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

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Research

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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 γ-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 haa 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 molecues 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□ and CRKL could synergistically increase RANKL-induced osteoclast differentiation [31]. However, the precise roles and underlying mechanisms of CRKL and CRK□ in leukemic cell differentiation are still not reported.

In our study, we investigated the effect of CRKL and CRK□ 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 ^{o}C with 5% CO_2. 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 ^{o}C with 5% CO_2.

Benzidine staining assay

1×10^5 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% CO_2 at 37 ^{o}C 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% H_2O_2, 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 Na_3VO_4, 1 μg/ml leupeptin and 0.5 mM PMSF. The supernatant was collected by centrifugation at 12000 rpm for 15 min at 4 ^{o}C. 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 ^{o}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

3×10^7 shRNA-NC-K562 and shRNA-CRKL-K562 group cells were harvested in at least three independent experiments, and centrifugated 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 supernatant was collected by centrifugation at12000g for 15 min at 4 ^{o}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 $2^{-\triangle\triangle CT}$ 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

1×10^7 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 RNeasy 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% CO_2.

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 *γ-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 *γ-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 ($P < 0.05$) 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, γ-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□ has no effect on erythroid differentiation of K562 cells

CRK□ may be unimportance for erythroid differentiation, to confirm the effect of CRK□ on erythroid differentiation, we transiently transfected K562 cells with siCRK□ to knockdown CRK□. CRK□ protein and mRNA levels were decreased by 53.6% (*P* = 0.0015) and 43.5% (*P* = 0.0059) in siRNA-CRK□-K562 cells compared with siRNA-NC-K562 cells (Fig. 7A), providing a control study for the downregulation effect of CRK□ on K562 cell erythroid differentiation. qRT-PCR detected the expression level changes of erythroid genes after CRK□ knockdown, there were no obvious changes in *γ-globin* (*P* = 0.088) and *GPA* (*P* = 0.133) mRNA expression levels between siRNA-CRK□-K562 and siRNA-NC-K562 cells (Fig. 7B). Meanwhile, to show that CRK□ 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□ knockdown by WB. No changes were observed for p-Raf, p-MEK, p-ERK1/2 (Fig. 7C). Clearly, our results further demonstrated CRK□ has no effect on erythroid differentiation of K562 cells.

To further confirm the effect of CRK□ on erythroid differentiation, we transiently transfected siCRK□ in shRNA-CRKL-K562 cells with CRKL stable knockdown. Compared with shRNA-CRKL-K562 + siNC cells, the CRK□ protein expression level decreased by 42.5% in shRNA-CRKL-K562 + siCRK□ cells (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□ cells (Fig. 7F). Interestingly, there were no obvious changeS in *γ-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□ 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□ 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, polychromatic 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, CRKⅡ enhances osteoclast differentiation by activating Rac1, the overexpression of CRKⅡ and CRKL significantly enhances RANKL-induced osteoclast differentiation, and the downregulation of CRKⅡ and CRKL synergistically decreases RANKL-induced osteoclast differentiation [31]. The effect of CRKL and CRKⅡ on leukemia cell differentiation has not been reported, in our study we investigated the potential role of CRKL and CRKⅡ 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 thalasssemia 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 CRKⅡ was not changed in hemin-induced erythroid differentiation of K562 cells (Fig. 2D) and CRKⅡ downregulation did not affect erythroid differentiation of K562 cells (Fig. 7B). Moreover, we further investigated the effect of CRKⅡ knockdown on erythroid differentiation of K562 cells by transiently transfecting siCRKⅡ in shRNA-CRKL-K562 cells, interestingly, consistent with the

above results, CRKⅡ knockdown in K562 cells with CRKL downregulation (Fig. 7F). Collectively, CRKⅡ is not associated with erythroid differentiation of K562 cells. Although CRKII 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 CRKII 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 CRKII 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 CRKII 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*, *γ-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, CRKⅡ downregulation could not affect the Raf/MEK/ERK pathway (Fig. 7C, G), further indicating CRKⅡ 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 CRKⅡ and CRKL indicates that they may serve differently in leukemogenesis. Our findings point to CRKL rather than CRKⅡ 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

Acknowledgements

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 approvaland 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.

