successfully eradicated with PARPi, without cytoxicity in BRCA–proficient counterparts [176]. Additionally, inhibition of JAK1/2 in JAK2(V617F) MPN by TKi (ruxolitinib) and targeting of BCR-ABL1 in CML by TKi (imatinib) resulted in deficiencies of BRCA1/2 and RAD51, respectively, together with down-regulation of Ligase IV [177, 178]. Thus, the synergistic treatment with TKi and PARPi can be capable of eradicating both proliferating and quiescent malignant cells. All these promising results have made up a rationale for clinical trials with PARPi in patients with certain hematological malignancies (reviewed in [179]).

1.6.5 Resistance against PARPi–induced synthetic lethality

PARPi have performed with extraordinary efficacy against BRCA/HR–deficient tumor cells, without cytotoxicity to non-malignant counterparts. Several PARP inhibitors have also been approved by FDA as a targeted therapy to treat advanced/metastatic *BRCA*–mutated breast/ovarian cancers. However, resistance to PARPi-induced synthetic lethality can sometimes be acquired in BRCA/HR–deficient tumor cells. The first, and also the most common, mechanism inducing resistance to PARPi is restoration of HR pathway in BRCA–mutated tumor cells. In detail, secondary mutations in *BRCA1/2* have been associated with abrogation of chain terminator/frameshift resulting from initial mutations, thus leading to restoration of full-length *BRCA1/2* open reading frame (ORF). This leads to normal protein expression and restarts the proficient functions of BRCA1/2 in HR pathway [180, 181]. The HR restoration mediated by secondary mutations in *BRCA1/2* has been the most common acquired mechanism of PARPi resistance, clinically observed in 46% of *BRCA*–mutated cancer patients, refractory against platinum-based chemotherapy [182]. Besides the resistance mediated by additional mutations in *BRCA1/2*, reduction/loss of BRCA1 promoter methylation has been documented to restore functional activity of BRCA1 in HR pathway, thereby leading to PARPi resistance. Indeed, PARPi–sensitive primary breast cancer cells exhibited elevated *BRCA1* promoter methylation, associated with impaired BRCA1 expression. Meanwhile, decreased promoter methylation and proficient BRCA1 have been observed in individuals who did not respond to PARPi treatment [183].

The second well-established mechanism of PARPi resistance in BRCA–deficient tumor cells occurs due to deficiency/loss of PARP1 expression, leading to failure of PARP1 trapping by PARPi. Expression level of PARP1 was remarkably reduced in colorectal carcinoma HCT116 clones refractory against PARPi and temozolomide [184]. Moreover, missense mutation (1771C > T) in *PARP1* in patient ovarian cancer has been described to induce *de novo* attenuation of PARPi [185]. Another common mechanism related to PARPi resistance has been associated with alteration of DSB end re-section, related to HR pathway. In this case, suppression of DSB end re-section leads to inhibition of HR mediated by 53BP1. Hence, inhibition of 53BP1 in *BRCA1*–null murine embryonic stem cells reverses deficiency of HR, protecting cells against cytotoxic effect of PARPi or DNA damaging agents [186].

In addition to described mechanisms that result in either HR restoration or abrogation of PARP1 expression, another mechanistic pathway is HR restoration–independent and is induced by protection of replication forks (RFs). Besides function in the HR pathway, BRCA1 and BRCA2 have been reported to also perform roles in maintaining the integrity RFs [187, 188]. Therefore, acquired resistance to PARPi in BRCA1–deficient cells can also be caused by decreased expression of a nuclease, MRE11, at stalled RFs, leading to a protective effect toward RFs [188]. Additionally, in a case of resistance to PARPi in BRCA2–deficient tumor cells, the integrity of RFs was preserved by reduction of other nuclease, MUS81, at stalled RFs, which was mediated by down-regulation of EZH2 [189].

1.7 Bone marrow microenvironment (niche) – a potentially novel mechanism of resistance to PARPi in leukemia

Resistance to PARPi-induced synthetic lethality in BRCA–deficient tumor cells has been documented in numerous papers, and several mechanisms were briefly described above. However, because the approach of PARPi-induced synthetic lethality has just recently been used and validated in BRCA–deficient leukemias and other hematopoietic malignancies, intrinsic mechanisms of resistance to PARPi specifically in leukemias have not been well understood. Recently, our group has shown that activation of OTK c-KIT (N822K mutant in *KIT* receptor) restored proficiencies of BRCA1/2, therefore, inducing resistance to PARPi (olaparib) in BRCA–deficient AML1-ETO–positive AML. Conversely, inhibition of oncogenic c-KIT re-sensitized leukemia cells to PARPi [190]. In the presented thesis, we demonstrate a

novel and constitutive mechanism inducing resistance to PARPi in BRCA–deficient leukemias. Leukemic cells start development in the bone marrow and bone marrow microenvironment (BMM) (also known as BM niche) has been associated with protection supporting the LSCs/LPCs against cytotoxic effect of TKi or chemotherapeutic agents. Thus, we hypothesized that BMM-mediated signaling could be a novel, intrinsic mechanism of resistance to PARPi in BRCA–deficient leukemia.

1.7.1 Bone marrow microenvironment of HSCs and LSCs

HSCs originate from a specific hematopoietic tissue – the bone marrow (BM). Anatomically, the bone marrow (BM) is formed by various types of stromal cells in the BM (including mesenchymal stromal/stem cells (MSCs) and endothelial cells (ECs), as two most common stromal cell components), together with sympathetic nerve-related cells, macrophages, osteoclasts, fibroblasts, megakaryocytes etc., all functioning in hypoxic conditions (Figure 1.10). The term "HSC niche" has been introduced in 1978 by Ray Schofield, to initially elicit the importance of hematopoietic tissues, such as BM and spleen, for HSCs' biology [191]. This has led to numerous studies conducted during the past four decades to validate function of BMM in supporting development and retention of HSCs (reviewed in [192]). Overall, BMM plays a significant role in maintenance, retention and differentiation of HSCs, while molecular interactions between HSCs and cellular components of BMM are set to maintain the balance between self-renewal and differentiation of HSCs.

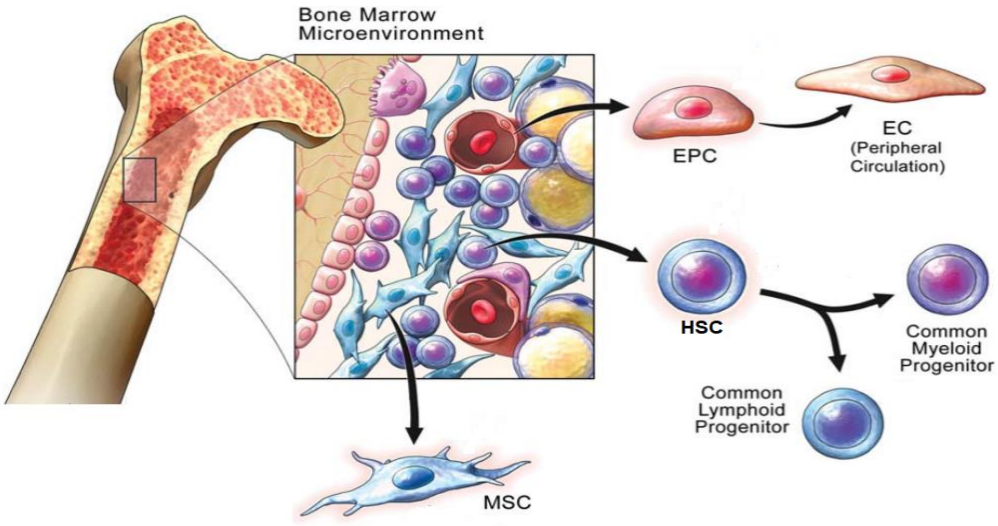

Figure 1.10. Hematopoietic stem cells (HSC) locate in the bone marrow microenvironment constituted by stromal cells in hypoxia [193-modified]. **MSC**: mesenchymal stromal/stem cell, **EPC**: endothelial progenitor cell, **EC**: endothelial cell.

The best known BMM-mediated signaling pathway that supports HSCs is mediated by stromal cells-derived factor 1α (SDF-1α/CXCL12). The main axis of this pathway is CXCL12 interaction with its receptor CXCR4 expressing on HSCs, to maintain quiescent state of HSCs pool by home nesting in BM [194, 195]. In fact, CXCL12 released by CXCL12-abundant reticular (CAR) cells enhanced hematopoietic migration and localization within the BM. Meanwhile, in a study conducted with use of conditional knock-out of CXCL12 and stem cell factor (SCF) in HSCs, the maintenance and self-renewal of knock-out HSCs were supported by co-culture with perivascular, nestin-positive, immature MSC and endothelial cells. This suggests that all mentioned types of cells produced sufficient CXCL12 and SCF (crucial for HSCs maintenance in the BM) upon co-culture with HSCs with knock-out of CXCL12 and SCF [195, 196]. In addition to CXCL12, another BM-derived cytokine, also capable of inducing quiescent state of HSCs pool, is transforming growth factor β (TGF-β) [197, 198], which is mostly produced by two types of BM stromal cells including megakaryocytes and Schwann cells in sympathetic nerves within normal hematopoietic niche [199]. Moreover, hypoxic conditions (below 2% of oxygen) of the BMM stabilize the hypoxia inducible factor 1α (HIF-1α) and HIF-2α transcription factors, which are involved in regulation of HSCs maintenance [199].

BMM performs an extremely vital role in maintenance and self-renewal of HSCs, thus also leading to a reciprocal relationship between leukemia stem cells (LSCs) and BMM. LSCs are responsible for leukemogenesis, as well as reprogramming of the BMM, and in turn, BMM has been documented as essential for maintenance and survival of LSCs [200, 201]. Moreover, as a hematopoietic malignancy initiate in the BM, LSCs also efficiently exploit constitutive signals in the BMM to acquire enhanced survival and proliferation. Therefore, the protective effect of the BMM towards LSCs often renders malignant cells refractory to chemotherapeutic agents and TKi [202-204]. Additionally, after engrafting into BM of host animal, engrafted $CD34^{+}$ leukemia cells remodeled the BM and bone marrow stromal cells, switching to an unfavorable microenvironment for normal HSCs, but pro-survival for LSCs [205]. LSCs-induced alteration of stromal cells was also documented in a proteomic study, that revealed up-regulation of survival–related proteins including GSKA, STAT1, STAT5, PP2A, CDKN1A, and CDK4 in MSCs of AML–remodeled BMM [206]. Furthermore, gene

expression analysis of MSCs from AML-remodeled microenvironment showed remarkable overexpression of *CXCL12* and complement-related genes such as *C4A, C4B* and *Serpin G1* as well as inhibitor of osteoblast differentiation, *IGFBP5*. In addition to supporting role of the BMM, stromal cells harboring genetic alterations have also been documented to initiate leukemogenesis. For example, MPN and MDS developed in mice with conditional deletion of retinoic acid receptor γ and Dicer1 in BM stromal cells and osteoprogenitors, respectively [207, 208]. Another published example has been associated with activation of β-catenin and FOXO1 in osteoblasts in the BM which resulted in AML transformation, confirming BM niche-derived oncogenesis [209, 210]. In conclusion, though the reciprocal relationship between LSCs and BMM leads to growth and survival of leukemia, it also constitutes a weakness of LSCs that can be targeted to eliminate hematopoietic malignant cells in the BMM.

1.7.2 Molecular signaling pathways in BMM–induced drug resistance

To examine impact of the molecular signaling pathways in the BMM on survival and therapy-resistance of leukemia cells, Konopleva *et al.* mimicked BMM condition *in vitro,* via a co-culture system of human leukemic cell lines/primary leukemic cells with murine primary MSCs [203]. The BMM-mimicking conditions have been later "upgraded" by introduction of human primary MSCs from healthy donors (which replaced murine counterparts) and including the hypoxic conditions [211]. The availability of human and murine bone marrow stromal cell lines (HS-5 and OP-9) has improved studies on the relationship between leukemia/LSCs and the BMM. *In vivo* studies, in which human leukemic cells are engrafted in immunodeficient mice, are more complicated than *in vitro* due to the incompatibility between human leukemic cells and murine BM stromal cells. Therefore, several groups have developed methods to establish a humanized BM niche by engraftment of human BM-derived stromal cells before transplantation of leukemia cells [212-215]. Due to the massive progress in generation of BMM conditions both *in vitro* and *in vivo*, numerous cell signaling pathways in the BMM have been well understood. In this Introduction, the most dominant signaling pathway that promotes drug resistance in the BMM, as well as another signaling pathway of the BMM (related to DSB repair and DNA damage response) which could be potentially exploited for PARPi-mediated synthetic lethality, are described.

1.7.2.1 CXCL12–CXCR4 signaling pathway

In the hematopoietic niche, CXCL12 is a factor secreted into the microenvironment by several types of stromal cells. Moreover, CXCR4, which is expressed on normal hematopoietic, as well as leukemic stem cells, interacts with CXCL12 to activate its downstream signaling pathways. This leads to a supportive effect of the CXCL12-CXCR4 axis towards both normal and malignant hematopoietic cells. Specifically, the CXCL12-CXCR4 axis stimulates G-coupled receptor (GRK) to phosphorylate its substrates, inducing a Ca^{2+} channel which enhances cell migration and homing of HSCs/LSCs within the BM (Figure 1.11). Additionally, phosphorylation of GRK kinase results in activation of RAS/RAF/ERK and PI3K/AKT/mTOR signaling pathways, maintaining cell proliferation and survival [216]. Therefore, the CXCL12-CXCR4 pathway provides pro-survival signals and shelters with leukemic cells, thus generating malignant cells refractory to apoptosis triggered by chemotherapy or TKi. For example, AML cells, including FLT3(ITD)-positive leukemia, remained resistant against chemotherapy and FLT3 inhibitors, after they were supported by constitutively active pathways of the BMM [217-220]. Moreover, imatinib–treated BCR-ABL1–positive CML cells have been reported to overexpress CXCR4, enhancing their susceptibility to SDF-1 mediated signals. Therefore, this induced failure of imatinib against CML cells in the BMM conditions [110, 221].

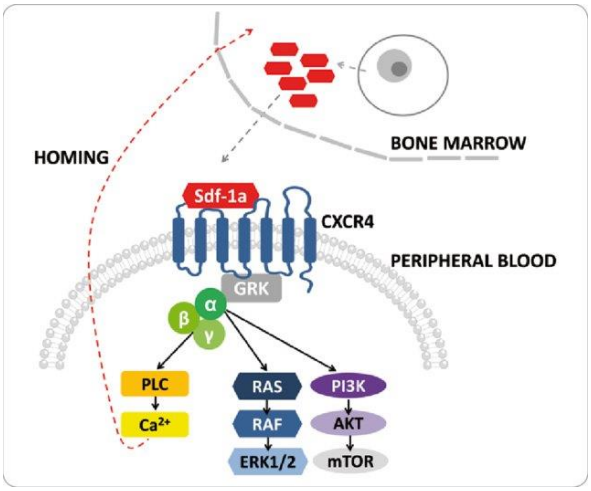

Figure 1.11. Molecular signaling pathway of CXCL12(SDF-1α) - CXCR4 in the bone marrow microenvironment [222].

More importantly, one of the critical BMM components - the hypoxia (oxygen concentration lower than 2%) - has been related to stabilization of HIF-1α and other factors, leading to up-

regulation of CXCL12 in ECs [223]. In addition, hypoxia has been associated with overexpression of CXCR4 in AML cell lines and primary patient-derived AML cells [224]. As CXCL12-CXCR4 pathway induces the BMM-mediated resistance to chemotherapy, as well as TKi in leukemias, a novel targeted therapy has been proposed to re-sensitize leukemias via abrogation of the pathway, by CXCR4 inhibitors or antagonists [217, 225-227]. Currently, the most studied CXCR4i is AMD3100 (also known as plerixafor), approved by the FDA in 2008 for autologous transplantation in affected individuals with Non-Hodgkin's Lymphoma or multiple myeloma [228]. Currently, novel and more potent CXCR4 inhibitors, including LY2510924 and BL8040, are under evaluation in clinical trials [226, 227]. Notably, until now, the CXCL12-CXCR4 pathway has not been documented to be involved in alteration/promotion of neither DNA damage response nor DSB repair.

1.7.2.2 TGF-β signaling pathway

Transforming Growth Factor beta (TGF-β) is an important cytokine that modulates variety of cellular processes, including cell growth, differentiation, apoptosis, motility, invasion, extracellular matrix (ECM) production, angiogenesis, and immune response [229]. In cancer biology, the TGF-β signaling pathway performs a dual role. It acts as a tumor suppressor in early stages of cancer, but it has an opposite role as a tumor promoter in advanced stages of cancer. Modulation of tumor microenvironment (TME) is supposedly involved in that function (comprehensively reviewed in [229]). High level of TGF-β was found in tumor tissues of cancer patients with poor prognosis [230, 231]. An immunosuppressive effect towards T cells can be exerted by stromal cells–derived TGF-β [232, 233]. To induce molecular signaling, TGF-β family members (including TGF-β1, 2 and 3) oligodimerize with type II TGF-β serine/threonine kinase receptor (TGFβR2). This leads to activation of serine/threonine kinase activity of TGFβR1 (also known as ALK5), which phosphorylates SMAD2/3 (pSMAD2/3) in the canonical pathway. Afterwards, a complex of pSMAD2/3 and SMAD4 is formed, followed by its translocation into the cell nucleus, to regulate transcription of targeted genes (Figure 1.12B) [234]. Moreover, in the non-canonical pathway, TGFβR1 kinase, after being recruited by TGFβR2, activates several protein kinase effector pathways, including PI3K/AKT, RAS/RAF/ERK, PAK/Rho and TAK1/p38 [235].

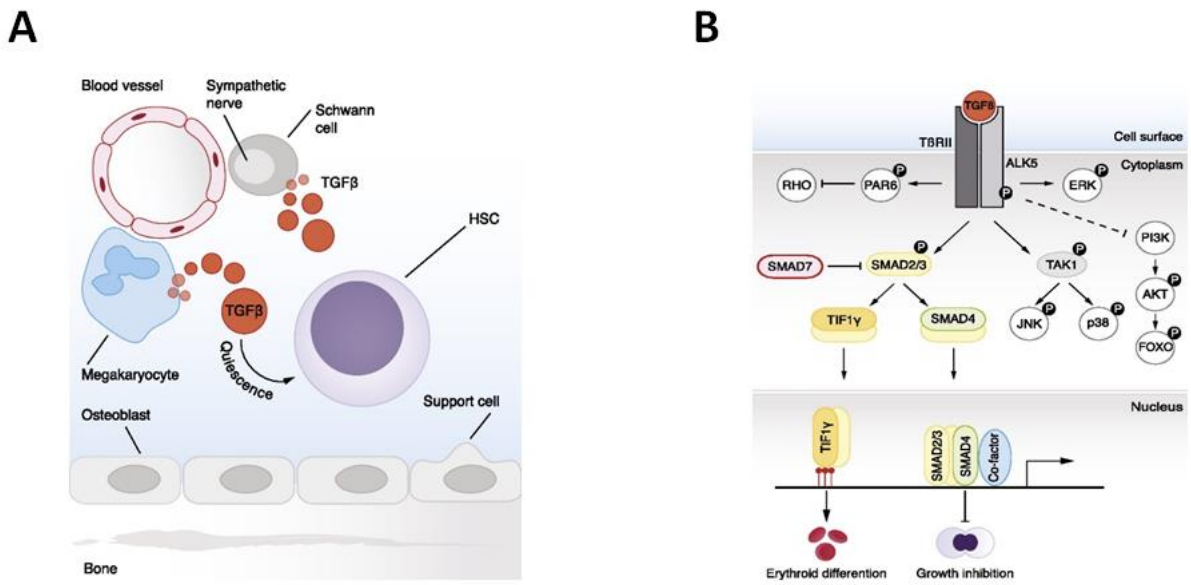

Figure 1.12. TGF-β signaling pathway in the bone marrow niche [199]. **A**. TGF-β is mostly produced by two types of BM stromal cells including megakaryocytes and sympathetic nerve–dependent Schwann cells in bone marrow niche to induce quiescent state of HSCs. **B**. Molecular signaling cascade of TGF-β – TGFβR pathway.

In BM, TGF-β is mostly produced by two types of BM stromal cells including megakaryocytes and Schwann cells in sympathetic nerves (Figure 1.12A). Due to expression of two TGFβRs in HSCs, activation of TGF-β – TGFβR pathway induces cell growth arrest via SMAD2/3/4 complex, to maintain HSCs in the quiescent state. On the other hand, when quiescent HSCs are supposed to enter the cell cycle, the non-canonical pathway is switched on in HSCs to trigger cell proliferation, survival and motility (reviewed in [199]). Since TGFβRs are also spontaneously expressed by LSCs and leukemia cells, the TGF-β pathway can be exploited by malignant cells to maintain their quiescence, as well as induce pro-survival signals to escape from apoptosis induced by chemotherapy and TKi [198, 236]. One of the most typical examples was coming from AML cells in the BMM. As AML cells reprogramed the BM niche by inducing osteogenic differentiation in MSCs, MSCs in turn released elevated amounts of TGF-β, to further supported leukemic cell stemness and resistance against chemotherapy in the BMM [237]. Therefore, inhibition of TGF-β signaling pathway by neutralizing antibody (1D11) or TGFβR kinase inhibitor, individually or in combination with CXCR4i, has been reported to restore efficacy of chemotherapy against leukemic cells in the BMM [238-240]. More importantly, TGF-β pathway has been associated with enhancement of DSB repair capacity in head and neck squamous cell carcinoma [241] and other cancer types [242], as

well as DNA damage response in epithelial cells [243, 244], breast cancer [245], prostate cancer [246], non-small cell lung cancer [247] and glioblastoma [248-250]. This suggests that TGF-β signaling pathway can be considered a potential target in the BM niche, also to restore efficiency of PARPi in case of PARPi resistance in the BMM.

2. AIMS

Previous report of our group has shown that down-regulation of BRCA1/2 (BRCAness phenotype) in leukemia patient's specimens, selected by Gene Expression and Mutation Analysis (GEMA) strategy, triggered efficacy of PARPi, used either as a single therapy or in combination with tyrosine kinase inhibitor (TKi)/chemotherapy. Therefore, those leukemia cells were eliminated by PARPi–induced synthetic lethality both *in vitro* and *in vivo* [176]. Moreover, other studies of our group have reported that TKis, including JAK1/2i (ruxolitinib) and ABL1i (imatinib), caused deficiencies in DSB repair proteins such as BRCA1/2, RAD51 and Ligase IV. As a result, such defects enhanced sensitivity to PARPi of JAK2(V617F)–positive and BCR-ABL1–positive leukemic cells [177, 178]. These data supported our further studies in which we propose to verify that another inhibitor of leukemic tyrosine kinase oncoprotein - FLT3i (quizartinib-also known as AC220) induces "BRCAness" phenotype, leading to sensitivity to PARPi-induced synthetic lethality in FLT3(ITD)–positive leukemia cells.

The stromal cells-mediated bone marrow microenvironment (BMM) has been documented to promote resistance against TKi and chemotherapy in leukemias [202-204]. Additional treatment with PARPi reversed the resistance to TKi in BMM [251]. However, the efficacy of individual PARPi in BMM was less profound than in normal blood condition.

The preliminary data leads to the hypothesis that the stromal cells–mediated BMM induces resistance to PARPi in BRCA1/2-deficient leukemias.

Therefore, in concordance with this hypothesis, three main objectives are proposed:

1. Studies of FLT3 inhibitor effect on FLT3(ITD) oncogenic receptor, induction of "BRCAness" phenotype and enhanced efficacy of PARP inhibitors on leukemia cells *in vitro* and *in vivo*.
2. Verification role of the bone marrow microenvironment, constituted by stromal cells in hypoxia, in induction of the resistance to PARPi in leukemia cells.
3. Investigation the molecular mechanism responsible for the bone marrow microenvironment–mediated resistance to PARPi in leukemia cells *in vitro* and *in vivo*.

3. MATERIALS AND METHODS

3.1 Cell lines and primary cells

3.1.1 Cell lines

3.1.1.1 FLT3(ITD)–positive cell line MV-4-11

To test a FLT3(ITD)–positive cell line with [PARPi + FLT3i], MV-4-11 cells were obtained from ATCC (#CRL-9591), which has been described to express FLT3(ITD) oncogenic receptor [252]. The cell is a human cell line characterized from the blast cells of a 10-year-old male with biphenotypic B-myelomonocytic leukemia and growing as lymphoblastic suspension cells. To maintain cells in culture, medium was prepared from RPMI 1640 (Corning #10-040-CV) plus 10% heat inactivated fetal bovine serum (FBS) (Hyclone #SH30071.03HI) and antibiotic cocktail (Gibco #15240062) with 3 x 10^5 cells/mL as initial density and the medium was renewed every 3 days.

3.1.1.2 FLT3(wild-type) cell lines REH and HL-60

To obtain negative controls for FLT3(ITD)–positive cells, REH (#CRL 8286) and HL-60 (#CCL-240) were purchased from ATCC. Those cell lines have been reported as typical negative controls for FLT3(ITD)–positive leukemia [253]. In details, REH is acute lymphocytic leukemia without T and B cells and maintained in culture as lymphoblastic suspension cells. Meanwhile, HL-60 is acute promyelocytic leukemia, growing as myeloblastic suspension cells, obtained by leukopheresis from a 36-year-old Caucasian female with acute promyelocytic leukemia. To culture these cell lines, a medium with RPMI 1640 plus 10% heat inactivated FBS and antibiotic cocktail was used and refreshed every 2-3 days.

3.1.1.3 WEHI-3

WEHI-3 is a murine cell line provided by ATCC (#TIB-68) prominently producing interleukin-3 (IL-3) into conditioned medium. Because IL-3 is an important growth factor to isolate and maintain murine leukemias in culture, the conditioned medium derived from WEHI-3 cell culture can be considered as an alternative source of murine recombinant IL-3. Originally, the cell line was characterized from peripheral blood of murine BALB/s strain with leukemia disease and observed as suspension cells. To obtain conditioned medium, WEHI-3 cells were

maintained in RPMI 1640 plus 10% heat inactivated FBS and antibiotic cocktail with 3 x 10^5 cells/mL as initial density. After 3 days, cell culture was centrifuged at 1,500 rpm in 10 minutes and collectively filtered supernatant was considered murine IL-3 supplementary source.

3.1.1.4 BaF3

Besides human FLT3(ITD)–positive cell line, to obtain murine FLT3(ITD)–positive cell line, BaF3 cell line was purchased from DSMZ (#ACC 300), which has been reported an effective host to express oncogenic tyrosine kinase via viral transduction [254, 255]. In details, BaF3 is murine IL-3-dependent pro B cell line, appears as suspension cells (sometimes clumps) with the origin remains unknown. The cells were maintained in Iscove's Dulbecco's Modified Eagle Medium (IMDM) (Corning #10-016-CV) plus 10% heat inactivated FBS, 10% WEHI-3–derived conditioned medium and antibiotic cocktail with initial 3 x 10^5 cells/mL. Clonal selection was conducted to obtain FLT3(wild-type) and FLT3(ITD) cells and the selection was confirmed by polymerase chain reaction (PCR).

3.1.1.5 Human bone marrow stromal cell line (HS-5)

To establish bone marrow microenvironment (BMM) for human leukemias, HS-5 cell line was purchased from ATCC (#CRL-11882). In details, the cells were characterized from 30 year-old Caucasian male, growing as fibroblasts and expression of HPV-positive E6/E7 proteins has been sufficient for cell immortalization without malignant transformation [256]. To culture these cells, HS-5 were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Corning #10-017-CV) plus 10% heat inactivated FBS and antibiotic cocktail with initial cell density 2 x 10^4 cells/cm^2 in 37^{o}C with 5% CO_2. The medium was refreshed every 3-4 days and the cells were trypsinized after a week in culture or until reaching completely confluent. Besides HS-5 cells from ATCC, we obtained HS-5 cells with CRISPR/Cas9 knock-down of *TGF-β1* gene to reduce TGF-β1 production in BMM. The cells were generated by Dr. Jian Huang laboratory at Department of Pathology and Laboratory Medicine, Temple University School of Medicine. To select cells with knock-down of *TGF-β1* gene, puromycin (1 μg/mL) was added into medium and the reduction of TGF-β1 level in BMM was confirmed by ELISA method done by Julian Swatler at Laboratory of Cytometry, Nencki Insititute of Experimental Biology.

3.1.1.6 Murine bone marrow stromal cell line (OP-9)

OP-9 cell line was obtained from ATCC (#CRL-2749) to establish BMM for murine leukemias. The cells were isolated from embryos of C57BL/6 mouse strain, growing in culture as fibroblast-like cells. Medium with IMDM plus 20% heat inactivated FBS and antibiotic cocktail was used to culture the cells with initial 1.5 x 10^4 cells/cm^2. The medium was refreshed every 3-4 days and the cells were trypsinized after a week in culture or until reaching completely confluent.

3.1.1.7 K562-parental and K562-CRISPR/Cas9 cells

K562 is a CML cell line in terminal of blast crisis phase, commercially available from ATCC (#CCL-243). The cell line was characterized from 53-year old female CML patient and growing as lymphoblastic suspension cells. Up to now, as K562 has been commonly using in CML studies, in current study, besides parental K562 purchased from ATCC, a K562 cell line with genetic down-regulation of TGFβR2 by CRISPR/Cas9 was obtained from Applied Biological Materials Inc. (Vancouver, Canada) (#NM_003242). In terms of cell culture, both cell lines could be maintained in DMEM plus 10% heat inactivated FBS and antibiotic cocktail. As for CRISPR/Cas9 cell line, puromycin (1.1 μg/mL) and G418 (500 μg/mL) were added in medium to conduct the selection.

3.1.1.8 Kasumi-1

Kasumi-1 is an acute myeloblastic leukemia cell line characterized from 7-year old Japanese male. Genetically, the cell line contains a translocation between chromosome 8 and 21 (t8;21) to constitute a fusion oncogenic protein AML1-ETO (also known as AML1-MTG or RUNX1-CBF2T1). The translocation oncogene suppresses CEPBA in all mRNA, protein and DNA binding activity, therefore it blocks granulocytes differentiation. The cell line now is commercially provided by ATCC (#CRL-2724) and growing as myeloblastic suspension cells. RPMI-1640 plus 20% heat inactivated FBS and antibiotic cocktail are required to culture the cells with initial 3 x 10^5 cells/mL.

3.1.1.9 BHK-21

Baby Hamster Kidney (BHK) 21 cell line has been considered as stem cell factor (SCF) producer by releasing the growth factor into conditioned medium [257]. The cell line was achieved from healthy one-day old hamster and grows as adhered fibroblast and distributed by ATCC (#CCL-10). The cells were remained in DMEM plus 10% heat inactivated FBS and antibiotic cocktail with initial 1.2 x 10^4 cells/cm^2. After 4 days, conditioned medium was collected and filtered, which can be considered an alternative SCF source for murine recombinant SCF.

3.1.2 Primary cells

3.1.2.1 Murine primary cells

3.1.2.1.1 Leukemia-like cells from bone marrow

At first, the dedicated medium to culture and isolate murine primary leukemia–like cells, contains IMDM plus 20% heat inactivated FBS and antibiotic cocktail with mandatory growth factors including 20% IL-3 supplement from WEHI-3, 20% SCF supplement from BHK-21 and 12.5 ng/mL recombinant IL-6. In terms of murine primary FLT3(ITD)-positive AML–like cells, FLT3(ITD)$^{+/+}$ knock-in mice were obtained from Jackson Laboratory. The mice were crossed with FLT3(WT) mice to get FLT3(ITD)$^{+/-}$ and mice genotype was confirmed by PCR. In BCR-ABL1-positive CML, the cells were originated from GFP(+) BCR-ABL1 CML–bearing mice. Animal procedure protocols were approved by Institutional Animal Care and Use Committee of Temple University. GFP(+) BCR-ABL1–positive CML-like cells were sorted at Temple University School of Medicine flow core facility.

As for other primary leukemia cells including FLT3(ITD); FLT3(ITD);Tet2-/-, AML1-ETO, mice with these genotypes were generated at Division of Oncology, Department of Medicine, Washington University School of Medicine, Saint Louis, MO, USA. Murine femoral specimens were obtained to isolate bone marrow cells. Animal studies were also approved by Institutional Animal Care and Use Committee of Washington University at St. Louis.

To isolate leukemia–like cells in bone marrow of femoral specimens, total bone marrow cells in those specimens were flushed into 1X phosphate buffer saline (PBS) by needle syringes, followed by removing red blood cells by ACK lysis buffer (Thermo Fisher #A1049201),

washing twice and re-suspending into dedicated medium described above. Furthermore, to obtain murine primary cells for treatment assays, linage-negative and c-Kit (CD117)-positive population was sorted by EasySep mouse hematopoietic progenitor cell isolation kit (Stem Cell #19856) and CD117 (cKIT) positive selection kit (Stem Cell #18757). The entire process was followed the instructions of manufacture.

3.1.2.1.2 Autologous bone marrow stromal cells

Total bone marrow cells from mice with BCR-ABL1 or FLT3(ITD);Tet2-/- genotype obtained from femoral samples were cultured in medium dedicated for murine primary cells. After 2 days, all suspension cells were aspirated and remaining adherent cells were continuously maintained in fresh medium and co-cultured with leukemia-like cells from corresponding mice to establish autologous BMM.

3.1.2.2 Human primary cells

In terms of FLT3(ITD)–positive AML, peripheral blood and bone marrow samples from patients with newly diagnosed AMLs were obtained from the Department of Internal Medicine III, University of Ulm; the Department of Internal Medicine (Hematology, Oncology, Hemostaseology, and Stem Cell Transplantation), RWTH Aachen University; and the Department of Internal Medicine I, Medical University of Vienna. Furthermore, FLT3(ITD);TET2mut, and AML1-ETO–positive primary AML samples were from the ECOG-ACRIN E1900 clinical trial [258]. BRCA1/2-deficient AML samples were previously described [176]. BCR-ABL1–positive CML-CP samples were obtained from the Department of Internal Medicine I, Division of Hematology & Hemostaseology, Medical University of Vienna, Austria. Samples of normal hematopoietic cells were purchased from StemCell Technologies (Vancouver, Canada) and cultured in provided medium from manufacturer. All procedures of human studies were approved by Temple University Institutional Review Boards and met all requirements of the Declaration of Helsinki. Each type of human primary leukemia cells were cultured in their distinct medium described below.

