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ORIGINAL ARTICLE

Basic Study

GATA binding protein 2 mediated ankyrin repeat domain containing 26 high expression in myeloid-derived cell lines

Yang-Zhou Jiang, Lan-Yue Hu, Mao-Shan Chen, Xiao-Jie Wang, Cheng-Ning Tan, Pei-Pei Xue, Teng Yu, Xiao-Yan He, Li-Xin Xiang, Yan-Ni Xiao, Xiao-Liang Li, Qian Ran, Zhong-Jun Li, Li Chen

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Abstract

BACKGROUND

Thrombocytopenia 2, an autosomal dominant inherited disease characterized by moderate thrombocytopenia, predisposition to myeloid malignancies and normal platelet size and function, can be caused by 5′-untranslated region (UTR) point mutations in ankyrin repeat domain containing 26 (ANKRD26). Runt related transcription factor 1 (RUNX1) and friend leukemia integration 1 (FLI1) have been identified as negative regulators of *ANKRD26*. However, the positive regulators of *ANKRD26* are still unknown.

AIM

To prove the positive regulatory effect of GATA binding protein 2 (GATA2) on *ANKRD26* transcription.

METHODS

Human induced pluripotent stem cells derived from bone marrow (hiPSC-BM)

and urothelium (hiPSC-U) were used to examine the *ANKRD26* expression pattern in the early stage of differentiation. Then, transcriptome sequencing of these iPSCs and three public transcription factor (TF) databases (Cistrome DB, animal TFDB and ENCODE) were used to identify potential TF candidates for *ANKRD26*. Furthermore, overexpression and dual-luciferase reporter experiments were used to verify the regulatory effect of the candidate TFs on *ANKRD26*. Moreover, using the GENT2 platform, we analyzed the relationship between *ANKRD26* expression and overall survival in cancer patients.

RESULTS

In hiPSC-BMs and hiPSC-Us, we found that the transcription levels of *ANKRD26* varied in the absence of RUNX1 and FLI1. We sequenced hiPSC-BM and hiPSC-U and identified 68 candidate TFs for *ANKRD26*. Together with three public TF databases, we found that GATA2 was the only candidate gene that could positively regulate *ANKRD26*. Using dual-luciferase reporter experiments, we showed that GATA2 directly binds to the 5′-UTR of *ANKRD26* and promotes its transcription. There are two identified binding sites of GATA2 that are located 2 kb upstream of the TSS of *ANKRD26*. In addition, we discovered that high *ANKRD26* expression is always related to a more favorable prognosis in breast and lung cancer patients.

CONCLUSION

We first discovered that the transcription factor GATA2 plays a positive role in *ANKRD26* transcription and identified its precise binding sites at the promoter region, and we revealed the importance of ANKRD26 in many tissue-derived cancers.

Key Words: Ankyrin repeat domain containing 26; GATA binding protein 2; Thrombocytopenia 2; Transcriptional regulation; Myeloid-derived cell lines

Core Tip: The 5'-untranslated region mutation of ankyrin repeat domain containing 26 (ANKRD26) plays an important role in the pathology of thrombocytopenia 2 (THC2). Considering the predisposition of THC2 patients to myeloid malignancies, further revealing the molecular mechanism of *ANKRD26* transcription is warranted. Although Runt related transcription factor 1 and friend leukemia integration 1 have been shown to negatively regulate *ANKRD26* expression, no known positive regulators have been reported. Here, we first revealed that GATA binding protein 2 mediates high *ANKRD26* expression by binding to its promoter region. We discovered that high *ANKRD26* expression was always associated with favorable overall survival. Our study provides insights into the regulatory network of *ANKRD26* and the pathological process of THC2.

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INTRODUCTION

Ankyrin repeat domain containing protein 26 (ANKRD26) acts as a regulator of adipogenesis and is involved in the regulation of feeding behavior[1-3]. The *ANKRD26* gene is located on chromosome 10 and shares regions of homology with the primate-specific gene family *POTE*. According to the Human Protein Atlas database, the ANKRD26 protein is localized to the Golgi apparatus and vesicles, and its expression can be detected in nearly all human tissues[4]. Moreover, UniProt annotation revealed that ANKRD26 is localized in the centrosome and contains coiled-coil domains formed by spectrin helices and ankyrin repeats[5,6].

The most common disease related to *ANKRD26* is thrombocytopenia 2 (THC2), which is a rare autosomal dominant inherited disease characterized by lifelong mild-to-moderate thrombocytopenia and mild bleeding[7-9]. Caused by the variants in the 5′-untranslated region (UTR) of *ANKRD26*, THC2 is defined by a decrease in the number of platelets in circulating blood and results in increased bleeding and decreased clotting ability[8,10]. Due to the point mutations that occur in the 5′-UTR of *ANKRD26*, its negative transcription factors (TFs), Runt related transcription factor 1 (RUNX1) and friend leukemia integration 1 (FLI1), lose their repression effect[11]. The persistent expression of *ANKRD26* increases the activity of the mitogen activated protein kinase and extracellular signal regulated kinase 1/2 signaling pathways, which are potentially involved in the regulation of thrombopoietin-dependent signaling and further impair proplatelet formation by megakaryocytes (MKs)[11]. However, the positive regulators of *ANKRD26*, which might be associated with THC2 pathology, are still unknown.

In this study, we demonstrated that GATA binding protein 2 (GATA2) functions as a positive regulator of *ANKRD26* by binding to its promoter region and identified its precise binding sites. Furthermore, we scanned the expression levels of *ANKRD26* in human cancers and reported that high *ANKRD26* expression was linked to more favorable overall survival. In summary, this is the first study in which the regulatory effect of GATA2 on *ANKRD26* in THC2 cells has been investigated. The results of this study will enhance our understanding of the regulatory network of *ANKRD26* and the pathological process of THC2.

MATERIALS AND METHODS

Cell lines

K562 and HEL cells were cultured in RPMI 1640 medium (Gibco, C11875500BT) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. All human induced pluripotent stem cells (hiPSCs) were cultured in Matrigel-coated 6-well cell culture plates with Essential 8 (E8) medium (Cellapy, CA1014500). hiPSCs derived from bone marrow (hiPSC-BM) (SHAMUi001-A, https://hpscreg.eu/cell-line/SHAMUi001-A) were induced as previously described, and hiPSCs derived from the urothelium (hiPSC-U) were obtained from Cellapy (hiPSC-U1)[12].

