

RESEARCH ARTICLE

Voltage-Gated K⁺ Channel, K_v3.3 Is Involved in Hemin-Induced K562 Differentiation

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Abbreviations: K_v channels, voltage-gated potassium channels; CREB, cAMP response element-binding protein; MAPK, mitogen activated

Abstract

Voltage-gated K⁺ (K_v) channels are well known to be involved in cell proliferation. However, even though cell proliferation is closely related to cell differentiation, the relationship between K_v channels and cell differentiation remains poorly investigated. This study demonstrates that K_v3.3 is involved in K562 cell erythroid differentiation. Down-regulation of K_v3.3 using siRNA-K_v3.3 increased hemin-induced K562 erythroid differentiation through decreased activation of signal molecules such as p38, cAMP response element-binding protein, and c-fos. Down-regulation of K_v3.3 also enhanced cell adhesion by increasing integrin β3 and this effect was amplified when the cells were cultured with fibronectin. The K_v channels, or at least K_v3.3, appear to be associated with cell differentiation; therefore, understanding the mechanisms of K_v channel regulation of cell differentiation would provide important information regarding vital cellular processes.

Introduction

Voltage-gated K⁺ (K_v) channels are well-established ion channels in excitable cells, where they serve as regulators of membrane potential and neuronal activities; however, these channels are also found in non-excitable cells, including cancer cells [1–3]. Previous studies have revealed cellular functions of K_v channels that include cell proliferation, apoptosis, and oxygen sensing [4–9]. Specifically, the modulation of certain K_v channel subunits, such as K_v1.1, K_v1.3, K_v4.1, K_v10.1, and K_v11.1, significantly affects cancer cell proliferation [8, 10–13]. Nevertheless, even though a relationship is known to exist between cell proliferation and cell differentiation [14–16], a function for K_v channels in cell differentiation has not been well established. However, K_v channels may be involved in a series of cell differentiation mechanisms, and specific K_v channel subunits may have direct effects on cell differentiation.

K562 cells are human immortalized myelogenous leukemia cells obtained from the pleural fluid of patients with chronic myeloid leukemia in blast crisis [17]. These cells have been useful for studying hematopoietic cell proliferation and differentiation [18] and can differentiate into an erythroid lineage when treated with differentiation-inducing reagents such as hemin, sodium butyrate, and nicotinic acid [19, 20]. The induced cells produce hemoglobin, and differentiation can be validated by benzidine staining or hemoglobin quantification [18, 21–23].

protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2.

K562 cells also can differentiate into megakaryotic lineages when treated with megakaryotic differentiation-inducing reagents, such as phorbol 12-myristate 13-acetate [24, 25].

K562 cell differentiation involves the mitogen-activated protein kinase (MAPK) and cAMP response element-binding protein (CREB) signaling pathways; extracellular signal-regulated kinase 1/2 (ERK1/2), CREB, and p38 have been specifically identified as important factors in K562 erythroid differentiation and hemoglobin synthesis [26–29]. In addition, certain K_v channels have close links to signaling molecules including CREB and CBP (CREB binding protein); they modulate K_v channel expression [30, 31].

Taken together, the available evidence suggests that K_v channels may be involved in the cell differentiation process through a range of signal pathways. An understating of the relationship between K_v channels and cell differentiation mechanisms might therefore suggest a new paradigm for cell differentiation research. In the present study, we investigated the roles of K_v channels and underlying signal mechanisms in the differentiation of K562 cells.

Materials and Methods

2.1. Cell culture and hemin-induced cell differentiation

K562 cells obtained from Korean Cell Line Bank were cultured in RPMI1640 medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO) at 37°C incubation with 5% CO_2 . T25 flasks (SPL Life Sciences, Gyeonggi-do, Korea) were used for culturing the cells. When sufficient growth was achieved, 1×10^5 cells were plated into a new T25 flask (SPL Life Sciences, Gyeonggi-do, Korea) and incubated with 50 μM hemin (Sigma, St. Louis, MO) to induce erythroid differentiation.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNeasy Micro Kit (Quiagen, Valencia, CA) according to the manufacturer's instructions. The cDNA was synthesized by reverse transcribing 1 μg of extracted RNA using random hexamers and an M-MLV reverse transcription kit (Promega, Madison, WI). The PCR reaction was performed with 2 μl of cDNA, 1 \times GoTaq[®] green master mix (Promega), and target K_v channel specific primers using the following reaction conditions: initial denaturation at 94°C for 5 min, 35 cycles of cycling process (94°C for 40 s, the indicated annealing temperature (Table 1) for 40 s, 72°C for 1 min, and an extension at 72°C for 1 min), and a final extension at 72°C for 7 min (Table 1). All PCR products were subjected to electrophoresis on 1.6% agarose gel and analyzed using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA) to confirm their amplified sequences.

