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Novel tumor suppressor function of KLF4 in pediatric T-cell acute lymphoblastic leukemia

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Abstract

Acute lymphoblastic leukemia (ALL) is the most common hematological malignancy in pediatric patients. Despite advances in the treatment of this disease, many children with T-cell ALL (T-ALL) die from disease relapse due to low responses to standard chemotherapy and the lack of a targeted therapy that selectively eradicates the chemoresistant leukemia-initiating cells (LICs) responsible for disease recurrence. We recently reported that the reprogramming factor KLF4 has tumor-suppressive function in children with T-ALL. KLF4 silencing by promoter DNA methylation in patients with T-ALL leads to aberrant activation of MAP2K7 kinase and the downstream JNK pathway that controls the expansion of leukemia cells via c-JUN and ATF2. This pathway can be inhibited with small molecules and therefore has the potential to eliminate LICs and eradicate disease in combination with standard therapy for patients with refractory and relapsed disease. The present review summarizes the role of the KLF4-MAP2K7 pathway in T-ALL pathogenesis and the function of JNK and MAP2K7 in carcinogenesis and therapy.

T-cell acute lymphoblastic leukemia: a model of multi-step leukemogenesis

T-ALL is an aggressive hematological cancer that originates from lymphoid progenitor cells via malignant transformation into pre-leukemic or leukemic-initiating cells (LICs) caused by driver mutations. LICs are then subjected to secondary genetic alterations that increase the proliferation of leukemia T cells and self-renewal of LICs. The overall survival of ALL patients has improved significantly over the decades (80–90%) owing to a combination of the risk-based assignment of treatment, efficient multi-agent chemotherapy, and prophylactic CNS therapy (1). Unfortunately, the prognosis for children who relapse is poor; their complete remission rates drop to 44% for second marrow relapse and 27% for third marrow relapse (2). The pathobiology of pediatric T-ALL encompasses several genetic alterations such as activating mutations of the NOTCH1 receptor, inactivating mutations of the ubiquitin ligase FBXW7 involved in the proteasomal degradation of the intracellular domain of NOTCH1, gene deletion of the CDKN2A locus, aberrant activation of transcription factors with oncogenic activity in the T-cell lineage (e.g., TAL1, TAL2, LYL1, NKX2,

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TLX1, TLX3, and MYC), genetic mutations or deletions in genes involved in histone methylation (e.g., EZH2, SUZ12, JARID2, and UTX), histone acetylation (e.g., CREBBP, EP300, and HDAC), and the loss of tumor suppressors (e.g., PTEN and NF1) (1, 3, 4). Up to sixty-five percent of patients exhibit gain-of-function alleles of the NOTCH1 gene that encodes a single-pass transmembrane receptor involved in T-cell differentiation. Upon ligand activation, NOTCH1 undergoes sequential proteolysis, releasing the intracellular domain of NOTCH1 that activates the genes involved in T-cell differentiation (5–9). Mutations in the heterodimerization domain and/or the PEST domain lead to ligand-independent activation and/or a longer half-life of the intracellular NOTCH1 domain. Despite the high frequency, the strength of NOTCH1 signaling elicited by mutants found in patients is insufficient to trigger overt leukemia in mouse models, suggesting that NOTCH1 activation may initiate leukemia but needs cooperative genetic alterations to cause full-blown leukemia (10). In fact, the emerging paradigm of clonal selection throughout disease evolution proposes the acquisition of additional genetic alterations in initial malignant clones as part of the leukemogenic process and selective pressure (11, 12). Therefore, these cooperative events in patients with refractory and relapse disease might be more appropriate targets for adjunctive therapy.