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Tables

Table 1
Synthesized sequences of primers for targeting genes

Targeting gene	Primer sequence
CRKL	F:5′-GTGCTTATGACAAGACTGCCT-3′
	R:5′-CACTCGTTTTCATCTGGGTTT-3′
CRK	F: 5′-CTATGCCCAACCCAGCGTCA-3′
	R: 5′-CGTTTGCCATTACACTCCCC -3′
γ-globin	F:5′-GCAGCTTGT-CACAGTGCAGTTC-3′
	R:5′-TGGCAAGAAGGTGCT-GACTTC-3′
GPA	F:5′-GACAAATGATACGCACAAACGG-3′
	R:5′-TCCAATAACACCAGCCATCAC-3′
CD41	F:5′-TTCGACGGGGATCTCAACACT-3′
	R:5′-CCACTGAATGCCCAAAATACGAC-3′
CD61	F:5′-ACTCTGCCTCCACTACCATG-3′
	R:5′- CAGCTCGATTTAGAACGGAT-3′
Elk-1	F:5′-TCTCCTGGACTTCACGGCAT-3′
	R:5′-CGAACTTCTGGCCGCTCAC-3′
GATA-1	F:5′-CTGCGGCCTCTATCACAAGATG-3′
	R:5′-ACTGAGTACCTGCCCGTTTACTGAC-3′
HMGB2	F:5′- TGTCCTCGTACGCCTTCTTC-3′
	R:5′-CCTCCTCATCTTCTGGTTCG-3′
ACTB	F:5′-AGGCCAACCGCGAGAAG-3′
	R:5′-ACAGCCTGGATAGCAACGTACA-3′

