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Analysis and targeting of cell surface receptors in myeloid leukemia

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Emily Diaz

Committee in charge:

Professor Tannishtha Reya, Chair
Professor Catriona Jamieson
Professor Robert Signer
Professor David Traver
Professor Jin Zhang

2024

The Dissertation of Emily Diaz is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

Dedication

To the family and friends who kept me sane.

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List of abbreviations

AML	acute myeloid leukemia
bcCML	blast crisis chronic myeloid leukemia
CML	chronic myeloid leukemia
CRISPR	clustered regularly interspaced short palindromic repeats
CRO	contract research organization
CSC	cancer stem cell
FACS	fluorescence activated cell sorting
GFER	growth factor, augmenter of liver regeneration
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cell
HUVEC	human umbilical vein endothelial cell
KLS	cKit+Lin-Sca1+
LSC	leukemia stem cell
mAb	monoclonal antibody
MDS	myelodysplastic syndrome
MPS	myeloproliferative syndromes
Msi2	Musashi2
NSG	NOD-scid IL2Rgamma^{null}
qRT-PCR	quantitative real-time polymerase chain reaction
shRNA	short hairpin RNA
Tspan3	Tetraspanin3

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Abstract of the dissertation

Analysis and targeting of cell surface receptors in myeloid leukemia

by

Emily Diaz

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2024

Professor Tannishtha Reya, Chair

Despite advances in cancer treatment, relapse remains a critical factor in the overall mortality of patients. Relapse is often driven by a rare population of cells within the tumor that have the ability to evade treatment, self-renew, and repopulate a tumor. The initiating cell in leukemia is often a normal hematopoietic stem cell that accumulates mutations over time, causing aberrant function. These cells, often referred to as cancer stem cells, co-opt stem cell programs. These cancer stem cells maintain their ability to interact with and manipulate the stem cell niches within the bone marrow microenvironment to further

propagate disease. To understand the programs that are necessary for successful leukemia engraftment and progression, the Reya lab conducted an *in vivo* CRISPR screen. From the dropouts of this screen, we targeted GFER. GFER, a secreted protein, could potentially influence the surrounding niche to make it more permissive to the cancer stem cell. We found that the inhibition of GFER led to mislocalization within the bone marrow as well as a decrease in leukemia blasts in the bone marrow and spleen. This could be evidence that the cancer stem cell relies on the niche and, without it, they are left vulnerable. To further target the cancer stem cell and its relationship with the niche, we developed a monoclonal blocking antibody that binds to Tspan3. This anti-Tspan3 antibody reduced colony forming ability of myeloid leukemia patient samples, both *in vitro* and *in vivo*. We found that inhibition of Tspan3, by either blocking antibody or shRNA, led to alterations in stem programs that could push the cancer stem cell to differentiate. This would leave the cancer stem cell more susceptible to traditional cancer treatments. Indeed, we found that the anti-Tspan3 antibody synergized with imatinib, a front-line tyrosine kinase inhibitor for the treatment of CML. To further develop the antibody, we used humanized variants to test against myeloid leukemia cells. These humanized constructs similarly inhibited colony formation and they would be less immunogenic when delivered to patients in the future. The studies contained here illustrate a basis for targeting the interface between cancer stem cells and their microenvironment for the development of novel therapies.

Chapter 1 Introduction

1.1 The cell of origin in hematological malignancies

Cancer arises when a series of mutations and genetic alterations occur in a cell, giving rise to the ability to survive and grow nearly endlessly[1, 2]. These changes can accrue over a lifetime, gradually transforming a normal cell into the cell of origin, the first cell of the tumor. This cell of origin has been implicated to be the normal stem or progenitor cells of the tissue in question[3-5]. Given the hierarchy within blood cell differentiation and the long life of the hematopoietic stem cell (HSC), hematological malignancies can often be traced to a cell of origin in a hematopoietic stem or early progenitor cell (HSPC).

HSCs are expected to generate billions of blood cells over a person's lifetime[6]. HSCs, and stem cells throughout the body, persist through means of self-renewal and asymmetric cell division where a single stem cell can create a more differentiated daughter cell while preserving its stem cell identity[7, 8]. Through the duration of the stem cell's lifetime, mutations can spontaneously occur, some with no consequences and some with dire consequences. These mutations can exist for years or decades before the cell turns malignant[9, 10]. Once transformed, and the stem cell has become cancerous, it can go on to complete normal stem cell functions such as asymmetric division to give rise to additional transformed cancer cells while preserving its stem identity[8, 11-13].

Depending on the mutations and the specific cell of origin, be it the HSC or an early progenitor of myeloid or lymphoid lineage, different hematological malignancies can form. This includes the BCR-ABL fusion protein that is a driver of chronic myeloid leukemia (CML), whose cell of origin is often the HSC or an early myeloid progenitor[14]. The BCR-ABL fusion product is a

tyrosine kinase protein with elevated activity that acts as an onco-protein by activating other signaling pathways that contribute to further transformation[15]. These pathways include Myc[16], Ras[17], Jnk[18], Jak/Stat[19], PI-3K/Akt[20], and NF-kB[21]. The activation of these pathways and more can lead to increased proliferation, decreased apoptosis, and decreased adhesion to the bone marrow microenvironment[22]. These changes to CML cell function contribute to the progression of disease. These additional mutations and molecular changes to CML cells can lead to accelerated disease progression, eventually reaching the blast crisis phase of CML (bcCML). bcCML is much more treatment resistant, often overcoming therapies that target the driver fusion protein BCR-ABL, such as imatinib. Imatinib revolutionized the treatment of CML , leading to a 10 year survival rate of over 80% with a complete cytogenic response seen in 82% of patients[23]. While a small population of patients with a deep molecular response can cease imatinib treatment, other patients progress to bcCML[23]. bcCML progression is linked to a variety of additional molecular changes, leading to a more heterogenous population of cancer cells[22, 24]. This more closely resembles another type of myeloid leukemia, acute myeloid leukemia (AML), rather than the original CML.

AML is a devastating type of leukemia for a variety of reason. AML can arise from many different mutational hits such as KIT[25], FLT3[26], CEBPA[27], NRAS[28], DNMT3a[29], and TET2[30] as well as fusion products such as AML1-ETO[25], PML-RARa[31], CBFb-MYH11[31], and MLL-fusions[31]. These are most often found to be in the HSC which then acts as the cell of origin for AML[32]. This heterogeneity prevents the creation of a singularly specific targeted therapy, similar to imatinib, because there are too many mutations to target within the same tumor. Since AML is such a heterogenous and undifferentiated disease, it is very complex to treat and often results

in relapse even after aggressive treatments such as a chemotherapy and radiation, with or without an HSC transplant[33]. The cause of residual disease that gives rise to relapse is often the cancer stem cell (CSC), more specifically here the leukemia stem cell (LSC), that can enter a quiescent state during the stress of chemotherapy, then arise and begin proliferating once more[34].

1.2 Cancer stem cells in leukemia and other cancers

There are certain stem cell programs that CSCs appropriate to maintain its stemness. These programs include signaling pathways such as WNT, Notch, and Shh that promote self-renewal in cancers such as colon carcinoma[35], leukemia[36, 37], cervical cancer[38], and medulloblastoma[39]. CSCs also induce increased expression of ABC transporters and ALDH activity that protect the cell from chemotherapeutic drugs used to treat renal cell cancer[40], breast cancer[41], and non-small cell lung carcinoma[42, 43]. This leads to residual disease where the bulk of the tumor may have been removed by treatment, but CSCs remains[44]. Once the selective pressure of the chemotherapeutic regimen has been lifted, it can once again divide into more cancerous cells eventually causing the patient to relapse. This secondary disease is often more aggressive and difficult to treat, as the cells that survived had to have some advantage that allowed them to persist[45].

For LSCs, there are a number of altered pathways and markers that can be exploited as a therapeutic target. For example, LSCs have a very different response to stress and metabolic profile than normal cells. LSCs rely on constitutively activated NF-kB that is reinforced through the secretion of TNFα by the bulk of the leukemia burden[46]. This feedback loop further

promotes the production of additional LSCs in the overall tumor which can increase the difficulty in treating and the incidence of relapse[46]. In addition to this response to inflammation and stress, LSCs have altered mitochondrial function which changes their overall metabolism. LSCs are particularly reliant upon oxidative phosphorylation as their primary source of energy, as opposed to other tumor types that preferentially rely upon glycolysis[47, 48]. Therefore, disruption to mitochondrial function by either small molecule against BCL-2[48] or mitochondrial protein translation[49] can lead to the preferential death of LSCs in comparison to the bulk of the tumor and normal cells. However, these targets are likely to have a narrow therapeutic window as mitochondrial function is necessary for every other cell type as well. While mitochondrial function may have an enriched effect on LSCs, there may be off target effects.

In addition to differences in stress response and metabolism, there are also changes to the cell surface markers of LSCs. LSCs have some similar features to normal HSCs. They share a similar surface marker profile that can include being $CD34^{+}CD38^{-}$ while giving rise to more differentiated $CD38^{+}$ cells[50]. These markers, typically found on normal HSCs, were the first forays into identifying LSCs. However, markers are not enough to definitively label something an LSC. The functional ability to propagate a tumor *in vivo*, similar to a transplantation assay for an HSC[51], is required to demonstrate that a certain cell is in fact an LSC[50].

One of the first cell surface markers to be found enriched on LSCs, but not HSCs, was CD123[52]. In the years since this discovery, many efforts have been made to target CD123, particularly by monoclonal antibody (mAb). In preclinical studies, targeting CD123 proved to be an effective strategy to preferentially target LSCs[53]. In fact, several antibody based therapies targeting CD123 have entered clinical trials for the treatment of AML[47]. However, CD123 isn't

the only potential candidate for targeting LSCs. Other targets include CD33[54], CD47[55], and CD25[56]. Each of these targets have antibody based therapies currently in clinical trials for the treatment of AML[47]. These targets may be more ideal given the relative lack of these markers on normal cells. However, their expression is not completely absent which can also lead to off target effects. Some newer therapeutic antibodies rely on targeting two markers at once to reduce these off target effects[47]. While these potential therapeutic targets are on the LSCs themselves, there are also opportunities to target the niche in conjunction with the LSC itself.

1.3 The role of the niche in leukemia initiation and progression

As the normal HSC transforms, the components of the niche will also change into a structure that is more permissive to the LSCs. The LSCs and normal HSCs compete for resources within the niche[57-60]. This often leads to the impairment of the normal HSC, further compromising the health of the bone marrow and blood[61, 62]. In leukemia, LSCs do not cause the death of normal HSCs by pushing them out of the niche, but rather hinders their ability to produce normal blood cells, leading to bone marrow failure[62]. This shows how important niche regulation is because without it, normal HSCs begin to fail as LSCs begins to flourish. And there is increasing evidence that the surrounding bone marrow microenvironment can also predispose patients to hematological malignancies[63]. A more permissive bone marrow microenvironment can create a welcoming “soil” for the initial “seed”[64]. It has been previously shown that altering the bone marrow stromal cells can induce abnormal hematopoiesis that can eventually develop into leukemia.

For example, myeloproliferative syndromes (MPS), including myelofibrosis and myelodysplastic syndrome (MDS), can be induced in RARγ null mice, independent of mutations in the HSC[65]. While the RARγ null mice never gave rise to AML, the common end stage of MDS, other alterations to the bone marrow microenvironment have. It was found that an activating β-catenin mutation in osteoblasts can give rise to *de novo* AML[66]. The mutant osteoblasts were able to alter the differentiation potential of myeloid and lymphoid progenitors, leading to the development AML with chromosomal aberrations on a mouse ortholog to a human chromosome that are commonly found in patients[66]. These niche abnormalities can serve as initiators of leukemia, especially when combined with mutations to the HSC as mentioned above.

While mutations in the niche can give rise to *do novo* MDS or AML, most studies looking at the leukemic niche utilize a transplantation model. With this model, it has been found that the leukemia cells disrupt both the bone marrow microenvironment cells as well as the remaining normal HSC function. Leukemia transplantation has been shown to significantly impact endothelial cell function in particular[57, 67, 68]. Upon transplantation, leukemia cells will home to vascular niches, similar to their normal HSC counterparts[57]. However, once they engraft, they alter key chemoattractants such as SDF-1 and SCF that lead to the mislocalization of normal HSCs over time as the leukemia progresses[57]. Transplanted leukemia cells can also destroy normal vasculature while inducing the growth of abnormal vasculature through the upregulation of pro-angiogenic factors such as VEGF[67, 68]. This abnormal vasculature can induce hypoxia in the bone marrow microenvironment and lead to the increased production of nitric

oxide[68]. Both of these factors significantly contribute to treatment failure through vascular leakiness which leads to poor distribution of chemotherapy drugs.

Endothelial cells aren't the only bone marrow microenvironment cells that are radically altered during leukemia engraftment and progression. Sympathetic nerves, osteoblasts, mesenchymal stromal cells, and adipocytes have all been shown change in response to leukemia initiation and progression[69]. It has been shown in MLL-AF9 model of AML that there are rapid changes made to the density of sympathetic nerves in the bone marrow[70]. Sympathetic nerves are critical for maintaining mesenchymal stromal cell quiescence and without them, the mesenchymal stromal cells expand and begin osteoblastic differentiation[70]. These new osteoblasts have high expression of osteopontin whose major receptor, integrin $\alpha_V\beta_3$, is required for leukemogenesis of MLL-AF9 AML[71].

The aberrant expansion of osteoblasts also contributes to a permissive niche in other forms of myeloid leukemia. In a BCR-ABL$^+$ model of myeloproliferative neoplasia, the number of osteoblasts significantly increased and this was associated with increased collagen deposition, leading to bone marrow fibrosis[72]. The leukemia cells, through both secretion of soluble factors and physical contact, induced mesenchymal stromal cells to overproduce aberrantly acting osteoblasts. This led to further bone marrow fibrosis which negatively impacts the normal HSCs while leaving the LSCs largely unaffected[72]. This creates a self-reinforcing niche with osteoblast expansion and increased fibrosis, leading to clonal expansion of LSCs, and the cycle begins again when the new leukemia cells spread and reach new areas of the bone marrow. This permissive leukemic niche supports the propagation of leukemia at the expense of normal HSC function.

Mesenchymal stromal cells can also produce adipocytes that can contribute to the leukemic niche. The bone marrow is rich in adipose tissue that has multiple functions in supporting leukemia cells[73]. They serve as a source of fatty acids that provide energy to leukemia blasts to grow and proliferate further[73, 74]. They function as a shield from chemotherapy as adipocytes preferentially uptake and metabolize drugs leading to a decreased concentration of chemotherapeutics in adipose rich tissues like the bone marrow[73]. The lowered concentration of drugs in the bone marrow, in addition to LSC quiescence, further reduces the efficacy of existing chemotherapies. There is a dire need for novel therapeutics for the treatment of leukemia to overcome these challenges.

1.4 Targeting the leukemia stem cell and the leukemic niche

Given the role of the LSCs and the niche in relapse, many research groups are attempting to target both. Targeting LSCs would be cutting the tumor off at the source. While targeting the niche could supplement existing leukemia treatments. With how changed both LSCs and the niche becomes, there are many aberrant targets that aren't expressed, or are minimally expressed, by normal tissues.