Emerging role of KLF4 in hematological malignancies

The Krüppel-like factor 4 (KLF4) protein contains both activating and repressing domains and regulates gene expression by different mechanisms: direct DNA binding to unmethylated and methylated promoters and recruitment of co-activators or co-repressors, protein-to-protein interactions, organization of higher chromatin structures, and regulation of the epigenome (13). This tissue-dependent dual function as activators and repressors of gene expression might drive KLF4 to function as a tumor suppressor in the gastrointestinal tract and as an oncogene in breast cancer (14, 15). Aside from solid tumors, the role of KLF4 has not been well defined in hematological malignancies, although KLF4 is known to regulate blood formation, homeostatic proliferation of naïve T cells, and monocytic differentiation (13, 16–18). Previous reports of gene expression and mutations have positioned KLF4 as a potential negative regulator of leukemogenesis. Low expression levels have been found in B-cell non-Hodgkin and Hodgkin lymphomas, multiple myeloma, and acute myeloid leukemia, although high KLF4 levels were associated with a poor prognosis in pediatric Burkitt lymphoma (19–23). In addition, a recent study using single-cell RNA sequencing revealed high KLF4 expression during the acceleration of chronic lymphocytic leukemia (CLL) that reoccurs at relapse (24). In myeloid leukemias, low KLF4 levels were associated with altered differentiation via deregulation of gene and microRNA networks (25) and KLF4 expression inversely correlated with HDAC1 activity (26). Inducible expression of KLF4 in Jurkat cell lines and primary T-ALL cells induced apoptosis via the BCL2/BCLXL pathway (27). Finally, two inactivating mutations in the zinc-finger of KLF4 have been found in childhood ALL, one in the 3'-UTR-abolishing miR-2909 regulatory domain and a second on a zinc-finger motif that inactivates transcriptional capacity (28). These findings suggest that KLF4 may not be a classical tumor suppressor but rather a passenger genetic event that cooperates with driver mutations to induce full-blown leukemia (15).

Our group recently described a novel tumor suppressor function for KLF4 in NOTCH1-induced T-ALL and identified a cellular pathway aberrantly activated in pediatric T-ALL that can be targeted to eradicate the leukemia-initiating cell population (29). We initially hypothesized that KLF4 may be inactivated in T-cell leukemogenesis because of its inhibitory role in the proliferation of T cells (16). Supporting this model, the analysis of multi-center international studies showed that KLF4 is significantly downregulated in pediatric T-ALL compared with normal bone marrow, particularly in more aggressive subtypes (ETP-ALL, TLX) associated with the worst prognosis (29). Alterations in DNA methylation are a hallmark of cancer and an important mechanism of gene silencing (30, 31). Furthermore, the emerging paradigm is that epigenetic modifications can contribute to leukemogenesis and clonal heterogeneity in addition to recurring chromosomal aberrations and gene mutations (12, 32). Our analysis of promoter DNA methylation by targeted next-generation sequencing in a genomic region containing the proximal promoter and exons 1–2 of KLF4 revealed that most of the T-ALL patients displayed CpG hypermethylation—ranging from 10–20% to nearly 100%, particularly in the proximal promoter—in contrast to low or no methylation in normal bone marrow and T cells and patients with B-ALL (29). Supporting a correlation between KLF4 promoter methylation and gene expression, the treatment of T-ALL cell lines with 5-azacytidine (5-AZA) restored the expression of KLF4 and consequently inhibited cell proliferation and induced apoptosis. KLF4 inactivation by DNA methylation has also been described in numerous cancer types such as esophageal squamous cell carcinoma, gastric, oral carcinoma, cervical, urothelial, renal carcinoma, lymphoma, medulloblastoma, colorectal carcinoma, and, more recently, chronic lymphocytic leukemia (19, 33–42).