Chemicals and antibodies

The following antibodies were used for western blot and chromatin immunoprecipitation (ChIP) analyses: Rabbit anti-ANKRD26 (GeneTex, GTX128255), rabbit anti-GATA2 (Cell Signaling Technology, #79802S), rabbit anti-RUNX1 (Abcam, ab272456), rabbit anti-FLI1 (Abcam, ab133485), mouse anti-GAPDH (Beyotime, AF0006) and rabbit anti-normal IgG (Cell Signaling Technology, #2729P).

Western blot analysis

Cells were lysed with ice-cold western and IP cell lysis buffer (Beyotime, P0013) containing protease inhibitors. After protein quantification, equal amounts of protein from each sample were separated by sodium-dodecyl sulfate gel electrophoresis (Solarbio, P1200-1/P1200-2) and transferred to polyvinylidene fluoride membranes (Millipore, 0000227526). Then, the membranes were incubated with primary antibodies. The samples were incubated overnight at 4 °C, followed by three washes with TBST. Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies [goat anti-rabbit IgG/HRP (Solarbio, SE134) and goat anti-mouse IgG/HRP (Solarbio, SE131)] for 1 h at room temperature. Blots were visualized using SuperSignal™ West Atto Chemiluminescent Substrate (Thermo Scientific, A38555).

Dual-luciferase reporter assay

The promoter region (2 kb upstream of the translation start site) of ANKRD26 was amplified by polymerase chain reaction (PCR) and cloned with a luciferase reporter vector (P2000). The promoter regions of the P2000 GATA2M1 and P2000 GATA2M2 vectors were constructed by mutating all the bases 821-825 bp and 1685-1689 bp upstream of the *ANKRD26* gene TSS to adenine (A). These two mutated promoters were amplified by PCR and cloned with luciferase reporter vectors. Then, K562 cells were cotransfected with luciferase reporter vectors and the *GATA2* overexpression plasmid using Lipofectamine 3000 transfection reagent (Invitrogen, L3000-05). After 48 h of incubation, a dual-luciferase reporter assay was performed using the Dual-Luciferase Reporter Assay System (Promega, E1910). Renilla luciferase activity was used to normalize the transfection efficiency.

ChIP assay

ChIP assays were conducted using a SimpleChIPPlus Enzymatic Chromatin IP Kit (Magnetic Beads, Cell Signaling Technology, 9005 S) with an anti-GATA2 antibody (Cell Signaling Technology, #79802S). Assays were performed using chromatin prepared from K562 cells. The cells were first crosslinked with 1% formaldehyde in phosphate buffered saline (PBS) at room temperature for 10 min, quenched with 2.5 M glycine at room temperature for 5 min and washed with ice-cold PBS three times. Sonication was used for DNA fragmentation. The supernatants were immunoprecipitated by incubation with 5 μL of anti-GATA2 (Cell Signaling Technology, #79802S), 10 μL of anti-histone H3 (D2B12) XP rabbit mAb (Cell Signaling Technology, #4620) as a positive control and 2 μL of rabbit anti-normal IgG (Cell Signaling Technology, #2729) as a negative control at 4 °C for 16 h. Then, the immunocomplexes were rotationally incubated with 30 μL of ChIP-Grade Protein G Magnetic Beads for 2 h at 4 °C and then washed three times with low-salt wash buffer and one time with high-salt wash buffer at 4 °C for 5 min per wash. Chromatin was eluted by ChIP elution buffer for 30 min at 65 °C with gentle vortex mixing (1200 rpm), and crosslinking was reversed by treatment with 5 M NaCl and proteinase K overnight at 65 °C. The samples were then incubated with RNase at 37 °C for 1 h. ChIP DNA was purified and subsequently quantified by quantitative real-time PCR (RT-qPCR). The sequences of primers used for *ANKRD26* are listed in Supplementary Table 1.

RT-qPCR

The primer sequences for *ANKRD26*, *GATA2*, *RUNX1*, *FLI1* and the housekeeping gene *GAPDH* were predicted using Primer3 and can be found in Supplementary Table 1. mRNA isolation, reverse transcription, and RT-qPCR were performed as previously described[13]. Total RNA was extracted using TRIzol Reagent (TaKaRa, 9109) according to the manufacturer's instructions. First-strand cDNA was synthesized from l μg of RNA using the PrimeScript RT Reagent Kit