The niche has changed into an environment that preferentially supports LSCSs. This is partially due to adhesion molecule that help LSCs physically remain in the niche. These adhesion molecules include CD44, integrins, selectins, and immunoglobulin-like cell adhesion molecules like VCAM-1[75]. Proteins such as these connect LSCs to the surrounding cells and extracellular matrix of the niche. CD44 expressed on LSCs binds to hyaluronan and plays a key role in homing and engrafting to the bone marrow[76]. CD44 facilitates LSC adhesion to the bone

marrow microenvironment through VLA-4[77]. Targeting CD44 by antibody has been shown promote differentiation of AML cells as well as induce apoptosis[78-81]. There are clinical trials currently active for anti-CD44 antibody treatment for not only AML, but also solid tumors that express CD44 on their CSC population[78, 82, 83].

On the niche side, E-selectin and VCAM-1 are expressed on endothelial cells, which are a major cellular component of the niche[75]. AML cells have been shown to upregulate E-selectin on endothelial cells within the niche. This in turn shields the AML cells from chemotherapy by initiating pro-survival signaling in the AML cell[84, 85]. Similar to CD44, leukemia cells interact with VCAM-1 through VLA-4, leading to a feedback loop between leukemia cells and the bone marrow stroma, further changing the niche to support LSCs[86]. These cell surface proteins facilitate interactions between the bone marrow microenvironment and LSCs. Due to their location on the cell surface, they can potentially be targeted by therapeutic blocking antibody, thereby removing LSCs from the niche and increasing the efficacy of treatment.

LSCs have been shown to upregulate CXCR4, the receptor for SDF-1 which is a key homing signal to the niche[87]. There have been efforts made to target the CXCR4/SDF-1 signaling axis for the treatment of AML[88]. A drug targeting this axis, AMD3100, has been used previously in combination with G-CSF to promote HSC mobilization in healthy patients[89]. It has now also been shown to increase leukemia blasts in circulation, indicating it was successful in removing leukemia cells from their place in the niche[88]. By removing the leukemia cells from the niche, they were sensitized to the other chemotherapy drugs that were also used which improved leukemia burden and overall survival in a mouse model[90]. CXCR4, and CD44, can also be targeted through the use of a small molecule, ARV-825[91]. Gene expression analysis showed that

ARV-825 also induced a downregulation of genes associated with stemness, indicating it can target both cell intrinsic and extrinsic functions[91]. There have been other attempts to target the microenvironment itself, but they have largely failed in clinical trials[69]. It is clear that more work needs to be done for the treatment of aggressive myeloid leukemias, particularly those that are largely undifferentiated.

Chapter 2 Altered spatiotemporal dynamics of myeloid leukemia in response to GFER inhibition

2.1 Abstract

Myeloid leukemia is a cancer that originates in the hematopoietic stem and progenitor cells that leads to the rapid production of malignant, immature myeloid cells. In more aggressive subtypes, such as acute myeloid leukemia (AML) and blast crisis phase of chronic myeloid leukemia (bcCML), they are also characterized by low therapeutic response and consistent relapse. Current therapies remain ineffective, highlighting the need for the development of novel strategies. To address this, the Reya lab conducted an *in vivo* CRISPR screen to identify key regulatory pathways in bcCML. While the intrinsic properties of aggressive myeloid leukemia are fairly well known, the extrinsic properties of how the niche supports the leukemia cells are not. To this end, I took the significant hits from the *in vivo* CRISPR screen and filtered them for genes expressed on the surface or secreted, as they could potentially facilitate interactions between the leukemia cells and the niche cells. One of the more interesting candidates was the secreted protein Growth Factor, Augmenter of Liver Regeneration (GFER). GFER has been implicated in liver regeneration and hematopoietic stem cell maintenance. In preliminary studies, I found that knock down of GFER impairs growth of human bcCML cell lines *in vitro* and of bcCML in a mouse model *in vivo*. Moreover, using dynamic *in vivo* imaging, I observed a difference in localization and mobility of bcCML cells when GFER is knocked down. This suggests that they are mislocalized to the vasculature instead of the marrow space, and they exhibit increased speed as they travel in the bloodstream without the ability to extravasate. Given the impact of GFER knockdown on leukemia cells *in vitro*, there appears to be a cell intrinsic mechanism. However, there may also be a cell extrinsic

mechanism where GFER could be impacting the microenvironment to create a more supportive niche. Therefore, in the absence of GFER, the myeloid leukemia cells will be unable to localize to and anchor within the niche, leading to decreased growth. The identification of key regulators that support and maintain leukemia cells within the niche provides new opportunities to disrupt these critical interactions. This may lead to development of new therapeutic approaches for this deadly disease.

2.2 Introduction

Among hematologic malignancies, aggressive myeloid leukemias remain among the most difficult to control. This includes blast crisis phase chronic myeloid leukemia (bcCML) and acute myeloid leukemia (AML). Chronic myeloid leukemia (CML) arises from a constitutively active kinase fusion protein, BCR-ABL, in hematopoietic stem cells (HSCs). CML progresses to bcCML through the accumulation of additional mutations such as loss of p53 or translocations such as AML-EVI1 or Nup98-Hoxa9[92, 93]. AML arises from a variety of mutations in the HSC such as RAS family members including NRAS and KRAS, KIT, and FLT3[94]. While imatinib has dramatically changed outcomes for CML, bcCML and AML remain a therapeutic challenge. The median survival of CML patients is 25 to 30 years, while that for bcCML is 7 to 11 months[95]. This closely mirrors the disease progression of AML where the five-year survival rate is a mere 28.7%[96, 97].

While these cancers are driven by distinctly different oncogenic hits, they are similar in that they are highly undifferentiated and aggressive. The highly undifferentiated state of these leukemias is a major reason for their aggressiveness and therapy resistance[98]. These cells often

flourish at the expense of normal HSCs, leading to decreased production of normal blood cells and increased production of leukemic blasts[62]. In order to better define new approaches to controlling myeloid leukemia, Bajaj et al conducted an *in vivo* CRISPR screen[99]. This screen was done to uncover novel regulatory pathways and to investigate the role of the bone marrow microenvironment on leukemia progression. They were able to sequence mouse bcCML generated from a Cas9-eGFP mouse after the cells had been allowed to engraft and proliferate *in vivo*. Through that sequencing, they could identify which guides had been lost, indicating that they were necessary for bcCML establishment and progression. Because the screen was carried out *in vivo* it was, in part, designed to identify molecules that mediate interactions of leukemia cells within the microenvironment. Here I further focused on genes that would be expressed on the cell surface or secreted. These genes have the ability to interact with other cells of the microenvironment.

From this screen, I identified several genes of interest but ultimately pursued Growth Factor, Augmenter of Liver Regeneration (GFER). GFER has two functional isoforms, secreted and mitochondrial[100, 101]. Secreted GFER was originally discovered as a growth factor due to its role in liver regeneration[102]. As a secreted protein, it could be acting through autocrine, paracrine, or even endocrine signaling pathways to stimulate regeneration[103]. The exact mechanism and receptor GFER works through has yet to be identified. However, it has been reported to regulate and interact with several pathways including AP-1 through JAB1, COP9, MAPK, and EGFR[100, 104, 105]. Thus far, the majority of the work investigating secreted GFER is in the liver, and its function in cancer has largely been unexplored.

In addition to the secreted isoform, there is a highly conserved mitochondrial isoform. GFER has a *S. cerevisiae* ortholog, Erv1, where it functions as a mitochondrial redox protein[106]. This mitochondrial function appears to be critical to maintaining a variety of stem cell populations such as mouse embryonic stem cells, HSCs, and neural stem cells[106-108]. In the hematopoietic system, it appears to play a significant role in HSC proliferation[109]. Sankar and Means have reported that GFER expression induces HSC quiescence so aberrant GFER expression may result in increased proliferation. This would leave the HSCs vulnerable to developing the mutations that cause leukemia. Therefore, GFER's role in regeneration and stem cells makes it an interesting target for cancer stem cells (CSCs) as well as leukemia in general.

2.3 Materials and methods

Mice

C57BL6/J mice were uses as transplant donors and recipients. dsRed (B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J) mice were used as transplant recipients All mice were between 8-16 weeks of age. Mice were bred and maintained in the animal care facility at University of California San Diego. All animal experiments were performed according to protocols approved by University of California San Diego Institutional Animal Care and Use Committees.

Generation of leukemic mice

Bone marrow KLS ($cKit^+Lin^-Sca1+$) cells were sorted and cultured overnight. To generate bcCML, the cells were cultured in X-VIVO 15 Serum-free Hematopoietic Cell Medium (Lonza) supplemented with 10% HyClone fetal bovine serum (Cytiva), 1% Penicillin-Streptomycin

(Gibco), 50uM 2-Mercaptoethanol (Sigma-Aldrich), murine stem cell factor (mSCF, 100 ng/ml, R&D Systems), and murine thrombopoietin (mTPO, 20ng/ul, R&D Systems) overnight. Subsequently, the cells were retrovirally infected with MSCV-BCR-ABL-IRES-huNGFR and MSCV-NUP98-HOXA9-IRES-huCD2. Cells were collected 48 hours post infection and sorted. For primary bcCML, 1,000-5,000 BCR-ABL$^+$/NUP98-HOXA9$^+$ cells were transplanted retro-orbitally in sub-lethally irradiated (6 Gy) C57BL6/J mice. Transplanted mice were analyzed as previously reported[8].

Cell culture

K562 cells (ATCC) were maintained in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (GeminiBio) and 1% penicillin-streptomycin (Gibco) in a 5% CO_2 incubator at 37°C.

shRNA mediated knockdown in K562 cells

shRNA sequences were designed using the Broad Institute's Genetic Perturbation Platform (GPP). These sequences were then cloned into the pLV-hU6-EF1a-green backbone (Biosettia) according to manufacturer's instructions. K562 cells were nucleofected with these constructs using a Cell Line Nucleofector™ Kit L (Lonza). After nucleofection, positively transfected cells were sorted with a FACSAria III (BD).

In vitro colony forming assay

For a K562 methylcellulose colony forming assay, cells containing the shRNA construct were plated in methylcellulose media:IMDM (Methocult H4434 Classic, StemCell Technologies) supplemented with 2% fetal bovine serum at 300 cells/well. Resultant colonies were evaluated and counted at 7-10 days post plating.

Retroviral constructs and production

Short hairpin RNA (shRNA) constructs were designed and cloned into the MSCV/LTRmiR30-PIG (LMP) vector from Open Biosystems according to their instructions. Virus was produced in 293T cells (ATCC) transfected using X-tremeGENE HP (Roche) with the plasmid of interest, VSV-G, and gag-pol. Viral supernatants were collected for two to three days and ultracentrifuged at 20,000g for two hours at 4°C.

In vivo leukemia burden

Primary murine bcCML was isolated from the spleens of transplanted mice by FACS on a FACSAria III (BD). Cells were infected with retroviruses that were either empty or contained shRNA sequences against GFER. Positively infected cells were sorted using FACS and transplanted into sublethally irradiated (6 Gy) B6 mice. The spleen and bone marrow cells were isolated and analyzed 14 days post-transplant for leukemia burden on a FACSAria III (BD).

Bone marrow sectioning and staining

B6 mice were sublethally (6 Gy) irradiated and injected with either PBS (Gibco) or murine bcCML cells. The bcCML cells were allowed to engraft and proliferate over the course of

one week. At that point, the mice were sacrificed and their femurs were removed for sectioning. Femurs were fixed with 4% paraformaldehyde (PFA, Thermo Scientific) overnight at 4°C on a rotator. The bones were washed with PBS several times before decalcification using Cal-Ex™ Decalcifier (Fisher Scientific). After washing the bones with PBS, they were added to a 30% sucrose (Sigma) solution and incubated overnight at 4°C on a rotator. The bones were dried and frozen in OCT (Sakura) over dry ice before being stored in the -80°C freezer. For sectioning, I used the CryoJane Tape-Transfer System in combination with a CM3050 S Cryostat (Leica).

To stain the sections, slides were first blocked with 10% normal goat serum (Vector Laboratories), 5% bovine serum albumin (Sigma), and 0.1% Triton (Sigma) for 1 hour at room temperature. After blocking, slides were incubated with an anti-GFER antibody (Proteintech) as a primary antibody at a 1:500 dilution in blocking buffer, overnight at 4°C. Slides were then washed three times with PBS-T for 5 minutes. An Alexa Fluor™ 488 goat anti-rabbit secondary (Invitrogen) was used at a 1:1000 dilution for 1 hour at room temperature. Slides were washed 3 times with PBS for 5 minutes and then mounted using Fluoromount-G™ with DAPI (Invitrogen). The mounting media was allowed to cure overnight. Images were captured on a Confocal Leica TCS SP5 II (Leica Microsystems).

In vivo imaging

Prior to imaging, dsRed mice were transplanted with either normal cKit+ hematopoietic stem and progenitor cells (HSPCs) stained with CFSE (Invitrogen), GFP+ control bcCML cells, or GFP+ shGFER bcCML cells. These mice were imaged as previously described[110]. Briefly, mice

were anaesthetized by an intraperitoneal injection of a ketamine and xylazine cocktail (100 and 20 mg/kg, respectively). After confirming anesthetic plane by toe pinch, VE-Cadherin conjugated to Alexa Fluor 647 (eBiosciences) was administered and the hair on the scalp was removed with Nair (Church & Dwight Co.). Then the scalp was removed, exposing the calvarium. Mice were secured into a mouse stereotactic holder (Stoelting Co.) and the top layer of bone was removed using a Dremel 3000 with a 1/16" engraving cutter bit. A 1 cm rubber O-ring (Fine Science Tools Inc.) was attached to the calvarium to hold a droplet of water for the water immersion lens. Images were acquired with the Leica LAS AF on a TCS SP5 upright DM600 CFS Leica confocal system using an HCX APO L 20x/1.00W Leica Plan Apochromat objective.

Quantitative analysis

Images were analyzed by ImageJ. The distance traveled by cell was measured and the velocity (μm/sec) was calculated using the time elapsed during imaging. Statistical analyses were done on Prism version 10.1.1 (GraphPad Software Inc.). All data shown is mean $\pm$ standard deviation. Either a one-way ANOVA with Bonferroni multiple-comparison correction or an unpaired two-tailed t-test was used to calculate statistical significance where applicable.

2.4 Results

Screening key regulators of leukemia progression

In the Reya lab's previous studies, Bajaj et al identified several key regulators of bcCML engraftment and propagation[99]. From this list of genes that are implicated in bcCML progression, I decided to focus on cell surface and secreted proteins. In these preliminary

studies, I first concentrated on those targets to allow for potential targeting by blocking antibody. This included several genes such as: GHDC, MXRA8[111], COCH[112], DSPP[113], GFER[100, 104], TRPM7[114, 115], and ATP6AP2[116] with SDC1[117] as a positive control (Figure 2.1A, n = 3-6 technical replicates, ** = $P < 0.01$, one-way ANOVA with a Bonferroni correction was used). While a number of these shRNA constructs were shown to inhibit colony forming ability in K562, I selected GFER as our gene of interest. Given GFERs established role in embryonic, neural, and hematopoietic stem cells, as well as liver regeneration, I was interested in understanding its role in cancer stem cells[102, 106-108]. I confirmed that the shRNA constructs repressed GFER expression in K562 cells by qRT-PCR and moved forward investigating its role in myeloid leukemia (Figure 2.1B, n = 3 technical replicates, * = $P < 0.05$, **** = $P < 0.0001$, one-way ANOVA with a Bonferroni correction was used).