Mechanism of KLF4 tumor suppressor function in T-ALL

A mouse model of T-ALL generated by combining NOTCH1-mediated retroviral transformation of hematopoietic progenitor cells with somatic deletion of the *Klf4* gene followed by transplantation in cytoablated mice was used to test the effect of the gene silencing seen in patients (29). The compound mutant NOTCH1-L1601P- P was utilized for transformation because of its high frequency in T-ALL patients and weak leukemogenic strength, which allows studying the role of KLF4 in disease onset and progression (10). Gene deletion in hematopoietic cells was achieved by crossing *Klf4* floxed mice with transgenic mice expressing Cre-recombinase under the control of the *Vav* promoter (43); a model we have used to examine the role of KLF4 during normal hematopoiesis (16, 18, 44). We showed that hemizygous and homozygous loss of KLF4 in hematopoietic progenitors transformed with the gain-of-function NOTCH1 allele resulted in accelerated disease onset and worsened the penetrance in transplanted mice (29). The study of blood and bone marrow from leukemic mice at early and late stages of disease (moribund) revealed increased G1-to-S phase transition in the absence of KLF4 measured by the incorporation of BrdU and levels of phosphorylated Rb, Cyclin E, and Ki67. Of note, the early stage of disease in this study was defined as the time where wild-type and KLF4-deficient T-ALL mice do not show signs of leukemia but exhibit similar expansion of GFP-positive cells co-expressing CD4 and CD8 in the bone marrow (29). A concern of analyzing cell proliferation in both groups at an early stage of disease development is a potential increased frequency of clonal disease in the

absence of KLF4. However, the determination of D β 1J β 1 and D β 2J β 2 rearrangements in the bone marrow of mice transplanted with NOTCH1-L1601P- P-transformed *Klf4*^{f/f} (control) or *Klf4*^{-/-} (KLF4 knockout) cells showed similar frequency of polyclonal leukemia cells at the early stage of disease and clonal expansion at the time of death in both groups. Finally, a similar level of the NOTCH1 intracellular domain in control and KLF4-deficient T-ALL cells suggested that KLF4 controls the G1-S transition in leukemia T cells independently of NOTCH1 signaling and that inactivation of KLF4 likely has a global effect on T-ALL cells irrespective of the initiating driver mutation.

In the model described above, transformation occurred in hematopoietic progenitor cells with *Klf4* deletion, although gene inactivation could occur in cells already transformed in the leukemogenic process. Using the ROSA-CreER^{T2} system, we showed that *Klf4* deletion after transformation and transplantation also accelerated disease progression, suggesting that KLF4 inactivation could represent a secondary event in pre-leukemic/malignant cells. In fact, the loss of KLF4 in T-ALL mice was associated with a significant increase in the frequency of immunophenotypic LIC defined as CD4⁻ CD8⁻ CD25⁺ IL7R α ⁺ and CD25⁺ c-Myc⁺(c-Myc-GFP knock-in) leukemic cells (29, 45). An increase in the LIC frequency (9-fold) was confirmed by limiting-dose transplantation of control and KLF4-deficient bone marrow cells isolated from leukemic mice, likely caused by the increased self-renewal of this leukemia-feeding population. These findings are significant for clinics because LICs are a rare cellular subset of leukemia cells responsible for refractory disease and relapses given their capacity to dodge cytotoxicity caused by standard chemodrugs.

To unravel the mechanism underlying KLF4 inactivation in mouse models and T-ALL patients, we performed genomic analysis of gene expression (control versus KLF4-deficient) and KLF4 binding to the genome in leukemic bone marrow cells, revealing that KLF4 represses the gene encoding the mitogen-activated kinase kinase MAP2K7 (also called MKK7). MAP2K7 was among a handful of genes that overlapped in a comparative analysis between genes deregulated in the T-ALL mouse model and pediatric T-ALL patients. As described in more detail, MAP2K7 is a 'kinase kinase' activated in response to cellular stress that leads to the downstream activation of JNK, c-Jun, and ATF2. In the mouse model, deletion of the *Klf4* gene was associated with increased levels of total and phosphorylated MAP2K7 and downstream targets JNK and ATF2 (29). Interestingly, the increased phosphorylation of JNK and ATF2 was also detected in murine T-ALL LICs lacking KLF4 (29). Most importantly, lymphoblasts from children with T-ALL showed barely detectable levels of KLF4 at diagnosis, increased levels of MAP2K7 and phosphorylation of MAP2K7, JNK, ATF2, and c-JUN.

