ANKRD26 is a new regulator of type I cytokine receptor signaling in normal and pathological hematopoiesis

Francesca Basso-Valentina,[1,2*] Alessandro Donada,[1,2*] Vladimir T. Manchev,[1] Manuel Lisetto,[1] Nathalie Balayn,[1] Jean Edouard Martin,[1,2] Delphine Muller,[1] Cecilia Paola Marin Oyarzun,[1] Hélène Duparc,[1] Brahim Arkoun,[1] Alessandro Cumin,[1,3,4] Lionel Faivre,[5] Nathalie Droin,[1] Ida Biunno,[6,7] Alessandro Pecci,[8,9] Alessandra Balduini,[10,11] Najet Debili,[1] Iléana Antony-Debré,[1] Caroline Marty,[1] William Vainchenker,[1] Isabelle Plo,[1] Remi Favier[1,12] and Hana Raslova[1]

[1]INSERM, UMR 1287, Gustave Roussy, Université Paris Saclay, Equipe Labellisée Ligue Nationale Contre le Cancer, Villejuif, France; [2]Ecole Doctorale Hematopoïèse, Oncogénèse et Biothérapie, Université Paris Diderot, Université Sorbonne Paris Cité, Paris, France; [3]Dipartimento di Scienze della Vita, University of Trieste, Trieste, Italy; [4]University of Paris Diderot, Paris, France; [5]Unité Thérapie Cellulaire, Hôpital St Louis, Assistance Publique-Hôpitaux de Paris, Paris, France; [6]Integrated Systems Engineering, Milan, Italy; [7]Institute for Genetic and Biomedical Research-CNR, Milan, Italy; [8]Department of Internal Medicine, University of Pavia, Pavia, Italy; [9]General Medicine 1, IRCCS Policlinico San Matteo Foundation, Pavia, Italy; [10]Department of Molecular Medicine, University of Pavia, Pavia, Italy; [11]Department of Biomedical Engineering, Tufts University, Medford, OR, USA and [12]Centre de Référence des Pathologies Plaquettaires, Hôpital Armand Trousseau, Assistance Publique-Hôpitaux de Paris, Paris, France

**FB-V and AD contributed equally as first authors.*

Correspondence: H. Raslova
hana.raslova@gustaveroussy.fr

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Abstract

Sustained ANKRD26 expression associated with germline *ANKRD26* mutations causes thrombocytopenia 2 (THC2), an inherited platelet disorder associated with a predisposition to leukemia. Some patients also present with erythrocytosis and/or leukocytosis. Using multiple human-relevant *in vitro* models (cell lines, primary patients' cells and patient-derived induced pluripotent stem cells) we demonstrate for the first time that ANKRD26 is expressed during the early steps of erythroid, megakaryocyte and granulocyte differentiation, and is necessary for progenitor cell proliferation. As differentiation progresses, ANKRD26 expression is progressively silenced, to complete the cellular maturation of the three myeloid lineages. In primary cells, abnormal ANKRD26 expression in committed progenitors directly affects the proliferation/differentiation balance for the three cell types. We show that ANKRD26 interacts with and crucially modulates the activity of MPL, EPOR and G-CSFR, three homodimeric type I cytokine receptors that regulate blood cell production. Higher than normal levels of ANKRD26 prevent the receptor internalization that leads to increased signaling and cytokine hypersensitivity. These findings afford evidence how ANKRD26 overexpression or the absence of its silencing during differentiation is responsible for myeloid blood cell abnormalities in patients with THC2.

Introduction

ANKRD26 (ankyrin repeat domain containing 26) is the ancestor of a family of primate-specific genes termed POTE (Prostate-, Ovary, Testis-, and placenta-Expressed genes) with a gene expression restricted to a few normal tissues and a larger number of pathological tissues.[1,2] It encodes for a protein of 192 kDa, containing both spectrin helices and ankyrin repeats, protein domains known to interact with cytoskeletal and signaling proteins.[3-5] Germline mutations in the regulatory region of the gene encoding ANKRD26 are associated with thrombocytopenia 2 (THC2).[6] All THC2 patients present with moderate thrombocytopenia and mild bleeding, while a smaller number of patients present with erythrocytosis and/or leukocytosis.[7] Importantly, 10% of THC2 patients develop myeloid malignancies, classifying THC2 as an inherited thrombocytopenia predisposing to leukemia.[7-9] We have previously demonstrated that ANKRD26 is indeed involved in modulating thrombopoietin (TPO)-dependent signaling[10] and its expression increases the intensity of mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)1/2 activation. Coupled with clinical observations, we made the hypothesis that ANKRD26 plays a broader role in hematopoiesis by modifying cytokine-mediated cell signaling.

Type I receptors, particularly the homodimeric ones, play

a fundamental role in myeloid blood cell production. Granulocyte colony-stimulating factor receptor (G-CSFR) activation drives neutrophil differentiation,[11] erythropoietin (EPO)/erythropoietin receptor (EPOR) signaling is indispensable for red blood cell production,[12] and TPO/TPO receptor (MPL) signaling is essential for megakaryocyte differentiation[13] and hematopoietic stem cell quiescence.[14] Loss- or gain-of-function mutations of the homodimeric receptors are associated with several inherited or acquired diseases,[15-18] as are mutations in proteins directly modulating ligand/receptor signaling.[19]

These receptors are traditionally activated through ligand binding, and their cell surface density is tightly regulated to prevent aberrant activation.[20] After ligand binding, Janus kinases (JAK) activate and phosphorylate the receptor cytoplasmic domain. This phosphorylation allows the recruitment of several signaling molecules such as the signal transducer and activator of transcription (STAT) proteins, which in turn are phosphorylated by JAK.[21,22] Other pathways commonly activated by type I cytokine receptors which are also dependent on JAK activation are the phosphoinositide 3-kinase (PI3K)/AKT pathway and the ERK pathway, both of which are essential for the control of cell proliferation, cell survival and differentiation.[23,24] To protect cells from over-stimulation, several mechanisms exist to attenuate signal transduction: recruitment of tyrosine phosphatases to inactivate JAK;[15] synthesis of suppressor of cytokine signaling (SOCS) proteins that can inactivate JAK;[25] receptor internalization and subsequent degradation through the proteasome and lysosome pathways;[26,27] and, finally, the modification of ligand-receptor kinetic parameters.[28] There is therefore strong interest in identifying other proteins able to modulate the intensity of these crucial signaling axes. Using different human-relevant models, we identified a crucial role for ANKRD26 in modulating TPO-, EPO- and G-CSF-dependent cell signaling, and thereby in the generation of the hematopoietic cells controlled by these cytokines.

Methods

Study approval

Blood samples from patients and healthy subjects were collected after informed written consent and obtained in accordance with the Declaration of Helsinki. The study was approved by the Comité de Protection des Personnes (CPP 2020T2-02) and by AP-HP, Hôpital Saint-Louis, Unité de Thérapie Cellulaire, CRB-Banque de Sang de Cordon, Paris, France (AC-2016-2759).

Animal experiments were performed in accordance with 2010/63/UE European legislation and decree N 2013-118 of French legislation and recorded under protocol number APAFIS# 2016-008-7175.

Primary cell culture

CD34$^+$ cells were isolated from umbilical cord blood or peripheral blood by positive selection using an immunomagnetic bead cell-sorting system (AutoMacs; Miltenyi Biotec).

UT7 cell lines

Human UT7 megakaryoblastic cells expressing HA_MPL[29] and HA_EPOR[30] have been previously reported, and a human UT7 line expressing HA_G-CSFR was obtained by the transduction of UT7 parental cells with a retrovirus encoding HA_G-CSFR.

Generation and expansion of induced pluripotent stem cells

Patients' CD34$^+$ cells were expanded in serum-free medium containing EPO (1 U/mL), FLT3L (10 ng/mL), G-CSF (20 ng/mL), interleukin (IL)-3 (10 ng/mL), IL-6 (10 ng/mL), stem cell factor (SCF; 25 ng/mL), TPO (10 ng/mL) and GM-CSF (10 ng/mL) for 6 days and transduced with VSV-G pseudotyped retroviruses encoding for the OSKM combination (OCT4, SOX2, KLF4 and c-MYC). Colonies with an ES-like morphology were manually isolated, expanded for a reduced number of passages and frozen. The induced pluripotent stem cell (iPSC) control cell lines C1, C2 and C3 were already established and previously characterized.[31-33]

Clonogenic potential of primary cells in semi-solid culture

Methylcellulose culture assay

Hematopoietic progenitors (CD34$^+$CD43$^+$ for iPSC and CD34$^+$ for primary cells) were plated in human methylcellulose medium H4434 (Stemcell Technologies), containing recombinant human cytokines and scored for the presence of colonies 14 days after.

Fibrin clot assay

To assess colony-forming unit - megakaryocyte (CFU-MK) potential, CD34$^+$GFP$^+$ or CD34$^+$Cherry$^+$ sorted cells were seeded at 1.5x10^3 cells/mL in triplicate in fibrin clot medium, as previously described.[32]

Additional information

Other protocols are detailed in the *Online Supplementary Materials and Methods* and all the primers, antibodies and other reagents are listed in *Online Supplementary Table S1.*

Statistics

All data are shown as mean ± standard deviation unless specified differently. The statistical analyses were performed using PRISM software. Statistical significance was established using a Student *t* test, as specified in the legends, or one-way analysis of variance (ANOVA), followed by all pairwise multiple comparison procedures. Differences were considered statistically significant for *P* values <0.05.