3.1.2.2.1 Acute/Chronic myeloid leukemia cells

Human CML/AML primary cells from bone marrow of peripheral blood were maintained in StemSpan SFEM II (Stem Cell #09655) plus 10% heat inactivated FBS, antibiotic and cocktail of required human recombinant growth factors including 100 ng/mL SCF, 100 ng/mL FLT3, 20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL G-CSF and 100 ng/mL TPO. In advance of treatment assays, linage–negative and CD34–positive cells were sorted by EasySep human hematopoietic progenitor cell enrichment kit (Stem Cell #14056) and CD34 positive selection kit (Stem Cell #17856). The entire procedures were followed the instructions of manufacture.

3.1.2.2.2 Autologous bone marrow stromal cells

Human autologous primary stromal cells were obtained from aspirated bone marrow specimens of AML patients with FLT3(ITD);TET2mut by the method to isolate murine primary stromal cells described at 3.1.2.1.2.

3.1.2.2.3 Bone marrow mesenchymal stromal cells (allogeneic stromal cells)

Human primary bone marrow mesenchymal stromal cells (MSCs) were purchased from Stem Cell (#70022), which was isolated from bone marrow monoclear cells in culture and the first cell passage was cryopreserved in prior delivery. In terms of genetic profile, surface antigenic markers revealed definite indication of hMSCs including over 90% expression of CD105, CD73 and CD90, meanwhile markers of human hematopoietic cells such as CD34, CD45, CD14 are lower than 5%. To maintain cells in culture, Mesencult^{TM} Proliferation Kit (Stem Cell #05411) was also purchased, the medium was renewed every 3-4 days and cells were then co-cultured with human leukemias to establish allogeneic BMM.

3.2 Reagents

3.2.1 Inhibitors

All inhibitors demonstrated in cell treatment are purchased from Selleckchem (Houston, TX, USA) including: TGFβ receptor inhibitors: SB435142 (#S1067) and Galunisertib (#S2230), CXCR4 inhibitor/antagonist: AMD3100 (#8030) and WZ811 (#S2912), PARP inhibitors: Olaparib (#S1060) and Talazoparib (#S7048), FLT3 inhibitor: Quizartinib (#S1526), ABL1

inhibitor: Imatinib (#S2475), DNA topoisomerase II inhibitor: Doxorubicin (#S1208), SMAD3 inhibitor: SIS3 (#S7959), JAK1/2 inhibitor: Ruxolitinib (#S1378), PI3K inhibitor: Buparlisib (#S2247), RAF1 inhibitor: LY3009120 (#S7842), PAK1 inhibitor: IPA-3 (#S7093), TAK1 inhibitor: Takinib (#S8663) and TGFβ-1,2,3 neutralizing antibody 1D11.16.8 delivered by Invitrogen (Waltham, MA, USA) (#16-9243-85).

3.2.2 Primary antibodies

Total information of primary antibodies purchased for protein detection are clearly described in the table below (Table 3.1).

Table 3.1. List of primary antibodies.

Protein	Company	Catalogue Number	Host	Working concentration	Blocked solution in TBST	Diluted solution in TBST	Solution of secondary Ab in TBST (1:10,000)	Molecular weight (kDa)
TGFβR1	Santa Cruz	sc-518018	Mouse	1:500	5% Milk	5% BSA	5% BSA	53
TGFβR2	Invitrogen	701683	Rabbit	1:1000	5% Milk	5% BSA	5% BSA	65
pSMAD2	Cell Signaling	5339S	Rabbit	1:1000	5% Milk	5% BSA	5% BSA	60
SMAD2	Cell Signaling	3108S	Rabbit	1:1000	5% Milk	5% BSA	5% BSA	60
pSMAD3	Cell Signaling	9520S	Rabbit	1:1000	5% BSA	5% BSA	5% BSA	52
SMAD3	Cell Signaling	9523S	Rabbit	1:1000	5% BSA	5% BSA	5% BSA	52
pAKT	Cell Signaling	9271S	Rabbit	1:1000	5% Milk	5% BSA	5% BSA	60
AKT	Cell Signaling	2920S	Mouse	1:1000	5% Milk	5% BSA	5% BSA	60
pERK1/2	Cell Signaling	4370S	Rabbit	1:1000	5% Milk	5% BSA	5% BSA	42/44
ERK1/2	Cell Signaling	4696S	Mouse	1:1000	5% Milk	5% BSA	5% BSA	42/44
pSTAT5	Cell Signaling	9359S	Rabbit	1:1000	5% Milk	5% BSA	5% BSA	90
STAT5	Cell Signaling	25656	Rabbit	1:1000	5% Milk	5% BSA	5% BSA	90
p-p38/MAPK	Cell Signaling	9216S	Mouse	1:1000	5% Milk	5% BSA	5% BSA	43

Protein	Company	Catalogue Number	Host	Working concentration	Blocked solution in TBST	Diluted solution in TBST	Solution of secondary Ab in TBST (1:10,000)	Molecular weight (kDa)
p38/MAPK	Invitrogen	33-1300	Mouse	1:500	5% Milk	5% BSA	5% BSA	43
BRCA1	Calbiochem	OP92	Mouse	1:250	5% Milk	5% BSA	5% BSA	260
BRCA2	Abcam	ab75335	Rabbit	1:500	5% Milk	5% Milk	5% Milk	384
PALB2	Abcam	ab202970	Rabbit	1:2000	5% Milk	5% Milk	5% Milk	131
ATM	Santa Cruz	sc-135663	Mouse	1:1000	5% Milk	5% Milk	5% Milk	370
ATR	Santa Cruz	sc-515173	Mouse	1:500	5% Milk	5% Milk	5% Milk	250
DNA-PKcs	Bethyl	A300-518A	Rabbit	1:2000	5% Milk	5% Milk	5% Milk	460
Ligase IV	Santa Cruz	sc-271299	Mouse	1:250	5% BSA	5% BSA	5% BSA	95
RAD51	Santa Cruz	sc-8349	Rabbit	1:500	5% Milk	5% Milk	5% Milk	37
Ku80	Invitrogen	MA5-15873	Mouse	1:500	5% Milk	5% BSA	5% BSA	80
Ku70	Santa Cruz	sc-17789	Mouse	1:500	5% Milk	5% BSA	5% BSA	70
53BP1	Abcam	ab-21083	Rabbit	1:1000	5% Milk	5% BSA	5% BSA	350
CtIP	Invitrogen	PA5-20963	Rabbit	1:1000	5% Milk	5% BSA	5% BSA	125
PARP1	Santa Cruz	sc-74470	Mouse	1:500	5% Milk	5% BSA	5% BSA	116
Ligase III	Santa Cruz	sc-135883	Mouse	1:500	5% Milk	5% BSA	5% BSA	95
Pol θ	Invitrogen	PA5-69577	Mouse	1:250	5% Milk	5% Milk	5% Milk	290
Lamin	Abcam	ab-16048	Rabbit	1:500	5% Milk	5% BSA	5% BSA	70
Histone H3	Thermo Fisher	AHO1432	Mouse	1:1000	5% Milk	5% BSA	5% BSA	17
β-actin	Santa Cruz	sc-47778	Mouse	1:500	5% Milk	5% BSA	5% BSA	45

3.2.3 Secondary antibodies

There were two types of secondary antibodies in this study. At first, to develop protein in X-ray film for western blotting analysis, goat HRP-conjugated anti-rabbit (MerckMillipore #12-348) and anti-mouse (MerckMillipore #12-349) IgG antibodies were obtained. Meanwhile, in terms of immunofluorescence, FITC-conjugated anti-rabbit (#11034) and anti-mouse (#A11001) were purchased from Invitrogen.

3.2.4 Recombinant cytokines

All recombinant cytokines required for culture of primary cells and treatment assays are listed in the table below (Table 3.2). All of them were delivered in dry powder and re-suspended in 1X PBS (sterilized) + 0.1% BSA to obtain expected concentration.

Table 3.2. List of recombinant cytokines.

Recombinant cytokine	Host	Company	Catalogue Number
TGF-β1	Human	Invitrogen	PHG9204
TGF-β1	Mouse	R&D Systems	7666-MB-005
SCF	Human	Stem Cell Tech	78155.2
SCF	Mouse	Stem Cell Tech	78064.2
FLT3	Human	Stem Cell Tech	78137.2
IL-3	Human	Stem Cell Tech	78040.2
IL-3	Mouse	Pepro Tech	213-13
IL-6	Human	Stem Cell Tech	78050.2
G-CSF	Human	Stem Cell Tech	78012.2
TPO	Human	Pepro Tech	300-18

3.3 Cell cultured conditions

3.3.1 Bone marrow microenvironment

3.3.1.1 Bone marrow microenvironment constituted by stromal cell lines

To mimic bone marrow microenvironment (BMM) condition, at first, cell line stromal cells (HS-5 or OP-9) were cultured with initial density as 2 x 10^4 cells/cm^2 in serum-supplied medium within 24 hours for murine cells and 48 hours for human cells to generate a monolayer stromal cells. After 24/48 hours, initial medium was removed and stromal layer were rinsed once with 1X PBS to eliminate any remaining serum.

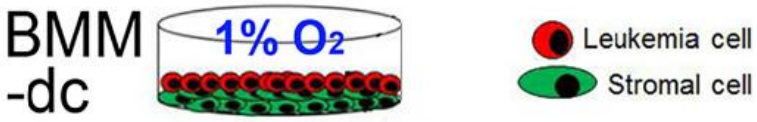

Figure 3.1. Bone marrow microenvironment with direct contact between stromal cells and leukemia cells (BMM-dc).

The step was followed by addition of Lin^-CD34^+ human cells or Lin^-cKIT^+ murine cells (ratio 1:1 with stromal cells in 24-well plate or 0.5 x 10^6 cells/mL in 12-well and 6-well plates) in serum–deprived medium (StemSpan SFEM II plus required growth factors) to co-culture with stromal cells. The plates were then maintained in a chamber supplying 1% oxygen and 5% CO_2 in 37^{o}C to establish hypoxia condition in 24 hours to completely establish BMM condition with direct contact between stromal cells and leukemia cells (BMM-dc) (Figure 3.1).

3.3.1.2 Allogeneic/autologous bone marrow microenvironment

In terms of allogeneic BMM, we purchased human bone marrow mesenchymal stromal cells from Stem Cell Technologies as mentioned above. The cells were cultured in treated plate with provided medium from manufacture for two days or until reaching confluent. After obtaining stromal monolayer, initial medium was removed and human Lin^-CD34^+ leukemia cells were added and the co-culture was then remained in hypoxic condition for 24 hours to achieve allogeneic BMM-dc. On the other hand, to establish autologous BMM-dc with primary stromal cells, 1x10^6 total bone marrow cells of FLT3(ITD);Tet2-/- bearing mice and FLT3(ITD);TET2mut AML patients were cultured in one well of 24-well plate with serum-

sufficient medium plus required grow factors in 48-72 hours. After stromal monolayer was generated, all suspension cells were aspirated and primary stromal cells were washed by 1X PBS and followed by addition of 4 x 10^4 corresponding Lin^-cKIT^+ murine leukemias and Lin^-CD34^+ human leukemias in 1 mL serum-starved medium. The mixture was incubated in hypoxia for 24 hours before further assays.

3.3.1.3 Bone marrow microenvironment without contact between stromal cells - leukemia cells

Besides BMM-dc condition with direct contact of stromal cells with leukemia cells, to obtain BMM without this contact (indirect contact) (BMM-idc), a PET culture insert (Millipore #MCHT12H48) was hanged inside each well of 12-well plate containing stromal monolayer in 1.5 mL serum-starved medium.

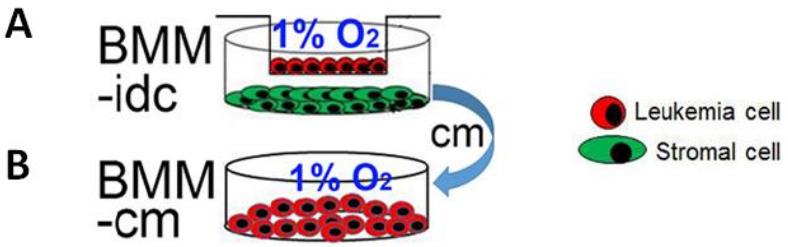

Figure 3.2. Bone marrow microenvironment without direct contact of stromal cells – leukemia cells (BMM-idc) (**A**) and with conditioned medium derived from BMM-idc to culture leukemia cells in hypoxia (BMM-cm) (**B**).

To accomplish this cultured condition, 2 x 10^4 cells/mL (for clonogenic assay) or 5 x 10^5 cells/mL (for other assays) Lin^-CD34^+ human cells or Lin^-cKIT^+ murine cells were introduced into the insert with 500 µL serum-depleted medium (Figure 3.2A). Similar to other BMM conditions, this indirect co-culture was maintained in hypoxia in 24 hours in advance of later assays.

3.3.1.4 Bone marrow microenvironment with conditioned medium produced in BMM-idc

In this condition of BMM, leukemia and stromal cells were maintained in BMM-idc for two days. The medium in BMM-idc was then obtained and thoroughly centrifuged to eliminate any remaining cells or debris. This supernatant was considered conditioned medium (cm)

from BMM-idc and used to culture leukemia cells in hypoxic condition in 24 hours to accomplish BMM-cm (Figure 3.2B).

3.3.2 Peripheral blood microenvironment

In terms of peripheral blood microenvironment (PBM) condition, the BMM -equal number of Lin^-CD34^+ human leukemia cells or Lin^-cKIT^+ murine leukemia cells were maintained in serum–deprived medium in an ordinary cultured incubator performing normoxia condition with 17% oxygen and 5% CO_2 supplement in 24 hours before further treatment.

3.4 Cell treatment assays

3.4.1 Clonogenic assay

In terms of clonogenic test in normal condition, FLT3(ITD) -positive leukemia cells were treated by PARPi (olaparib/talazoparib) +/- FLT3i quizartinib in 96 hours and the procedure was continued with later steps described below with additional plating cells in methylcellulose. About the clonogenic assays in established BMM/PBM, leukemic cells were treated by PARPi (olaparib/talazoparib) +/- TKi (quizartinib/imatinib). The treatment was maintained under BMM/PBM in 72 hours. This step was followed by plating all cells into methylcellulose (Methocult H4230, Stem Cell #04230) with medium containing 10X growth factor cocktail. Number of colony forming in methylcellulose was counted after 7-10 days. Percentage of colony was calculated based on colony number of untreated group considered as 100%.

For the assay with pre-treatment of TGFβRi or CXCR4i, the first dose of inhibitors was added immediately after introducing leukemia cells into microenvironment. After 24 hours to completely establish BMM/PBM, the second dose was added simultaneously with [PARPi +/- TKi].

3.4.2 γ-H2AX and cell viability assay

In cells, when DSBs are introduced, ATM kinase is activated as a DSB response to phosphorylate itself at Ser1981 [129]. Intracellular pathway triggers signaling transduction from phosphorylated ATM to phosphorylation of Histone H2AX at Ser139 - also known as γ-H2AX [130]. From activation of γ-H2AX, DSB repair proteins are recruited to repair the DNA

lesions, suggesting that γ-H2AX is considered a marker of DSBs in cells. In details of this assay, cells were treated with [PARPi +/- TKi] and collected after 24 and 48 hours. After treatment, cells were centrifuged and wash with 1X PBS, re-suspended in 500 µL diluted Fixable Viability Dye eFluour 780 (BD Biosciences #565388) and incubated in dark cold condition in 15 minutes. The assay was continued by fixing cells in 4% formaldehyde (Fisher Scientific #S25329) for 10 minutes in room temperature (RT) and nuclear permeable step in 100% methanol (Fisher Scientific #A412-500). The cells - methanol mixture was chilled at -20^{o}C in at least an hour. After chilling period, cells were wash with 1X PBS + 0.1% Triton X-100 (Sigma Aldrick #9002-93-1) before addition of PE -conjugated γ-H2AX antibody (BD BioSciences #562377) in 1X PBS + 1% BSA buffer (Sigma-Aldrich #A9205). The antibody incubation was maintained in dark cold condition for 30 minutes. In the last step, cells were washed and re-suspended in 1X PBS + 0.1% Triton-X100 and analyzed in flow cytometry.

3.4.3 Neutral comet assay

Neutral comet assay is a technique to directly measure DSBs in cells through cell-agarose electrophoresis in neutral condition buffer (Tris-Acetic acid-EDTA buffer) (Figure 3.3A). In this assay, DSBs are measured as percentage of tail DNA calculated by ratio (tail DNA/total cell DNA) x 100 (Figure 3.3B).

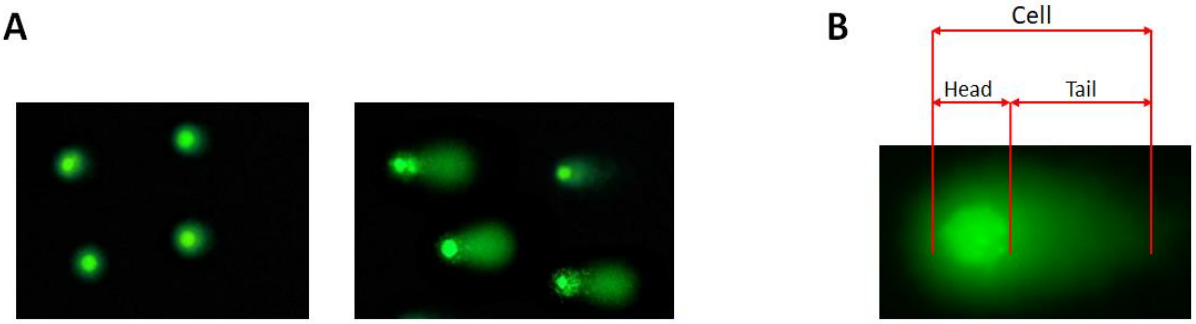

Figure 3.3. Outcome of neutral comet assay. **A**. Untreated cell without comet tail DNA (left) and treated cells with comet tail DNA (right). **B**. Calculation of DSBs based on length of tail DNA.

To do this method, at first, an OxiSelect Comet Assay Kit (3-well slides) was purchased from Cell Biolabs (#STA-351). In terms of procedure, leukemia cells were treated by [PARPi +/- TKi] in normoxia or BMM vs PBM or BMM +/- SB431542 in 24 hours, the step was followed by collecting and washing cells in 1X PBS before doing cell lysis in agarose and loading this

mixture into each well of slide according to protocol of manufacturer. The cell-agarose electrophoresis was run in 1X TAE buffer with 1.2 voltage/cm in 30 minutes for primary cells and 15 minutes for cell lines. After electrophoresis, slides were washed by deionized water and ethanol 70% before adding Vista Green DNA dye and comet tail was observed by FITC channel of fluorescent microscope. Percentage of tail DNA is calculated by Open Comet ImageJ software version 1.3.1. About electrophoresis running buffer, 50X TAE buffer was made from 242g Tris base, 57.1 mL glacial acetic acid, 100 mL 500 mM EDTA (pH 8.0) in 1 L distilled water and diluted 50 times to obtain 1X buffer before running the electrophoresis.

3.4.4 DNA double strand break repair assay

The purpose of this assay is to determine DSB repair activities of DSB repair pathways including homologous recombination (HR), canonical non-homologous end joining (D-NHEJ) and PARP1 -dependent alternative end joining (Alt-NHEJ). To do this method, initial materials include three plasmid reporters representing for these three DSB repair pathways.

3.4.4.1 *I-sce*I endonuclease digestion and DNA clean up

In details, all three reporters contain restricted site of endonuclease *I-sce*I in Green Fluorescent Protein (*GFP*) gene and the enzyme (Thermo Fisher #ER1771) was used to digest three plasmids to generate DSB in *GFP*. In the experiment, 25 µg of each DNA reporter was digested in 37^{o}C in 3 hours and linearized DNA after digestion was purified by DNA clean up micro kit (Thermo Fisher #K0831) and DNA concentration and purification were measured in NanoDrop machine.

3.4.4.2 DNA-cell co-nucleotransfected and DSB repair analysis

Linearized DNA after *I-sce*I enzymatic digestion was co-nucleotransfected with Red Fluorescent Protein (RFP) -expressed plasmid (DsRed) into murine primary $Lin^{-}cKIT^{+}$ FLT3(ITD);TET2-/- AML -like cells via Mouse Macrophage Nucleofector Kit (Lonza #VPA-1009). In details, 2 µg of linearized HR reporter or 0.5 µg of linearized D-NHEJ/Alt-NHEJ was co-transfected with 0.1 µg DsRed reporter in one cuvette provided in the kit by Lonza nucleo-transfection machine. After transfection, leukemia cells were maintained in BMM-dc with/without addition of TGFβRi SB431542 in 72 hours. Survival cells were analyzed in flow

cytometry with FITC for GFP(+) cells and PE for RFP (+) counterparts. The DSB repair activity, in which DSB repair restored expression of GFP, was calculated by ratio between total restored GFP(+) cells/total transfected RFP(+) cells.

3.4.5 Quiescent stem cell assay

Leukemia stem cells (LSCs) ($Lin^-CD34^+CD38^-$ cells) occupy a quite small proportion of total leukemia population but they are the most refractory, responsible for chemotheurapeutic/cytotoxic resistance, enhancing DNA repair, evading immune system, overturning apoptosis etc [259-262]. TGF-β signaling pathway has been reported to induce quiescent state of leukemic cells in BMM [197-199], therefore, identification of quiescent LSCs between PBM and BMM or in BMM with/without addition of TGFβRi SB435142 is essential to evaluate effect of BMM and TGFβR–mediated pathway promoting quiescent LSCs refractory against [PARPi +/- TKi].

3.4.5.1 Cell labelling and treatment

In this approach, initially, human primary leukemia cells were labelled with eBioscience Cell Proliferation Dye eFluor 670 (Invitrogen #65-0840) at 37^oC in dark condition for 20 minutes and maintained in cultured conditions in 24 hours. In the following day, the first doses of inhibitors were added. After 72 hours, the second doses were introduced and the procedure was prolonged in following 48 hours to totally obtain 120-hour treatment.

3.4.5.2 Survival cell counting and flow cytometric analysis

After the treatment, total living cells in each treated group were recorded via tryphan blue in automated cell counting machine (BioRad). Viable cells were then analyzed in flow cytometry with FITC for linage antibody (BD BioSciences #340546), PerCP-Cy5.5 for CD34 antibody (BD BioSciences #347203), PE for CD38 antibody (BD BioSciences #555460) and APC for Cell Proliferation Dye eFluor 670. Percentage between $Lin^-CD34^+CD38^-$ APC^{max}/total living cells was calculated via cytometric analysis. This percentage was then applied to the total living cells of tryphan blue counting to calculate total number of $Lin^-CD34^+CD38^-APC^{max}$ cells after treatment.

3.5 Cell cycle analysis

This method was conducted to evaluate discrepancies in cell cycle distribution between BMM and PBM as well as BMM-dc with/without addition of SB435142. In both circumstances, cells were maintained in PBM/BMM or BMM-dc +/- SB431542 in 48 hours. The cells were then collected and washed twice with 1X PBS before getting fixed by 4% formaldehyde in 10 minutes at RT and permeable by ethanol 80% in 2 hours at -20^{0}C. In the last step, fixed and permeable cells were incubated with PE-conjugated PI/RNase staining buffer (BD BioSciences #550825) for 15 minutes at RT before analyzing in flow cytometry. As the outcome, proportion of G_o-G_1, S and G_2-M phases were distinguished in concordance to instruction of manufacture protocol.

3.6 DNA cloning

This method was carried out to demonstrate dominant negative mutant of *SMAD3* (D407E) on sensitivity of leukemia to [PARPi +/- TKi] in BMM. In details, DNA fragments containing sequence of [FLAG;SMAD3(WT)] and [FLAG;SMAD3(D407E)] from pcDNA3 were re-cloned into pMIG-IRES-GFP in order to sort cells with SMAD3 mutant and WT by expression of GFP.

3.6.1 Bacterial competent cells preparation

To perform entire DNA cloning procedure, bacterial competent cells from *E. coli* is a prerequisition because it is the initial material for heat shock transformation and DNA plasmid amplification. To obtain bacterial competent cells, *E. coli* DH5α was restored in 3-5 mL liquid LB medium overnight in shaking incubator in 12-16 hours, in the following day, 1 mL bacterial culture was transferred into a new autoclaved flask with 50 mL LB medium and *E. coli* continues expanding in shaking incubator until optical density at 600 nm ($OD_{600\ nm}$) reached 0.4-0.6. Bacterial pellet was obtained by centrifugation and re-suspended in autoclaved cold $CaCl_2$ 0.1 M with an incubation in ice in 30 minutes before centrifugation to obtain bacterial pellet and this step was repeated with 15-minute ice incubation. In the final step, *E. coli* pellet was re-suspended in 1 mL cold $CaCl_2$ 0.1 M with 1 mL autoclaved 60% glycerol and the bacterial solution was then aliquoted into 1.5 mL eppedorf tubes, rapidly frozen by liquid nitrogen and stored in -80^{o}C. LB medium was prepared in 10 g trypton, 5 g yeast extract, 5 g NaCl, 20 g agar (only for solid medium) in 1 L distilled water and autoclaved before usage.

3.6.2 Bacterial heat shock transformation and DNA plasmid amplification

From two initial DNA plasmids including [pCNA3;FLAG;SMAD3(WT)] and [pCNA3;FLAG;SMAD3(D407E)] obtained from Dr. Mitsuyasu Kato [263] as generous gifts, they were heat shock transformed into *E.coli* DH5α competent cells at 42^{o}C in 45 seconds. Bacterial cells were recovered in liquid SOC medium (Thermo Fisher #15544034) in one hour and cell pellet were spread into agar LB medium plus selected antibiotic, ampicillin (Thermo Fisher #11593027) 100 µg/mL. Single bacterial colony was picked up from agar LB medium and cultured in 5 mL liquid LB medium plus selected ampicillin in 12-16 hours. *E. coli* pellet were then used to extract DNA plasmid according instruction of NucleoSpin Plasmid (NoLid) kit (Macherey-Nagel #740588). Concentration and purification of DNA plasmid were measured in NanoDrop machine.

3.6.3 Restricted endonuclease digestion and DNA ligation

Two amplified pcDNA3 DNA plasmids above were digested by FastDigest *Xho*I (Thermo Fisher #FD0695) and *BamH*I (Thermo Fisher #FD0054) and targeted DNA fragments were then excised from agarose gel after DNA elctrophoresis and purified by GeneJET Gel Extraction Kit (Thermo Fisher #K0691). In terms of pMIG-IRES-GFP plasmid, because this plasmid does not contain restricted site of *BamH*I in multi-cloning site, therefore, we used *Bgl*II because sticky end of *BamH*I can be ligated with the counterpart *Bgl*II. Therefore, pMIG-IRES-GFP was digested by FastDigest *Xho*I and *Bgl*II (Thermo Fisher #FD0084), linearized DNA was also cleaned up by GeneJET Gel Extraction Kit. To perform ligated reaction of [FLAG;SMAD3(WT)] and [FLAG;SMAD3(D407E)] with linearized pMIG-IRES-GFP, all DNA concentrations were measured and ligated reaction was prepared based on standard protocol of Rapid DNA Ligation Kit (Thermo Fisher #K1422). Ligated products were then heat shock transformed into *E. coli* competent cells to amplify recombinant DNA plasmids. Because the restricted sites of *BamH*I and *Bgl*II cannot be re-generated after ligation, to confirm efficacy of ligated reaction, amplified DNA plasmids were digested by *Xho*I and *Hind*III (Thermo Fisher #FD0504) whose restricted site locates outside pMIG-IRES-GFP's multi-cloning site. In the last step, after confirming effectiveness of DNA ligation, DNA plasmids were amplified in high concentration by NucleoBond Xtra Maxi Kit (Macherey-Nagel #740414) to fulfill materials for retroviral infection into host cells.

3.7 Immunofluorescent analysis

To do this analysis, after 48 hours maintaing in cultured conditions including PBM, BMM-dc, BMM-idc, and hypoxia, leukemia cells were centrifuged and washed twice by 1X PBS. The cell pellets were re-suspended in 1X PBS + 1% BSA and blocked by Human TruStain FcX Solution (Bio Legend #422302) in 10 minutes at RT. Primary antibodies were incubated with cells in one hour at $4^{O}C$. This step was followed by washing cells with 1X PBS , re-suspended in 1X PBS + 1% BSA and FITC-conjugated secondary antibodies was incubated with cells in one hour at $4^{O}C$ in dark before washing, re-suspending cells and analyzing in flow cytometry.

3.8 Protein level analysis

3.8.1 Protein extraction

To detect expression level of DSB repair proteins, cellular nuclear lysate was extracted. In details, in terms of FLT3(ITD)–positive leukemia cells, 6 x 10^6 cells were treated with DMSO as vehicle control, FLT3i, JAK1/2i, PI3Ki and RAF1i in 24 hours in normoxic condition. On the other hand, in BMM-dc, 6 x 10^6 leukemia cells were co-cultured with stromal cell line in 12 mL serum-deprived medium treated by DMSO as vehicle control, TGFβRi, SMAD3i, PI3Ki, RAF1i, PAK1i, TAK1i. After 48 hours treatment of TGFβRi, SMAD3i and 24 hours of remaining inhibitors, leukemia cells were collected and washed by cold 1X PBS. Cell nucleus was obtained by nuclear lysis buffer made from 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT and 1X protease inhibitor cocktail (Thermo Fisher #78429). Cell nucleus was 15-minute incubated and frequently vortexed in buffer with 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM DTT and 1X protease inhibitor cocktail to extract nuclear proteins. Protein concentration was measured by Bradford method using Coomassie Blue, 30 µg protein was loaded in one well of SDS-PAGE gel. On the other hand, whole cell protein lysate was also extracted in terms of receptor proteins or other proteins to validate efficacy of kinase inhibitors. In this circumstance, after washing with 1X cold PBS, cells were re-suspended in RIPA buffer (Thermo Fisher #89900) plus 1X protease inhibitor cocktail and remained in ice for 10 minutes. Cell lysate was centrifuged at 8000 rpm in 12 minutes, whole cell lysate was obtained as the supernatant and 100 µg protein was used in each well of SDS-PAGE gel.

3.8.2 SDS-PAGE electrophoresis

Nuclear or whole cell protein was mixed with 5X SDS-PAGE sample loading buffer (Thermo Fisher #39000) to dilute the buffer to 1X. The mixture was then boiled at 95-100^{O}C in 5 minutes to denature protein and introduced into 4-20% Mini-PROTEAN TGX Precast Protein Gels (BioRad #4561094). The electrophoresis was run in 1X Tris-Glycin-SDS buffer (5X solution made from 15.1 g Tris Base, 72 g Glyncin, 50 mL SDS 10% in 1 L distilled water) at 75 V in 20 minutes for protein stacking and continued with 105 V in 90 minutes for protein separation.

3.8.3 Western blot

After SDS-PAGE electrophoresis, proteins in gel were transferred into nitrocellulose membrane (Thermo Fisher #88018) by transferring buffer made from 18.5 g Tris base, 86.4 g Glycin, 800 mL Methanol in 6 L distilled water. The transferring process was carried out in cold condition with 105 V in one hour or until entire proteins were completely transferred to membrane. After transferring period, membrane was washed twice in 5 minutes with 1X TBST buffer (10X solution made from 80 g NaCl, 2 g KCl, 30 g Tris base, pH 7.4 and 10 mL Tween 20 in 1 L distilled water) before blocked by 5% BSA or fat-free milk in 1X TBST buffer with gently shaking in one hour. The blocked step was followed by washing membrane three times with 1X TBST and addition of primary antibody. The incubation of the membrane in primary antibody solution was maintained overnight with gentle shaking in cold condition. The membrane was then washed three times by 1X TBST and incubated with solution of secondary antibody in one hour with gentle shaking. The procedure was continued by washing membrane three times with 1X TBST and using Pierce ECL Western Blotting Substrate (Thermo Fisher #32106), shaking in 2 minutes in dark condition. The final step was developing of protein blots in Premium X-Ray Film (Phenix #F-BX57) in dark room with western blotting developer machine. All the steps from block of membrane to using primary and secondary antibodies were followed the information listed in Table 3.1.

3.9 *In vivo* experiment

3.9.1 Testing [PARPi +/- FLT3i] in human FLT3(ITD)–positive AML cells *in vivo*

3.9.1.1 Subcutaneous injection

To establish experiment to examine anti-leukemia effect of [PARPi + FLT3i], primary FLT3(ITD)–positive AML patient cells were obtained from bone marrow sample. The cells were expanded in medium and sorted by Lin^-CD34^+ kits as described above for human primary cells. In the meantime, in terms of animal model, total 40 NOD.$Rag1^{-/-}$;γc^{null} (NRGS) mice expressing human IL-3, GM-CSF, and SCF were purchased from Jackson Laboratory and divided into 4 groups (10 mice/group) of treatment regimen including (1) DMSO as vehicle control, (2) PARPi BMN673, (3) FLT3i AC220 and (4) BMN673 + AC220. To engraft leukemia into mice, one day before injection, mice were sub-lethally irradiated at 200 Gy and 1 x 10^6 cells were subcutaneously injected in one mouse with no more than 100 µl of cell culture in one syringe.

3.9.1.2 Leukemia engraftment confirmed via human CD45–positive cells in tail vein blood

Since the engraftment of human leukemia in mice is validated by expression of cell surface antigenic marker CD45 [264], to evaluate the efficiency of human leukemia engraftment in mice, percentage of CD45–positive cells was detected in tail vein blood via flow cytometry. In details, every one week after the subcutaneous injection, tail vein blood was taken from 5 mice in a random cage. The blood was lysed with ACK lysis buffer in 3 minutes to remove red blood cells and remaining white blood cells were washed twice with 1X PBS. The cell pellet was re-suspended in 1X PBS + 1% BSA and cells were blocked by Human TruStain FcX Solution in 10 minutes at RT. PE–conjugated anti-human CD45 antibody (BD BioSciences #555483) was incubated with cells in one hour at 4^OC in dark. The cells finally washed and re-suspended in 1X PBS and analyzed in flow cytometry. In the analysis, negative control was cells without adding CD45 antibody and positive control was the human primary AML cells. The percentage of hCD45–positive cells should be at least 5% in prior to start treatment regimen in mice.

3.9.1.3 *In vivo* treatment regimen with inhibitors

In total three weeks after leukemia injection, percentage of hCD45–positive cells reached to approximately 5-8% in tail vein blood and mice in four groups were treated for 7 consecutive days with vehicle, BMN673 (0.33 mg/kg/day) [176], AC220 (10 mg/kg/day) [265] and BMN673 + AC220 (Figure 3.4A).

