CRKL but not CRKII Inhibits Erythropoiesis and Megakaryopoiesis of CML via Inactivating Raf/MEK/ERK/Elk-1 Pathway

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Abstract

**Background:** As members of the CT10 regulation of kinase (CRK) adaptor protein family, CRK-like (CRKL) and CRKII are involved in cell proliferation, survival, adhesion, migration and differentiation. However, the exact role and underlying mechanism of CRKL and CRKII in leukemic cell differentiation are still unknown.

**Methods:** Quantitative real-time qPCR (qRT-PCR) was used to detect the expression levels of CRKL and CRKII in chronic myeloid leukemia (CML) patients and complete remission (CR) patients; Western blotting (WB) was used to measure the expression levels of CRKL and CRKII during hemin-induced erythroid differentiation of K562 cells; Benzidine staining, isobaric tags for relative and absolute quantitation (iTRAQ) proteomic analysis, cDNA microarray assay, qRT-PCR and WB were used to examine the effects of CRKL and CRKII deregulation on erythroid and megakaryocyte differentiation of K562 cells; PD98059 was used to investigate the underlying mechanism of CRKL in erythropoiesis and megakaryopoiesis.

**Results:** CRKL was found to be overexpressed in chronic myeloid leukemia (CML) patients compared with normal samples, while its expression level was lower in CR patients than in corresponding CML patients. The CRKL expression level was significantly decreased during the erythroid differentiation of K562 cells following hemin treatment. Moreover, CRKL downregulation promoted erythroid and megakaryocyte differentiation of K562 cells accompanied by increased expression level of the erythroid differentiation markers $\gamma$-globin, glycophorin (GPA) and the megakaryocyte differentiation markers CD41, CD61. Furthermore, gene microarray and iTRAQ quantitative proteomic analysis showed that CRKL downregulation increased hemoglobin (HB) molecules HBD, HBA1, HBA2, HBZ, HBE1, HBG1 and globin transcription factor 1 (GATA1), high-mobility group protein (HMGB2) expression levels. Mechanistically, CRKL inhibited erythroid and megakaryocyte differentiation of K562 cell via inactivating Raf/MEK/ERK/Elk-1 pathway. Conversely, CRKII was only slightly overexpressed in CML patients and had no effect on erythroid differentiation of K562 cells.

**Conclusions:** Taken together, our results demonstrate that CRKL but not CRKII contributes to development, progression, erythropoiesis and megakaryopoiesis of CML, providing novel insights into effective diagnosis and therapy for CML patients.

**Background**

Hematopoiesis is a precisely modulated multi-step process including hematopoietic stem cell (HSC) self-renewal and hematopoietic stem/progenitor cell differentiation [1, 2]. Erythropoiesis and megakaryopoiesis are important parts of hematopoiesis [3, 4]. Normal erythropoiesis produces about $10^{11}$ new red blood cells (RBCs) every day in an adult human through the commitment of hematopoietic stem cells into erythroid progenitors, which subsequently differentiate into mature RBCs [5, 6]. Megakaryopoiesis is responsible for blood platelets [7]. Destruction of erythropoiesis and megakaryopoiesis processes leads to various diseases, including thrombocytopenia, anemia and
leukemia. Understanding the regulatory mechanisms of erythropoiesis and megakaryopoiesis can lead to characterizing novel modulators and developing new methods for treatment of blood related diseases.

Chronic myeloid leukemia (CML) is a clonal myeloproliferative pluripotent hematopoietic stem cell malignancy disorder characterized by the expression of \( BCR/ABL1 \) (B-cell receptor/v-abl Abelson murine leukemia viral oncogene) fusion gene \([8, 9]\), which is generated from the Philadelphia chromosome translocation of chromosome 9 to 22 \([10, 11]\). \( BCR-ABL \) is the molecular hallmark of CML with tyrosine kinase activity that can potentially activate multiple signal transduction pathways, resulting in abnormal cell proliferation, apoptosis, migration, invasion and differentiation \([12–14]\). The delay of differentiation and maturation is considered to be a characteristic of leukemia, and inducing leukemia cell differentiation and breaking through the barrier of differentiation and maturation haas become a research hotspot in basic medical research and its clinical translation \([15]\). The K562 cell is human leukemia cell derived from the pleural effusion of a CML woman patient in terminal blast crisis \([16, 17]\). K562 cells behave more like undifferentiated early pluripotent hematopoietic progenitors, and have been widely used as a model for studying hematological cell differentiation due to its ability to express specific markers of granulocytic, monocytic, erythroid and megakaryocytic lineages \([18, 19]\).

The CT10 regulation of kinase (CRK) adapter protein family is involved in intracellular signal transduction. Members of the CRK family were the first identified adaptor proteins, which connect with upstream molecules through their C-terminal SH2 domain and with downstream molecules through their N-terminal SH3 domain \([20]\). CRK consists of cellular homologs CRKI, CRKII and CRK-like (CRKL) which are ubiquitously expressed and conserved across eukaryotic organisms \([21]\). CRKI and CRKII were originally described as splice variants, while CRKL is encoded by another homologous gene. CRKI is composed of one SH2 domain and one SH3 domain, CRKII and CRKL are composed of one SH2 domain, one SH3N and one SH3C domains \([21–23]\). CRKII and CRKL are highly similar in sequence and both possess tyrosine phosphorylation sites that can be phosphorylated by \( BCR-ABL \) to activate signaling pathways \([21, 22]\), suggesting CRKII and CRKL have overlapping functions. CRKII and CRKL contains a variety of linkages for docking \( BCR-ABL \), p130Cas, Dock180, GAB, ABL-1, Pax, GEF, C3G and SOS to form localized complexes critical for cell proliferation, survival, adhesion, migration and invasion \([24–26]\). CRKII and CRKL deregulation has been proved to be involved in the development and progression of a variety of cancers \([27, 28]\). Nevertheless, a few studies report the association of CRK with differentiation: CRK could induce pheochromocytoma PC12 cell differentiation \([29, 30]\); CRK\(\text{I} \) and CRK\(\text{I} \) could synergistically increase RANKL-induced osteoclast differentiation \([31]\). However, the precise roles and underlying mechanisms of CRKL and CRK\(\text{I} \) in leukemic cell differentiation are still not reported.

In our study, we investigated the effect of CRKL and CRK\(\text{I} \) on erythroid and megakaryocyte differentiation using the K562 cell lines as a model system. Interestingly, we found CRKL was overexpressed in CML patients compared with normal samples, while its expression level was lower in complete remission (CR) patients than in corresponding CML patients. Moreover, CRKL was down-regulated in hemin-induced erythroid differentiation of K562 cells. Furthermore, we have demonstrated, for the first time, that CRKL downregulation promoted K562 cell erythroid and megakaryocyte differentiation via the
Raf/MEK/ERK/Elk-1 pathway. Conversely, CRKII was slightly overexpressed in CML patients and had no effect on erythroid differentiation of K562 cells. Our results provide novel insights into CRKL regulates erythroid and megakaryocyte differentiation through the Raf/MEK/ERK/Elk-1 pathway, and suggest that CRKL may serve as a potential target for therapeutic treatment and prognosis of CML disease.

Materials And Methods

Patients and blood samples

A total of 33 CML patient samples, 5 CR patient samples and 13 healthy subject normal samples were collected from the Department of Hematology, The Second Affiliated Hospital of Dalian Medical University, Dalian, China. The mononuclear bone marrow (BM) and peripheral blood (PB) cells were separated from the CML patients and healthy subject normal samples, respectively. All mononuclear cell specimens were frozen in liquid nitrogen immediately after separation and stored at -80 ºC prior to RNA isolation. The study protocol was approved by the Medical Ethics Committee of Dalian Medical University and informed consent was obtained from all patients. All experiment methods were performed in accordance with the relevant guidelines and regulations.

Cell culture

Human CML K562 cells were obtained from American Type Culture Collection (ATCC, VA) and cultured in 85% RPMI-1640 (Gibco, USA) supplemented with 15% fetal bovine serum (FBS, TransGen, China), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, USA) in a humidified incubator at 37 ºC with 5% CO₂. shRNA-NC-K562 and shRNA-CRKL-K562 cells, which our group previously constructed, were grown in RPMI-1640 medium supplemented with 15% FBS and 250 µg/ml G418 in a humidified incubator at 37 ºC with 5% CO₂.