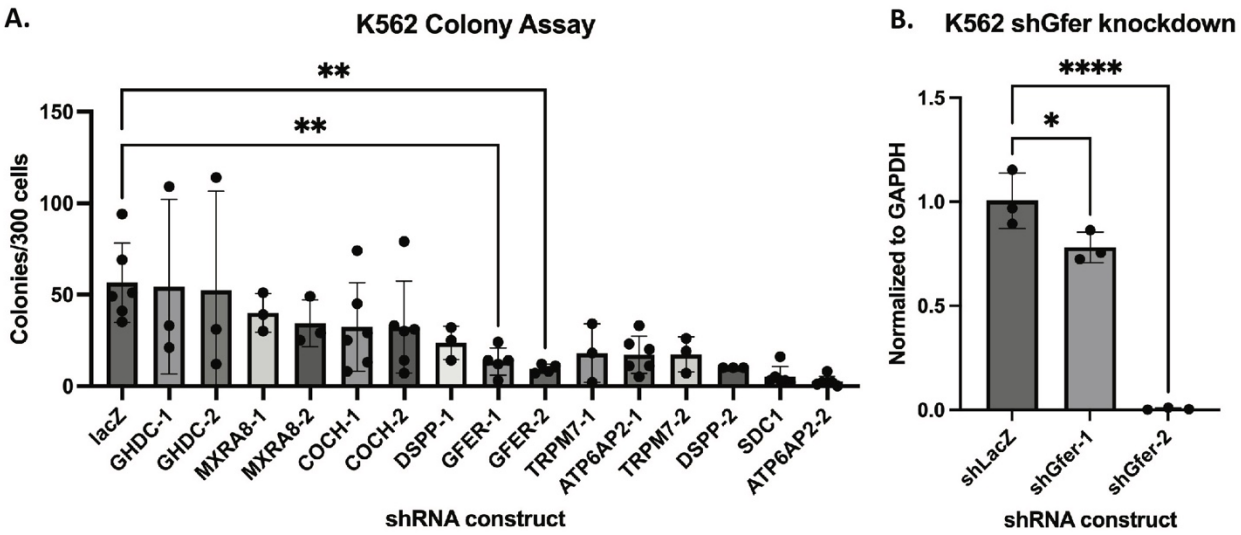


Figure 2.1 GFER is a key regulator of leukemia growth.

(A) Colony forming assay of K562 cells with shRNA against cell surface and secreted proteins (n = 3-6 technical replicates, ** = $P < 0.01$, one-way ANOVA with a Bonferroni correction was used). (B) shRNA mediated knock down of Gfer normalized to B2M (n = 3 technical replicates, * = $P < 0.05$, **** = $P < 0.0001$, one-way ANOVA with a Bonferroni correction was used).

GFER expression in the bone marrow after engraftment

Given GFER's secreted isoform, I was interested in seeing where in the bone marrow it is expressed, with and without bcCML engraftment. Upon staining bone marrow sections with an antibody against GFER, I found that leukemia engraftment and proliferation increased GFER expression in the bone marrow compared to bone marrow that doesn't have bcCML (Figure 2.2A-I). While there are cells expression GFER at a baseline in the bone marrow (Figure 2.2D-F), this number significantly increases in the presence of bcCML (Figure 2.2G-I). The source of GFER is still unknown, but it could be secreted from either bone marrow stromal cells, the bcCML cells, or both.

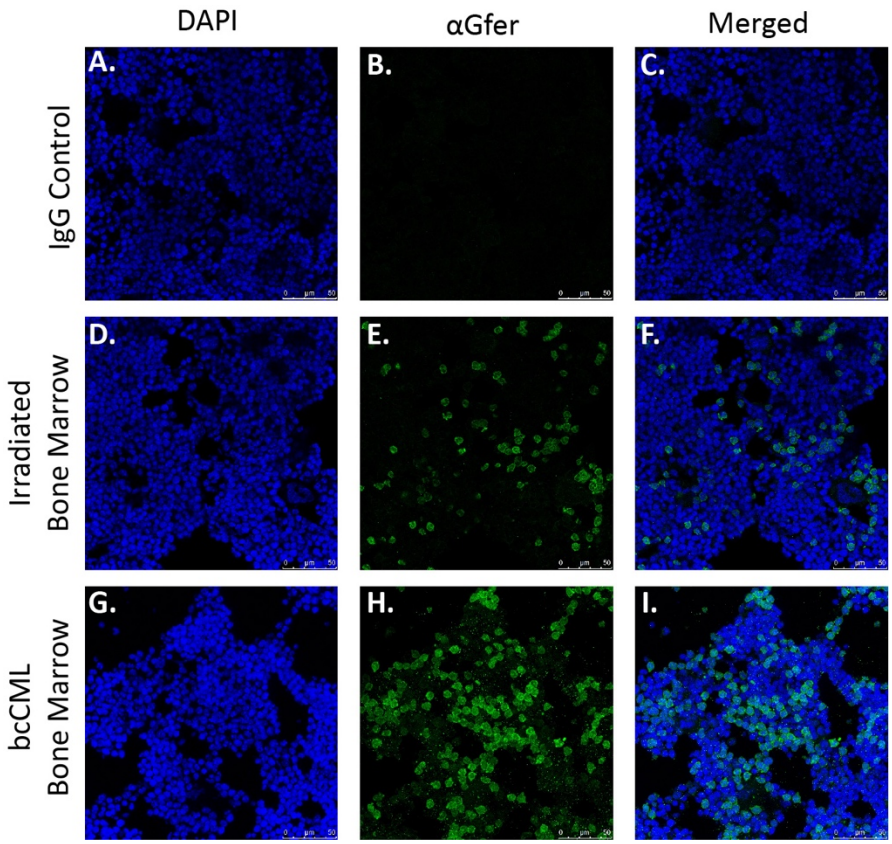


Figure 2.2 GFER expression in the bone marrow after bcCML engraftment.

(A-C) IgG control staining of bone marrow. (D-F) GFER staining of irradiated bone marrow. (G-I) Gfer staining of irradiated bone marrow transplanted with bcCML.

Leukemia burden in vivo after GFER knockdown by shRNA

Following our *in vitro* investigation into the dependency of K562 cells on GFER, I wanted to attempt to recapitulate those findings *in vivo*. I used two independent shRNA constructs to inhibit GFER expression, confirmed the knockdown, and then transplanted infected bcCML cells *in vivo* (Figure 2.3A, n = 3 technical replicates, *** = $P < 0.001$, **** = $P < 0.0001$, one-way ANOVA with Bonferroni correction was used). After allowing the cells to engraft and begin proliferation, I isolated the spleen and bone marrow for preliminary analysis. Upon isolation, the control bcCML spleen was noticeably larger than the shGFER transplanted spleens (Figure

2.3B). In line with that, I found that there was a reduced number of leukemia blasts in the bone marrow and the spleen (Supplementary Figures 2.5A-B).

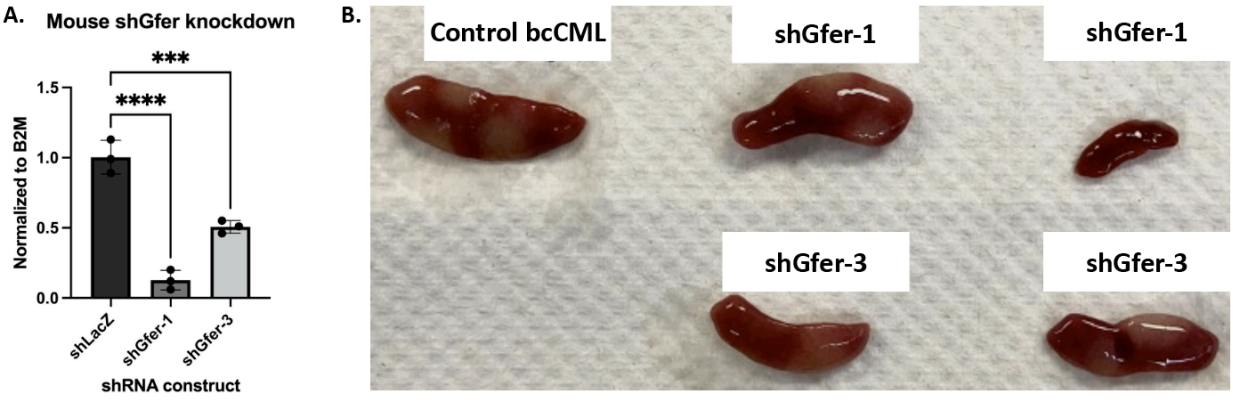


Figure 2.3 The effect of shRNA mediated knockdown of Gfer on murine bcCML growth *in vivo*. (A) Confirmation of GFER knockdown in mouse cells normalized to B2M (n = 3 technical replicates, *** = $P < 0.001$, **** = $P < 0.0001$, one-way ANOVA with Bonferroni correction was used). (B) *S*pleens from mice transplanted with shControl or shGfer bcCML.

Spatiotemporal dynamics of bcCML after GFER knockdown by shRNA

I was interested in how Gfer may be contributing to the spatiotemporal dynamics of myeloid leukemia growth *in vivo*. To study this, I used an *in vivo* imaging technique to visualize the bone marrow of the calvarium. The dynamics of normal HSCPs (Figure 2.4A-C), shLacZ bcCML cells (Figure 2.4D-F), and shGFER bcCML cells (Figure 2.4G-I) were captured over the course of nearly an hour. Based on these preliminary observations, I calculated how many cells were moving in each condition and how fast they were moving. When GFER is knocked down, I found that there were more mobile cells and that they were moving faster as well (Figure 2.4J-K, n = 23 or 64 technical replicates, **** = $P < 0.0001$, unpaired two-tailed t-test was used). The shGFER bcCML cells also appeared to be in vessels being pushed along by the bloodstream

more than the control bcCML cells. This mislocalization indicates that the shGFER cells were mislocalized from the niche, potentially leaving them weaker.

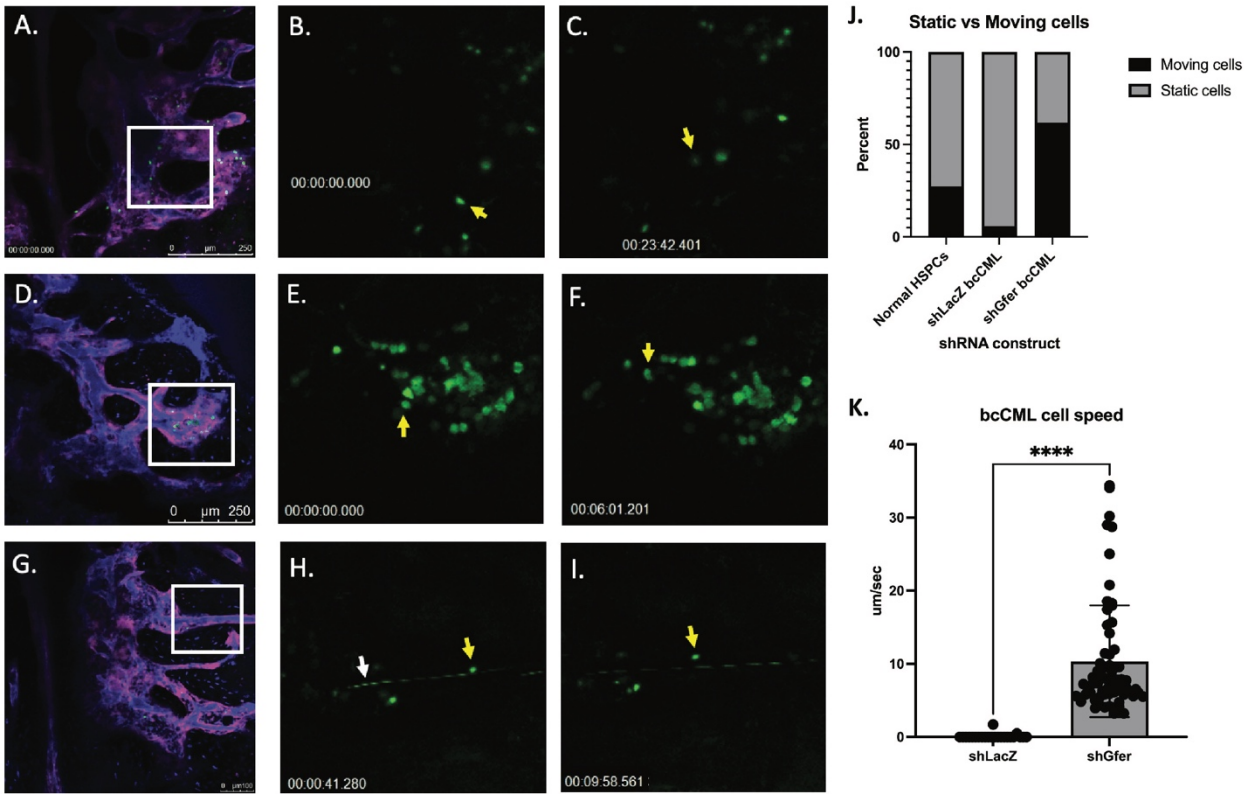


Figure 2.4 Altered spatiotemporal dynamics of murine bcCML *in vivo* after shRNA mediated knockdown of GFER

(A-C) *in vivo* imaging of normal hematopoietic stem and progenitor cells. (B) Yellow arrow indicates cell start position. (C) Yellow arrow indicates cell end position. (D-F) *in vivo* imaging of control bcCML cells. (E) Yellow arrow indicates cell start position. (F) Yellow arrow indicates cell end position. (G-I) *in vivo* imaging of shGFER bcCML cells. (H) Yellow arrow indicates cell start position; white arrow indicates cell smear. (I) Yellow arrow indicates cell end position. (J) The ratio of cells that are static to those that are moving. (K) bcCML cell speed in control bcCML and shGFER bcCML (n = 23 or 64 technical replicates, **** = $P < 0.0001$, unpaired two-tailed t-test was used).

2.5 Discussion

Following an *in vivo* CRISPR screen, I identified several key genes that may play a role in mediating the interactions between leukemia and the bone marrow microenvironment. Of

these genes, I selected GFER as a potential candidate to explore. It is known that GFER has two isoforms, a mitochondrial isoform linked to stemness[109] and a secreted isoform that is involved in liver regeneration[103]. Both of these isoforms could contribute to the dependency of bcCML on GFER. The mitochondrial isoform may influence cell intrinsic mechanisms, while the secreted may influence cell extrinsic mechanisms.

Cell extrinsic signals from the niche have the ability to affect both the normal hematopoietic stem and progenitor cells (HSPCs) as well as the cancer stem cells (CSCs). In turn, CSCs can influence the niche, building a self-reinforcing system for the propagation of leukemia[57, 67, 72, 118-120]. In these preliminary studies, I found that knocking down GFER expression led to increased egress from the niche as well as increased speed once out of the niche. Similar to stem cells, CSCs function in a supportive niche[121]. The niche supplies key signals through soluble growth factors as well as physical contact that help maintain the cells stemness[121]. Therefore, it stands to reason that removing the cell from its supportive microenvironment would lead it to differentiation and potentially increased susceptibility to chemotherapeutic drugs. With this in mind, it is possible to create a blocking antibody against secreted GFER that could someday yield improved patient outcomes if their cancer cells are weaker to traditional treatment.