We propose a model of T-ALL leukemogenesis in which inactivation of the KLF4 gene via DNA methylation causes upregulation of MAP2K7 and activation of downstream targets involved in the proliferation and self-renewal of leukemic cells and LICs (Figure 1). This model requires the presence of pre-leukemic cells generated by a driver mutation, such as activated alleles of NOTCH1 in our model. It is not yet clear how MAP2K7 is activated in normal and leukemic T cells or whether KLF4 epigenetic silencing is associated with patient outcome. That T-ALL patients showed the same aberrant activation of the MAP2K7

pathway and LICs from leukemic mice showed activation of this signaling pathway suggest that pharmacological inhibition can be an alternative approach for LIC eradication.

MAP2K7 in normal and malignant cellular function

The dual specificity mitogen-activated protein kinase kinase 7 (MAP2K7) mediates stress signals as part of a three-tiered signaling unit consisting of MAP3K, MAP2K (MAP2K7) and MAPK (JNK) (Figure 2) (46). Phosphorylation of the serine and threonine residues within the S-K-A-K-T motif of the MAP2K7 kinase domain leads to the downstream activation of JNK and JNK-activated transcription factors or pro-apoptotic proteins (46). It has been shown that MAP2K7 mediates JNK activation during the deletion of CD4 and CD8 double-positive thymocytes (47), MAP2K7 is an essential regulator of stress-induced SAPK/JNK activation in mast cells and negatively regulates proliferation in hematopoietic cells (48), and MAP2K7 sequesters JNK1 in the cytosol, preventing pro-survival functions in Jurkat cells (49). The development of the loss-of-function mouse models with cell type-specific and inducible somatic *Map2k7* deletion allowed further studies on MAP2K7 function, particularly in cancer, because embryonic *Map2k7* deletion results in embryonic lethality (50). As described in more detail, the inactivation of *MAP2K7* accelerated tumor development in breast cancer (51), pancreatic cancer (52), and lung cancer mouse models (51). By contrast, an oncogenic role has been suggested from the up-regulation of MAP2K7 in colon cancer and liver metastases, as well as from our work in T-ALL patients (29, 53). Thus, similar to KLF4, MAP2K7 can act as a tumor suppressor and an oncogene depending on specific tissues and the cellular context.

As a tumor suppressor, the deletion of *MAP2K7* in murine mammary epithelial cells (MECs) induced the earlier onset of NeuT-driven mammary tumors, suggesting a tumor suppressor role of *MAP2K7* (51). MECs containing the *MAP2K7* deletion impaired p53 activation and resulted in failure to engage in G2/M arrest in response to DNA damage (51). Only the p53 protein levels were reduced in the presence of *MAP2K7* deletion, indicating that the mechanism of MAP2K7-driven tumor suppression involves stabilization of the p53 protein (51). Loss of *MAP2K7* in the pancreas of KrasG12D transgenic mice accelerates the formation of intraepithelial neoplasia (mPanIN), a common precursor lesions in pancreatic ductal adenocarcinoma (PDAC) (52). Deletion of *MAP2K7* in addition to *MAP2K4* further accelerated mPanIN formation via the increased phosphorylation of STAT3 (52). Activation of STAT3 via IL-6 promoted the formation of mPanINs, leading to the development of PDAC, while the inactivation of STAT3 signaling impaired the formation of mPanIN lesions (54). The tumor suppressor role of *MAP2K7* and downstream signaling components in PDAC stem from the inhibition of STAT3 activation. Finally, the conditional deletion of *MAP2K7* in lung tissue in mice transgenic for inducible KrasG12D resulted in the accelerated formation of lung adenocarcinomas (51). MAP2K7-deficient adenocarcinomas exhibited reduced levels of p53 protein although *p53* mRNA expression remained unchanged (51). While deletion of *p53* accelerates tumor formation in a KrasG12D-driven lung cancer model (55), deletion of *p53* in conjunction with the loss of *MAP2K7* failed to further accelerate tumor growth, indicative of an epistatic relationship between MAP2K7 and p53 (51). The overexpression of p53 in mice with MAP2K7 deficiency normalized the

tumor burden to that of MAP2K7 wild-type animals, demonstrating that MAP2K7's tumor suppressor function stabilizes p53 in lung cancer (51).