Results

ANKRD26 regulates the early steps of megakaryopoiesis

We have previously shown that the overexpression of ANKRD26 in megakaryocytes of THC2 patients leads to thrombocytopenia in these patients as a result of deregulation of the TPO/MPL signaling axis[10] at the late stages of megakaryocyte differentiation. Indeed, the abnormal presence of ANKRD26 in the terminal phases of megakaryocyte maturation and consequent stronger MAPK-pathway activation cause a strong reduction in the number of proplatelet-forming megakaryocytes in THC2 patients.

We have transduced cord blood CD34$^+$ progenitor cells with a lentiviral vector encoding ANKRD26 (*Online Supplementary Figure S1A*), in order to assess the impact of this protein on the early steps of megakaryopoiesis. ANKRD26 overexpression only slightly increased the number of megakaryocyte progenitors at 10 ng of TPO (Figure 1A) without affecting their proliferation (Figure 1B) but, as previously shown, completely prevented proplatelet formation (Figure 1C, D) without disturbing MPL expression level at the megakaryocyte cell surface (*Online Supplementary Figure S1B*).

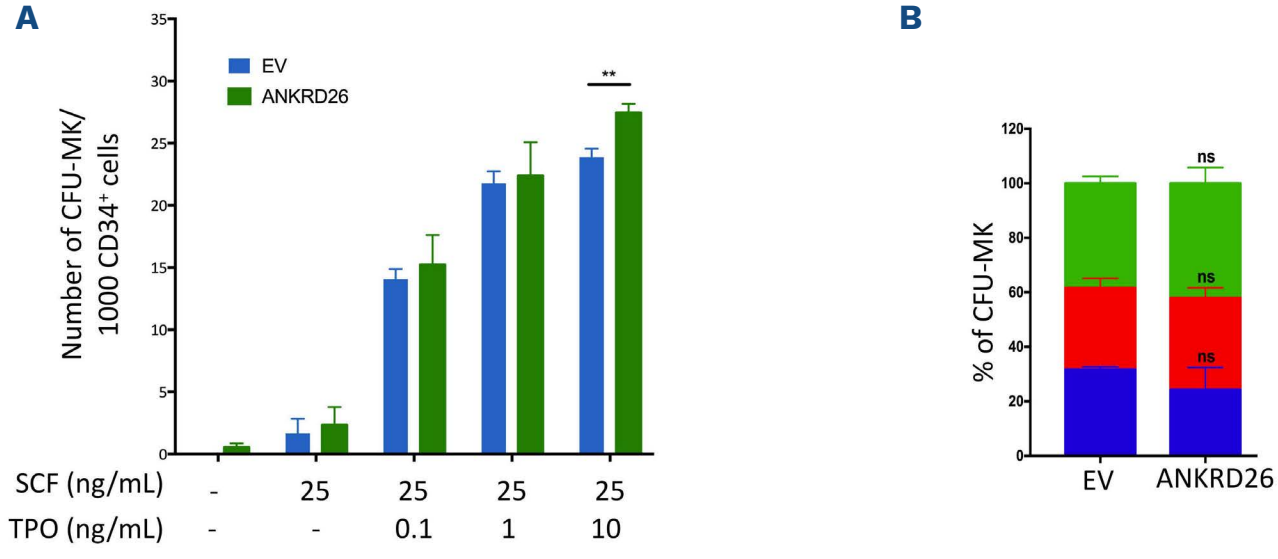


Figure 1. ANKRD26 overexpression alters late but not early stages of megakaryopoiesis. (A, B) Primary CD34$^+$ cells were transduced with empty lentivirus (EV) or lentivirus encoding *ANKRD26_V5* (ANKRD26) and Cherry. CD34$^+$-Cherry cells were sorted at day 2 after transduction and cultured in semi-solid medium (fibrin clot medium) in the presence of stem cell factor (SCF) and thrombopoietin (TPO). Colonies derived from megakaryocyte progenitors were assessed by anti-CD41 antibody labeling. (A) Plating efficiency of megakaryocyte progenitors (CFU-MK) was only slightly increased after ANKRD26 overexpression in the presence of 10 ng/mL TPO. (B) The proliferative rate of CFU-MK was not affected by increased ANKRD26 level. Proliferation was assessed according to the size of the colonies (three types of colonies were scored: <20 cells/colony, <50 cells/colony and >50 cells/colony), each colony corresponding to one progenitor. The averages of three independent experiments are shown as the mean ± standard error of mean. **P<0.01; paired t test, ns: non-significant. (C, D) Primary CD34$^+$ cells were transduced with EV or lentivirus encoding *ANKRD26_V5* (ANKRD26) and a gene resistant to hygromycin B. ANKRD26 overexpression in primary CD34$^+$ cells cultured in the presence of SCF and TPO and hygromycin B prevented proplatelet formation, evaluated by inverted light microscopy at day 14 of culture. (C) Representative pictures of one experiment are shown. (D) The histogram represents the averages of five independent experiments as mean ± standard deviation. ***P<0.005; paired t test. MK: megakaryocytes.

ANKRD26 has been shown to be expressed more in CD34⁺ progenitor cells than in mature megakaryocytes, suggesting that its expression is necessary during the early stages of hematopoiesis[10,34] (*Online Supplementary Figure S2A, B*). As overexpression had no major effect on CD34⁺ cells, we decided to investigate whether ANKRD26 downregulation at the CD34⁺ progenitor level could affect the number of megakaryocyte progenitors (CFU-MK). We transduced cord blood CD34⁺ hematopoietic progenitors with lentiviruses encoding short hairpin RNA (sh) against *ANKRD26* (shANK_1 or shANK_2) (*Online Supplementary Figure S1C*) and cultured them in fibrin clot medium, in the presence of SCF and increasing doses of TPO. We observed a 50% decrease in CFU-MK plating efficiency (Figure 2A), as well as a reduction of their proliferative rate, as attested by the lower number of megakaryocytes per colony (Figure 2B). This result was confirmed in liquid culture in the presence of SCF and TPO (*Online Supplementary Figure S1D, E*). Interestingly, the reduction in ANKRD26 also led to a lower frequency of mature megakaryocytes (CD41⁺CD42⁺) at day 10 of culture

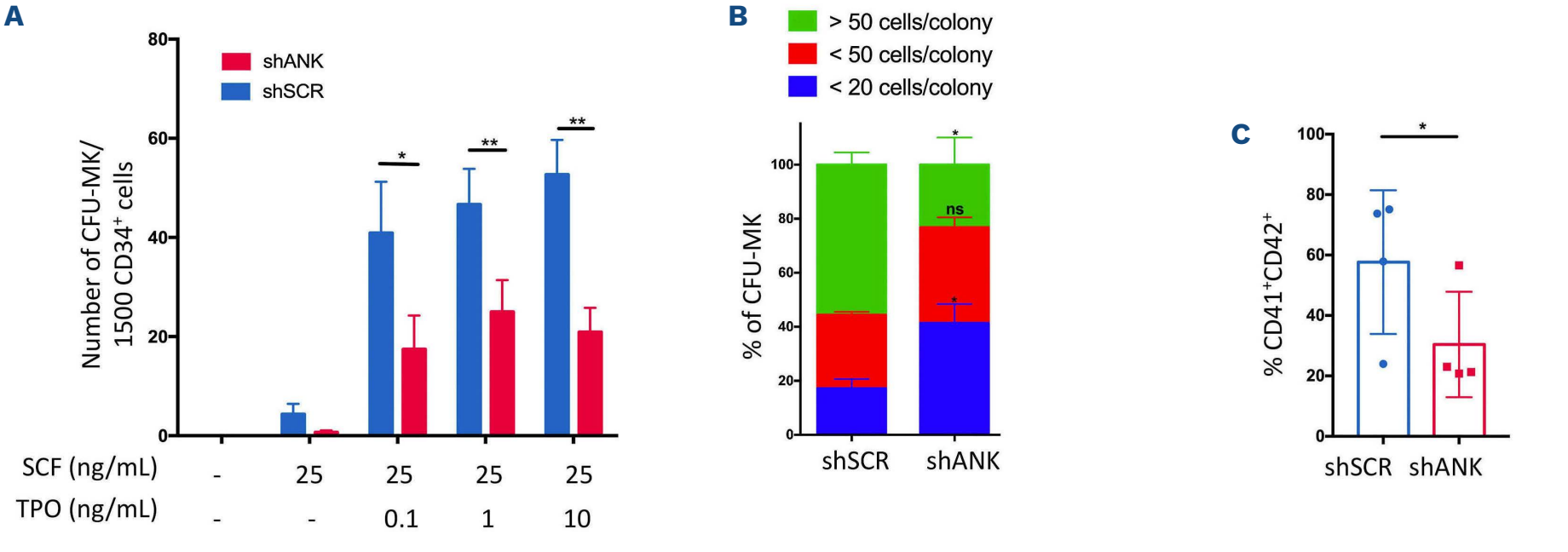


Figure 2. ANKRD26 is necessary for early but not late stages of megakaryopoiesis. (A-F) CD34⁺ cells were transduced with shSCR or shANK and GFP encoding lentiviruses, sorted 2 days after transduction and cultured in semi-solid medium (fibrin clot medium) in the presence of stem cell factor (SCF) and thrombopoietin (TPO) (A, B) or in liquid medium (C-F). (A, B) Colonies derived from megakaryocyte progenitors were assessed by anti-CD41 antibody. (A) Plating efficiency of megakaryocyte progenitors (CFU-MK) was decreased after inhibition of ANKRD26 (shANK), in the presence of different TPO doses. (B) The proliferation rate of megakaryocyte progenitors was decreased after ANKRD26 inhibition, as shown by the increase of colonies composed of less than 20 cells and the decrease of those with more than 50 cells. The averages of three independent experiments are shown as the mean ± standard error of mean. *P<0.05; **P<0.01; ns: non-significant; paired t test. (C) The inhibition of ANKRD26 led to a decreased frequency of megakaryocytes (CD41⁺CD42⁺ cells) at day 10 of culture. (D-F). In contrast, an increase in the ploidy level (D) and in the percentage of proplatelet-forming megakaryocytes was detected after ANKRD26 inhibition (E, F). (E) Representative pictures of one experiment are shown. (C, D, F) The histograms represent the averages of three (D), four (C) or six (F) independent experiments as the mean ± standard deviation, *P<0.05; ****P<0.001, paired t test. All the ANKRD26 inhibition experiments were performed at least twice with shANK_1 and once with shANK_2. MK: megakaryocytes.