2.3. Western blotting

K562 cells were lysed with 1X passive lysis buffer (Promega, Madison, WI) and total protein was quantified with a BCA protein assay kit (Pierce, Rockford, IL). The extracted proteins were separated on a 10% SDS-PAGE and then transferred to Nitrocellulose membranes (Whatman, Maidstone, Kent). After blocking in 1x TBS-Tween 20 containing 5% nonfat milk (5% TTBS) (Difco Franklin Lakes, NJ), membranes were probed with specific antibodies (in 5% TTBS) for $K_v2.1$, $K_v3.3$, p38, phospho-p38 (Abcam, Cambridge, MA), $K_v1.2$, $K_v1.3$, CREB, phospho-CREB (Millipore, Billerica, MA), ERK, phospho-ERK (Cell Signaling Technology, Inc, Danver, MA), $K_v9.3$, c-fos or β -actin (Santa Cruz Biotechnology, CA, USA). After overnight incubation, membranes were treated with horseradish peroxidase-conjugated goat, anti-rabbit secondary

Table 1. RT-PCR primers.

Subtype	Accession No.	Size (bp)	Primer sequence (Forward/Reverse)	Annealing (°C)
K _v 1.1	L02750	498	5'-ACATTGTGGCCATCATTCT-3'	55
			5'-GCTCTTCCCCTCAGTTTCT-3'	
K _v 1.2	NM_004974.3	200	5'-ATGAGAGAATTGGGCCTCT-3'	58
			5'-CCCCTATCTTTCCCCTAAT-3'	
K _v 1.3	NM_002232.3	177	5'-TGTCATGGCATCTCTTGC-3'	60
			5'-TGCATTGGGATTCATTT-3'	
K _v 1.4	NM_002233.3	170	5'-ACGAGGGCTTTGTGAGAGAA-3'	58
			5'-GGTTCCAGGCAAAGATGA-3'	
K _v 1.5	M55513	917	5'-TGCATCATCTGGTTCACCTTCG-3'	60
			5'-TGTTCCAGCAAGCCTCCATTCC-3'	
K _v 2.1	L02840	451	5'-GGAAGCCTGCTGTCTTCTTG-3'	65
			5'-CTTCATCTGAGAGCCCAAGG-3'	
K _v 3.3	AF055989	284	5'-CCTCATCTCCATCACCACCT-3'	60
			5'-CGAGATAGAAGGGCAGGATG-3'	
K _v 3.4	M64676	631	5'-TTCAAGCTCACACGCCACTTCG-3'	65
			5'-TGCCAAATCCCAAGGCTGAGG-3'	
K _v 4.3	AF048712	349	5'-TGAGCTGATTGCCTCAACG-3'	60
			5'-GTTCTCCGAGTCGTTGTCGT-3'	
K _v 9.3	NM_002252.3	200	5'-CAGTGAGGATGCACCAGAGA-3'	60
			5'-TTGCTGTGCAATTCTCCAAG-3'	

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antibody (Santa Cruz Biotechnology, CA, USA) and visualized using an enhanced chemiluminescent detection kit (iNtron Biotechnology, Gyeonggi-do, Korea)

2.4. Real-time RT-PCR

A standard curve and primer efficiency were analyzed from the standard curve prepared from diluted cDNAs (2 or 10 fold) using a primer of GAPDH, a house keeping gene, as a reference. The real-time RT-PCR reaction was performed with 2 µl of cDNA, 1x SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and 0.2 µM forward and reverse primers (Table 2) in the following reaction: initial step 95°C 30 s, then 40 cycles of the at 95°C for 5 s, and either