As for the mitochondrial isoform, Singh et al discovered that it plays a role in the distribution of copper within the mitochondria[101]. Removing GFER from leukemia stem cells led to increased differentiation and lower viability[101]. This may explain the cell intrinsic inhibition of growth seen here *in vitro*. Therefore, further work may be done to understand the cell extrinsic properties of GFER.

2.6 Acknowledgements

Chapter 2 contains unpublished material coauthored with Reya, T. The dissertation author was the primary researcher and author of this paper.

2.7 Supplemental figures

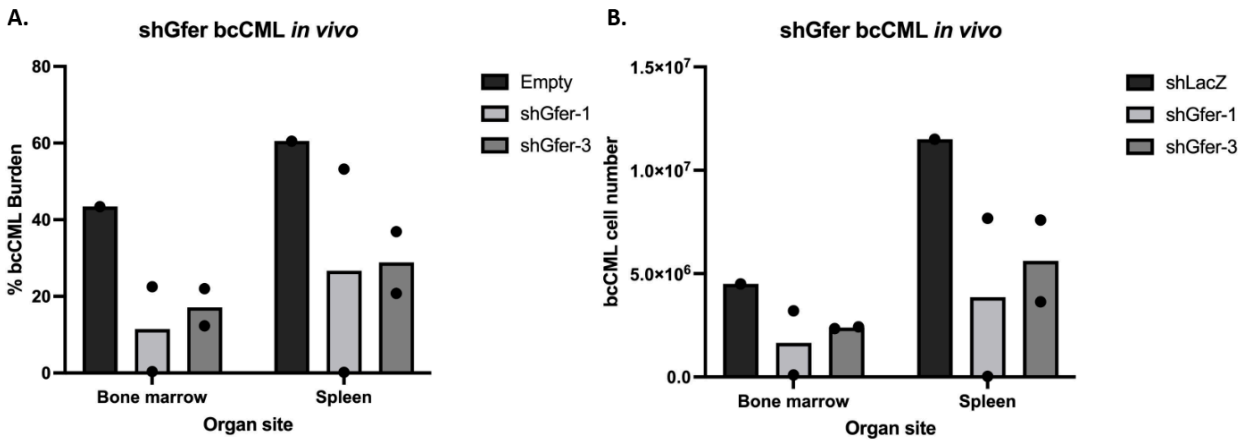


Figure 2.5 shRNA mediated knock down of GFER impaired growth *in vivo*

(A) bcCML burden in the bone marrow and spleen. (B) bcCML cell numbers in the bone marrow and spleen.

Chapter 3 Targeting Tspan3 by blocking antibody for the treatment of myeloid leukemia

3.1 Abstract

Acute myeloid leukemia (AML) and blast crisis chronic myeloid leukemia (bcCML) are characterized by the rapid expansion of immature myeloid cells. While these leukemias are distinct, they are both highly aggressive and relatively resistant to existing therapies. Therefore, identifying new approaches to target AML and bcCML is critically important. Our lab has previously shown that Musashi2 (Msi2) is a key driver of leukemia self-renewal. To understand the mechanisms by which Msi2 drives leukemia, our lab carried out a genome wide transcriptional analysis of changes that occur in myeloid leukemia when Msi2 is inhibited. We identified Tetraspanin3 (Tspan3) as a key downstream target of Msi2. Given that Tspan3 is expressed on the cell surface, we developed a blocking antibody to inhibit Tspan3 function as a potential therapeutic strategy against aggressive myeloid leukemias. The candidate Tspan3 monoclonal antibodies (mAbs) bound to human Tspan3, were able to inhibit AML and bcCML patient sample growth *in vitro,* and decreased leukemia burden in xenografts *in vivo*. In addition, delivery of Tspan3 mAbs did not affect the viability of normal CD34+ human hematopoietic stem cells nor triggered pathological abnormalities in other tissues. We were also able to build a library of potential biomarkers for the stratification of patients, as well as determined that the mAbs synergize with existing frontline therapies for the treatment of myeloid leukemia. This indicates that targeting Tspan3 by blocking antibody may be an ideal supplement to existing therapies to improve patient outcomes. Future work is still needed to fully understand the mechanism of action, but the impact of the Tspan3 mAbs on myeloid leukemia is clear.

3.2 Introduction

While therapeutic response in a variety of hematological cancers has drastically improved upon the introduction targeted therapies such as imatinib for chronic myeloid leukemia (CML), there remains challenges for other subtypes of leukemia[122]. In the case of acute myeloid leukemia (AML) and blast crisis chronic myeloid leukemia (bcCML), there are minimal therapeutic options available for treatment outside of chemotherapy and hematopoietic stem cell (HSC) transplant[123, 124]. Even with those options, treatment often fails, leading relapse and death in a majority of patients[125]. While genotypically distinct, AML and bcCML have similar mechanisms of action marked by the uncontrolled growth of immature myeloid cells [126, 127]. These immature myeloid cells arise from the dysregulated self-renewal of leukemia cells with stem cell like properties, cancer stem cells (CSCs)[126]. These CSCs co-opt pathways and mechanisms used by normal stem cells in order to further propagate the tumor. CSCs are uniquely capable of evading treatment as well as detection, so novel therapies that target them specifically could lead to advancements in treatment and prevention of relapse in patients with leukemia that has a high CSC burden such as AML and bcCML[128].

To understand the underlying mechanisms of aggressive myeloid leukemia, we have focused on pathways involved in stemness. One such pathway is regulated by the RNA-binding protein, Musashi2 (Msi2). Msi2 expression has been shown to predict poor outcomes in AML and bcCML[129]. We analyzed the differential gene expression between CSCs with or without Msi2 in both bcCML and AML and found that tetraspanin3 (Tspan3) was a downstream target of Msi2[130]. Tspan3 is a member of the tetraspanin family of integral membrane proteins with four membrane spanning domains and a large extracellular domain[131]. Tetraspanins have previously

been investigated for their role in cellular processes such as cell adhesion, proliferation, and antigen presentation[132]. They are able to regulate these many functions by forming microdomains on the cell surface with other tetraspanins and cell surface proteins such as integrins and signaling proteins[132]. Although expression of tetraspanin proteins is fairly ubiquitous across tissue types, they are fairly unstudied, especially in the context of cancer and disease[133]. Some individual tetraspanins have been studied more rigorously, but often independently rather than how they function in complex with each other and other cell surface proteins[133].

We have previously shown that Tspan3 plays a role in AML self-renewal and propagation, both *in vitro* and *in vivo*[130]. In line with the goal of developing novel therapies for the treatment of aggressive myeloid leukemia, we furthered our investigation into Tspan3 by developing a monoclonal antibody (mAb) against it. This allows for the direct targeting of CSCs by therapeutic intervention. Growth and propagation of myeloid leukemia cells was blocked upon incubation with the mAb, both *in vitro* and *in vivo*, similar to the genetic inhibition we previously established. While investigating its function *in vitro*, we found that there may be an adhesion defect with patient samples when incubated with the mAb targeting Tspan3. Finally, upon sequencing myeloid cell lines after incubation with the antibody or after targeting Tspan3 by shRNA mediated knockdown, we found that stem programs were significantly impacted. Together, these results suggest that Tspan3 may play a role in regulating stemness and interactions with the bone marrow microenvironment.

3.3 Materials and methods

Generation and validation of a monoclonal antibody against Tetraspanin 3

Our previous studies indicate that targeting Tspan3 could be a viable route for the generation of a novel therapeutic for the treatment of myeloid leukemia[130]. In order to pursue this, we developed blocking mAbs against Tspan3 in conjunction with a contract research organization (CRO). The CRO designed an immunization strategy to generate diverse antibodies. They immunized mice with human Tspan3 DNA and supplemented with human Tspan3-expressing cells over the course of approximately 15 weeks. This led to the creation of three independent cohorts of mice from different strain backgrounds (NZBW, CD-1, Balb/C). Serum from these mice were tested by FACS and three mice showed a strong immune response by day 92 of the immunization protocol (Figure 1A).

Two hybridoma libraries were generated from the most reactive mice, and then re-screened for reactivity to human Tspan3 expressing cells by FACS. Of the two hybridoma libraries, one showed more favorable binding to human Tspan3 and was used to create antibody clones. Six individual antibody clones were generated and screened for binding to cell surface Tspan3. Of the six mAb clones, five were tested for the ability to block the growth of eight independent, primary patient myeloid leukemia samples (five AML, three bcCML) in a colony forming assay. All five mAbs against Tspan3 showed significant impact on growth as measured by colony formation (Figure 1B, n = 12-24, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, one-way ANOVA with Bonferroni correction was used). Upon sequencing, clones five and six were shown to have the same amino acid sequence. Clone two was unable

to be sequenced, in addition to some toxicity issues (data not shown), we moved forward with the clone six (M6) blocking mAb.

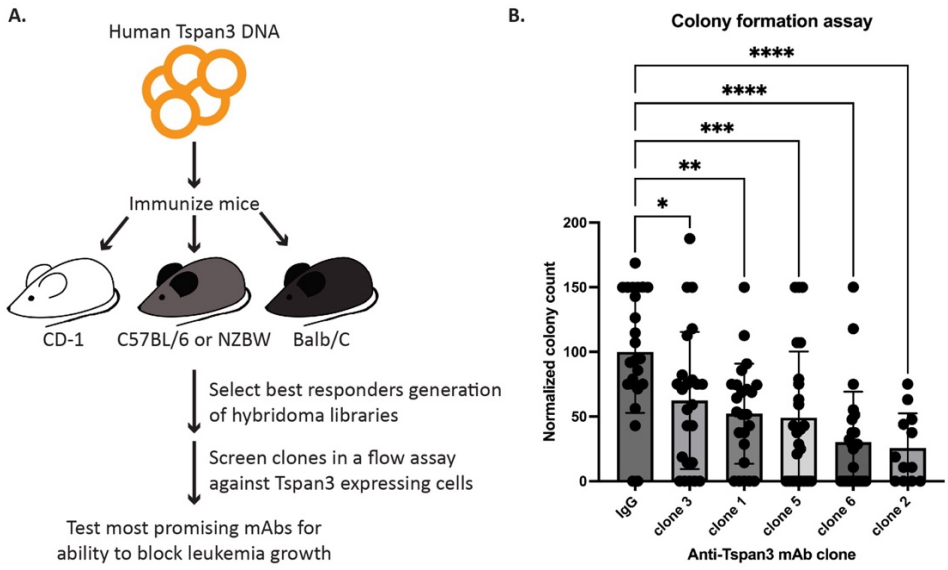


Figure 3.1 Generation and validation of a monoclonal antibody against Tetraspanin 3

(A) Schematic of antibody generation. (B) Colony forming assay of AML and bcCML patient samples treated with different antibody clones (n = 12-24, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, one-way ANOVA with Bonferroni correction was used).

Mice

C57BL6/J were uses as transplant donors and recipients. Human patient samples were transplanted into NSG mice (NOD-scid IL2Rgamma^{null}). All mice were between 8-16 weeks of age. Mice were bred and maintained in the animal care facility at University of California San Diego. All animal experiments were performed according to protocols approved by University of California San Diego Institutional Animal Care and Use Committees.

Generation of leukemic mice

Bone marrow KLS (cKit^{+}Lin^{-}Sca1^{+}) cells were sorted and cultured overnight. To generate bcCML, the cells were cultured in X-VIVO 15 Serum-free Hematopoietic Cell Medium (Lonza)

supplemented with 10% HyClone fetal bovine serum (Cytiva), 1% Penicillin-Streptomycin (Gibco), 50uM 2-Mercaptoethanol (Sigma-Aldrich), murine stem cell factor (mSCF, 100 ng/ml, R&D Systems), and murine thrombopoietin (mTPO, 20ng/ul, R&D Systems) overnight. Subsequently, the cells were retrovirally infected with MSCV-BCR-ABL-IRES-huNGFR and MSCV-NUP98-HOXA9-IRES-huCD2. Cells were collected 48 hours post infection and sorted. For primary bcCML, 1,000-5,000 BCR-ABL$^+$/NUP98-HOXA9$^+$ cells were transplanted retro-orbitally in sub-lethally irradiated (6 Gy) C57BL6/J mice. Transplanted mice were analyzed as previously reported[8].

Cell line culture conditions

Human AML cell line Kasumi-6 (ATCC) was maintained in RPMI 1640 medium (Gibco) with 20% fetal bovine serum (GeminiBio) and 1% Penicillin-Streptomycin (Gibco), supplemented with human recombinant granulocyte macrophage colony stimulating factor (hGM-CSF, 2ng/ml, R&D Systems). Human AML cell line Kasumi-1 (ATCC) was maintained in RPMI 1640 medium (Gibco) supplemented with 20% fetal bovine serum (GeminiBio). Human bcCML cell lines KCL22-s, LAMA84-s, and K-562 (ATCC) were maintained in RPMI 1640 medium with 10% fetal bovine serum and 1% Penicillin-Streptomycin. Human Biphenotypic B Myelomonocytic Leukemia cell line MV-4-11 (ATCC) was maintained in in IMDM medium (Gibco) with 10% fetal bovine serum and 1% Penicillin-Streptomycin. Human epithelial kidney cell line 293T (ATCC) was maintained in DMEM medium (Gibco) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. Human endothelial cell line HUV-EC-C (ATCC) was maintained in F-12K

medium (ATCC) supplemented with 0.1 mg/ml heparin (Sigma), 10% fetal bovine serum (GeminiBio), and 30 μg/ml endothelial cell growth supplement (Fisher Scientific).

A murine Tspan3 overexpressing cell line, T3 OE, was generated using the P3x63Ag.653 cell line as a transfection host by Antibody Solutions. T3 OE cells were maintained in HyClone IMDM (Cytiva) with 10% HyClone Characterized fetal bovine serum (Cytiva). Puromycin (4.5μg/ml, Sigma) was added to maintain the overexpression of Tspan3.

Human leukemia patient samples

Fifteen patient samples were obtained from Singapore General Hospital, the Fred Hutchinson Cancer Research Center, and UC San Diego Moores Cancer Center from institutional review board-approved protocols with written informed consent in accordance with the Declaration of Helsinki. Leukemia cells were thawed in a 37°C water bath and resuspended in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin (Gibco). Freezing medium was removed, and cells were resuspended in human AML media composed of IMDM (Gibco), 20% BIT 9500 serum substitute (StemCell Technologies), 2mM GlutaMAX (Gibco), 1% Penicillin-Streptomycin (Gibco), 50uM 2-Mercaptoethanol (Sigma-Aldrich), and 17ug/ml LDL (Calbiochem 437644). Media was supplemented with human stem cell factor (hSCF, 100ng/ul, R&D Systems) and human thrombopoietin (hTPO, 100ng/ul, R&D Systems) immediately prior to culture. Cells were allowed to recover at 37°C overnight.

In vitro methylcellulose colony formation assays with the Tspan3 monoclonal antibody

For murine methylcellulose colony forming assays, BCR-ABL^{+}/NUP98-HOXA9^{+} bcCML cells were isolated, incubated with the anti-Tspan3 monoclonal antibody for 3 hours at 37°C, and plated in methylcellulose media:IMDM medium (Methocult GM M3434, StemCell Technologies) supplemented with 2% fetal bovine serum at 500 cells/well. Resultant colonies were evaluated and counted at 7-10 days post plating.

For human cell line methylcellulose colony forming assays, cells were incubated with the anti-Tspan3 monoclonal antibody for 3 hours at 37°C and plated in methylcellulose media:IMDM (Methocult H4434 Classic, StemCell Technologies) supplemented with 2% fetal bovine serum at 300 cells/well. Resultant colonies were evaluated and counted at 7-10 days post plating.

