FLT3 inhibition upregulates OCT4/NANOG to promote proliferation and TKI resistance of FLT3-ITD+ acute myeloid leukemia

Qi Zhou  
Lanzhou University

Xiaojun Shu  
First Hospital of Lanzhou University

Pingping Zhao  
Lanzhou University

Zijian Li  
First Hospital of Lanzhou University

Huiyuan Chu  
Gansu University of Traditional Chinese Medicine

Yongyu Guan  
Gansu University of Traditional Chinese Medicine

Yihong Chai  
Lanzhou University

Yaming Xi  
xiyaming022@163.com

First Hospital of Lanzhou University

Research Article

Keywords: Acute myeloid leukemia, OCT4, NANOG, FLT3-ITD, Tyrosine kinase inhibitor

Posted Date: April 29th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4310376/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: No competing interests reported.
Abstract

Background: Up to 30% of acute myeloid leukemia (AML) patients face unfavorable outcomes due to the FMS-like receptor tyrosine kinase-3 (FLT3) internal tandem duplication (ITD) mutation. Although FLT3 inhibitors show encouraging outcomes in treatment, they fail to eliminate leukemia stem cells, the origin of persistent and resistant lesions. Exploration of the mechanism in FLT3-ITD⁺ AML maintenance and chemoresistance is crucial for the development of novel therapeutic approaches. The manifestation of pluripotency transcription factors (TFs) and their link to clinical outcomes have been documented in various tumors. This study investigates the correlation between core pluripotency TF and treatment in AML.

Results: We discovered that FLT3 inhibition induced upregulation of OCT4 and NANOG in FLT3-ITD⁺ AML cells. Subsequently, we demonstrated that downregulation of OCT4 or NANOG inhibited cell growth, promoted apoptosis, and induced G0/G1 cell cycle phase arrest in FLT3-ITD⁺ AML cells. Importantly, downregulation of OCT4 or NANOG increased responsiveness to FLT3-tyrosine kinase inhibitor (TKI) (gilteritinib), implying that OCT4 and NANOG may contribute to TKI resistance in FLT3-ITD⁺AML.

Conclusion: Our study verifies the involvement of OCT4 and NANOG in regulating TKI sensitivity, and targeting them may improve the cytotoxicity of FLT3 TKIs in FLT3-ITD⁺ AML.

Highlights

OCT4 and NANOG were upregulated after FLT3 inhibition.

Downregulation of OCT4 or NANOG inhibited cell proliferation and induced apoptosis.

OCT4 or NANOG silencing in FLT3-ITD⁺ AML cells improved TKI sensitivity.

OCT4 and NANOG may be potential targets to overcome TKI resistance.

Introduction

Acute myeloid leukemia (AML) is a hematologic malignancy affecting the whole body [1]. Despite growing research on the pathogenesis of AML and the emergence of targeted medicines such as FMS-like receptor tyrosine kinase 3 (FLT3) tyrosine kinase inhibitors (TKIs) [2-4], isocitrate dehydrogenase (IDH) inhibitors [5-7], and B-cell leukemia/lymphoma 2 (BCL2) inhibitors [8], most patients still rely on conventional chemotherapy and hematopoietic stem cell transplantation (HSCT) [9]. As the most frequent mutant subtype, the FLT3-internal tandem replication (ITD) mutation in AML leads to ongoing activation of subsequent signaling pathways and increases the risk of recurrence [10]. New-generation FLT3 inhibitors, such as gilteritinib, only partially inhibit AML cell growth and temporary clinical responses when employed as a single agent [11]. Consequently, it is urgent to explore the potential mechanisms of chemotherapy resistance in FLT3-ITD⁺ AML to find targets that restore cancer sensitivity.
Transcription factors (TFs) are key regulators of cancers [12, 13]. Octamer-binding transcription factor 4 (OCT4), NANOG Homeobox (NANOG), SRY-box transcription factor 2 (SOX-2), and Krüppel-like factor 4 (KLF4) are among the most important stemness-associated TFs that regulate the stemness properties of cancer cells, including processes of chemotherapy-induced dormancy and reactivation [14, 15]. OCT4 and NANOG are pluripotency markers that not only regulate gene expression during embryonic growth but also preserve the self-renewal and pluripotency of stem cells [16, 17]. Deregulation of OCT4 and NANOG has been detected in numerous malignant tumors, leading to malignant progression [18, 19].