Table 2. Real-time RT-PCR primers.

fig	Accession No.	Size (bp)	Primer sequence (Forward/Reverse)	Annealing (°C)
K _v 2.1	NM_004975.2	173	5'-GTTGGCCATTCTGCCACT-3'	60
			5'-GCAAAGTGAAGCCCAGAGAC-3'	
K _v 3.3	NM_004977.2	147	5'-CCTTCCTGACCTACGTGGAG-3'	60
			5'-CGAGATAGAAGGGCAGGATG-3'	
K _v 3.4	NM_004978.4	178	5'-AATATCCCAGGGTGGTGACA-3'	60
			5'-GGTCTTCAAAGCTCCAGTGC-3'	
K _v 9.3	NM_002252.3	200	5'-CAGTGAGGATGCACCAGAGA-3'	60
			5'-TTGCTGTGCAATTCTCCAAG-3'	
integrin β1	NM_002211.3	209	5'-CATCTGCGAGTGTGGTGTCT-3'	55
			5'-GGGGTAATTTGTCCGACTT-3'	
integrin β3	NM_000212.2	176	5'-GCAATGGGACCTTTGAGTGT-3'	55
			5'-GTGGCAGACACATTGACCAC-3'	

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60°C ($K_v3.3$) or 55°C (integrin) for 45 s. A dissociation protocol was used to confirm that paired primers produced only a single product. All of the procedures were performed using an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). The relative mRNA expressions of the K_v channel gene were normalized to the GAPDH gene and expressed as a fold change relative to the control group.

2.5. Benzidine staining

Cultured cells were collected and centrifuged at 400 g for 5 min to obtain cell pellets. The cell pellets were washed using PBS and recentrifuged for 5 min. A benzidine working solution was prepared by mixing 20 μ l of hydrogen peroxide solution (Sigma, St. Louis, MO) in 1 ml benzidine stock solution [0.2% 3,3'-dimethoxybenzidine (Sigma, St. Louis, MO) dissolved in 3% glacial acetic acid solution (Sigma, St. Louis, MO)]. The cell pellets were incubated at room temperature with the solution for 2 min and passed through the washing step again. Benzidine positive cells were analyzed by light microscopy. The K562 cells that were cultured in fibronectin coated wells underwent the washing and staining steps without centrifugation.

2.6. Hemoglobin quantification

Cell pellets were collected for protein extraction. Extracted protein was quantified using a BCA protein assay kit (Pierce, Rockford, IL). Quantitative analysis of hemoglobin was performed using a QuantiChrom™ Heme Assay Kit (BioAssay Systems, Hayward, CA) following manufacturer's instructions. The unit-hemoglobin contents (ng Hb/ μ g Protein) were calculated by dividing the amount of hemoglobin by the total amount of protein.

2.7. Patch clamp recordings

Poly-L-lysine coated 12 mm coverslips (SPL) was put into the recording chamber to allow the cells to be attached on the bottom of the chamber. K562 cells were centrifuged and then resuspended with bath solution to be transferred to the chamber. The cells were visualized by the differential interference contrast video microscopy (OLYMPUS, Tokyo, Japan). Patch pipettes were pulled from the borosilicate glass capillaries (1.7 mm diameter; 0.5 mm wall thickness) (World Precision Instruments, Sarasota, FL); the range of the seal resistance was from 8 to 10 M Ω . The internal pipette solution (in mM concentration) was consist of 135 K-gluconate, 5 KCl, 20 HEPES, 0.5 CaCl₂, 5 EGTA, and 5 ATP-Mg and the bath solution is consist of 126 NaCl, 26 NaHCO₃, 5 KCl, 1.2 NaH₂PO₄, 2.4 CaCl₂, 1.2 MgCl₂, and 10 glucose. The currents were recorded in the whole cell configuration by using Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Electric signal was filtered at 1 kHz and digitized at 10 kHz using analog-digital converter (Digidata 1320A, Axon Instruments) and pClamp software (Version 9.0, Axon Instruments). For the voltage-clamp mode, following protocol was used: cells were hyperpolarized by -90 mV pulse for 320 ms and the membrane currents were activated by depolarizing pulse for 400 ms from a holding potential -80 mV to the test potential which are ranged from -70 to 40 mV in 10 mV increments.