Table 2
Gene microarray 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

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

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

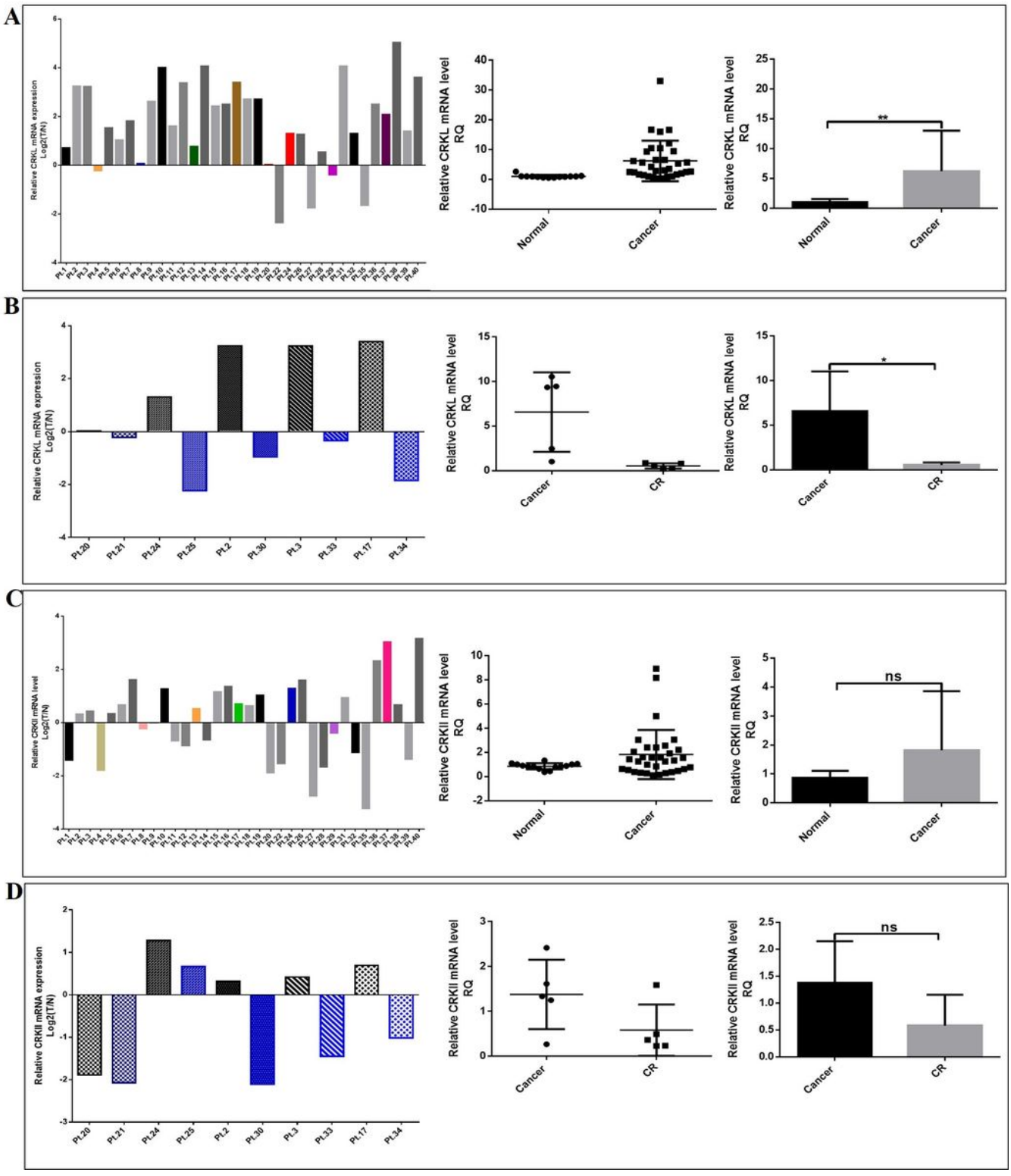

Figure 1

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.

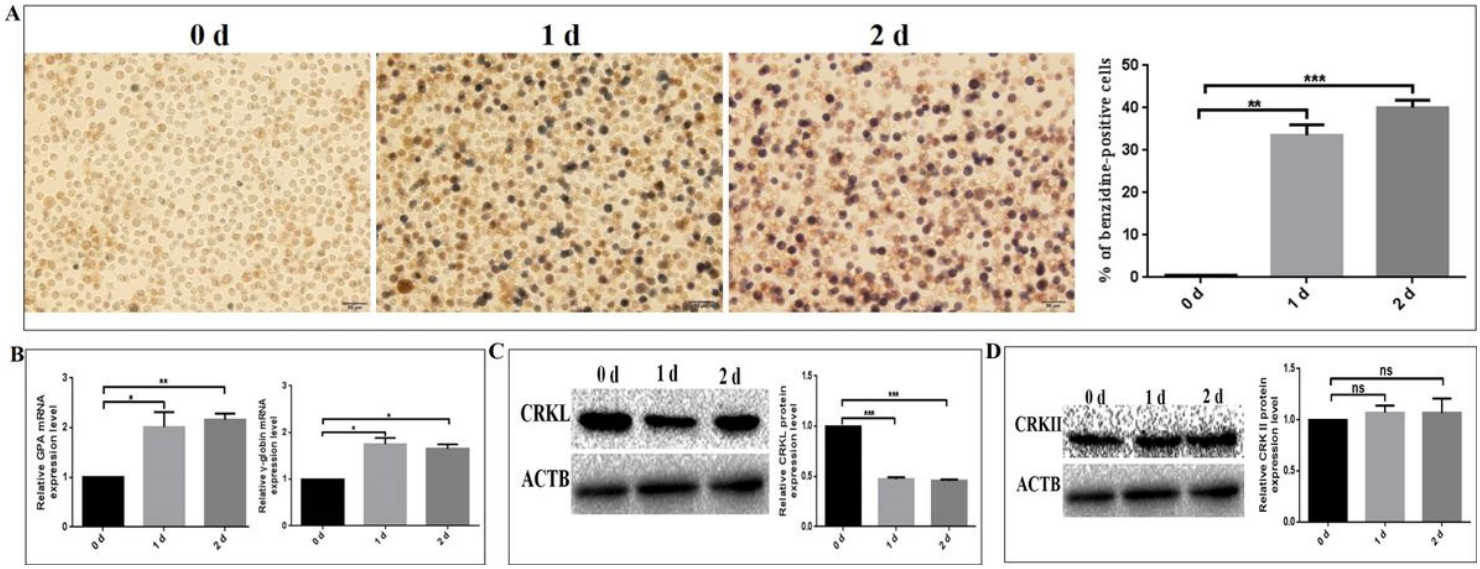

Figure 2

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.