Benzidine staining assay

1 × 10⁵ K562 cells in 2 ml RPMI-1640 medium supplemented with 15% FBS were seeded into a well of a 6-well plate, then treated with 20 µM hemin (Sigma-Aldrich, Japan) in a humidified incubator with 5% CO₂ at 37 ºC for 0, 1 and 2 d before harvesting and washing once with PBS. Benzidine dihydrochloride solution (Beyotime, China) was prepared with 0.5% acetic acid containing 0.1% H₂O₂, then 9 µl benzidine dihydrochloride solution was directly added to 81 µl cell suspension, incubated at room temperature (RT) for 5 min, and immediately imaged by an upright light microscope (Olympus, Japan) with 100× magnification. Benzidine-positive cells were stained blue, while benzidine-negative cells were light yellow.

Western blotting (WB) assay

Each group of cells was harvested and washed with PBS, then total protein was extracted using RIPA buffer (50 mM pH 8.0 Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with 1 mM Na₃VO₄, 1 µg/ml leupeptin and 0.5 mM PMSF. The supernatant was collected by centrifugation at 12000 rpm for 15 min at 4 ºC. Equal amounts of each protein sample to be
determined by Bradford assay were boiled for 5 min in loading buffer and separated by 10% SDS-PAGE. The protein bands were transferred onto a nitrocellulose membrane (PALL, USA), blocked with 5% (w/v) skim milk (BD, USA) in TBST (pH 7.5; 100 mM NaCl, 50 mM Tris and 0.1% Tween-20) for 3 h at RT and incubated with primary antibodies at 4 °C overnight. The primary antibodies were CRKL (1:1000, Genex, USA), Ras (1:500, Ruiying, China), Raf (1:500, Cell Signaling, USA), p-Raf (1:500, pTyr341, Cell Signaling, USA), p-MEK1/2 (1:500, pSer217/221, Cell Signaling, USA), ERK1/2 (1:1000, Cell Signaling, USA), p-ERK1/2 (1:500, pThr202/pTyr204, Cell Signaling, USA), GATA-1 (Globin transcription factor 1, 1:500, TransGen, China), HMGB2 (High-mobility group protein, 1:1000, TransGen, China), CRK (1:1000, Santa Cruz Biotechnology, USA), ACTB (1:4000, TransGen, China). The nitrocellulose membrane was then washed with TBST for 3 × 10 min, incubated with the secondary antibody conjugated for 3 h at RT and washed again with TBST for 3 × 10 min. Protein bands were visualized by ECL (Advansta, USA) and analyzed using Bio-Rad ChemiDoc™ MP system (Bio-Rad, USA). ACTB was used as internal references.

Isobaric tags for relative and absolute quantitation (iTRAQ) proteomic analysis

3 × 10⁷ shRNA-NC-K562 and shRNA-CRKL-K562 group cells were harvested in at least three independent experiments, and centrifuged at 1000 rpm for 5 min and then the cell pellets were washed with ice-cold PBS. Total protein was extracted from each group of cells using SDT buffer. The SDT buffer was added to the sample and boiled for 15 min, then the supematant was collected by centrifugation at 12000g for 15 min at 4 °C and quantified by a BCA Protein Assay Kit (Bio-Rad, USA). 20 µg protein samples were mixed with 5× loading buffer, boiled for 15 min and separated by 12.5% SDS-PAGE. Protein bands were visualized by Coomassie Blue R-250 staining. The subsequent steps including filter-aided sample preparation (FASP Digestion), iTRAQ labeling, peptide fractionation with strong cation exchange (SCX) chromatography and LC-MS/MS analysis were performed by the Research Center for Proteome Analysis, Shanghai Institutes for Biological Sciences according to the standard method [32].

Quantitative real-time RT-PCR (qRT-PCR) assay

Total RNA was extracted from patient samples and K562 cells using Trizol™ reagent (Invitrogen, USA) and reversely transcribed into cDNA using PrimeScript™ RT Kit with gDNA Eraser (Takara, Japan). qRT-PCR was then performed using FastStart universal SYBR Green Master (ROX) (Roche, USA) with an Mx3005P Real-time PCR System (Agilent, USA). ACTB was used as internal reference. The relative expression levels of CRKL, CRK, γ-globin, GPA (glycophorin), CD41, CD61, Elk-1, GATA-1, HMGB2 in different groups of cells and in CML patient samples were analyzed using the 2^−ΔΔCT method. The specific primers of CRKL, CRK, γ-globin, GPA, CD41, CD61, Elk-1, GATA-1, HMGB2 and ACTB are provided in Table 1.

cDNA microarray assay

1 × 10⁷ shRNA-NC-K562 and shRNA-CRKL-K562 group cells were harvested for total RNA extraction using Trizol™ reagent (Life Sciences). The RNA concentration and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo) and 1.5% denaturing agarose gel electrophoresis. cDNA was
synthesized using SuperScript II kit and purified by QIAGEN RNase Mini Kit. cRNA was created using a Genechip IVT labeling kit. The biotin-labeled fragmented cRNA (≤ 200 nt) was hybridized at 45 °C for 16 h on a Affymetrix Genechip (Human Transcriptome Array 2.0). All the arrays were imaged by a 3000 7G Scanner and processed by Affymetrix Genechip Operating Software. The random variance model (RVM) t-test was performed to screen the differentially expressed genes between the shRNA-NC-K562 and shRNA-CRKL-K562 group cells.

siRNA design and Transient transfection

For CRK− knockdown, targeting siRNAs (small interfering RNA) were designed based on the CRK− sequence (Genbank: NM_016823.3, siCRK−-1: 5’-CCAGAATGGGCCCATATAT-3’, siCRK−-2: 5’-GCGAGTCCCCAATGCCTAC-3’) using Invitrogen, siDirect and Whitehead software. Meanwhile, one siRNA with non-targeting sequence (5’-TTCTCCGAACGTGTCACGT-3’) was designed as a negative control (NC). One day before transfection, 3 × 10^5 cells/well in 2 ml RPMI-1640 supplemented with 15% FBS were seeded into a 6-well plate, and the siRNA mixtures (3 µl siCRK−-1 + 3 µl siCRK−-2) were transfected into K562 cells using 5 µl Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer’s instructions for 48 h at 37 °C with 5% CO₂.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. The data were presented as mean ± SD of at least three independent experiments. Student’s t-test was performed to measure the differences between two groups. Differences with P< 0.05 were considered statistically significant.

Results

The expression patterns of CRKL and CRK− in CML patients

To investigate the potential role of CRKL in CML, we examined the expression pattern of CRKL in 33 CML patient BM samples, 5 CML CR patient BM samples and 13 normal PB samples by qRT-PCR. Our results showed that CRKL was almost universally overexpressed in CML patient BM samples (29/33), the mRNA expression level of CRKL was upregulated 6.2-fold in CML patient BM samples compared with normal samples (P = 0.009, Fig. 1A). We further compared the mRNA expression level of CRKL in 5 pairs of CML primary and CR patient samples. Interestingly, the mRNA expression level of CRKL was lower in CR patient samples than in the corresponding CML primary patient samples, the mRNA expression level of CRKL was downregulated 47.1% in CR patient samples compared with CML patient samples (P = 0.0165, Fig. 1B). Our results indicate that CRKL is highly expressed in CML and plays a crucial role in the development and progression of CML, it may be a potential diagnostic and therapeutic biomarker for CML.

Meanwhile, we examined the expression pattern of CRK− in 33 CML patient BM samples, 5 CR patient BM samples and 13 normal PB samples. Our results showed that CRK− was slightly overexpressed in CML
patient BM samples (20/33), although there was no statistically significant difference, the mRNA expression level of CRK was upregulated 1.8-fold in CML patient BM samples compared with normal samples ($P = 0.0855$, Fig. 1C). We further compared the mRNA expression level of CRK in 5 pairs of CML primary and CR patient samples. The mRNA expression level of CRK was lower in CR patient samples than in the corresponding CML primary patient samples, the mRNA expression level of CRK was downregulated 41.9% in CR patient samples compared with CML patient samples, but there was no significant difference ($P = 0.1014$, Fig. 1D). Our results indicate that CRK is only slightly overexpressed in CML and may not play a very important role in the development and progression of CML.