(Figure 2C), but with a higher ploidy level (Figure 2D) and enhanced proplatelet formation capacity (Figure 2E, F). Together these results demonstrate that ANKRD26 is required for optimal proliferation and differentiation of megakaryocyte progenitors, but that its downregulation is required for thrombopoiesis.

ANKRD26 regulates the early steps of granulopoiesis

ANKRD26 expression decreases not only during normal megakaryocyte differentiation but also during erythroid and granulocytic differentiation (*Online Supplementary Figure S2A, B*). To confirm that ANKRD26 deregulation could be responsible for the leukocytosis detected in some THC2 patients, we studied granulopoiesis in primary patients' cells (*Online Supplementary Table S2*). Although ANKRD26 expression was not different in progenitors (CD34⁺ cells) isolated from the peripheral blood of THC2 patients, a significant increase in ANKRD26 levels was de-

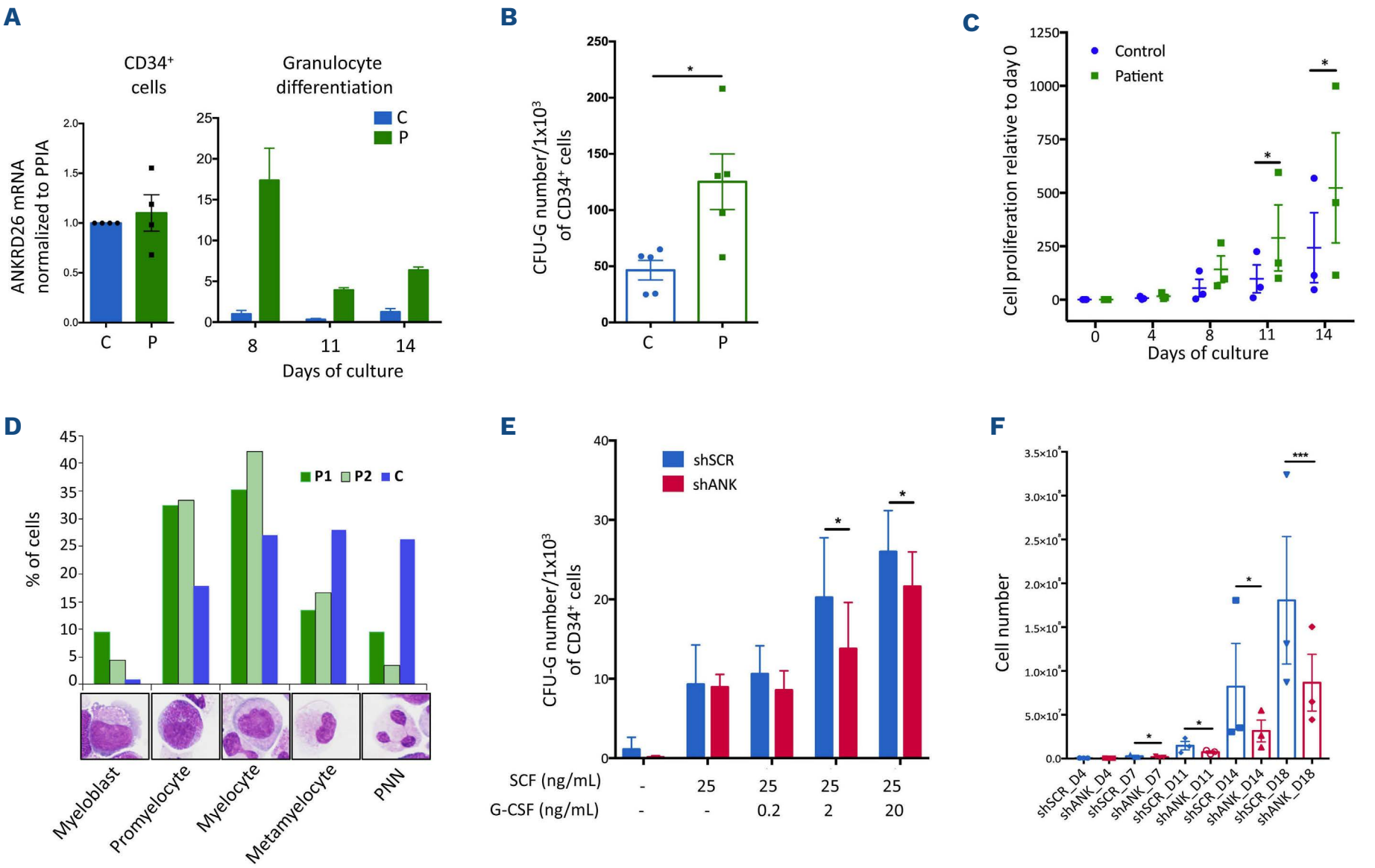


Figure 3. ANKRD26 regulates early stages of granulopoiesis. Primary CD34⁺ cells obtained from peripheral blood of patients with thrombocytopenia 2 with different 5' untranslated region mutations were induced to granulocytic differentiation in the presence of granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3) and stem cell factor (SCF). (A) ANKRD26 expression in patients' CD34⁺ cells [P] was similar to that in control CD34⁺ cells [C] obtained from healthy individuals, but increased during *in vitro* granulocytic differentiation, with a peak at day 8 of culture. *ANKRD26* transcript level was normalized to *PPIA*. Averages are shown for four (for CD34⁺ cells) and two (for granulocytic differentiation) independent experiments. (B) The number of patients' myeloid progenitors (CFU-G) was significantly higher, compared to control progenitors, as assessed by a methylcellulose assay. The averages of five independent experiments are shown as mean ± standard error of mean. **P*<0.05; *t* test with Mann-Whitney correction. (C) Proliferation assay performed in liquid culture supplemented with G-CSF, SCF and IL-3 showed a significant increase in cell number for patients' samples at days 11 and 14 of culture. The cell count was normalized to day 0. Averages of three independent experiments are shown as mean ± standard error of mean, **P*<0.05; ***P*<0.01; paired *t* test. (D) May-Grünwald Giemsa staining of samples from two patients and one control at day 15 of culture showed an increase in the proportion of immature cells (myeloblasts, promyelocytes and myelocytes) and a decrease in the proportion of more mature cells (metamyelocytes and polynuclear neutrophils). (E, F) Effect of ANKRD26 inhibition on the granulocytic lineage. CD34⁺ cells were transduced with lentiviruses encoding shSCR and shANK (shANK_1 or shANK_2, respectively). (E) CD34⁺-GFP cells were sorted 2 days after transduction and grown in semi-solid medium (methylcellulose) in the presence of 25 ng/mL SCF and different doses of G-CSF. Granulocytic progenitors (CFU-G) were enumerated at day 14 of culture. (F) CD34⁺-GFP cells were sorted 2 days after transduction and grown in liquid medium in the presence of 25 ng/mL SCF, 10 ng/mL IL-3 and 20 ng/mL G-CSF. Proliferation assays showed a significant decrease in shANK transduced cell number at days 7, 11, 14 and 18. The averages of three independent experiments are shown as mean ± standard error of mean, **P*<0.05; ***P*<0.01; paired *t* test. PNN: polynuclear neutrophils.

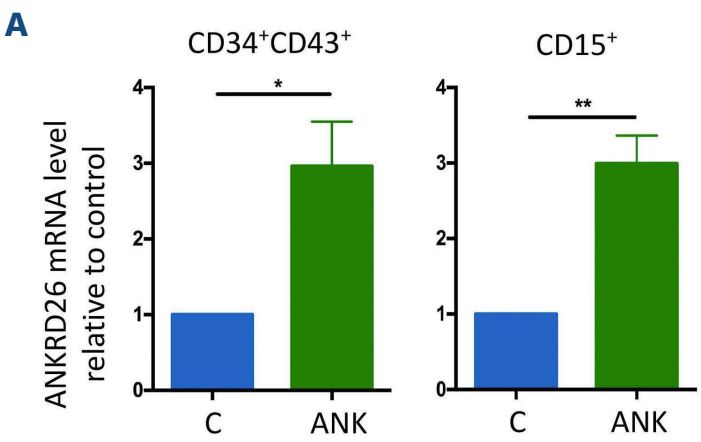
tected through *in vitro* granulocyte differentiation (Figure 3A). To evaluate the biological effects of increased ANKRD26 expression, we assessed the colony-forming potential of patients' $CD34^+$ cells in semi-solid medium and their proliferative rate in the presence of SCF, IL-3 and G-CSF.[35] We detected a more than two-fold increase in granulocyte colony (CFU-G) numbers (Figure 3B), as well as an increased proliferative capacity (Figure 3C), compared to healthy controls. We performed May-Grünwald Giemsa staining at day 15 of culture for two patients, and detected an increased frequency of immature cells (myeloblasts, promyelocytes and myelocytes) compared to controls, while the percentages of metamyelocytes and polynuclear neutrophils were clearly decreased (Figure 3D, *Online Supplementary Figure S3A*). Nevertheless, the percentages of mature granulocytes for patients' cells increased over time (*Online Supplementary Figure S3B*), suggesting a maturation delay due to the increased proliferation, and not a complete blockage. Overall, these results show that ANKRD26 overexpression leads to defective granulopoiesis in THC2 patients, with increased proliferation and delayed maturation. It should be noted that different subtypes of acute myeloid leukemia express higher ANKRD26 levels as compared to the corresponding committed progenitors (*Online Supplementary Figure S2B*) suggesting that ANKRD26 overexpression may play an important role in the proliferative phenotype of leukemic cells. To further confirm the role of ANKRD26 in the granulocytic lineage, we transduced the cord blood $CD34^+$ progenitors with lentiviruses encoding shANK and cultured them in increasing doses of G-CSF. We observed a reduction in the number of granulocytic progenitor-derived colonies (CFU-G) in methylcellulose assay in the presence of 2 and 20 ng of G-CSF (Figure 3E), and a decreased proliferation rate in liquid culture (Figure 3F) with accelerated differentiation (*Online Supplementary Figure S3C*), and no effect on apoptosis (*Online Supplementary Figure S3D*).