For human patient samples, cells were incubated with the anti-Tspan3 monoclonal antibody for 3 hours at 37°C and plated in methylcellulose media:IMDM (Methocult H4434 Classic, StemCell Technologies) supplemented with 2% fetal bovine serum at between 8,000-74,000 cells/well. Resultant colonies were evaluated and counted at 10-14 days post plating.

For normal human CD34+ bone marrow cells, cells were incubated with the anti-Tspan3 antibody for 3 hours at 37°C and plated in methylcellulose media:IMDM (Methocult H4434 Classic, StemCell Technologies) supplemented with 2% fetal bovine serum at 1,000 cells/well and counted at 10-14 days after plating.

Normal human CD34⁺ cell viability assay

Normal human CD34⁺ bone marrow cells (Stem Cell Technologies) were thawed according to manufacturer's protocol. Once thawed, cells were treated with either IgG or anti-Tspan3 antibody for 24 hours in liquid culture. Viability was measured by trypan blue exclusion.

Western blot

LAMA84-s cells, 293t cells, and TSPAN3-P3-C1 cells were collected and washed with PBS. Cells were then lysed in RIPA buffer (Thermo Fisher Scientific). Protein concentrations were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) on a Varioskan™ LUX microplate reader. Equivalent amounts of protein were mixed with Laemmli sample buffer (Bio Rad) and boiled at 95°C to denature the proteins. Once the proteins were prepared, the general western blot protocol from Bio Rad was used with the Bio Rad system to separate proteins by gel electrophoresis and transfer the proteins to a PVDF membrane. The membrane was then blocked by with BSA in TBST. After blocking, the membrane was incubated with primary antibodies against Tspan3 (Novus Biologics) and beta-Actin (Biolegend) overnight at 4°C. Peroxidase goat anti-mouse secondaries were used to label the proteins of interest and it was developed using the Pierce™ ECL Western Blotting Substrate (Thermo Scientific) kit. The blot was imaged on a Bio Rad ChemiDoc™ MP Imaging System and analyzed with ImageJ.

qRT-PCR analysis

RNA was isolated using the RNeasy micro kit (Qiagen) and the RNA was converted to cDNA using iScript™ Reverse Transcription Supermix (Bio Rad). Quantitative real-time PCRs were run on an CFX Opus 96 Real-Time PCR System (Bio Rad) by combining cDNAs, iQ SYBR

Green Supermix (Bio Rad) and primers against target genes. Gene expression for human samples were normalized to the levels of GAPDH and gene expression for mouse samples were normalized to B2M. Primers were retrieved from PrimerBank (https://pga.mgh.harvard.edu/primerbank/).

Fluorescent secondary with the anti-Tspan3 antibody

We stained cells with the indicated concentration of the anti-Tspan3 mAb at a cell density of 0.5e6 cells/ml in PBS containing 1% BSA (Sigma) and 0.005% sodium azide (Sigma). The cells and mAb were incubated for 30 minutes on an orbital shaker at room temperature. After primary incubation, unbound mAb was washed away and then incubated for another 30 minutes with a fluorescent secondary (Jackson ImmunoResaerch Laboratories Inc, 115-096-071) at 1:400 in the dark. The cells were washed once again and analyzed by flow cytometry on a FACSAria™ III (BD). Collected data was subsequently analyzed by FlowJo software (Tree Star Inc.).

FITC-antibody conjugation of the anti-Tspan3 antibody

The anti-Tspan3 antibody was covalently labeled with FITC using the Lightning-Link FITC Conjugation Kit (abcam) according to manufacturer's protocol. Once conjugated, cells were incubated with the conjugated antibody for 30 minutes, washed once, and then analyzed by flow cytometry on a FACSAria™ III (BD). Collected data was subsequently analyzed by FlowJo software (Tree Star Inc.).

Cytospins

To make cytospins for staining, 5e5 cells per slide were mixed with 75ul cells in media and 75ul fetal bovine serum. This cell suspension was added to a Cytofunnel™ (Epredia) inserted into a Cytoclip™ (Epredia) with a Cytoslide™ (Epredia). The Cytoclips were loaded onto a Cytospin 4 centrifuge (Thermo Scientific). Slides were fixed with 4% paraformaldehyde (Thermo Scientific) for 10 minutes then washed with PBS (Gibco) twice for 5 minutes. Then slides were allowed to air dry at room temperature overnight.

To stain the cytospins, slides were first blocked with 10% normal goat serum (Vector Laboratories), 5% bovine serum albumin (Sigma), and 0.1% Triton (Sigma) for 1 hour at room temperature. After blocking, slides were incubated with the anti-Tspan3 antibody as a primary antibody at a 1:500 dilution in blocking buffer, overnight at 4°C. Slides were then washed three times with PBS-T for 5 minutes. An Alexa Fluor™ 488 goat anti-mouse secondary (Invitrogen) was used at a 1:1000 dilution for 1 hour at room temperature. Slides were washed 3 times with PBS for 5 minutes and then mounted using Fluoromount-G™ with DAPI (Invitrogen). The mounting media was allowed to cure overnight. Images were captured on a Confocal Leica TCS SP5 II (Leica Microsystems).

Annexin V staining for apoptosis

Following a pre-treatment with 20μg/ml IgG2b or 20μg/ml anti-Tspan3 antibody for 3 hours at 37°C, cells were collected and stained using the FITC Annexin V Apoptosis Detection Kit with PI according to manufacturer's protocol (BioLegend). Once stained, cells were analyzed by

flow cytometry on a FACSAria™ III (BD). Flow cytometry data was analyzed with the FlowJo software (Tree Star Inc.).

BrdU staining for proliferation

A BrdU staining kit (BD Biosciences) was used to stain proliferating LAMA84-s cells. LAMA84-s cells were pulsed with 10μM BrdU for 24 hours. Cells were collected and stained according to the manufacturer's protocol with a Pacific Blue™ BrdU monoclonal antibody (Invitrogen). Analysis was conducted on a FACSAria III (BD). Collected data was subsequently analyzed by FlowJo software (Tree Star Inc.).

HUVEC in vitro adhesion assay

HUVEC cells (ATCC) were plated and allowed to become confluent in 48-well plates before being activated with 10ng/ml TNFA (Gibco). The following day, LAMA84-s cells or patient samples were pretreated with IgG2b or anti-Tspan3 antibody for 3 hours at 37°C in a 5% CO_2 incubator. After this pretreatment, 5000 LAMA84-s cells or patient sample cells were added to the HUVECs and incubated at 37°C in a 5% CO_2 incubator for 2 hours. In addition to the pretreatment conditions, anti-VCAM1 blocking antibody was added as a positive control before plating on the HUVECs. After the 2-hour incubation, the wells were washed 3 times with PBS (Gibco) to remove unattached cells. The remaining cells were collected by adding trypsin. These cells were then stained for CD45 to label the adherent leukemia cells and analyzed on a FACSAria III (BD). Collected data was subsequently analyzed by FlowJo software (Tree Star Inc.).

In vitro synergy with anti-Tspan3 antibody and imatinib

Similar to the *in vitro* colony forming assay set up above, LAMA84-s cells were pretreated with IgG2b or anti-Tspan3 antibody for 3 hours. Imatinib was added to the methylcellulose at the time of adding the cell suspension. Colonies were counted after 7-10 days. The resultant colony counts were used to calculate percent inhibition and the web application SynergyFinder+ was used to calculate the synergy between imatinib and IgG2b or anti-Tspan3 antibody using the Loewe model[134].

Human cell line biomarker analysis

Transcriptomic information of each cell lines was collected from the Broad Institute's Cancer Dependency Map (DepMap). For biomarker analysis, Gene Set Enrichment Analysis (GSEA)[135] was performed with the Bioconductor GSVA[136] and the Bioconductor GSVAdata c2BroadSets gene set collection, which is the C2 collection of canonical gene sets from MsigDB3.0[135]. Briefly, GSEA evaluates a ranked gene expression dataset against previously defined gene sets. GSEA was performed with the following parameters: mx.diff = TRUE, verbose = TRUE, parallel.sz = 1, min.sz = 5, max.sz = 500, rnaseq = F.

shRNA mediated knockdown of Tspan3 for RNA-sequencing

Lentiviral shRNA constructs were designed and cloned into the pLV-hU6-EF1a-green backbone (Biosettia) according to manufacturer's instructions. LAMA84-s cells were infected with shRNA against LacZ or Tspan3 for 72 hours and positively infected cells were sorted using a FACSAria III (BD). Knockdown was confirmed by qRT-PCR as described above. The cells were

collected and used for either RNA extraction using the RNeasy Micro Plus kit (Qiagen) or for plating a colony forming assay, as described above.

The RNA was subsequently used for an RNA-seq. Its quality was assessed by Agilent Bioanalyzer and RIN scores were all above 9. The Illumina TruSeq Stranded mRNA kit was used to generate the library and the final amplification replaced the Illumina TruSeq PCR enzyme with KAPA HiFi HotStart Ready Mix. The libraries were sequenced on an Aviti following the manufacturer's instructions.

RNA-seq fastq files were processed into transcript-level summaries using *kallisto*[137], an ultrafast pseudo-alignment algorithm with expectation maximization. Transcript-level summaries were processed into gene-level summaries by adding all transcript counts from the same gene. Gene counts were normalized across samples using *DESeq* normalization[138] and the gene list was filtered based on mean abundance. Differential expression was assessed with an *R* package *limma*[139] applied to $\log_2$-transformed counts. Statistical significance of each test was expressed in terms of local false discovery rate (*lfdr*)[140] using the *limma* function *eBayes*[141]. *lfdr*, also called posterior error probability, is the probability that a particular gene is not differentially expressed, given the data.

LAMA84-s antibody response signature analysis

LAMA84-s cells were incubated with either 20μg/ml IgG2b or anti-Tspan3 antibody for 72 hours. After this incubation, viability was assessed by trypan blue. Total RNA was isolated using the RNeasy Micro Plus kit (Qiagen). Total RNA quality was assessed using an Agilent

Tapestation. RNA libraries were generated using the TruSeq Stranded mRNA Sample Prep Kit (Illumina) according to manufacturer's instructions.

RNA-seq fastq files were processed into transcript-level summaries using *kallisto*[137], an ultrafast pseudo-alignment algorithm with expectation maximization. Transcript-level summaries were processed into gene-level summaries by adding all transcript counts from the same gene. Gene counts were normalized across samples using *DESeq* normalization[138] and the gene list was filtered based on mean abundance. Differential expression was assessed with an *R* package *limma*[139] applied to $\log_2$-transformed counts. Statistical significance of each test was expressed in terms of local false discovery rate (*lfdr*)[140] using the *limma* function *eBayes*[141]. *lfdr*, also called posterior error probability, is the probability that a particular gene is not differentially expressed, given the data.

IgG2b and M6 mAb treated LAMA84-s RNA-seq fastq files in were processed into transcript-level summaries using kallisto[137]. Transcript-level summaries were processed into gene-level summaries and differential gene expression was performed using sleuth with the Wald test[142]. GSEA was performed as detailed above[135]. Gene ontology analysis was performed using Metascape using a custom analysis with GO biological processes and default settings with genes with a FDR < 5% and a beta value > 0.5.

Notch inhibitor colony forming assay

LAMA84-s cells were used to set up an *in vitro* colony forming assay as described above. DAPT (StemCell Technologies) or DBZ (StemCell Technologies) was added to the cell suspension, and this was added to methylcellulose. The colonies were counted after 7-10 days of incubation

at 37°C in a 5% CO_2 incubator. To test synergy between DAPT and imatinib, both compounds were added to the cell suspension and subsequently added to the methylcellulose. The resultant colonies were counted after 7-10 days at 37°C in a 5% CO_2 incubator.

In vivo anti-Tspan3 monoclonal antibody treatment

For human leukemia cell lines and patient samples, 100,000 cells were transplanted into sublethally irradiated (2.75Gy) NSG mice. The anti-Tspan3 monoclonal antibody was administered intraperitoneally two days post-transplant at 15mg/kg and continued three times a week for three to four weeks. Bone marrow and spleens were isolated and analyzed by FACS. Bones were crushed by mortar and pestle and rinsed with Hanks' balanced salt solution (HBSS) (Gibco) containing 5% fetal bovine serum and 2 mM EDTA. Spleens cells were isolated and resuspended with Hanks' balanced salt solution (HBSS) (Gibco) containing 5% fetal bovine serum and 2 mM EDTA. Once the bone marrow cells and the spleen cells were isolated and in suspension, red blood cells were lysed using RBC Lysis Buffer (eBioscience) before staining for human leukemia cells. Leukemia chimerism was measured by antibodies against hCD45 (Invitrogen) for human leukemia. Following staining and washing, propidium iodide was added to the cell solution, and the samples were analyzed on a FACSAria II. Collected data was subsequently analyzed by FlowJo software (Tree Star Inc.).

Antibody humanization

We partnered with another CRO, Curia, to use their protocol for humanization. In brief, the murine antibody sequence is analyzed, and the 3D structure is modeled for human

homology. They identified key positions supporting the complementarity-determining region (CDR) loop structure and the heavy chain and light chain interface. From this, they designed 3 humanized heavy chains and 3 humanized light chains. They assessed the humanness of these variants by the T20 humanness score[143]. After confirming the humanization of the sequence was successful, they generated nine humanized antibodies comprised of the three heavy chains and three light chains, as well as a chimera, with CHO cells. Then they used FACS to measure the EC50 for each individual humanized antibody.

Statistical analysis

Statistical analyses were done on Prism version 10.1.1 (GraphPad Software Inc.). All data shown is mean $\pm$ standard deviation. Either a one-way or two-way ANOVA with Bonferroni correction was used to calculate statistical significance where applicable.

3.4 Results

In vitro application of the anti-Tspan3 mAb on myeloid leukemia

Once we determined which antibody clone to utilize, we applied it to different forms of myeloid leukemia. Given the high homology between human and murine Tspan3, we were interested in determining if there was any species cross-reactivity. We found that the M6 mAb was able to significantly block the growth of murine bcCML *in vitro* by up to 2-fold compared to IgG control (Figure 3.2A, n = 5-12 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, one-way ANOVA with Bonferroni correction). It was also effective against different myeloid leukemia cell lines, to varying degrees. Cells that did not respond were identified as

low responders. Those that showed a trend to respond were identified as mid responders. Those that showed a significant response were identified as high responders. While the M6 mAb was not significantly effective at preventing the growth of the AML cell line KCL22-s (Figure 3.2B, n = 2-6 technical replicates, p = NS, one-way ANOVA with Bonferroni correction) or the Biphenotypic B Myelomonocytic Leukemia cell line MV411 (Figure 3.2C, n = 3-9 technical replicates, p = NS one-way ANOVA with Bonferroni correction), the bcCML cell line K562 (Figure 3.2D, n = 3-11 technical replicates, p = NS, one-way ANOVA with Bonferroni correction) showed a trend of responding to the antibody treatment, though non-significant. However, the M6 mAb was effective against other myeloid leukemia cell lines. For the AML cell line Kasumi-6 (Figure 3.2E, n = 6-9 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, one-way ANOVA with Bonferroni correction) and the bcCML cell LAMA84-s (Figure 3.2F, n = 5-9 technical replicates, * = $P < 0.05$, one-way ANOVA with Bonferroni correction), there was a significant reduction in growth, between 28% and 48%. The difference in response was then used to generate a preliminary biomarker profile between low responders, mid responders, and high responders.