**CRKL was down-regulated during hemin-induced erythroid differentiation of K562 cells**

K562 cells can be differentiated into erythroid cells by treatment with hemin, so we investigated the expression pattern of CRKL during hemin-induced erythroid differentiation of K562 cells. After treatment with hemin, K562 cells showed significant increases in the number of benzidine-positive cells in a time-dependent manner. The benzidine-positive rates of K562 cells induced by hemin for 0, 1, 2 d were 0.4%, 32.9% ($P = 0.0026$) and 40.3% ($P = 0.0009$), respectively (Fig. 2A). Meanwhile, the mRNA expression levels of erythroid differentiation markers $\gamma$-globin and glycophorin (GPA) were also increased in K562 cells after treatment with hemin (Fig. 2B), indicating the erythroid differentiation of K562 cells was successfully induced by hemin. Then we measured the protein expression level of CRKL during erythroid differentiation of K562 cells. WB results showed that the CRKL protein expression level was significantly downregulated by 52.7% ($P = 0.0007$) and 54.5% ($P = 0.0004$) in K562 cells following treatment with hemin for 1 and 2 d (Fig. 2C), respectively. Taken together, CRKL expression is downregulated during erythroid differentiation of K562 cells, indicating a potential role for CRKL in erythroid differentiation.

Meanwhile, we investigated the expression pattern of CRK during erythroid differentiation of K562 cells. No obvious protein level change was observed for CRK during erythroid differentiation of K562 cells. CRK protein expression level was only slightly upregulated by 7.0% ($P = 0.2893$) and 6.8% ($P = 0.5675$) in K562 cells following treatment with hemin for 1 and 2 d (Fig. 2D), respectively, indicating CRK might not be involved in erythroid differentiation of K562 cells.

**CRKL knockdown promoted erythroid differentiation of K562 cells**

To gain insight into the role of CRKL in erythroid differentiation, we used monoclonal cell lines previously successfully constructed by our group (shRNA-NC-K562 and shRNA-CRKL-K562) to investigate the effect of endogenous CRKL on erythroid differentiation. The CRKL protein and $CRKL$ mRNA levels were decreased by 84.2% ($P = 0.0005$) and 91.0% ($P = 0.0002$) by RNAi (Fig. 3A). The establishment of monoclonal shRNA-CRKL-K562 cells with stable CRKL knockdown enabled the investigation of CRKL in erythroid differentiation of K562 cells, which provide a control study for the downregulation effect of CRKL on erythroid differentiation of K562 cells. shRNA-CRKL-K562 cells showed increased numbers of benzidine-positive cells than shRNA-NC-K562 cells. The benzidine-positive rates of shRNA-CRKL-K562
and shRNA-NC-K562 cells were 10.5% and 3.4%, respectively, the benzidine-positive rates of shRNA-CRKL-K562 cells increased by 2.1-fold compared with shRNA-NC-K562 cells \((P = 0.0239\) \(, \)Fig. 3B\)). Meanwhile, the erythroid genes \(\gamma\)-globin and GPA mRNA expression levels also increased by 96.9% \((P = 0.0006\) and 59.35% \((P = 0.0096\) in shRNA-CRKL-K562 compared to shRNA-NC-K562 cells \(\)Fig. 3C\). Our results showed that CRKL downregulation promotes erythroid differentiation of K562 cells.

We further screened the differentially expressed genes between shRNA-CRKL-K562 and shRNA-NC-K562 cells by gene microarray. A total of 549 mRNAs were identified as up- or down-regulated over 1.5-fold in shRNA-CRKL-K562 cells compared with shRNA-NC-K562 cells. Among these targeting genes, we focused on the molecules associated with erythroid differentiation. We found that Hemoglobin \(\)HB\) molecules \(HBD\), \(HBA1\), \(HBA2\) and \(HBZ\) were upregulated 1.6-, 2.2-, 2.3- and 2.5-fold in shRNA-CRKL-K562 cells compared to shRNA-NC-K562 cells \(\)Table 2\), respectively.

Moreover, we screened the differentially expressed proteins between shRNA-CRKL-K562 and shRNA-NC-K562 cells by iTRAQ quantitative proteomic analysis. A total of 215 proteins were identified as up- or down-regulated over 1.2-fold \((P < 0.05\) \(, \)Fig. 4A\) in shRNA-CRKL-K562 cells compared with shRNA-NC-K562 cells. Among these differently expression proteins, 53 proteins were up-regulated and 162 proteins were down-regulated in shRNA-CRKL-K562 cells compared to shRNA-NC-K562 cells, these proteins were clustered as shown in Fig. 4A. Gene ontology analysis was performed on the differentially expressed proteins, these differentially expressed proteins were related to positive regulation of erythrocyte differentiation, leukocyte differentiation, positive regulation of megakaryocyte differentiation, hemoglobin complex, regulation of erythrocyte differentiation and megakaryocyte differentiation, indicating CRKL deregulation is associated with differentiation of K562 cells.

Among these targeting proteins, we also focused on the molecules associated with erythroid differentiation. Consistently with microarray results, we found that hemoglobin molecules \(HBE1\), \(HBD\), \(HBZ\), \(HBG1\), erythroid specific transcription factor GATA-1 and HMGB2 were upregulated 1.2-, 1.3-, 1.2-, 1.2-, 1.3- and 1.2-fold in shRNA-CRKL-K562 cells compared to shRNA-NC-K562 cells \(\)Table 2\), respectively. We detected the expression level of GATA-1 and HMGB2 by WB and qRT-PCR to validate the proteomic analysis results. The protein expression levels of GATA-1 and HMGB2 were upregulated 63.0% \((P = 0.004)\) and 54.0% \((P = 0.0391)\) in shRNA-CRKL-K562 cells compared to shRNA-NC-K562 cells \(\)Fig. 4B\), respectively, while, the mRNA expression levels of \(GATA-1\) and \(HMGB2\) were upregulated 29.5% \((P = 0.0041)\) and 44.1% \((P = 0.0012)\) in shRNA-CRKL-K562 cells compared to shRNA-NC-K562 cells \(\)Fig. 4C\), respectively. The expression profile was consistent with proteomic analysis results, indicating the proteomic analysis results were believable. Our results indicated that CRKL downregulation promoted hemoglobin molecules expression, which resulted in erythroid differentiation of K562 cells. Taken together, the gene microarray and iTRAQ quantitative proteomic analysis further confirmed that CRKL downregulation promotes erythroid differentiation of K562 cells.

**CRKL knockdown promoted megakaryocyte differentiation of K562 cells**
In addition, we also evaluated the effect of CRKL knockdown on megakaryocyte differentiation of K562 cells. K562 cell approximates to the megakaryocyte-erythrocyte progenitor stage, which has the potential to differentiate into megakaryocytes. As shown in Fig. 5A, compared to shRNA-NC-K562 cells, shRNA-CRKL-K562 cells exhibited typical characters of megakaryocyte differentiation with an increase in cell size, polyploidization and the presence of vacuoles. The percentage of megakaryocyte cells in the shRNA-CRKL-K562 group was higher than in the shRNA-NC-K562 group. The megakaryocyte surface differentiation markers CD41 and CD61 were also determined by qRT-PCR. The mRNA expression levels of CD41 and CD61 were significantly increased 73.6% (P = 0.0302) and 47.15% (P = 0.0234) in shRNA-CRKL-K562 compared to shRNA-NC-K562 cells, respectively (Fig. 5B). Our results indicated that CRKL downregulation also promotes megakaryocyte differentiation of K562 cells.

**CRKL knockdown promoted erythroid and megakaryocyte differentiation of K562 cells via activating the Raf/MEK/ERK/Elk-1 signaling pathway**

The underlying molecular mechanisms of CRKL on erythroid and megakaryocyte differentiation is unknown. Current work links CRKL downregulation to promoting K562 cell erythroid and megakaryocyte differentiation via activating the Raf/MEK/ERK/Elk-1 signaling pathway.

Since the Raf/MEK/ERK pathway is involved in erythropoiesis, we hypothesized that CRKL affects K562 cell differentiation via the Raf/MEK/ERK pathway. We found that CRKL downregulation activated the Raf/MEK/ERK pathway. CRKL downregulation increased the protein expression levels of Raf, p-Raf, p-MEK and p-ERK1/2, the protein expression levels of Raf, p-Raf, p-MEK, p-ERK1/2 were upregulated 61.9% (P = 0.045), 43.1% (P = 0.041), 43.9% (P = 0.028) and 2.1-fold (P = 0.045) in shRNA-CRKL-K562 cells compared with shRNA-NC-K562 cells (Fig. 6A). No changes were observed for Ras and ERK1/2 (Fig. 6A). Elk-1 is the downstream molecule of ERK, which is associated with erythroid differentiation. Our results showed that CRKL downregulation increased the mRNA expression levels of Elk-1, the mRNA expression level of Elk-1 was upregulated 44.1% (P = 0.0131) in shRNA-CRKL-K562 cells compared with shRNA-NC-K562 cells (Fig. 6A). Clearly, CRKL mediates the K562 cell erythroid and megakaryocyte differentiation might be via the Raf/MEK/ERK/Elk-1 signaling pathway.