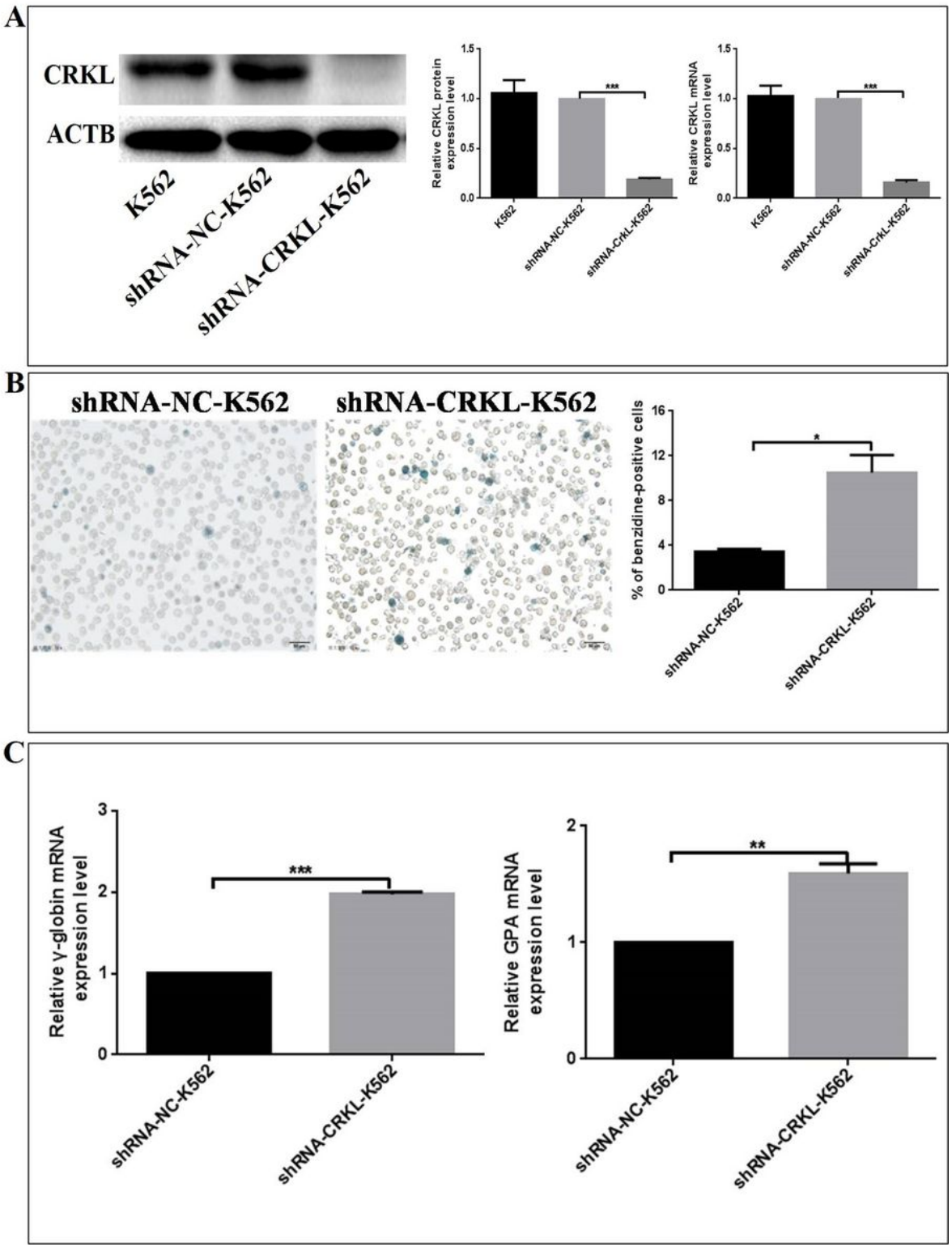

Figure 3

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. (C) 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.