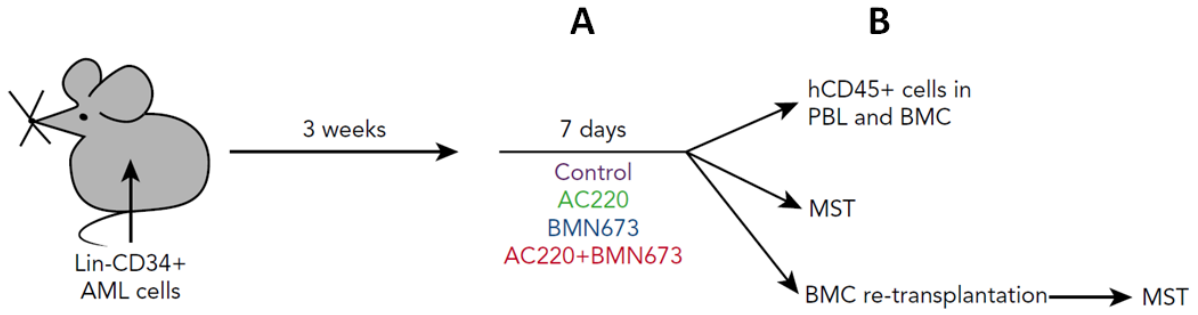

Figure 3.4. Design of *in vivo* treatment of [AC220 +/- BMN673] in mice bearing human primary FLT3(ITD)–positive AML cells. **A**. Four groups of 7-day treatment regimen. **B**. Identification of engrafted leukemia cells in mice after treatment regimen and survival of leukemia-bearing mice. PBL: peripheral blood leukocytes, BMC: bone marrow cells, MST: median survival time.

At the end of treatment, hCD45–positive cells were detected in peripheral blood leukocytes (PBL) and bone marrow cell (BMC) via flow cytometry (Figure 3.4B). Moreover, 1 x 10^6 bone marrow cells were re-transplanted into the sub-lethally irradiated recipients to evaluate the effect of treatment on LSCs. Median survival time (MST) of the initial and secondary recipients was also determined. All animal procedures were approved by Institutional Animal Care and Use Committee of Temple University.

3.9.2 Testing TGFβRi +/- [PARPi + TKi] in murine primary AML/CML–like cells *in vivo*

3.9.2.1 GFP(+) murine primary leukemias preparation

To prepare leukemia cells for *in vivo* experiment, murine primary BCR-ABL1–positive CML–like and FLT3(ITD);TET2-/- AML–like cells were retrovirally infected with pMIG-IRES-GFP as described before [176]. The cells were then sorted by flow cytometry to obtain GFP(+) CML/AML–like cells.

3.9.2.2 Subcutaneous injection

In the meantime of recovery and expansion of GFP(+) leukemias after sorting, about animal model, total 80 severe combined immunodeficient (SCID) mice were ordered from Jackson Laboratory and a half (40 mice) were used for each leukemia. In each 40 mice, they were divided into 4 groups of treatment (10 mice per group): (1) vehicle control, (2) TGFβRi SB431542, (3) PARPi talazoparib + TKi (imatinib or quizartinib), (4) SB431542 + talazoparib + TKi (imatinib or quizartinib). One day in advance of subcutaneous injection, every SCID mice were sub-lethally irradiated at 200 Gy and injected with 2 x 10^6 GFP(+) leukemia cells per mouse with no more than 100 µl of cell culture in one syringe.

3.9.2.3 Leukemia engraftment confirmed via GFP(+) cells in tail vein blood

One week after the injection, to confirm leukemia engraftment in SCID mice, peripheral blood of 5 injected mice and 2 non-injected mice as negative controls was taken from tail vein. The blood was lysed with ACK lysis buffer in 3 minutes to remove red blood cells and remaining white blood cells were washed twice with 1X PBS before analyzing GFP(+) leukemia–like cells in flow cytometry. The percentage of GFP(+) cells should be at least 10% in order to conduct treatment regimen.

3.9.2.4 *In vivo* treatment with inhibitors

In details of treatment regimen, SCID mice bearing GFP(+) BCR-ABL1 CML-like or GFP(+) FLT3(ITD);Tet2-/- AML-like cells (approximately 15-20% GFP(+) cells in tail vein peripheral blood) were treated for 7 consecutive days with vehicle, TKi (100 mg/kg imatinib or 10 mg/kg quizartinib) + PARPi (0.33 mg/kg talazoparib) as described before [176], TGFβRi (10 mg/kg SB431542) [266] and the combination of [TKi + PARPi + TGFβRi] (Figure 3.5A). GFP(+) leukemia cells were detected by flow cytometry in peripheral blood leukocytes (PBL), splenocytes (SPL) and bone marrow cells (BMC) 3 days after the end of treatment (Figure 3.5B). Moreover, 1 × 10^6 femoral bone marrow cells were re-transplanted into the sub-lethally irradiated recipients to evaluate the effect of treatment on LSCs. Median survival time (MST) of initial and secondary recipients was also determined. All animal procedures were approved by Institutional Animal Care and Use Committee of Temple University.

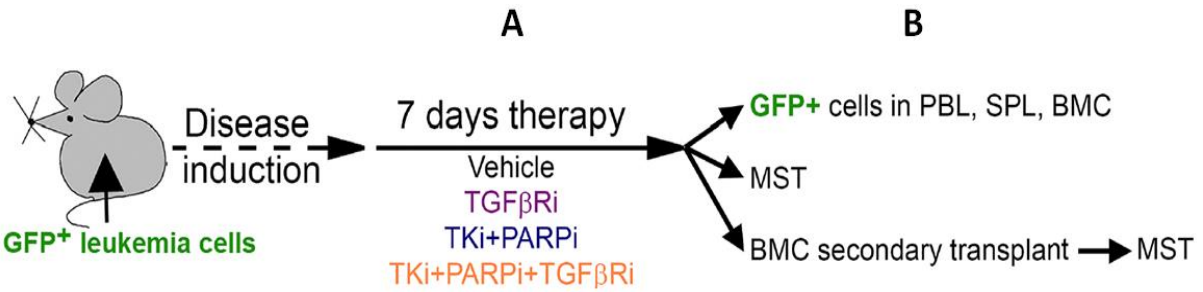

Figure 3.5. Design of *in vivo* treatment of TGFβRi SB435142 +/- [PARPi + TKi] in mice bearing GFP(+) murine primary CML/AML-like cells. **A**. Four groups of 7-day treatment regimen. **B**. Identification of engrafted leukemia cells in mice after treatment regimen and survival of leukemia-bearing mice. PBL: peripheral blood leukocytes, SPL: splenocytes, BMC: bone marrow cells, MST: median survival time.

3.10 Data statistical analysis

All methodologies were designed at least in three independent experiments. The Mean and Standard Deviation were calculated by Microsoft Excel from raw data and graphs were made in SigmaPlot version 11.0 delivered by Temple University. P-value was calculated by student t-test tool of SigmaPlot. Data is considered statistical significance once p-value is lower than 0.05.

4. RESULTS

4.1 Inhibition of the FLT3(ITD) oncogenic receptor elicits the possibility of PARPi–induced synthetic lethality in FLT3(ITD)–positive leukemia cells

PARP inhibitors (PARPi)-induced synthetic lethality has been effectively applying to eradicate *BRCA*–mutated cancers such as breast/ovarian tumor cells. As in general, leukemias have not been directly associated with *BRCA1/2* mutagenesis, it was obviously questionable to propose PARPi to eliminate leukemia cells. However, our recent studies have reported that expression of oncogenic BCR-ABL1 led to down-regulation of BRCA1 in chronic myeloid leukemia (CML) cells. Moreover, leukemia samples from patients containing BRCA deficiency were sensitive to PARP inhibitors (olaparib and talazoparib) *in vitro* and *in vivo* [2, 176], suggesting that leukemias should be considered as cancers with possible BRCA deficiency and sensitivity to PARP inhibitors. Moreover, the current study is supported by studies in which inhibition of another oncogenic tyrosine kinase associated with hematopoietic malignancies – JAK2 by JAK1/2 inhibitor (ruxolitinib) triggered PARPi-induced synthetic lethality in JAK2(V617F)–positive myeloproliferative neoplasm (MPN) cells [177]. Taking into account the mutation in FLT3 cell membrane tyrosine kinase [FLT3(ITD)], which also belongs to the oncogenic tyrosine kinases (OTKs) are related to leukemias [e.g. acute myeloid leukemia (AML)], we hypothesized that FLT3(ITD) oncogenic receptor inhibitor (AC220/quizartinib) could be exploited to sensitize FLT3(ITD)–positive leukemia cells to PARP inhibitors.

4.1.1 Inhibition of FLT3(ITD) receptor induces “BRCAness” phenotype revealing down-regulation of DSB repair proteins in FLT3(ITD)–positive leukemia cells

To determine whether inhibition of FLT3(ITD) oncogenic receptor is associated with down-regulation of DSB repair proteins (Figure 4.1A), FLT3(ITD)-positive BaF3 cells and their parental counterparts were treated with FLT3i (AC220), which selectively targets oncogenic FLT3(ITD) and spares wild-type FLT3 receptor, for 24 hours in the presence of IL-3. The efficacy of AC220 against FLT3(ITD) was confirmed by decrease of phosphorylated STAT5 (pSTAT5) in FLT3(ITD) –positive counterparts (Figure 4.1B). After analysis of DSB repair protein expression level by western blot, down-regulation of proteins involved in HR (BRCA1, BRCA2, PALB2, RAD51) and D-NHEJ (LIG4) pathways of DSB repair but not B-NHEJ

(PARP1, LIG3) was observed in FLT3(ITD)-positive BaF3 cells treated with AC220 (Figure 4.1B). On the other hand, in the parental BaF3 cells, expression of these DSB repair proteins remained unaffected during AC220 treatment, confirming that AC220 did not exert effect on FLT3(WT) cells based on the unaffected pSTAT5.

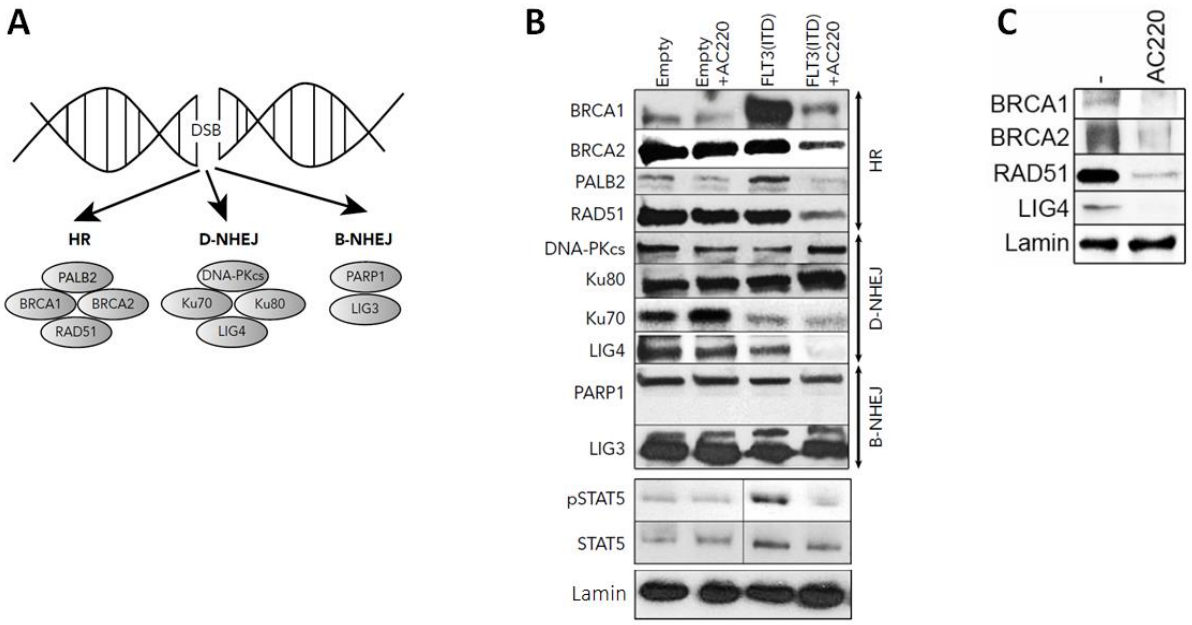

Figure 4.1. DSB repair protein analysis with/without treatment of AC220. **A**. Scheme of examined proteins in DSB repair pathways. **B**. Western blotting analysis of all proteins in parental and FLT3(ITD)-expressed BaF3 cells, untreated or treated with AC220. **C**. Western blot analysis of targeted proteins in primary FLT3(ITD)–positive leukemia cells from AML patient, untreated or treated with AC220. Lamin was used as a loading control.

After selection of five DSB repair proteins with down-regulation due to effect of AC220, we evaluated the efficacy of AC220 on human primary FLT3(ITD)–positive AML cells from patient sample. As expected, expression levels of BRCA1, BRCA2, PALB2, RAD51 and LIG4 were decreased in the malignant cells under treatment of AC220 (Figure 4.1C).

It is known that activation of FLT3(ITD) oncogenic receptor leads to transduction of downstream protein kinase effectors including JAK1/2-STAT5, PI3K/AKT and RAS/RAF/ERK [39]. To determine the impact of JAK1/2, PI3K and RAF1 protein kinase signaling pathways on expression of DSB repair proteins, inhibitors of JAK1/2, PI3K and RAF1 were added to the human primary FLT3(ITD)–positive AML cells for 24 hours, to verify alterations in the expression levels of the five investigated proteins (BRCA1/2, PALB2, RAD51, LIG4). The activity of three inhibitors was confirmed via decreased phosphorylation of STAT5, AKT and

MEK, respectively (Figure 4.2). BRCA1 down-regulation was observed under inhibition of JAK1/2, meanwhile inhibition of JAK1/2 and PI3K reduced expression levels of RAD51 and LIG4. RAF1i did not alter expression of investigated DSB repair proteins.

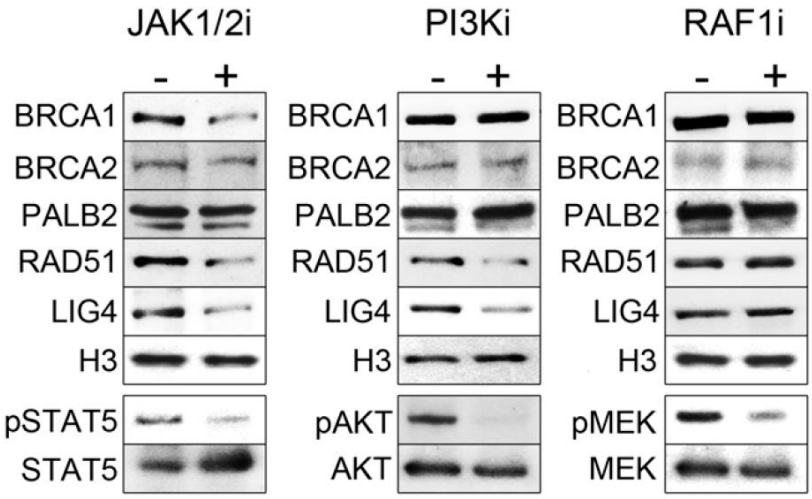

Figure 4.2. Western blotting analysis of targeted DSB repair proteins under inhibition of JAK1/2, PI3K and RAF1.

To validate the AC220–induced down-regulation of DSB repair proteins, we examined AC220-affected DSB repair activities of HR and D-NHEJ pathways in FLT3(ITD)–positive leukemia cell line MV-4-11 and FLT3(WT) leukemia cell line REH.

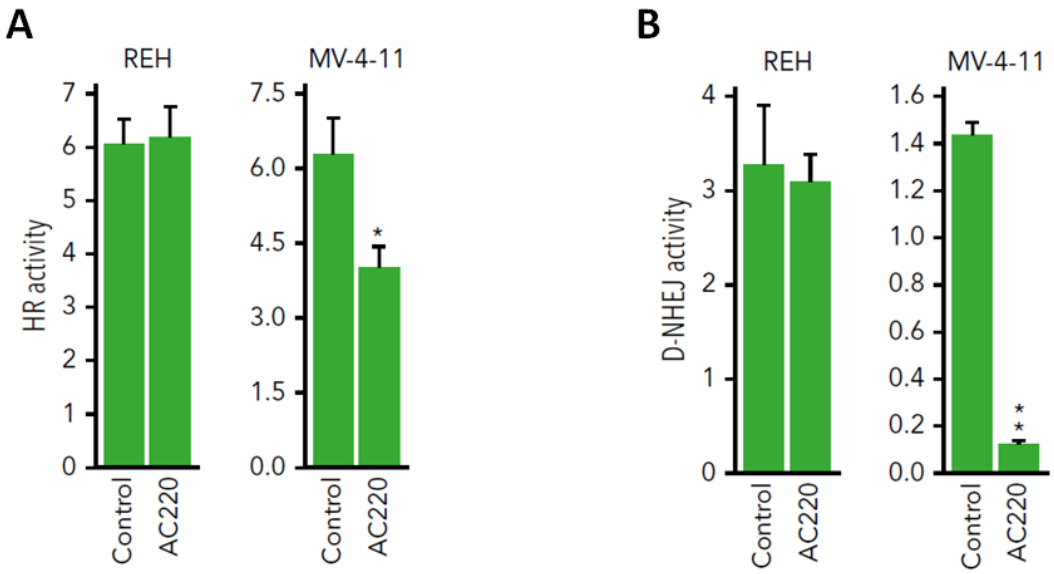

Figure 4.3. Measurement of AC220-affected DSB repair activities in FLT3(WT) leukemia REH and FLT3(ITD)–positive leukemia MV-4-11. **A**. DSB repair activity of HR pathway. **B**. DSB repair activity of D-NHEJ pathway. DSB repair activity was calculated as % of restored GFP(+) cells. * P = 0.02, ** P< 0.001.

To do this, two specific DSB repair pathways' reporter systems were used, in which the restoration of GFP expression was considered as DSB repair efficiency. We found that under the AC220, the repair activities of both DSB repair pathways in FLT3(ITD)–positive MV-4-11 leukemia cells were remarkably reduced, what was especially visible in D-NHEJ (Figure 4.3). On the other hand, HR and D-NHEJ repair activities remained unaffected in REH FLT3(WT) cells after incubation with AC220 (Figure 4.3). This data illustrates a convincing evidence supporting the AC220-dependent down-regulation of proteins and activities of HR and D-NHEJ in FLT3(ITD)–positive leukemia cells.

4.1.2 FLT3i sensitizes FLT3(ITD)–positive leukemia cells to PARPi–induced synthetic lethality

Results at Chapter 4.1.1 have shown down-regulation of proteins involved in HR and D-NHEJ pathways of DSB repair, together with reduction of DSB repair activities in HR and D-NHEJ pathways under inhibited efficacy of AC220 in FLT3(ITD)–positive leukemia cells. Therefore, theoretically, in those malignant cells with DSB repair defects in HR and D-NHEJ pathways, PARP1–dependent B-NHEJ pathway is required for survival. Therefore, inhibition of PARP1 in cells with activated FLT3(ITD) receptor should result in strong induction of lethal DSBs. From this, we determined the accumulation of DSBs by neural comet assay and elevated DSB response via detection of γ-H2AX [130] in FLT3(ITD)–positive leukemia cells, or FLT3(WT) HL-60 and REH cells, in the combinational treatment with FLT3(ITD) inhibitor and PARPi (BMN673) [PARPi + AC220], or upon with single treatments.

Combinational treatment with PARPi (BMN673) and AC220 for 24 hours induced remarkable accumulation of unrepaired DSBs (considered as percentage of tail DNA versus total cell DNA as described in Materials and Methods) in comparison with individual AC220 or BMN673 treatments. This also caused elevated nuclear DSB response verified by the presence of marker protein - γ-H2AX in FLT3(ITD)–positive MV-4-11 cells but not in FLT3(WT) leukemia REH and HL-60 cells (Figure 4.4A and Figure 4.4B). Furthermore, as consistency, the enhancement of DSB accumulation and response coincided with increased cell death of MV-4-11 incubated with [BMN673 + AC220] for 96 hours (Figure 4.4C).

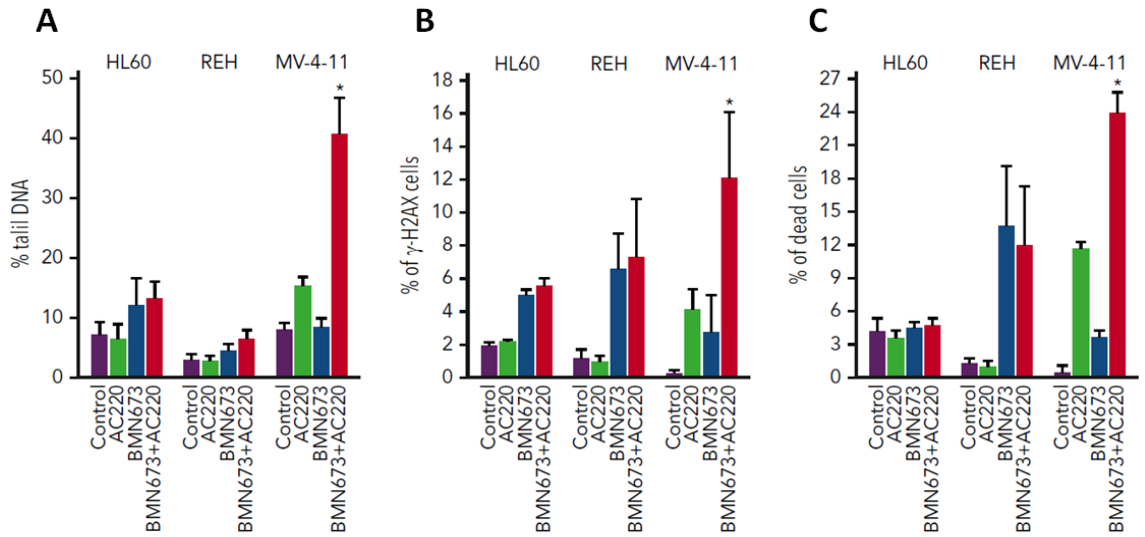

Figure 4.4. Effect of AC220 on accumulation of PARPi-induced DSBs in FLT3(ITD)-positive leukemia cell line MV-4-11 and FLT3(WT) leukemia cell lines REH and HL-60, treated as indicated. **A**. DSBs measurement by neutral comet assay. % tail DNA was calculated as ratio of tail DNA on total cell DNA. **B**. DSB response detection via γ-H2AX. **C**. Cell death analysis by detection of Annexin-V-positive cells. * $P < 0.05$.

To further determine the contribution of FLT3(ITD) inhibition by AC220 to the effectiveness of PARPi against FLT3(ITD)–positive leukemia cells, at first, we used the model cell lines including FLT3(ITD) and FLT3(WT) BaF3 together with parental counterparts which were treated with two different PARP inhibitors (olaparib or BMN673) +/- AC220, followed by analysis of cell survival by Tryphan blue viable cell counting.

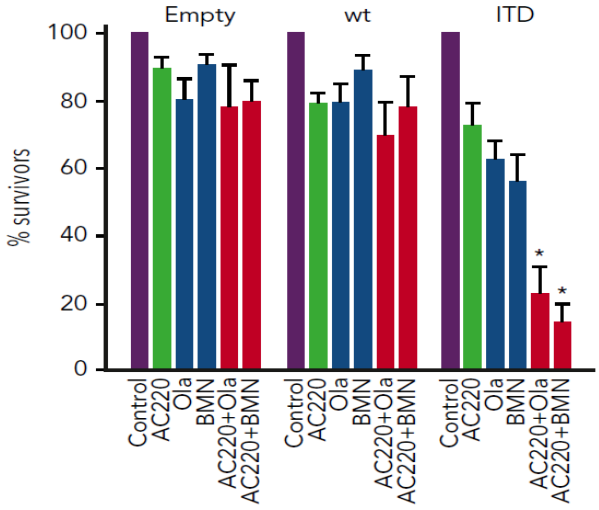

Figure 4.5. AC220 enhanced efficacy of PARP inhibitors against FLT3(ITD)–positive BaF3 but not BaF3 parental and FLT3(WT) BaF3 cells. Survival cells were counted by tryphan blue in automate cell counter and number of survival cells in control group was considered 100%. * $P < 0.05$.

We observed that individual treatment with PARP inhibitors or AC220 did not reduce cell survival in FLT3(WT) and parental cells while a modest but not significant decrease in cell survival was observed in FLT3(ITD)-positive BaF3 cells. Whereas remarkable anti-leukemia effect was obtained by combinational treatment of [AC220 + PARPis] selectively in BaF3 cells with expression of oncogenic FLT3(ITD) (Figure 4.5). Such effect was observed for both PARP inhibitors (olaparib and BMN673).

Besides using cell line expressing FLT3(ITD), the second examined model was murine primary cells obtained from mice expressing FLT3(ITD)$^{+/-}$, obtained by us by crossing ordered FLT3(ITD)$^{+/+}$ with FLT3^{WT} mice. To test the effect of combinational treatment of AC220 and PARPi, Lin^{-}Sca1^{+}cKIT^{+} bone marrow cells from FLT3(ITD)$^{+/-}$, FLT3(ITD)$^{+/-}$, and FLT3^{WT} mice were incubated with [AC220 +/- olaparib], followed by γ-H2AX immunostaining and plating in methylcellulose. As consistent to the results obtained before of MV-4-11 cells, the combinational treatment of AC220 and olaparib resulted in enhanced accumulation of DSB response marker γ-H2AX in FLT3(ITD)$^{+/+}$ and FLT3(ITD)$^{+/-}$ cells but not in FLT3^{WT} cells (Figure 4.6A).

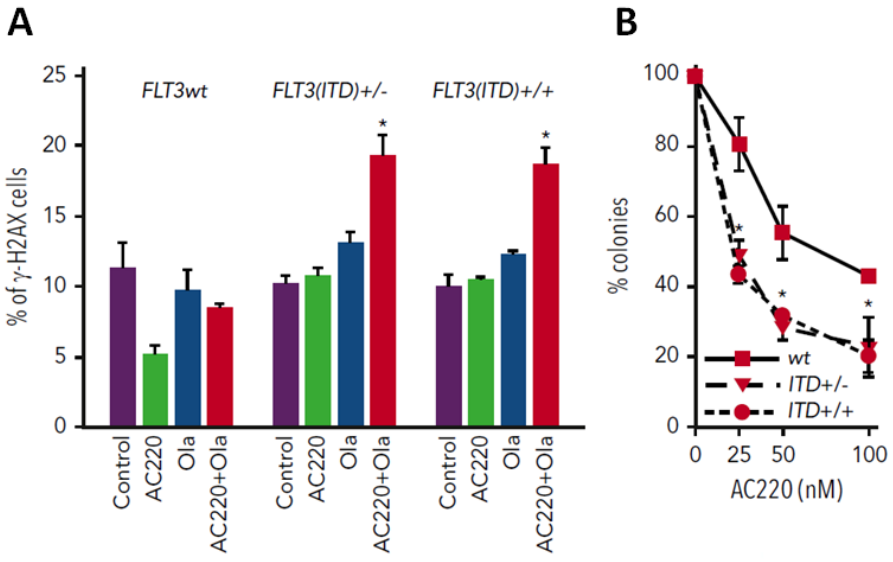

Figure 4.6. AC220 sensitized both murine primary FLT3(ITD)$^{+/-}$ and FLT3(ITD)$^{+/+}$ cells but not FLT3^{WT} counterparts to PARPi. **A**. AC220 causes accumulation of γ-H2AX in FLT3(ITD)$^{+/-}$ and FLT3(ITD)$^{+/+}$ primary cells. **B**. AC220 in a dose dependent-manner reduced colony number of PARPi (olaparib)–treated FLT3(ITD)$^{+/-}$ and FLT3(ITD)$^{+/+}$ primary cells. * $P < 0.05$.

Moreover, clonogenic test of PARPi–treated FLT3(ITD)$^{+/+}$ and FLT3(ITD)$^{+/-}$ cells showed a dose–dependent reduced clonogenic properties after treatment with 25, 50 and 100 nM

AC220 in comparison with PARPi–treated FLT3^{WT} cells (Figure 4.6B). Altogether, the potent anti-leukemia effect of combinational treatment with AC220 and PARPi does not depend on patterns of FLT3(ITD) genotypes between homozygosity and heterozygosity, and confirms the efficiency and potential of the combinational treatment.

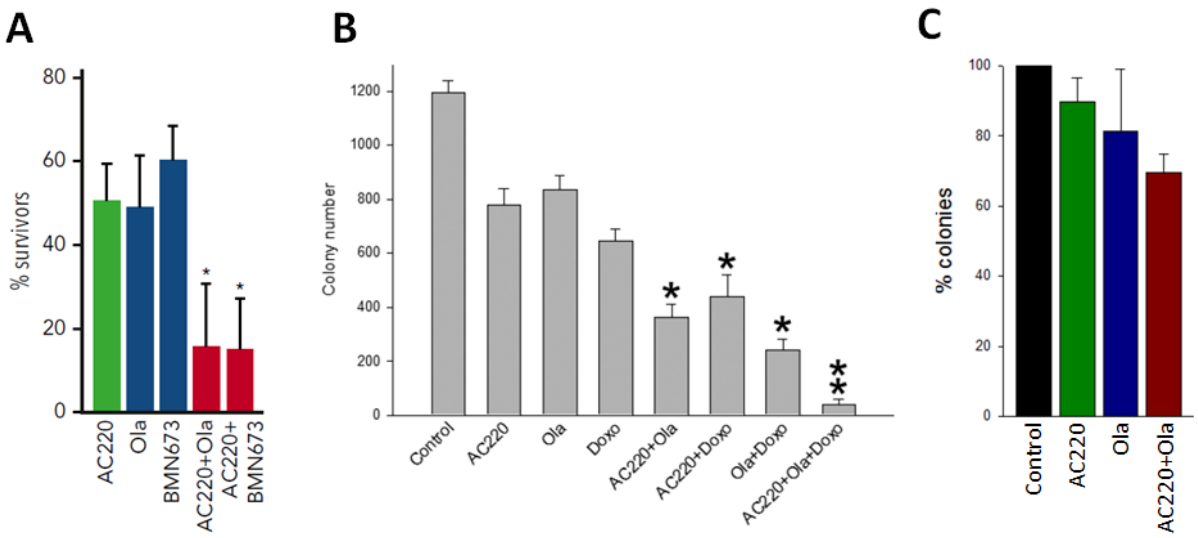

Figure 4.7. AC220 promoted sensitivity of FLT3(ITD)–positive AML patient cells to PARP inhibitors. **A**. AC220 enhanced efficacy of both olaparib and BMN673 against FLT3(ITD)–positive AML patient cells (3 patients). Survival cells were counted by Tryphan blue in automate cell counter and number of survival cells in control group was considered 100%. **B**. [AC220 + olaparib] plus Doxorubicin eradicated FLT3(ITD)–positive AML cells from patient samples (3 patients). Colony number indicated total colonies forming after treatment. **C**. [AC220 + olaparib] caused non-significant cytotoxic effect on hematopoietic samples from healthy donors (3 individuals). % colonies was calculated as total number of colonies in control group as considered 100%. * $P < 0.02$, ** $P < 0.001$.

The last and also the most important leukemia model were human primary FLT3(ITD)–positive cells obtained from AML patients. As results presented before in Figure 4.1C have shown that AC220 caused down-regulation of BRCA1, BRCA2, PALB2, RAD51 and LIG4 in human primary FLT3(ITD)–positive AML cells, the combinational treatment of AC220 with PARP inhibitors (olaparib/BMN673) dramatically eliminated number of survival human Lin$^-$ CD34$^+$ FLT3(ITD)–positive AML cells, as expected (Figure 4.7A). Similarly as before, the individual treatment with AC220/Olaparib/BMN673 only moderately reduced viable cells (Figure 4.7A). Moreover, with addition of DNA topoisomerase II inhibitor (doxorubicin), which has been commonly being utilized as a chemotherapeutic agent for AML patients, AC220 also enhanced efficacy of doxorubicin. Furthermore, combination of [AC220 +

olaparib + doxorubicin] eradicated almost completely the total clonal number of human Lin^-CD34^+ FLT3(ITD)–positive AML cells (Figure 4.7B).

Combinational treatment of AC220 with PARPi exerts great anti-leukemia effect on FLT3(ITD)–positive leukemia cells. However, to verify whether this strategic therapy is possible to enter clinical trials, we examined the cytotoxic effects of treatment on Lin^-CD34^+ cells obtained from healthy donors. We observed that AC220 did not significantly enhance efficacy of olaparib, meanwhile, as expected, AC220 or olaparib incubated individually did not induce cytotoxicity on normal hematopoietic cells (Figure 4.7C), suggesting [AC220 + PARPi] therapy is totally capable for entering clinical demonstration as a precision medicine.

4.1.3 FLT3i enhances anti-leukemia effect of PARPi (BMN673) *in vivo*

AC220 has been shown to enhance effectiveness of PARPi to eradicate FLT3(ITD)–positive leukemia cells *in vitro*, therefore, it was essential to test the FLT3i *in vivo* in NOD.$Rag1^{-/-}$;γc^{null} (NRGS) mice engrafted with human FLT3(ITD)–positive leukemia from patients, which was described at Chapter 3.9.1.3 of Materials and Methods (Figure 3.4).

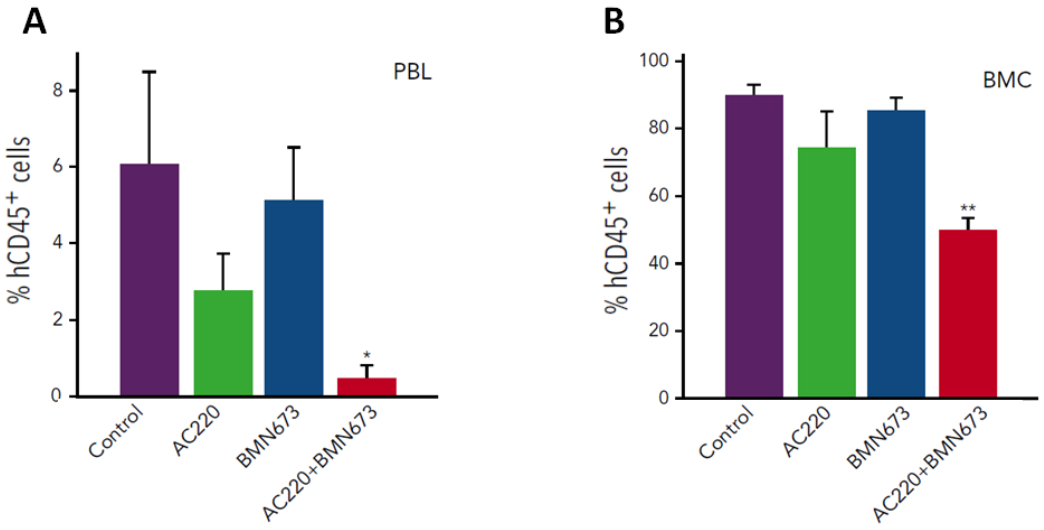

Figure 4.8. AC220 enhanced anti-leukemia effect of PARPi (BMN673) in peripheral blood leukocytes (PBL) (**A**) and bone marrow cells (BMC) (**B**) of leukemia-bearing mice. The percentage of human CD45(+) cells was estimated by flow cytometry in PBL or BMC populations. * $P < 0.01$, ** $P = 0.002$.