The linkage of the Raf/MEK/ERK/Elk-1 pathway to CRKL-mediated K562 cell differentiation was further validated using PD98059, a specific pERK1/2 inhibitor, for specific signaling blocking. The treatment of shRNA-CRKL-K562 cells with 20 µM PD98059 resulted in p-ERK1/2 protein expression decreasing by 44.65% (Fig. 6B, P = 0.0093) and Elk-1 mRNA expression level reducing by 30.15% (Fig. 6B, P = 0.0082), while no significant change was observed for ERK1/2 (Fig. 6B, P = 0.1018), indicating the Raf/MEK/ERK/Elk-1 signaling pathway was successfully blocked by PD98059.

We further detected the expression level changes of erythroid and megakaryocyte differentiation related markers GPA, γ-globin, CD41 and CD61 after blocking the Raf/MEK/ERK/Elk-1 signaling pathway by
PD98059. The treatment of shRNA-CRKL-K562 cells with 20 µM PD98059 resulted in **GPA**, \(\gamma\)-globin, **CD41** and **CD61** mRNA expression levels decreasing by 33.8% (Fig. 6C, \(P = 0.0106\)), 30.8% (Fig. 6C, \(P = 0.0459\)), 66.1% (Fig. 6C, \(P = 0.0351\)) and 62.1% (Fig. 6C, \(P = 0.0259\)), respectively. These results indicate that CRKL regulates K562 cell erythroid and megakaryocyte differentiation via the Raf/MEK/ERK/Elk-1 pathway.

**CRK\(\hat{\alpha}\) has no effect on erythroid differentiation of K562 cells**

CRK\(\hat{\alpha}\) may be unimportance for erythroid differentiation, to confirm the effect of CRK\(\hat{\alpha}\) on erythroid differentiation, we transiently transfected K562 cells with siCRK\(\hat{\alpha}\) to knockdown CRK\(\hat{\alpha}\). CRK\(\hat{\alpha}\) protein and mRNA levels were decreased by 53.6% (\(P = 0.0015\)) and 43.5% (\(P = 0.0059\)) in siRNA-CRK\(\hat{\alpha}\)-K562 cells compared with siRNA-NC-K562 cells (Fig. 7A), providing a control study for the downregulation effect of CRK\(\hat{\alpha}\) on K562 cell erythroid differentiation. qRT-PCR detected the expression level changes of erythroid genes after CRK\(\hat{\alpha}\) knockdown, there were no obvious changes in \(\gamma\)-globin (\(P = 0.088\)) and **GPA** (\(P = 0.133\)) mRNA expression levels between siRNA-CRK\(\hat{\alpha}\)-K562 and siRNA-NC-K562 cells (Fig. 7B). Meanwhile, to show that CRK\(\hat{\alpha}\) downregulation did not affect the Raf/MEK/ERK pathway, we measured the expression level changes of p-Raf, p-MEK, p-ERK1/2 after CRK\(\hat{\alpha}\) knockdown by WB. No changes were observed for p-Raf, p-MEK, p-ERK1/2 (Fig. 7C). Clearly, our results further demonstrated CRK\(\hat{\alpha}\) has no effect on erythroid differentiation of K562 cells.

To further confirm the effect of CRK\(\hat{\alpha}\) on erythroid differentiation, we transiently transfected siCRK\(\hat{\alpha}\) in shRNA-CRKL-K562 cells with CRKL stable knockdown. Compared with shRNA-CRKL-K562 + siNC cells, the CRK\(\hat{\alpha}\) protein expression level decreased by 42.5% (Fig. 7D, \(P = 0.0038\)), while the CRKL protein expression level had no significant change (Fig. 7E, \(P = 0.0974\)). We compared the erythroid differentiation ability between shRNA-CRKL-K562 + siNC and shRNA-CRKL-K562 + siCRK\(\hat{\alpha}\) cells (Fig. 7F). Interestingly, there were no obvious changes in \(\gamma\)-globin (\(P = 0.7762\)) and **GPA** (\(P = 0.1261\)) mRNA expression levels between the two groups. Meanwhile, we measured the expression levels change of ERK1/2 (\(P = 0.9171\)) and p-ERK1/2 (\(P = 0.1207\)) after CRK\(\hat{\alpha}\) knockdown in shRNA-CRKL-K562 cells (Fig. 7G). Consistent with the above results, no changes were observed for ERK1/2 and p-ERK1/2. Collectively, the results further demonstrate that CRKL can inhibit erythroid differentiation of K562 cells, while CRK\(\hat{\alpha}\) has no effect on erythroid differentiation of K562 cells.

**Discussion**

Hematopoiesis is a highly and precisely regulated multistage process by which all of the different cell lineages (erythroid cells, lymphocytes and myeloid cells) that form the immune and blood systems originate from pluripotent stem cells [33, 34]. Erythropoiesis happens in human red bone marrow after kidneys responses to low levels of oxygen by releasing erythropoietin [35]. Erythropoiesis is a multi-step cellular course by which a primitive multipotent HSC experiences a series of differentiations resulting in production of erythroid lineage, undergoing erythroid progenitors (colony-forming unit erythroid [CFU-E] and burst-forming unit erythroid [BFU-E]), normoblasts, proerythroblasts, early basophilic erythroblasts, late basophilic erythroblasts, polychromatric erythroblasts, orthochromatic erythroblasts, reticulocytes, ultimately differentiating to mature erythrocytes [5, 6]. Megakaryopoiesis occurs through a hierarchical
series of progenitor cells, multipotent progenitor (MPP), common myeloid progenitor (CMP) and megakaryocyte-erythroid progenitor (MEP), megakaryocyte progenitor (MKP), ultimately differentiating to mature megakaryocytes [36]. The two dynamic processes are mediated by a balance of intrinsic and extrinsic factors, containing transcription factors, growth factors and miRNAs, and destruction of the two dynamic processes leads to CML. Tyrosine kinase inhibitors (TKIs) targeting BCR-ABL for CML therapy have effectively improved the survival of CML patients, however, about 20% of CML patients have not been benefited from TKIs treatment, commonly due to TKIs resistance which lead to disease relapse and progression [37–39]. Therefore, it is urgent to seek more efficient therapeutic strategies to overcome the problem. Deeper study of the molecular mechanisms governing the development, progression and differentiation of CML can lead to finding novel therapeutic targets and improving the therapy effects for CML patients.

CRK proteins are the predominant phosphorylation substrates for BCR-ABL, which is found in over 95% of CML and 25% of acute lymphoblastic leukemias (ALL) [40]. Although CRKII and CRKL share a high degree of homology within their functional domains, CRKL is the major tyrosine-phosphorylated protein in BCR-ABL-driven CML patient neutrophils [40]. The preferential binding of BCR-ABL to CRKL, even in the presence of CRKII [41], suggests disparity in interaction properties and differential regulation of CRK proteins by BCR-ABL or ABL tyrosine kinases. These finds imply that CRKII and CRKL may play different role in CML, so in this work we investigated the exact effect of CRKII and CRKL on erythropoiesis and megakaryopoiesis of CML. The current study illustrated for the first time that CRKL but not CRKII inhibits erythroid and megakaryocyte differentiation via the inactivating Raf/MEK/ERK/Elk pathway.