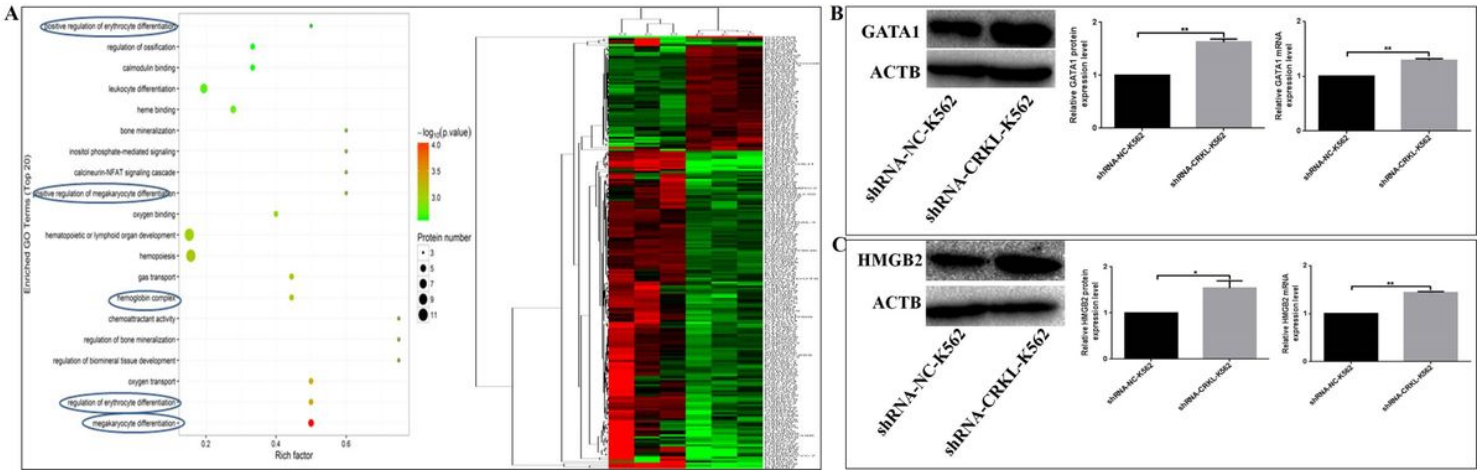

Figure 4

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.