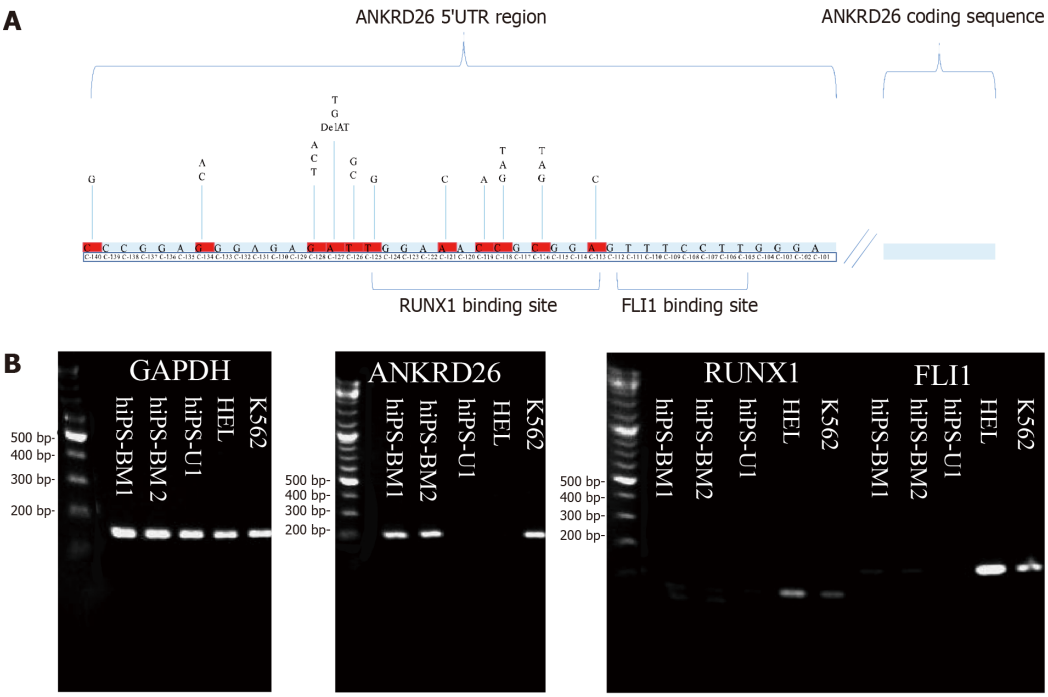

Figure 1 The expression of ankyrin repeat domain containing 26 varied in induced pluripotent stem cells derived from bone marrow and the urothelium. A: Schematic structure of the 5'-untranslated region ankyrin repeat domain containing 26 (ANKRD26) region and its relationship with the Runt related transcription factor 1 (RUNX1) and friend leukemia integration 1 (FLI1) binding sites. The bases in red represent the mutation sites reported in the literature; B: Agarose gels after polymerase chain reaction to assess the expression of ANKRD26, RUNX1 and FLI1 in HEL, K562, human induced pluripotent stem cells derived from bone marrow, and human induced pluripotent stem cells derived from urothelium. UTR: Untranslated region; ANKRD26: Ankyrin repeat domain containing 26; RUNX1: Runt related transcription factor 1; FLI1: Friend leukemia integration 1; hiPSC-BM: Human induced pluripotent stem cells derived from bone marrow; hiPSC-U: Human induced pluripotent stem cells derived from urothelium.

with gDNA Eraser (TaKaRa, RR047A). qPCR was performed in triplicate in 20-μL reactions containing SYBR Premix Ex Taq II (TaKaRa, RR820A). The reaction protocol was as follows: Heating for 30 s at 95 °C, followed by 40 cycles of amplification (5 s at 95 °C and 30 s at 60 °C).

Transcriptome sequencing and data analysis for iPSCs

Total RNA was extracted from bone marrow- and urothelium-derived iPSCs using TRIzol reagent, as previously described[14]. Then, the RNA quality and quantity were analyzed by an Agilent 2100 Bioanalyzer. After the cDNA libraries for all the samples were prepared using the Illumina RNA Prep with Enrichment Kit (Illumina), they were sequenced on the Illumina NovaSeq 6000 platform with the PE150 strategy. Next, the output of sequencing data (fastq format) was cleaned using SOAPnuke and quality controlled by FastQC, as previously described[15]. HISAT2 and Stringtie were used to align the clean reads to the human reference genome (GRCH38) and to profile gene expression for each sample[16]. The fragments per kilobase per million mapped reads (FPKM) method was used to normalize the gene expression in each sample. Differential expression analysis was performed as previously described[15]. HISAT2 and Stringtie were used to align the clean reads to the human reference genome (GRCH38) and to profile gene expression for each sample[16]. The FPKM method was used to normalize the gene expression in each sample. Differential expression analysis was performed as previously described[15].

Statistical analysis

All the statistical analyses were performed using GraphPad Prism 9.0 software. The mean ± SD method was used to present the values of replicates, and P values were calculated using Student's t test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

ANKRD26 gene expression is different in iPSCs derived from bone marrow and urothelium

Sustained ANKRD26 expression in the late stage of megakaryopoiesis is due to point mutations in the 5'-UTR of *ANKRD26*, which is in or close to the binding sites of RUNX1 and FLI1 (Figure 1A)[11,17]. To investigate whether there are positive regulators of ANKRD26, we examined its expression, together with its two negative regulators, RUNX1 and FLI1, in two classic THC2 model cell lines, HEL and K562. RUNX1 and FLI1 expression was greater in HEL cells than in K562 cells. ANKRD26 expression was greater in K562 cells than in HEL cells (Figure 1B). Next, we employed hiPSC-BM