CRKL deregulation is linked to the development and progression of a variety of cancers [26–28]. As we summarized in our review [25], abnormal CRKL expression is associated with gastric cancer, glioblastoma multiforme, hepatocellular carcinoma, bladder cancer, lung cancer, colon cancer, ovarian cancer, leukemia, breast cancer, head and neck cancer, rhabdomyosarcoma and neuroblastoma. It is of promise as an indicator for cancer development, invasion and metastasis as well as an attractive target for the diagnosis and prognosis of cancer. CRKL is a major tyrosine-phosphorylated protein in CML cells, pCRKL plays a special role in CML pathogenesis, and the constitutive phosphorylation of CRKL is unique to CML, which makes it a useful target for therapeutic intervention [42–44]. We previous reported that CRKL is associated with proliferation, migration and invasion of hepatocarcinoma and clear cell renal cell carcinoma cells [45–50]. However, the exact role of CRKL in CML is unknown. Our current work showed that the upregulation of CRKL potentially promotes the clinical development and progression of CML patients and enhances CML cell aggressiveness. CRKL was universally overexpressed in CML patient samples compared with normal samples (Fig. 1A). Interestingly, CRKL expression level was lower in CR patient samples than in corresponding CML patient samples (Fig. 1B). Our results indicate that CRKL is a tumor promoter playing a vital role in the development and progression of CML. To the best of our knowledge, this work is the first reporting the expression pattern of CRKL in CML patients, CR patients and normal samples. CRK deregulation is also linked to the development and progression of a variety of cancers [26–28], But our results show that CRK is only slightly overexpressed in CML (Fig. 1C, D), and that it may not play an important role in the development and progression of CML.
It is known that the CRK family plays important roles in the regulation of cell differentiation. v-CRK overexpression can increase rat pheochromocytoma PC12 cell differentiation [29], and both SH2 and SH3 domains of the CRK protein are required for neuronal differentiation of PC12 cells [30]. Moreover, CRK enhances osteoclast differentiation by activating Rac1, the overexpression of CRK and CRKL significantly enhances RANKL-induced osteoclast differentiation, and the downregulation of CRK and CRKL synergistically decreases RANKL-induced osteoclast differentiation [31]. The effect of CRKL and CRK on leukemia cell differentiation has not been reported, in our study we investigated the potential role of CRK and CRKL in erythroid and megakaryocyte differentiation of K562 cells. Hemin is an iron-containing porphyrin which is involved in oxygen delivery and used to treat acute porphyria and thalassemia intermedia, and is also a relatively strong inducer for heme biosynthesis of K562 cell erythroid differentiation [51]. Using K562 cells as a model, we found that CRKL expression level was downregulated in hemin-induced erythroid differentiation of K562 cells (Fig. 2C), indicating CRKL might play an important role in erythroid differentiation of K562 cells. In order to verify the hypothesis, we selected previously successfully constructed CRKL stably downregulated monoclonal cell lines to investigate the effect of endogenous CRKL on erythroid differentiation. We further found that CRKL downregulation promoted erythroid differentiation of K562 cells with more benzidine-positive cells and higher mRNA expression levels of γ-globin and GPA (Fig. 3). Moreover, CRKL downregulation enhanced megakaryocyte differentiation of K562 cells with increased number of megakaryocyte cells and higher mRNA expression levels of CD41 and CD61 (Fig. 5). Our results first demonstrate CRKL is a new regulator of erythroid and megakaryocyte differentiation of K562 cells. Furthermore, we screened the differentially expressed molecules between shRNA-CRKL-K562 and shRNA-NC-K562 cells using gene microarray and iTRAQ quantitative proteomic analysis. Results showed hemoglobin molecules HBD, HBA1, HBA2, HBZ, HBE1 and HBG1 were more upregulated in shRNA-CRKL-K562 than in shRNA-NC-K562 cells (Tables 2 and 3). Moreover, GATA-1 and HMGB2 expression were increased in shRNA-CRKL-K562 than in shRNA-NC-K562 cells (Fig. 4), which are crucial for erythrocyte and megakaryocyte lineages. The zinc-finger transcription factor GATA-1 binds to GATA/AATC consensus elements in the globin gene clusters and other erythroid or megakaryocytic cell-specific genes [52]. The zinc-finger proto-oncogene Gfi-lb is an erythroid-specific transcription factor that plays an important role in erythropoiesis [53], Gfi-1B gene disruption results in embryonic death of mice due to failure to produce red blood [54]. The Gfi-1B promoter contains 2 tandem sites which includes both a GATA-1- and an Oct-1-binding sequence [55]. HMGB2 bends DNA at the Gfi-1B promoter by binding to the Gfi-1B promoter to facilitate the binding of Oct-1 to the Gfi-1B promoter [56], subsequently enhancing the binding of GATA-1 to the AATC sites of Gfi-1B promoter and activating the transcription of Gfi-1B [57]. Our results show that CRKL regulates erythroid and megakaryocyte differentiation of K562 cells by upregulating GATA-1 and HMGB2 expression.

However, the expression level of CRK was not changed in hemin-induced erythroid differentiation of K562 cells (Fig. 2D) and CRK downregulation did not affect erythroid differentiation of K562 cells (Fig. 7B). Moreover, we further investigated the effect of CRK knockdown on erythroid differentiation of K562 cells by transiently transfecting siCRK in shRNA-CRKL-K562 cells, interestingly, consistent with the
above results, CRK\(^\text{II}\) knockdown in K562 cells with CRKL downregulation (Fig. 7F). Collectively, CRK\(^\text{II}\) is not associated with erythroid differentiation of K562 cells. Although CRK\(^\text{II}\) and CRKL have a high degree of similarity in sequence, the two isoforms vary in ligand affinities and specificity, and the 3-dimensional structures of CRK\(^\text{II}\) and CRKL differ to engage key signaling partners [21, 28]. The respective knockout mice have distinct phenotypes but both proteins are required for embryonic development [58, 59]. So CRK\(^\text{II}\) and CRKL might function differently in leukemogenesis, erythropoiesis and megakaryopoiesis of CML, which deserves more attention to understand the differences between the two CRK adapter proteins. Our results are also consistent with the previous report that CRKL expression level is highest in adult hematopoietic tissues and low in epithelial tissues, whereas CRK\(^\text{II}\) exhibits the highest expression in the brain, lung, kidney and low expression in bone marrow [60].

The Ras/Raf/MEK/ERK signaling pathway is involved in erythropoiesis which mainly promotes growth, differentiation and prevents apoptosis of hematopoietic cells [61–63]. MASL1 could induce erythroid differentiation in CD34 cells through the Raf/MEK/ERK signaling pathway [64]. G protein expression levels increased and ERK1/2 activated during hemin-induced differentiation of K562 cells [65]. Inhibition of the MEK/ERK signaling pathway promoted erythroid differentiation and reduced HSCs engraftment in ex-vivo expanded haematopoietic stem cells [66]. The ERK/MAPK pathway is involved in megakaryocytic differentiation of K562 cells induced by 3-hydrogenkwadaphnin [67]. Our results showed that CRKL affected the expression levels of Raf/MEK/ERK-Elk-1 pathway-related molecules (Fig. 6), we speculated CRKL might mediate erythroid and megakaryocyte differentiation of K562 cells by regulating the Raf/MEK/ERK-Elk-1 pathway. We validated the potential involvement of Raf/MEK/ERK-Elk-1 using a specific ERK inhibitor PD98059. The expression levels of GPA, \(\gamma\)-globin, CD41 and CD61 decreased after blocking the Raf/MEK/ERK-Elk-1 pathway with the ERK inhibitor PD98059 (Fig. 6). CRKL regulates erythroid and megakaryocyte differentiation of K562 cell via inactivating the Raf/MEK/ERK/Elk-1 pathway. However, CRK\(^\text{II}\) downregulation could not affect the Raf/MEK/ERK pathway (Fig. 7C, G), further indicating CRK\(^\text{II}\) has no effect on erythroid differentiation of K562 cells.

Taken together, we have illustrated for the first time that CRKL can inhibit erythroid and megakaryocyte differentiation of K562 cells via inactivating the Raf/MEK/ERK/Elk pathway. The novel action mechanism is outlined in Fig. 8. CRKL downregulation promotes the expression of Raf, p-Raf, p-MEK, p-ERK1/2 and Elk-1, then HMGB2 binds to the \(Gfi-1B\) promoter and enhances its transactivation by promoting the binding of Oct-1 and GATA-1 to the \(Gfi-1B\) promoter, which induces erythroid and megakaryocyte differentiation of K562 cells by increasing globin, hemoglobin and differentiation-specific genes expression. Taken together, we have established a new functional role and molecular pathway for CRKL during hematopoietic differentiation. These findings could be fundamental to the development of a novel potential diagnostic biomarker and therapeutic target for CML patients.

Conclusions

In conclusion, the different features of CRK\(^\text{II}\) and CRKL indicates that they may serve differently in leukemogenesis. Our findings point to CRKL rather than CRK\(^\text{II}\) as a biomarker associating with
differentiation of CML. Their different functions in CML cells may result from different preferential interactions with binding partners, thereby activating different signaling pathways leading to different roles in CML.