Human CD45 cell surface antigen was considered in peripheral blood leukocytes (PBL) and bone marrow cells (BMC) populations as marker for the engraftment efficiency as well as human leukemia development in mice. We found that, combinational treatment with AC220

and BMN673 significantly reduced the percentage of hCD45(+) cells PBL (Figure 4.8A) and BMC (Figure 4.8B) in comparison with individual treatments. Moreover, as consistent to reduction of hCD45(+) cells in PBL and BMC under [AC220 + BMN673] treatment, the combinational usage of two inhibitors remarkably prolonged survival of leukemia–bearing mice after initial engraftment and treatment (Figure 4.9A). Importantly, after secondary transplantation with cells derived from bone marrow of animals from initial treatment, mice engrafted with [AC220 + BMN673]–treated bone marrow cells were able to survive significantly longer than mice injected with cells from remaining groups (Figure 4.9B). This clearly showed that [AC220 + BMN673] combinational therapy is also capable to eliminate leukemia stem cells, which are responsible for self-renewal of leukemia, causing disease relapse after delaying treatment.

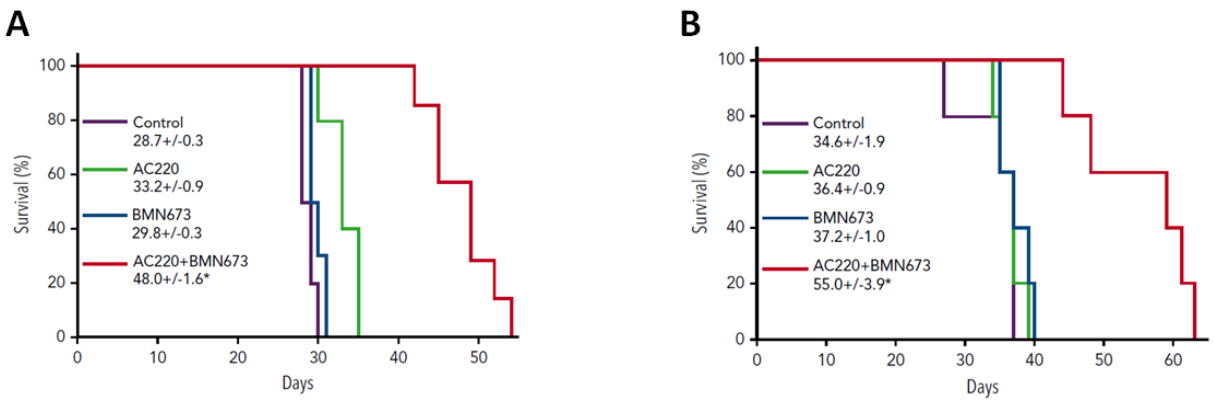

Figure 4.9. [AC220 + BMN673] prolonged survival of leukemia-bearing mice in initial transplantation after treatment regimen (**A**) and secondary transplantation using BMCs in mice from initial engraftment (**B**). * P < .002.

Altogether, the data obtained in this Chapter (4.1) show that although BRCA1 and BRCA2 are both proficient in FLT3(ITD)–positive leukemia cells, the inhibition of FLT3(ITD) oncogenic receptor by FLT3i (AC220) triggers PARPi-induced synthetic lethality in the leukemia cells *in vitro* and *in vivo*. The effectively synergistic therapy results from the fact that AC220 induced "BRCAness" phenotype due to rendering deficiencies of BRCA1, BRCA2, RAD51 and LIG4 in malignant cells.

4.2 Bone marrow microenvironment induces resistance to PARPi–mediated synthetic lethality in leukemias

The resistance phenomenon against PARPi–induced synthetic lethality has been observed and reported in both preclinical studies and clinical trials in *BRCA1/2*–mutated cancers and solid tumors via several mechanisms briefly described in Chapter 1.6.5 of Introduction. Although therapies in leukemia treatment have been exploiting advantages of PARPi to eliminate malignant cells based on leukemia–associated oncogenes or tyrosine kinase inhibitors causing deficiencies in BRCA1/2, the resistance against PARPi-mediated synthetic lethality in leukemias is unavoidable. Excluding all previously reported mechanisms inducing PARPi resistance, we proposed a completely novel mechanism of the resistance in leukemias mediated by the bone marrow microenvironment (BMM), with contribution of BM-derived stromal cells in hypoxia, where leukemia cells initiate, maintain and progress before spreading into bloodstream. In fact, in initially preliminary data, our group have recently verified the usage of PARPi in BMM to attenuate the BMM–dependent resistance against TKi (imatinib) in BCR-ABL1-positive CML cells [251] and observed that efficacy of PARPi was also limited in BMM compared to normal cultured condition. Therefore, it is quite paramount to investigate and understand the mechanism inducing resistance to PARPi in leukemia in BMM. To address this hypothesis, the advanced cell culture models were established to mimic the BMM in different conditions described in details in Chapter 3.3 of Materials and Methods.

4.2.1 Bone marrow microenvironment mediated by stromal cells attenuates efficacy of PARP inhibitors in leukemias

To test the impact of BMM on efficacy of PARPi-mediated synthetic lethality, we generated two *in vitro* cell cultured models to mimic BMM and PBM conditions, which were described in details in Chapter 3.3 of the Materials and Methods. Briefly, in the BMM condition, leukemia cells were co-cultured on the monolayer of epithelial (bone marrow stromal) cells in hypoxic condition (1% oxygen) (Figure 3.1). Whereas, in the PBM, leukemia cells was maintained as suspension cells in medium with sufficient oxygen intake (17%).

In term of examined leukemia cells, previously identified BRCA1/2-deficient leukemias (e.g., BCR-ABL1–positive BRCA1-deficient CML, AML1-ETO–positive BRCA2-deficient AML and

BRCA1/2-deficient AML [1, 2, 176]) and FLT3(ITD);TET2mut-positive AML (TET2mut was associated with BRCA1-deficiency and enhanced sensitivity to DNA damaging agents [267]) were incubated in BMM or PBM with PARPi (olaparib). To evaluate susceptibility of leukemias to olaparib in BMM, HS-5/OP-9 cell line was co-cultured with human/murine leukemias, respectively, in hypoxic condition to establish BMM.

We found that in PBM, as consistency with previous results and distinction of PARPi-mediated synthetic lethality, malignant cells were sensitive to olaparib in a dose–dependent manner (Figure 4.10A). Conversely, in BMM, leukemia cells became refractory to PARPi in every olaparib concentration (Figure 4.10A). In terms of human normal hematopoietic cells, cells did not respond to olaparib and there was no significant difference between PBM and BMM with HS-5 as stromal cells, as expected (Figure 4.10B).

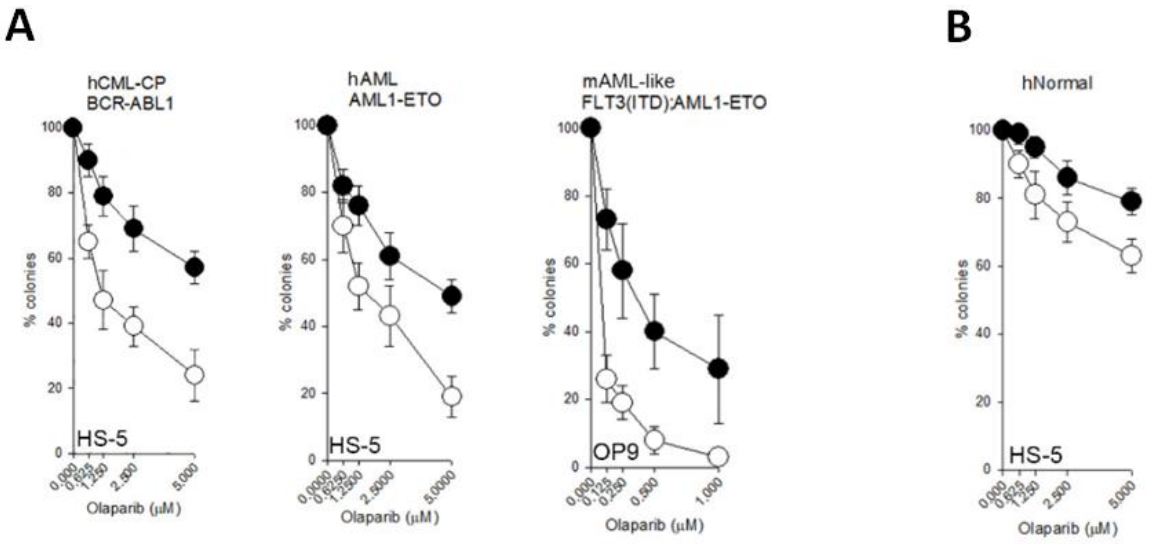

Figure 4.10. Resistance to PARPi (olaparib) in BRCA–deficient leukemias was obtained in BMM constituted by stromal cell lines in hypoxia (black circle) in comparison with sensitive effect of PARPi in PBM (white circle). **A**. Testing olaparib sensitivity between BMM and PBM in BRCA–deficient leukemias. HS-5 or OP9 stromal cells were used in co-cultures as indicated. $P < 0.05$. **B**. Effect of olaparib in normal human $CD34^+$ cells in BMM and PBM. $P > 0.05$.

Since we have observed that the BMM-like stromal cell lines induced resistance against olaparib in leukemias, it would be more reliable and convincing to test efficacy of olaparib in BMM constituted by primary stromal cells in hypoxia. In this case, allogeneic BMM was established from human mesenchymal stromal cells purchased from StemCell Technologies and autologous BMM was accomplished from stromal cells isolated from leukemia-

engrafted mice or leukemia patient aspirated bone marrow specimens. In terms of leukemias, again, human BCR-ABL1–positive BRCA1-deficient CML cells or human FLT3(ITD);TET2mut and murine FLT3(ITD);TET2-/- AML–like cells were used. We have confirmed that, similarly to BMM containing stromal cell lines, leukemia cells were refractory to olaparib in both, allogeneic and autologous BMM conditions (Figure 4.11).

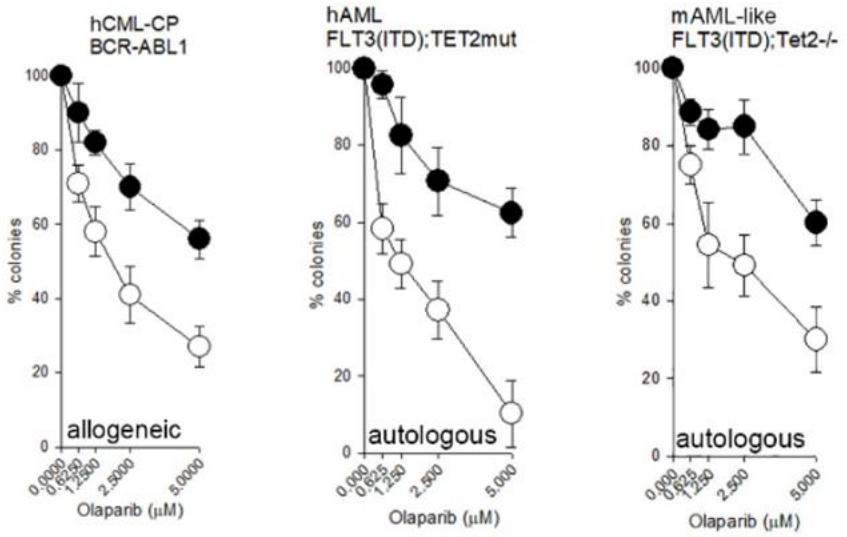

Figure 4.11. Resistance to PARPi (olaparib) in BRCA–deficient leukemias was obtained in BMM constituted by primary stromal cells in hypoxia (black circle) in comparison with potent effect of PARPi in PBM (white circle). $P < 0.05$.

Talazoparib is the second generation of PARPi with the best active PARPs trapping, which has been already approved by FDA. It has also been applied in a clinical trials with approximately 100 times higher potency than the first generation PARPi (olaparib) [162, 163]. Therefore, testing efficacy of talazoparib on BRCA–deficient leukemias in BMM is essential since we observed resistance to olaparib within BMM.

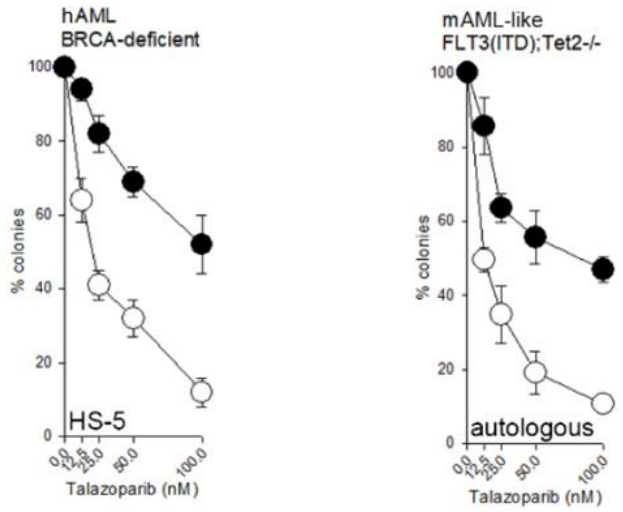

Figure 4.12. Resistance to PARPi (talazoparib) in BRCA–deficient leukemias was obtained in BMM (black circle) in comparison with potent effect of PARPi in PBM (white circle). $P < 0.05$.

Thus, human BRCA-deficient AML cells were treated with talazoparib in HS-5 cell line-mediated BMM or murine FLT3(ITD);TET2-/- AML–like cells were examined in autologous BMM as described earlier. Similarly to olaparib, both BMM conditions protected malignant cells from efficacy of talazoparib (Figure 4.12). This data also showed that resistance to PARPi induced by stromal cells–mediated BMM does not depend on categories of PARP inhibitors, as no difference was seen between PARPs catalytic inhibition (olaparib) and PARPs trapping (talazoparib).

Resistance to PARPi–induced synthetic lethality has been described to associate with restoration of HR pathway of DSB repair, making malignant cells no longer dependent on PARP1 to survive [180, 181, 183]. In our study, BMM, which is established by stromal cell lines or primary stromal cells, has been observed to cause resistance to PARPi, thus, we hypothesized that BMM promotes DSB repair efficiency in leukemia cells to reduce PARPi–induced DSBs. To investigate accumulation of DSBs induced by [PARPi +/- TKi], we conducted neutral comet assay, in which acquired DSBs in cells were lysed with agarose and DNA electrophoresis was performed to measure DSBs as percentage of tail DNA versus cell DNA (described at Chapter 3.4.3 of Materials and Methods). In terms of leukemia model, we used $Lin^{-}KIT^{+}$ (stem cells-like phenotype) primary cells obtained from mice model of AML, selected based on FLT3(ITD);Tet2-/- AML–like genotype.

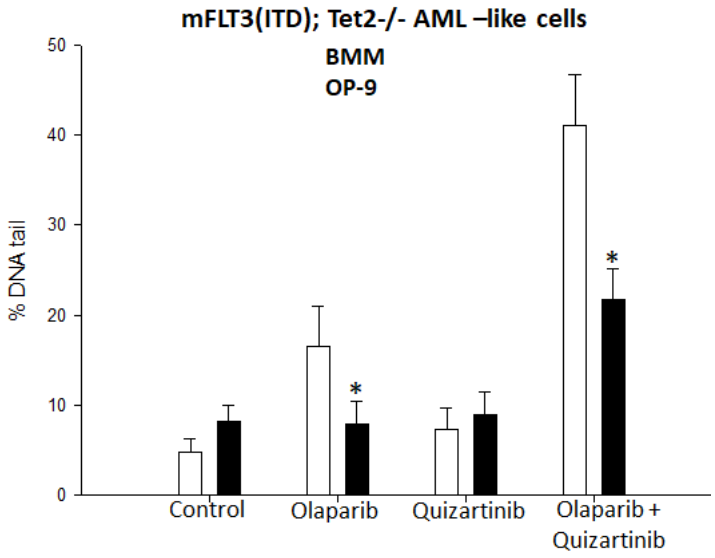

Figure 4.13. BMM (black bars) reduced accumulation of [olaparib +/- FLT3i (AC220/quizartinib)]–induced DSBs in BRCA –deficient leukemia cells occurring in PBM (white bar). For BMM conditions, murine leukemia cells were co-cultured with OP9 stromal cells. * $P < 0.05$.

We found that accumulation of olaparib–induced DSBs detected in PBM was considerably higher than the counterparts in the BMM conditions containing stromal OP-9 cells (Figure 4.13), which corresponds to efficacy of PARPi. Moreover, FLT3i (AC220/quizartinib), which has been shown earlier to cause DSB repair defects in FLT3(ITD)–positive AML cells (presented in 4.1.1), when used in combination with olaparib in PBM, dramatically enhanced DSBs in comparison with individual olaparib incubation. On the contrary, even with the participation of the FLT3i, accumulation of DSBs in BMM was remarkably reduced compared to PBM (Figure 4.13).

4.2.2 Bone marrow stromal cells–derived factors/cytokines play a major role in PARPi resistance in leukemias

After confirming the protective effect of BMM containing either stromal cell lines or primary stromal cells, against PARPi-mediated synthetic lethality, the following critical step was to identify the component in the BMM that mediates the resistance and is involved in the stroma-leukemia direct contact and stromal cells-derived factors/cytokines. To mimic BMM conditions and to separate two ways of intercellular communication including 1) the direct contact between stromal cells-leukemia cells and 2) the indirect BM stromal cells–derived

signaling, the standard BMM with co-culture of leukemia and stromal cells was named the BMM-direct contact (BMM-dc) and the BMM condition preventing the direct interaction between stromal cells and leukemia cells by using transwell cell culture insert system (0.4 µm cell culture insert), was considered BMM-indirect contact (BMM-idc). The tested leukemia cells including human BCR-ABL1-positive BRCA1–deficient CML and human/murine FLT3(ITD);TET2mut/knock-out AML were treated with PARPi (olaparib) in either both types of BMM conditions as described above or PBM condition as a control.

We observed that in BMM-dc, as expected, malignant cells were refractory to olaparib when compared to PBM. Moreover, leukemia cells maintained in BMM-idc did not respond to olaparib and there was no significant difference in survival observed as percentage of cell colonies between two BMM conditions (Figure 4.14A), suggesting that stromal cells–derived secretory factors/cytokines are the major mediators of resistance against PARPi in leukemia cells in BMM. Furthermore, to comprehensively validate the contribution of secreted stromal cells-derived factors/cytokines, we used the BMM-conditioned medium (BMM-cm) obtained from stromal cells by method described in Chapter 3.3.1.4. In this case, BMM-idc was replaced by BMM-cm and the obtained result corresponded with the data in the previous experiment comparing the BMM-dc and BMM-idc (Figure 4.14B) (done by Dr. Paulina Podszywalow-Bartnicka, Nencki Institute).

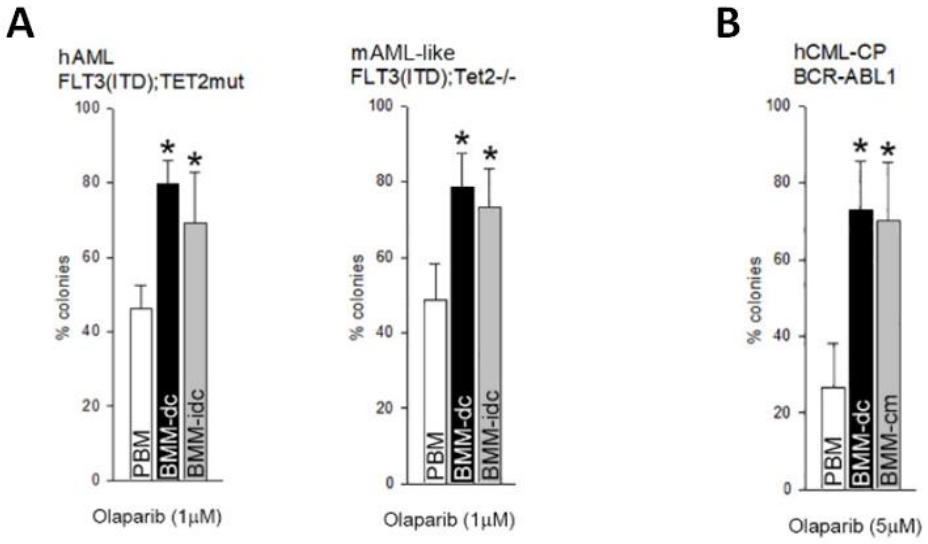

Figure 4.14. Comparison of olaparib efficacy on BRCA–deficient leukemias between BMM conditions with direct and indirect contact between leukemia and stromal cells (BMM-dc and BMM-idc, as indicated) (**A**) and influence of conditioned medium from stromal cells (BMM-dc and BMM-cm) (**B**). * $P < 0.05$.

Altogether, this data clearly confirmed that the bone marrow stromal cells–derived factors/cytokines play the major role in the BMM–dependent resistance to PARPi in leukemias.

The last essential approach, which verified the protective effect of the BMM against PARPi-induced synthetic lethality and validated the major function of stromal cells–derived factors/cytokines in the resistance, was the quiescent stem cell assay conducted in PBM, BMM-dc and BMM-idc conditions.

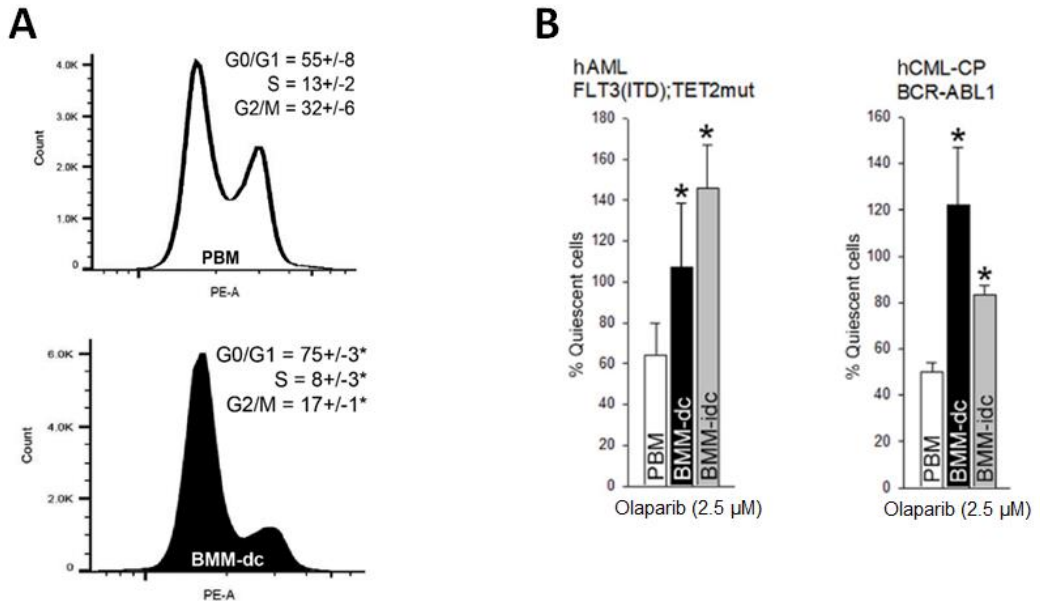

Figure 4.15. Quiescent stem cell assay in BMM versus PBM. **A**. Cell cycle analysis of leukemia cells cultured in PBM or BMM-dc. Mean distribution of cell cycle phases is indicated. **B**. survival of quiescent LSCs in PBM, BMM-dc and BMM-idc after treatment with olaparib. * $P < 0.05$.

Numerous studies on human hematopoietic malignancies have reported the resistance against chemotherapeutic/cytotoxic agents or TKis and disease relapse deriving from the quiescent leukemia stem cells (LSCs), characterized with the specific phenotype ($Lin^-CD34^+CD38^-CFSE^{max}$), and residing in the bone marrow (reviewed in [204]). Moreover, cell cycle analysis illustrated that our established BMM-dc condition maintained leukemia cells in the quiescent state, visible as increased percentage of cells in the G_0-G_1 phase (Figure 4.15A). Next, we performed the quiescent stem cell assay (described in Chapter 3.4.5 of Materials and Methods). The total survival of $Lin^-CD34^+CD38^-CFSE^{max}$ cells was analyzed by flow cytometry using cell proliferation dye (CFSE) and fluorchrome-conjugated primary

antibodies of remaining markers, in two human leukemias including BCR-ABL1–positive BRCA1–deficient CML and FLT3(ITD);TET2mut-positive AML. To be compatible with the earlier results in this Chapter, BMM-dc and BMM-idc induced $Lin^{-}CD34^{+}CD38^{-}CFSE^{max}$ cells refractory against olaparib, comparing with leukemia cells incubated in PBM. The number of $Lin^{-}CD34^{+}CD38^{-}CFSE^{max}$ cells found between two BMM conditions was not statistically different (Figure 4.15B), highlighting the significant role of the stromal cells–derived factors/cytokines in resistance to PARPi in BMM, that was acquired not only by proliferative LPCs, but also by quiescent LSCs.

Altogether, the data obtained in this Chapter (4.2) reveal that the BMM condition, established by involvement of BM stromal cells in hypoxia, induces the resistance to PARPi–mediated synthetic lethality in BRCA–deficient leukemia cells and BM stromal cells–derived factors/cytokines are prevalently responsible for this BMM-dependent resistance.

4.3 Molecular mechanism of the bone marrow microenvironment-dependent resistance to PARPi in leukemias

Results presented in Chapter 4.2 have shown that stromal cells–mediated BMM promotes the resistance to PARPi–induced synthetic lethality in leukemias via BM stromal cells–derived factors/cytokines. As described in the Introduction, in hematopoietic BM niche, CXCL12 and TGF-β have been reported the most abundant factors produced by stromal cells and TGF-β-induced signaling pathway has been associated with DSB repair as well as DNA damage response. Moreover, simultaneous inhibition of CXCL12– and TGF-β–mediated signaling pathways has been described to re-sensitize leukemias to chemotherapy in BMM [238, 240]. Therefore, we proposed that resistance against PARPi in BMM might be mechanistically supported by one or both of these signaling pathways.

4.3.1 CXCL12–CXCR4 signaling pathway does not induce resistance to PARPi in leukemias in the bone marrow microenvironment

The CXCL12–CXCR4 axis is the most well-known signaling pathway in BMM, that causes resistance against chemotherapy and TKi in leukemia cells (described in Introduction chapter 1.7.2.1). Moreover, abrogation of this pathway by CXCR4 inhibitor/antagonist has been reported to reverse the resistance. Therefore, we examined the effect of CXCR4

inhibitor/antagonist on the efficacy of PARPi in BMM as well as on the potency of TKi as a positive control, using human and mouse models of BRCA-deficient AML cells, co-cultured with stromal cell lines in hypoxic condition to mimic BMM-dc. On the other hand, we inhibited TGF-β receptor (TGFβR) by TGFβR inhibitor (SB431542), or blocked the cytokine by using TGF-β1 neutralizing antibody (1D11).

As expected, inhibition of CXCL12-CXCR4 pathway by CXCR4i (AMD3100) and CXCR4 antagonist (WZ811) re-sensitized human FLT3(ITD);TET2mut and murine FLT3(ITD);TET2-/- leukemia cells to TKi (quizartinib), as previously described [217]. However, it did not restore sensitivity of leukemia cells to PARPi (olaparib) in BMM (Figure 4.16). Meanwhile, TGFβR inhibitor (SB431542) and TGF-β1 neutralizing antibody (1D11) re-sensitized leukemia cells to both PARPi and TKi. However, simultaneous inhibition of CXCR4 and TGF-β signaling pathway did not enhance efficacy of both PARPi and TKi, suggesting a unique role of TGF-β1 - TGFβR pathway in the molecular mechanism of resistance to PARPi in leukemia cells in BMM.

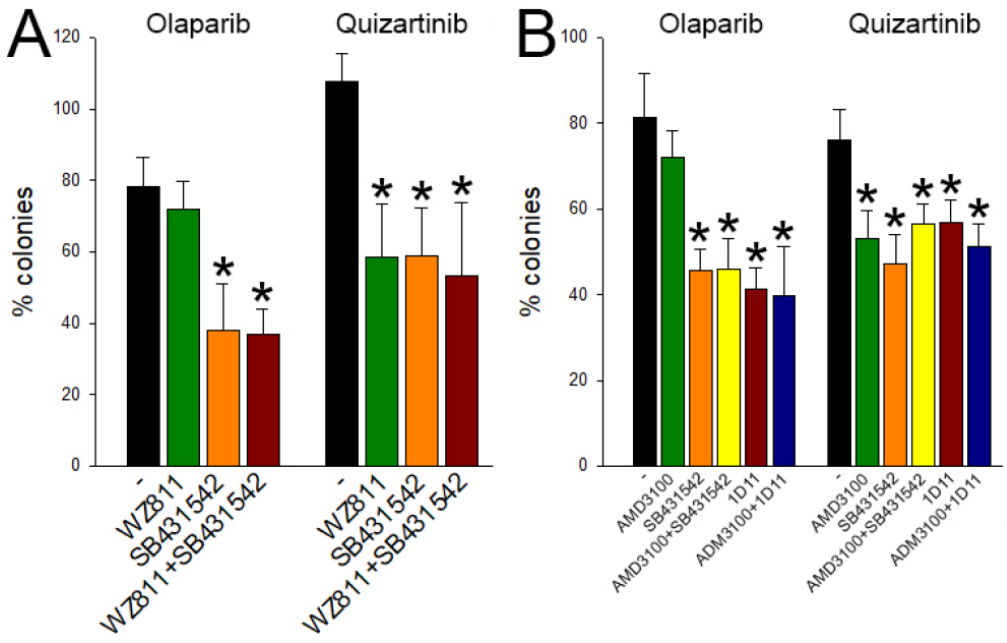

Figure 4.16. Determining role of CXCL12 CXCR4 and TGFβ1–TGFβR signaling pathways in resistance to PARPi of leukemia cells in BMM. **A**. Effect of CXCR4 antagonist (WZ811) +/- TGFβRi (SB435142) on human FLT3(ITD);TET2mut in HS-5–mediated BMM. **B**. Effect of CXCR4i (AMD3100) +/- TGFβRi [SB435142/TGF-β1 neutralizing antibody (1D11)] on murine FLT3(ITD);TET2mut in OP-9–mediated BMM. * $P < 0.05$.

4.3.2 TGFβ1–TGFβR signaling pathway is activated in the bone marrow microenvironment in leukemia cells

Besides the CXCL12-CXCR4 pathway, Chapter 1.7.2.2 of the Introduction reveals the important role of the TGF-β signaling pathway in promoting resistance to chemotherapy in leukemia cells in BMM, together with the regulatory function in DSB repair and DNA damage response of solid tumors. This enables the TGF-β signaling pathway as a potential mediator of resistance against PARPi in leukemia cells in BMM. Taken together, we hypothesized that the TGFβ1–TGFβR signaling might cause the PARPi resistance in BMM. The preliminary data presented earlier showing that abrogation of TGF-β signaling pathway by TGFβRi (SB431542) or TGF-β1 neutralizing antibody (1D11) restored sensitivity of leukemia cells to olaparib and quizartinib, supported our hypothesis. Thus, at first, we checked whether the TGF-β1 is produced in our established BMM models. Using the conditional media from cultured stromal cells in hypoxia and TGF-β1 ELISA test (done by Julian Swatler, Nencki Institute), we confirmed that TGF-β1 is produced and secreted by HS-5, OP-9 and primary stromal cells (Figure 4.17).

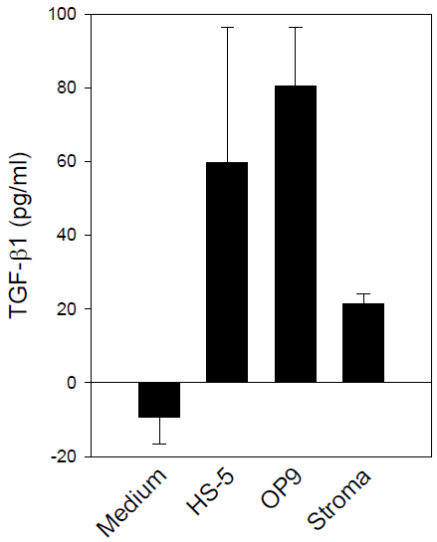

Figure 4.17. Measurement of TGF-β1 production in conditioned medium derived from bone marrow stromal human HS-5, murine OP9 cell lines or primary stromal cells by ELISA test.

Hematological malignancies induce extensive “remodeling” of the bone marrow niche and TGF-β1 is a major cytokine involved in the leukemic bone marrow niche “remodeling” process [237, 268, 269]. The above results confirmed that TGF-β1 is secreted by stromal cells in our established BMM. Moreover, by using immunofluorescence followed by flow

cytometry and western blot analysis, we assessed the expression levels of TGFβR2 kinase. We found that human $Lin^{-}CD34^{+}$ FLT3(ITD);TET2mut primary AML and BCR-ABL1–positive CML K562 cells, showing 2-3 fold higher level of TGFβR2 kinase in autologous/stromal cell line BMM-dc versus PBM (Figure 4.18A). This data shows that the BMM increases expression of TGFβR2 kinase in leukemic cells, what is not the case in PBM conditions.

