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Exp Cell Res. Author manuscript; available in PMC 2019 October 28.

Published in final edited form as:

Exp Cell Res. 2018 September 15; 370(2): 551–560. doi:10.1016/j.yexcr.2018.07.020.

TCEA1 regulates the proliferative potential of mouse myeloid cells

Taomei Yang[a], **Haoran Cui**[a], **Mingxin Wen**[b], **Johannes Zuber**[c,d], **Scott C Kogan**[e,*], **Guangwei Wei**[a,*]

[a]Department of Cell Biology and Key Laboratory of Experimental Teratology, Ministry of Education, Shandong University School of Medicine, Jinan, Shandong, China;

[b]Department of Anatomy and Key Laboratory of Experimental Teratology, Ministry of Education, Shandong University School of Medicine, Jinan, Shandong, China;

[c]Research Institute of Molecular Pathology (IMP), Vienna BioCenter (VBC), 1030 Vienna, Austria;

[d]Medical University of Vienna, Vienna BioCenter (VBC), 1030 Vienna, Austria;

[e]Department of Laboratory Medicine, University of California San Francisco, CA, USA;

Abstract

Leukemia is a malignance with complex pathogenesis and poor prognosis. Discovery of new regulators is amenable to leukemia could be of value to gain insight into the pathogenesis, diagnosis and prognosis of leukemia. Here, we conducted a large-scale shRNA library screening for functional regulators in the development of myeloid cells in primary cells. We identified eighteen candidate regulators in the primary screening. Those genes cover a wide range of cellular functions, including gene expression regulation, intracellular signaling transduction, nucleotide excision repair, cell cycle control and transcription regulation. In both primary screening and validation, shRNAs targeting *Tcea1*, encoding the transcription elongation factor A (SII) 1, exhibited the greatest influence on the proliferative potential of cells. Knocking down the expression of *Tcea1* in the 32Dcl3 myeloid cell line led to enhanced proliferation of myeloid cells and blockage of G-CSF induced myeloid differentiation. In addition, silencing of *Tcea1* inhibited apoptosis of myeloid cells. Thus, *Tcea1*, was identified as a gene which can influence the proliferative potential, survival and differentiation of myeloid cells. These findings have implications for how transcriptional elongation influences myeloid cell development and leukemic transformation.

Keywords

Myelopoiesis; TCEA1; Proliferative potential; Differentiation

*Authors to whom correspondence should be addressed: gwwei@email.sdu.edu.cn (G.W.), Tel./Fax: +86-531-8838-2308; scott.kogan@ucsf.edu (S.K.), Tel./Fax: +1-415-514-1590/+1-415-514-3433.

Author Disclosure Statement

No potential conflicts of interest was disclosed.

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Introduction

Acute myeloid leukemia (AML), an aggressive hematological malignancy arising in hematopoietic stem and progenitor cells, is identified with distinct pathogenesis, clinical behavior, and prognosis [1,2]. AML generally has poor prognosis. Until now only approximately 35%−40% of patients younger than 60 years old could be cured, and the cure rate decreases with ageing [3]. AML is a heterogeneous clonal malignancy [3,4] characterized by the expansion of undifferentiated myeloid precursors, resulting in impaired hematopoiesis. In recent years, accompanied with the development of molecular biology and genetics, various genetic and molecular alterations including acquired genetic mutation or deregulation of gene expression were easily identified. These alterations have proven to be useful molecular tools for diagnosis and risk stratification of myeloid leukemia [5–7], but the discovery and validation of new genes that regulate proliferative potential of myeloid cells could be of value to improve the understanding of leukemogenesis, to predict outcome, and to provide novel targets for therapy.

Using custom libraries of shRNAs targeting known genes has proven to be an effective approach to find novel genes for regulating myeloid cells [8–11]. Focusing on such candidates, we used a shRNA screen library [12,13] to select for shRNAs capable of accelerating the proliferative potential of myeloid cells. Ultimately, we identified *Tcea1* as a functional gene regulating the process of myeloid cell expansion. *Tcea1* encodes the type 1 of SII, one of the best characterized transcription elongation factors. *In vitro*, S-II is a stimulatory protein of RNA polymerase II [14]. It directly binds RNA polymerase II, allowing it to read through various transcription arrest sites including poly T stretches [15,16]. S-II has three distinct isoforms which are conserved in frog, mouse, and human. The type 1, also known as TCEA1, is ubiquitously expressed [16]. To further clarify the function of TCEA1, we analyzed survival/proliferation, cell cycle, differentiation and apoptosis of myeloid cells after the down-regulation of TCEA1 *in vitro* using the 32Dcl3 myeloid cell line. The 32Dcl3 cells represent a simplified *in vitro* model of normal granulocytic differentiation [17,18]. 32Dcl3 cells cultured in G-CSF undergo granulocytic differentiation, which is morphologically characterized by the condensation of chromatin [19], the reducing of nucleo-cytoplasmic ratio [20], then nuclear segmentation and appearance of granules proteins [20–22], and different granules proteins are synthesized at different stages of maturation [23,24].

In this study, we identified *Tcea1* as a potential functional gene in the development of myeloid cells by the means of shRNA library screening. We further found that the down-regulation of TCEA1 promoted the survival/proliferation of myeloid cells, while, losing function of TCEA1 impaired differentiation and inhibited apoptosis of myeloid cells *in vitro*.

Materials and Methods

shRNA screen

FVB/n mice were purchased from Jackson ImmunoResearch Laboratories. All mice were bred and maintained at the University of California at San Francisco, and the animal experiments were approved by the Institutional Animal Care and Use of Committee. The

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used shRNA library was sub-cloned into LMS (MSCV based vector). Adult FVB/n mice were injected with 150 mg/kg 5-FU 5 days before harvest their BM cells. Harvested BM cells were transduced with shRNA library retrovirus packaged by BOSC23 cells. For the screening, 2 million myeloid cells were infected with 1 million shRNA viral particles, on average, each BM cell expressed a single shRNA. After one day, $GFP^{+}7AAD^{-}$ cells were sorted and cultured in methylcellulose for 5 days for colony formation. Colonies were collected and re-plated. Re-plated cells were incubated in methylcellulose for another 7 days. Colonies that appeared in the second plated were collected for DNA extraction.

Methylcellulose colony-forming assay

BM cells transduced with shRNA library retrovirus were cultured in methylcellulose (Stem Cell Technologies, Canada) supplemented with recombinant murine G-CSF at 100 ng/ml and SCF at 100 ng/ml. Cells were cultured at 37°C 5% CO_2 for 5 days. For sorting cells which are capable of proliferative potential, colonies were collected and subjected to secondary colony assay culturing in methylcellulose.