**Abbreviations**

HSC: Hematopoietic stem cell; RBCs: Red blood cells; CML: Chronic myeloid leukemia; BCR/ABL1: B-cell receptor/v-abl Abelson murine leukemia viral oncogene; CRK: CT10 regulation of kinase; CRKL: CRK-like; CR: Complete remission; BM: Bone marrow; PB: Peripheral blood; ATCC: American Type Culture Collection; FBS: Fetal bovine serum; RT: Room temperature; WB: Western blotting; GATA-1: Globin transcription factor 1; HMGB2: High-mobility group protein; Itraq: Isobaric tags for relative and absolute quantitation; FASP: Filter-aided sample preparation; SCX: Strong cation exchange; qRT-PCR: Quantitative real-time RT-PCR; GPA: Glycophorin; RVM: Random variance model; siRNAs: Small interfering RNA; NC: Negative control; CFU-E: Colony-forming unit erythroid; BFU-E: Burst-forming unit erythroid; MPP: Multipotent progenitor; CMP: Common myeloid progenitor; MEP: Megakaryocyte-erythroid progenitor; MKP: Megakaryocyte progenitor; TKIs: Tyrosine kinase inhibitors; ALL: Acute lymphoblastic leukemias.

**Declarations**

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Not applicable.

**Author contributions**

CMG and QLZ conceived the study and designed the experiments; QLZ, CMG, XXL and SLZ performed the experiments; CMG wrote the manuscript; CMG, QSY, FTG, SQL and MZS modified the manuscript. All authors read and approved the final manuscript.

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**Availability of data and material**

The data supporting the conclusions of this study are included within the article.

**Ethics approval and consent to participate**

This study has been conducted in accordance with ethical standards and according to the Declaration of Helsinki and national and international guidelines, and has been approved by the authors’ institutional
review board. The study protocol was approved by the Medical Ethics Committee of Dalian Medical University.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Jagannathan-Bogdan M, Zon LI. Development. 2013;140(12): 2463-7.
2. Poller WC, Nahrendorf M, Swirski FK. Hematopoiesis and cardiovascular disease. Circ Res. 2020;126(8):1061-85.
3. Park SJ, Bejar R. *Clonal hematopoiesis in cancer*. Exp Hematol. 2020;83: 105-12.
4. Cheng H, Sun GH, Cheng T. Hematopoiesis and microenvironment in Hematological malignancies. Cell Regen. 2018;7(1): 22-6.
5. Eggold JT, Rankin EB. Erythropoiesis, EPO, macrophages, and bone. Bone. 2019;119: 36-41.
6. Vinchi F. Erythroid differentiation: a matter of proteome remodeling. *Hemasphere*. 2018;2(1): e26.
7. Noetzli LJ, French SL, Machlus KR. *New insights into the differentiation of megakaryocytes from hematopoietic progenitors*. Arterioscler Thromb Vasc Biol. 2019;39(7): 1288-300.
8. Zhang X, Ren R. Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocytemacrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. Blood. 1998;92(10): 3829-40.
9. Hemin D, Ebnothere M, Favre G. Chronic myeloid leukemia-update 2020. Ther Umsch. 2019;76(9): 503-9.
10. Shimoda K. Chronic myeloid leukemia: CML. Rinsho Ketsueki. 2019;60(3): 230-1.
11. Qin YZ, Huang XJ. Molecular detection of BCR-ABL in chronic myeloid leukemia. Methods Mol Biol. 2016;1465: 1-15.
12. Naughton R, Quiney C, Turner SD, Cotter TG. Bcr-Abl-mediated redox regulation of the PI3K/AKT pathway. Leukemia. 2009;23(8): 1432-40.
13. Baum KJ, Ren R. Effect of Ras inhibition in hematopoiesis and BCR/ABL leukemogenesis. J Hematol Oncol. 2008;1: 5.
14. Hantschel O, Warsch W, Eckelhart E, Kaupe I, Grebien F, Wagner KU, Superti-Furga G, Sexl V. BCR-ABL uncouples canonical JAK2-STAT5 signaling in chronic myeloid leukemia. Nat Chem Biol. 2012;8(3): 285-93.
15. Bruserud O, Gjertsen BT, Huang TS. Induction of differentiation and apoptosis-a possible strategy in the treatment of adult acute myelogenous leukemia. Oncologist. 2000;5(6): 454-62.

16. Lozzio BB, Lozzio CB. Properties of the K562 cell line derived from a patient with chronic myeloid leukemia. Int J Cancer. 1977;19(1): 136.

17. Andersson LC, Nilsson K, Gahmberg CG. K562-a human erythroleukemic cell line. Int J Cancer. 1979;23(2): 143-7.

18. Tsiftsoglou AS, Vizirianakis IS, Strouboulis J. Erythropoiesis: Model systems, molecular regulators and developmental programs. IUBMB life. 2009;61(8): 800-30.

19. Wan LM, Zhang SK, Li SB, Li W, Ji SP, Gong L, Yun ZM, Zhang X, Gao HW, Zhong H, Wei CW, Bian LH, Zhuo HL, Luo Q, Li JP, Tan YX, Gong F. Heparanase facilitates PMA-induced megakaryocytic differentiation in K562 cells via interleukin 6/STAT3 pathway. Thromb Haemost. 2020;120(4): 647-57.

20. Brige RB, Kalodimos C, Inagaki FT, Tanaka S. Crk and CrkL adaptor proteins networks for physiological and pathological signaling. Cell Commun Signal. 2009;7: 1-23.

21. Feller SM. Crk family adaptors-signaling complex formation and biological roles. Oncogene. 2001;20(44): 6348-71.

22. Gelkop S, Babichev Y, Kalifa R, Tamir A, Isakov N. Involvement of crk adapter proteins in regulation of lymphoid cell functions. Immunol Res. 2003;28(2): 79-91.

23. Nichols GL, Raines MA, Vera JC. Identification of CRKL as the constitutively phosphorylated 39-kD tyrosine phosphoprotein in chronic myelogenous leukemia cells. Blood. 1994;84(9): 2912-8.

24. Johnson KJ, Griswold IJ, O’Hare T, Corbin AS, Loriaux M, Deininger MW, Druker BJ. A BCR-ABL mutant lacking direct binding sites for the GRB2, CBL and CRKL adapter proteins fails to induce leukemia in mice. PLoS One. 2009;4(10): e7439.

25. Rhodes J, York RD, Tara D, Tajinda K, Druker BJ. CrkL functions as a nuclear adaptor and transcriptional activator in Bcr-Abl-expressing cells. Exp Hematol. 2000;28(3): 305-10.

26. Guo CM, Liu SQ, Sun MZ. The role of CT10 regulation of kinase-like in cancer. Future Oncol. 2014;10(16): 2687-97.

27. Bell ES, Park M. Models of crk adaptor proteins in cancer. Genes Cancer. 2013;3(5-6): 341-52.

28. Sriram G, Birge RB. Emerging roles for crk in human cancer. Genes Cancer. 2011;1(11): 1132-9.

29. Hempstead BL, Birge RB, Fajardo JE, Glassman R, Mahadeo D, Kraemer R, Hanafusa H. Expression of the v-crk oncogene product in PC12 cells results in rapid differentiation by both nerve growth factor- and epidermal growth factor-dependent pathways. Mol Cell Biol. 1994;14: 1964-71.

30. Tanaka S, Hattori S, Kurata T, Nagashima K, Fukui Y, Nakamura S, Matsuda M. Both the SH2 and SH3 domains of human CRK protein are required for neuronal differentiation of PC12 cells. Mol Cell Biol. 1993;13: 4409-15.

31. Kim JH, Kim K, Kim I, Seong S, Nam KI, Lee SH, Kim KK, Kim N. Role of CrkII signaling in RANKL-induced osteoclast differentiation and function. J Immunol. 2016;196(3): 1123-31.
32. Moulder R, Lonnberg T, Elo LL, Filn JJ, Rainio E, Corthals G, Oresic M, Nyman TA, Aittokallio T, Lahesmaa R. Quantitative proteomics analysis of the nuclear fraction of human CD4+ cells in the early phases of IL-4-induced Th2 differentiation. Mol Cell Proteomics. 2010;9(9):1937-53.

33. Sun JL, Camargo FD, Ramos A, Le L, Chapman B, Hofmann O, Johnnidis J. Clonal dynamics of native haematopoiesis. Nature. 2014;514(7522): 322-7.

34. Ebdon C, Batty P, Smith G. Haematopoiesis and red blood cells. Surg Oncol. 2013;31(5): 200-5.

35. Dzierzak E, Philipsen S. Erythropoiesis: development and differentiation. Cold Spring Harb Perspect Med. 2013;3(4): a011601.

36. Debili N, Coulombel L, Croisille L, Katz A, Guichard J, Breton-Gorius J, Vainchenker W. Characterization of a bipotent erythro-megakaryocytic progenitor in human bone marrow. Blood. 1996;88(4): 1284-96.

37. Aladağ E, Haznedaroğlu İC. Current perspectives for the treatment of chronic myeloid leukemia. Turk J Med. 2019;49(1): 1-10.