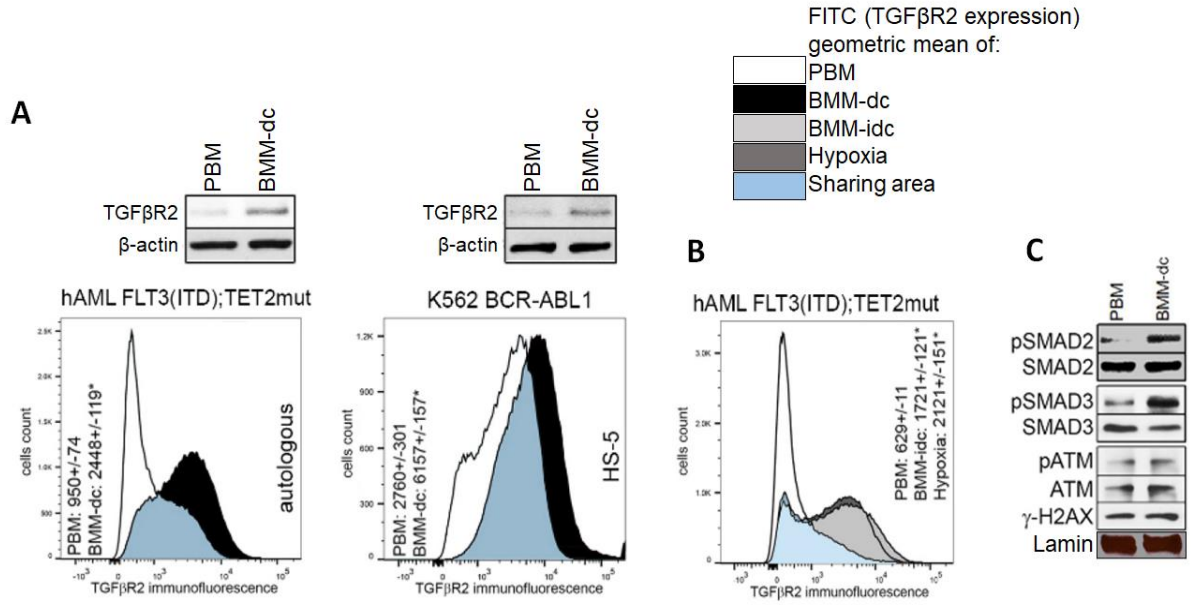

Figure 4.18. TGFβR2 overexpression and activation of TGF-β signaling pathway in BMM. **A**. TGFβR2 expression levels in PBM and BMM-dc detected by western blot and immunofluorescence followed by flow cytometry. Representative overlays of histograms are presented. **B**. TGFβR2 expression levels in PBM, BMM-idc and hypoxia measured by immunofluorescence followed by flow cytometric analysis. Representative overlay of histograms is presented. **C**. Validating activation of TGF-β signaling pathway via pSMAD2 and pSMAD3 without inducing DSB response between PBM and BMM-dc via ATM, phospho-ATM (pATM) and γH2AX. For fluorescence of FITC (TGFβR2 expression) geometric means are presented on the side of all histograms. For western blotting analysis, β-actin, and Lamin were used as loading controls. *P < 0.05.

More interestingly, the overexpression of TGFβR2 kinase was also found in BMM-idc and especially in hypoxic condition without stromal cells (Figure 4.18B). Additionally, the effect was triggered by hypoxia and was not associated with elevated DNA damage including DSBs (no increase of γ-H2AX, ATM and phospho-ATM detected) (Figure 4.18C) as reported before in epithelial cells [244]. Taken together, our data suggest that the hypoxia-induced TGFβR2 overexpression was stimulated by stromal cells-derived TGF-β1 to activate the entire TGFβ1-

TGFβR signaling pathway in BMM. The activation was confirmed by enhanced phosphorylated forms of SMAD2 and SMAD3 in BMM (Figure 4.18C).

4.3.3 Activated TGFβ1–TGFβR signaling pathway in the bone marrow microenvironment induces resistance to PARPi in leukemias

4.3.3.1 Genetic targeting TGFβR2 and TGF-β1 restores efficacy of PARPi in leukemia cells in the bone marrow microenvironment

To obtain the direct evidence that the TGF-β1 → TGFβR signaling pathway regulates efficacy to PARPi in BMM, TGFβR2 was genetically manipulated by CRISPR/Cas9 in BCR-ABL1–positive K562 cells. Because clonal selection was not conducted to obtain complete TGFβR2-negative cells, a few K562 clones maintained normal expression of TGFβR2. Therefore, we obtained down-regulation of TGFβR2 in K562 cells, which was confirmed by western blot (Figure 4.19A).

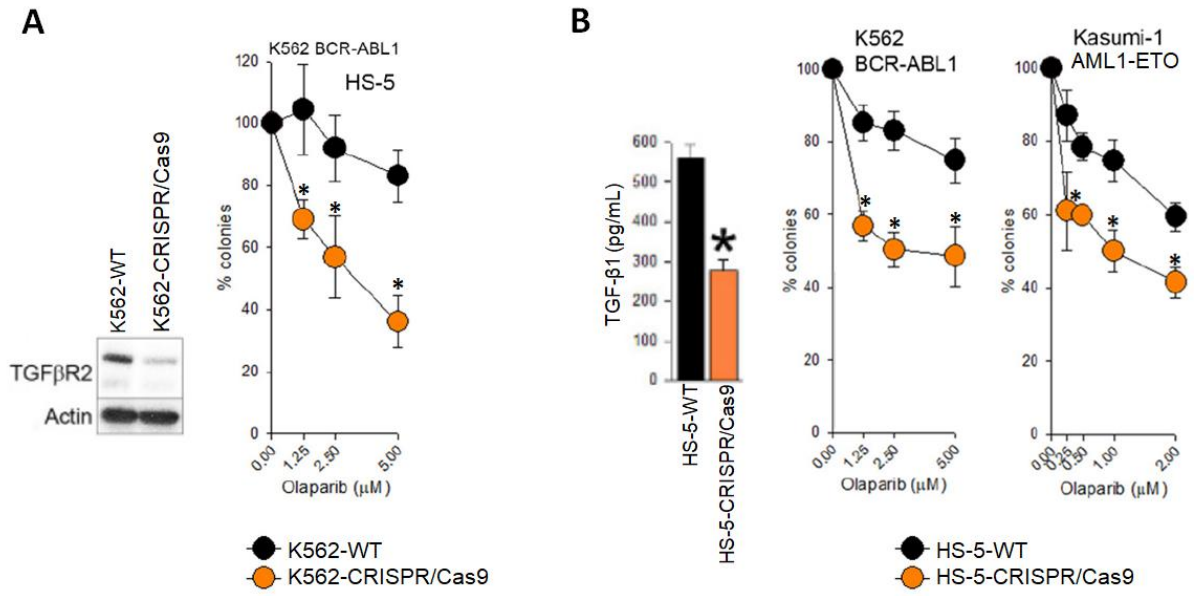

Figure 4.19. Genetic targeting TGF-β1 – TGFβR signaling pathway sensitized leukemia cells to PARPi in BMM. **A**. TGFβR2 down-regulation by CRISPR/Cas9 in BCR-ABL1–positive cell line K562 was confirmed by western blot. TGFβR2–downregulated K562 cells were eliminated by olaparib as estimated by colonies test. **B**. Targeting TGF-β1 by CRISPR/Cas9 in HS-5 cells was validated by reduction of TGF-β1 produced in BMM via ELISA. BRCA–deficient leukemias co-cultured with TGF-β1 –CRISPR/Cas9 HS-5 were sensitive to olaparib. WT: wild-type. * $P < 0.05$.

To verify sensitivity to olaparib, survival of colonies was estimated. We found that, BCR-ABL1–positive CML K562 cells with strongly decreased expression of TGFβR2 serine/threonine receptor kinase became sensitive to olaparib, meanwhile, as expected, K562-WT cells with normal expression of TGFβR2 kinase remained refractory against olaparib in BMM-dc (Figure 4.19A). This data highlights the importance of hypoxia–induced overexpression of TGFβR2 in TGFβ1–TGFβR pathway as crucial part of the PARPi resistance in BMM. Besides down-regulation of TGFβR2 expression on K562 CML cells, next we considered the *TGF-β1* gene in stromal cells as a genetic target to reduce stromal cells - derived TGF-β1 production in BMM. Thus, the *TGF-β1* gene in HS-5 cells was down-regulated by CRISPR/Cas9 (done by our collaborator, Dr. Jian Huang's laboratory, Temple University School of Medicine) and the reduced level of TGF-β1 ligand in BMM produced by CRISPR/Cas9 HS-5 cells was confirmed by ELISA (done by Julian Swatler, Nencki Institute) (Figure 4.19B). BCR-ABL1–positive K562 cells and AML1-ETO–positive Kasumi-1 cells were tested in BMM with HS-5 parental or HS-5 without expression of TGF-β1. We found that together with the decreased amount of TGF-β1 secreted in BMM, sensitivity of leukemias to olaparib was restored, while malignant cells co-cultured with parental HS-5 remained PARPi resistant (Figure 4.19B). Taken together, the data clearly illustrates that both, stromal cells–derived TGF-β1 and hypoxia–mediated overexpression of TGFβR2 kinase receptor on leukemic cells, are two indispensable components to trigger entire TGF-β signaling pathway in BMM, leading to resistance against PARPi in leukemia cells.

Additionally, to validate the PARP1-dependent survival of BRCA-deficient leukemia in functional and inactivated TGFβR-mediated signaling pathway in BMM, we utilized cells obtained from the PARP1 double conditional knock-out mice model. Thus, the murine FLT3(ITD);Tet2-/- and FLT3(ITD);Tet2-/-;PARP1-/- AML-like cells were incubated with TGFβR inhibitor (TGFβRi) as SB435142. To study the resistance, cell colony assay was performed as before. Consequently, the TGFβR kinase inhibitor was capable to eradicate cell colony number of PARP1–null leukemia cells in OP-9-mediated BMM but incapable in PARP1–proficient AML-like cells (Figure 4.20). This data emphasizes the importance of PARP1 for survival of BRCA –deficient leukemia once TGFβR is inhibited in BMM. In other words, it can be thought that targeting PARP1 together with impairment of the TGFβR–mediated signaling

pathway will eliminate survival of leukemia cells in BMM and might be considered as novel therapeutic strategy.

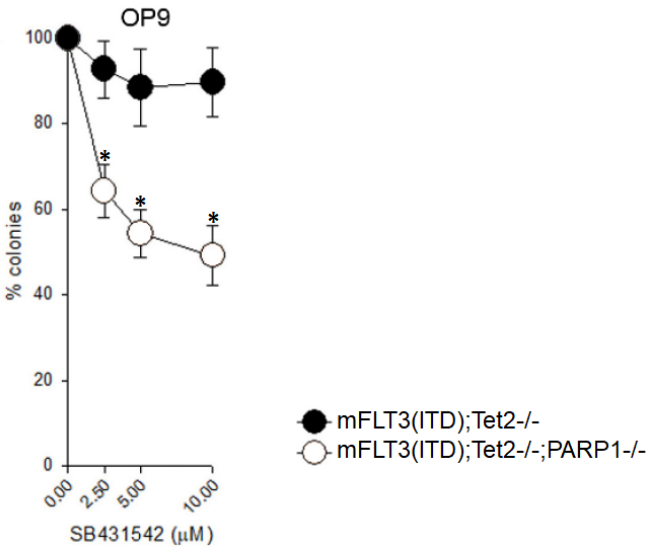

Figure 4.20. The interplay between PARP1 and TGFβR-mediated signaling pathway in BRCA–deficient leukemia in BMM. Cells were treated with different concentrations of TGFβR inhibitor (SB435142). * P< 0.05.

4.3.3.2 External recombinant TGF-β1 triggers resistance to PARPi in leukemia cells in hypoxia

We have shown before (Chapter 4.3.2) that the stimulation of stromal cells-derived TGF-β1 on hypoxia -induced overexpression of TGFβR2, activates the TGFβ1–TGFβR signaling pathway in BMM. Alternatively, to use a different approach and additionally confirm our findings, the TGF-β1 cytokine from human/murine recombinant protein (rTGF-β1) was externally added into medium in the absence of BM stromal cells. By this way, we wanted to confirm the importance of the stromal cells–derived TGF-β1 in supporting resistance to PARPi in BMM.

Activation of the TGFβR–mediated signaling pathway by addition of rTGF-β1 was detected by enhancement of phosphorylated forms of SMAD2 and SMAD3 (Figure 4.21B). We observed that, the presence of human rTGF-β1 (3ng/mL) protected human FLT3(ITD);TET2mut cells from elimination of [olaparib +/- quizartinib] in hypoxic condition. Whereas, without addition of the recombinant cytokine, as expected, the malignant cells were very sensitive to the drugs also in hypoxia (Figure 4.21A).

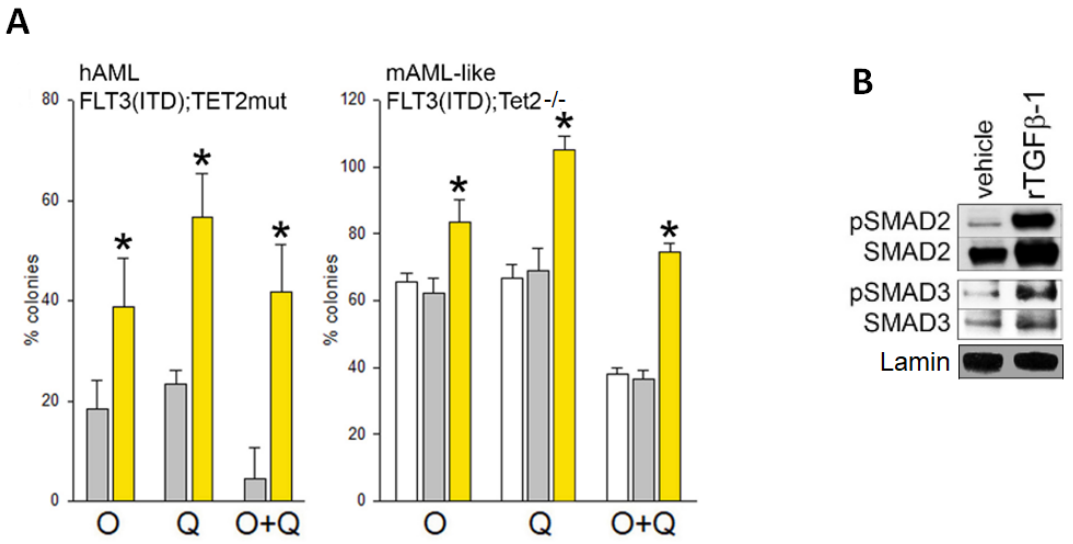

Figure 4.21. Supplement of external recombinant TGF-β1 (rTGF-β1) rescued BRCA–deficient leukemia cells from efficacy of [PARPi +/- TKi]. **A**. Clonogenic test of BRCA –deficient leukemia cells in normoxia (white bar), hypoxia (grey bar) and hypoxia with rTGF-β1 (yellow bar). **B**. Validation of rTGF-β1-dependent pathway activity by detection of pSMAD2/3 levels. Lamin was used as loading control. * $P < 0.05$. O: Olaparib, Q: Quizartinib.

Turning into murine FLT3(ITD);Tet2-/- AML-like cells, the same observation was achieved with involvement of the normoxic condition as another negative control (Figure 4.21A). This data, once again, validates the essential role of the stromal cells-derived TGF-β1 ligand in BMM, and confirms that without the TGF-β1 ligand, the resistance against PARPi-induced synthetic lethality is not effectively induced, even with overexpression of TGFβR2 kinase in hypoxia.

4.3.4 Pharmacological inhibition of TGF-β signaling pathway restores efficacy of [PARPi + TKi] against leukemias in the bone marrow microenvironment

4.3.4.1 TGFβR kinase inhibitor (SB431542) restores efficacy of [PARPi +/- TKi] in leukemias only in the bone marrow microenvironment

The TGFβ1–TGFβR signaling pathway has been shown above as the major component that induced resistance to PARPi in leukemia cells in BMM. We presented that reduced secretion of TGF-β1 from bone marrow stromal cells and down-regulation of TGFβR2 in leukemia cells by CRISPR/Cas9 re-sensitized malignant cells to PARPi (olaparib). Also, pharmacological inhibition of the TGFβ serine/threonine receptor kinase by small molecule inhibitors (SB431542 or clinical trials-approved galunisertib) was demonstrated to recover efficacy of

PARPi in BRCA–deficient leukemia cells. Furthermore, we have described above that quizartinib (AC220)-treated FLT3(ITD)-positive AML cells and previously reported imatinib-treated BCR-ABL1-positive CML cells displayed “acute” deficiencies in HR and D-NHEJ and were highly sensitive to PARPi-triggered synthetic lethality [178].

In the next step we found that the combinational treatment with PARPi (olaparib) +/- TKi (quizartinib, imatinib) reduced the clonogenic survival of human $Lin^{-}CD34^{+}$ (stem cells-like phenotype) FLT3(ITD);TET2mut-positive AML cells and BCR-ABL1–positive CML K562 cells in PBM. As predicted, TGFβRi (SB431542) did not alter the sensitivity in PBM, because TGFβ1–TGFβR signaling pathway is low/not activated, and therefore, SB435142 did not affect the level of phosphorylated SMAD2 (Figure 4.22A).

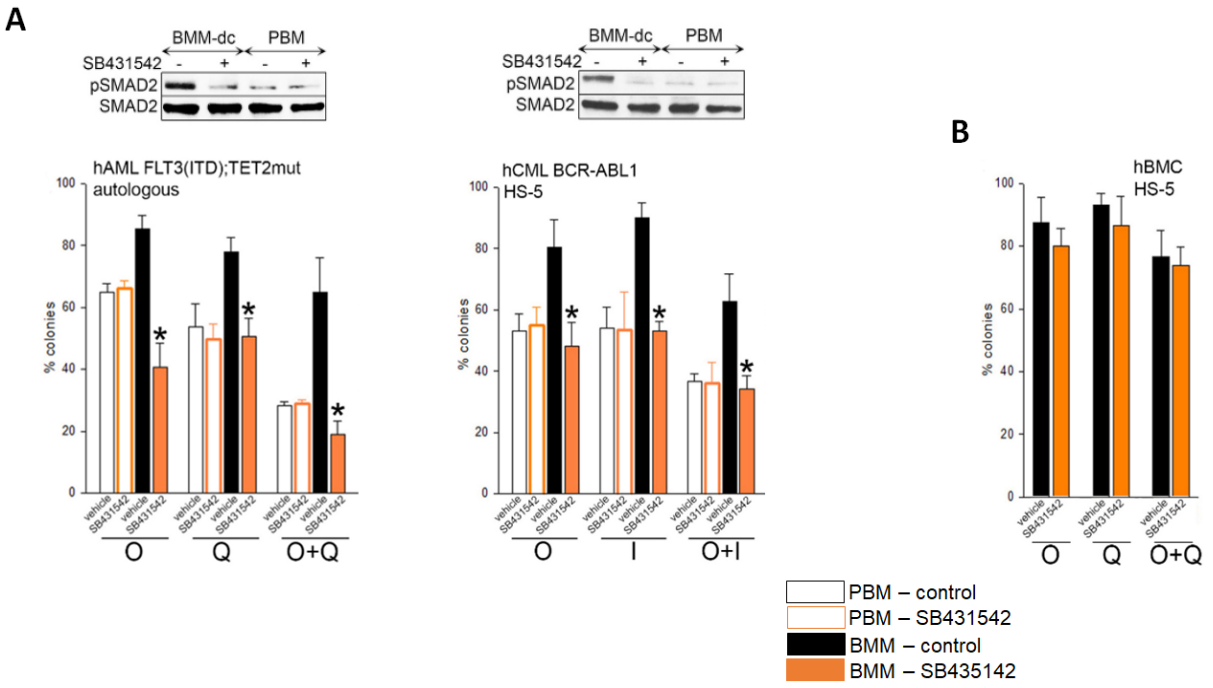

Figure 4.22. Pharmacological inhibition of TGFβR-mediated signaling pathway by TGFβR kinase inhibitor (SB431542) restored efficacy of PARPi against BRCA–deficient leukemias only in BMM (**A**) and did not cause cytotoxicity to normal human bone marrow cells (hBMC) in BMM (**B**). Inhibition of TGFβR-mediated signaling by SB431542 was verified by analysis of pSMAD2 level by western blot in PBM or BMM conditions. O: Olaprib, Q: Quizartinib, I: Imatinib. * $P < 0.05$.

On the other hand, in BMM, leukemia cells expectedly remained refractory to [PARPi +/-TKi], however, inhibition of TGFβR kinase by SB431542 (validated by down-regulation of

phosphorylated SMAD2) dramatically re-sensitized malignant cells to [PARPi +/- TKi] (Figure 4.22A). Notably, SB431542 did not enhance the cytotoxic efficacy of [PARPi +/- TKi] to Lin^-CD34^+ bone marrow cells from healthy donors (Figure 4.22B). Altogether, this data suggests that TGFβRi is only capable to inhibit activated TGFβ1–TGFβR signaling pathway in BMM because of the involvement of the stromal cells-derived TGF-β1 and overexpression of TGFβR2 in hypoxic condition. More importantly, without enhanced cytotoxicity of [PARPi +/- TKi] on human Lin^-CD34^+ normal hematopoietic cells even in BMM conditions, TGFβR kinase inhibitor is potentially possible to enter clinical trials for patients currently receiving PARPi and/or TKi, to prevent the resistance.

4.3.4.2 TGF-β signaling pathway inhibitors sensitize leukemias to [PARPi +/- TKi] in the bone marrow microenvironment

The therapy with TGFβR kinase inhibitor has just been described above to possibly enter clinical trials on leukemia patients receiving PARPi and/or TKi. Therefore, a FDA clinical trials-approved TGFβRi (galunisertib) was used to investigate this strategy. Moreover, TGF-β1 neutralizing antibody (1D11) has been successfully verified in BMM to regain sensitivity of leukemia cells to chemotherapy in BMM [238].

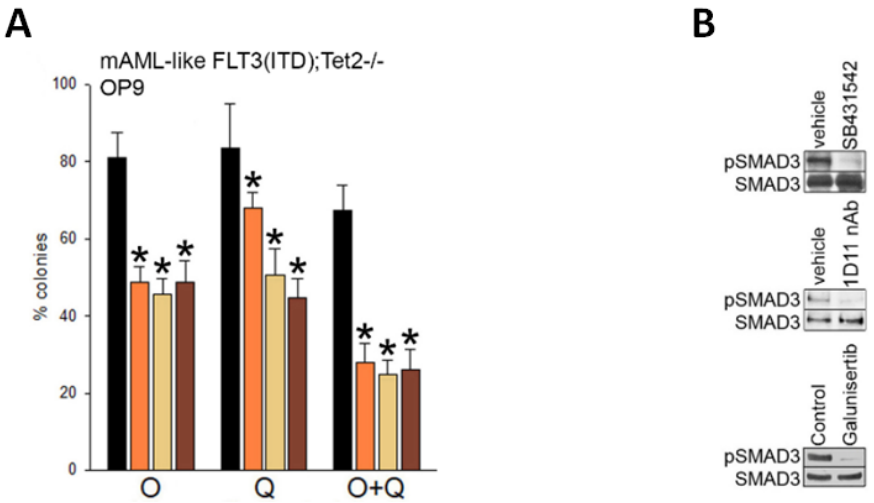

Figure 4.23. Pharmacological TGFβR inhibitors including SB435142 (orange bars), TGF-β1 neutralizing antibody 1D11 (yellow bars) and galunisertib (brown bars) sensitized BRCA–deficient leukemia cells to [PARPi +/- TKi] in BMM in comparison with untreated group (black bars) (**A**). Activities of these inhibitors were verified by decrease of pSMAD3 detected by western blotting analysis. Total SMAD3 was used as loading control (**B**). O: Olaprib, Q: Quizartinib. * $P < 0.05$.

Therefore, it was considered to examine efficacy of PARPi on leukemia cells in BMM. Besides galunisertib and 1D11 neutralizing antibody, SB431542 was added as a positive control. The activities of these TGFβ signaling pathway inhibitors were confirmed by down-regulation of phosphorylated SMAD3 (Figure 4.23B). We observed that galunisertib and neutralizing antibody (1D11) remarkably re-sensitized murine FLT3(ITD);Tet2-/- AML–like cells to [olaparib +/- quizartinib] in OP-9–mediated BMM and the same outcome was obtained after treatment with SB431542 (Figure 4.23A). This also suggested that restored efficacy of [PARPi +/- TKi] in leukemia cells in BMM thanks to inhibition of TGF-β signaling pathway, is independent on categories of TGFβR kinase inhibitor or TGF-β1 neutralizing antibody.

After we successfully validated potency of TGFβR kinase inhibitors to re-sensitize leukemias to [PARPi +/-TKi] in BMM constituted by stromal cell lines, to be consistent with examination of PARPi efficacy in BMM constituted by primary stromal cells, as the next step, we conducted the usage of TGFβR kinase inhibitors (SB435142 and galunisertib) in autologous/allogeneic BMM. In this case, leukemia cells were tested, including human primary Lin^-CD34^+ BCR-ABL1–positive CML together with murine primary Lin^-cKIT^+ FLT3(ITD);Tet2-/- AML- and BCR-ABL1–positive CML–like cells.

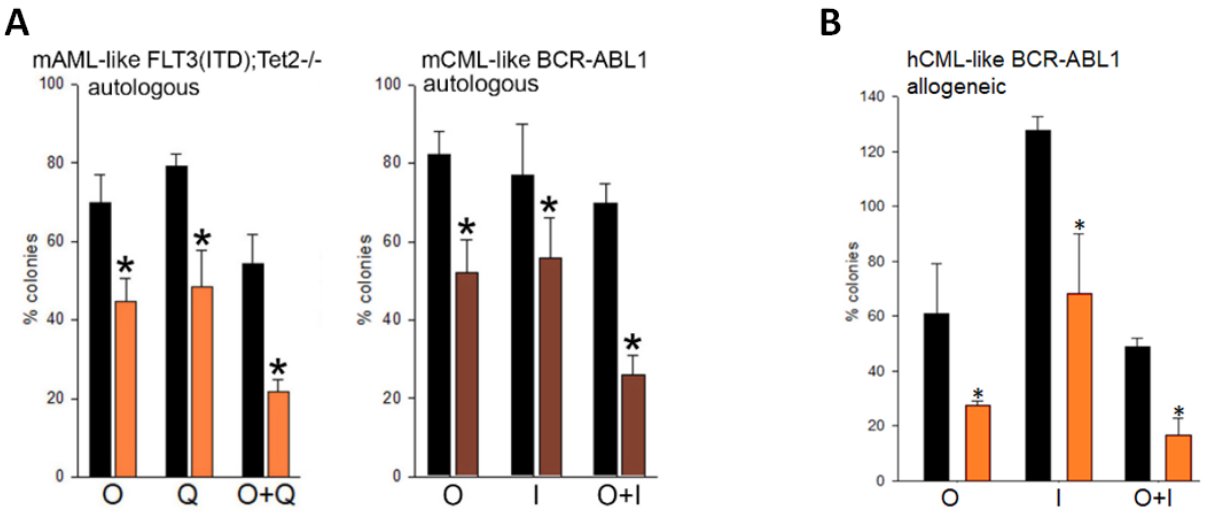

Figure 4.24. Two TGFβR kinase inhibitors including SB431542 (orange bars) and galunisertib (brown bars) recovered efficacy of [PARPi +/- TKi] against BRCA–deficient leukemias in autologous BMM (**A**) and allogeneic BMM (**B**) in comparison with untreated group (black bar). O: Olaprib, Q: Quizartinib, I: Imatinib. * $P < 0.05$.

Obtained data showed that in terms of autologous BMM-dc, both SB435142 and galunisertib recovered sensitivity of leukemias to [PARPi +/- TKi] in BMM (Figure 4.24A). Meanwhile, in

allogeneic BMM-dc with human mesenchymal bone marrow stromal cells, SB435142 re-sensitized human $Lin^{-}CD34^{+}$ BCR-ABL1–positive CML cells to [olaparib +/- imatinib] (Figure 4.24B).

4.3.4.3 TGFβRi (SB431542) combined with [PARPi + TKi] reduces quiescent LSCs in the bone marrow microenvironment

Since the result shown above in Chapter 4.2.6, presented both BMM-dc and BMM-idc induced quiescent LSCs refractory to PARPi (olaparib), determining the effect of SB431542 on survival of quiescent LSCs in combinational treatment with [PARPi + TKi] is necessary. This can confirm that TGF-β1 – TGFβR signaling pathway inducing quiescent LSCs, responsible for resistance to [PARPi + TKi]. In concordance with previous report [270], BMM promoted quiescent state of leukemia cells, manifested by accumulation of cells in G_0/G_1 cell cycle phase (Figure 4.15A). Quiescent LSCs are refractory to TKi and therefore, responsible for resistance against treatment therapies and/or disease relapse [261, 262].

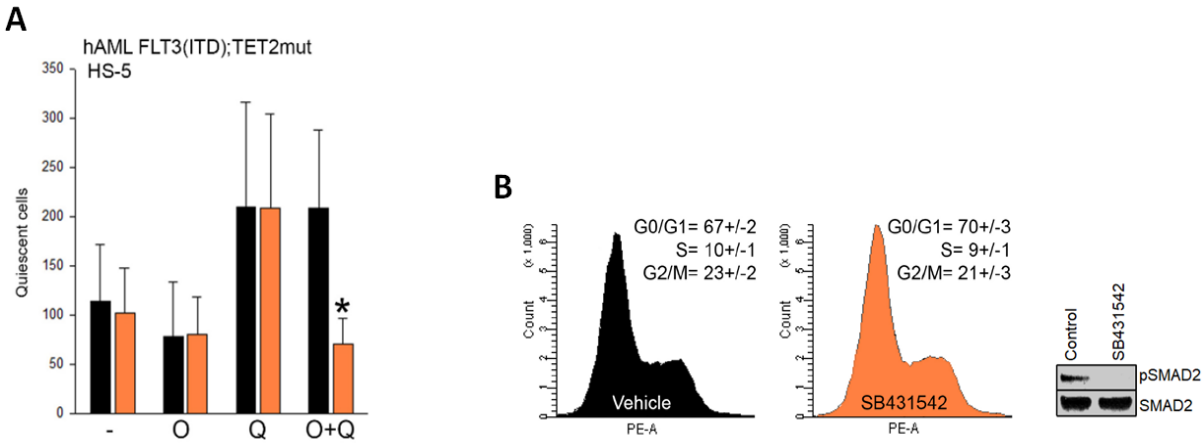

Figure 4.25. SB431542 (orange bar/histogram) enhanced the efficacy of [PARPi + TKi] against quiescent LSCs (**A**) but did not alter cell cycle distribution as estimated of leukemia cells in BMM. Effectiveness of SB431542 was confirmed by down-regulation of pSMAD2 level determined by western blotting analysis and total SMAD2 was used as loading control (**B**). O: Olaprib, Q: Quizartinib. * $P < 0.05$.

Here we verified that BMM-dc protected human FLT3(ITD);TET2mut AML cells with phenotype of $Lin^{-}CD34^{+}CD38^{-}CFSE^{max}$, against elimination of [PARPi + TKi]. Remarkably, the addition of SB431542 in [PARPi + TKi] dramatically reduced survival of quiescent LSCs in BMM (Figure 4.25A). Moreover, SB435142 did not alter cell cycle distribution of primary FLT3(ITD);TET2mut AML cells from patient sample in BMM-dc (Figure 4.25B), suggesting

additional treatment of SB435142 is capable to enhance efficacy of [PARPi + TKi] to eliminate quiescent LSCs. Importantly, the TGFβRi did not induce accumulation of cells in G_2/M phase, which may lead to reduction of cells in G_o/G_1 phase of cell cycle.

4.3.5 TGFβRi (SB431542) promotes anti-leukemic effect of [PARPi + TKi] *in vivo*

With given evidences above of TGFβR kinase inhibitor in restoring efficacy of [PARPi + TKi] on leukemias in BMM established by stromal cell line and primary stromal cells *in vitro*, it is rational to verify the influence of TGFβRi on anti-leukemia effect of [PARPi + TKi] *in vivo*. Notably, we previously reported and described above that TKi-treated BCR-ABL1–positive CML cells and FLT3(ITD)-positive AML cells display profound HR/D-NHEJ deficiencies, which make them especially vulnerable to PARPi-based therapies *in vivo* [176]. To interpret this hypothesis, combined treatment: [TKi (imatinib for BCR-ABL1 and quizartinib for FLT3(ITD)) + PARPi (talazoparib)] +/- TGFβRi (SB431542) was examined in SCID mice bearing GFP(+) BCR-ABL1–positive CML-like or GFP(+) FLT3(ITD);TET2-/- AML-like leukemias. Short and aggressive 7-day treatment regimen was applied to create a better opportunity to detect beneficial effect of TGFβRi. The entire procedure of *in vivo* leukemic engraftment and inhibitor treatment regimen was described at Chapter 3.9.2 of Materials and Methods (Figure 3.5). SCID mice were employed as hosts to exclude the impact of PARPi-mediated immune modulation on therapeutic effect [271].

4.3.5.1 TGFβRi enhances efficacy of [PARPi + TKi] against leukemias in splenocytes and bone marrow cells of xenograft mice

Since SB431542 does not inhibit TGFβR-mediated signaling pathway and alter leukemia susceptibility to [PARPi +/- TKi] in PBM condition *in vitro*, at first, we analyzed GFP(+) leukemia-like cells in peripheral blood leukocytes (PBL) of engrafted mice after treatment regimen. As expected, [imatinib + talazoparib] and [quizartinib + talazoparib] provided remarkable anti-leukemia effect in PBL of mice bearing CML-like and AML-like leukemias, respectively, and addition of SB431542 did not enhance this effect (Figure 4.26). The data is very consistent to the result we obtained *in vitro*, in which TGFβRi (SB431542) did not promote efficacy of [PARPi + TKi] against leukemias in PBM.

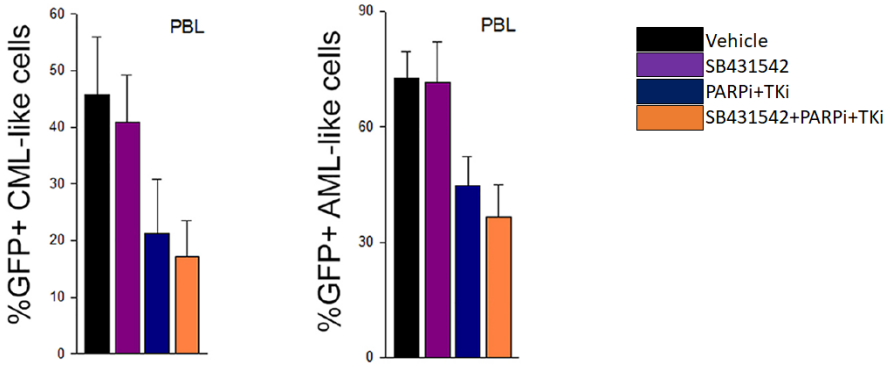

Figure 4.26. SB431542 did not enhance anti-leukemia effect of [PARPi + TKi] in peripheral blood leukocytes (PBL) in leukemia–bearing mice, treated as indicated. The percentage of GFP(+) leukemic cells was estimated by flow cytometry in PBL population.