Re-cloning of shRNAs identified in the screen.

The amplified polymerase chain reaction (PCR) products from each colony were digested with restriction enzyme (XhoI, EcoRI). The shRNA-containing fragments were then re-sub-cloned into the MSCV-LTR-miR30-SV40-GFP (MLS) shRNA expression vector. The shRNA expression plasmids were transfected into the BOSC23 packaging cell line using a lipo2000 transfection Kit (Invitrogen). Viral particles were concentrated as described previously [25].

Cell culture and transduction

32Dcl3 cells were maintained in RPMI 1640 containing 10% FBS (Fetal Bovine Serum), 100 u/ml penicillin, 100 μg/ml streptomycin and 1 ng/ml IL-3 (Peprotech, Rocky Hill, USA). In the text, this medium is referred to as Growth medium [26,27]. To induce differentiation, cells were washed three times with PBS (Phosphate Buffered Saline) and transferred into RPMI 1640 with 50 ng/ml G-CSF (Peprotech, Rocky Hill, USA). In the text, this medium is referred to as Differentiation medium [26]. The harvested BM cells and BOSC23 cells were maintained as described [28]. Viruses were collected at 48 hours and filtered through 0.45 um filters. The 32Dcl3 cells were transduced by incubation with virus containing 2 ug/ml Polybrene (Sigma-Aldrich, Saint Louis, USA) and centrifugation at 2400 rpm for 2 hours; the transduced procedure was repeated the next day. Two days later, colonies were selected with 2 ug/ml puromycin.

Western immunoblots

Cells in the logarithmic phase were washed twice in ice-cold hanks' balanced slat solution (Beyotime, Shanghai, China) and lysed in RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with 1 × protease inhibitor cocktail (Roche, Penzberg, Germany). Protein concentrations were determined using the Bio-Rad BCA protein assay kit (Thermo Scientific, Schwerte, Germany). For Western immunoblot, 30 ug of protein extracts per lane were electrophoresed with denaturing SDS-polyacrylamide gels (10%), transferred to PVDF

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(polyvinylidene difluoride) membranes (Millipore, Darmstadt, Germany). The membranes were blocked in TBST (Tris-Buffered Saline and Tween 20) containing of 5% BSA (Albumin Bovine Serum) for 2 hours at room temperature, and incubated with TCEA1 (Abcam, London, England) antibody at 1:1000 dilution, then washed three times with TBST followed by incubation with HRP-conjugated secondary antibody at 1:10000. The signal was visualized by using ECL detection reagent (Millipore, Darmstadt, Germany) and FluorChem E system (Proteinsimple, California, USA).

Cell proliferation assay

32Dcl3 stable expression shRNA of *Tcea1* were cultured in Growth medium for 7 days or in Differentiation medium for 5 days, cell proliferation assay and viability examination were performed by using Trypan Blue dye exclusion method every day, all experiments were performed in triplicate.

Cell cycle analysis

Cells cultured in medium with 1 ng/ml IL-3 or 50 ng/ml G-CSF were collected and washed twice with PBS, and then fixed with 70% ice-cold ethanol. The fixed cells were resuspended in PBS containing 200 ug/mL RNase A (Thermo Scientific, Schwerte, Germany), and incubated at 37°C for 30 minutes, then stained with 50 ug/mL propidiumiodide (Sigma-Aldrich, Saint Louis, USA) for 5 minutes on ice. Cells were subjected to flow cytometric analysis of DNA content using a flow cytometer (Beckman Coulter, Inc). The percentages of cell cycle distribution were calculated by Cell Quest software (MultiCycle for Windows 32-bit).

Quantitative real-time PCR

Vector-transduced cells or the cells expressing TCEA1 shRNA were exposed to 50 ng/ml G-CSF for one day and total RNA was extracted with TRIzol Reagent (Thermo Scientific, Schwerte, Germany). cDNA was prepared from RNA using a First Strand Synthesis kit (Thermo Scientific, Schwerte, Germany). Quantitative PCR was carried out using 50 ng of each cDNA using SYBR Green supermix (Thermo Scientific, Schwerte, Germany). The used qPCR primers [29] and sequence were summarized in Supplement Table 2.

Morphologic characterization

Cells were washed twice with medium without IL-3, and transfered to Differentiation medium for 5 days, changing 40% medium every day. After 5 days, cells were collected and subjected to make cytospin slides. Morphologic differentiation was assessed by cytospin, followed by Wright-Giemsa staining and microscopic examination (Olympus, Japan).

Cell apoptosis assay

Phosphatidylserine externalization was quantified by flow cytometry with a commercially available annexin-V PE apoptosis detection kit (eBioscience, San Diego, CA) according to the manufacturer's guideline. In brief, cells were seeded in 6-well flat-bottom and cultured for 48 hours in medium with 1 ng/ml IL-3 or 50 ng/ml G-CSF. The cells were harvested and resuspended in 100 μl annexin-V binding buffer with 5μl PE-annexin V and 1 μl 7-AAD

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working solution. The resuspended cells were incubated in the dark for 15 minutes at room temperature. Next additional 400 μl binding buffer was added, and cells were analyzed immediately by flow cytometry (BD Pharmingen, San Diego, CA, USA).

Statistical analysis

Values shown in each figure represent the mean plus and minus the standard deviation (SD). Differences were assessed by using Prism. Comparisons between different groups were calculated with the Student *t*-test. The criteria of statistical significances were P values less than 0.05 or 0.01.

Results

shRNA library screening for functional genes that regulate proliferative potential of myeloid cells

Proliferative potentials of myeloid cells are important for maintaining the pool size of functional immune cells and dysregulation of proliferation and maturation of myeloid cells may lead to accumulation of immature cells, which might place myeloid cells at a higher risk of leukemic transformation. To further understand the regulation of myelopoiesis, a shRNA library was used to find genes that potentially regulate the proliferative potentials of myeloid cells [30,31]. This library contains 2300 shRNAs targeting about 1000 cancer-related mouse genes (2 or 3 different shRNAs per gene) [30].

To set up the screening system, we first used a culture condition for the differentiation and maturation of BM cells, which was modified from a previously described system [32]. 5-FU treated FVB/n mouse BM cells were cultured in methylcellulose containing 100 ng/ml G-CSF and 100 ng/ml SCF. Under this culture condition, all cells are differentiated and become mature myeloid cells as assessed by their morphology 5 days after plating. (Figure 1A).