38. Özgür Yurttaş N, Eşkazan AE. Novel therapeutic approaches in chronic myeloid leukemia. Leuk Res. 2020;91: 106337.

39. Gambacorti-Passerini C, Piazza R. Imatinib-a new tyrosine kinase inhibitor for first-line treatment of chronic myeloid leukemia in 2015. JAMA Oncol. 2015;1(2): 143-4.

40. Oda T, Heaney C, Hagopian JR, Okuda K, Griffin JD, Druker BJ. Crkl is the major tyrosinephosphorylated protein in neutrophils from patients with chronic myelogenous leukemia. J Biol Chem. 1994;269(37): 22925-8.

41. Jankowski W, Saleh T, Pai MT, Sriram G, Birge RB, Kalodimos CG. Domain organization differences explain Bcr-Abl's preference for CrkL over CrkII. Nat Chen Biol. 2012;8(6): 590-6.

42. ten Hoeve J, Kaartinen V, Fioretos T, Haataja L, Voncken JW, Heisterkamp N, Groffen J. Cellular interactions of CRKL and SH2-SH3 adaptor protein. Cancer Res. 1994;54(10): 2563-7.

43. Lucas CM, Harris RJ, Giannoudi A, Knight K, Watmough SJ, Clark RE. BCR-ABL1 tyrosine kinase activity at diagnosis, as determined via the pCrkL/CrkL ratio, is predictive of clinical outcome in chronic myeloid leukaemia. Br J Haematol. 2010;149(3): 458-60.

44. Singer CF, Hudelist G, Lamm W, Mueller R, Handl C, Kubista E, Czerwenka K. Active (p)CrkL is overexpressed in human malignancies: potential role as a surrogate parameter for therapeutic tyrosine kinase inhibition. Oncol Rep. 2006;15: 353-359.

45. Lin QY, Sun MZ, Guo CM, Liu SQ. CRKL overexpression suppresses in vitro proliferation, invasion and migration of murine hepatocarcinoma Hca-P cells. Biomed Pharmacother. 2015;69: 106337.

46. Shi J, Meng LL, Sun MZ, Guo CM, Sun XJ, Lin QY, Liu SQ. CRKL knockdown promotes in vitro proliferation, migration and invasion, in vivo tumor malignancy and lymph node metastasis of murine hepatocarcinoma Hca-P cells. Biomed Pharmacother. 2015;71: 84-90.

47. Abdul S, Majid A, Wang JX, Liu LQ, Sun MZ, Liu SQ. Bidirectional interaction of IncRNA AFAP1-AS1 and CRKL accelerates the proliferative and metastatic abilities of hepatocarcinoma cells. J Adv Res.
Wang JX, Wang CY, Li Q, Guo CM, Sun WB, Zhao DT, Jiang SX, Hao LH, Tian YX, Liu SQ, Sun MZ. miR-429-CRKL axis regulates clear cell renal cell carcinoma malignant progression through SOS1/MEK/ERK/MMP2/MMP9 pathway. Biomed Pharmacother. 2020;127: 110215.

Guo CM, Gao C, Zhao DT, Li JH, Wang JX, Sun XJ, Liu QL, Hao LH, Greenaway FT, Tian YX, Liu SQ, Sun MZ. A novel ETV6-miR-429-CRKL regulatory circuitry contributes to aggressiveness of hepatocellular carcinoma. J Exp Clin Cancer Res. 2020;39(1): 70.

Guo CM, Zhao DT, Zhang QL, Liu SQ, Sun MZ. miR-429 suppresses tumor migration and invasion by targeting CRKL in hepatocellular carcinoma via inhibiting Raf/MEK/ERK pathway and epithelial-mesenchymal transition. Sci Rep. 2018;8(1): 2375.

Baliga BS, Mankad M, Shah AK, Mankad VN. Mechanism of differentiation of human erythroleukaemic cell line K562 by hemin. Cell Prolif. 1993;26(6): 519-29.

Shimizu R, Yamamoto M. GATA-related hematological disorders. Exp Hematol. 2016;44(8): 696-705.

Loic G, Catherine L, Fedor S, Jean-Pierre LC, Jean-Luc V, William V, Dominique D. Gfi-1B plays a critical role in terminal differentiation of normal and transformed erythroid progenitor cells. 2005;105(4): 1448-55.

Saleque S, Cameron S, Orkin SH. The zinc-finger proto-oncogene Gfi-1b is essential for development of the erythroid and megakaryocytic lineages. Genes Dev. 2002;16(3): 301-6.

Nishimura S, Takahashi S, Kuroha T, Suwabe N, Nagasawa T, Trainor C, Yamamoto M. A GATA box in the GATA-1 gene hematopoietic enhancer is a critical element in the network of GATA factors and sites that regulate this gene. Mol Cell Biol. 2000;20(2): 713-23.

Laurent B, Randrianarison-Huetz V, Marechal V, Mayeux P, Dusanter-Fourt I, Dumenil D. High-mobility group protein HMGB2 regulates human erythroid differentiation through trans-activation of GFI1B transcription. Blood. 2010;115(3): 687-95.

Huang DY, Kuo YY, Chang ZF. GATA-1 mediates auto-regulation of Gfi-1B transcription in K562 cells. Nucleic Acids Res. 2005;33(16): 5331-42.

Guris DL, Fantes J, Tara D, Druker BJ, Imamoto A. Mice lacking the homologue of the human 22q11.2 gene CRKL phenocopy neurocristopathies of DiGeorge syndrome. Nat Genet. 2001;27(3): 293-8.

Park TJ, Boyd K, Curran T. Cardiovascular and craniofacial defects in Crk-null mice. Mol Cell Biol. 2006;26(16): 6272-82.

de Jong R, Haataja L, Voncken JW, Heisterkamp N, Groffen J. Tyrosine phosphorylation of murine Crkl. Oncogene. 1995;11(8):1469-74.

Zhang J, Lodish HF. Constitutive activation of the MEK/ERK pathway mediates all effects of oncogenic H-ras expression in primary erythroid progenitors. Blood. 2004;104: 1679-87.

Platanias LC. MAP kinase signaling pathways and hematologic malignancies. Blood. 2003;101: 4667-79.
63. Deathridge J, Antolović V, Parsons M, Chubb JR. Live imaging of ERK signaling dynamics in differentiating mouse embryonic stem cells. 2019;146(2): 1-12.

64. Kumkhaek C, Aerbajinai W, Liu W, Zhu J, Uchida N, M Hsieh M, Tisdale JF, Rodgers GP. MASL1 induces erythroid differentiation in human erythropoietin-dependent CD34+ cells through the Raf/MEK/ERK pathway. Blood. 2013;121(6): 3216-27.

65. Kucukkaya B, Arslan DO, Kan B. Role of G proteins and ERK activation in hemin-induced erythroid differentiation of K562 cells. Life Sci. 2006;78: 1217-24.

66. Zarrabi M, Afzal E, Asghari MH, Mohammad M, Es HA Ebrahimi M. Inhibition of MEK/ERK signaling pathway promotes erythroid differentiation and reduces HSCs engraftment in ex vivo expanded haematopoietic stem cells. J Cell Mol Med. 2018;22(3): 1464-74.

67. Azadeh M, Razieh Y. Involvement of ERK/MAPK pathway in megakaryocytic differentiation of K562 cells induced by 3-hydrogenkwadaphnin. Toxicol In Vitro. 2008;22(6): 1503-10.