The overexpression of OCT4 and NANOG was positively associated with higher grades, metastasis formation, and poor survival of tumors, underscoring their clinical importance [20, 21]. OCT4 expression was increased in AML patients, which was linked to the FLT3-ITD mutation, complex karyotypes, and poor risk categorization [22]. Elevated levels of OCT4 expression independently forecasted poor overall survival (OS) and event-free survival (EFS). Whereas OCT4 expression was reduced in patients who achieved complete response (CR) compared with non-CR patients [22]. However, the direct regulating mechanisms of OCT4 and NANOG in AML are unclear.

Increased OCT4/NANOG expression shows a positive association with chemoresistance in cancer patients [21, 23]. OCT4 and NANOG expression is thought to be correlated with cisplatin resistance in gastric cancer cells [26]. Knockdown of OCT4 and NANOG improved the cisplatin sensitivity of head and neck squamous cell carcinoma and breast cancer [23, 24]. There are no studies on whether OCT4 and NANOG have an influential role in the TKI resistance of AML cells.

The aim of our study is to investigate the underlying mechanisms of TKI response and the role of pluripotency TFs in FLT3-ITD+ AML. We examined the alteration in the expression levels of pluripotency factors at transcript and protein levels after FLT3 inhibition in FLT3-ITD+ AML cells. In addition, the role of OCT4 and NANOG on cancer survival and TKI treatment was investigated by knocking down OCT4 or NANOG expression in FLT3-ITD+ AML cells. We propose that simultaneous targeting of FLT3 and pluripotency TFs may be an effective strategy to improve outcomes in AML patients.

Materials and Methods

2.1 FLT3 inhibitor

Gilteritinib (ASP2215) (Selleck Chemicals, USA) was mixed into dimethyl sulfoxide (Macklin, China) and subsequently diluted using RPMI-1640 medium to working concentrations.

2.2 Cells and cell culture

Human FLT3-ITD+ AML cell lines (MV4-11 and MOLM-13) were bought from American Type Culture Collection (ATCC, USA). Cells underwent cultivation in RPMI-1640 (Gibco, USA), which included 10% FBS
and 1% penicillin/streptomycin (Solaribio, China). Cells underwent cultivation in humidified environment at 37°C and 5% CO2.

### 2.3 Lentiviral transduction

ShRNAs were used for stable knockdown of OCT4 and NANOG expression. MV4-11 and MOLM-13 cells were transfected using lentiviral shOCT4 (TranSheepBio, China) targeting OCT4 and shNANOG (TranSheepBio, China) targeting NANOG. An empty lentiviral vector control shRNA (shCtrl) was serving as a control. The shRNA sequences are in Table 1. Prior to lentiviral transduction, a single-cell suspension was prepared post-centrifugation and resuspension with fresh medium containing 4 μg/ml polybrene (TranSheepBio, China), and seeded 4 × 10^5 cells in 24-well plates with a volume of 500 μL per well. Lentivirus was mixed and added to the cell culture medium, which underwent an overnight incubation. After 15 hours, the fresh medium was substituted. Successful transduction was verified through the detection of green fluorescent protein expression in fluorescence microscopy. For identifying shRNA-positive cells, they were grown in a full medium containing 1.5 μg/mL puromycin (Sparkjade, China) for a minimum of three days. The expression of target genes was assessed by qRT-PCR and western blotting.