As Myc was well-known to impair maturation and promote proliferation of myeloid cells [28,33], a degradation-resistant form of Myc, Myc^{T58A} [28,34], was used to set up the criteria for selecting functional genes that impair myelopoiesis [35]. There are five times more colonies cultured in the above-mentioned condition from Myc^{T58A} transduced mouse BM cells than that transduced with control vector 5 days after plating and more cells are morphologically immature (Figure 1B). All colonies were collected and replated in the same culture condition. 7 days after secondary culture, there are still colonies formed from Myc transduced BM cells and these cells showed immature phenotype (Figure 1B). Thus, we established our screen system for functional genes that may promote the proliferation and impair the mature of myeloid cells.

5-FU treated FVB/n mouse BM cells were collected and retrovirally transfected in a ratio of 2:1 as BM cells to retroviral particles of the shRNA library to insure that most BM cells were transfected by zero or one retroviral particle. After 1 day of culture, the $GFP^{+}7AAD^{-}$ cells were sorted into the above methylcellulose medium for the differentiation and maturation of BM cells and cultured for 5 days. After 5 days, cells were replated and grown in secondary culture for 7 days. We obtained a total of 28 secondary colonies. For

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identification of positive hits, the shRNA fragments from each colony were amplified by PCR amplification of the genomic DNA extracted from the 28 colonies. Specifically shRNA sequences were identified by sub-cloning and DNA sequencing, the experiment design was partly adapted as described by Hattori H [25] and summarized in Supplement Figure 1.

We developed criteria to score candidates to determine which were positive hits. First, the sequenced shRNA needed to match a specific gene. Second, the genes identified should be recurrent in our analyses. We thus identified shRNAs corresponding to 18 candidate genes that may increase the proliferative potential of myeloid cells (Table 1).

We reasoned that the shRNAs that featured the highest frequencies in the primary screening would be most likely to validate when tested as individual shRNAs. Meanwhile, considering the specific role of genes, we decided to retest the capacity of promoting the proliferative potential of shRNAs targeting *Tcea1*, *Eps8* and *Znf212* in 32Dcl3 cells. Those shRNAs were reintroduced into 32Dcl3 cells and the ability to promote proliferation was assessed by cell cycle analysis. Results showed that these shRNAs accelerated proliferation of 32Dcl3 cells, the shRNA targeting *Tcea1* showed the most significant capacity to increase the proliferation of cells (Supplement figure 2A). Survival of cells was also assessed by trypan blue staining. Results also showed that these shRNAs promoted the survival ability of 32Dcl3 cells, and shRNA targeting on *Tcea1* exhibited the highest promotion of cell survival (Supplement figure 2B). Overall, we preliminarily identified functional genes that regulate proliferative potential of myeloid cells by shRNA library screening. The shRNA targeting *Tcea1* exhibited excellent performance on the ability to promote the proliferative potential of myeloid cells. Thus, we next focused on *Tcea1*.

TCEA1 knockdown promotes proliferative capacity of myeloid cells *in vitro*

In the primary screening, shRNA targeting *Tcea1* showed the most prominent capacity to promote the proliferative potential of myeloid cells. To validate the shRNA library screening results *in vitro*, we generated additional shRNAs against *Tcea1* (Supplement Table 1) and tested their ability to promote the proliferative potential of myeloid cells. We established stable knockdown TCEA1 cell lines in 32Dcl3 by retroviral transfection. The levels of TCEA1 in resultant cell lines were verified by Western blotting. 32Dcl3-shTCEA1.1669 and 32Dcl3-shTCEA1.538 cell lines exhibited high efficient down-regulation of TCEA1 (Figure 2A). Then the following validation was mainly based on 32Dcl3-shTCEA1.1669 and 32Dcl3-shTCEA1.538 cell lines.

The proliferation was monitored by direct counting of viable cells (dead cells were excluded by trypan blue staining). Growth curves were made according to daily viable cell counts for cells transduced with control vector or the indicated shRNAs. Compared with cells receiving control vector, a significantly increased cell number were detected in 32Dcl3-shTCEA1.1669 and 32Dcl3-shTCEA1.538 cells both in medium with IL-3 (Figure 2B) or G-CSF (Figure 2C).

We also analyzed the cell cycle distribution using PI staining, which was a measurement of DNA content, to test the promotion of proliferation by *Tcea1* shRNA in 32Dcl3 cells. As shown in the results, down-regulation of TCEA1 significantly increased the percentage of S

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phase cells not only in IL-3 medium (Figure 2D) but also in the medium containing G-CSF (Figure 2E) compared with the control. Taken together, these results suggest that TCEA1 is an important regulator for the proliferation of myeloid cells.

TCEA1 knockdown impairs G-CSF induced granulocytic differentiation of myeloid cells

Cell proliferation and terminal differentiation are opposing processes. This opposing process is critical for normal development and keeping homeostasis in myeloid cells, while, it is frequently disrupted during tumorigenesis [36]. We showed that the down-regulation of TCEA1 promoted the proliferative potential of myeloid cells. To gain insight into the influence of shTCEA1 on the differentiation of myeloid cells, we analyzed the mRNA expression level of genes contributing to granulopoiesis [22,24,37,38] by quantitative real-time PCR. Silencing *Tcea1* elevated the expression of early neutrophil genes myeloperoxidase (*Mpo*), proteinase3 (*Prtn3*), and neutrophil elastase (*Elane*), markers of myeloblast and promyelocyte (Figure 3A). We also analyzed the mRNA expression level of genes expressed with neutrophil maturation, the secondary granule gene lactoferrin (*Ltf*), as well as the tertiary granule gene gelatinase (*Mmp9*) and the cysteine-rich secretory protein 3 (*Crisp3*). The results indicated that silencing *Tcea1* decreased the expression of differentiation markers of myeloid cells (Figure 3B).

For further confirming these findings morphology analysis by Wright–Giemsa stain was employed to assess the differentiation of 32Dcl3 cells transduced with *Tcea1* shRNAs or control vector. Consistent with our gene expression results, cells with silenced *Tcea1* exhibited an immature phenotype, with a single ovoid nuclei and a high nuclear:cytoplasmic ratio whereas cells transduced with control vector showed segmented nuclei, a feature of differentiation (Figure 3C). The morphologic analysis revealed that down regulation of TCEA1 resulted in an accumulation of immature cells and blocked the further differentiation of myeloid cells whereas cells transduced with control vector morphologically showed few immature cells and became segmented neutrophils. Taken together, these data indicated that TCEA1 can play an important role in the differentiation of myeloid cells.