In addition to the human leukemia cell lines, we also tested additional AML patient samples, a bcCML sample, and a myelodysplastic syndromes (MDS) sample. Here we are showing three representative AML patient samples treated with the M6 mAb (Figure 3.2G-J, n = 3 technical replicates of 3 biological replicates, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, one-way ANOVA with Bonferroni correction). We are also showing a representative bcCML patient sample (n = 3 technical replicates, ** = $P < 0.01$, one-way ANOVA with Bonferroni correction. MDS is often the precursor to AML so preventing MDS growth could indicate the Tspan3 mAbs could function as a preventative treatment to hinder disease

progression. We found that incubating the MDS sample with the M6 mAb significantly impacted its ability to form colonies by 60% (Figure 3.2K, n = 5 technical replicates, * = $P < 0.05$, one-way ANOVA with Bonferroni correction). Overall, we found that 11 out of 15 primary patient samples responded to the Tspan3 mAb *in vitro*. This spans a mixture of AML, bcCML, and MDS samples, indicating that targeting Tspan3 may be a viable treatment strategy for both prevention of progression and treatment of end stage disease.

Ideally, a cancer treatment will be able to target the cancer cells while leaving the normal cells alive and functional. To ensure that the normal hematopoietic stem and progenitor cells (HSPCs) of the bone marrow would be unaffected by the M6 mAb, we used normal human $CD34^+$ bone marrow cells in both a colony forming assay and viability assay. In the colony forming assay, there was a non-significant difference in growth when the cells were treated with either IgG2b or the M6 mAb (Figure 3.2L, n = 3 technical replicates, p = NS, one-way ANOVA with Bonferroni correction). When measuring the viability after 24 hours in culture, there was a slight (6%) reduction in viability at 50ug/ml, which is a 10-fold greater concentration than the minimally effective dose for myeloid leukemia response (Figure 3.2M, n = 3 technical replicates, * = $P < 0.05$, one-way ANOVA with Bonferroni correction). These results are encouraging as they indicate that targeting Tspan3 by blocking mAb won't affect the remaining normal HSPCs in the bone marrow.

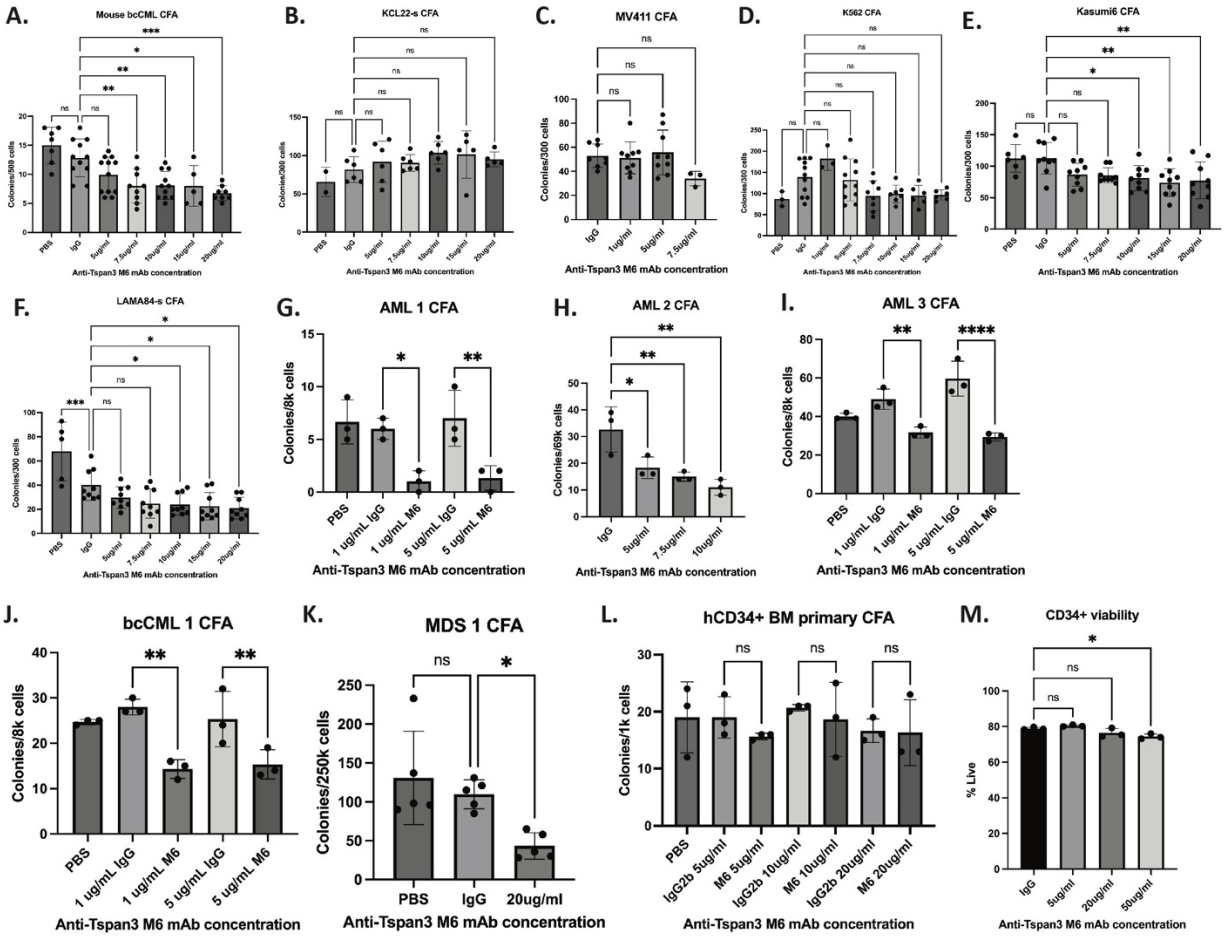


Figure 3.2 Inhibition of colony formation *in vitro* with the M6 mAb

(A) Significant inhibition of colony formation using primary murine bcCML (n = 5-12 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, one-way ANOVA with Bonferroni correction). (B-F) Colony formation of (B) human AML cell line KCL22-s (n = 2-6 technical replicates, p = NS, one-way ANOVA with Bonferroni correction). (C) Biphenotypic B Myelomonocytic Leukemia cell line MV411 (n = 3-9 technical replicates, p = NS one-way ANOVA with Bonferroni correction). (D) bcCML cell line K562 (n = 3-11 technical replicates, p = NS, one-way ANOVA with Bonferroni correction). (E) AML cell line Kasumi-6 (n = 6-9 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, one-way ANOVA with Bonferroni correction). (F) bcCML cell LAMA84-s (n = 5-9 technical replicates, * = $P < 0.05$, one-way ANOVA with Bonferroni correction). (G-I) Significant inhibition of colony formation using three independent AML patient samples (n = 3 technical replicates of 3 biological replicates, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, one-way ANOVA with Bonferroni correction). (J) Significant inhibition of colony formation using a bcCML patient sample (n = 3 technical replicates, ** = $P < 0.01$, one-way ANOVA with Bonferroni correction). (K) Significant inhibition of colony formation using an MDS patient sample (n = 5 technical replicates, * = $P < 0.05$, one-way ANOVA with Bonferroni correction). (L) No significant impact on colony formation using normal human CD34+ bone marrow cells (n = 3 technical replicates, p = NS, one-way ANOVA with Bonferroni correction). (M) Minimal impact on viability using normal human CD34+ bone marrow cells (n = 3 technical replicates, * = $P < 0.05$, one-way ANOVA with Bonferroni correction).

Validating the binding of the M6 mAb to human Tspan3

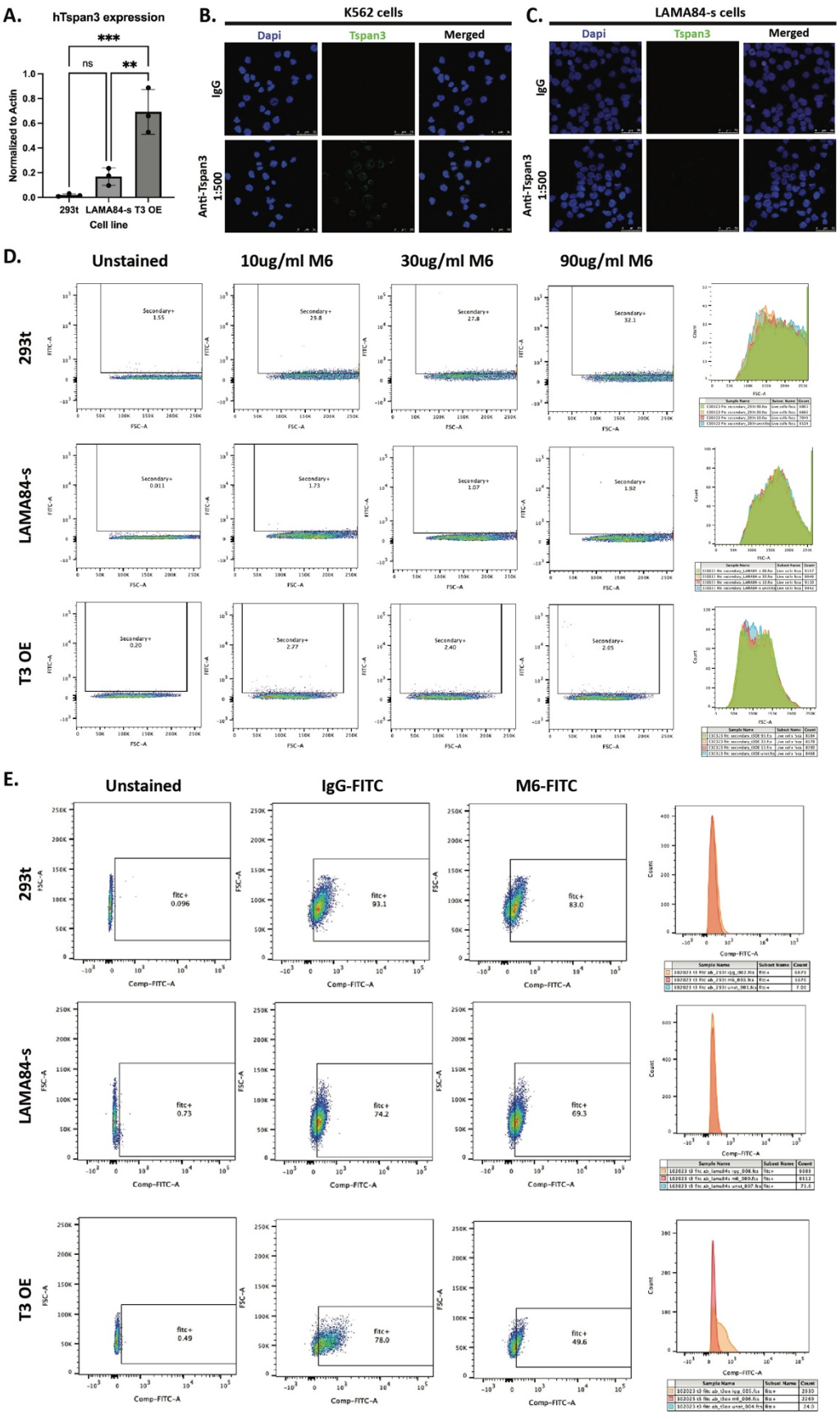
After confirming the effect of the M6 mAb on myeloid leukemia, we next turned to validating that it's binding to human Tspan3. To confirm human Tspan3 protein expression, we used a western blot with a commercially available antibody against Tspan3. For a negative control, we used 293Ts, an epithelial-like cell line that should not have any expression. For a positive control, we used a murine Tspan3 overexpressing cell line, T3 OE. With the use of these controls, we were able to determine that LAMA84-s cells do express human Tspan3 (Figure 3.3A, n = 3 technical replicates, ** = $P < 0.01$, *** = $P < 0.001$, one-way ANOVA with Bonferroni correction).

This western blot confirmed protein expression of Tspan3, but it used a commercially available antibody rather than the M6 mAb against Tspan3. To build more evidence of direct binding between the M6 mAb and human Tspan3, we used immunofluorescence (IF) staining on cytospins of LAMA84-s cells and K562 cells. K562 cells were positively labeled for Tspan3 by IF while LAMA84-s cells were not (Figure 3.3B-C). Though LAMA84-s cells are confirmed to express human Tspan3, there may have been too low of expression to be seen by IF.

In addition to IF staining, we also used the M6 mAb to measure Tpsan3 expression by flow cytometry analysis. Given that the M6 mAb is unconjugated, first we attempted to use a fluorescent secondary to bind to M6 as the primary antibody (Figure 3.3D). This strategy did not yield very conclusive results as not even the T3 OE Tspan3 overexpressing cells were labeled. To have a more direct way to label Tspan3 by antibody for flow cytometry, we covalently conjugated the M6 mAb to FITC. This conjugated M6 mAb was then used to stain different cell lines and once again, it did not yield conclusive staining (Figure 3.3E).

Figure 3.3 Validating the binding of the M6 mAb to human Tspan3

(A) Confirmation of human Tspan3 expression in 293t cells, LAMA84-s cells, and Tspan3 overexpressing cells (T3 OE) by western blot (n = 3 technical replicates, ** = $P < 0.01$, *** = $P < 0.001$, one-way ANOVA with Bonferroni correction). (B-C) Immunofluorescent staining of (B) K562 cell cytospins and (C) LAMA84-s cell cytospins using the M6 mAb. (D) FACS analysis using a fluorescent secondary to bind to M6 mAb as the primary antibody. (E) FACS analysis using covalently conjugated FITC to the M6 mAb.


A.
hTspan3 expression
***
ns
**
Normalized to Actin
1.0
0.8
0.6
0.4
0.2
0.0
293t
LAMA84-s T3 OE
Cell line
B.
K562 cells
Dapi
Tspan3
Merged
IgG
Anti-Tspan3 1:500
C.
LAMA84-s cells
Dapi
Tspan3
Merged
IgG
Anti-Tspan3 1:500
D.
Unstained
10ug/ml M6
30ug/ml M6
90ug/ml M6
293t
LAMA84-s
T3 OE
Secondary+ 1.55
Secondary+ 29.8
Secondary+ 27.8
Secondary+ 32.1
Secondary+ 0.011
Secondary+ 1.73
Secondary+ 1.07
Secondary+ 1.92
Secondary+ 0.20
Secondary+ 2.77
Secondary+ 2.40
Secondary+ 2.05
FITC-A
FSC-A
Count
E.
Unstained
IgG-FITC
M6-FITC
293t
LAMA84-s
T3 OE
fitc+ 0.096
fitc+ 93.1
fitc+ 83.0
fitc+ 0.73
fitc+ 74.2
fitc+ 69.3
fitc+ 0.49
fitc+ 78.0
fitc+ 49.6
FSC-A
Comp-FITC-A
Count

Functional assays using the anti-Tspan3 mAb on human myeloid leukemia

While the binding capacity of the M6 mAb proved to be difficult to fully confirm, we had previously confirmed that the antibody was affecting the myeloid cells given the response to *in vitro* treatment with the M6 mAb. However, we are still interested in understanding how the M6 mAb is functionally impacting the leukemia cells. To begin with, we first checked overall viability and cell counts after a 72-hour incubation with the M6 mAb with trypan blue. There was no significant difference in either overall viability or cell number (Figure 3.4A-B, n = 3 technical replicate, p = NS, one-way ANOVA with Bonferroni correction). To examine the effect of the M6 mAb more closely on cell health, we used Annexin V and propidium iodide (PI) staining to analyze cell death by flow cytometry. This allows us to differentiate between viable cells, early apoptotic, and late apoptotic cells. Using LAMA84-s cells, we found no significant difference between IgG2b and M6 mAb antibody treatment for any stage of cell death (Figure 3.4C, n = 3 technical replicates, p = NS, one-way ANOVA with Bonferroni correction). In addition to looking closer at cell death, we also looked at a more sensitive assay for proliferation, BrdU staining. After a pulsing with BrdU, we found no significant difference in cell proliferation between IgG2b or M6 mAb treatment (Figure 3.4D, n = 3 technical replicates, p = NS, one-way ANOVA with Bonferroni correction).