#### Table 1 ShRNA sequence

<table>
<thead>
<tr>
<th>shRNA</th>
<th>Sense Sequence</th>
<th>Antisense Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>CAACAAGATGAAGAGCACAA</td>
<td>TTGGTGCTCTTCATCTTGGTG</td>
</tr>
<tr>
<td>shOCT4-1</td>
<td>GAGATTGATAACTGGTGTGTT</td>
<td>AACACACCAGTTATCTTC</td>
</tr>
<tr>
<td>shOCT4-2</td>
<td>GAGGATCACCCTGGGATATAC</td>
<td>GTATATCCCAGGGGTGATC</td>
</tr>
<tr>
<td>shNANOG-1</td>
<td>GCATGCAGTTCCAGCAAATT</td>
<td>AATTTGGCTGGAACTGC</td>
</tr>
<tr>
<td>shNANOG-2</td>
<td>GCTTTGAAAGCTCCCAATT</td>
<td>TACAGTGGATCTTCAAAGC</td>
</tr>
</tbody>
</table>

### 2.4 Cell Counting Kit (CCK)-8 proliferation analysis

MV4-11 and MOLM-13 cells were counted at 1 × 10^4 in 96-well plates in triplicate. Each experimental group had at least three replicated wells. Cell viability was monitored at the times of 24, 48, 72, 96, and 120 hours. Post-treatment for specified durations, each well received 10 μL of CCK-8 solution (Beyotime Institute of Biotechnology, China), was incubated for an additional 3 hours, and the absorbance at 450 nm was gauged using the BioTek microplate reader (USA). For every group, the optical density (OD) of each well was calculated by subtracting the OD value of the blank control from that of each well.

### 2.5 RNA extraction and qRT-PCR
Total RNA from MV4-11 and MOLM-13 cells was obtained using a TRIzol reagent (Sparkjade, China). RNA was reverse transcribed to complementary DNA (cDNA) using the HiScript™ III RT SuperMix (Vazyme, China). For qRT-PCR analysis, ChamQ SYBR qPCR Master Mix (Vazyme, China) was used on DA7600 Real-time Nucleic Acid Amplification Fluorescence Detection System (Bio-Rad Laboratories Inc, USA). Gene expression was normalized by GAPDH. The determination of mRNA levels was expressed as the mean, and qRT-PCR experiments were repeated with at least three independent assays. PCR primer sequences are in Table 2. The primer sequences were synthesized by General Biol (Anhui, China). The final analysis was relatively quantified by $2^{\Delta\Delta CT}$ method.

**Table 2** PCR primer sequence

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence (5’-3’)</th>
<th>Reverse Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT4</td>
<td>CCGAAGAGAAGGAGCGAACC</td>
<td>CCCCTGAGAAAGGAGACCA</td>
</tr>
<tr>
<td>NANOGR</td>
<td>CTAAGAGGTGCAGAAAACA</td>
<td>CTGGTGATAGGAAGTAAGGG</td>
</tr>
<tr>
<td>KLF4</td>
<td>GGCTGCGGCAAACCACCC</td>
<td>CGGGCGAATTTCCATCCAC</td>
</tr>
<tr>
<td>SOX2</td>
<td>GGAGGGGTGGGAGAGGAGGAGG</td>
<td>TCCCCAAAAAGAAGTCCAGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACTTCAACAGCGACACCCACT</td>
<td>GCCAAATTCGTTGTCATACCAG</td>
</tr>
</tbody>
</table>

### 2.6 Western blotting analysis

Total proteins were extracted from MV4-11 and MOLM-13 cells using RIPA buffer (Beyotime, China), which contained protease inhibitors (Beyotime, China). The protein concentration in the samples was measured using the BCA kit (Epizyme, China). Proteins were isolated through 10% SDS-PAGE and electro-transferred onto PVDF membranes (Merck Millipore, USA). Subsequently, PVDF membranes underwent blocking using 5% skim milk for two hours, followed by an overnight incubation with primary antibodies at 4°C. PVDF membranes underwent a TBST wash followed by a 2-hour incubation with horseradish peroxidase-tagged secondary antibodies. The detection of the protein bands was achieved through a highly sensitive ECL chemiluminescence substrate (Biosharp, China). Using GAPDH as a control for OCT4 and β-actin as a control for NANOGR. All primary antibodies were bought from Proteintech (Wuhan, China), and secondary antibodies were from Epizyme (Shanghai, China). All Western blot analyses were performed at least three times.