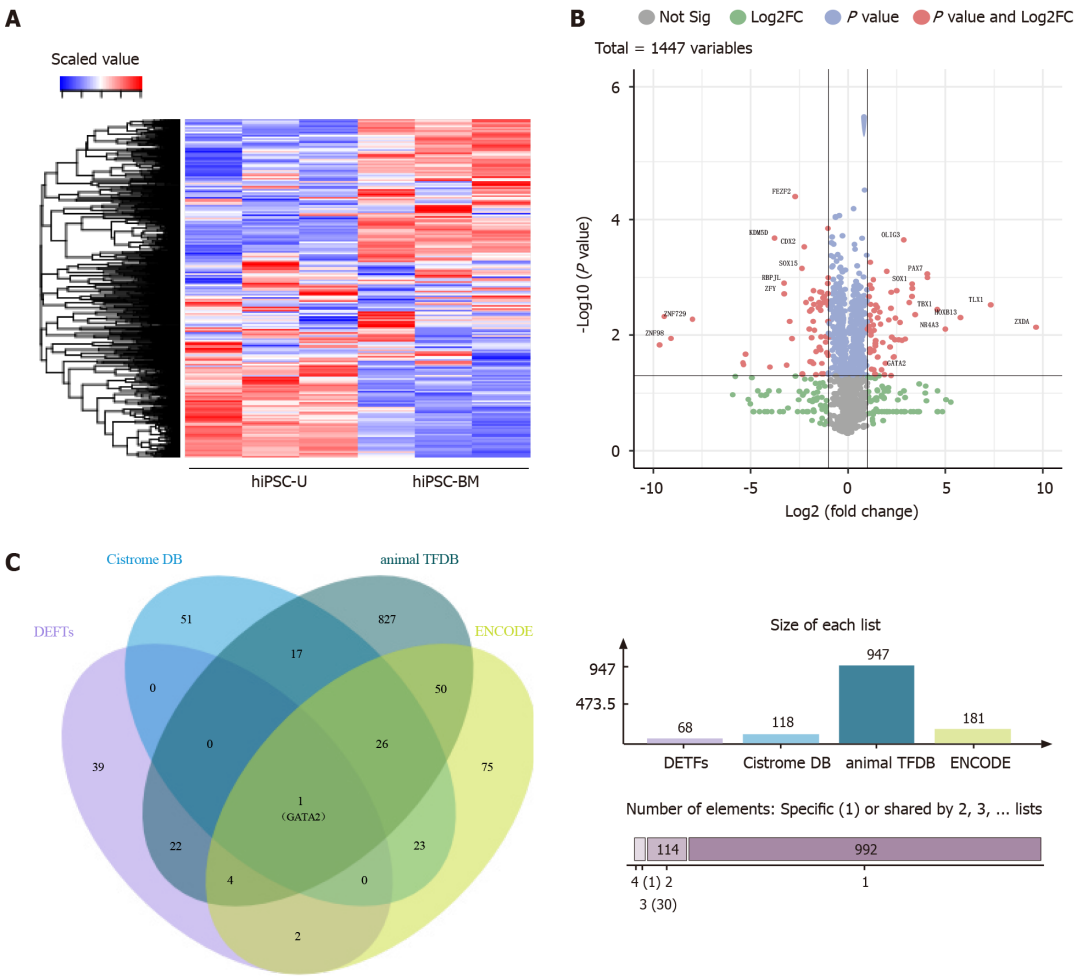

Figure 2 The transcription factor GATA binding protein 2 is potentially involved in regulating ankyrin repeat domain containing 26 expression. A: A heatmap showing the expression patterns of transcription factor (TF) genes in human induced pluripotent stem cells derived from bone marrow (hiPSC-BM) and human induced pluripotent stem cells derived from urothelium (hiPSC-U); B: A volcano plot showing the differentially expressed TF genes in hiPSC-BM and hiPSC-U. Red dots represent TF genes with significant differential expression; C: Venn diagram showing overlaps of ankyrin repeat domain containing 26 (ANKRD26) related transcription factors from the Cistrome DB, animal TFDB, ENCODE and transcriptome (differentially expressed transcription factors)[41]. DETFs: Differentially expressed transcription factors; GATA2: GATA binding protein 2; hiPSC-BM: Human induced pluripotent stem cells derived from bone marrow; hiPSC-U: Human induced pluripotent stem cells derived from urothelium.

and hiPSC-U cells to obtain further insights into the regulatory mechanisms of ANKRD26 at an early stage and examined the expression of RUNX1, FLI1 and ANKRD26 in these cells. In the present study, RUNX1 and FLI1 were not detected in these iPSCs, but ANKRD26 was detected only in the hiPSC-BM (Figure 1B). Thus, we proposed that there might be other positive regulators of ANKRD26.

GATA2 is a potential regulator of ANKRD26

Then, we performed transcriptome sequencing and screened the expression of all TF genes in both kinds of iPSCs. A heatmap (Figure 2A) and a volcano plot (Figure 2B) were used to show the differential expression of TF genes in hiPSC-BM and hiPSC-U. We detected significant differences in the expression of some TF genes ($P < 0.05$, fold change > 2) in the two types of iPSCs, such as *FEZF2*, *OLIG3*, *PAX7* and *GATA2*. In total, 68 TF genes were more highly expressed in hiPSC-BM cells than in hiPSC-U cells. Next, we searched for potential TFs for ANKRD26 in three databases, namely, the Cistrome DB, animal TFDB and ENCODE[18-20]. Interestingly, GATA2 was the only TF identified by our transcriptome sequencing and the three datasets (Figure 2C and Supplementary Table 2), which strongly supports it as a potential regulator of ANKRD26.

ANKRD26 expression is upregulated by GATA2 overexpression

To verify the regulatory effect of GATA2 on the transcription of the *ANKRD26* gene, we analyzed the expression of GATA2 and ANKRD26 in HEL, K562, hiPSC-BM and hiPSC-U cells. Although GATA2 was expressed in HEL cells, ANKRD26 was not detected, probably due to the dominant repressive effect of its suppressors RUNX1 and FLI1 (Figures 1B and 3A). In other cells, such as K562 and hiPSC-BM cells, we found that GATA2 was co-expressed with ANKRD26 (Figures 1B and 3A). To study the positive regulatory effect of GATA2 on ANKRD26 expression, we transfected GATA2 plasmids into K562 cells and observed that ANKRD26 expression was significantly increased at both the mRNA and