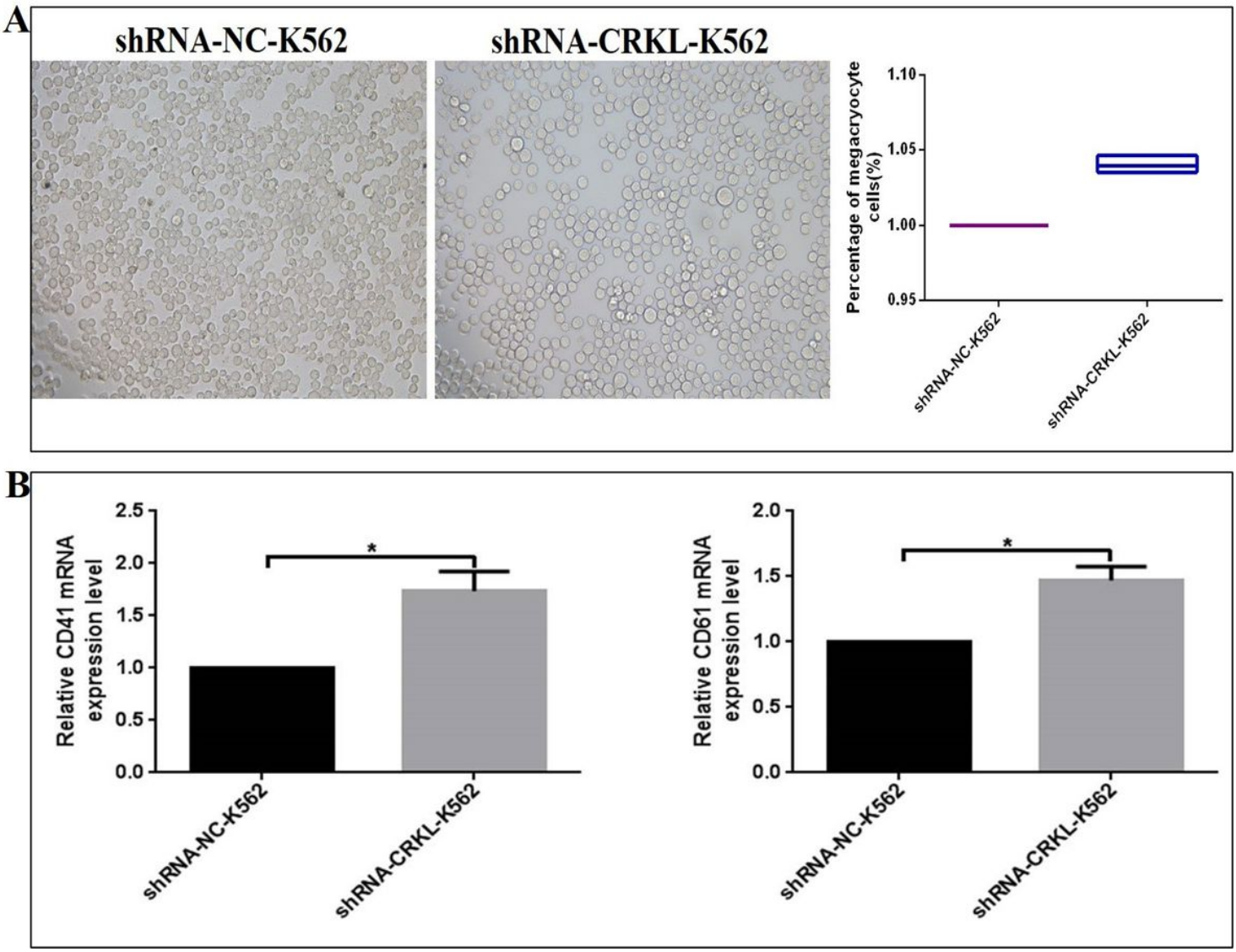

Figure 5

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.