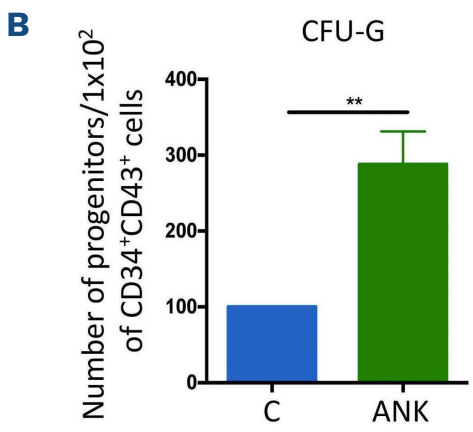
Patient-derived induced pluripotent stem cells recapitulate the defective granulopoiesis

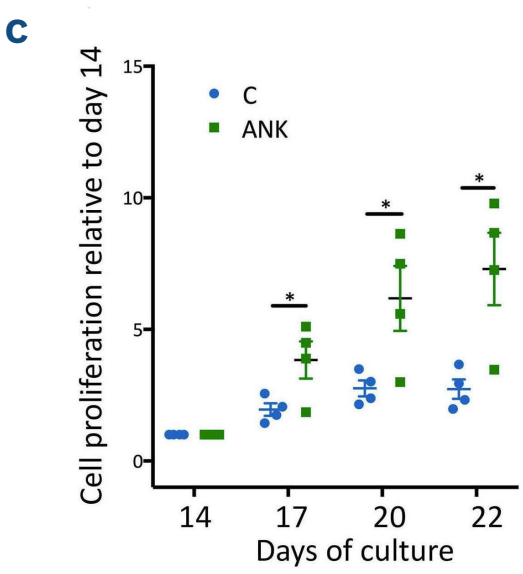
To overcome the limitations associated with the rarity of THC2 patients, we derived iPSC lines from two patients harboring two different mutations: c.-118A>C (ANK1) and c.-127delAT (ANK2) (*Online Supplementary Table S3*). The iPSC lines were generated from $CD34^+$ cells isolated from peripheral blood via integrative reprogramming and were characterized for their phenotypic and functional pluripotency (*Online Supplementary Figure S4*). Three different clones were studied for each patient-derived iPSC. As a control, we used three different iPSC lines derived from healthy individuals.[31-33]

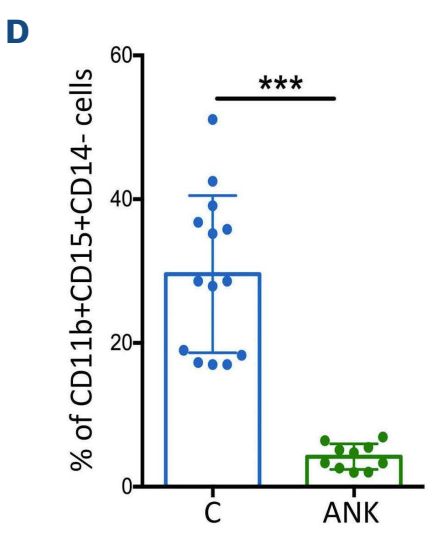
We used a serum-free, xeno-free differentiation protocol (*Online Supplementary Figure S5*). We isolated the hematopoietic progenitors after 14 days of culture ($CD34^+CD43^+$), a time described to give a bias towards granulo-monocytic differentiation output.[33] ANKRD26 expression in patients' iPSC-derived $CD34^+CD43^+$ cells was three times higher than in controls (Figure 4A); this difference with respect to ANKRD26 expression in cord blood-derived early progenitors ($CD34^+$ cells) can be explained by the fact that iPSC-derived $CD34^+CD43^+$ cells are developmentally different from adult $CD34^+$ cells and are already committed to the myeloid lineage. We detected three times more CFU-G colonies in semi-solid medium compared to the numbers from healthy controls (Figure 4B). We also found an increased proliferative rate in liquid culture in the presence of SCF, IL-3 and G-CSF (Figure 4C). $CD15^+$ granulocytic cells derived from $CD34^+CD43^+$ patients' cells expressed higher levels of ANKRD26 as compared to control $CD15^+$ cells (Figure 4A). Flow cytometry and analysis of May-Grünwald Giemsa staining revealed a delay in granulocytic differentiation at days 17-23 of culture (*Online Supplementary Figure S6A*): myeloid cells derived from patients' iPSC displayed less than 10% of mature $CD11b^+CD15^+CD14^-$ cells, compared to about 30% for the control cell lines at day 23 (Figure 4D, *Online Supplementary Figure S6A*). Overall, these results clearly show defective granulopoiesis, similar to that observed for primary cells. To gain more insights into this defect, we investigated the transcriptomic profile of the patients' granulocytic progenitors: iPSC-derived granulocytic progenitors ($CD43^+CD11b^+CD14^-$) were sorted and profiled by RNA sequencing. We found 24 genes significantly upregulated and seven significantly downregulated ($P<1x10^{-5}$) in patients' cells compared to controls (Figure 4E, *Online Supplementary Table S4*). Gene set enrichment analysis performed with two different databases (KGEA and GOBP) revealed a significant enrichment in the JAK/STAT signaling pathway in patient-derived iPSC progenitors (Figure 4F) with a tendency to increased granulocytic count and delayed myeloid development (*Online Supplementary Figure S6B*) confirming the results obtained with primary patients' cells and with shANK in cord blood-derived progenitors.

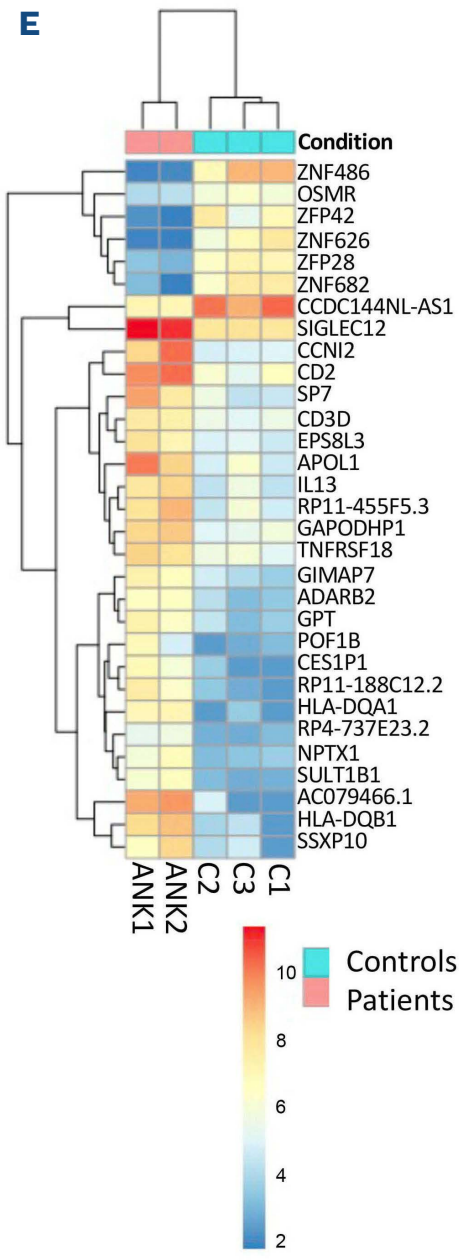
Within the significantly upregulated genes, the *CCNI2* gene (Cyclin I family member 2), a cyclin responsible for cyclin-dependent kinase 5 (CDK5) activity,[36] caught our attention. CDK5 is generally not directly involved in cell cycle regulation and has been described to phosphorylate NOXA thereby promoting its cytosolic sequestration and suppression of apoptosis in leukemic cells.[37] However, depletion of CCNI2 has been shown to inhibit cell cycle progression and proliferation.[36] CCNI2 expression was almost completely absent from control iPSC-derived $CD43^+CD11b^+CD14^-$ cells. After validation of CCNI2 upregulation in patients' iPSC-derived progenitors by quantitative