The pro-oncogenic functions caused by the aberrant activation of MAP2K7 have been less studied. Human primary colon cancer tumors from patients with liver metastasis exhibited increased levels of MAP2K7, suggesting a pro-metastatic role in colon cancer (53). Distant colon cancer metastases primarily colonize the liver, contributing to the severity of disease and complicating treatment options (56). Mice transplanted with *MAP2K7*-silenced colon cancer cells exhibited decreased metastases in the liver, supporting MAP2K7's role as a facilitator of colon cancer metastasis, although the mechanism is yet to be identified (53). As described in this review, we reported, for the first time, oncogenic MAP2K7 activation in a hematological malignancy: human T-ALL lymphoblasts featured elevated levels of *MAP2K7* transcripts compared with healthy bone marrow and T cells (29). Activation of MAP2K7 and its downstream targets may increase the proliferation and self-renewal of T-ALL cells via basal c-Jun regulation of T-ALL cell survival and proliferation (57). The downstream consequences of MAP2K7 activation include the activation of oncogenic signals, indicating MAP2K7's oncogenic role in the context of T-ALL. Because of the limited availability of specific MAP2K7 inhibitors, we tested, as proof-of-principle, the inhibition of downstream JNK in T-ALL.

From T-ALL to JNK

The c-Jun NH₂-terminal kinase (JNK) was first identified as a protein kinase that is activated in rat liver after cycloheximide injection (58). An independent study later showed that JNK is activated by stress such as transforming oncogenes and UV light and, in turn, phosphorylates the amino-terminal activation domain of c-Jun (59). Subsequent studies demonstrated JNK as one of the mitogen-activated protein kinases (MAPKs) activated by pro-inflammatory signals, environmental and genotoxic stresses to regulate cell proliferation, survival and differentiation (60–62). For these reasons, the JNK pathway is also called the stress-activated protein kinase pathway.

JNK proteins are encoded by three genes—*JNK1*, *JNK2* and *JNK3*—which produce at least ten isoforms through alternative splicing (63). JNK1 and JNK2 are ubiquitously expressed in almost all cells, while JNK3 is specifically expressed in the brain, testis and heart (64). JNK is activated by dual phosphorylation of the Thr-Pro-Tyr motif located in the activation loop by upstream MAPK kinases (MAP2Ks) (65), which are activated by MAP3Ks. Two upstream MAP2Ks that can activate JNKs (MAP2K4 and MAP2K7) have been identified thus far. MAP2K4 can phosphorylate both JNKs and p38 MAPK, while MAP2K7 only activates JNK (66, 67). These two MAP2Ks are not functionally redundant because the deletion of either gene leads to embryonic lethality (50, 68). Furthermore, they exhibit distinct affinity towards the Thr-Pro-Tyr motif of JNK proteins with MAP2K4 and MAP2K7 preferentially phosphorylating the tyrosine and threonine residues, respectively, suggesting that the optimal activation of JNK requires both MAP2Ks (69, 70). MAP2K4 and MAP2K7 have been shown to have different functions in response to extracellular stimuli because the deletion of both genes is required to block JNK activation in mouse embryonic fibroblasts in response to environmental stress such as UV irradiation and anisomycin, whereas the

deletion of MAP2K7 alone is sufficient to inhibit JNK activation induced by pro-inflammatory cytokines such as TNF α and IL1 α (71). Upon phosphorylation, the JNK proteins phosphorylate and activate various downstream molecules, including the transcription factors AP1, ATF2, Elk1, c-Myc, and p53 and Bcl2 family proteins, to regulate cell proliferation, survival and differentiation (64).