On the contrary to PBM, in BMM condition, SB431542 showed impact to inhibit TGFβR–mediated signaling pathway, restoring sensitivity of leukemias to [PARPi +/- TKi] *in vitro*. Thus, in the *in vivo* study, GFP(+) leukemia-like cells from splenocytes (SPL) and bone marrow cells (BMC) of engrafted mice were obtained. Remarkably, while anti-leukemia effect of [PARPi + TKi] was limited in SPL and BMC, addition of SB431542 greatly enhanced this effect (Figure 4.27). Furthermore, immunohistochemical analysis of GFP(+) cells in the bone marrow of femoral specimens of leukemia-engrafted mice (done by our collaborator, Prof. Mariusz A. Wasik's laboratory, Fox Chase Cancer Center, Temple Health) supported the conclusion that TGFβRi (SB435142) facilitated targeting of CML-like and AML-like leukemia cells by [TKi + PARPi] in BMM (Figure 4.28).

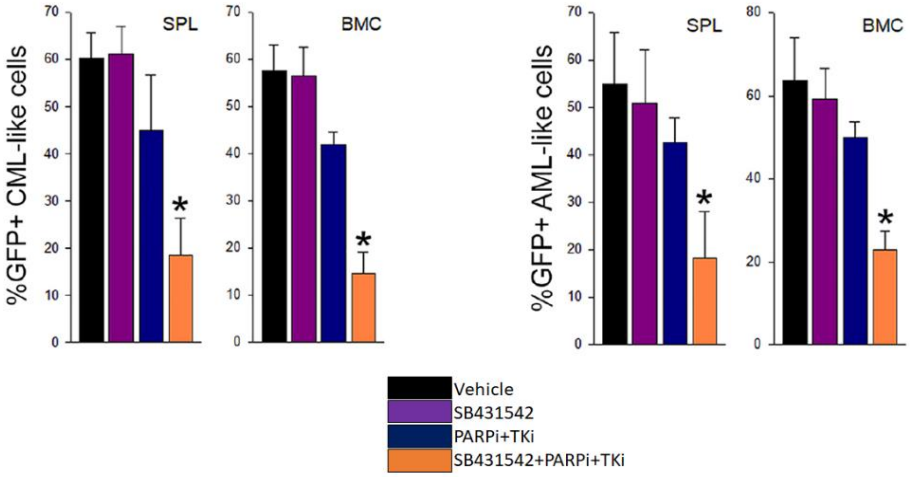

Figure 4.27. TGFβRi (SB435142) promoted anti-leukemia effect of [PARPi + TKi] in splenocytes (SPL) and bone marrow cells (BMC) of leukemia–bearing mice. The percentage of GFP(+) leukemia cells was estimated by flow cytometry in SPL or BMC population. * $P < 0.05$.

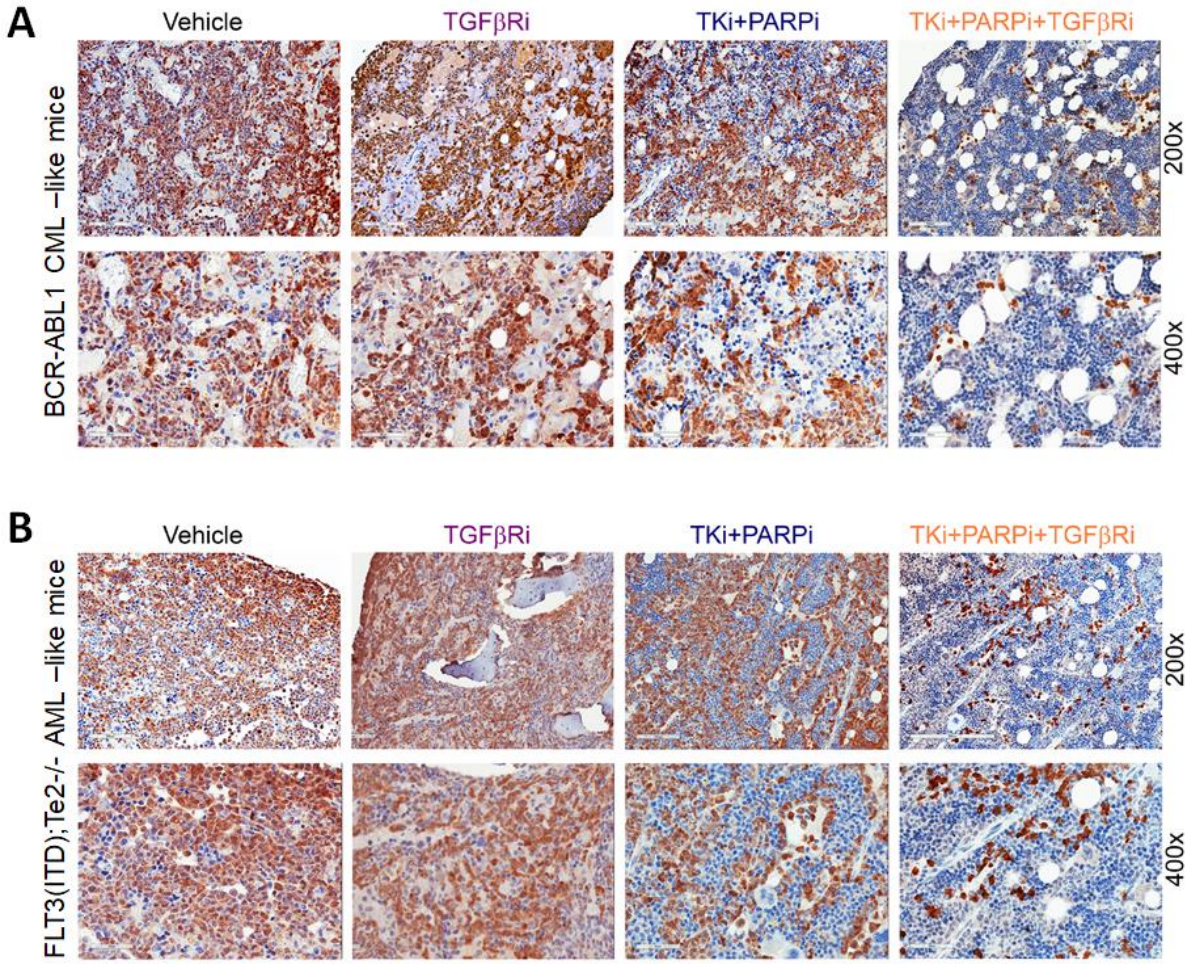

Figure 4.28. Immunohistochemical analysis of bone marrow sections from femoral specimens revealed that TGFβRi (SB435142) enhanced anti-leukemia effect of [PARPi + TKi] in mice engrafted with GPF(+) BCR-ABL1 CML–like (**A**) and FLT3(ITD);Tet2-/- AML–like (**B**) cells. GFP(+) leukemia–like cells were stained with anti-GFP antibody and visualized in brown color. Blue color illustrated Eosin-Hematoxylin staining.

4.3.5.2 TGFβRi prolongs survival of leukemia–engrafted mice in the treatment with [PARPi + TKi]

After observation of SB431542 enhanced anti-leukemia effect of [PARPi + TKi] in SPL and BMC, we proposed that survival of leukemia-transplanted mice treated with [PARPi + TKi + SB431542] should be extended compared to vehicle control and individual SB431542 or [PARPi + TKi] treatments. We found that, SB435142 prolonged the survival of CML-like and AML-like leukemia-bearing mice receiving [TKi + PARPi], indeed (Figure 4.29A).

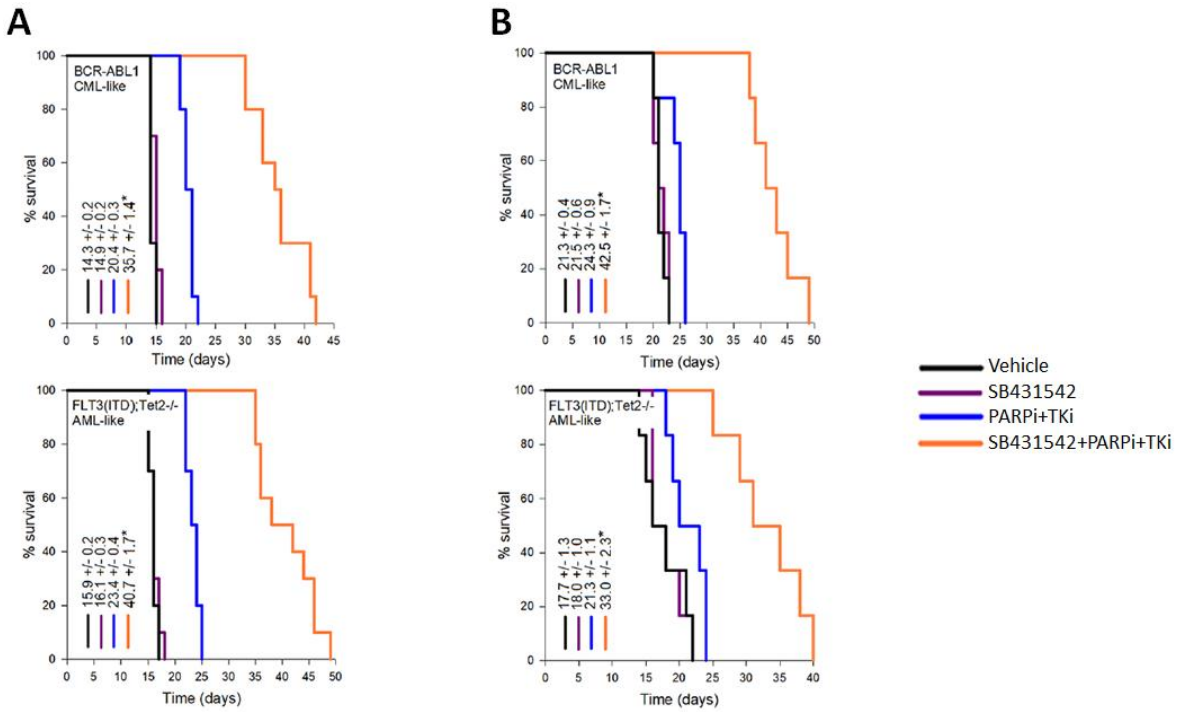

Figure 4.29. SB431542 significantly prolonged survival of leukemia–bearing mice in initial treatment (**A**) and after bone marrow cells -derived secondary transplantation (**B**). * P < 0.05.

The secondary transplantation of bone marrow cells allows to test the self-renewal capacity of hematopoietic stem cells *in vivo* and better estimates whether the treatment also targeted leukemia stem-like or progenitor cells. Thus, we performed experiments in which secondary recipients obtained bone marrow cells from the initial experiments. Importantly, secondary recipients of BMCs obtained from initial [PARPi + TKi + SB435142] treatment presented significantly longer lifespan than the counterparts injected with BMCs from mice individually treated by [TKi + PARPi] or SB431542 (Figure 4.29B). It suggests that addition of TGFβRi (SB435142) into [PARPi + TKi] eradicates LSCs in bone marrow niche. Taken together, these data clearly elucidates our hypothesis that TGF-β1 – TGFβR signaling pathway induces resistance to PARPi in BMM. Conversely, inhibition of TGFβR kinase restores efficacy of PARPi *in vitro* and specific leukemia microenvironments (bone marrow and spleen) *in vivo*.

4.3.6 TGFβR kinase inhibitor (SB431542) causes "BRCAness/DNA-PKness" phenotype in leukemias in the bone marrow microenvironment

4.3.6.1 TGFβRi causes DSB repair deficiencies in leukemia cells in the bone marrow microenvironment

As TGFβ1–TGFβR signaling pathway has been shown in this study to induce resistance to PARPi in BRCA–deficient leukemias in BMM, we investigated on mechanism, in which PARPi resistance is induced by TGF-β signaling pathway. We proposed that inhibition of TGFβR kinase causing defections of HR and/or D-NHEJ pathways in DSB repair, leads to highly addictive dependence of malignant cells on PARP1 to repair DSBs and survive.

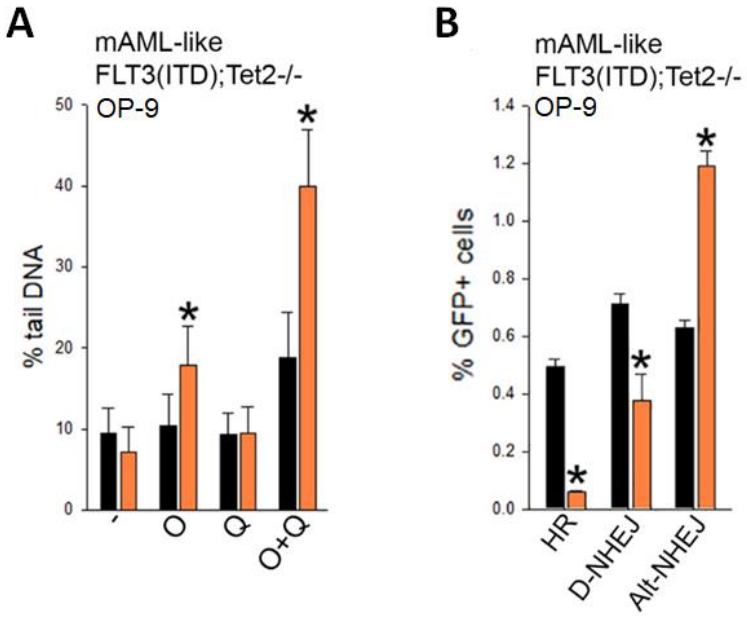

Figure 4.30. SB431542 (orange bars) caused deficiencies of HR and D-NHEJ pathways in DSB repair of leukemia cells in BMM, leading to dependence of DSB repair on PARP1–mediated Alt-NHEJ. **A**. Inhibition of TGFβR kinase by SB435142 resulted in accumulation of [PARPi +/- TKi]–induced DSBs in leukemia in BMM. DSBs were measured in the neutral comet assay as % of tail DNA **B**. SB435142 reduced DSB repair activities of HR and D-NHEJ pathways but promoted the activity of PARP1–dependent Alt-NHEJ of leukemia cells in BMM. Percentage of cells with restoration of GFP(+) was considered DSB repair activity. O: Olaparib, Q: Quizartinib. * $P < 0.05$.

At first, in direct measurement of DSBs via neutral comet assay, we observed inhibition of TGFβR kinase by SB431542 was associated with elevated accumulation of DSBs in olaparib- and [olaparib + quizartinib]–treated murine $Lin^{-}cKIT^{+}$ FLT3(ITD);Tet2-/- AML-like cells co-cultured with OP-9 cells in BMM-dc (Figure 4.30A), which coincided with enhanced

sensitivity of leukemia cells to PARPi (Figure 4.23A). Moreover, because TKi has been reported not to stimulate lethal DSBs [272], even with presence of SB431542, the level of quizartinib-induced DSBs in leukemia cells remained unchanged.

Because the elevated accumulation DSBs in response to treatment with [PARPi + TKi] is well believed to be related to deficiencies of HR and/or D-NHEJ pathways in DSB repair and sustainable dependence on PARP1–mediated Alt-NHEJ, we conducted an assay to directly calculate activities of all DSB repair pathways. In details, the impact of TGFβRi (SB431542) on activities of DSB repair pathways in murine $Lin^{-}cKIT^{+}$ FLT3(ITD);Tet2-/- AML–like cells was examined using three specific reporter cassettes representing for HR, D-NHEJ and Alt-NHEJ pathways of DSB repair as described before [176].

Incubation with SB431542 caused approximately 9-fold and 2-fold reduction of HR and D-NHEJ activities, respectively, whereas, DSB repair efficiency of Alt-NHEJ was elevated by approximately 2-fold (Figure 4.30B). The data is completely consistent with accumulation of olaparib- or [olaparib + quizartinib]–induced DSBs under effect of SB431542 in neutral comet assay. In which, due to deficiencies of HR and D-NHEJ repair activities, DSB repair requirement is switched to PARP1–dependent Alt-NHEJ pathway. Therefore, inhibition of PARP1 eliminates leukemia cells in BMM which corresponds to the result obtained for PARP1–null leukemia cells and described earlier in Chapter 4.3.3.1.

4.3.6.2 TGFβRi and down-regulation of TGFβR2 cause negative modulation of DSB repair proteins in leukemias in the bone marrow microenvironment

Once we observed the elevated accumulation of [PARPi + TKi]–induced DSBs and reduction of DSB repair activities of HR and D-NHEJ pathways caused by TGFβRi (SB431542) in BMM, analysis of the expression levels of DSB repair proteins was essential. In this approach, DSB repair proteins in murine $Lin^{-}cKIT^{+}$ FLT3(ITD);Tet2-/- AML–like cells incubated with SB431542 and human BCR-ABL1–positive CML K562 cells with down-regulation of TGFβR2 by CRISPR/Cas9 were examined in BMM-dc. Analysis of protein levels by western blot revealed that SB435142-mediated modulation of DSB repair was associated with down-regulation of ATM kinase (Figure 4.31A) which is involved in DSB response, meanwhile, the expression of SSB response protein, ATR kinase, remained unaffected during the inhibition (Figure 4.31A).

In addition, BRCA1 and BRCA2 (HR proteins) and DNA-PKcs and Ligase IV (D-NHEJ proteins) were significantly (below the detection level) down-regulated in murine FLT3(ITD);Tet2-/- AML-like cells in BMM-dc after inhibition of TGFβR kinase by SB431542 (Figure 4.31A). On the other hand, SB431542 did not negatively affect on expression levels of Alt-NHEJ proteins (PARP1, Ligase III, Polθ). Importantly, these TGFβRi-induced alterations in DNA damage/repair were not dependent on the cell cycle as the inhibitor did not modify the cell cycle distribution of leukemia cells, what we presented before (Figure 4.25B). Additionally, ATM, BRCA1, BRCA2, DNA-PKcs and Ligase IV, but not Alt-NHEJ proteins were also significantly down-regulated in BCR-ABL1-positive CML K562-Cr cell line (in which, expression of TGFβR2 was reduced by CRISPR/Cas9), when co-cultured with HS-5 in BMM-dc (Figure 4.31B).

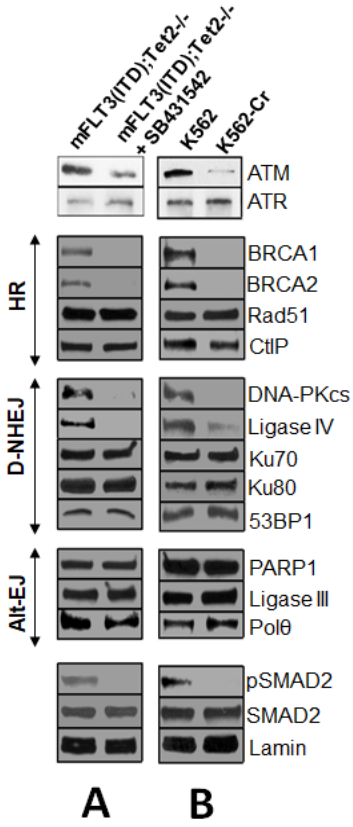

Figure 4.31. Western blotting analysis on whole panel of DSB repair proteins in BMM. Inhibition of TGFβR -mediated signaling pathway by SB431542 (**A**) or down-regulation of TGFβR2 by CRISPR/Cas9 (-Cr) (**B**) down-regulated proteins in DSB response (ATM), HR (BRCA1, BRCA2) and D-NHEJ (DNA-PKcs, Ligase IV) pathways of DSB repair but did not alter expression of proteins in Alt-NHEJ (PARP1, Ligase III, Polθ), SSB response (ATR) and other proteins in HR (Rad51, CtIP), D-NHEJ (Ku70, Ku80, 53BP1) pathways in leukemias in BMM.

The obtained data using CML cell line with down-regulation of TGFβR2 due to CRISPR/Cas9 can be considered a convincing confirmation that inhibition of TGFβR kinase down-regulates DSB repair proteins in both HR and D-NHEJ pathways and shifts DSB repair requirement to PARP1-dependent Alt-NHEJ in BMM.

4.3.7 TGFβR–SMAD3 signaling induces resistance to [PARPi +/- TKi] in leukemia cells in the bone marrow microenvironment

We have revealed above that inhibition of TGFβ serine/threonine kinase receptor sensitized leukemia cells to [PARPi + TKi] in BMM *in vitro* and *in vivo*. In this Chapter, to further investigate on molecular mechanism of the resistance to PARPi in BMM and in concordance with previous reports, we proposed that the resistance against PARPi in BMM could be mediated by TGF-β–downstream mechanisms. These mechanisms includes TGFβR–miRNA-182 regulatory link, TGFβR–SMAD3 signaling and TGFβR non-canonical protein kinase effectors.

4.3.7.1 TGFβR–miRNA-182 axis does not involve PARPi resistance in leukemias in the bone marrow microenvironment

The most recent paper reporting the association between TGF-β signaling pathway and PARPi–mediated synthetic lethality has investigated on head and neck squamous cell carcinoma [241]. It has shown that the blockage of TGFβR–mediated signaling pathway by HPV–positive E6/E7 proteins which antagonizes TGFβRs or TGFβR kinase inhibitor, releases overexpression of oncogenic miRNA-182, inhibiting BRCA1 and FOXO3, a required element for activation of ATM kinase. Therefore, this led to triggering of PARPi-mediated synthetic lethality in the tumor cells. Because we have presented here that both, BRCA1 and ATM were significantly down-regulated by TGFβRi (SB431542) and reduced expression of TGFβR2 by CRISPR/Cas9 in BMM, it was rational to determine expression of miRNA-182 in leukemias in BMM after incubation with SB435142 and down-regulation of TGFβR2 by CRISPR/Cas9. As miRNA controls, we evaluated the expression levels of miR-183, which belongs to the same miRNA family with miRNA-182, and miRNA-103 which does not join the same miRNA cluster with miRNA-182/1983. In terms of loading control, we utilized UniSp6 RNA template to verify the efficiency of the experiment. The RT-qPCR experiment, which quantified relative expression of miRNAs, was conducted by a given order to external industrial company.

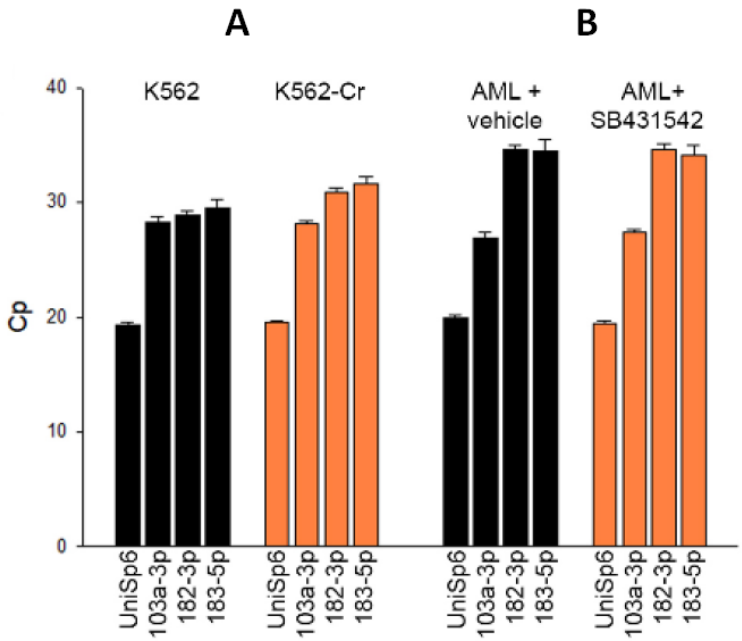

Figure 4.32. Verification of relative expression levels of miRNAs by reversed transcription-qPCR. Inhibition of TGFβR-mediated signaling pathway (orange bar) by down-regulation of TGFβR2 via CRISPR/Cas9 (-Cr) in K562 CML cells (**A**) and TGFβRi (SB431542) in murine FLT3(ITD);Tet2-/- AML–like cells (**B**) did not alter expression of oncogenic miRNA-182 and its miRNA family member, miRNA-183. Whereas, the unrelated miRNA to miRNA-182/183, miRNA-103 was also unaffected. The expression of loading control, UniSp6, remained completely similar throughout cell types and inhibitions, representing the integrity of experiments. Cp was mean of relative expression. $P > 0.05$.

We found that in murine primary FLT3(ITD); Tet2-/- AML-like cells, expression of miRNA-182 and another miRNA member in this cluster, miRNA-183, remained unaffected, when TGFβR -mediated signaling pathway was deactivated by SB431542 (Figure 4.32B). Similarly to leukemia cells incubated with TGFβR kinase inhibitor, the same observation was also obtained for BCR-ABL1–positive CML cells with down-regulation of TGFβR2 due to CRISPR/Cas9 (Figure 4.32A). Besides two miRNAs above, miRNA-103a was analyzed as a negative control, which does not belong to miRNA-182/-183 cluster as well as TGF-β signaling. As expected, the expression of miRNA-103a remained unchanged throughout every groups. This data excluded the involvement of miRNA-182/183 in the regulatory link with TGF-β observed in resistance against PARPi in leukemias in BMM.

4.3.7.2 Pharmacological inhibition of SMAD3 restores efficacy of [PARPi +/- TKi] against leukemia cells in the bone marrow microenvironment

Inhibition of TGFβR kinase down-regulates expression of DBS proteins in HR and D-NHEJ pathways, enhancing accumulation of [PARPi +/- TKi]–induced DSBs and therefore, restores sensitivity of leukemia to [PARPi +/- TKi] in BMM. From this, after excluding effect of the TGF-β–miRNA-182 regulatory link, we proposed the downstream mechanism of TGFβ1–TGFβR signaling pathway which might be directly involved in activation of the PARPi resistance in BMM.

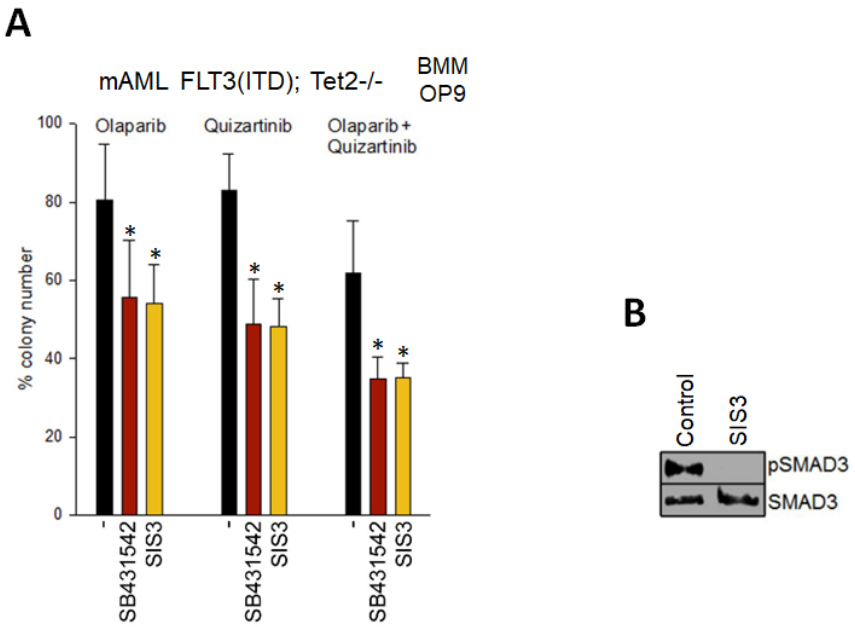

Figure 4.33. Inhibition of TGFβR–SMAD3 signaling, via suppression of phosphorylated SMAD3 (pSMAD3) restored efficacy of [PARPi +/- TKi] against leukemia cells in BMM (**A**); the activity of SMAD3i (SIS3) was confirmed by decrease of pSMAD3. The levels of pSMAD3 and total SMAD3 (as the loading control) were verified by Western blotting analysis (**B**). * $P < 0.05$.

In fact, TGFβR kinase activates numerous downstream signaling effectors including transcriptionally active SMAD2/3/4 complex as the canonical pathway and several kinases (e.g., PI3K, RAF1, PAK1, TAK1) as non-canonical downstream signals [273]. At first, to investigate on TGFβR–mediated canonical downstream mechanisms via SMAD2/3/4 in PARPi resistance, murine $Lin^{-}cKIT^{+}$ AML-like FLT3(ITD);Tet2-/- AML-like cells were incubated with [olaparib +/- quizartinib] in OP-9-mediated BMM-dc with addition of SMAD3 inhibitor (SMAD3i-SIS3) alongside SB435142 as a positive control. The activity of SIS3 was confirmed

by down-regulation of phosphorylated SMAD3 (Figure 4.33B). Remarkably, SIS3 re-sensitized leukemia cells to [olaparib +/- quizartinib] and the effect was similar to SB431542 (Figure 4.33A). This data strongly supported the role of TGFβ1-TGFβR-SMAD3 signaling in the investigated process.

After obtaining positive effectiveness of SMAD3i to reduce clonal survival of leukemia cells upon the treatment with [PARPi +/- TKi] in BMM, we investigated the possible effects on the expression levels of DSB repair protein. We found that SIS3 fully recapitulated the inhibitory effect of TGFβRi (SB435142) on all five analyzed DSB repair proteins including BRCA1, BRCA2, ATM, DNA-PKcs and Ligase IV (Figure 4.34A). Importantly, the putative SMAD2/3/4 binding sites were found in promoter regions of five genes encoding these DSB repair proteins (done by our collaborator, Dr. Michal Dąbrowski, Nencki Institute, Laboratory of Bioinformatics) (Figure 4.34B). Altogether, similarly to TGFβRi (SB431542), SMAD3i (SIS3) down-regulated proteins associated with DSB response (ATM) and HR (BRCA1/2) and D-NHEJ (DNA-PKcs, Ligase IV) pathways in DSB repair and "shifted" the DSB repair requirement to PARP1–dependent Alt-NHEJ in BMM. Therefore, it restores sensitivity of malignant cells to PARPi in BMM.

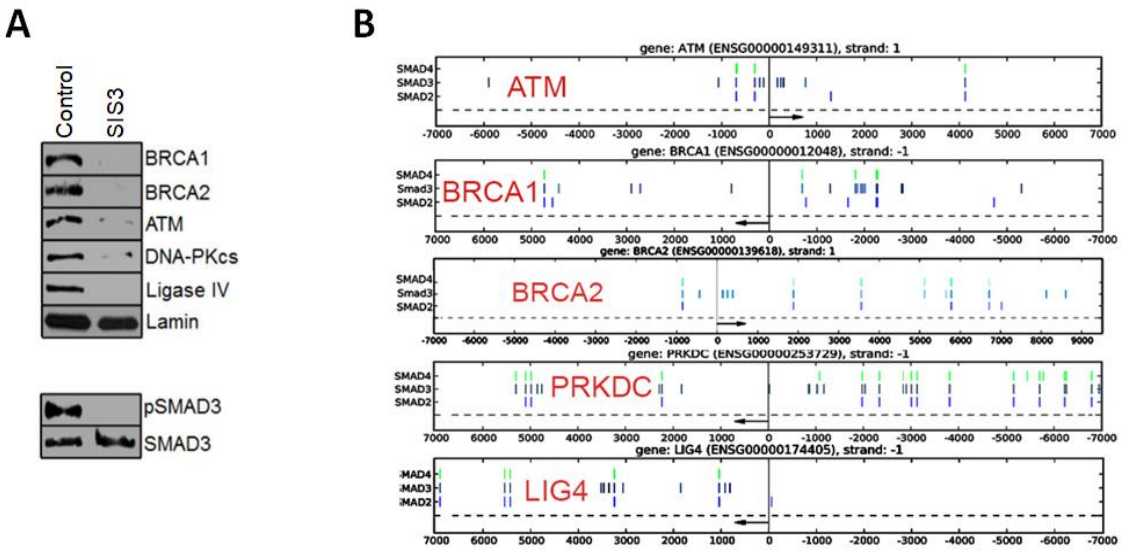

Figure 4.34. Inhibition of SMAD3 by SIS3 completely down-regulated ATM, BRCA1, BRCA2, DNA-PKcs and Ligase IV in leukemia cells in BMM. Cells were left untreated (control) or treated with SIS3 and protein levels were estimated by western blotting analysis (**A**) and putative binding sites of SMAD3 were detected in promoters of genes encoding these five proteins, the black arrows indicate start of transcription binding sites +/- 2,000 bp (**B**).

4.3.7.3 Dominant negative mutant SMAD3(D407E) sensitizes leukemia cells to [PARPi +/- TKi] in the bone marrow microenvironment

In concordance with previous publication describing that the dominant negative SMAD3(D407E) mutant completely blocks the TGFβR–SMAD3 signaling pathway via reduction of phosphorylated forms of both SMAD2 and SMAD3 [263], we forwarded to express the SMAD3 mutant and SMAD3 wild-type (WT) form in murine $Lin^{-}cKIT^{+}$ FLT3(ITD);Tet2-/- AML-like cells.

At first, two DNA fragments including sequence of FLAG-SMAD3(WT) and FLAG-SMAD3(D407E) were obtained from pcDNA3 plasmid by digestion of *BamH*I and *Xho*I. The DNA fragments were then re-cloned into pMIG-IRES-GFP retroviral construct by molecular cloning techniques, which were described at Chapter 3.6 of Materials and Methods. The DNA ligation into pMIG-IRES-GFP plasmid was confirmed by endonuclease digestion of *Xho*I and *Hind*III (Figure 4.35A).

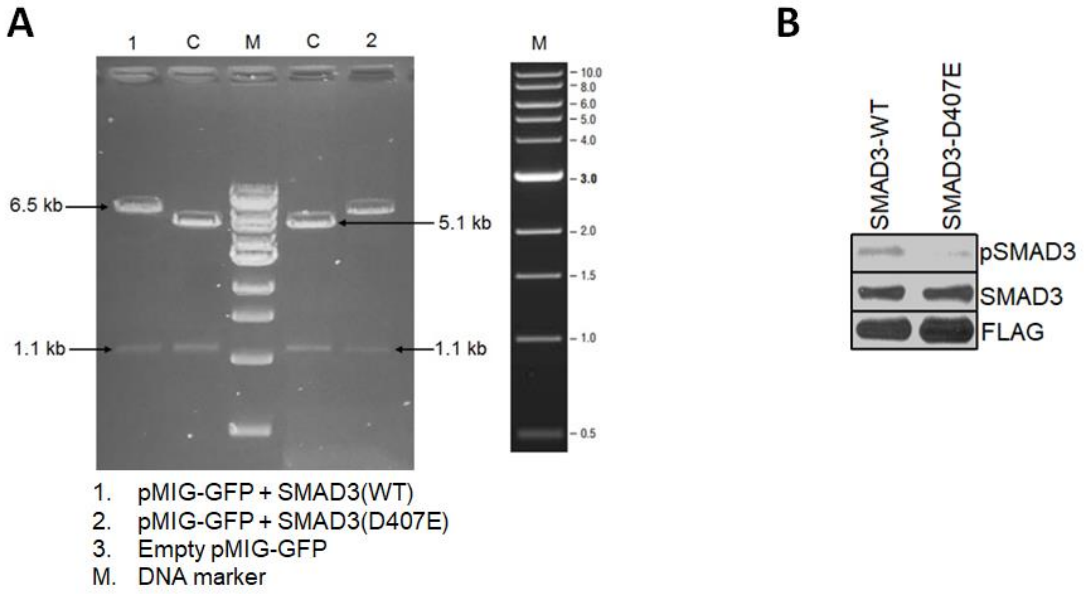

Figure 4.35. Expression of SMAD3(WT) and SMAD3(D407E) via pMIG-IRES-GFP in leukemia cells. **A**. Successful re-clone of [FLAG-SMAD3(WT)] and [FLAG-SMAD3(WT)] from pcDNA3 to pMIG-IRES-GFP. **B**. Verifying expression of SMAD3 negative dominant mutant and FLAG tag in leukemia cells.