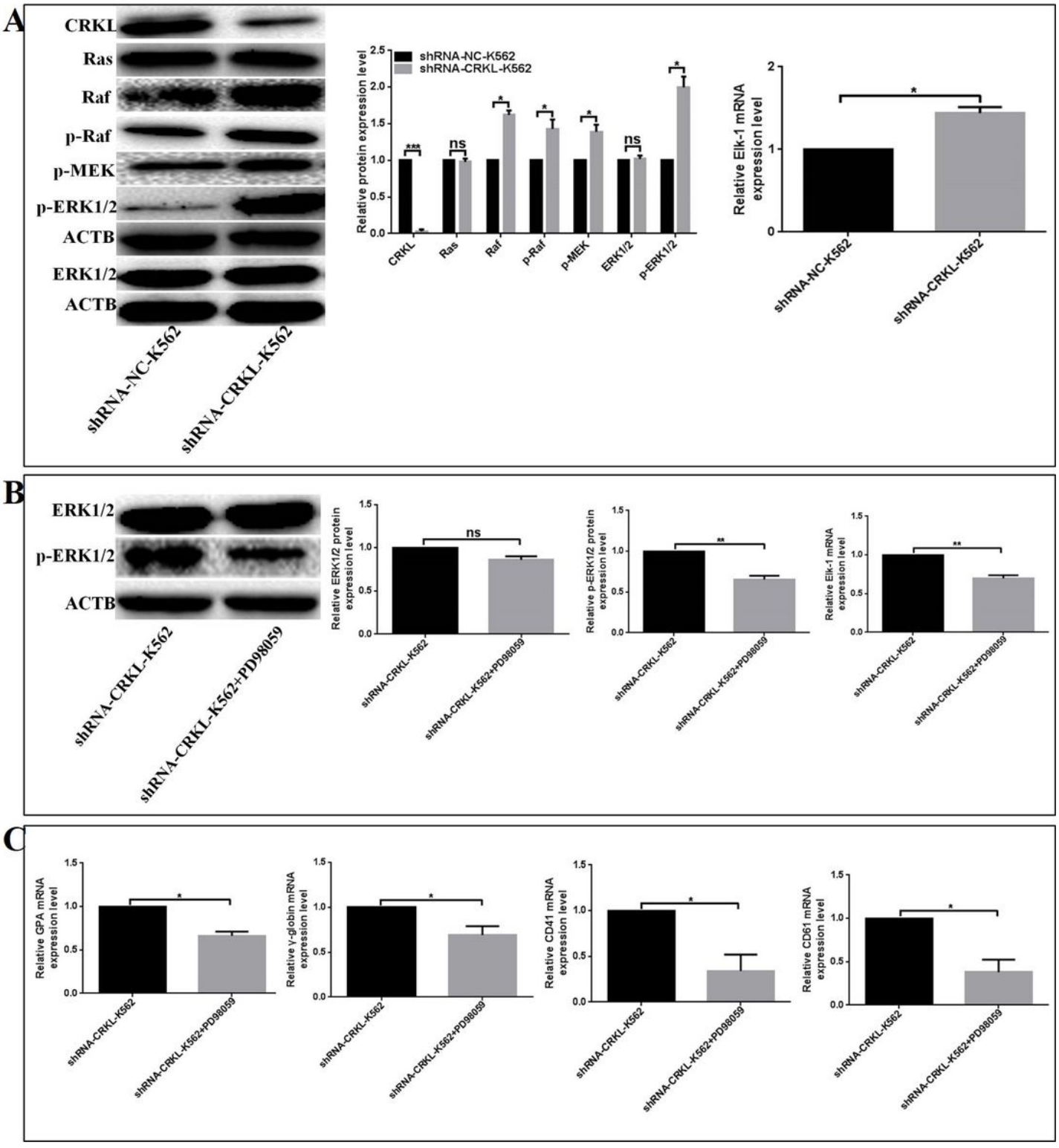

Figure 6

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.

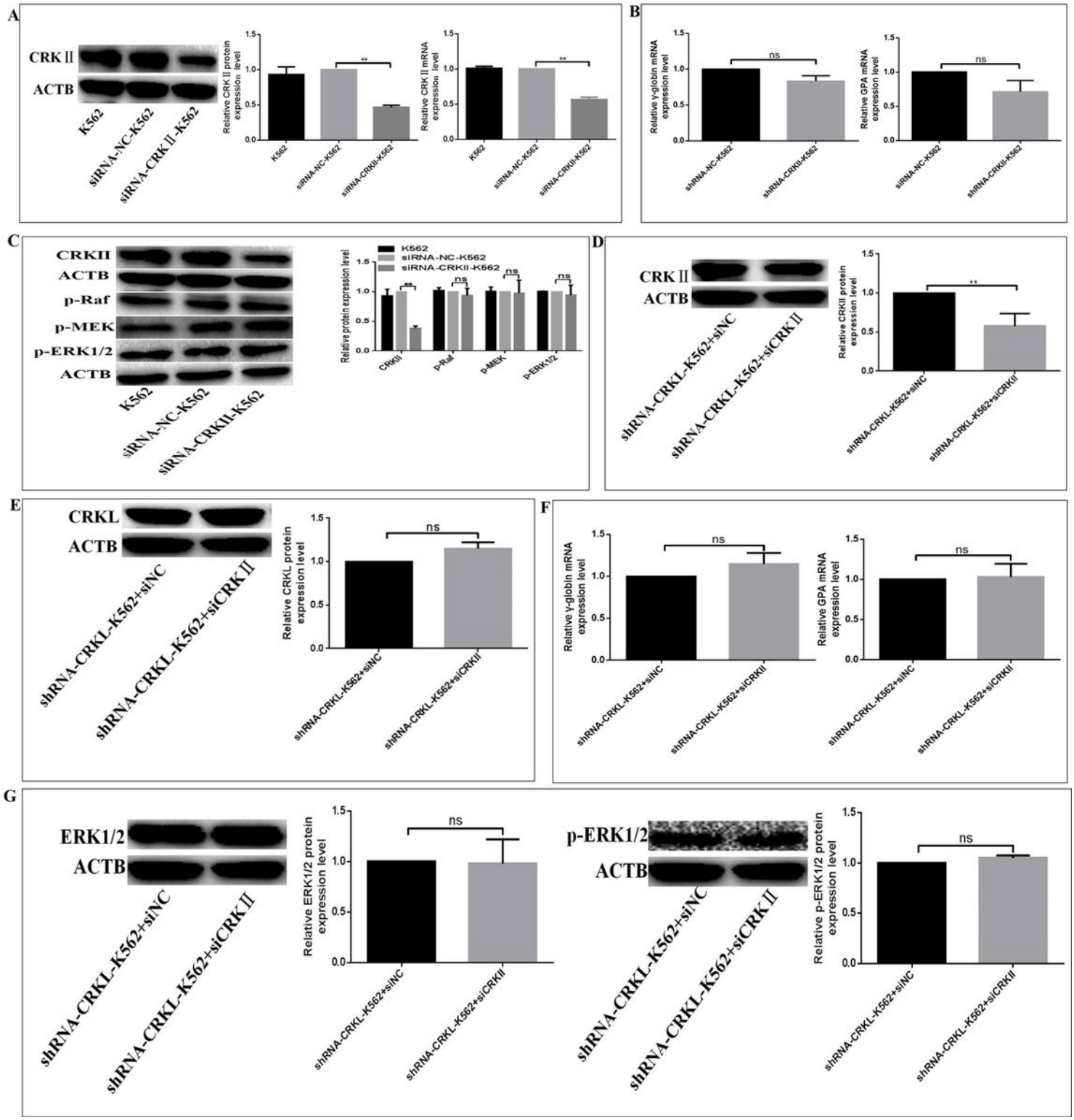

Figure 7

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-CRK-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-CRK-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.