Down-regulation of TCEA1 inhibits the apoptosis of myeloid cells

Apoptosis is a genetically regulated cell death process that involves a series of biochemical changes. Proliferation, differentiation and apoptosis are important elements for myeloid cell development. The effect of TCEA1 depletion on proliferation and differentiation of myeloid cells was explored above. Next, to explore the effect caused by TCEA1 depletion on myeloid cell apoptosis, we measured the fraction of apoptotic annexin V-stained cells by flow cytometric analysis. After 48 hours in the medium containing G-CSF, 32Dcl3-shTCEA1.1669 and 32Dcl3-shTCEA1.538 cells showed 16.5% and 22.63% apoptosis rate respectively, while, 54% apoptosis rate was found in 32Dcl3 cells transduced with control vector (Figure 4A). A similar decrease of apoptosis was observed during cells cultured in medium containing IL-3 (32Dcl3-shTCEA1.1669 3.36%, 32Dcl3-shTCEA1.538 4.76% and 32Dcl3 control vector 11.53%; Figure 4B). These results showed that silencing *Tcea1* inhibited apoptosis of myeloid cells.

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Discussion

The insufficient transcription elongation of blood genes caused by decreasing of elongation activity led to anemia and embryonic lethality during cell differentiation and development [39,40]. Moreover, a translocation partner of mixed lineage leukemia (*MLL*) gene, which rearrangement was always related with the rise of aggressive leukemia, was found to coexist in a super elongation complex that including the positive transcription elongation factor b (p-TEFb) and eleven-nineteen lysine-rich leukemia (ELL) [41–43]. In the study, through screening a 1000 gene shRNA library, TCEA1, a transcription elongation factor, was identified as a new regulator in the myeloid cell development, specifically, silencing TCEA1 impaired the differentiation of myeloid cells. In mouse fetal liver cells, homozygous inactivation of TCEA1 was also showed to impair the definitive erythropoiesis and resulted in severe anemia [44]. To the best of our knowledge, this is the first study to identify *Tcea1* as a functional gene in granulopoiesis. Our experimental evidence showed that silencing TCEA1 decrease the maturation of neutrophils and increase the accumulation of immature myeloid cells that may contribute to the leukemic transformation.

To gain insight into the expression level of TCEA1 in normal and cancer cells, we reanalyzed the publicly accessible data of Gene Expression Omnibus database (GEO), the GSE47044 database [45], we found that the expression level of TCEA1 was significantly down-regulated in lymphoma cells compared with germinal center B cells (Supplement Figure 3). Decreasing TCEA1 in lymphoma indicates the potential relationship between TCEA1 function and hematopoietic malignance. Besides, lymphoma, tumors of hematopoietic and lymphoid tissue, were caused by various of possible mechanisms which were associated with blockage of differentiation [46]. In this study, TCEA1 silencing significantly blocked myeloid cells differentiation both in molecular and morphological level. Therefore, both GEO analysis data and our findings supports a speculation that inactivation of TCEA1 may disrupt normal development of cells and has a risk of tumor transformation.

The regulation of transcription elongation is a critical step for the control of gene expression. For example, approximately 30% of genes experience transcription initiation but show no detectable transcripts some of these showed no indication of elongation [47]. This result indicate that elongation is essential for gene expression. However, RNA polymerase II (Pol II) stalling relative with insufficient elongation is a widespread phenomenon in gene expression [47,48]. TCEA1, the first general transcription factor reported to associate with Pol II [49], is required for the release of paused polymerase RNA II by stimulating arrested polymerase RNA II to cleavage nascent transcript to promote elongation of gene transcription [50,51]. TCEA1 facilitates independently its role by promoting transcript cleavage, in additional, is also found to overcome obstacle by the cooperation with distinct factors such as positive transcription elongation factor b (p-TEFb) [52–54].

We know there is a link between transcription elongation with hematopoietic cell fates. Transcriptional intermediary factor 1 gamma (TIF1γ) deficiency promotes myelopoiesis while inhibiting erythropoiesis by regulating the elongation of blood genes [40]. Ikaros, a lineage-specific transcription factor was reported to facilitate productive transcription

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elongation at *c-Kit* and *Flt3* gene, and loss function of Ikaros decreased the colony formation activity and different cell fates of hematopoietic progenitor cells [55]. In the study, we observed a blockage of granulopoiesis with silencing TCEA1, it showed that TCEA1 is required for myeloid maturation from an early stage. Transcription factors have been showed to be the key determinants in the orchestration of myeloid identity and differentiation fates [56–58]. Different transcription factors drive a certain set of linage-specific genes, thereby instructing a certain lineage commitment. The mutation or dysregulation of transcription factors could easily result in rapidly malignant transformation [59–61]. It is a well-known fact that there is a tightly controlled balance between the production and destruction of elements in the blood. For differentiation, the expression of genes maintaining proliferation should be switched off, whereas, the expression of genes controlling differentiation should be switched on. The dysregulation of transcripts in cells might disrupt the balance between the production and destruction, thereby shifting towards cancer. Taken together, our observation that loss of TCEA1 impaired myelopoiesis suggests that as a transcription elongation factor, TCEA1 may influence cell fates.

Of note, knocking down of TCEA1 was reported to inhibit cancer cell proliferation and induce apoptosis in breast, lung and pancreatic cancer cell lines [62]. However, differential effects of TCEA1 knockdown were observed in cancerous MCF7 and non-cancerous MCF10A cells, TCEA1 knockdown increased the p53 and c-myc levels in MCF7 cells but not in MCF10A cells [62]. Both the expression level of TCEA1 and the context of cells in which it is expressed may influence the function of TCEA1.

Reanalyzing GSE31912 database [63] revealed that silencing TCEA1 influenced numerous transcription factors in MCF-7 cells, such as C/EBPα, C/EBPε, GFI-1 and IRF8 (Supplement Figure 4A–D). Data showed that silencing TCEA1 resulted in concomitant increasing of C/EBPα and GFI-1, whereas decreasing of C/EBPε and IRF8. C/EBPα and GFI-1 have function in self-renewal of hematopoietic stem cells, moreover, C/EBPα also have vital roles in regulating common myeloid progenitors (CMPs) towards to granulocyte-monocyte progenitors (GMPs) [57]. Reports also showed that GFI-1 is essential for MYB' ability to block myeloid cells differentiation [64]. Thus, increase of GFI-1 may contribute to the blockage of granulopoiesis. C/EBPε, another CCAAT/enhancer-binding protein, were responsible for inducing cell cycle arrest and supporting terminal differentiation [65], low level of C/EBPε will support cell proliferation whereas inhibit differentiation. Studies have showed that C/EBPε knocking down cells contained increasing granulocytic progenitors, and most of cells were promyelocytes and myelocytes compared with wild type in which cells contained different phenotype from promyelocytes to neutrophils [66]. Similarly, IRF8 deficient mice exhibited uncontrolled clonal expansion of undifferentiated cells and IRF8 loss was frequently observed in human myeloid leukemia patients [67]. It is reasonable to speculate that attenuating in C/EBPε and IRF8 resulted by loss function of TCEA1 may play role in expansion of undifferentiated cells. Therefore, aberrant expression of transcription factors resulted by silencing TCEA1 could be a possible explanation for cell expansion and differentiation blockage due to inactivation of TCEA1 in myeloid cells.