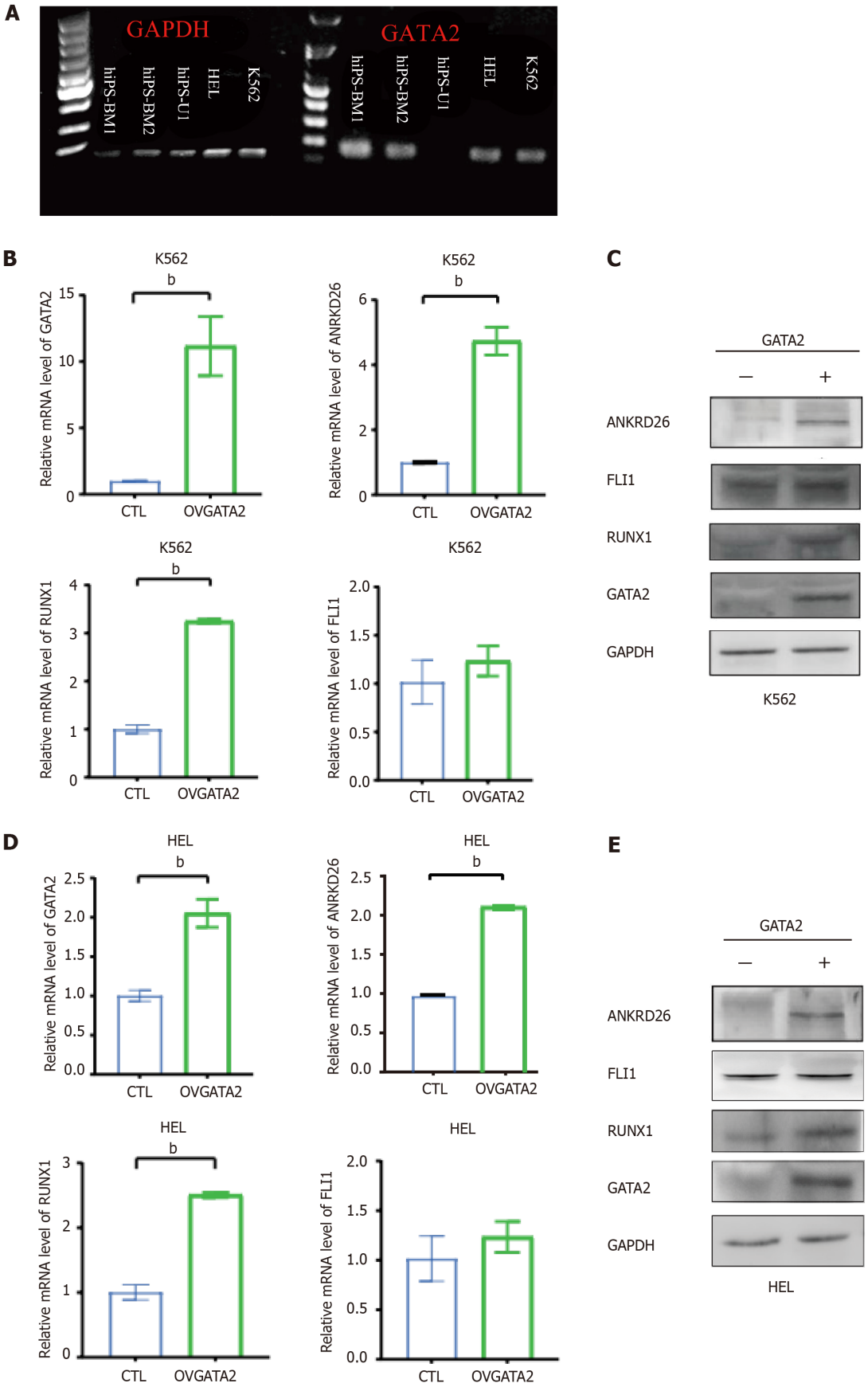

Figure 3 The expression of ankyrin repeat domain containing 26 is upregulated by the overexpression of GATA binding protein 2. A: Agarose gels after polymerase chain reaction (PCR) to assess the expression of GATA binding protein 2 (GATA2) in HEL, K562, human induced pluripotent stem cells derived from bone marrow (hiPSC-BM) and human induced pluripotent stem cells derived from urothelium (hiPSC-U); B: Quantitative real-time PCR (RT-qPCR) analyses showing the promoting effect of GATA2 on ankyrin repeat domain containing 26 (*ANKRD26*) mRNA expression. K562 cells were transfected with GATA2

plasmids and control plasmids; C: Western blots showing the effect of GATA2 on promoting ANKRD26 protein expression in K562 cells; D: RT-qPCR analyses showing the effect of GATA2 on promoting *ANKRD26* mRNA expression. HEL cells were transfected with GATA2 plasmids and control plasmids; E: Western blots showing the effect of GATA2 on promoting *ANKRD26* expression at the protein level in HEL cells. The error bars represent the means ± SD of triplicate samples. $^{b}P < 0.01$, calculated by Student's *t* test. GATA2: GATA binding protein 2; ANKRD26: Ankyrin repeat domain containing 26; RUNX1: Runt related transcription factor 1; FLI1: Friend leukemia integration 1; hiPSC-BM: Human induced pluripotent stem cells derived from bone marrow; hiPSC-U: Human induced pluripotent stem cells derived from urothelium.

protein levels (Figure 3B and C). Interestingly, the expression of RUNX1 was significantly increased at the mRNA and protein levels (Figure 3B and C), while the expression of FLI1 mRNA and protein was also increased but not significantly different in K562^{GATA2+} cells (Figure 3B and C). Then, we transfected the GATA2 plasmids into HEL cells and examined the expression levels of GATA2, ANKRD26, RUNX1 and FLI1. Unexpectedly, the expression patterns of these genes in HEL cells following GATA2 overexpression were the same as those in K562 cells following GATA2 overexpression (Figure 3D and E). This might suggest that the positive regulation by the overexpression of GATA2 would take over the repressive effect of RUNX1 and FLI1.

Because the expression of ANKRD26 varied between hiPSC-BMs and hiPSC-Us (Figure 1B), we next transfected the GATA2 plasmids into bone marrow (THP-1 and MOLM13)- and urothelium (LNCaP and DU145)-derived cell lines. The results showed that in these cell lines, overexpressed GATA2 can consistently stimulate the expression of ANKRD26 at both the mRNA and protein levels (Figure 4). Taken together, these findings indicate that GATA2 does not increase the expression of ANKRD26 *via* the downregulation of RUNX1 or FLI1.

GATA2 promotes ANKRD26 expression by binding to its promoter region

Next, we established a dual-luciferase reporter covering the 2 kb region upstream of the TSS of the *ANKRD26* gene (Figure 5A). K562 cells were co-transfected with the GATA2 expression plasmids and these dual-luciferase reporter constructs. Compared with that in the empty vector group, luciferase activity was significantly greater in the GATA2 overexpression group, indicating that GATA2 binds to this region (Figure 5B). Furthermore, using bioinformatics tools, we identified 3 potential consensus binding motifs for GATA2 in the promoter region of *ANKRD26* (Figure 5C). These motifs were searched against the 5′-UTR of *ANKRD26*, and 8 hits were found (Figure 5D). PCR primers encompassing these sites were designed, ChIP assays were performed, and two potential sites were identified (Figure 5E).

To further confirm the binding sites of GATA2 in the *ANKRD26* promoter region, we performed a site-directed mutagenesis experiment by modifying the bases in the two binding sites to disrupt the binding of GATA2 (Figure 5F). Then, we found that the luciferase activities of both mutated dual-luciferase reporters were significantly decreased (Figure 5G). Taken together, our findings reveal that GATA2 promotes *ANKRD26* transcriptional activity by directly binding to two sites in the *ANKRD26* promoter region and that these two sites are indispensable for *ANKRD26* gene transcription.

The importance of ANKRD26 in cancers

We next explored the expression patterns of ANKRD26 in other human diseases, such as cancers. We downloaded *ANKRD26* gene expression profiles (from the GPL570 and GPL96 platforms) from the GENT2 database and compared the expression levels of ANKRD26 in normal and cancer tissues of various cancer types (Figure 6A)[21]. ANKRD26 expression was significantly different ($P < 0.05$) between cancer and normal tissues of multiple cancer types (15 and 16 cancer types from the GPL570 and GPL96 platforms, respectively), including brain, breast, lung, and blood (Supplementary Table 3). Next, we analyzed the relationship between ANKRD26 expression and the overall survival rate of cancer patients. Notably, in breast and lung cancers, high expression of ANKRD26 was significantly associated with survival, although its expression might not be significantly different in patients with different tumor grades (Figure 6B). Furthermore, considering its association with THC2, we analyzed the expression of ANKRD26 in blood cancers (leukemia, lymphoma, and myeloma) (Figure 6C). Interestingly, lymphoma and myeloma were found to have the highest and lowest expression of ANKRD26, respectively. The overall survival rate of patients with lymphoma was much greater than that of patients with leukemia (Figure 6D); the reason might be complicated, as the factors influencing the two subtypes of blood cancer are excessive. However, the potential function of ANKRD26 in hematopoietic differentiation might also be a factor, and further experiments are needed.