In cancer, JNKs can function as oncogenes or tumor suppressors depending on the cellular context. Moreover, specific JNK members can even exert opposite functions in certain cancer types. Therefore, understanding the roles of each JNK protein is particularly important to design targeted therapies. Activation of the JNK/Jun pathway is observed in human patient samples such as human hepatocellular carcinoma cells (72), multiple myeloma cells (73) and lung cancer cells (74). Consistent with those findings, several *in vitro* studies have revealed that the JNK/Jun pathway is required for efficient transformation by other oncogenes. For example, H-Ras induces the phosphorylation of the same serine residue of c-Jun that is phosphorylated by JNK, and activated c-Jun, in turn, cooperates with Ras to fully transform cells (75). The fusion protein BCR-ABL found in leukemia patients can activate the JNK/Jun pathway in hematopoietic cells, and a dominant-negative mutant of c-Jun impairs BCR-ABL transformation activity (76). The requirement of an activated JNK/Jun pathway is observed in cellular transformation by oncogenes such as Met and Fos (77, 78). The oncogenic roles of JNK have been recapitulated in mouse models. In the Apc^{min} intestinal cancer mouse model, the transcriptional activity of c-Jun is dependent on activated JNK, and the conditional deletion of c-Jun or expression of a dominant-negative c-Jun reduces the tumor size and prolongs the lifespan (79). *Jnk1* deletion decreases lung tumor development induced by tobacco smoke (74) and hepatocellular carcinoma development induced by diethylnitrosamine in mice (80). *Jnk2* deficiency reduced the tumor burden in a 12-O-tetradecanoylphorbol-13-acetate-induced skin cancer mouse model (81). Hyperactivation of JNK has also been reported in T-ALL associated with increased MAP2K7 levels, and inhibition of the JNK pathway by small-molecule inhibitors suppresses primary human leukemic cells growth *in vivo* (29). Studies in one human T-ALL cell line (CEM cells) showed that the knockdown of JNK1, but not that of JNK2, inhibits cell growth and induces apoptosis *in vitro* (57), suggesting that JNK1 might be the predominantly activated JNK member in T-ALL. The JNK/Jun pathway has also been implicated in drug resistance. The efflux activity of multidrug transporter protein (MRP) is abrogated in human acute myeloid leukemia cells expressing dominant-negative c-Jun, leading to increased sensitivity to chemodrugs (82). Taken together, these data suggest an oncogenic role of JNK in transformation, tumorigenesis and treatment resistance, which is mainly mediated through the activation of its downstream target c-Jun.

In addition to the oncogenic role described above, JNKs have been implicated in tumor suppression. Fibroblasts that are *Jnk* deficient form more tumor nodules *in vivo* after being transformed by Ras (83). *Jnk1*-deficient mice, in contrast to *Jnk2*-deficient mice mentioned above, are more susceptible to skin tumor development induced by 12-O-tetradecanoylphorbol-13-acetate (84). Moreover, it has been shown that mice with inactivated JNK spontaneously develop intestinal tumors (85). In a Trp53 heterozygous breast cancer model, JNK deficiency significantly increased the incidence of mammary carcinomas (86). The tumor suppressor function of JNK is thought to be mediated through

its capacity to induce apoptosis in response to genotoxic and oncogenic stress. JNK is activated by stress such as UV irradiation to induce apoptosis through the release of Cytochrome C (87), and this process requires pro-apoptotic Bax family proteins (88). The induction of apoptosis in multiple myeloma cells is associated with the activation of JNK and the release of Smac from the mitochondria to the cytosol (89). In addition, JNK2 has been shown to phosphorylate and stabilize p53, leading to apoptosis in cancer cells (90). In addition to its ability to induce apoptosis, JNKs play a tumor-suppressive role by promoting differentiation. In human leukemic cells, the activation of JNKs mediates monocytic differentiation induced by 1,25-dihydroxyvitamin D₃ (1,25D) (91). Further studies revealed that JNK1 promotes 1,25D-induced differentiation, while JNK2 antagonizes the differentiation signal (92), suggesting that the JNK2-specific inhibitor could be used as an anti-leukemia drug to enhance differentiation.