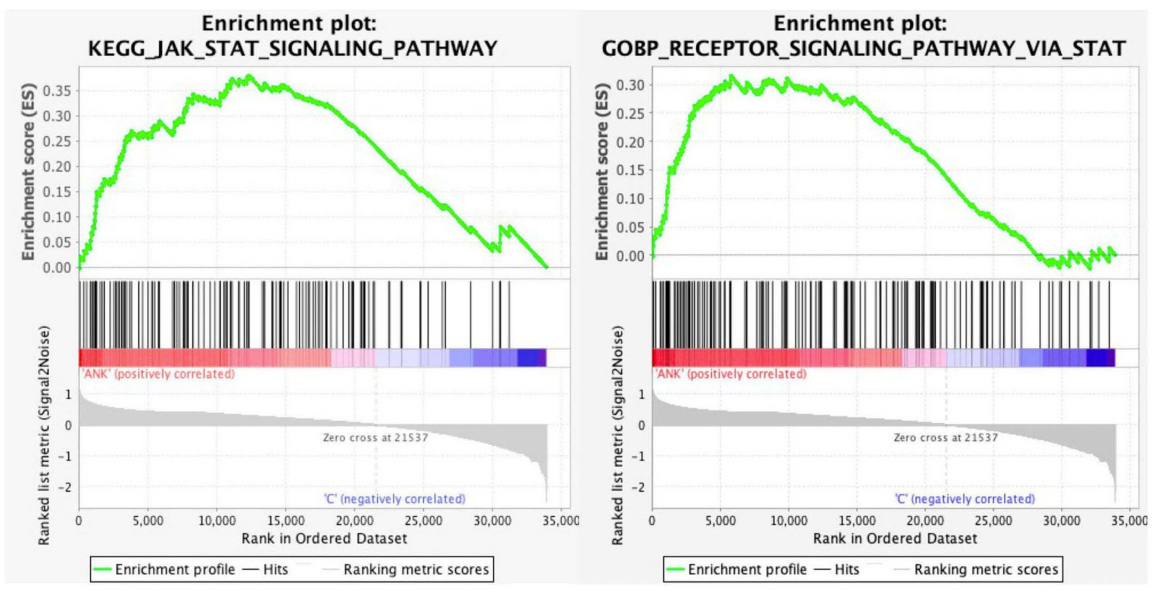


Figure 4. Increased ANKRD26 expression level enhances proliferation of granulocyte progenitors in a model of induced pluripotent stem cells, through an enhanced JAK/STAT pathway. $CD34^+CD43^+$ progenitors derived from induced pluripotent stem cells (iPSC) from patients [ANK] and controls [C] were differentiated into granulocytes in the presence of granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3) and stem cell factor (SCF). (A) ANKRD26 expression in $CD34^+CD43^+$ progenitors (n=4) and in $CD11b^+CD15^+CD14^-$ cells (n=6) derived from patients' iPSC was increased when compared to that in controls. *ANKRD26* transcript was normalized to *PPIA*. Results are shown as mean ± standard deviation, **P*<0.05; ***P*<0.01; paired *t* test. (B) The number of patients' granulocytic progenitors (CFU-G) derived from iPSC was significantly higher than the control, as assessed by methylcellulose assay. The averages of seven independent experiments are shown as mean ± standard deviation. ***P*<0.01; paired *t* test. (C) The proliferation rate was significantly increased at days 17, 20 and 22 of culture for patients' cells as compared to controls. The averages of four independent experiments are shown as the mean ± standard error of mean. **P*<0.05; paired *t* test. (D) $CD11b^+CD15^+CD14^-$ cell frequency analyzed at days 21-23 reflected a delay in maturation for patients' cells. Averages of 14 experiments for controls and ten for patients' cells are shown as the mean ± standard error of mean. ****P*<0.005, unpaired *t* test. (E) An RNA-sequencing assay was performed on $CD43^+CD11b^+CD14^-$ progenitors sorted at day 16 of culture. Seven downregulated and 24 upregulated genes in patients' cells (n=3 for each, ANK1 and ANK2) ($P<1x10^{-5}$) were identified compared to controls (n=1 for C1 and C3, n=3 for C2). (F) Gene set enrichment analysis for the KEGG_JAK_STAT_signaling pathway and for the receptor signaling pathway via STAT (GO:0007259, GOBP_RECEPTOR_SIGNALING_PATHWAY_VIA_STAT) in ANK *versus* control iPSC. NES: normalized enrichment score; FDR: false discovery rate.

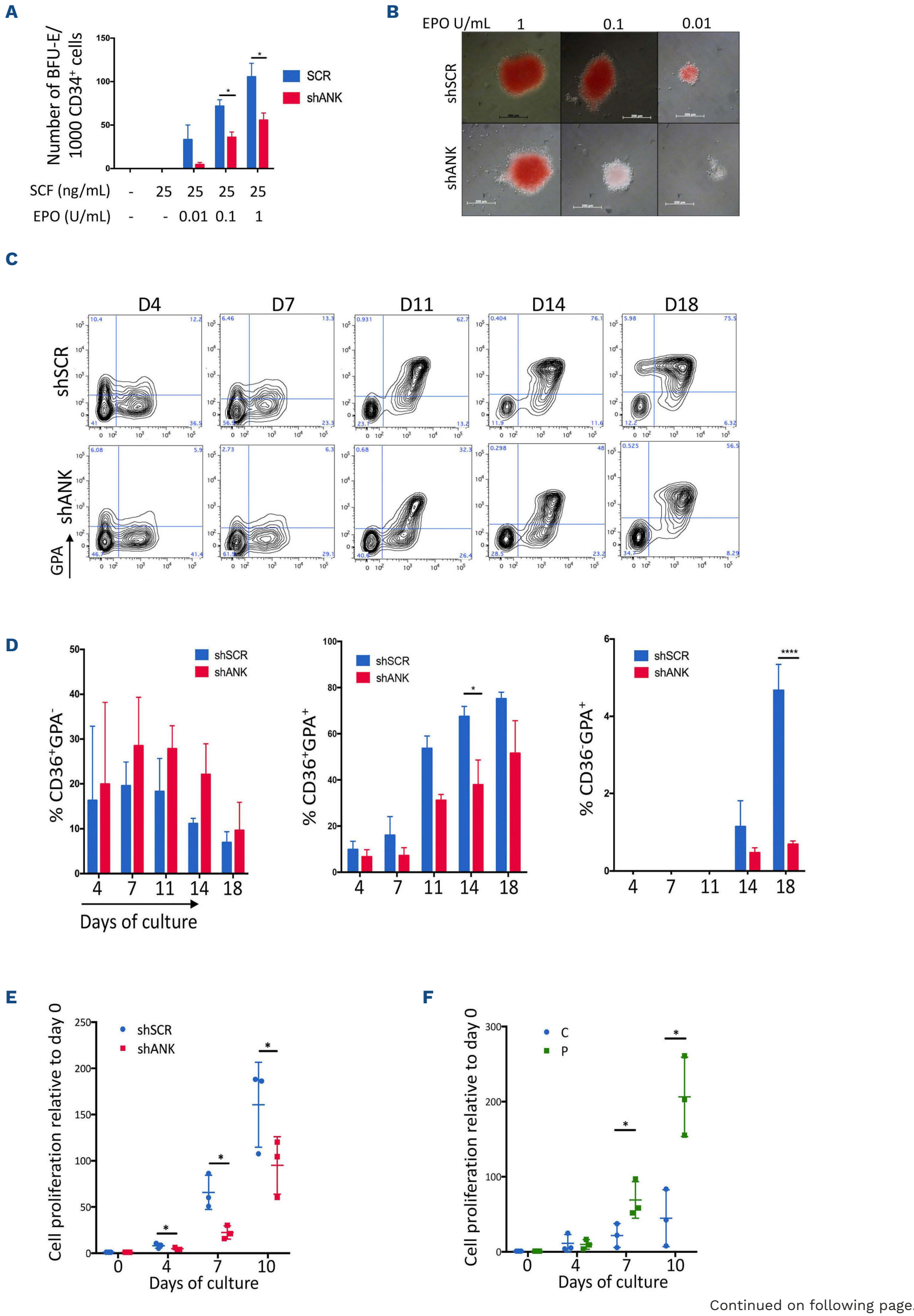


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Figure 5. ANKRD26 regulates the early stage of erythropoiesis. (A, B) CD34$^+$ cells were transduced with lentiviruses encoding shSCR or shANK and erythroid progenitors (BFU-E) were grown in semi-solid medium (methylcellulose) in the presence of 25 ng/mL stem cell factor (SCF) and different doses of erythropoietin (EPO) and enumerated at day 14 of culture. (A) ANKRD26 inhibition led to a significant decrease in BFU-E number. Averages of three independent experiments are shown as the mean ± standard deviation. *P<0.05; one-tailed t test with Mann-Whitney correction. (B) Representative pictures of BFU-E colonies showing that ANKRD26 inhibition led to a lack of hemoglobinization of BFU-E-derived colonies, both at 0.1 and 0.01 U/mL of EPO. (C-F) Transduced CD34$^+$ cells (C-E) or primary patients' CD34$^+$ progenitors (F) were cultured in liquid medium in the presence of EPO (1 U/mL), SCF and interleukin-3 for 18 days. (C, D) Kinetics of erythroid differentiation assessed by fluorescence activated cell sorting (C) showed that ANKRD26 inhibition leads to a delay in differentiation (D). CD36$^-$GPA$^-$ cells represent immature, CD36$^+$GPA$^+$ intermediate and CD36$^-$GPA$^+$ mature erythroid cells. Statistical analysis of different populations is shown as the average of three independent experiments (mean ± standard deviation, *P<0.05, ***P<0.005, 2-way analysis of variance with multiple comparisons). (E, F) ANKRD26 expression level affected proliferation of CD34$^+$ cells grown in erythroid conditions. (E) Inhibition of ANKRD26 led to a significant decrease in the proliferation of transduced CD34$^+$ cells cultured in erythroid conditions. The average of three independent experiments is shown as mean ± standard deviation, *P<0.05; paired t test. (F) Proliferation rate of primary CD34$^+$ cells from patients with thrombocytopenia 2 cultured in erythroid conditions was significantly higher compared to controls. The average of three independent experiments is shown as mean ± standard deviation, *P<0.05; t test with Mann-Whitney correction. D: day; GPA: glycophorin A.

real-time polymerase chain reaction analysis (*Online Supplementary Figure S6C*), we transduced the CD34$^+$CD43$^+$ progenitors at day 14 of culture with a lentivirus encoding shCCNI2 (shCCNI2_1 and shCCNI2_2), sorted the GFP$^+$ cells 48 hours later and measured their proliferative rate in granulocytic conditions at days 19, 21 and 23. The inhibition of CCNI2 by shRNA significantly reduced the number of granulocytes in patient-derived iPSC (*Online Supplementary Figure S6D*). Although an *in silico* analysis of the *CCNI2* promoter revealed the presence of two STAT5 binding sites (*data not shown*), the implication of this in the observed phenotype needs to be investigated further.