DISCUSSION

In hematopoietic stem and progenitor cells, FLI1 plays a key role in proliferation and differentiation[22]. RUNX1 is indispensable for the establishment of definitive hematopoiesis, and studies of iPSCs derived from patients with familial platelet disorder with a predisposition to acute myeloid leukemia (FPD/AML) have revealed that RUNX1 mutations also cause megakaryopoiesis defects[23,24]. Bluteau *et al*[11] reported that RUNX1 and FLI1 are two critical transcription factors that regulate ANKRD26 expression, and losing the binding of these two critical TFs causes persistent ANKRD26 expression, which is the most important factor that contributes to THC2 expression. It is reasonable to assume that ANKRD26 plays an important role in the downstream pathway of RUNX1 because the clinical features of FPD/AML are similar to those of THC2, and RUNX1 is an essential regulatory factor of *ANKRD26*. To observe the relationship between ANKRD26 and these two TFs (RUNX1 and FLI1) at an early stage, in our study, we measured the expression of

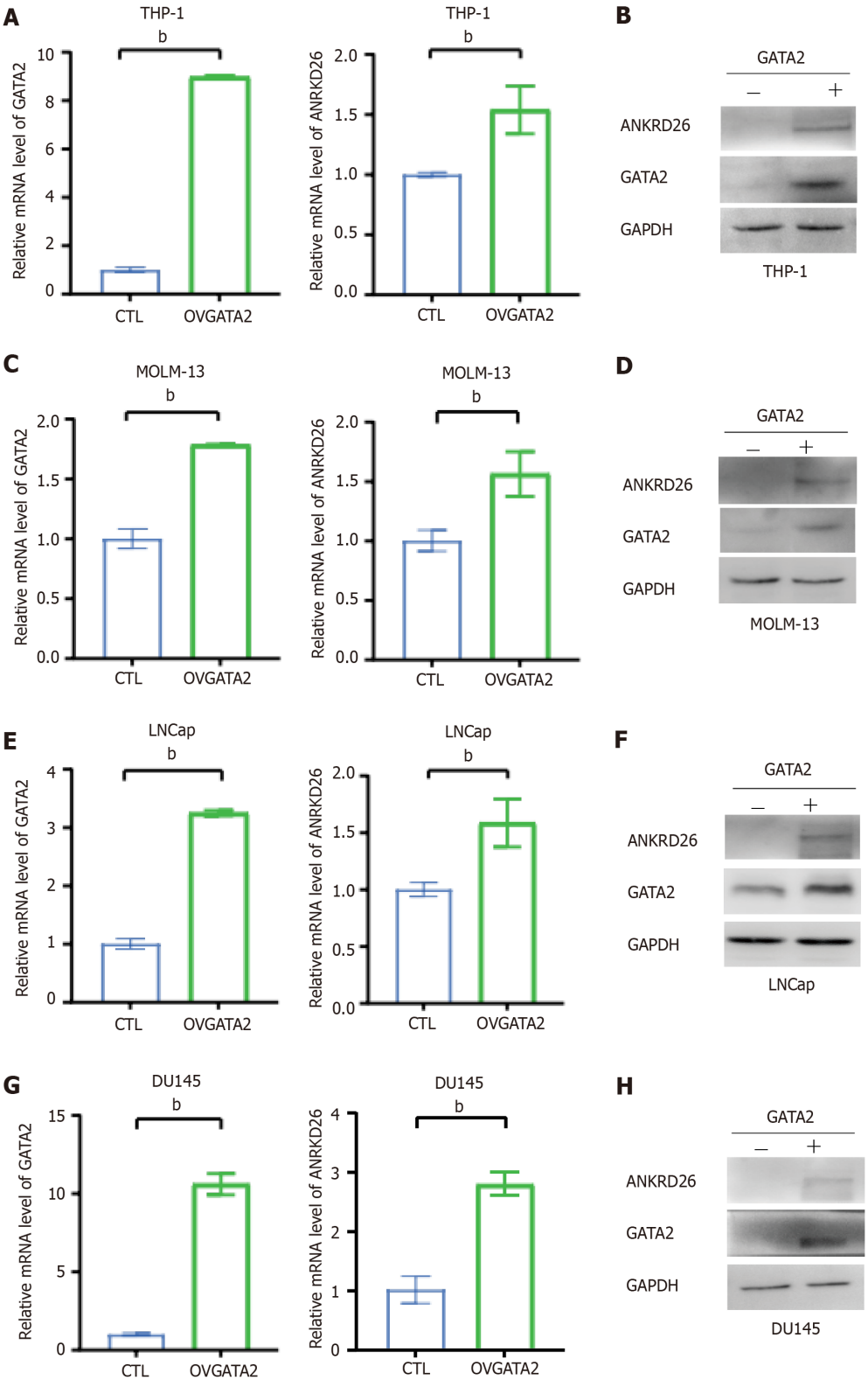

Figure 4 The regulation of ankyrin repeat domain containing 26 by GATA binding protein 2 overexpression in bone marrow- and urothelium-derived cell lines. A-D: Quantitative real-time polymerase chain reaction (RT-qPCR) and western blot analyses showing that GATA binding protein 2 (GATA2) promoted ankyrin repeat domain containing 26 (ANKRD26) expression in two bone marrow-derived cell lines (THP-1 and MOLM-13); E-H: RT-qPCR and western blot analyses showing that GATA2 promoted ANKRD26 expression in two urothelium-derived cell lines (LNCaP and DU145). The error bars represent the means ± SD of triplicate samples. [a]*P* < 0.05; [b]*P* < 0.01, calculated by Student's *t* test. GATA2: GATA binding protein 2; ANKRD26: Ankyrin repeat domain containing 26.