### 2.7 Cell apoptosis analysis

The cells were collected through centrifugation, cleansed using chilled PBS (Phosphate Buffered Saline, Servicebio, China) at 4 °C, and then reconstituted in 500 μL of binding buffer to modify the concentration to $1 \times 10^7$ /mL. The cells underwent incubation with 5μL of Annexin V-FITC and 5μL of propidium iodide (PI) (Biolegend, USA) for a quarter-hour at ambient temperature in darkness. Subsequently, leukemia cells
underwent apoptosis analysis at an excitation wavelength (Ex) of 488 nm and an emission wavelength (Em) of 585 ± 21 nm, utilizing CytoFLEX (Beckman, USA). The FlowJo 7.6 software was utilized to assess the proportion of apoptosis cells.

2.8 Cell cycle analysis

The cells underwent a cleansing process using PBS and were then reconstituted in a staining solution with 50 μg/mL PI (KeyGEN, China) and PBS, inclusive of 100 μg/mL RNase A. These suspensions were then left to incubate at 4°C overnight. Data on the distribution of cell cycles were acquired through the use of CytoFLEX (Beckman, USA). The cell cycle distribution was examined using FlowJo 7.6 software.

2.9 Statistical analysis

Every experiment was conducted a minimum of three times. The GraphPad Prism software (Version 9.0, San Diego, CA, USA) was utilized for visualizing data and conducting statistical analyses. The variances among the experimental groups were examined through either a paired or unpaired student t-test, along with a two-way variance analysis incorporating multiple tests. A p-value below 0.05 was deemed to hold statistical significance.

Results

3.1 OCT4 and NANOG expression upregulated after gilteritinib treatment

MV4-11 and MOLM-13 cells carry the FLT3-ITD mutation. FLT3 inhibitors are used for the treatment of FLT3-ITD+ AML. We evaluated the expression of four core pluripotency TFs, including OCT4, NANOG, KLF4, and SOX-2, in both cell lines after gilteritinib (an FLT3 inhibitor) treatment. Remarkably, OCT4 and NANOG transcript levels were significantly increased after gilteritinib (5 nM) exposure, while KLF4 and SOX-2 changed little (Fig. 1A, B). We next examined the alterations in OCT4 and NANOG expression after different concentrations of gilteritinib at 0, 2, 5, and 10 nM. Levels of both OCT4 (Fig. 1C, D) and NANOG (Fig. 1E, F) increased in response to upregulation of the administered concentration. Protein expression levels in OCT4 (Fig. 1H) and NANOG (Fig. 1I) were dose-dependent when MV4-11 and MOLM-13 (Fig. 1G) cells were treated with gilteritinib at different concentrations.

3.2 Downregulation of OCT4 suppresses growth and induces apoptosis in FLT3-ITD+ AML cells
To further investigate the role of OCT4 in FLT3-ITD+ AML progression, we evaluated the effect of OCT4 downregulation on FLT3-ITD+ AML cell proliferation and apoptosis. We first generated stable OCT4 knockdown and control vector cell lines in MV4-11 and MOLM-13 cells by lentiviral (shOCT4-1, shOCT4-2, and shCtrl) transduction. It is showing that OCT4 protein expression decreased when lenti-OCT4 was infected with MV4-11 and MOLM-13 cells (Fig. 2A-D). Notably, knockdown of OCT4 suppressed MV4-11 and MOLM-13 cell growth (Fig. 2E, F) and induced apoptosis (Fig. 2G-I) compared with the control. These results suggest that OCT4 confers cell viability and that OCT4 downregulation inhibits the malignant proliferation of FLT3-ITD+ AML cells.