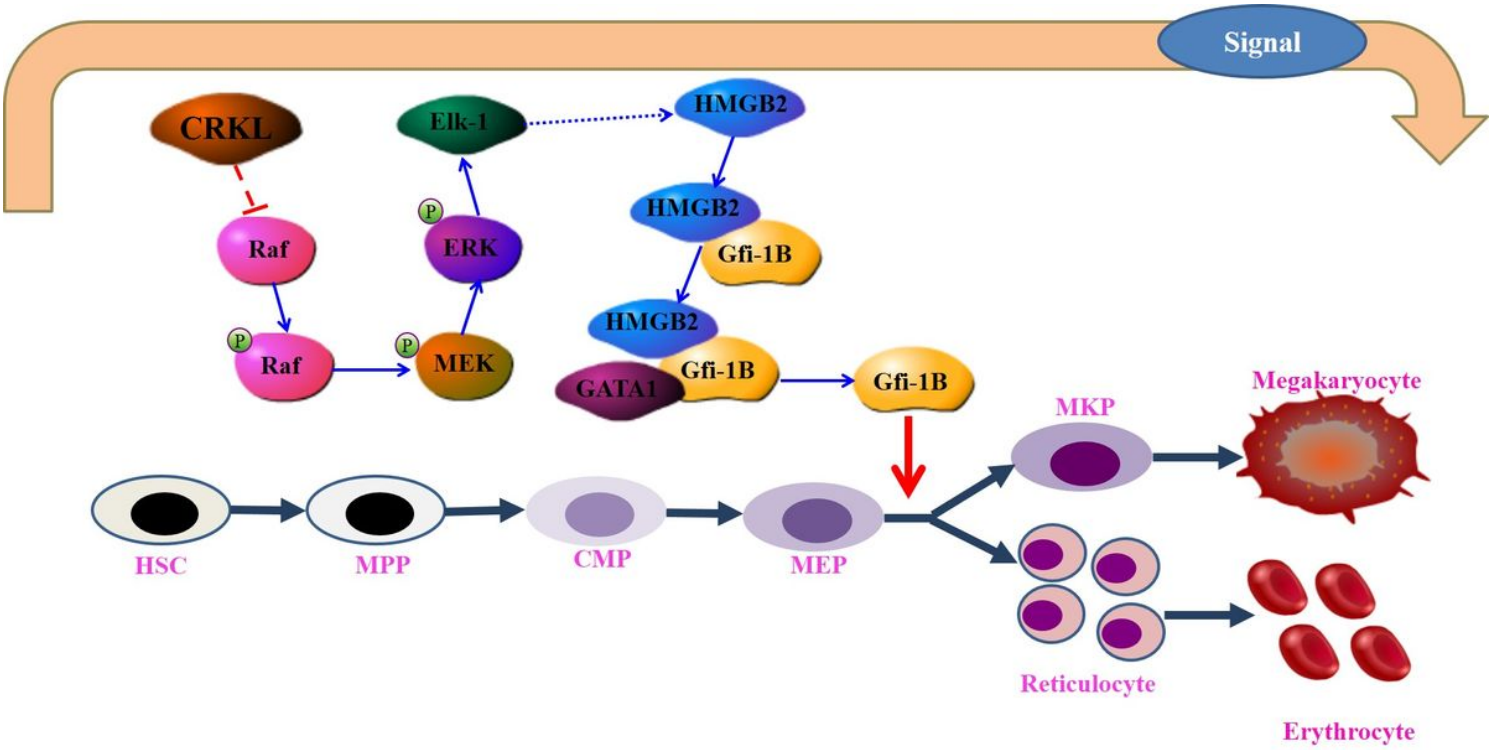

Figure 8

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.