ANKRD26 regulates the early steps of erythropoiesis

The role of ANKRD26 in the regulation of erythropoiesis was investigated in cord blood CD34$^+$ cells. ANKRD26 downregulation led to a reduction in the plating efficiency of erythroid progenitors (BFU-E) in semi-solid assay, in the presence of SCF and increasing doses of EPO (Figure 5A). Moreover, colonies from shANK progenitors were paler, indicating a decrease in hemoglobin content at lower EPO doses (0.01 and 0.1 U/mL), a sign of impaired differentiation (Figure 5B). A delay of erythroid differentiation in the presence of lower ANKRD26 levels was confirmed in a kinetics assay in liquid medium, in the presence of EPO, SCF and IL-3 (Figure 5C, D). Finally, cord blood CD34$^+$ cells transduced with shANK and cultured in erythroid conditions showed reduced proliferation compared to the control (Figure 5E). This is consistent with the increased proliferation observed for THC2 patient-derived cells cultured in the same conditions (Figure 5F). We measured the serum EPO levels in seven THC2 patients and detected normal values for three patients without erythrocytosis. In four patients with erythrocytosis, the serum EPO levels were slightly lower and either below or at the lower limit of normal values, suggesting that erythrocytosis was cell autonomous (*Online Supplementary Table S5*).

ANKRD26 regulates MPL, G-CSFR and EPO-mediated signaling

To further understand how ANKRD26 could modify the proliferation and differentiation of hematopoietic progenitors under TPO, G-CSF and EPO stimulation, we investigated whether it may interfere with the signaling of their cognate receptors. For these studies we used two cell lines, the murine pro-B Ba/F3 and the human erythro-MK UT7 cell lines which were rendered dependent on TPO, G-CSF or EPO via retrovirus encoding *MPL, G-CSFR* or *EPOR*. In the Ba/F3 cell line we overexpressed human ANKRD26 and in the UT7 cell line expressing a high level of human ANKRD26, we downregulated its expression by two different shRNA.

ANKRD26 levels did not affect MPL, G-SCFR and EPOR levels at the cell surface either at the steady state (Figure 6A, *Online Supplementary Figures S7, S8A* and *S9A*) or after overnight starvation (Figure 6B, *Online Supplementary Figures S7, S8B,* and *S9B*). We confirmed our previous observation[10] that higher ANKRD26 levels induced stronger STAT5, ERK, AKT and also STAT3 phosphorylation at 10 ng/mL of TPO (Figure 6C, *Online Supplementary Figure S10A*). Moreover, we observed that a very low TPO dose (0.01 ng/mL) was still able to stimulate TPO/MPL signaling in cells expressing higher levels of ANKRD26 (Figure 6D). To determine whether ANKRD26 increases MPL activation and signaling by different ligands, we assessed the response to the TPO-mimetic eltrombopag which, in contrast to TPO, binds to the transmembrane region involving H499,[38] inducing downstream signaling in a different conformation than that done by TPO.[39] Higher levels of ANKRD26 led to the same hypersensitivity for eltrombopag as observed for TPO (*Online Supplementary Figure S10B, C*), suggesting that the main effect of ANKRD26 is to stabilize MPL surface expression without changing its conformation.

Using UT7 cells, we showed that the higher ANKRD26 levels led to stronger G-CSF-mediated STAT3 and AKT phosphorylation, with a similar tendency for ERK1/2 (*On-*

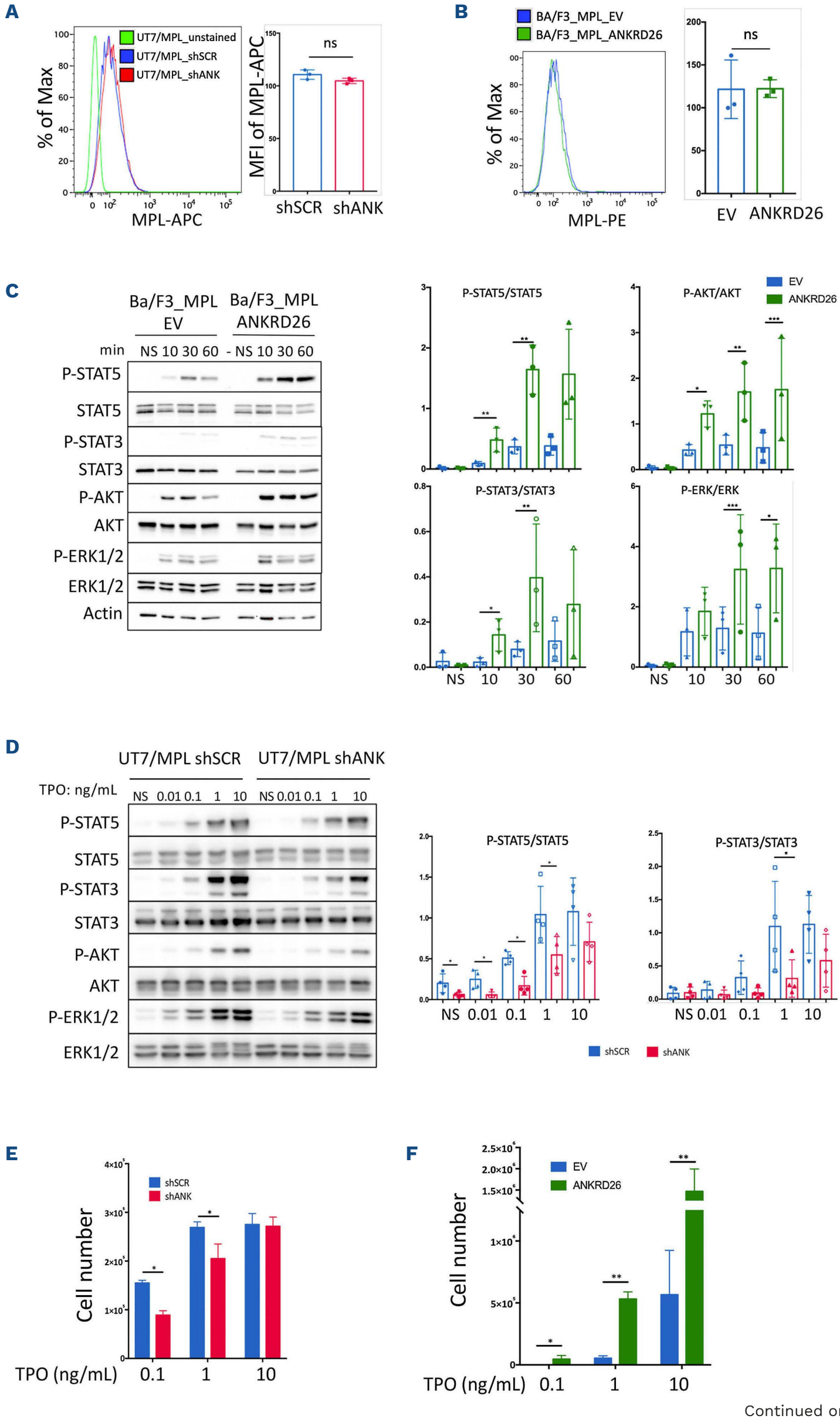

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Figure 6. ANKRD26 regulates MPL-mediated signaling. UT7 or Ba/F3 cell lines expressing MPL were transduced with lentiviruses harboring control scramble shRNA (shSCR), shANKRD26 (shANK), ANKRD26 cDNA or empty vector (EV). (A, B) Downregulation (A) or upregulation (B) of *ANKRD26* expression level did not affect the expression of MPL measured with anti-MPL antibody. The receptor levels are presented as median fluorescence intensity at the cell surface. The averages of three independent experiments are shown as mean ± standard deviation (2 with shANK1_1 and 1 with shANK_2). ***P*<0.01; *****P*<0.001; ns: non-significant, *t* test with Mann-Whitney correction. (C, D) One of at least three independent western blot (WB) analyses on signaling proteins in Ba/F3 and UT7 cells, at different times after stimulation with 10 ng/mL of thrombopoietin (TPO) (C), and different TPO doses (D) at 10 min. The histograms show quantification of the WB representing averages of three or four independent experiments as mean ± standard deviation. **P*<0.05; ***P*<0.01; ****P*<0.005; paired *t* test. (E, F) Number of UT7/MPL (E) and Ba/F3_MPL (F) cells measured at day 4 of culture, with three different doses of TPO are shown as mean ± standard error of mean of three independent experiments, **P*<0.05, ***P*<0.01; ns: non-significant, *t* test with Mann-Whitney correction. APC: allophycocyanin; MFI: median fluorescence intensity, PE: phycoerythrin; NS: not stimulated.

line Supplementary Figure S8C). The overexpression of human ANKRD26 in the Ba/F3 cell line expressing G-CSFR did not affect STAT3 activation but led to enhanced ERK and AKT phosphorylation (*Online Supplementary Figure S8D*). Using UT7/G-CSFR cells, we also observed the activation of STAT3 at a lower G-CSF dose (0.2 ng/mL), in the presence of higher ANKRD26 levels (*Online Supplementary Figure S8E*).