3.3 Downregulation of NANOG suppresses growth and induces apoptosis in FLT3-ITD+ AML cells

To further investigate the role of NANOG in FLT3-ITD+ AML progression, we evaluated the effect of NANOG downregulation on FLT3-ITD+ AML cell growth and apoptosis. We first stably transfected MV4-11 and MOLM13 cells using shRNA (shNANOG-1 and shNANOG-2) targeting NANOG or shCtrl as controls. NANOG protein levels were reduced in both cell lines compared to controls (Fig. 3A-D). NANOG knockdown significantly suppressed cell growth (Fig. 3E, F) and induced apoptosis in both cell lines (Fig. 3G, H) compared to controls. The findings verify that NANOG downregulation inhibits the malignant proliferation of FLT3-ITD+ AML cells.

3.4 The effect of reduced OCT4/NANOG on cell cycle distribution

Subsequently, we examined whether reducing OCT4/NANOG levels influences the cell cycle in FLT3-ITD+ AML cells. Flow cytometry was conducted to evaluate OCT4/NANOG function in the cell cycle distribution. The results demonstrate that reduced OCT4 causes MV4-11 (Fig. 4A) and MOLM-13 (Fig. 4C) cell cycle arrest at the G0/G1 phase and a shortened S phase. As compared with 52.75% in cells treated with shCtrl, the G0/G1 phase was 63.84% and 64.63% in MV4-11 cells treated with shOCT4-1 and shOCT4-2 (Fig. 4B). As compared with 48.59% in cells treated with shCtrl, the G0/G1 phase was 74.62% and 68.38% in MOLM-13 cells treated with shOCT4-1 and shOCT4-2 (Fig. 4D). Similarly, cell cycle analysis was conducted to evaluate the role of NANOG in cell cycle distribution. The results show that reduced NANOG causes MV4-11 (Fig. 4E) and MOLM-13 (Fig. 4G) cell cycle arrest at the G0/G1 phase and a shortened S phase. As compared with 49.21% in the control cells, the proportion of G0/G1 phase in MV4-11 cells undergoing treatment with shNANOG-1 and shNANOG-2 was 64.40% and 63.93% (Fig. 4F). As compared with 45.38% in the control cells, the proportion of G0/G1 phase was 71.21% and 66.72% in MOLM-13 cells undergoing treatment with shNANOG-1 and shNANOG-2 (Fig. 4H). Taken together, OCT4/NANOG knockdown inhibited AML cell growth by inducing G0/G1 phase arrest of the cell cycle.
These results confirmed that OCT4 and NANOG contribute to enhancing the inherent carcinogenicity and malignant transformation of FLT3-ITD⁺ AML cells.

### 3.5 Downregulation of OCT4 enhances gilteritinib inhibition of FLT3-ITD⁺ AML

Given the involvement of deregulated OCT4 and NANOG in cancer chemoresistance, it was hypothesized that upregulating OCT4 and NANOG in FLT3-ITD⁺ AML cells subsequent to the inhibition of FLT3 could be significantly influential in counteracting the effect of TKIs on eradicating leukemia. We downregulated OCT4 expression in cells through lentivirus vectors of OCT4 shRNA (shOCT4-1 and shOCT4-2) or shCtrl. The suppression of OCT4 induced apoptosis of two cell lines, and the anti-leukemia effect was more significant when in combination with gilteritinib in MV4-11 (Fig. 5A) and MOLM-13 cells (Fig. 5B). Knockdown of OCT4 (shOCT4-1 and shOCT4-2) increased the apoptosis rate of MV4-11 cells treated with gilteritinib by 35.79% and 33.23% (Fig. 5A). Knockdown of OCT4 (shOCT4-1 and shOCT4-2) increased the apoptosis rate of MOLM-13 cells after gilteritinib treatment by 34.39% and 32.35% (Fig. 5B). Knockdown of OCT4 increased gilteritinib sensitivity of both cell lines to gilteritinib (Fig. 5C, D). Downregulation of OCT4 activity significantly increased gilteritinib-induced apoptosis and growth suppression in FLT3-ITD⁺ AML cells. These data support that OCT4 expression level is associated with TKI susceptibility in FLT3-ITD⁺ AML cells.