Leukemia cells containing the retroviral construct were sorted with GFP(+) channel in flow cytometry as the pMIG-IRES-GFP retroviral construct contains GFP cassette and the expression of WT/mutant on SMAD3 and FLAG tag were confirmed in western blotting

analysis (Figure 4.35B). Next, we performed clonogenic test and found that cells expressing SMAD3(D407E) were sensitive to [olaparib +/- quizartinib] ,whereas, cells with SMAD3(WT), as expected, remained refractory to the drugs in BMM (Figure 4.36). To sum up, this result can be considered a very convincing validation of data obtained with SMAD3i.

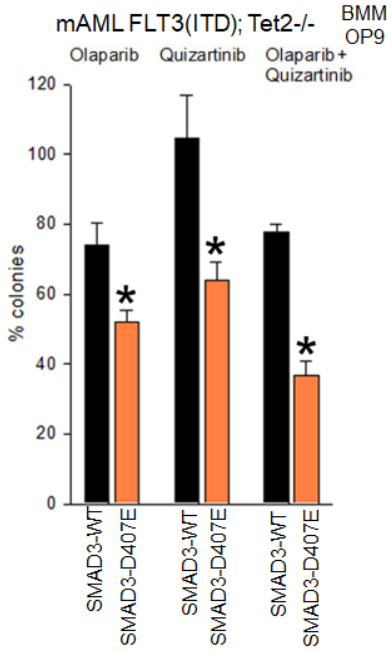

Figure 4.36. Expression of SMAD3(D407E) sensitized leukemia cells to [PARPi +/- TKi] in BMM. AML-like leukemia cells expressing either wt (SMAD3-WT) or mutant (SMAD3-D407E) form of SMAD3 were co-cultured with OP9 cells and treated with olaparib +/- quizartinib. * $P < 0.05$.

4.3.7.4 TGFβR–mediated non-canonical protein kinase signals do not induce resistance to PARPi in leukemia cells in the bone marrow microenvironment

Although canonical TGFβR–SMAD3 signaling directly induces resistance to PARPi in BMM, we also investigated the non-canonical effector protein kinases of TGFR–mediated signaling pathway, to exclude their possible involvements.

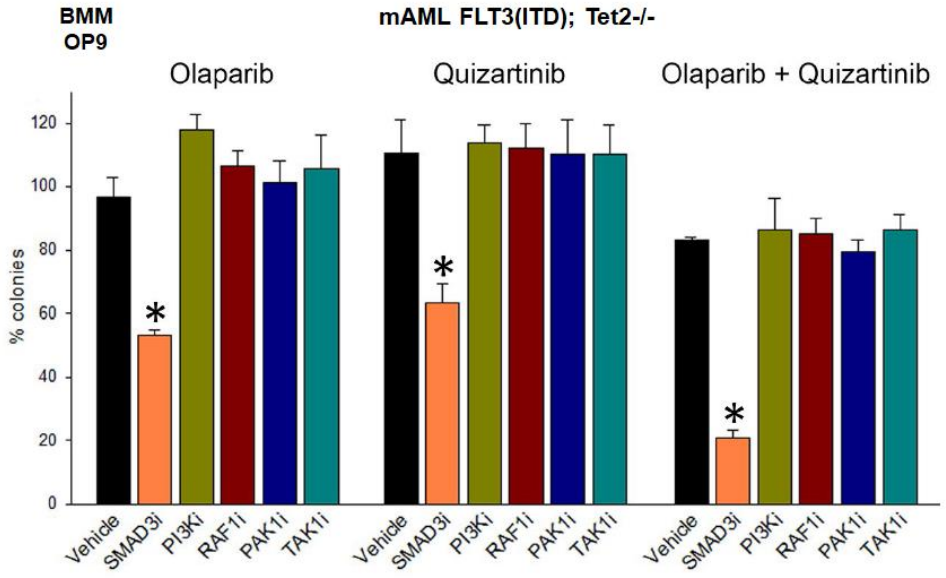

Figure 4.37. Inhibition of TGFβR–mediated non-canonical effector kinases by PI3Ki, RAF1i, PAK1i and TAK1i did not alter BMM–dependent [PARPi +/-TKi] resistance in leukemia cells in BMM in comparison with SMAD3i. * $P < 0.05$.

Therefore, inhibitors of TGFβR–mediated non-canonical effector kinases including PI3K, RAF1, PAK1 and TAK1 were used in both clonogenic test with [olaparib +/- quizartinib] and western blotting analysis of DSB repair proteins in murine primary $Lin^{-}cKIT^{+}$ FLT3(ITD);Tet2-/- AML–like cells, alongside SMAD3i as a positive control. The activities of protein kinase inhibitors were confirmed by suppression of phosphorylated AKT, ERK1/2, and p38/MAPK (Figure 4.38).

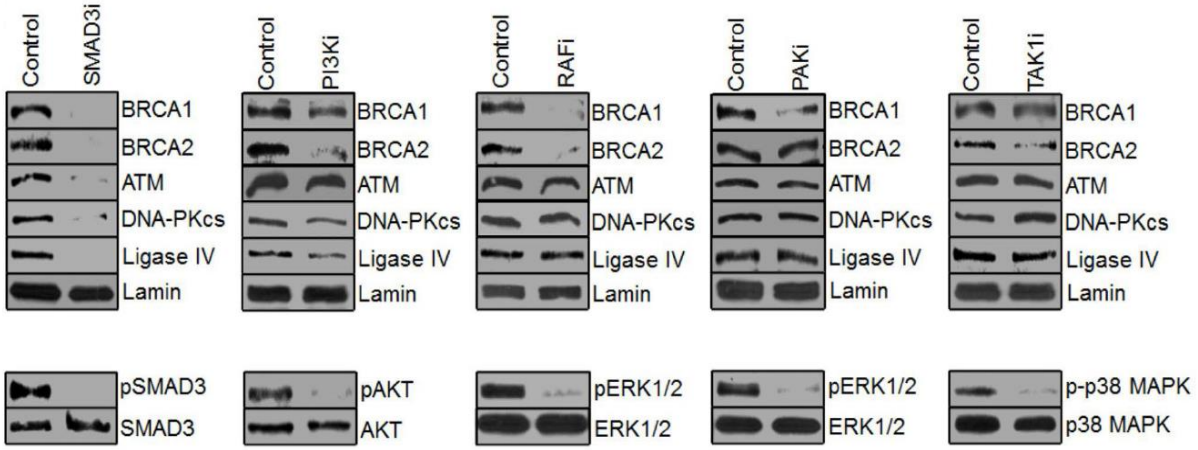

Figure 4.38. Inhibition of TGFβR–mediated non-canonical effector kinases by PI3Ki, RAF1i, PAK1i and TAK1i did not simultaneously down-regulate expression of BRCA1, BRCA2, ATM, DNA-PKcs and Ligase IV in leukemia cells in BMM.

We observed by clonogenic test that, inhibition of PI3K, RAF1, PAK1 and TAK1 did not re-sensitize leukemia cells to [olaparib +/- quizartinib] (Figure 4.37). This was validated by western blotting analysis of expression levels of ATM, BRCA1, BRCA2, DNA-PKcs and Ligase IV. It illustrated that these inhibitors did not down-regulate all five targeted DSB repair proteins simultaneously, what was observed upon SMAD3 inhibition (Figure 4.34A). This data show that the TGFβR-mediated non-canonical signaling pathways via four effector protein kinases do not perform potential role in resistance to PARPi in leukemia cells in BMM.

Altogether throughout the study, our all obtained data indicated that although FLT3(ITD)–positive leukemia (occupies approximately 23% of AML cases) does not carry mutations/deficiencies of *BRC1/2*, inhibition of FLT3(ITD) oncogenic receptor by FLT3i (AC220) induced "BRCAness" phenotype, triggering PARPi–mediated synthetic lethality in the leukemia cells. Therefore, FLT3i remarkably sensitized FLT3(ITD)–positive leukemia cells to PARPi *in vitro* and *in vivo*. Unfortunately, likely to *BRCA*–mutated solid tumors, BRCA–deficient leukemia cells are capable to acquire resistance against PARPi. In the current study, we have discovered a novel and constitutive mechanism involved in this protection and described that, the TGFβ1-TGFβR-SMAD3 signaling, activated in the bone marrow niche, plays a role in the acquired resistance to PARPi–induced synthetic lethality in leukemias. Importantly, our results confirmed that pharmacological inhibition of TGFβR serine/threonine kinase by the inhibitor, which makes a strong background for therapeutic intervention to sensitize CML/AML cells to PARP inhibitors.

5. DISCUSSION

In this study, we report a novel synergistically possible therapy to enhance efficacy of PARPi-mediated synthetic lethality in leukemia cells, carrying BRCA1/2 deficits, but lacking *BRCA1/2* mutations. We found that FLT3 tyrosine kinase inhibitor (AC220) induced DSB repair defects and sensitized FLT3(ITD)-positive leukemia cells to PARP inhibitors *in vitro* and *in vivo*. Moreover, because leukemia cells are derived from the bone marrow, we examined effectiveness of PARPi in BRCA-deficient leukemias in a condition mimicking bone marrow microenvironment (BMM). We identified a novel mechanism that is induced by stromal cells in the BMM and leads to resistance to PARPi in leukemia. Furthermore, we discovered that the resistance is mediated by the TGFβ1-TGFβR-Smad3 signaling pathway. Genetic or pharmacological targeting this signaling pathway restored/enhanced efficiency of PARPi in leukemia cells in the BMM *in vitro* and *in vivo*.

5.1 The significance of FLT3i (AC220) triggering PARPi-induced synthetic lethality on FLT3(ITD)-positive leukemia cells

Although tyrosine kinase inhibitor AC220 (quizartinib) targets activated FLT3(ITD) receptor, which functions as an oncoprotein in a large subgroup of AML, the treatment, even if combined with cytotoxic drugs, is not curative in the vast majority of cases. The major reason is that leukemia cells are able to induce protective responses to DNA damage induced by cytotoxic agents and apoptotic effect caused by TKi [274-277].

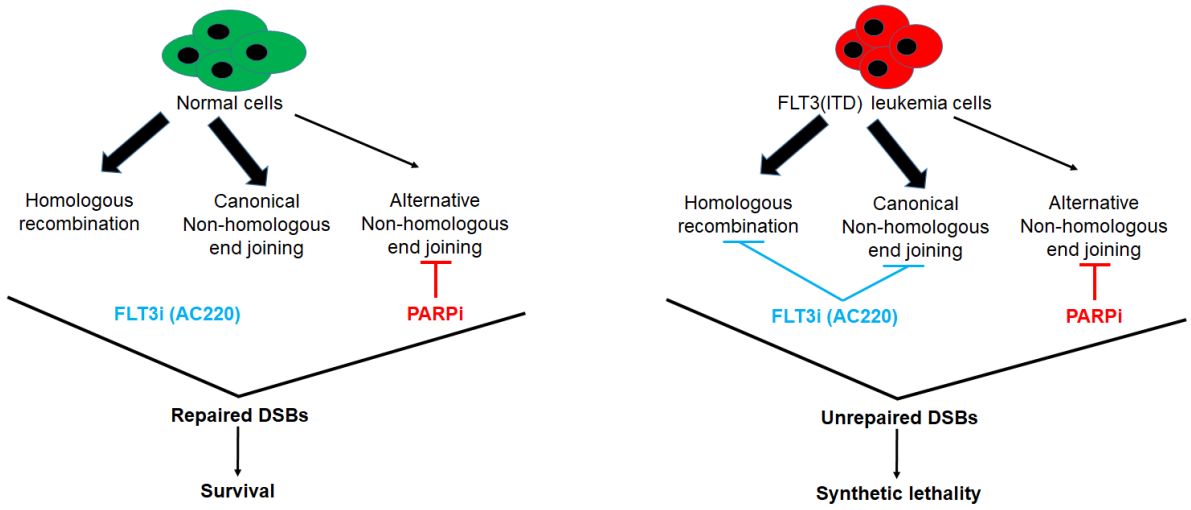

Figure 5.1. Model of exploitation on FLT3i (AC220) to sensitize FLT3(ITD)-positive leukemia to PARPi-induced synthetic lethality.

Therefore, we postulated that specific inhibition of DNA repair may dramatically improve the therapeutic outcome of patients with FLT3(ITD)-positive AML (Figure 5.1). The success of PARP inhibitors observed in patients with *BRCA1/2*-mutated breast and ovarian carcinomas supported our hypothesis [278].

5.1.1 The role of FLT3i leading to DSB repair defects in FLT3(ITD)-positive leukemia cells

Despite of the fact that mutations in genes whose products are responsible for DNA repair are rather infrequent in leukemias [23], we and other investigators have reported that several leukemia-related oncogenes (eg. *AML1-ETO, BCR-ABL1, PML-RARA, TCF3-HLF*) have been associated with BRCA1/2 and/or DNA-PKcs deficiencies and therefore, triggerred sensitivity of leukemias to synthetic lethality induced by PARP inhibitors [1, 2, 176, 279, 280]. On the other hand, since activation of FLT3(ITD) oncogenic receptor has been reported to enhance the BRCA1-RAD51–dependent HR, it is unlikely that FLT3(ITD)–positive leukemia cells are vulnerable to PARP inhibitors [277, 281]. In concordance, we detected that FLT3(ITD)-positive cells displayed only modest sensitivity to PARP inhibitors, what is probably due to the accumulation of ROS-mediated DNA damage and/or inhibition of Ku70 protein involved in D-NHEJ [253, 282].

Because AC220 promotes quiescence rather than cytotoxicity [118], it seems to be important to combine AC220 with another compound, leading to a combinational therapy capable to eliminate proliferating and non-proliferating cells. Remarkably, inhibition of FLT3(ITD) receptor by AC220 caused early and dramatic down-regulation of selected proteins in HR (BRCA1, BRCA2, PALB2, and RAD51) and D-NHEJ (LIG4) pathways of DSB repair, what resulted in simultaneous inhibition of HR and D-NHEJ activities (“BRCAness/DNA-PKness” phenotype). Numerous major intracellular signaling pathways, including PI3K/AKT, RAF1/MEK, and JAK2/STAT5, are activated in FLT3(ITD)-positive AML cells [283, 284]. Our studies using selective inhibitors: PI3Ki, RAF1i, JAK1/2i suggest that active JAK2/STAT5 stimulates expression of BRCA1, RAD51, and LIG4; PI3K/AKT promotes expression of RAD51 and LIG4; and RAF1/MEK pathway does not regulate expression of these DSB repair proteins in FLT3(ITD)-positive cells. The mechanisms responsible for FLT3i-mediated down-regulation of BRCA2 and PALB2 have not been understood and require further interpreted investigations. Additionally, AC220–induced deficiencies of DSB repair

proteins were validated by AC220–dependent reduction of DSB repair activities in both HR and D-NHEJ.

5.1.2 The role of FLT3i sensitizing FLT3(ITD)-positive leukemia cells to PARPi *in vitro* and *in vivo*

We have recently reported that OTK inhibitors including JAK1/2i (ruxolitinib) and ABL1i (imatinib) caused defects of HR and D-NHEJ pathways in DSB repair, which eventually leads to elevated efficacy of PARPi–induced synthetic lethality in JAK2(V617F)–positive MPN cells [177] and BCR-ABL1–positive CML cells [178], respectively. In the current study, as consistency, another tyrosine kinase inhibitor (TKi) - FLT3i (AC220) induced down-regulation of DSB repair proteins in both HR and D-NHEJ pathways, and as a consequence, sensitized FLT3(ITD)–positive leukemia cells to PARP inhibitors (olaparib, BMN673). This observation was confirmed by increased DSB response via γ-H2AX, accumulation of DSBs and cell death. The AC220-mediated effective PARPi sensitivity was obtained in every cell types including cell lines as well as murine and human primary cells expressing FLT3(ITD). Moreover, the anti-leukemia effect of combined treatment [AC220 + PARPi] was enhanced by addition of standard chemotherapy used in AML such as doxorubicin. More importantly, combinational treatment of AC220 with PARPi did not cause cytotoxic effect on normal hematopoietic cells from healthy donors, suggesting that the strategic therapy of [AC220 + PARPi] can be potentially applied for clinical trials.

It has been reported that G_0-arrested FLT3(ITD)-positive leukemia cells display inappropriate RAD51 expression and HR activity [277]. Therefore FLT3i-mediated inhibition of RAD51 in HR pathway in these cells may cause the “BRCAness” phenotype and initiate PARP1i-mediated synthetic lethality in FLT3(ITD)-positive quiescent leukemia cells. Not only *in vitro*, but also *in vivo*, AC220 promoted the cytotoxic effect of PARPi (BMN673) against leukemia in both peripheral blood leukocytes and bone marrow cells, and prolonged the survival of leukemia-bearing mice receiving individual BMN673. However, the anti-leukemia effects of individual AC220 and [PARPi + AC220] were limited in bone marrow cells of leukemia-xenograft mice. This phenomenon can be elucidated that the bone marrow microenvironment (BMM) with supportive involvement of stromal cells in hypoxia, protects leukemias against TKi [110, 220, 221]. Although the murine BM niche may contain mismatches with human leukemia cells,

expression of essential human growth factors in bone marrow of immunodeficient NRGS mice possibly results in attenuation of the TKi. Therefore, in BMM condition, additional treatment of PARPi is absolutely necessary to reverse the BMM-dependent resistance against FLT3i. Moreover, since LSCs reside in the bone marrow [200, 201, 285], the hCD45-positive cells eliminated in the bone marrow may likely contain LSCs. Such anticipation was validated by the extensive lifespan of secondary recipients re-engrafted with bone marrow cells from mice treated by [AC220 + BMN673] in the initial treatment.

In conclusion, we postulated that AC220-induced deficiencies of DSB repair pathways sensitize FLT3(ITD)-positive AML cells to synthetic lethality triggered by PARP inhibitors. Therefore, activation of FLT3(ITD) oncogenic receptor could be considered a precision medicine marker to identify patients with AMLs possibly getting beneficial from a combined therapeutic regimen of the FLT3i with PARPi. Moreover, TKi-mediated inhibition of DSB repair, which sensitizes malignant cells to PARP1-triggered synthetic lethality, can also be demonstrated in tumors expressing oncogenic tyrosine kinases. This speculation is supported by our recent study reporting that JAK1/2i (ruxolitinib) causes the "BRCAness/DNA-PKness" phenotype, sensitizing MPN cells to PARPi-mediated synthetic lethality [177].

5.2 The significance of bone marrow microenvironment in the resistance to PARPi–mediated synthetic lethality

Experimental results and clinical trials have indicated that tumor cells possibly acquire PARPi resistance during treatment, which is usually associated with restoration of HR, loss of PARP1 expression and/or loss of DSB end resection regulation (reviewed in [286]). Here we reported that BRCA-deficient leukemia cells, which are theoretically sensitive to PARPi in peripheral blood microenvironment (PBM) *in vitro* and peripheral blood leukocytes *in vivo*, but remained refractory to PARPi in conditions mimicking BMM. In our established BMM conditions, stromal cells-derived TGF-β1 stimulated hypoxia-induced overexpression of TGFβR2 on leukemia cells, to activate TGFβR serine/threonine kinase-dependent SMAD2/3 canonical signaling pathway in malignant cells. Inhibition of TGFβR kinase and SMAD3, down-regulated key proteins in DSB response (ATM), HR (BRCA1, BRCA2) and D-NHEJ (DNA-PKcs, Ligase IV) pathways. This reduced HR and D-NHEJ activities but promoted efficiency of

PARP1-dependent Alt-NHEJ and therefore, restored sensitivity of leukemias to PARPi-induced synthetic lethality in the bone marrow microenvironment (Figure 5.2). We obtained consistent and reproducible results, starting from verification of the BMM-induced resistance to PARPi in leukemias by BM stromal cells to understanding the molecular mechanism of the resistance via TGFβ1-TGFβR signaling pathway in BMM. To clearly interpret and validate the proposed hypothesis, we discuss all the obtained data below.

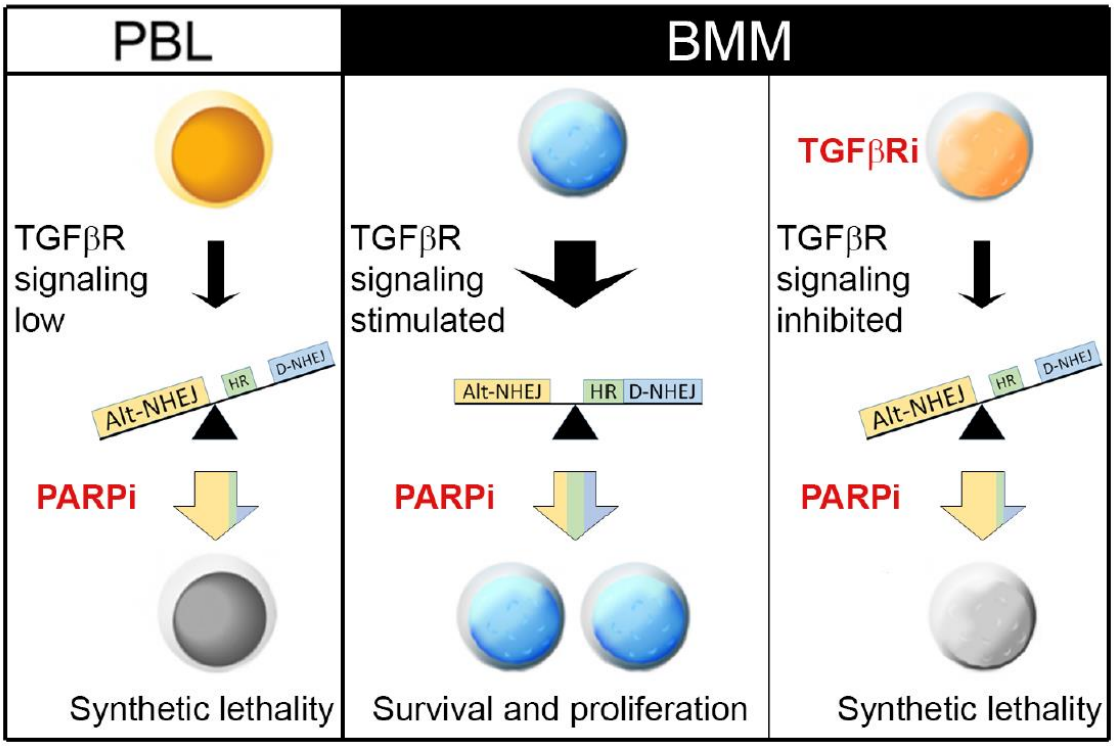

Figure 5.2. Model of exploitation on inhibition of TGFβR–mediated signaling pathway to restore efficacy of PARPi–induced synthetic lethality in leukemia cells in the BMM. **PBL**: Peripheral blood leukocytes, **BMM**: Bone marrow microenvironment.

5.2.1 Bone marrow microenvironment is a novel mechanism inducing resistance to PARPi in leukemias

Resistance against PARPi-induced synthetic lethality has been reported in several different mechanisms including deficiency of PARP1 expression due to mutations, gaining proficient HR and/or D-NHEJ and emerging alternative pathways to repair DSBs [286]. Therefore, in this study, to demonstrate a novel mechanism leading to PARPi resistance in leukemias mediated by BMM, clonogenic test was simultaneously performed in PBM and BMM-dc to assess a direct comparison between these two culture conditions. In terms of cell types, we used human/murine primary leukemias associated with deficiencies of BRCA1/2, that are

theoretically sensitive to PARPi in normoxic condition. Indeed, as expected, the malignant cells were eliminated by PARP inhibitors (olaparib and talazoparib) in PBM accomplished with sufficient oxygen uptake (17%). On the contrary, in BMM-dc, every leukemias were refractory to PARP inhibitors, verifying that BMM uniquely induces resistance to PARPi in leukemias without unexpected impact in PBM. Additionally, when it comes to categories of PARP inhibitors, initial observation of the resistance was obtained with olaparib which is the first FDA-approved PARPi. Besides olaparib, talazoparib is the next generation FDA-approved PARPi with stronger potency and higher selectivity than olaparib because of PARPs trapping capacity. Our results showed that leukemias were also did not respond to talazoparib inBMM-dc but very sensitive in PBM, suggesting that BMM-induced PARPi resistance is independent on the distinct functionality of PARPi such as inhibition of PARylation catalysis exerted by olaparib and PARPs trapping capacity in talazoparib.

Numerous reports described leukemias, including AML and CML, as malignances remodeling bone marrow niche by regulating the physiological and differentiated characteristics of normal BM-derived stromal cells, to support leukemic stem cell phenotype, survival, progression and chemotherapeutic resistance [217, 236, 237, 285, 287]. In the current studies, healthy bone marrow stromal cell lines (HS-5, OP-9) and primary human bone marrow mesenchymal stromal cells (hBMSCs), mediated PARPi resistance in BMM. On the other hand, HS-5 did not significantly promoted the survival of human $Lin^{-}CD34^{+}$ normal hematopoietic cells, refractory against PARPi in BMM. Taken together, it is possible that malignant cells regulated the normal stromal cells (HS-5, OP-9, hMSCs), leading to reprogramming of BM niche to protect leukemia cells against PARP inhibitors. Moreover, the BMM constituted with primary leukemia stromal cells derived from leukemia patients/mice (autologous BMM) also induced resistance to PARPi. This confirms a discussion in which primary leukemia cells exploit the support of autologous leukemic stromal cells in the BM niche to survive under cytotoxic effect of PARPi.

Tumor microenvironment has been reported to enhance DNA repair efficiency supported by alterations of cell signaling pathways in tumor cells, especially cancer stem cells [260]. Since bone marrow is a specific leukemia microenvironment [285], it is possible to promote DNA repair in leukemia cells. Our data showed that treatment with either individual PARPi (olaparib) or in combination with FLT3i, quizartinib (AC220), caused less accumulation of

DSBs in murine primary FLT3(ITD);Tet2-/- AML–like cells in BMM compared with PBM. Moreover, the percentage of DSBs in both PBM and BMM coincided to PARPi sensitivity of leukemia cells in clonogenic assay. Taken together, it is clearly thought that BMM enhances the DSB repair activities in leukemia, leading to independence of malignant cells on PARP1-dependent Alt-NHEJ to survive. Therefore, leukemias remained refractory against PARP inhibitors in the bone marrow microenvironment.

5.2.2 The significance of bone marrow stromal cells-derived cytokines in PARPi resistance in leukemias

In general, BM niche supports survival, self-renewal and differentiation of HSCs. Two major components involved in such remodeling include: (1) direct stromal cells-HSCs contact which promotes bone marrow homing of HSCs mediated by cell migration and HSCs maintenance [194, 196], (2) bone marrow-derived cytokines which regulate self-renewal and differentiation of HSCs into other hematopoietic cells as well as maintain the balance of proliferation and differentiation (reviewed in [192]). As hematopoietic malignancies, leukemia cells have capacity to exploit these two important factors of BMM to enhance the stemness phenotype, survival, progression and especially refractory against chemotherapy/TKi. For example, in OTK-positive leukemias, direct contact of BM stromal cells with leukemia cells have been reported to cause resistance to TKi. The blockage of that interaction restored sensitivity of leukemia to TKi [110, 217, 218, 220, 221]. As BMM-dc was observed to induce resistance to PARPi-mediated synthetic lethality in BRCA-deficient leukemia cells, the direct contact of stromal cells with leukemia cells and/or bone marrow-derived cytokines are likely responsible for the resistance. To address this hypothesis, BMM-dc is considered to contain the supporting role of both BMM components and BMM-idc with transwell cell culture insert was established to evaluate the unique impact of stromal cells-derived cytokine in PARPi resistance. We found that both BMM conditions induced resistance to PARPi, but without a significant difference in survival of leukemia cells between these two BMM conditions. This suggests that bone marrow-derived cytokine is the major component contributing in the resistance to PARPi in leukemia in BMM. This conclusion was later validated by analysis of BMM-cm in comparison with BMM-dc, leading to a convincing evidence that further suported direction of our studies to understand the mechanism of PARPi resistance in BMM.

Leukemia cells have been reported to regulate normal bone marrow stromal cells to enhance quiescent leukemia stemness phenotype [262]. Furthermore, LSCs have a dual role in hematopoietic cancer progression as disease-initiated and therapy-refractory cells [259]. Additionally, one of the major features of tumor microenvironment to support cancer stem cells is enhancing DNA repair [260]. In our study, cell cycle analysis revealed elevated accumulation of G_0/G_1 cells in BMM-dc, meanwhile G_2/M cells in PBM were almost doubled to the counterparts in BMM-dc. As consistency, both BMM-dc and BMM-idc induced quiescent LSCs refractory against PARPi. Moreover, the number of quiescent LSCs between BMM-dc and BMM-idc was relatively equal. Taken together, bone marrow stromal cells-derived cytokine can be rationally considered as the major inducer of resistance to PARPi not only in proliferating LPCs but also in quiescent LSCs. From this translational data, based on the abundance of CXCL12 and TGF-β1 cytokines produced in both healthy BM niche and remodeled leukemic BMM, together with their involvement in BMM-dependent resistance to chemotherapy and TKi [238, 240], it was sensible to investigate molecular mechanism of resistance to PARPi in BMM via TGFβ1-TGFβR and CXCL12-CXCR4 signaling pathways.

5.3 Molecular mechanism of resistance to PARPi in leukemias in the bone marrow microenvironment

5.3.1 CXCL12-CXCR4 signaling pathway in the bone marrow microenvironment

After confirming that bone marrow stromal cell-derived cytokines play the major role promoting PARPi resistance in BMM, we investigated the most dominant cytokine-receptor signaling pathway reported in normal hematopoietic niche – the CXCL12-CXCR4 axis. In fact, CXCL12 is the most abundant stromal cells–derived cytokine in hematopoietic bone marrow, prevalently produced by CXCL12-abundant reticular (CAR) cells, together with the second source coming from vascular endothelial grow factor (VEGF) derived from endothelial cells [196, 288, 289]. Moreover, the signaling pathways triggered by the stimulation of CXCL12 to corresponding receptor, CXCR4, have been described to induce failure of chemotherapy and TKi in CML/AML co-cultured with monolayer stromal cells in hypoxia [110, 217, 218, 220, 221]. As the consequence, blockage of the signaling pathway by CXCR4 inhibitors or antagonists restores the sensitivity of leukemia cells. Additionally, besides CXCL12, TGF-β1 has been shown a very important stromal cells-derived cytokine in hematopoietic BM niche.

Moreover, the TGF-β signaling is also involved in diverse physiological processes of hematopoiesis [199]. The combination of CXCR4 inhibitor/antagonist with TGF-β signaling inhibitor has been an effective therapeutic strategy to sensitize leukemia cells to TKi and chemotherapy in the BMM [238, 240]. In our study, as expected, CXCR4 inhibitor (AMD3100) or anatagonist (WZ811) regained efficacy of TKi (quizartinib) in FLT3(ITD);Tet2-/-(mutant) AML-like cells but was not capable to exert the similar effect on PARPi (olaparib). Alternatively, TGF-β signaling inhibitors re-sensitized leukemia cells to both olaparib and quizartinib. More interestingly, combinational therapy of CXCR4 inhibitor/antagonist with TGF-β signaling inhibitors did not promote potency of [olaparib +/- quizartinib]. Taken together, it can be apparently confirmed that CXCL12-CXCR4 signaling pathway does not induce resistance to PARPi in leukemias in BMM.

To interpret such observation, in terms of signaling cascade, the CXCL12-CXCR4 axis activates G-coupled receptor kinase which phosphorylates substrates to trigger Ca^{2+} channel as the canonical pathway. This promotes cell migration and homing retention in bone marrow, leading to elevated stromal cells – leukemia/hematopoietic cells interaction [216]. Moreover, the bone marrow-mediated cellular interaction has been shown not to be involved in PARPi resistance in leukemias, therefore, abrogation of CXCL12-CXCR4 signaling pathway rationally did not restore PARPi sensitivity in leukemia cells. Last but not least, up to now, inhibition of CXCL12-CXCR4 signaling pathway has not been reported to result in DSB repair deficiencies in neither leukemias nor other solid tumors yet. Instead, inhibition of CXCR4 has been revealed to cause increased reactive oxygen species (ROS) and oxidative stress, which may lead to oxidative DNA damages including DSBs without deficiencies in DSB repair pathways [290]. In our experiment, with DSB repair defects caused by quizartinib (AC220), together with elevated DSBs induced by CXCR4i, combinational treatment of olaparib and quizartinib significantly eliminated leukemia cells in BMM compared with individual quizartinib when CXCL12-CXCR4 axis was inhibited.