In addition, analysis on the change of important signaling proteins based on the GSE31534 database [63] showed that there was an increasing expression of *NOTCH2*, *NOTCHT3*,

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STAT1 and *STAT3* gene with silencing TCEA1 in A379 melanoma cells (Supplement Figure 5A–D). Of interest, NOTCH signaling have pleiotropic effects on progenitors which were determined by the signaling strength, duration and the context of cells [68]. For example, NOTCH signaling can influence cell growth and differentiation, alternatively, activation of NOTCH can protect cells from apoptosis [69]. Likewise, STATs, signaling transducers and transcription factors, exerts crucial role in cell proliferation, differentiation and apoptosis. The aberrant expression and activity of STAT proteins, particularly STAT3 have been described involving in tumor transformation [70,71]. In erythroleukemia cells, STAT1 and STAT3 were also observed constitutively activation and described as negative regulators of differentiation [72,73]. Hence, as results of TCEA1 depletion, signaling protein abnormality might be beneficial for TCEA1 to regulate myelopoiesis. Altogether, TCEA1 appears to play an important role in transcription factor expression and this may involve conventional signaling pathways. However, in our study, we showed the role of TCEA1 in the cell fate determination of myeloid cells, but we cannot provide a specific mechanism regulated by TCEA1. Future work will clarify the specific mechanism or pathway mediated by TCEA1 in myeloid cells.

In conclusion, our results indicate that TCEA1 play a critical role in the regulation of myeloid cell fates, and myeloid cells with inactivation of TCEA1 might tend to transform into leukemia. Our study also suggests that there should be a molecular mechanism regulated by TCEA1 responsible for myeloid cell fates. Clarifying this mechanism may provide valuable clues to develop new differentiation therapies for AML. Our study provides the first evidence that TCEA1 regulates myeloid cell fates and differentiation. In combination with prior work [39–43] the findings highlight the potential importance of regulation of transcriptional elongation in myelopoiesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Professor Scott W Lowe in Memorial Sloan Kettering Cancer Center for his contribution of the cancer 1000 shRNA library and technical assistance. This work was supported by National Natural Science Foundation of China (grant numbers 81472583, 31271461 to G.W.); The Taishan Scholar Program of Shandong Province (GW); and by the United States of America National Cancer Institute (grant number R01CA095274 to SCK). S.K and G.W. conceived the work. T.Y. performed most of the experiments and interpreted the experimental data. T.Y. wrote the manuscript, S.K and G.W. edited the manuscript. All authors have read and approved the final version of the manuscript for publication.

Abbreviations

BM	Bone Marrow
CRISP3	Cysteine-Rich Secretory Protein 3
ELANE	Neutrophil Elastase
G-CSF	Granulocyte-Colony Stimulating Factor

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GFP	Green Fluorescent Protein
IL-3	Interleukin 3
LTF	Lactoferrin
MMP9	Gelatinase
MPO	Myeloperoxidase
PVDF	Polyvinylidene Difluoride
PRTN3	Proteinase 3
SCF	Stem Cell Factor
SDS	Sodium Dodecyl Sulfate
TBST	Tris-Buffered Saline and Tween 20
TCEA1	Transcription Elongation Factor A (SII) 1

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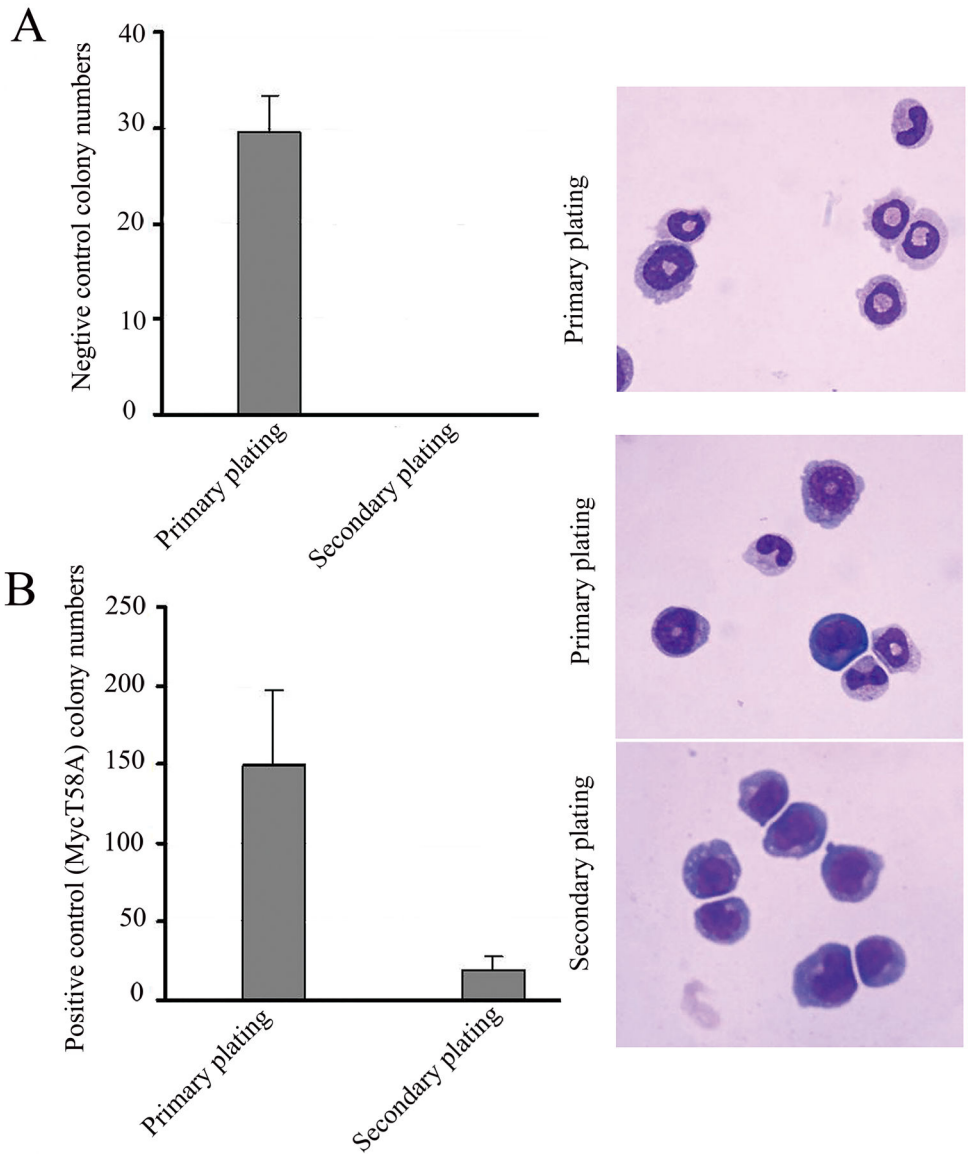