Finally, we showed that higher ANKRD26 levels led to stronger and more sustained EPO/EPOR-mediated activation of ERK1/2, AKT and, to a lesser extent, of STAT5 in UT7/EPOR cells (*Online Supplementary Figure S9C*). An increased level of AKT phosphorylation and the same tendency for ERK1/2 were also detected in the Ba/F3 cell line overexpressing ANKRD26 and EPOR (*Online Supplementary Figure S9D*). Moreover, a significantly increased level of STAT5 phosphorylation was detected at 0.1 U/mL of EPO in the presence of higher ANKRD26 levels (*Online Supplementary Figure S9E*).

To analyze the biological consequence of the TPO, G-CSF and EPO hypersensitivity, we measured the proliferation rate of UT7/MPL, UT7/G-CSFR and UT7/EPOR cells expressing shSCR and shANK at different cytokine concentrations. We observed that at 0.1 ng/mL of TPO only the UT7/MPL cells with higher ANKRD26 levels were able to proliferate, after 4 days of culture. At higher doses of TPO (10 ng/mL), no difference in the proliferation rate was detected (Figure 6E), a finding that corroborates the idea of receptor saturation at higher doses. A similar result was obtained in the presence of eltrombopag (*Online Supplementary Figure S10C*). In line with these results, the overexpression of ANKRD26 in murine Ba/F3_MPL cells led to an increased proliferation rate. At 0.1 ng/mL of TPO, only cells overexpressing ANKRD26 were able to grow (Figure 6F).

In a similar manner, UT7/G-CSFR cells expressing higher levels of ANKRD26 were able to proliferate in the presence of 0.2 ng/mL of G-CSF, unlike cells expressing lower ANKRD26 levels. Using higher doses of G-CSF (2 ng/mL), ANKRD26 downregulation led to a decreased proliferation rate, suggesting a progressive saturation of the cell surface pool of available G-CSFR (*Online Supplementary Figure S8F*). Finally, UT7/EPOR cells expressing higher ANKRD26 levels proliferated more at low EPO concentration (0.1 U/mL) than the control. This proliferation gap was absent at higher EPO doses (1 U/mL) (*Online Supplementary Figure S9F*).

Overall, these results demonstrate that higher ANKRD26 levels lead to MPL, G-CSF and EPO hypersensitivity.

ANKRD26 interacts with the homodimeric type I receptors

We then explored whether this effect of ANKRD26 was a direct or indirect effect on receptor signaling. First we investigated whether ANKRD26 interacts with MPL, G-CSFR and EPOR respectively. To this end, HEK293T cells were transduced with retroviruses encoding, respectively, HA_MPL, HA_G-CSFR or HA_EPOR and with a lentivirus encoding ANKRD26_V5. The interaction of ANKRD26 with each of these three homodimeric receptors was demonstrated by co-immunoprecipitation assays (Figure 7A-C). As a proof of concept, the ANKRD26 and MPL interaction was confirmed by proximity ligation assays in the UT7 cell line (*Online Supplementary Figure S11*) and in γ2A cells, in which it was shown that JAK2 was dispensable for this interaction, although its presence slightly enhanced it (Figure 7D-F).

Second, as ANK repeat-containing proteins have been shown to interact with different receptors and to participate in their internalization, one possible mechanism was that ANKRD26 affects receptor-mediated signaling by interfering with their internalization. To investigate this hypothesis, we used the murine Ba/F3 cell line overexpressing ANKRD26 and MPL, G-CSFR or EPOR, as this cell line is a better model for internalization studies than UT7 cells.[40] After overnight starvation, ANKRD26 did not modify receptor expression at the cell surface (Figure 6B, *Online Supplementary Figures S8B* and *S9B*). The MPL-expressing Ba/F3 cells were then exposed to 50 ng/mL of TPO. In the absence of human ANKRD26, about 40% of MPL was internalized 15 min after TPO exposure and additional exposure only slightly increased the quantity of internalized MPL. In contrast, the presence of human ANKRD26 almost completely abrogated MPL internalization, even after 60 min of TPO exposure (Figure 8A). Similarly, the overexpression of human ANKRD26 in the BaF/3 cell line expressing G-CSFR or EPOR led to a defect in G-CSFR or EPOR internalization at 15 and 30 min of stimulation of starved cells with G-CSF and EPO, respectively (Figure 8B, C). These results demonstrate that receptor internalization is finely regulated by ANKRD26 levels.

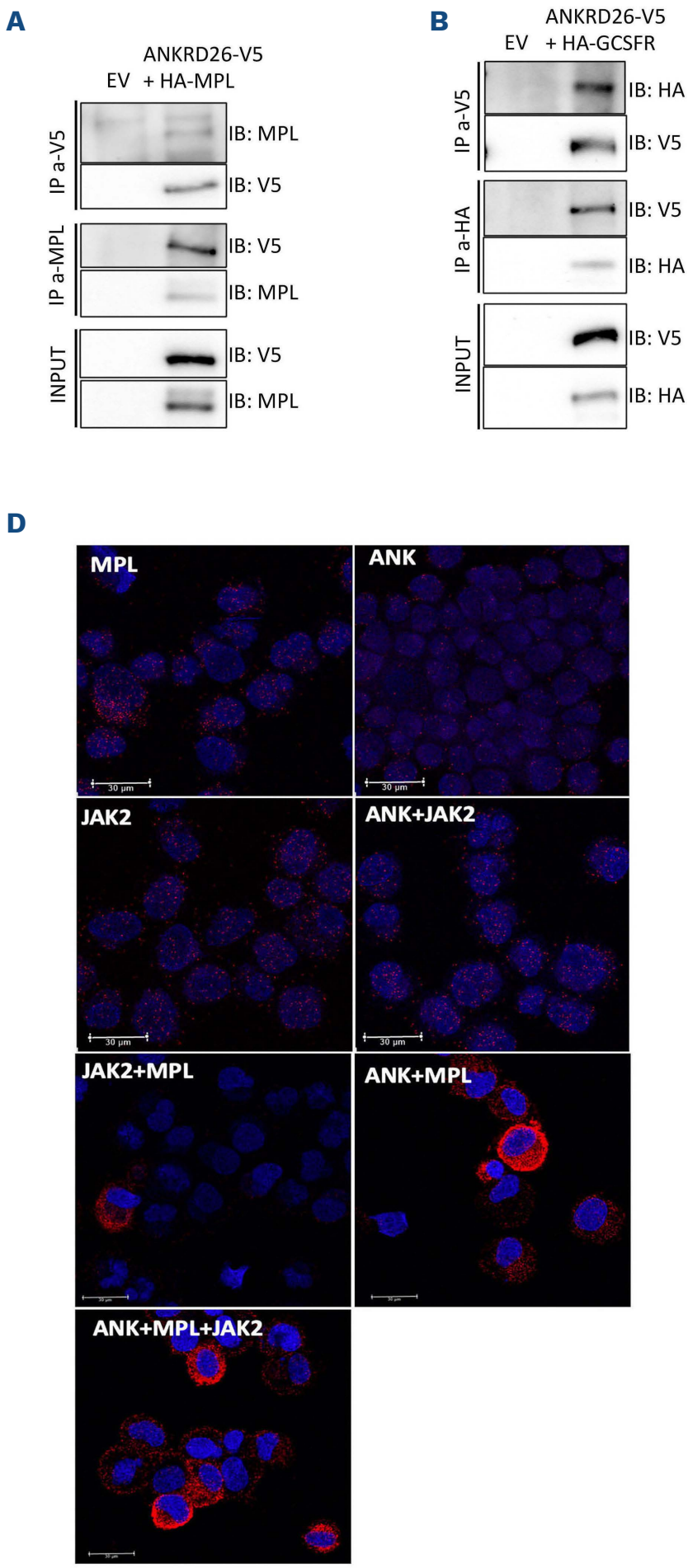

Figure 7. ANKRD26 interacts with homodimeric type I receptors. (A-C) Co-immunoprecipitation assay performed in HEK293 cells showing the presence of ANKRD26 and MPL (A), ANKRD26 and G-CSFR (B), and ANKRD26 and EPOR (C) in the same protein complex. For each receptor, one of three independent experiments with similar results are shown. Input represents western blot analysis of cells expressing empty vectors or cells co-expressing ANKRD26_V5 and HA_MPL (A), ANKRD26_V5 and HA_G-CSFR (B), or ANKRD26_V5 and HA_EPOR (C). The antibodies used were anti-V5 (for ANKRD26_V5), anti-MPL (for HA-MPL), and anti-HA (for HA-G-CSFR and HA-EPOR). (D-F) Proximity ligation assay for the ANKRD26 and MPL interaction. FLAG_ANKRD26 (ANK), HA_MPL (MPL) and JAK2 were overexpressed in γ2A cells (cells not expressing endogenous Jak2). Monoclonal anti-FLAG antibody was used for ANKRD26 and polyclonal anti-HA for MPL. (D) Representative pictures of the proximity ligation assay for the ANKRD26 and MPL interaction. The red staining represents the ANKRD26/MPL interaction, scale bar = 30 µM. (E) Data represent the mean of two independent experiments. (F) Data represent the number of dots per positive cell. At least 40 positive cells were analyzed for each condition. ***P<0.005, ****P<0.001, t test with Mann-Whitney correction. IP: immunoprecipitation, IB: immunoblot.

Discussion

In this work, we thoroughly demonstrate the role of ANKRD26 in the regulation of three myeloid lineages by modulating the activity of three type I cytokine receptors that are essential in normal hematopoiesis.