5.3.2 Activated TGFβ1-TGFβR signaling pathway in the bone marrow microenvironment induces resistance to PARPi in leukemias

After validating that CXCL12-CXCR4 signaling pathway is not associated with the resistance to PARPi in BMM, the TGFβ1-TGFβR signaling pathway becomes the most potential

candidate for the resistance based on preliminary results of TGFβRi (SB431542) and TGF-β1 neutralizing antibody (1D11), which restored PARPi sensitivity of leukemia cells. We confirmed that TGF-β1 is produced by stromal cells in our established BMM condition but the amount of TGF-β1 was not one of the top secreted cytokines (data not shown in this thesis). Therefore, the existence of an overexpressed TGFβR kinase is required, which can be stimulated by TGF-β1, to activate the entire signaling pathway in BMM. Indeed, TGFβR2 was overexpressed on leukemia cells in BMM-dc. Furthermore, additionally to BMM-dc condition, TGFβR2 was also overexpressed in BMM-idc and especially in hypoxia, without presence of stromal cells. This suggests that the overexpression of TGFβR2 in leukemia cells is not regulated in a stromal cell-dependent manner, however, it only depends on the level of oxygen intake. Consistently, hypoxia has been reported to increase TGFβR1/pSMADs signaling pathway through the hypoxia–induced factor 1/2α (HIF-1/2α) in dermal fibroblasts [291] and especially in lung and renal carcinoma cells [292, 293]. Meanwhile, in our data, for the first time, low oxygen (1%) has been described to induce overexpression of TGFβR2, which is directly oligodimerized by BM stromal cells-derived TGF-β1 to activate entire TGFβR-mediated signaling pathway. In BMM, with stimulation of TGF-β1 produced by stromal cells intoh hypoxia-induced overexpression of TGFβR2 on leukemic cells, the TGFβ1-TGFβR signaling pathway is activated in BMM-dc via enhancement of phosphorylated SMAD2 (pSMAD2) and SMAD3 (pSMAD3) as TGFβR-mediated canonical pathway.

Tumor microenvironment (TME) has been considered an unfriendly condition for cancer cells because of its common and important component as hypoxia, which has been proved to induce genomic instability by elevation of DNA damages including DSBs (reviewed in [294, 295]). To respond to the elevated lethal DSBs derived from hypoxic condition in TME, tumor cells, including cancer stem cells, exploit activation of their intracellular signaling pathway to alter/promote DSB repair pathway to survive under an unfavorable environment [296]. DSB response is mediated by ATM kinase by phosphorylating itself at Ser1981 (pATM), leading to signal transduction to phosphorylation of histone H2AX at Ser139, also known as γ-H2AX [130]. Upon introduction of DSBs, entire pathway of DSB response is activated to recruit essential proteins to repair spontaneous DSBs in TME. In our result, the ATM, pATM and γ-H2AX levels remained unchanged between PBM and BMM-dc, suggesting over-activation of intracellular signaling pathways in BMM, which enhance DSB repair activity. Moreover,

TGFβ1-TGFβR signaling pathway has been reported to be involved not only in DSB response mediated by ATM [244, 297], but also in γ-irradiated sensitivity [243, 245-247, 249, 250] as well as DSB repair pathways [241, 242], in both normal and solid tumor cells. Therefore, it is possible that TGFβ1-TGFβR signaling pathway promotes DSB repair activities to maintain equal expression of DSB response proteins between PBM and BMM-dc, which will be further discussed in later section.

Since TGFβ1-TGFβR signaling pathway is activated in our established BMM, together with the preliminary observation of TGFβRi (SB431542) and TGF-β1 neutralizing antibody (1D11) re-sensitizing leukemia to PARPi (olaparib), we examined genetic down-regulation of each component in the signaling pathway in BRCA-deficient leukemias. Moreover, external recombinant TGF-β1 was used in hypoxia as an alternative source of bone marrow stromal cells–derived TGF-β1. According to reduction of stromal cells-derived TGF-β1 production in BMM or down-regulation TGFβR2 on leukemias which restored efficacy of PARPi in BMM, as well as supplementation of recombinant TGF-β1 in hypoxia inducing [PARPi +/- TKi] resistance in leukemia cells, it is clearly obvious that resistance to PARPi in BMM requires simultaneous involvement of stromal cells-derived TGF-β1 and hypoxia-induced overexpression of TGFβR2 to trigger the entire TGF-β signaling pathway. Additionally, the supporting effect of recombinant TGF-β1 ligand in hypoxic condition highlights the indispensable role of stromal cells–derived TGF-β1 in the resistance in BMM because even in hypoxia, TGFβR2 was overexpressed but malignant cells were still eliminated by [PARPi +/- TKi].

5.3.3 Therapeutic potential of TGF-β signaling pharmacological inhibitors to PARPi in leukemias

We are the first group describing the involvement of TGF-β signaling pathway inducing resistance to PARPi-mediated synthetic lethality in leukemias in BMM. In which, TGF-β1 and TGFβR are indispensable to activate entire TGF-β signaling pathway and responsible for the resistance to PARPi in leukemias in BMM. Moreover, pharmacological inhibitor against TGFβR serine/threonine kinase is commercially available and has been approved by FDA in clinical trials of some solid tumorigenic cancers. From this, we tested the influence of TGFβR inhibitor (SB431542) on sensitivity of leukemias to PARPi in BMM. In peripheral blood, TGF-β

signaling pathway has been inactive not only in leukemia patients [298], but also in individuals with multiple sclerosis [299]. Therefore, TGFβRi (SB431542) was not effective in PBM, but exerted inhibitory effect against the signaling pathway in BMM. This result is absolutely essential to verify that TGFβ1-TGFβR signaling pathway uniquely induces resistance to PARPi in leukemia cells in BMM. Meanwhile, in PBM, without activation of the signaling, the malignant cells were eliminated by PARPi.

Besides verifying SB431542, using of other TGFβRi (galunisertib) also restored efficacy of PARP inhibitors (olaparib and talazoparib). This suggests that restoration of PARPi efficiency mediated by inhibition of TGF-β signaling pathway, is independent on different categories of TGFβR kinase inhibitors. In addition, inhibition of TGFβR kinase recovered effectiveness of PARPi in both stromal cell line and allogeneic/autologous BMM. Furthermore, in the BMM, AML cells have been reported to induce osteoblast differentiation from mesenchymal stromal cells (MSCs) and in turn, MSCs produce elevated amount of TGF-β1 to support stemness phenotype of AML cells and resistance to chemotherapy [237]. Altogether, it can be well predicted that in our experiment, leukemia cells reprogrammed BMM and regulated stromal cells to enhance TGFβ1-TGFβR signaling pathway to induce resistance to [PARPi +/- TKi] in BMM.

The TGF-β signaling pathway, has been not only associated with DSB repair shown in our study and other cancers [241, 242], but also involved in DNA damage response induced by γ-irradiation. In fact, inhibition of TGFβR kinase by LY2109761 or LY364947 has been reported to enhance radio-sensitivity of solid tumors including glioblastoma [248-250], non-small cell lung cancer [247] and breast cancer [245]. Taken together, with the activation of TGFβ1–TGFβR signaling pathway in BMM, TGFβR inhibitors can be verified in BRCA-deficient solid tumors metastasizing into bone marrow to improve efficacy of PARPi on those tumor cells.

Getting along with targeting TGFβR by pharmacological inhibitors, TGF-β1 has been also eliminated in BMM by TGF-β1 neutralizing antibody (1D11) to deactivate entire signaling cascade. The neutralizing antibody has combined with CXCR4 antagonist to re-sensitize AML cells to cytarabine in the co-cultured condition with bone marrow mesenchymal stromal cells [238] or using individually to promote radio-sensitivity against prostate cancer [246]. In our study, with addition of the neutralizing antibody in culture, sensitivity of leukemia cells to [PARPi +/- TKi] was restored in BMM, suggesting that besides TGFβR kinase inhibitors,

1D11 neutralizing antibody is also a very effective weapon to reverse the resistance to [PARPi +/- TKi] in leukemias in BMM.

TGFβRi (SB435142) successfully re-sensitized leukemias to [PARPi +/- TKi] in BMM, meanwhile, significantly, the inhibitor did not induce cytotoxic effect of [PARPi +/- TKi] on bone marrow cells from healthy donors. This observation can be elucidated as healthy bone marrow cells are theoretically resistant to PARPi, therefore, the cells do not depend on activated TGFβ1-TGFβR signaling pathway in the BMM to survive under cytotoxic effect of PARPi. Thus, this promising result potentially leads to development of a novel strategic therapy that can be applied in clinical trials without cytotoxic effect on healthy cells. Moreover, another TGFβRi (galunisertib), which has been verified in clinical trials in several solid tumors [300-305], also restored PARPi sensitivity of leukemia in BMM. Taken together, it is well possible to introduce TGFβR kinase inhibitor (galunisertib), into leukemia patients currently receiving PARPi in treatment to enhance therapeutic outcome and prevent drugged resistance as well as disease relapse.

We have found that BMM conditions induced quiescent LSCs, remaining refractory to PARPi (olaparib). In comparison between PBM and BMM, quiescent LSCs population increased in BMM, however, the cell cycle distribution remained unaffected with/without addition of SB431542 in BMM. Moreover, SB431542 reduced number of quiescent LSCs under treatment of [PARPi + TKi] in BMM. Taken together, [SB431542 + PARPi + TKi] eliminated quiescent LSCs in BMM, which are responsible for resistance to [PARPi + TKi]. LSCs have been described to have dual role in leukemia progression when they act as disease-initiated and therapy-refractory cells [259] and upon responsive effect against TKi, quiescent LSCs have been illustrated to induce resistance to TKi in BMM [118]. In our study, as expected, in individual treatment with TKi (quizartinib), quiescent LSCs were increased in BMM and SB435142 was incapable to alter this resistance. However, under combination [TKi + SB431542 + PARPi], quiescent LSCs were remarkably reduced, whereas, without SB435142, quiescent LSCs maintained refractory to [TKi + PARPi]. It is suggested that the inhibition of TGFβR kinase by SB435142 in BMM in combinational treatment of [PARPi + TKi], eliminates the most refractory population of leukemia cells. This leads to enhanced efficacy of [PARPi +/- TKi] in BMM as well as an effective prevention against disease relapse. The importance of the eradication against LSCs will be discussed later, together with *in vivo* data.

5.3.4 The significance of TGFβRi (SB431542) in anti-leukemia effect of [PARPi + TKi] in the bone marrow *in vivo*

Currently, to examine efficacies of chemotherapy, TKi or even PARPi in eliminating leukemia or other hematopoietic malignancies *in vivo*, human leukemia/malignant cells are transplanted into NSG or NRSG mice expressing essential human growth factors supporting the human leukemic engraftment as well as survival and proliferation of transplanted cells in mice. However, such method evaluates the influential effects of inhibitors directly on the malignant cells, without supporting of stromal cells in bone marrow *in vivo* because the relationship between human hematopoietic malignant cells and murine bone marrow niche remains unknown. In fact, recently, to deal with this problem, several groups have demonstrated their methods to establish the humanized BM niche in mice [212-215], before transplantation of leukemia into the humanized niche. However, this method is very time consuming to completely accomplish the niche *in vivo* together with leukemia transplantation and treatment and was not possible to include into current study. Moreover, the reproducibility of this model can be very low, due to many different factors which can affect the final effect. Thus, even if we are currently working on establishment the humanized BM niche model, it will be demonstrated in another research project in the future. Therefore, in *in vivo* experiment of this study, to guarantee obtaining supportive effect of bone marrow stromal cells on leukemias in transplanted mice, the murine primary AML/CML-like cells were subcutaneously injected into immunodeficient animals (SCID mice).

The results presented here and recent reports from our group have shown tyrosine kinase inhibitors including quizatinib and imatinib, promoted efficacy of PARPi *in vivo* in FLT3(ITD)-positive AML and BCR-ABL1-positive CML [178], in peripheral blood leukocytes (PBL). On the other hand, the enhanced efficiency of PARPi was less profound in bone marrow, suggesting that BMM performs a protective effect against the treatment. Expectedly, in current study, we obtained similar anti-leukemia effect of [PARPi + TKi] in both PBL and bone marrow cells (BMC) but interestingly, with addition of SB435142 in [PARPi + TKi] treatment, engrafted leukemia cells were dramatically eliminated in BMC and splenocytes (SPL) which is also considered a leukemia microenvironment. On the other hand, SB431542 did not enhance effectiveness of [PARPi + TKi] against leukemias in PBL, revealing the consistency between

results obtained *in vitro* and *in vivo*. Thus, the result *in vivo* completely supports and validates all data obtained *in vitro*.

After verifying the impact of SB431542 on anti-leukemia effect of [PARPi + TKi] in in PBL, SPL and BMC, we examined the surival of leukemia-bearing mice after the treatment regime. In fact, engrafted mice treated with three inhibitors used in combination had significantly longer lifespan than the mice receiving [PARPi + TKi]. Additionally, the more important result was achieved in leukemia re-transplanted recipients, confirming effect on the LSCs. Previous publications with the re-engrafted *in vivo* studies have reported that LSCs, as leukemia-initiated cells, are responsible for leukemic relapse in animals in post-treatment [259, 306]. In our result of secondary leukemia transplantation with BMCs in mice from initial treatment regimen, survival of mice receiving BMCs treated by [PARPi + TKi] were not significantly different with the animals in control or individual SB431542, suggesting LSCs caused resistance to [PARPi + TKi], leading to disease relapse, shortening survival of secondary recipients. In contrast, re-transplanted mice with BMCs treated with [SB435142 + PARPi + TKi] had approximately doubled survival over mice injected with BMCs from [PARPi + TKi]-treated animals, suggesting inhibition of TGFβR kinase by SB435142 together with [PARPi + TKi] eliminates LSCs to prevent leukemic relapse after treatment postponement. As consistency, the data on re-transplanted mice *in vivo* entirely validates the result *in vitro*.

We observed that SB431542 enhanced anti-leukemia effect of [PARPi + TKi] in BMCs of leukemia-bearing mice, by collecting cells inside femoral samples and analyzing by flow cytometry. To physically confirm inhibitory effect of SB431542 in enhanced efficacy of [PARPi + TKi] against transplanted leukemia-like cells in the bone marrow niche of xenograft mice, immunohistochemical analysis was conducted to visualize GFP(+) leukemia cells inside femoral specimens via usage of anti-GFP antibody. This data provides a convincing evidence to confirm the *in vitro* results of which SB431542 regains sensitivity of leukemias to [PARPi +/ TKi] in BMM.

TGF-β signaling pathway has been described to perform a dual role in cancer cells, depending on stages of cancer [229]. In early-stage tumors, TGF-β pathway acts as a tumor suppressor to promote cell cycle arrest and apoptosis [307-309], meanwhile in advanced-stage tumors, TGF-β pathway performs a role to induce tumor progression and metastasis [307-309]. Besides advanced-stage tumor, in tumor microenvironment, as a TGF-β cytokine

produced by stromal cells, TGF-β signaling pathway provides pro-tumoral effects by altering cellular signal in tumor cells and mediating stromal cells-tumor cells interaction [310]. Therefore, there are conflicting reports about the impact of TGFβ1-TGFβR signaling pathway on DSB repair indicating positive and negative effects on expression of the genes and/or activities of HR and/or D-NHEJ/Alt-NHEJ pathways (reviewed in [311]). These discrepancies most likely depend on distinct epigenomes (distinct gene expression programs), tumor tissue type (hematologic malignancies, carcinomas or non-transformed cells) and tumor microenvironment conditions (normoxia vs. hypoxia, presence or absence of stromal cells) [312, 313]. In our study, in *in vitro* experiments with establishment of BMM by co-culture of leukemias with BM stromal cells in hypoxia, inhibition of TGFβR kinase restores sensitivity of leukemias to [PARPi +/- TKi]. Importantly, the *in vitro* data was validated by the demonstration that pharmacological inhibitor of TGFβR kinase (SB431542) enhanced anti-leukemia effect of [PARPi + TKi] in BMC but not in PBL of SCID mice *in vivo*. This data, again, emphasizes the important role TGF-β signaling pathway as cancer promoter in the BMM or generally in tumor microenvironment.

5.3.5 The significance of TGFβRi (SB431542) inducing "BRCAness/DNA-PKness" phenotype in leukemia cells in the bone marrow microenvironment

After obtaining the *in vitro* and *in vivo* data showing that inhibition of TGFβR kinase restores or enhances efficacy of [PARPi + TKi] against leukemias in BMM (*in vitro*) or bone marrow cells (*in vivo*), it is required to understand the distinct mechanism how TGFβRi sensitizes leukemias to [PARPi + TKi]. Because PARPi-induced synthetic lethality is directly associated with DSB repair pathways, we investigated the mechanism by measuring [PARPi + TKi]-induced DSBs, DSB repair activities and DSB repair protein expression under inhibition of TGFβR by SB431542 in BMM.

Inhibition of TGFβR kinase triggered by SB435142 in BMM caused down-regulation of DSB repair proteins in HR (BRCA1, BRCA2) and D-NHEJ (DNA-PKcs, Ligase IV) but not proteins in Alt-NHEJ (PARP1, Ligase III, Polθ), leading to reduced DSB repair activities of HR and D-NHEJ pathways. Therefore, total DSB repair responsibility was switched to PARP1-dependent Alt-NHEJ. Moreover, the down-regulation in DSB repair proteins in HR and D-NHEJ pathways by SB431542, leading to restoration of PARPi sensitivity of leukemias in BMM, is convincingly

validated and supported by the mechanism in which FLT3i (AC220) caused down-regulation of proteins in both HR and D-NHEJ and remarkably promoted anti-leukemia effect of PARPi in FLT3(ITD)-positive leukemia cells discussed at 5.1. More importantly, in BMM, SB435142 did not promote cells in G_2/M phase based on the cell cycle analysis presented in our current study as well as by other group [241]. Since enhancement of cell proliferation has been associated with increased genomic instability [314] and decreased DNA repair [315], therefore, down-regulation of DSB repair proteins in HR and D-NHEJ in BMM due to SB435142 is not dependent on cell cycle alteration-induced elevated DNA damages and DNA repair deficiencies.

There are couple of discrepancies in DSB repair protein down-regulation, between influences of FLT3i (AC220) in FLT3(ITD)–positive leukemia cells in normoxic condition and TGFβRi (SB431542) in BMM, reflecting the distinction of BMM. At first, TGFβ1-TGFβR signaling pathway has been shown to be activated in BMM. Moreover, the TGFβ1-TGFβR pathway has been reported to be involved in DSB response mediated by ATM [244, 297]. As consequences, SB431542 caused down-regulation of ATM in leukemia cells in BMM, leading to attenuation of ATM downstream signal via γ-H2AX. It is also the reason why we did not conduct assay to detect γ-H2AX in nucleus of leukemia cells in BMM with addition of SB431542. More interestingly, also in BMM, expression level of ATR, the protein involved in SSB response [316], remained unchanged under SB431542. Additionally, ATM-deficient lymphoid tumor cells have been eliminated by PARP inhibitors in both *in vitro* and *in vivo* [317, 318]. Altogether, it is suggested that TGFβRi negatively regulates ATM-mediated DSB response, which directly triggers PARPi-induced synthetic lethality in leukemia cells in BMM.

In terms of the second discrepancy in affected DSB repair proteins, we observed down-regulation of DNA-PKcs, which has a dual role as serving in DNA damage response and performing a major function in D-NHEJ pathway of DSB repair. Previous reports have shown PARP inhibitors eradicating DNA-PKcs–deficient leukemias in *in vivo* as well as *in vitro* [176, 319] and a novel DNA-PKcs inhibitor (AZD7648) enhancing radiation and PARPi sensitivity in ATM–deficient cells *in vitro* and *in vivo* [320]. Moreover, according to the cell cycle analysis performed in this study, quiescent (G_0/G_1) cells stage was induced in BMM. Specifically, the DSBs in quiescent cells are repaired only by D-NHEJ pathway. Therefore, down-regulation of DNA-PKcs due to inhibition of TGFβR kinase switched leukemia cells depending on PARP1-

mediated Alt-NHEJ to survive. This leads to enhanced anti-leukemia effect of [PARPi +TKi] *in vivo* and restoring efficacy of [PARPi + TKi] in BMM *in vitro*, especially in case of ATM down-regulation due to TGFβRi. The importance of DNA-PKcs and another protein in D-NHEJ pathway, Ligase IV, which was also down-regulated by SB431542, is discussed later.

It is worthy a consideration that genetic aberrations may dictate the role of TGFβ1-TGFβR kinase-dependent signaling pathway in DNA damage response. We postulate that TGFβRi reduces HR and D-NHEJ activity and re-sensitizes leukemias to [PARPi +/- TKi] in BMM. Of note, mutation-dependent disruptions of FANCD2, FANCA and FANCG [Fanconi Anemia (FA) pathway] have been revealed to cause transcriptional alteration resulting in hyperactive TGF-β signaling in hematopoietic stem cells, and inhibition of TGF-β pathway in FA cells diminished D-NHEJ but promoted HR activity [321]. Therefore, anti-leukemia effect of TGFβRi combined with PARPi may be limited in bone marrow of FA patients.

5.3.6 The role of TGFβR-downstream mechanism inducing resistance to PARPi in leukemia

5.3.6.1 The effect of TGFβR-miRNA-182 axis in leukemias in the bone marrow microenvironment

miRNA-182 has been implicated in TGFβR–mediated modulation of HR, leading to demonstration of PARPi-induced synthetic lethality in head and neck squamous cell carcinoma (HNSCC). In fact, inhibition of TGFβR kinase by TGFβRi or HPV-positive proteins (E6/E7) released overexpression of miRNA-182 in HNSCC, leading to suppressed activities of BRCA1 and FOXO3 – a required element for activation of ATM kinase, eventually triggering PARPi-induced synthetic lethality in the tumor cells [241]. Furthermore, miRNA-183 which belongs to the miRNA cluster together with miRNA-182, has been reported to modulate the TGFβ1-SMADs signaling pathway in cancer [322]. Since expression of neither miRNA-182 nor miRNA-183 was affected by CRISPR/Cas9-mediated down-regulation of TGFβR2 in CML-BC K562 cell line and inhibition of TGFβR kinase by SB435142 in AML primary cells in BMM-dc, we postulate that miRNA-182 and miRNA-183 do not associate with resistance to PARPi in leukemia cells in BMM induced by TGFβR–mediated signaling pathway. Moreover, in normoxic condition in which TGF-β signaling pathway is low/not activated, elevated expression of miRNA-182 has been shown to inhibit BRCA1, leading to PARPi sensitivity in two leukemia cell lines HL-60 and especially K562 [323]. Taken together, it can be

considered that the discrepancy between normoxia and BMM related to activation TGF-β signaling pathway, regulates expression of miRNA-182 to down-regulate BRCA1. Besides miRNA-182 and miRNA-183, miRNA-103a was analyzed as a control. In fact, it is a tumor suppressor miRNA, whose expression is changed in variety of cancers including myelodysplastic syndrome. However, it is not located in the cluster with miRNA-182/miRNA-183 and its relation to TGF-β signaling pathway has not been documented [324].

5.3.6.2 The role of TGFβR-SMAD3 in resistance to PARPi in leukemia cells in the bone marrow microenvironment

After excluding role of the TGFβR-miRNA-182 axis from the BMM-dependent resistance to PARPi in leukemias, in concordance with previous reports about relevance between TGFβ1-TGFβR signaling pathway and DNA damage response as well as DSB repair, the TGFβR-mediated canonical downstream signal via SMAD3 was considered a potential mediator for PARPi resistance in BMM. In fact, TGFβR-SMAD2/3 signaling pathway has been revealed to up-regulate Ligase IV to enhance D-NHEJ activity of DSB repair in cells treated by γ-irradiation [242, 243]. Moreover, SMAD2 has been reported to localize on irradiation-induced DSBs to activate ATM kinase activity, responding to the introduced DSBs [325]. Without availability of selective SMAD2 inhibitor, SMAD3i (SIS3) caused down-regulation of five DSB repair proteins (ATM, BRCA1, BRCA2, Ligase IV and DNA-PKcs) which were also decreased under the effect of TGFβRi (SB431542) in BMM, suggesting a novel discovery in which nuclear SMAD2/3/4 complex regulates transcription of genes encoding these five proteins. Therefore, it leads to overexpression of the five proteins when TGFβR-SMAD2/3/4 is activated in BMM. This transcriptional regulation of SMAD2/3/4 was validated by bioinformatics analysis of SMAD2/3/4 putative binding sites, found in promoters of five targeted genes.

In addition, besides associating with DSB repair in BMM, TGFβR-SMAD3 signaling pathway has been reported to be involved in immunosuppressive phenomenon against immune cells such as T cells, B cells and NK cells in tumor microenvironment (TME). While stromal cells-derived TGF-β is an important cytokine in TME to suppress immune cells, especially T cells to support survival of tumor cells [232], the TGFβR-SMAD3 signaling has been revealed to enhance expression of Programmed Cell Death Protein 1 (PD-1) in antigen–specific T cells,

which inhibits activation of T cells [326]. Moreover, SMAD3 has been described to inhibit development of NK cell mediated by E4BP4 to promote cancer progression [327]. Therefore, with single inhibition of SMAD3, two important targets can be simultaneously obtained in TME including enhancing both, efficacy of PARPi-mediated synthetic lethality and activation/development of immune cells to eradicate malignant cells. Furthermore, similarly to TGF-β cytokine, SMAD3 acts as both - positive and negative modulator of cancer progression, depending on cell type and clinical stage of the tumor (reviewed at [328]). In fact, to suppress tumorigenic progression, TGFβR-mediated SMAD3 has been documented to counteract BRCA1. This leads to enhanced radio-sensitivity of tumor cells in normal condition [329], reminding the distinction of BMM in which SMAD3 plays as a cancer promoter by up-regulating BRCA1 to response to DSBs.

Since SIS3 down-regulated DSB repair proteins in concordance with TGFβRi (SB435142), as expected, SIS3 re-sensitized leukemia cells to [PARPi +/- TKi] in BMM and the efficacy of combinational inhibitors was the same as SB435142. Additionally, TGFβR-SMAD2/3 signaling pathway has been described to be deactivated by a dominant negative mutant (D407E) on *SMAD3* gene, in which the mutant impairs both pSMAD2 and pSMAD3, leading to abrogation of TGFβR-mediated signaling pathway [263]. Within inhibitory effect on the pathway, SMAD3(D407E) can be considered a genetic targeting SMAD3, validating the usage of SMAD3i (SIS3). And in fact, expression of the SMAD3 mutant restored efficacy of [PARPi +/- TKi] against leukemia cells in BMM. SMAD3 but not SMAD2 has been illustrated a tumor suppressor protein because of direct involvement into TGF-β–mediated cell cycle arrest and growth inhibition. Therefore, mutants in *SMAD3* gene have been associated to T-ALL [330], gastric cancer [331], colorectal cancer [332]. In concordance with effect of dominant negative mutant of *SMAD3* on DSB repair in BMM, cancerous individuals harboring mutations on *SMAD3* can be potentially beneficial from effectiveness of synthetic lethality triggered by PARPi as a single agent or in combinational treatment with common chemotherapy in hematopoietic malignancies such as *SMAD3*-mutant T-ALL or other SMAD3-mutated solid tumor cells metastasizing into bone marrow to obtain better outcome in treatment regimen.

5.3.6.3 The effect of TGFβR-mediated non-canonical protein kinase signals in the bone marrow microenvironment

Besides TGFβR-SMAD3 canonical pathway, TGFβR non-canonical signals in BMM were also examined, whether they influence PARPi sensitivity as well as expression of DSB repair proteins. Although inhibition of PI3K, RAF1, PAK1 and TAK1 caused down-regulation of BRCA2 and/or BRCA1, these inhibitors were incapable to restore efficacy of [PARPi +/- TKi] against leukemia cells. This abnormal phenomenon in PARPi-induced synthetic lethality can be interpreted that leukemia cells in BMM were almost remained in quiescent state according to cell cycle analysis and DSBs in quiescent cells are only repaired by NHEJ pathway. Therefore, in BMM, it is probable that ATM, DNA-PKcs and Ligase IV are more important than BRCA1 and BRCA2 in terms of DSB repair. Moreover, inhibition of four protein kinases in TGFβR-mediated non-canonical pathway did not down-regulate ATM, DNA-PKcs and Ligase IV in BMM, therefore, efficacy of PARPi cannot be restored.

Inquiring more comprehensively in each protein kinase of TGFβR-mediated non-canonical signaling pathway, inhibition of mTOR, a downstream element in PI3K/AKT signaling, has been shown to down-regulate DNA repair proteins and enhance PARPi sensitivity in BRCA2–mutated breast cancer [333]. Moreover, our results have revealed PI3Ki-mediated down-regulation of RAD51 in FLT3(ITD)-positive leukemia cells. Also in other studies, inhibition of PI3K/AKT signaling pathway suppressed DNA repair in glioblastoma in response to ionized radiation [233]. In this study, as consistency, PI3K inhibitor also caused down-regulation of BRCA2 in HR pathway of DSB repair, however, did not regain efficiency of PARPi due to cell cycle distribution in BMM. The same phenomenon was observed in Rho/PAK1 pathway as inhibition of PAK1 effectively sensitized FA/BRCA-proficient breast cancer cells to PARPi [334] but did not restore efficacy of PARPi against leukemia in BMM in current study. On the other hand, PAK1i (IPA-3) has been reported to enhance sensitivity of BCR-ABL1–positive CML cells to TKi (imatinib) [335] but the enhancement was not obtained in BMM in our current study, highlighting the importance of BMM inducing resistance to TKi in OTK-positive leukemias. In terms of RAS/RAF/ERK in mitogen–activated protein kinase (MAPK) signaling pathway, surprisingly, inhibition of ERK leads to up-regulation of D-NHEJ pathway in DSB repair by increasing DNA-PKcs activity [336]. Furthermore, RAF1 inhibitor did not down-regulate DSB repair proteins in FLT3(ITD)-positive leukemia cells. These studies reveal the

irrelevance of inhibition of RAS/RAF/ERK pathway to DSB repair deficiency in normal condition. However, down-regulation of BRCA1 and BRCA2 induced by RAF1i in BMM have not been clearly understood. It may well derive from distinct cellular signaling pathway in BMM and therefore, a further investigation is required to elucidate RAF1i-induced BRCA1/2 deficiencies in BMM. Finally, TAK1/p38 MAPK signaling pathway is active as a branch of MAPK pathway and has not been described to be associated with DSB repair as well as PARPi sensitivity yet, suggesting that TAK1/p38 MAPK pathway also requires an inherited study to interpret inhibition of TAK1 causing down-regulation of BRCA2 in BMM.

6. SUMMARY AND CONCLUSION

Synthetic lethality triggered by PARPi has been successfully applied in *BRCA1/2*–mutated cancers such as breast and ovarian cancer. Although leukemia has not been commonly considered a *BRCA1/2*–mutated cancer, PARPi-induced synthetic lethality has been effectively verified in certain types of leukemia or other hematopoietic malignancies derived from oncoproteins, whose expression down-regulates BRCA1/2. In this study, we reported that FLT3i (quizartinib-also known as AC220) induces deficiencies of DSB repair in HR (BRCA1, BRCA2, RAD51, PALB2) and D-NHEJ (Ligase IV) pathways, resulting in "BRCAness" phenotype. This, therefore, sensitized FLT3(ITD)-positive leukemia to PARP inhibitors.

Since anti-leukemia efficacy of [PARPi + FLT3i] is less profound in bone marrow cells rather than in peripheral blood leukocytes *in vivo* and resistance to PARPi-induced synthetic lethality has been observed in a wide range of BRCA1/2-deficient cancers in both preclinical research and clinical treatment, we postulated that in BRCA-deficient leukemias, the bone marrow microenvironment (BMM) causes resistance against PARPi. Moreover, based on our studies, we proposed that the resistance is driven by the BM stromal cells-derived cytokines. TGF-β has been documented as a cytokine produced in BM niche and related to DSB repair as well as DNA damage response. Therefore, we hypothesized that BMM-dependent resistance to PARPi is mediated by the TGFβ1-TGFβR-SMAD3 signaling. Indeed, genetic and pharmacological targeting of individual component of this signaling pathway induced "BRCAness/DNA-PKness" phenotype in leukemia cells, described by down-regulation of HR (BRCA1, BRCA2) and D-NHEJ (DNA-PKcs, Ligase IV) proteins and DSB response (ATM). Thus, the used strategy restored sensitivity of leukemias to [PARPi +/- TKi] in BMM *in vitro*. Futhermore, inhibition of TGFβR serine/threonine kinase by TGFβRi promoted anti-leukemia efficacy of [PARPi + TKi] in bone marrow cells, splenocytes and finally prolonged the survival of leukemia-bearing mice. Therefore, the data *in vivo* has successfully validated the entire *in vitro* results.

The summary of the results obtained throughout the thesis:

1. *FLT3i, quizartinib (AC220), causes "BRCAness" phenotype, leading to DNA double-strand break (DSB) repair defects in FLT3(ITD)–positive leukemia cells.*
2. *[FLT3i + PARPi] causes elevated accumulation of DSBs, resulting in FLT3i enhanced PARPi sensitivity in FLT3(ITD)–positive leukemia cells in vitro.*

3. *FLT3i promotes anti-leukemia effect of PARPi in FLT3(ITD)-positive leukemia–bearing mice confirmed in peripheral blood leukocytes and bone marrow cells in vivo.*
4. *Bone marrow microenvironment (BMM) induces resistance to PARPi-mediated synthetic lethality in leukemias and the resistance depends on bone marrow stromal cells-derived cytokines.*
5. *Activated TGF-β1-TGFβR signaling pathway induces resistance to PARPi in leukemias in BMM.*
6. *Genetic/pharmacological targeting TGF-β1 and TGFβR induces "BRCAness" and "DNA-PKness" in leukemias, therefore, restoring efficacy of PARPi against leukemias in BMM in vitro.*
7. *TGFβR-mediated SMAD3 signaling induces PARPi resistance in leukemia cells in BMM and genetic/pharmacological targeting SMAD3 restores efficacy of PARPi.*
8. *FDA–approved TGFβR kinase inhibitor is possibly applied in clinical trials of leukemia patients currently receiving [PARPi +/- TKi] as [TGFβRi + PARPi + TKi] did not cause cytotoxic effect on healthy bone marrow cells.*
9. *TGFβRi (SB431542) enhances anti-leukemia effect of [PARPi + TKi] in bone marrow cells and splenocytes in vivo and the strategic combined therapy [TGFβRi + PARPi + TKi] eliminates leukemia stem cells and prolongs survival of leukemia-bearing mice.*

CONCLUSION

To conclude, our research highlighted an intrinsic weakness of leukemias caused by activation of oncogenic tyrosine kinases (OTKs), which can be exploited by PARPi–induced synthetic lethality. Although *BRCA1/2* mutations have not been common in leukemias, other post-transcriptional mechanisms or TKi-dependent down-regulated DSB repair proteins in HR and D-NHEJ pathway, promote PARP inhibitors to eliminate leukemia cells. Moreover, we discovered novel and constitutive mechanism of resistance to PARPi in BMM, which depends on activation of TGFβ1-TGFβR-SMAD3 signaling pathway, resulting in enhancement of HR and D-NHEJ pathways in leukemia cells residing in bone marrow. Therefore, we postulate that pharmacological inhibition of TGFβR serine/threonine kinase by TGFβRi may potentially improve therapeutic efficiency of leukemic individuals currently receiving PARP inhibitors.

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