Figure 1: Screen conditions and positive control.

(A) 5-FU treated BM cells cultured in methylcellulose containing 100 ng/ml G-CSF and 100 ng/ml SCF for 5 days showed maturation to neutrophils and could not be replated. (B) Myc^{T58A}, an active form of Myc, was used as a positive control. Cells expressing Myc^{T58A} exhibited immature phenotypes in the primary plating and formed secondary colonies.

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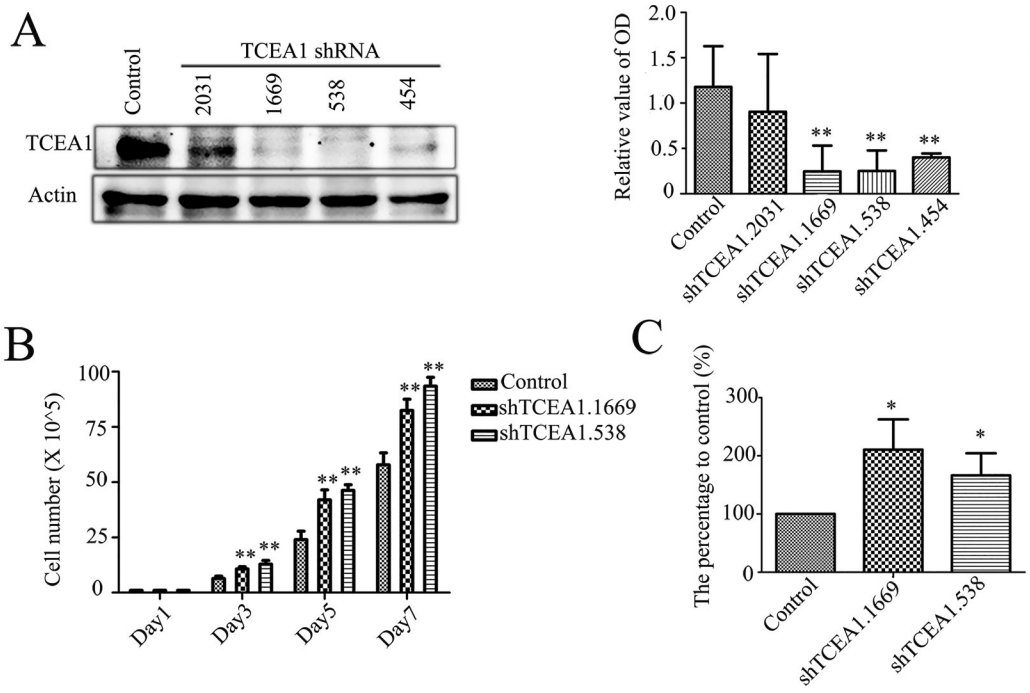

Figure 2: TCEA1 knockdown enhances 32Dcl3 cell survival/proliferation

(A) 32Dcl3 cells were transduced with retroviral vectors expressing TCEA1 shRNAs (2031, 1669, 538, 454) or with the control vector (Control), and transductants were selected with 2 ug/ml of puromycin. Total cellular proteins were subjected to Western blotting for the level of TCEA1. The relative amounts of TCEA1 were quantified using ImageJ software. The TCEA1 signals were normalized to the amount of housekeeping gene (actin) in each sample. Data presented are the means ± SD of 3 independent experiments. **, $P<0.01$ based on the Student *t* test. (B) Viable cell counts for the cells transduced with indicated shRNAs of TCEA1 or control vector at day 1, 3, 5, 7 in medium containing IL-3. Data presented are the means ± SD of 3 independent experiments, every group in each experiment had three wells. **, $P<0.01$ based on the Student *t* test. (C) Proportion of viable cells for the cells transduced with indicated shRNAs of TCEA1 or control vector at day 5 in medium containing G-CSF. Data presented are the means ± SD of 3 independent experiments, every group in each experiment had three wells. *, $P<0.05$ based on the Student *t* test.