### 3.6 Downregulation of NANOG improves gilteritinib-mediated inhibition of FLT3-ITD⁺ AML

The expression of NANOG was reduced in MV4-11 and MOLM-13 cells using lentiviruses (shNANOG-1, shNANOG-2, and shCtrl). The suppression of NANOG led to notable apoptosis in both cell lines, with its anti-leukemia impact being more intense when combined with gilteritinib (Fig. 6A, B). Knockdown of NANOG (shNANOG-1 and shNANOG-2) increased the apoptosis rate of MV4-11 cells treated with gilteritinib by 41.2% and 30.94% (Fig. 6A). Knockdown of NANOG (shNANOG-1 and shNANOG-2) increased the apoptosis percentage of MOLM-13 cells treated with gilteritinib by 27.81% and 18.2% (Fig. 6B). Knockdown of NANOG increased gilteritinib sensitivity of both cell lines to gilteritinib (Fig. 6C, D). Downregulation of NANOG significantly improved gilteritinib-induced apoptosis and growth inhibition of FLT3-ITD⁺ AML cells. These results indicate that NANOG is associated with TKI susceptibility in FLT3-ITD⁺ AML cells.

### Discussion

Despite advances in AML therapy with the development of FLT3 TKIs for the treatment of FLT3-ITD⁺ AML, TKIs have reported only limited effects as monotherapy in clinics [25]. Most FLT3-ITD⁺ AML patients
relapsed quickly, and 5-year survival remains poor [26]. Therefore, it is critically important to figure out the process of TKI resistance and develop novel approaches to eradicate FLT3-ITD+ AML cells. Several preclinical investigations indicate that TKI resistance may be driven by cellular adaptation mechanisms [27, 28]. Our study found that the pluripotency TFs OCT4 and NANOG were upregulated upon FLT3 inhibition.

Therapeutic agents associated with elevated TFs in pluripotency mainly fall into two categories, conventional chemoradiotherapy and TKIs [29-32]. Chemoradiotherapy results in an enrichment of cancer stem cells and an increase in cancer stem cell markers [33]. Studies of chemotherapy resistance in relation to OCT4 expression are confined to a few medicines, such as cisplatin, paclitaxel, doxorubicin, and tamoxifen [34-36]. Cisplatin, etoposide, doxorubicin, and paclitaxel γ-ray irradiation increase the expression level of OCT4 in lung cancer [37]. TKIs are another class of drugs that are prone to resistance. IL-6-DNMT3b-mediated OCT4 expression confers sorafenib resistance in hepatocellular carcinoma (HCC) patients [38]. We observed that OCT4 and NANOG were highly expressed in FLT3-ITD+ AML cells after TKI therapy in a dose-dependent manner, with a higher dose of gilteritinib (a common FLT3 TKI) inducing higher levels of OCT4 and NANOG expression. It suggests that OCT4 and NANOG may serve as molecular predictors of FLT3 inhibitor responses.

The malfunctioning of OCT4 and NANOG is pivotal in the development of tumors. Exogenous OCT4 expression increases tumorigenicity, while OCT4 knockdown reduces tumor malignancy. High OCT4 expression was positively linked with glioma grade [39]. OCT4 accelerates self-renewal, aggressiveness, and chemoresistance in HCC stem cells [40, 41]. High levels of OCT4 are linked to poor outcomes in hormone receptor-positive breast cancer patients [42]. Downregulation of OCT4 lowers stemness characteristics in germ cell cancers [43]. A multitude of studies have shown abnormal expression of NANOG in human cancers [44]. Colorectal cancer patients with high NANOG expression were linked to Dukes’ grade [45]. Gastric cancer patients with high NANOG levels have a poorer OS [46]. The Kaplan-Meier analysis demonstrated that the high expression of OCT4 and NANOG predicted a poor prognosis in pancreatic cancer [47]. It is critical to understand the function of the pluripotency TFs OCT4 and NANOG in FLT3-ITD+ AML.