With no apparent cell death phenotype, we hypothesized that there may be an adhesion defect to bone marrow stroma when *in vivo* and the antibody may interrupt that binding. To simulate one aspect of stroma, we used an endothelial cell line, HUVECs, to co-culture with LAMA84-s cells or AML patient sample cells. For LAMA84-s cells, there was no significant difference in adhered $hCD45^+$ leukemia cells (Figure 3.4HE, n = 3 technical replicates,

p = NS, one-way ANOVA with Bonferroni correction). For the AML patient samples, there was a 50% reduction in adhered hCD45^{+} leukemia cells when incubated with the M6 mAb compared to incubation with IgG2b (Figure 3.4F, n = 2 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, one-way ANOVA with Bonferroni correction). While it wasn't to the same depth of the positive control, VCAM-1, it does indicate that the M6 mAb can interrupt binding between leukemia cells and endothelial cells.

In addition to functional assays to understand the effect of the M6 mAb on leukemia cells, we were also interested in its ability to synergize with a frontline bcCML treatment, imatinib. A colony forming assay was used to establish the percent inhibition of colony forming ability when cells are treated with IgG2b or the M6 mAb in the presence or absence of imatinib (Figure 3.4G, n = 9 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, two-way ANOVA with Bonferroni correction). We used the web application, SynergyFinder+[134], to calculate the synergy using the Loewe model. We found that while IgG2b was not synergistic with imatinib, but the M6 mAb was (Figure 3.4H-I). The M6 mAb and imatinib received a synergy score exceeding 20 at 20μg/ml M6 mAb and 1μM imatinib, indicating there is synergy between the two treatments. Given that imatinib remains a frontline therapy for chronic myeloid leukemia, significantly synergizing with it could lead to increased patient response and improved patient outcomes.

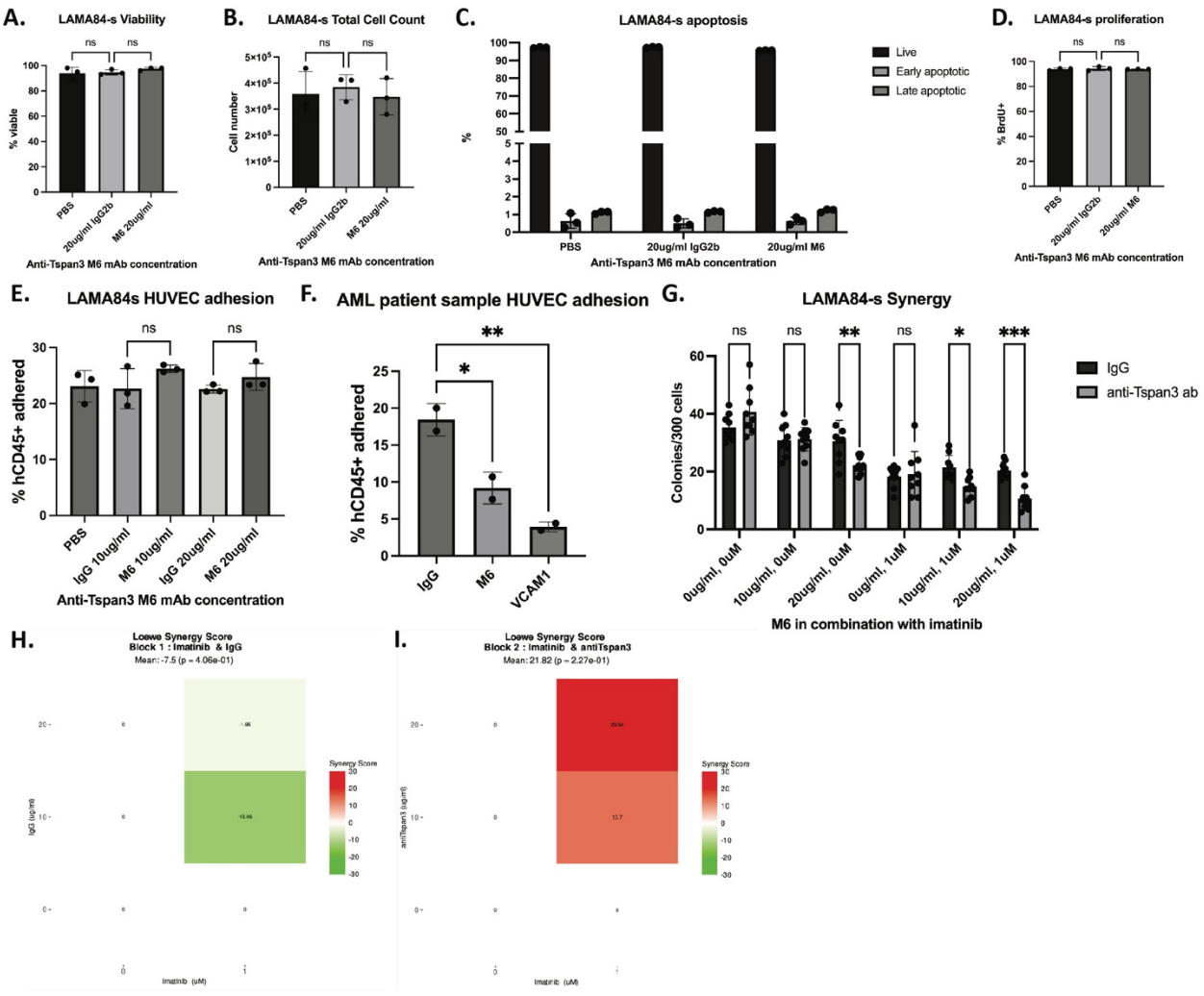


Figure 3.4 Functional assays using the M6 mAb on human myeloid.

(A-B) Measuring (A) viability and (B) cell count of LAMA84-s cells by trypan blue after incubation with either IgG2b or M6 mAb (n = 3 technical replicate, p = NS, one-way ANOVA with Bonferroni correction). (C) FACS analysis of apoptotic state by Annexin V and PI using LAMA84-s cells after incubation with either IgG2b or M5 mAb (n = 3 technical replicates, p = NS, one-way ANOVA with Bonferroni correction). (D) FACS analysis of proliferation by BrdU incorporation in LAMA84-s cells (n = 3 technical replicates, p = NS, one-way ANOVA with Bonferroni correction). (E-F) Percentage of (F) LAMA84-s cells (n = 3 technical replicates, p = NS, one-way ANOVA with Bonferroni correction) and (F) AML patient sample cells (n = 2 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, one-way ANOVA with Bonferroni correction) adhered to HUVECs after a wash-off assay. (G) Inhibition of colony formation with LAMA84-s cells using IgG2b or the M6 mAb in combination with imatinib (n = 9 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, two-way ANOVA with Bonferroni correction). (H-I) Loewe model synergy score with imatinib in combination with (H) IgG2b or (I) M6 mAb.

Transcriptomic landscape changes after Tspan3 inhibition

As mentioned previously, we used the differential response between human leukemia cell lines to generate a preliminary biomarker signature. We compared the transcriptomic profile of the cell lines that were low, mid, and high responders using the Broad Institute's Dependency Map (DepMap) Portal for the expression data of these cell lines. This expression data was used to run Gene Set Enrichment Analysis (GSEA) (Figure 3.5A). The analysis indicated that there was a significant difference in the expression of hematopoietic cell lineage markers (Figure 3.5B) that can potentially be used to differentiate between response.

To increase our understanding of the impact Tspan3 has on myeloid leukemia cells, we used an shRNA mediated knockdown of Tspan3 on LAMA84-s cells for RNA-sequencing. There are several key candidate genes, including FZD6 and MAP2K1, that are involved in maintaining stemness (Figure 3.5C). We found that these genes were downregulated upon Tspan3 knockdown which could lead to decreased stemness in the myeloid leukemia cells. We confirmed the downregulation of FZD6 and MAP2K1 by qPCR (Figure 3.5D, n = 3 technical replicates, ** = $P < 0.01$, one-way ANOVA with Bonferroni correction). The shRNA mediated knockdown of Tspan3 is a more specific, targeted means to decrease Tspan3 signaling than just using an anti-Tspan3 mAb. However, it does not explore the transcriptomic differences induced by incubation with the M6 mAb.

To further investigate the downstream, transcriptional response to the M6 mAb itself, we conducted an RNA-seq on LAMA84-s cells after a 72-hour incubation with either IgG2b or the M6 mAb. There were a number of downregulated genes that include transcription factors, other members of the tetraspanin family, and kinases (Figure 3.5E). Among the downregulated

genes were also several genes involved in processes including cell motility, binding to the extracellular matrix for adhesion, and cell-to-cell communication (Figure 3.5E). These genes include SEMA family members SEMA4C[144] and SEMA7A[145], ITGA2B[146], CELSR2[147], and CD81[148]. SEMA4C is the cell surface receptor for PLXNB2 and plays an important role in cell-to-cell signaling[144]. SEMA7A plays an important role in integrin-mediated signaling, motility, invasion and adhesion[145]. ITGA2B was originally identified as a receptor for collagens and laminins, though it has since been found to bind to several other extracellular matrix molecules[149]. CELSR2 has been implicated as a receptor involved in contact mediated communication[147]. CD81, another member of the tetraspanin family, is involved in cell migration and invasion[148]. Downregulation of these genes that facilitate interactions between the leukemia cell and other surrounding cells has may suggest that the inhibition of Tspan3 may lead to decreased ability of the leukemia cells to derive support from the niche. Additionally, by using GSEA, we found that there was also a reduction in stem cell programs (Figure 3.5F) after incubation with the M6 mAb, with multiple elements within the Notch pathway impacted, as well as transcription and chromatin programs (Figure 3.5G).

We followed up on the impact on the Notch pathways by indirectly inhibiting Notch signaling through gamma-secretase inhibition. Using two gamma-secretase inhibitors, DBZ (Figure 3.5H, n = 3 technical replicates, ** = $P < 0.01$, *** = $P < 0.001$, one-way ANOVA with Bonferroni correction) and DAPT (Figure 3.5I, n = 3 technical replicates, ** = $P < 0.01$, *** = $P < 0.001$, one-way ANOVA with Bonferroni correction), we found that they minimally suppressed colony forming ability of LAMA84-s cells. This suggests that Tspan3 may be interacting with Notch at the membrane to organize the signaling components at the membrane and reinforce

the signaling rather than Notch being directly involved.

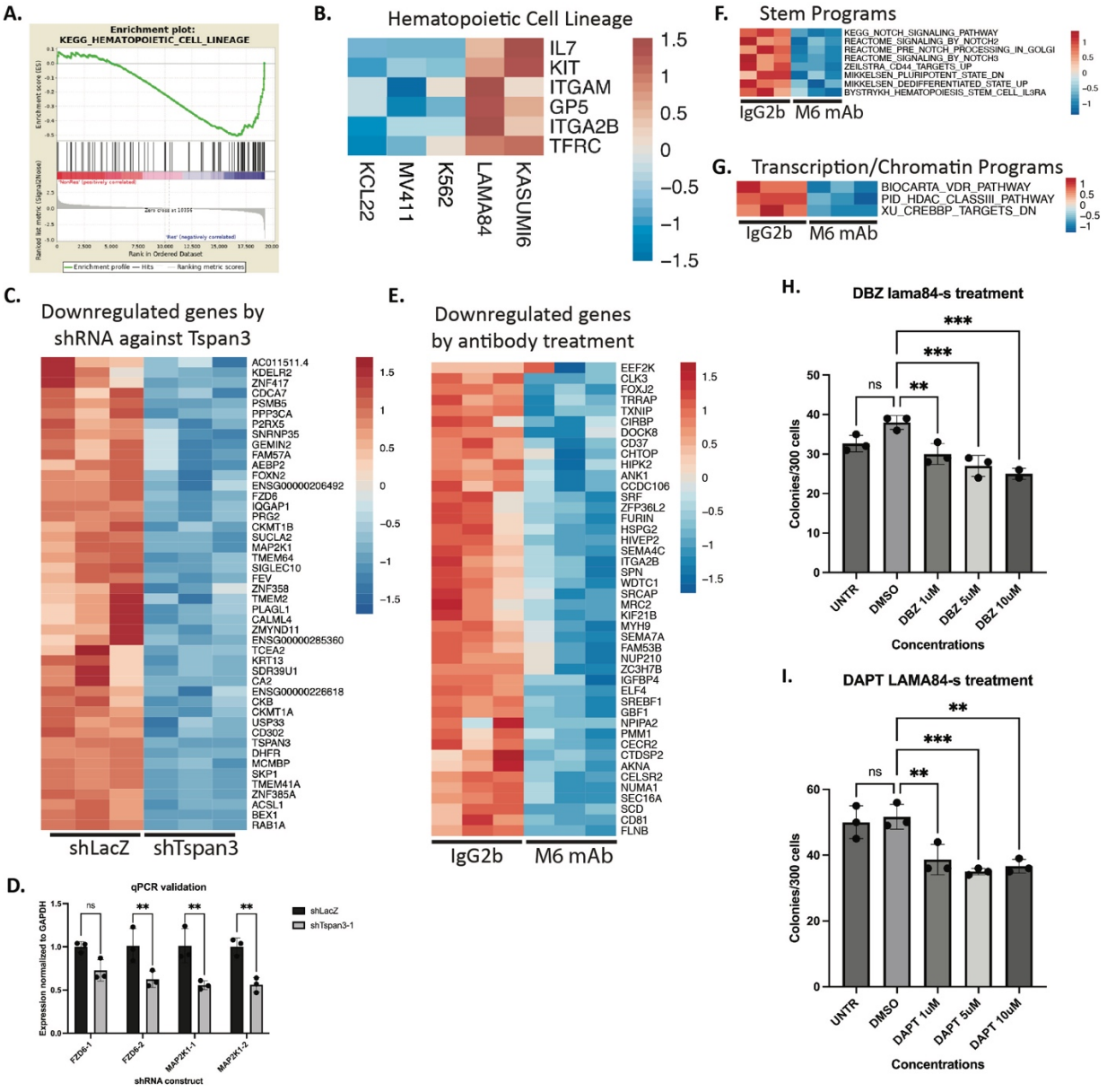


Figure 3.5 Impact of Tspan3 inhibition on transcriptional landscape of human myeloid leukemia

(A) GSEA of low, mid, and high responder cell lines with transcriptomic data from DepMap with a focus on hematopoietic cell lineage markers. (B) Hematopoietic cell lineage markers upregulated as samples move from low to mid to high responder cell lines. (C) Heatmap of downregulated genes in LAMA84-s cells after Tspan3 knockdown by shRNA. (D) qPCR validation of genes linked to stemness from the shTspan3 RNA-seq (n = 3 technical replicates, ** = $P < 0.01$, one-way ANOVA with Bonferroni correction). (E) Heatmap of downregulated genes in LAMA84-s cells after incubation with either IgG2b or M6 mAb. (F-G) GSEA of LAMA84-s cells treated with IgG2b or M6 mAb in (F) stem programs and (G) transcription and chromatin programs. (H-I) Inhibiting colony formation of LAMA84-s cells with gamma-secretase inhibitors (H) DBZ (n = 3 technical replicates, ** = $P < 0.01$, *** = $P < 0.001$, one-way ANOVA with Bonferroni correction) and (I) DAPT (n = 3 technical replicates, ** = $P < 0.01$, *** = $P < 0.001$, one-way ANOVA with Bonferroni correction).