We confirmed a role for ANKRD26 in the regulation of the megakaryocyte lineage, in which its presence is crucial in the early steps of differentiation, but gets in the way of correct terminal maturation. Indeed, shRNA-mediated decrease of ANKRD26 expression in $CD34^+$ progenitors greatly reduced plating efficiency and the proliferative potential of megakaryocyte progenitors. On the other hand, overexpression of ANKRD26 prevented correct proplatelet formation in megakaryocytes, which agrees with observations in THC2 patients.[10]

For the first time we show here that ANKRD26 also plays a crucial role in the granulocytic and erythroid lineages, the other two main myeloid lineages governed by type I homodimeric receptors, G-CSFR and EPOR, respectively. At the immature stage, hematopoietic stem and progenitor cells express basal levels of ANKRD26. Gene downregulation in these cells leads to decreased proliferation and clonogenic potential, for the megakaryocytic, granulocytic and erythroid lineages, suggesting an important role of this protein in committed progenitors. Physiologically, ANKRD26 is progressively silenced along the cellular differentiation and maturation of these lineages. Our results show that abnormal ANKRD26 expression in the granulocytic lineage of THC2 patients affects the normal process of cellular proliferation/differentiation, which could lead to the leukocytosis reported in some patients. Whether this mechanism could be responsible for the establishment of a fertile substrate prone to the acquisition of secondary mutations remains to be explored.

Previously we have shown that the sustained ANKRD26 expression in the late phase of megakaryopoiesis leading to a defect in proplatelet formation was due to a deregulation of TPO/MPL signaling.[10] Here we demonstrated that the modulation of ANKRD26 expression levels modifies TPO sensitivity. Interestingly, although ANKRD26 expression does not increase MPL level at the cell surface, its higher expression increases MPL signaling activity leading to enhanced proliferation. This observation prompted us to hypothesize that ANKRD26 could be part of a complex that regulates the internalization of the receptor. By different approaches, we collected evidence that ANKRD26 and MPL interact, although further studies will be needed to determine whether this is a direct or indirect process. Similarly we showed that both G-CSFR and EPOR interact with ANKRD26. Interestingly, thrombocytopenia rather than thrombocytosis is detected in THC2 patients. This observation could be explained by the fact that despite an amplification of megakaryocyte progenitors due to increased JAK2/STAT signaling at the early stage of megakaryopoiesis, the inactivation of the MAPK-pathway necessary for correct proplatelet formation is not achieved.[10] In erythroid and granulocytic lineages, ANKRD26 modulates the proliferation rate but not late

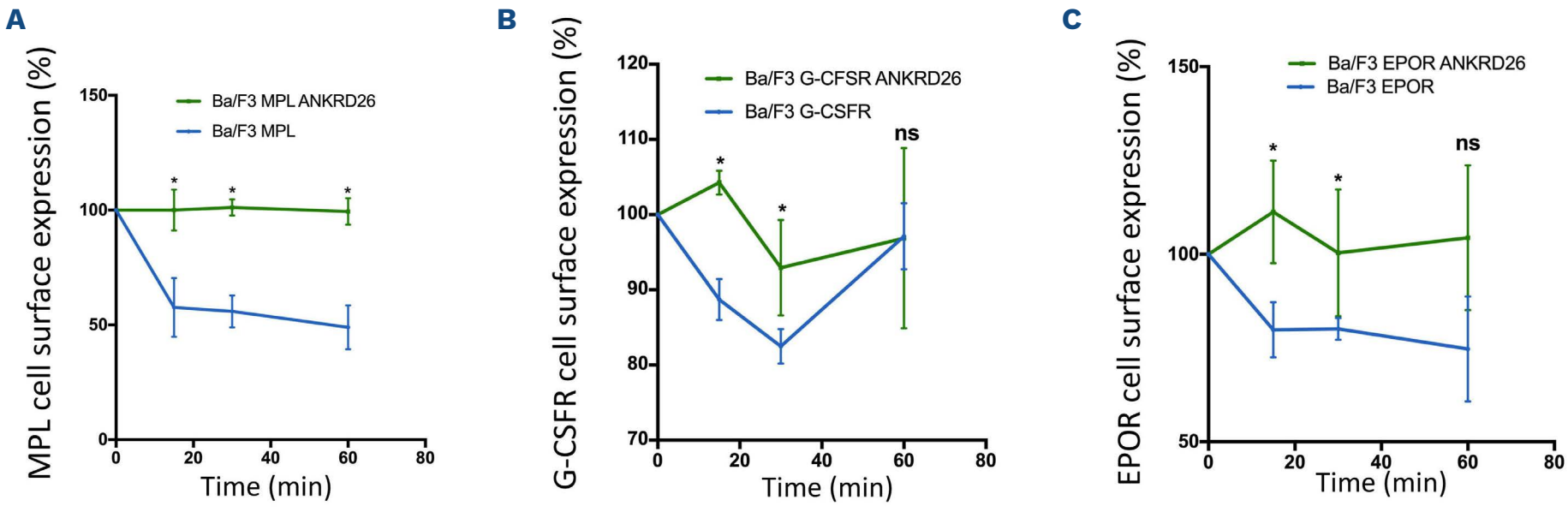

Figure 8. ANKRD26 regulates the internalization of homodimeric type I receptors. (A-C) Ba/F3 cells overexpressing the three receptors (MPL, G-CSFR, and EPOR) and transduced with either an empty vector or ANKRD26 cDNA encoding lentivirus were used for internalization assays. Internalization of MPL was measured with anti-MPL antibody (A), and that of G-CSFR (B) and EPOR (C) with anti-HA antibody. (A) In the absence of ANKRD26 overexpression, almost 50% of cell surface MPL was internalized as soon as 15 min after the addition of thrombopoietin, while in the presence of ANKRD26, MPL was not internalized. Mean fluorescence intensity (MFI) is normalized to that of Ba/F3/HA_MPL cells expressing empty vector. (B) ANKRD26 overexpression inhibited G-CSFR internalization at 15 and 30 min after stimulation of starved Ba/F3 cells with 20 ng/mL G-CSF. MFI is normalized to that of Ba/F3/HA_GCSFR cells expressing empty vector. (C) ANKRD26 overexpression significantly inhibited EPOR internalization at 15 and 30 min after starved Ba/F3 cell stimulation with 1 U/mL of EPO. MFI is normalized to that of Ba/F3/HA_EPOR cells expressing empty vector. The averages of three independent experiments are shown as mean ± standard deviation. *P<0.05; unpaired t test with Mann-Whitney correction.

stages of differentiation. Therefore, the severity of the phenotypes observed in THC2 patients is less pronounced and could be dependent on the mutation position that affects, to a variable extent, the binding of lineage-specific transcription factor(s) regulating ANKRD26 expression level in the granulocytic and erythroid lineages.

Leukocytosis and erythrocytosis are often a consequence of mutations that alter the correct signaling of G-CSFR and EPOR. *CSF3R* mutations are recurrent in chronic neutrophilic leukemia, which is characterized by excessive proliferation of the neutrophil lineage,[41-43] while *EPOR* mutations are a hallmark of familial erythrocytosis.[30,44] In both cases, the mutations may lead to the generation of truncated receptors that lack the specific domains that control the negative regulation of the signaling. We hypothesize that the persistent presence of ANKRD26 could act similarly to these mutations, by disturbing receptor internalization and shutdown. Several reports link ANK repeat-containing proteins to the interaction and regulation of signaling pathways and receptor internalization. Ankrd26, the mouse homolog of ANKRD26, is involved in the onset of obesity,[45-47] via regulation of signaling pathways,[48] and ANKRD26 was also described to interact with different proteins, including hyaluronan-mediated motility receptor (HMMR).[49]

How exactly ANKRD26 interferes with the regulation of type I cytokine receptor internalization and signaling is not completely clear. For example, ANKRD13A, 13B, and 13D were shown to bind to the ligand-activated EGFR through a ubiquitin-interacting motif (UIM), and direct rapid EGFR internalization, probably by connecting the receptor with the endocytic machinery via their ANK domain.[50] However, ANKRD26 does not contain the UIM domain and, according to the recently reported thrombocytopenia caused by WAC-ANKRD26 fusion, with a conserved C- but not N-terminal part containing ANK repeats,[34] only the C-terminal domain with a coiled-coil region seems to be necessary for the interactions with MPL, EPOR and G-CSFR. Further studies are necessary to clarify whether these interactions are direct or not and to identify precise mechanisms by which ANKRD26 prevents internalization.

In conclusion, we demonstrate a novel central role for ANKRD26 as responsible for the fine-tuning of the physiology of at least three different receptors. Small changes in their activity induce notable anomalies in proliferation of megakaryocytes, erythrocytes and granulocytes, and differentiation of megakaryocytes which cause thrombocytopenia, erythrocytosis and leukocytosis, respectively.

Disclosures

No conflicts of interest to disclose.

Contributions

FB-V and AD designed and performed experiments, analyzed data, and contributed to drafting the manuscript. VTM, ML, NB, CPMO, HD, BA, DM, J-EM and AC performed experiments and analyzed data. ND provided plasmid constructs, supervised RNA-sequencing and discussed results. IB analyzed induced pluripotent stem cell lines and discussed results. AB, ND, IA-D, CM, IP and WV designed experiments, discussed results and contributed to editing the manuscript. LF, AP and RF provided samples from patients and healthy controls and discussed results. HR designed and supervised the work and wrote the paper. All the authors gave final approval of the manuscript.

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Data-sharing statement

All data generated in this study are included in the article and its Online Supplementary File. The datasets used and analyzed during the current study are also available from the corresponding author on request.

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