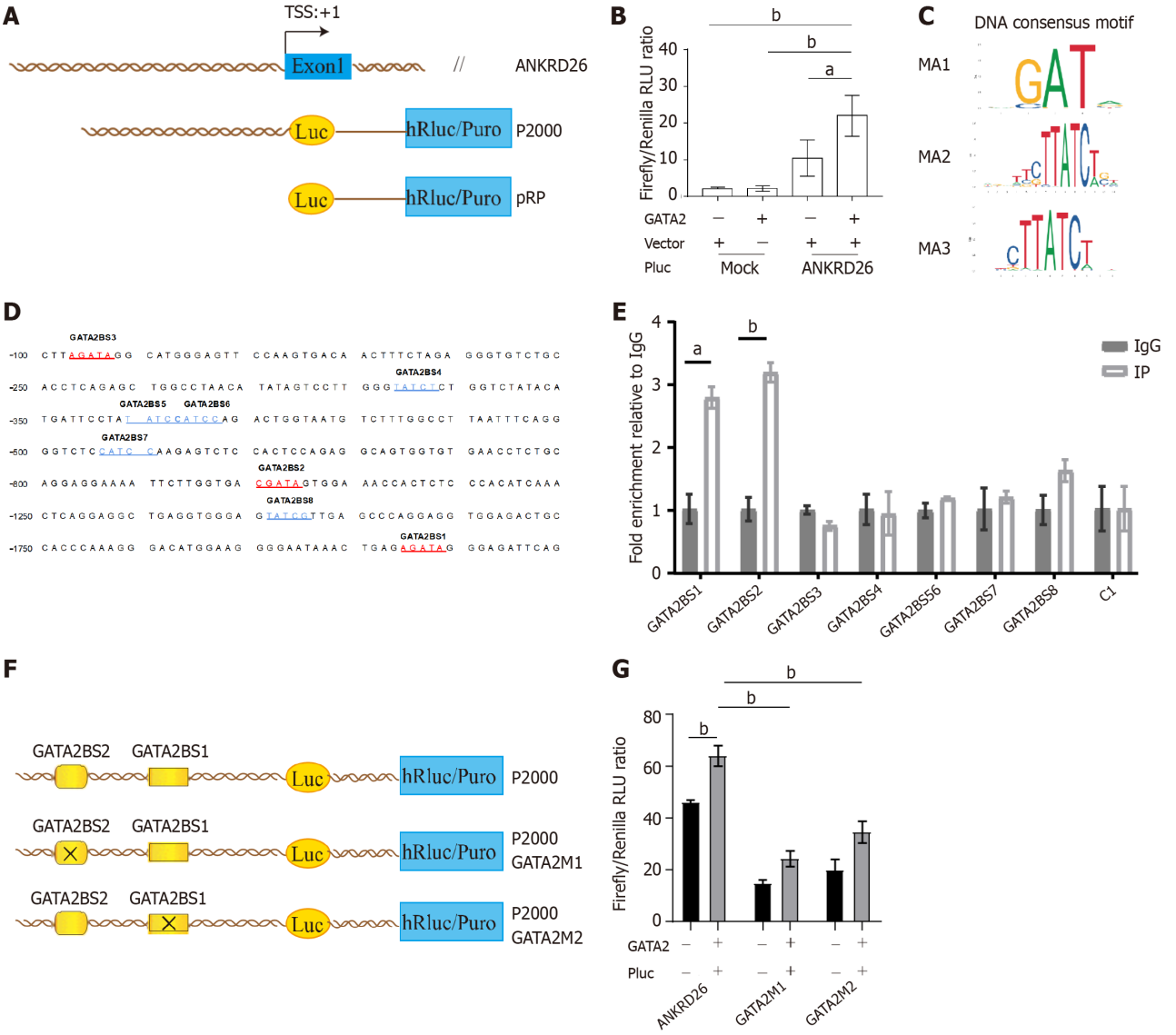

Figure 5 GATA binding protein 2 promotes ankyrin repeat domain containing 26 expression by binding to its promoter region. A: Schematic diagram of the ankyrin repeat domain containing 26 (*ANKRD26*) gene promoter reporter constructs. The constructs are named P-length. The open box shows the first exon of ANKRD26, and the positions relative to the major ANKRD26 transcription start site (+1) are indicated. The pRP represents an empty vector (mock); B: The empty (mock) or ANKRD26 (P2000) dual-luciferase reporter constructs were cotransfected into K562 cells with control plasmids or plasmids expressing GATA binding protein 2 (GATA2) in synergy. Cell extracts were analyzed for luciferase activity; C: DNA consensus motifs of GATA2; D: The eight potential GATA2 binding sites on the ANKRD26 promoter region. Red-marked sites are on the sense strand, and blue-marked sites are on the antisense strand; E: Chromatin immunoprecipitation assays performed in K562 cells show that GATA2 directly binds to the ANKRD26 promoter region. The binding sites of GATA2BS1 to GATA2BS8 encompass the 8 predicted binding sites in (D). C1, which encompasses the region without GATA2 binding sites, was used as the negative control; F: Schematic diagram of site-directed mutagenesis of GATA2 binding sites in the ANKRD26 promoter. The two potential GATA2 binding sites are indicated as open boxes (GATA2BS1 and GATA2BS2). The indicated point mutation is denoted by a cross; G: Dual-luciferase assays showing that two mutated GATA2 binding sites block GATA2 Luciferase-promoting activity. The error bars represent the means ± SDs of triplicate samples. $^{a}P < 0.05$; $^{b}P < 0.01$, calculated by Student's *t* test. GATA2: GATA binding protein 2.

ANKRD26, RUNX1 and FLI1 in K562 cells, HEL cells and two tissue-derived iPSCs (hiPSC-BM and hiPSC-U). With the same expression levels of RUNX1 and FLI1, we unexpectedly found that the ANKRD26 expression pattern differed between the two types of tissue-derived iPSCs. We first showed that GATA2 positively regulates ANKRD26 expression. Taken together, these three TFs (GATA2, RUNX1 and FLI1) contribute to the regulation of ANKRD26 expression.

In our study, we first identified the promoting effect of GATA2 on ANKRD26 expression by binding to its promoter region. It has been reported that GATA2 might regulate megakaryopoiesis at the level of MK progenitors[25]. By analyzing the gene expression data in the BloodSpot database (https://www.bloodspot.eu/), we also observed that the expression of GATA2 and ANKRD26 gradually decreased with MK differentiation and that the expression of RUNX1 and FLI1 did not obviously differ (Supplementary Figure 1)[26]. Therefore, we can speculate that GATA2 dominates *ANKRD26* regulation at an early stage of differentiation, which is consistent with its expression pattern. However, during the late stage of MK differentiation, GATA2 expression is decreased, and the repressive effect of RUNX1 and FLI1 regulates ANKRD26 expression. In addition, ANKRD26 expression was measured in HEL cells overexpressing GATA2.