It is speculated that JNKs exert context-dependent functions through the differential regulation of downstream targets. As discussed above, JNKs function as oncogenes mainly by activating downstream c-Jun, while JNKs suppress tumor development through their capability to induce apoptosis via mitochondria pathways. In summary, JNK proteins have oncogenic or tumor-suppressive functions depending on the cellular setting, highlighting the importance of understanding the roles of each JNK protein in different cancer types.

Towards novel targeted therapy in T-ALL: pharmacological inhibition of JNK

Considering the involvement of JNKs in various human diseases, scientists have designed JNK inhibitors with different inhibitory mechanisms, although most chemical compounds compete for ATP binding and, therefore, can inhibit other kinases. The anthrapyrazolone SP600125 is one of the first ATP-competitive inhibitors developed that can inhibit all three JNK proteins (93) and reduce neuron death via the inhibition of c-Jun in a Parkinson's disease mouse model (94). Although SP600125 has served as an important tool to study the JNK pathway, it inhibits other kinases such as Erk and p38 (Table 1) and showed low specificity for JNK proteins, an important caveat for cancer treatment where JNK1 and JNK2 can display opposite functions (95). Therefore, it is essential to develop selective inhibitors against specific JNK proteins to efficiently eliminate cancer cells with limited side effects. For example, two JNK2-specific peptide inhibitors showed the *in vivo* inhibition of migration in breast cancer cells through selective inhibition of JNK2 (96). AV7, an inhibitor selective for JNK1, has been tested in murine embryonic fibroblasts *in vitro* (97). Further studies are still required to test the pharmacodynamics and pharmacokinetics of different JNK inhibitors *in vivo* in combination with genetic studies to determine which JNK proteins should be targeted in specific cancers.

Recently, the irreversible inhibitor JNK-IN-8 was developed using a structure-based design to specifically inhibit JNK via the covalent modification of a unique cysteine residue highly conserved in JNK kinases, thus reducing significantly off-target effects (98). Although this inhibitor has not yet been tested in clinical trials, other chemical compounds have been tested in Phase I clinical trials for inflammatory endometriosis (oral inhibitor AS602801)

and Phase II trials for myeloid leukemia (second-generation ATP competitor CC401) (Table 1). These JNK inhibitors (JNK-IN-8, AS602801, and CC401) showed dose-dependent cytotoxicity in a panel of T-ALL cell lines that was associated with the inhibition of the downstream targets ATF2 and c-Jun (29). Moreover, AS602801 and CC-401 could control the leukemia burden in a cell-based xenograft model at concentrations that did not affect the health of tumor-bearing mice and in patient-derived xenograft models generated with diagnosis samples from T-ALL patients who later relapse (29). These proof-of-concept experiments confirmed the feasibility of the pharmacological inhibition of aberrantly activated JNK downstream of MAP2K7 in refractory and relapse disease.

MAPKs contain a docking site that binds to the scaffold proteins that brings upstream kinases and downstream targets of JNKs into proximity within the cell, thus optimizing the activation of the MAPK pathway (99). Therefore, the disruption of JNK docking to scaffold proteins provides another strategy to inhibit the JNK pathway. JNK-interacting protein-1 (JIP1) is a scaffold protein that binds to JNK through the conservative D domain and brings the upstream MAP2Ks and MAP3Ks and downstream substrates into proximity to coordinate the MAPK signaling cascade (100). A peptide, pepJIP1, which corresponds to the D domain (amino acids 153–163), inhibits JNK activity by competition with JIP1 for upstream kinases and substrates with limited effects on ERK and p38 activity (101, 102). Furthermore, a fusion protein that linked pepJIP1 to a carrier peptide derived from the human HIV-TAT sequence has been used as a delivery system into various tissues via intraperitoneal administration and showed *in vivo* activity by reducing insulin resistance in diabetic mice (103). Additional chemical compounds that also inhibit the JNK pathway by disrupting protein-protein interactions have been developed and proven effective to attenuate tumor growth in a mouse skin cancer model (104) and to block JNK-dependent liver damage induced with concanavalin A and restore insulin sensitivity in mouse models of type 2 diabetes (105).