Tables
### Table 1
Synthesized sequences of primers for targeting genes

| Targeting gene | Primer sequence          |
|----------------|--------------------------|
| CRKL           | F: 5'-GTGCTTATGACAAGACTGCCT-3'  
|                | R: 5'-CACTCGTTTTCTCTCTCCTTT-3'  |
| CRK           | F: 5'-CTATGCCCAACCACGCTCA-3'  
|               | R: 5'-CGTGTTCATTACACTCCC-3'  |
| γ-globin      | F: 5'-GCAGCTTGT-CACAGTCAGTTC-3'  
|               | R: 5'-TGGCAAGAGGTGCT-GACTTC-3'  |
| GPA           | F: 5'-GACAAATGATACGCACAAACGG-3'  
|               | R: 5'-TCCAATAACACCAGCCATAGCC-3'  |
| CD41          | F: 5'-TTGCACGGGATCTCAACACT-3'  
|               | R: 5'-CCACTGAATGCCCACACAGGAC-3'  |
| CD61          | F: 5'-ACTCTCCCTCCACACTCACC-3'  
|               | R: 5'-CAGCTCGATTTAGACGGAT-3'  |
| Elk-1         | F: 5'-TCTCCTGAAGATCGGCAACGG-3'  
|               | R: 5'-CGAACTTCTGCGGCTACGCA-3'  |
| GATA-1        | F: 5'-CTGCGGCCTGCTATCACAGAT-3'  
|               | R: 5'-ACTGAGTACCCGTTGACCTGAC-3'  |
| HMGB2         | F: 5'-TGTCCCTCTGTACGGCCTCTCTTC-3'  
|               | R: 5'-CCTCCTCATCTTCTGTCG-3'  |
| ACTB          | F: 5'-AGGCAAGCCGCGAGAAG-3'  
|               | R: 5'-ACAGCCTGGATAGCAACGTAC-3'  |

### Table 2
Gene microarry screened differentially expressed genes in shRNA-CRKL-K562 and shRNA-NC-K562 cells

| Gene Symbol | Description     | Fold change* |
|-------------|----------------|-------------|
| HBD         | Hemoglobin, delta | 1.6        |
| HBA1        | Hemoglobin, alpha1  | 2.2        |
| HBA2        | Hemoglobin, alpha2  | 2.3        |
| HBZ         | Hemoglobin, zeta  | 2.5        |
Table 3

iTRAQ quantitative proteomic screened differentially expressed genes in shRNA-CRKL-K562 and shRNA-NC-K562 cells

| Protein Symbol | Description                  | Fold change* | P       |
|----------------|------------------------------|--------------|---------|
| HBE1           | Hemoglobin, epsilon1         | 1.2          | 0.007134|
| HBD            | Hemoglobin, delta            | 1.3          | 0.00606 |
| HBZ            | Hemoglobin, zeta             | 1.2          | 0.0034  |
| HBG1           | Hemoglobin, gamma A          | 1.2          | 0.00802 |
| GATA-1         | Erythroid transcription factor | 1.3         | 0.03264 |
| HMGB2          | High mobility group protein B2 | 1.2        | 0.03107 |

*Refers to protein level changes of deregulated genes in shRNA-CRKL-K562 cells compared with shRNA-NC-K562 cells.

Figures
Figure 1

The expression patterns of CRKL and CRK in CML patient samples. (A) CRKL was overexpressed in CML patients compared with normal samples. (B) CRKL was downregulated in CML CR patients compared with the corresponding CML primary patients. (C) CRK was slightly overexpressed in CML patients compared with normal samples. (D) CRK was downregulated in CML CR patients compared with the
corresponding CML primary patients. ACTB was used as the internal reference, *, ** refer to P values <0.05, <0.01, ns refers to no statistical difference.

**Figure 1**

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Figure 2

CRKL is down-regulated during erythroid differentiation of K562 cells. (A) the benzidine-positive cells of K562 induced by hemin. (B) γ-globin and GPA mRNA expression levels were detected in hemin-induced K562 cells by qRT-PCR. (C) CRKL protein expression level was measured in hemin-induced K562 cells by WB. (D) CRK protein expression level was measured in hemin-induced K562 cells by WB. *, **, *** refer to P values <0.05, <0.01, <0.001, ns refers to no statistical difference.

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K562 cells by qRT-PCR. (C) CRKL protein expression level was measured in hemin-induced K562 cells by WB. (D) CRKL protein expression level was measured in hemin-induced K562 cells by WB. *, **, *** refer to P values <0.05, <0.01, <0.001, ns refers to no statistical difference.

Figure 3

CRKL downregulation promotes erythroid differentiation of K562 cells. (A) CRKL stably downregulated in K562 cells. (B) The benzidine-positive cells were counted in shRNA-CRKL-K562 and shRNA-NC-K562 cells.
γ-globin and GPA mRNA expression levels were detected in shRNA-CRKL-K562 and shRNA-NC-K562 cells by qRT-PCR. *, **, *** refer to P values <0.05, <0.01, <0.001.

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Figure 4

CRKL downregulation promotes erythroid differentiation molecule expression. (A) iTRAQ quantitative proteomic screened differentially expressed genes between shRNA-CRKL-K562 and shRNA-NC-K562 cells. (B and C) WB and qRT-PCR detected the protein and mRNA expression level of GATA-1 and HMGB2 in shRNA-CRKL-K562 and shRNA-NC-K562 cells. *, ** refer to P values <0.05, <0.01.
Figure 5

CRKL downregulation promotes megakaryocyte differentiation of K562 cells. (A) The percentage of megakaryocyte cells in shRNA-CRKL-K562 and shRNA-NC-K562 cells. (B) qRT-PCR detected the mRNA expression levels of CD41 and CD61 in shRNA-CRKL-K562 and shRNA-NC-K562 cells. *refers to P value <0.05.
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Figure 6

CRKL downregulation promotes erythroid and megakaryocyte differentiation of K562 cells via activating the Raf/MEK/ERK/Elk signaling pathway. (A) The effect of CRKL downregulation on the Raf/MEK/ERK/Elk signaling pathway. Comparative analysis of Ras, Raf, p-Raf, p-MEK, ERK1/2, p-ERK1/2 protein levels and Elk-1 mRNA expression level in shRNA-CRKL-K562 and shRNA-NC-K562 cells. (B) The influence of PD98059 on the Raf/MEK/ERK/Elk-1 signaling pathway. WB assay of ERK, p-ERK1/2 and
qRT-PCR assay of Elk-1 expression levels change in shRNA-CRKL-K562 cells with PD98059 treatment for 48 h. (C) qRT-PCR assay of GPA, γ-globin, CD41 and CD61 expression levels change in shRNA-CRKL-K562 cells with PD98059 treatment for 48 h. *, **, *** refer to P values <0.05, 0.01, 0.001, ns refers to no statistical difference.

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Figure 7

CRK-II downregulation has no effect on erythroid differentiation of K562 cells. (A) CRK-II downregulated in K562 cells by siCRK-II transient transfection interference. (B) γ-globin and GPA mRNA expression levels were detected in siRNA-CRK-II-K562 and siRNA-NC-K562 cells by qRT-PCR. (C) The effect of CRK-II downregulation on the Raf/MEK/ERK/Elk signaling pathway. Comparative analysis of p-Raf, p-MEK, p-ERK1/2 protein levels in siRNA-CRK-II-K562 and siRNA-NC-K562 cells. (D) Expression level comparisons of
CRKL in shRNA-CRKL-K562+siNC and shRNA-CRKL-K562+siCRKL cells. (E) Expression level comparisons of CRKL in shRNA-CRKL-K562+siNC and shRNA-CRKL-K562+siCRKL cells. (F) γ-globin and GPA mRNA expression levels were detected in shRNA-CRKL-K562+siNC and shRNA-CRKL-K562+siCRKL cells by qRT-PCR. (G) Expression level comparisons of ERK1/2 and p-ERK1/2 in shRNA-CRKL-K562+siNC and shRNA-CRKL-K562+siCRKL cells. ** refers to P value <0.01, ns refers to no statistical difference.

Figure 7
CRK downregulation has no effect on erythroid differentiation of K562 cells. (A) CRK downregulated in K562 cells by siCRK transient transfection interference. (B) γ-globin and GPA mRNA expression levels were detected in siRNA-CRKL-K562 and siRNA-NC-K562 cells by qRT-PCR. (C) The effect of CRK downregulation on the Raf/MEK/ERK/Elk signaling pathway. Comparative analysis of p-Raf, p-MEK, p-ERK1/2 protein levels in siRNA-CRKL-K562 and siRNA-NC-K562 cells. (D) Expression level comparisons of CRK in shRNA-CRKL-K562+siNC and shRNA-CRKL-K562+siCRK cells. (E) Expression level comparisons of CRKL in shRNA-CRKL-K562+siNC and shRNA-CRKL-K562+siCRK cells. (F) γ-globin and GPA mRNA expression levels were detected in shRNA-CRKL-K562+siNC and shRNA-CRKL-K562+siCRK cells by qRT-PCR. (G) Expression level comparisons of ERK1/2 and p-ERK1/2 in shRNA-CRKL-K562+siNC and shRNA-CRKL-K562+siCRK cells. ** refers to P value <0.01, ns refers to no statistical difference.

**Figure 8**

A schematic regulation mechanism of CRKL on erythroid and megakaryocyte differentiation of CML cell. CRKL inhibits erythropoiesis and megakaryopoiesis of K562 cell via inactivating the Raf/MEK/ERK/Elk pathway by inhibiting HMGB2 potentiates GATA-1-dependent transcription of Gfi-1B.
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