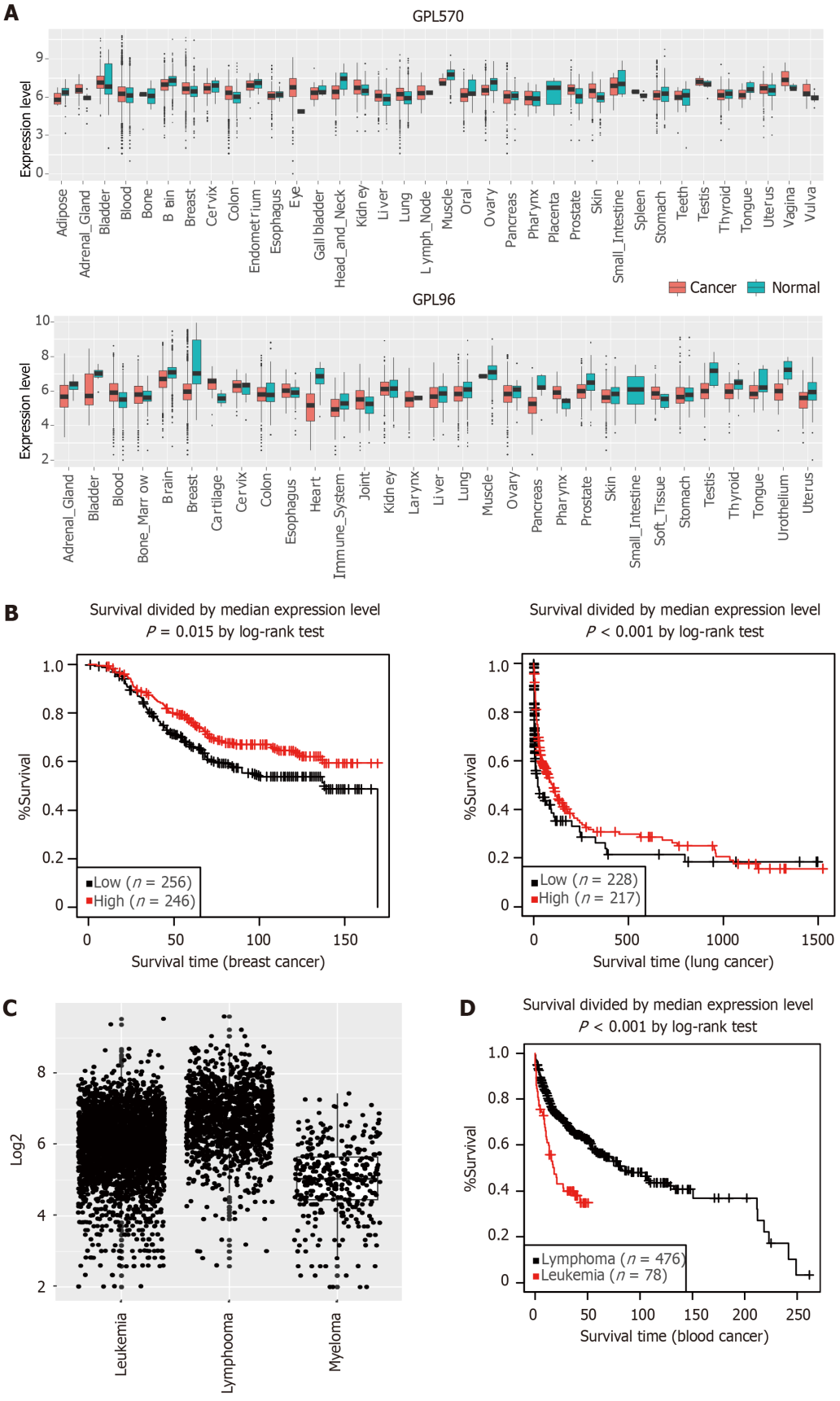

Figure 6 The importance of ankyrin repeat domain containing 26 in cancers. A: Two boxplots of ankyrin repeat domain containing 26 (*ANKRD26*) gene expression profiles from the GPL570 and GPL96 platforms. Red indicates a boxplot of cancer samples. Blue indicates a boxplot of normal samples; B: Kaplan-Meier plots by median cutoff showing an association between ANKRD26 expression and overall survival (left is in breast cancer, and right is in lung cancer); C: Box and dot plot of each subtype of blood cancer; D: Kaplan-Meier plots by subtypes showing an association between ANKRD26 expression and blood cancer subtypes.

This finding also proved that the repressive effect of RUNX1 and FLI1 would be disrupted if GATA2 expression was sufficient.

GATA2 was first reported in 1994 and was shown to encode an essential transcriptional regulator in multilineage hematopoiesis[27]. It is a transcriptional activator that regulates endothelin-1 gene expression in endothelial cells and binds to the consensus sequence[28]. In our study, we found that the GATA2 motif MA1 (5′-AGATA-3′) plays a major role in regulating *ANKRD26* (Figure 5C). As one of the six GATA family TFs, the pioneering TF GATA2 can facilitate the opening of heterochromatin and the subsequent binding of other TFs and further induce gene expression from previously inaccessible regions of the genome[29].

In addition to affecting THC2, ANKRD26 is related to many other diseases, such as diabetes, epidermodysplasia verruciformis, non-Langerhans cell histiocytosis and psychiatric disorders, although the underlying mechanism has yet to be elucidated[30-35]. Recent studies have shown that ANKRD26 is a distal appendage protein that plays an essential role in centrosome amplification[36,37]. Centrosome amplification is always observed in a variety of human cancers and promotes genome instability and tumor development[38,39]. To explore the role of ANKRD26 in cancer, we analyzed the change in the expression of ANKRD26 in cancers derived from different tissues by using the GENT2 platform. In our study, we showed that there is a significant difference in ANKRD26 expression in many cancer tissues, and patients with higher ANKRD26 expression had a more favorable prognosis in breast and lung cancers. In hepatocytes, ANKRD26 is regarded as an important factor in restricting polyploidization and preventing chronic injury because it can activate the PIDDosome-P53 axis after centrosome amplification[40]. Further investigation into whether ANKRD26 functions as a potential tumor suppressor by inducing apoptosis after centrosome amplification in cancer cells and whether the impairment of proplatelet formation in THC2 patients is caused by excessive ANKRD26, which limits MK polyploidy, is urgently warranted.

CONCLUSION

In conclusion, we first identified that GATA2 enhanced ANKRD26 expression, and we also identified the precise binding sites of GATA2 on the ANKRD26 promoter region. In addition, we discovered that high ANKRD26 expression was always linked to favorable overall survival. Hence, our study further revealed the transcriptional regulatory network of ANKRD26 and contributed to further exploration of the pathological process of THC2.

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FOOTNOTES

Author contributions: Chen L and Li ZJ collaboratively established the research framework, methodology, and provided comprehensive strategic guidance; Ran Q ensured seamless coordination throughout the entire research process; Jiang YZ and Hu LY as co-first authors, undertook the core experimental work; Chen MS, Yu T, He XY, and Li XL provided indispensable support for the experiment's execution; Chen MS, Xiang LX, and Xiao YN carried out the meticulous data collection and analysis; Xue PP, Wang XJ, and Tan CN delved into the data interpretation; Jiang YZ and Hu LY crafted the manuscript skillfully, reflecting their academic proficiency and expressive ability; and all authors collectively reviewed and approved the final outcome of this study.

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