In vivo treatment of myeloid leukemia with the anti-Tspan3 mAb

With the goal of eventually targeting Tspan3 by blocking antibody in the clinic, we wanted to understand how delivering the M6 mAb *in vivo* would impact both the leukemia cells and the normal tissues of the body. We designed a treatment strategy of one intraperitoneal injection every week for three weeks of 15mg/kg IgG2b or M6 mAb. With this strategy, we saw a reduction in chimerism, at most 44%, for both cell lines in the bone marrow and the spleen (Figure 3.6A,B, n = 4 technical replicates, * = $P < 0.05$, one-way ANOVA with Bonferroni correction). While the patient samples chosen to be transplanted all responded *in vitro*, there was only a non-significant trend of decreasing burden in both the spleen and bone marrow (Figure 3.6C, n = 8-11, p = ns, one-way ANOVA with Bonferroni correction).

In addition to treating xenografts *in vivo*, we also treated normal C57BL/6 mice with either IgG2b or the M6 mAb. Using the same treatment plan as above, there was no apparent off target effects on other tissues or toxicity. Upon a complete necropsy and hematology analysis, there were no uncommon lesions found in all the tissues examined (data not shown).

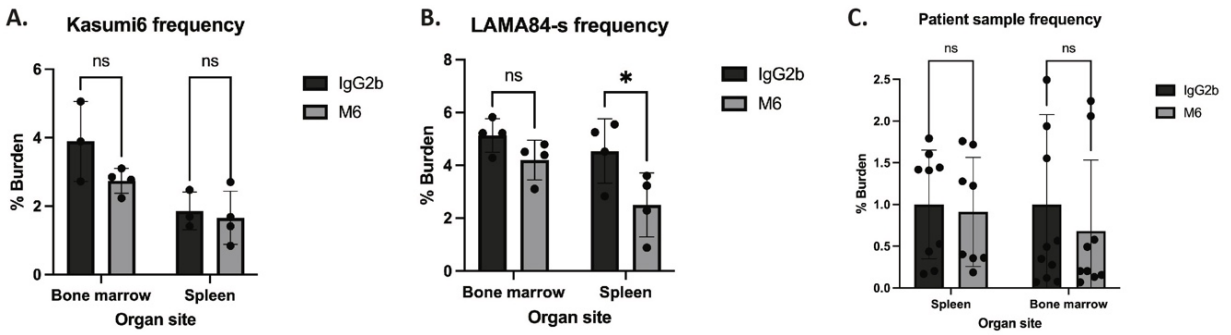


Figure 3.6 In vivo treatment of myeloid leukemia with the M6 mAb

(A-B) Leukemia burden in the spleen and bone marrow after transplantation of human cell lines (A) Kasumi6 (n = 3-4 technical replicates, p = NS, one-way ANOVA with Bonferroni correction) and (B) LAMA84-s (n = 4 technical replicates, * = $P < 0.05$, one-way ANOVA with Bonferroni correction). (C) Leukemia burden in the spleen and bone marrow after transplantation of AML patient samples (n = 8-11, p = NS, one-way ANOVA with Bonferroni correction).

Testing the impact of humanized anti-Tspan3 antibodies on human myeloid leukemia

Following the humanization strategy, we received eight humanized antibodies and one chimera. Of the eight we received, we used a colony forming assay to assess capacity of the humanized antibodies to inhibit colony formation. Five out of the eight humanized antibodies significantly inhibited colony forming of LAMA84-s cells (Figure 3.7A-H, n = 3 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, **** = $P < 0.0001$, one-way ANOVA with Bonferroni correction was used). The most effective humanized antibody was able to inhibit colony formation by up to 68% (Figure 3.7D, n = 3 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, **** = $P < 0.0001$, one-way ANOVA with Bonferroni correction was used). This improves upon the murine antibody which is encouraging for the further development of a blocking antibody targeting Tspan3.

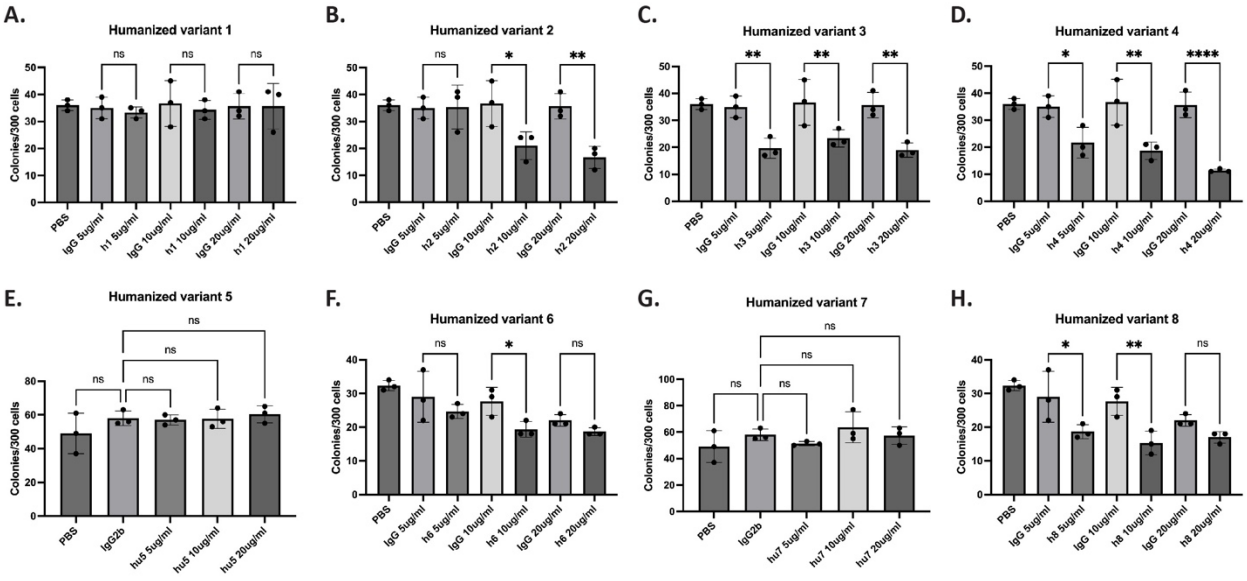


Figure 3.7 Colony forming assays using humanized variants of the anti-Tspan3 mAb

(A-H) Inhibition of colony formation using LAMA84-s with humanized antibody variants (n = 3 technical replicates, * = $P < 0.05$, ** = $P < 0.01$, **** = $P < 0.0001$, one-way ANOVA with Bonferroni correction was used).

3.5 Discussion

Building upon previous work that identified Tspan3 as critical regulator of myeloid leukemia[130], we developed a blocking monoclonal antibody (mAb) against human Tspan3. By using this mAb to block Tspan3 signaling, we found that we were able to significantly inhibit myeloid leukemia growth in both cell lines and patient samples *in vitro* and *in vivo*. We were able to validate the mAbs binding to human Tspan3 by immunofluorescence. With this confirmation of binding and of functional impact on myeloid leukemia, we were interested in determining how the mAb was affecting the cells directly. However, this proved to be elusive. Through sequencing both myeloid leukemia cells treated with an shRNA against Tspan3 and the Tspan3 blocking mAb, we found there were several alterations made to stem programs including Notch signaling. We followed up on the Notch signaling and determined that, while

Notch inhibitors led to a decrease in colony formation, there was no synergy with the Tspan3 mAb. However, all this work was done with a murine antibody, which can induce an immune reaction if used in patients[150-153]. To further our goal of eventually reaching the clinic with this mAb, we humanized the anti-Tspan3 blocking mAb and tested the different humanized constructs on a myeloid leukemia cell line. We found that a majority of them were able to block colony growth to an even greater depth than the murine antibody, which is very encouraging for the future development of this therapy.

Not only did the blocking mAb against Tspan3 lead to decreased myeloid leukemia growth, but it also did not significantly impact normal hematopoietic stem and progenitor cells (HSPCs) viability or their ability to form colonies. This suggests that the anti-Tspan3 antibody has limited toxicity and thus inform efforts to move this work forward into the clinic. As leukemia develops and progresses, normal HSPCs begin to falter in their normal function[61, 62]. This is partially due to CSCs outcompeting HPSCs for space and resources in the niche that helps support their function[57, 59, 60]. This competition both benefits CSCs while negatively impacting HSPCs. Therefore, it is critical to support whatever remaining normal HSPCs are present in the bone marrow during treatment for leukemia[154]. Given that it has been established that mice can be born without Tspan3 with no detectable impact to their bone marrow content at adulthood[130], it is likely that a blocking mAb against Tspan3 would not interfere with normal cells. This proved to be true as treatment with the blocking mAb both *in vitro* and *in vivo* did not impair normal HSPC function and there were no gross morphological or hematological differences in other tissues. This makes for an ideal cancer therapeutic where the impact on the cancer is clear while avoiding impacting normal, off-target cells.

However, given that Tspan3 is enriched in HSPCs over their more differentiated daughter cells, we were interested in understanding the role of Tspan3 on stemness. To accomplish this, we used RNA-sequencing to look at transcriptional changes after shRNA mediated knockdown of Tspan3. After Tspan3 knockdown, we that found several genes and pathways involved in stemness had reduced expression when Tspan3 is inhibited. Map2k1 and Fzd6 were among the genes downregulated by Tspan3 inhibition by shRNA. Map2k1 has been found to drive stem cell self-renewal[155]. Therefore, its downregulation would lead to decreased stem cell self-renewal, which is a key feature of CSCs[98, 156]. CSCs use self-renewal paired with asymmetric cell division to ensure the continuation of the tumor population[12, 157-159]. Fzd6 has also been implicated in maintaining stemness and serving as a prognostic marker in several cancers including neuroblastomas[160], triple negative breast cancer[161], glioblastoma[162], and chronic lymphocytic leukemia[163]. Fzd6 is a Wnt receptor and functions in both a canonical and non-canonical capacity in cancer[164]. With Fzd6's role in contributing to stemness, its repression by Tspan3 inhibition would lead to decreased stemness and increased differentiation. Given that CSCs are the leading cause of relapse in aggressive leukemias such as AML, targeting Tspan3 to induce increased differentiation would improve patient response duration[34].

In addition to the RNA-seq conducted on LAMA84-s cells after shRNA treatment, we also used RNA-seq to look at the transcriptomic changes after incubation with the M6 anti-Tspan3 mAb. Among the genes that were downregulated were several that are involved in mediating extracellular interactions between the leukemia cells and surrounding cells or the extracellular matrix. Included in the downregulated genes are SEMA family members SEMA4C[144] and SEMA7A[145], ITGA2B[146], CELSR2[147], and CD81[148]. Without these genes facilitating the interactions

between leukemia cells and their surroundings, the leukemia cell can become unattached and lose the support of the niche. This would leave them vulnerable to treatment as they exit the supportive niche which can also cause the CSCs to differentiate. This can render them sensitive to therapies that they were resistant to initially[165, 166].

Currently, there are many novel antibody based therapies that are being used to treat a variety of cancers[167, 168]. One of the first therapeutic monoclonal antibodies for the treatment of cancer, rituximab, changed the landscape of treatment for B-cell malignancies[169]. With immunotherapy becoming a leading component of cancer therapy regimens, there is an ongoing search for cell surface targets enriched on cancer cells. Our findings here indicate that targeting Tspan3 by mAb could be a new approach for treating aggressive myeloid leukemias.

3.6 Acknowledgments

Chapter 3, in part is currently being prepared for submission for publication of the material. Diaz E, Shima Y, Pettit H, Hamilton M, Quintero C, Kritzik M, Reya T. The dissertation author was the primary researcher and author of this material.

Conclusions

Unfortunately, the fate for many leukemia patients, particularly those with aggressive myeloid leukemia like acute myeloid leukemia (AML), is that of relapse and death. Current standard of care for AML is an aggressive regimen of chemotherapy and radiation, often followed by a hematopoietic stem cell transplant if possible[170-172]. Given that AML most often occurs in the very young and the very old, this treatment plan is not very well tolerated[172-174]. New advancements are required in order to improve patient outcomes in a disease that has not made significant progress in treatments in decades[175, 176]. The studies outlined here represent two novel targets, GFER and Tspan3, at the interface between the leukemia cell and the bone marrow.

One area of research that is becoming increasingly important to address, is the role of the bone marrow microenvironment on leukemia. It has been fairly well established at this point that, similar to solid tumors, liquid tumors like leukemia significantly rely on their surroundings to survive and propagate. This is especially true for cancer stem cells (CSCs). CSCs recapitulate many of the features of normal hematopoietic stem cells (HSCs) including residing within a supportive niche. The niche provides a variety of signals to HSCs to maintain their stem properties. This includes cytokines, exosomes, and physical connections to stromal cells and extracellular matrix proteins[177, 178]. When these signals are imbalanced or missing during leukemia initiation and progression, this can lead to HSC mislocalization, differentiation, and cell death. Therefore, it is critical to understand how the niche can be exploited to weaken CSCs and strengthen the remaining HSCs.

Here, we have identified GFER as a potential mediator of niche localization. We found that when GFER was knocked down, leukemia cells egressed from the niche into the vasculature. Once leukemia cells are out of the niche, they lose the support that was helping them maintain their stemness. This mislocalization also makes exposure to chemotherapeutics easier as leukemia cells within their niche are historically difficult to reach because of vascular failure induced by the leukemia cells. GFER, a secreted protein, has the potential to interact with the bone marrow microenvironment and create structural changes within the niche. This could impact the retention of CSCs within the niche that will further maintain its stemness, which can eventually lead to residual disease and relapse. Therefore, targeting CSCs and their interactions with the niche directly can improve patient outcomes by reducing the rate of relapse.

To target CSCs directly, we developed a monoclonal antibody against Tspan3. The utilization of the antibody led to decreased growth of primary myeloid leukemia samples and led to their detachment from endothelial cells. Inhibition of Tspan3, either by shRNA or monoclonal antibody, led to changes in stem programs. We also found that the anti-Tspan3 monoclonal antibody treatment did synergize with imatinib, a front-line targeted therapy for chronic myeloid leukemia (CML). By targeting CSCs itself, in combination with traditional targeted treatments or chemotherapy, it allows for general debulking of the tumor while also preventing it from growing back.

There is a significant amount of evidence building that shows the bone marrow microenvironment and CSCs are deeply intertwined. By targeting the bone marrow microenvironment and CSC directly, it can reduce the rate of relapse as CSCs are unable to

replenish the tumor after therapy. The work here outlines two potential candidates that can be targeted for the treatment of myeloid leukemia. Further work will be required to bring blocking antibodies against GFER and Tspan3 to the clinic, but these initial results are highly encouraging.

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