It is evident that JNK inhibitors have distinct properties in different types of cancer, perhaps partly due to the differential specificity to JNK isoforms, and display a range of off-target effects and associated toxicities. It is also important to consider that the JNK pathway is activated in T cells; therefore, JNK inhibition could have an anti-inflammatory effect in the immune system. From our work, KLF4 is significantly downregulated in ETP-ALL and TLX subtypes, which corresponds to patients with a poor outcome. In addition, we found in a different data set that MAP2K7 was significantly elevated in early immature (high risk) tumors (106). Although a preliminary analysis showed that patients with low KLF4 levels exhibit lower overall survival, additional work with a larger cohort of patients is required to evaluate the role of KLF4 and MAP2K7 expression with patient outcome and determine whether pharmacological inhibition of aberrantly activated MAP2K7 will have application to all patients with TALL or only those with a high risk of relapse. More research is needed to better understand the activation and regulation and to identify targets for the specific inhibition of the MAP2K7-mediated pathway.

Conclusions

Despite advances in T-ALL treatment, many children with T-ALL die from disease relapse due to low responses to standard chemotherapy and the lack of a targeted therapy that selectively eradicates chemoresistant LIC. The identification KLF4 as an inhibitor of T-ALL and the paradigm that activation of the MAP2K7/JNK pathway promotes the expansion of leukemia-feeding cells represent an important advance in our understanding of T-ALL pathobiology and provide a new Achilles' heel to develop LIC-targeted therapy. Because JNK plays a central role in inflammation that can affect disparate diseases, including cancer, it is imperative to identify inhibitors of MAP2K7 and upstream MAPKKK to increase the specificity while reducing the toxicity caused by off-target effects. Thus, more work is needed to bring LIC-targeted therapy to the clinic by identifying the appropriate chemical compound that can reduce the toxicities associated with multi-drug chemotherapy and, most importantly, improve the survival of patients with refractory and relapsed disease using a personalized therapeutic approach. A better understanding of the leukemogenic process will certainly bring us closer to this goal.

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Highlights

- Genetic loss or epigenetic silencing of the *KLF4* gene accelerates T-ALL.
- KLF4 represses the *MAP2K7* gene in T-ALL.
- JNK inhibition has anti-leukemic properties in T-ALL.

Figure 1.

Schematic representation of KLF4 repression in T-ALL. Inactivation of KLF4 by gene methylation in patients with T-ALL abrogates the repression of the MAP2K7 gene, leading to the aberrant activation of downstream targets JNK and c-Jun and ATF2. This pathway promotes the expansion (self-renewal) of LIC and bulk T-ALL cells. The model used to investigate this mechanism, which is independent of KLF4-mediated suppression, is the retroviral expression of an activated allele of NOTCH1.

Figure 2. Stress-activated MAPK pathway. Unknown stress signals or genomic stress mediate the activation of MAPKKK (e.g., Ask1 and Tak1) upstream of MAP2K7 and JNK as part of a three-tiered signaling unit stabilized by docking into the JNK-interacting protein (JIP). The JNK inhibitors JNK-IN-8, CC401, and AS602801 are indicated.

Table 1

Most common chemical compounds used to inhibit JNK

Chemical compound	Mechanism of inhibition	Specificity	Clinical trials
SP600125	ATP competitor	JNK1, JNK2, JNK3 (MKK4, MKK3, MKK6, PKB, PKCa, ERK2, p38)	No
JNK-IN-8	Irreversible inhibitor; covalently modifies cysteine in the ATP- binding site	JNK1, JNK2, JNK3	No
AS602801 (PGL5001)	ATP competitor oral inhibitor (also called Bentamapimod)	JNK1, JNK2, JNK3	Phase II: inflammatory endometriosis (ClinicalTrials.gov identifier: NCT01630252)
CC401	Second generation ATP- competitor	JNK1, JNK2, JNK3	Phase I: high-risk myeloid leukemia (ClinicalTrials.gov identifier: NCT00126893)