Studies on the molecular processes underlying the association between pluripotency TFs and AML are limited. The role of TFs such as OCT4 and NANOG in AML remains unknown. Our findings provide compelling evidence for the potential therapy of targeting OCT4 and NANOG in FLT3-ITD+ AML. We generated two FLT3-ITD+ AML cell lines with OCT4 or NANOG knockdown using stable lentivirus strains. By using cell viability, apoptosis, and cell cycle analysis, we found that knockdown of OCT4 or NANOG inhibited cell proliferation and promoted apoptosis. We then investigated the effect of downregulating OCT4 or NANOG on the sensitivity of FLT3-ITD+ AML cells to gilteritinib. A significant rise in the efficacy of TKI treatment was observed. This suggests that the presence of OCT4 or NANOG contributes to AML resistance to FLT3 inhibition. By targeting these genes, we can increase the sensitivity of FLT3 TKIs in
FLT3-ITD\(^+\) AML. This synergistic effect suggests that the combination of stemness gene silencing and FLT3i works together in a complementary mechanism to target FLT3-ITD\(^+\) AML more effectively.

In summary, the study investigated the therapeutic relevance of OCT4/NANOG in FLT3-ITD\(^+\) AML, focusing on the molecular mechanism by which they mediate AML progression and TKI sensitivity. The pluripotency TFs OCT4 and NANOG expression were shown to correlate with treatment and TKI resistance in FLT3-ITD\(^+\) AML. Overcoming this resistance will improve the prognosis of AML patients. In addition, it is helpful for the establishment of molecular biomarkers that predict the FLT3-ITD\(^+\) AML response to FLT3i before treatment. Our findings suggest that OCT4 and NANOG may be molecular biomarkers of the AML response to TKIs and potential targets to overcome resistance.

**Conclusions**

Our findings identify novel OCT4/NANOG targets that promote leukemia maintenance and mediate TKI resistance. Functionally, OCT4 or NANOG silencing in FLT3-ITD\(^+\) AML cells inhibited growth, promoted apoptosis, and significantly improved TKI treatment sensitivity. Given that mutations in tyrosine kinase are frequent genetic alterations in numerous tumors, our study may have implications for the development of therapeutic approaches. This also updates the role of pluripotency TFs in promoting the resistance of tumor cells to TKIs. Our study suggests that combining stemness gene intervention with TKIs may be a viable approach for treating FLT3-ITD\(^+\) AML and other tyrosine kinase-mutated leukemias.

**References**


30. Fang Hu, Changhui Li, Xiaoxuan Zheng, Hai Zhang, Yinchen Shen, Lin Zhou, Xiaohua Yang, Baohui Han and Xueyan Zhang. Lung adenocarcinoma resistance to therapy with EGFR-tyrosine kinase inhibitors is related to increased expression of cancer stem cell marker s SOX2, OCT4 and NANOG. Oncology reports 43(2):727-735.


cells. Glia 57(7):724-733.


Figures
FLT3 inhibition induces upregulation of OCT4 and NANOG. MV4-11 cells (A) and MOLM-13 cells (B) underwent a 24-hour treatment with vehicle (dimethyl sulfoxide) or gilteritinib (5 nM). QRT-PCR was employed to examine the transcription of the four primary transcription factors. MV4-11 (C, E) and MOLM-13 cells (D, F) underwent treatment with gilteritinib in concentrations of 0, 2, 5, and 10 nM for 24 hours. QRT-PCR was employed to examine the transcription of OCT4 (C, D) and NANOG (E, F). (G) Varying doses (0, 2, 5, 10 nM) of gilteritinib were administered to MV4-11 and MOLM-13 cells over a period of 48 hours, followed by western blot analysis to detect the indicated proteins. The protein expression of OCT4 (H) and NANOG (I) in MV4-11 and MOLM-13 cells underwent treatment with varying concentrations of gilteritinib (0, 2, 5, and 10 nM) for 48 hours using a western blotting assay. The statistical result is the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 2