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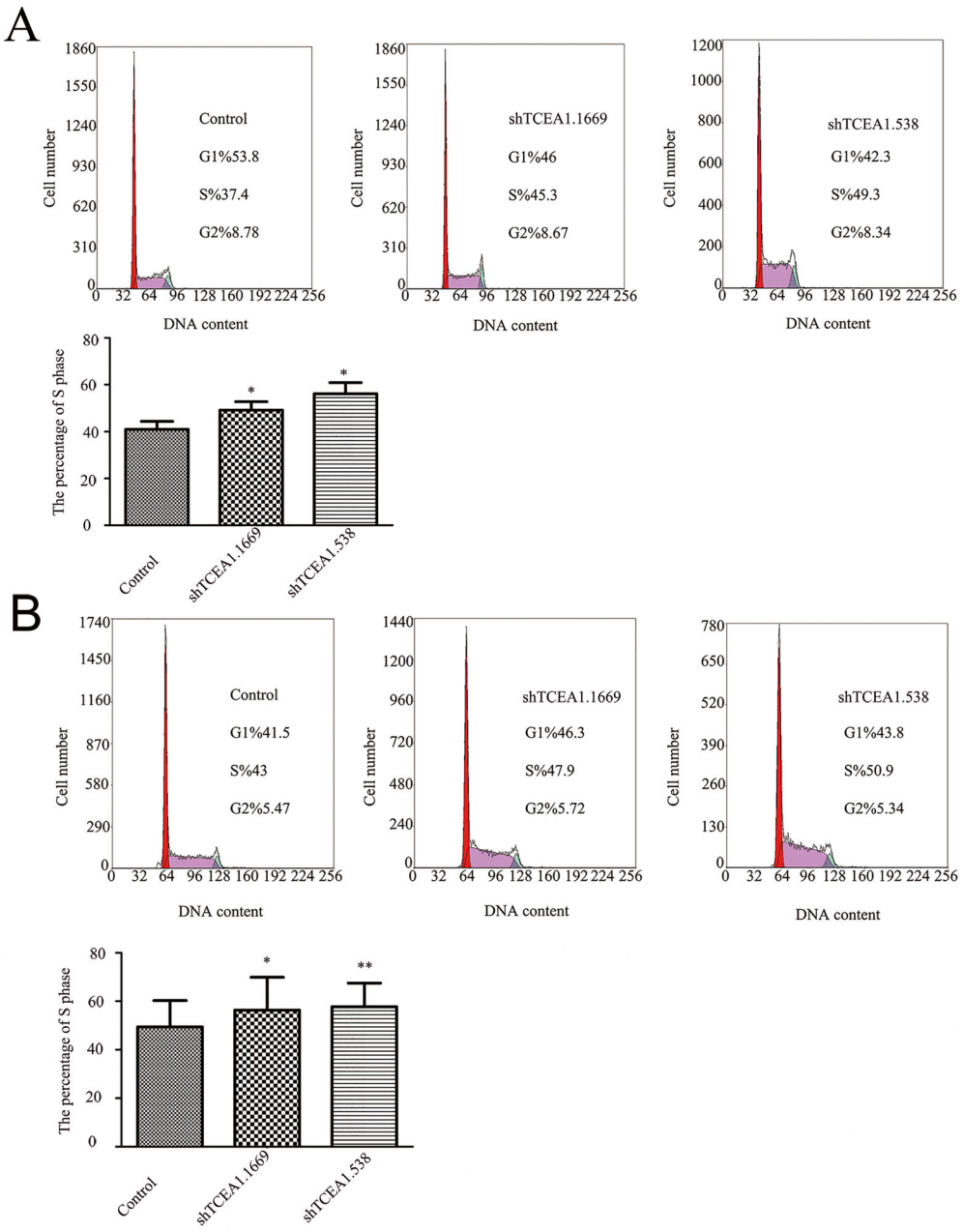

Figure 3: TCEA1 knockdown enhances 32Dcl3 survival/proliferation assayed by cell cycle

(A) Cells were grown in the medium containing IL-3, collected at log phase and stained with propidium iodide (PI), the cellular DNA content reflected by PI staining was analyzed by flow cytometer (Beckman). The percentage of cells in each cell-cycle phase on day 3 was determined by Cell Quest software (MultiCycle for Windows 32-bit). At least 10 000 cells were analyzed for data. Percentages of cells in G1, S and G2 phases were indicated. Three independent experiments were analyzed by Graphpad prism5. (B) Cells were grown in the medium containing G-SCF. Percentages of cells in G1, S and G2 phases on day 5 were determined as in (A). Three independent experiments were analyzed by Graphpad prism5. *, $P<0.05$; **, $P<0.01$ based on the Student t test.

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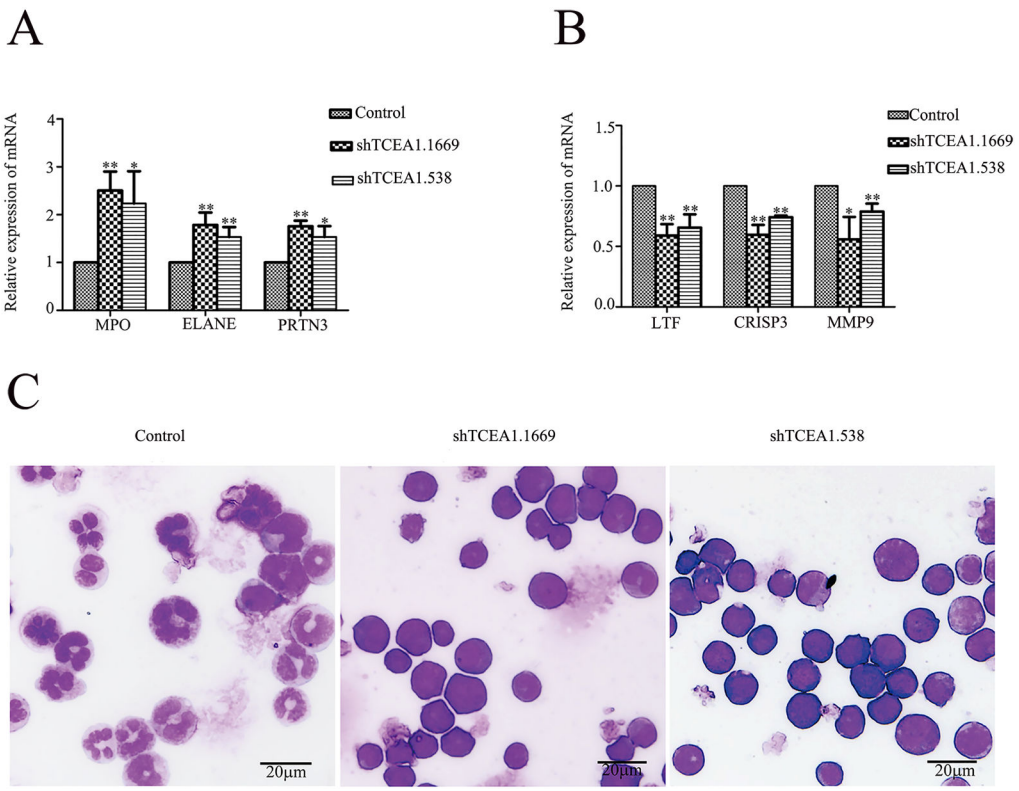

Figure 4: TCEA1 knockdown impairs G-CSF induced granulocytic differentiation of 32Dcl3 cells

(A) The vector-transfected 32Dcl3 cells or shTCEA1-transfected 32Dcl3 cells were transferred to medium containing 50 ng/ml G-CSF for 48 hours. Total cellular RNAs were prepared and converted to first-strand cDNA. The relative expression of myeloperoxidase (*Mpo*), neutrophil elastase (*Elane*) and proteinase 3 *(Prtn3*) normalized to ribosomal protein *S16* RNA were analyzed by real-time PCR analysis. Means for 3 independent RNA preparations are shown. *, $P<0.05$; **, $P<0.01$ for each RNA level relative to expression in the vector transduced cells. (B) The relative expression of lactoferrin (*Ltf*), cysteine-rich secretory protein 3 (*Crisp*3) and gelatinase (*Mmp9*) were also analyzed. Means for 3 independent RNA preparations are also shown. *, $P<0.05$; **, $P<0.01$ for each RNA level relative to expression in the vector transduced cells. (C) Shows the morphological characteristics of 32Dcl3 transduced with shTCEA1 or control vector after 5 days in differentiation medium. The morphology of cells was visualized using Wright-Giemsa staining.

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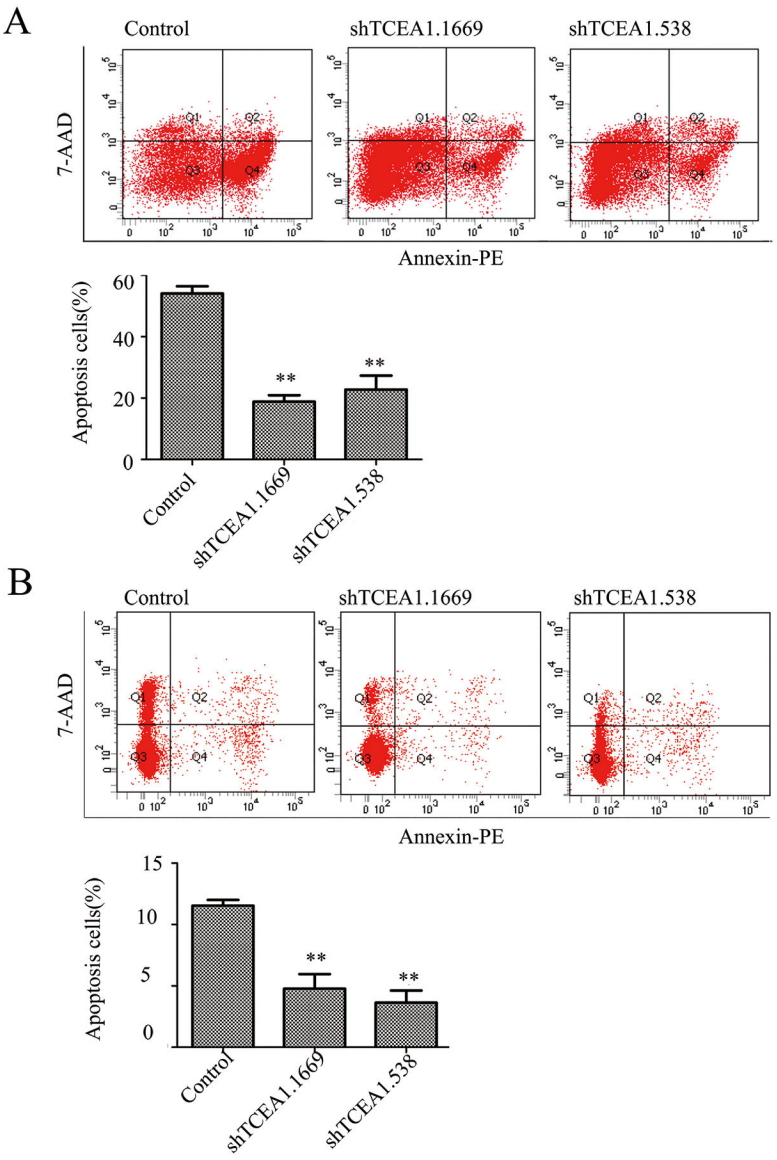

Figure 5: Down-regulation of TCEA1 inhibits the apoptosis of myeloid cells

(A) Control or shTCEA1 cells were cultured 48 hours in medium containing 50 ng/ml G-CSF then subjected to stain with Annexin-PE and 7-AAD for FACS analysis, representative data and a summary of 3 determinations are shown. *, $P<0.05$; **, $P<0.01$ based on the Student *t* test. (B) Control or shTCEA1 cells were cultured 48h in medium containing 1 ng/ml IL-3, then subjected to stain with Annexin-PE and 7-AAD for FACS analysis, representative data and a summary of 3 determinations are shown. **, $P<0.01$ based on the Student *t* test.

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Table 1:
The identified gene's list showed with the scores

The positive colonies that have been confirmed by the "methylcellulose colony-forming assay" (details in "Materials and methods") are indicated. Three digits represented respectively frequency appeared in (pool)·(pure BM colony)·(mixed BM colony).

Gene Name	Symbol	Function	Positive hits
Transcription elongation factor A (SII), 1	Tceal	Regulation of transcription elongation	(1.2.1)
Cyclin-dependent kinase inhibitor 1 B	Cdknlb, p27	Control the cell cycle	(1.3.0)
Tumor necrosis factor receptor superfamily, member 10 b	Tnfrsf10b	Transduce apoptosis signal	(1.3.0)
Topoisomerase (DNA) II alpha	Top2a	Target of several anticancer agents and associated with the drug resistance	(1.3.0)
Zinc finger protein 212	Znf1212	May be involved in transcriptional regulation	(1.3.0)
Epidermal growth factor receptor pathway substrate 8	Eps8	Functions as part of the EGFR pathway	(1.2.0)
Epidermal growth factor receptor	EGFR	Association with the proliferation	(1.1.1)
Nuclear receptor subfamily 4, group A, member 1	Nr4al	Translocation of the protein from the nucleus to mitochondria induces apoptosis	(1.1.1)
Actin related protein 2/3 complex, subunit 1A	Arpcla	Function as p41 subunit of the human Arp2/3 complex	(1.1.0)
Junction plakoglobin	Jup	Constituent common to sub membranous plaques	(1.1.0)
Secreted protein, acidic, cysteine-rich (osteonectin)	Sparc	Inhibits cell-cycle progression, and influences the synthesis of extracellular matrix (ECM)	(1.1.0)
Argase 2	Arg2	Catalyzes the hydrolysis of arginine to ornithine and urea	(1.1.0)
GLI family zinc finger 3	GH3	DNA-binding transcription factors	(1.1.0)
Jun oncogene	Jun	Regulation gene expression	(1.0.1)
Xeroderma pigmentosum, comple mentation group C	Xpc	Participation in the early steps of global genome nucleotide excision repair (NER)	(1.0.1)
Cyclin F	Ccnf	Regulators of cell cycle transitions	(1.0.1)
Notch homolog 4	Notch4	Affects the implementation of differentiation, proliferation and apoptotic programs	(1.0.1)
Vascular endothelial growth factor A	VEGFA	Vasculogenesis, endothelial cell growth, promoting cell migration, and inhibiting apoptosis	(1.0.1)