Knockdown of OCT4 suppresses FLT3-ITD⁺ AML cell growth and induces apoptosis. OCT4's suppression effectiveness was assessed using western blotting after transfecting MV4-11 (A-B) and MOLM-13 cells (C-D) with lentiviral vectors of OCT4 shRNA (shOCT4-1 and shOCT4-2) or control shRNA (shCtrl) for 48 hours. The growth of MV4-11 (E) and MOLM-13 (F) cells was evaluated by the CCK-8 assay. Examination of apoptosis via flow cytometry following 48 hours of MV4-11 (G) and MOLM-13 (H) cell transduction. (I) Apoptosis was examined through the application of annexin V/propidium iodide (PI) markers. The statistical result is the mean ± SEM. *p < 0.05, ****p < 0.0001.
Figure 3

Knockdown of NANOG suppresses FLT3-ITD⁺ AML cell growth and induces apoptosis. NANOG’s suppression effectiveness was assessed using western blotting after transfecting MV4-11 (A-B) and MOLM-13 (C-D) cells with lentiviral vectors of OCT4 shRNA (shNANOG-1 and shNANOG-2) or control shRNA (shCtrl) for 48 hours. The growth of MV4-11 (E) and MOLM-13 (F) cells was evaluated by the CCK-8 assay. Examination of apoptosis via flow cytometry following 48 hours of MV4-11 (G) and MOLM-13 (H) cell transduction. (I) Apoptosis was examined through the application of annexin V/propidium iodide (PI) markers. The statistical result is the mean ± SEM. *p < 0.05, ****p < 0.0001.
Figure 4

Knockdown of OCT4/NANOG causes G0/G1 phase arrest in FLT3-ITD^+ AML cells. MV4-11 (A, B) and MOLM-13 (C, D) cells were transferred with OCT4 shRNA (shOCT4-1 and shOCT4-2) or control shRNA (shCtrl). MV4-11 (E, F) and MOLM-13 (G, H) cells were transduced with lentiviral vectors of NANOG shRNA (shNANOG-1 and shNANOG-2) or control shRNA (shCtrl). Examination of the cell cycle via flow cytometry.
following 48 hours of cellular transduction. The statistical result is the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 5

Knockdown of OCT4 enhances gilteritinib-mediated elimination of FLT3-ITD⁺ AML. OCT4 shRNA (shOCT4-1 and shOCT4-2) or control shRNA (shCtrl) was transduced in MV4-11 and MOLM-13 cells. Cells were then treated with gilteritinib (2 nM) or vehicle (dimethyl sulfoxide) for 48 hours. Apoptosis was examined through the application of flow cytometry analysis. The ratio of apoptotic MV4-11 (A) and MOLM-13 (B) cells was measured. The viability of MV4-11 (C) and MOLM-13 (D) cells in treatment with varying doses of gilteritinib were examined by CCK-8 assay. The statistical result is the mean ± SEM. ****p < 0.0001.
Figure 6

Knockdown of NANOG enhances gilteritinib-mediated elimination of FLT3-ITD⁺ AML. NANOG shRNA (shNANOG-1 and shNANOG-2) or control shRNA (shCtrl) was transduced in MV4-11 and MOLM-13 cells. Cells were then treated with gilteritinib (2 nM) or vehicle (dimethyl sulfoxide) for 48 hours. Apoptosis was examined through the application of flow cytometry analysis. The ratio of apoptotic MV4-11 (A) and MOLM-13 (B) cells was measured. The viability of MV4-11 (C) and MOLM-13 (D) cells in treatment with varying doses of gilteritinib were examined by CCK-8 assay. The statistical result is the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.