2.8. Inducing cell differentiation after transfection with small interference RNA (siRNA)

Cells were transfected with siRNA- $K_v3.3$ using ON-TARGET plus SMART pool Human KCNC3 (Thermo Scientific Dharmacon, Lafayette, CO) and Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions for suspension cells. The ON-TARGET plus® Control pool (Thermo Scientific Dharmacon, Lafayette, CO)

was used as a control siRNA. The K562 cells (1×10^5) were plated in 6 well plates immediately prior to the transfection step in RPMI 1640 (Welgene, Daegu, Korea) containing 10% FBS without any antibiotics. After 24 h, the siRNA- $K_v3.3$ transfected cells were incubated with 50 μ M hemin in 6 well plates. The incubation time was 24 or 48 h for benzidine staining or hemoglobin quantification, respectively. To induce cell adhesion, 6 well plates containing 10 μ g/ml of fibronectin (Sigma, St. Louis, MO) dissolved in DPBS were incubated at 4°C for one day, and the coated plates were used for the experiment in place of uncoated plates.

2.9. $K_v3.3$ overexpressed K562 cell line establishment

HEK293T cells (GE lifesciences) were maintained in 10% fetal bovine serum and 1% penicillin/streptomycin in Dulbecco's modified Eagle medium (DMEM) at 37°C and 5% CO₂. 24 hours before transfection, 6×10^6 HEK293T cells were seeded into 100 mm dish. The following day, 50 μ L (47.5 μ g) of a Trans-lentiviral packaging mix encoding viral proteins Gag-Pol, Rev, and VSV-G and 42 μ g of lentiviral transgene plasmids were transfected into each well for lentivirus production using Calcium phosphate. 14 hours after transfection, the DNA-reagent mixture was removed and replaced with 5% FBS in 14 ml fresh DMEM. At 48 hours post-transfection, lentiviral supernatants were harvested and filtrated with 0.45- μ m filters. 1 volume of cold (4°C) PEG-it Virus Precipitation Solution (Systembio) was added to every 4 volumes of lentiviral particle-containing supernatant. The supernatant/PEG-it mixture was centrifuged at 1,500 x g for 30 minutes at 4°C. The viral pellet was resuspended and combined the lentiviral particles in 10 μ L using cold (4°C), DMEM media. To determine transduction efficiency, HEK293T cells (1×10^3 , of 96-well plate) were transduced with lentivirus harvested from HEK293T cell transfection. At 72 hours post-transduction, GFP-positive cells were observed by fluorescence microscope (NiKon). Lentivirus was added to K562 cell culture in RPMI media supplemented with 10% FBS. Lentivirus was then added to give a multiplicity of infection (MOI) of 1. After overnight incubation, lentivirus was removed and fresh media added. K562 cells were then transduced with KCNC3 (Openbiosystem, OHS5898-202623948) construct and selected by 2 μ g/ml Blastcidin to create K562-KCNC3.

2.10. Statistical analysis

All data are shown as means \pm standard error (SE) and the Student *t*-test or One-way ANOVA was used to analyze the data (GraphPad Prism version 5.0).

Results

3.1. Expression of K_v channels in K562 cells and the induction of K562 cell erythroid differentiation using hemin

RT-PCR analysis revealed that seven different K_v channels ($K_v1.2$, $K_v1.4$, $K_v2.1$, $K_v3.3$, $K_v3.4$, $K_v4.3$, and $K_v9.3$) were detected in K562 cells (Fig 1A). $K_v1.1$, $K_v1.3$, and $K_v1.5$ were also detected; however, the expression was too low. Western blot analysis demonstrated that $K_v2.1$, $K_v3.3$, $K_v3.4$, and $K_v9.3$ exist in K562 cells (Fig 1A). Hemin was used to induce K562 cell differentiation into erythroids (Fig 1B). Negative control data showed few stained cells. At an early stage of differentiation, the percentage of benzidine-positive cells, which were dyed black, rapidly increased and later reached a plateau (Fig 1C). The concentration of hemoglobin, on the other hand, showed no significant changes in the early stage of differentiation and started to increase between 24 and 48 h (Fig 1D).

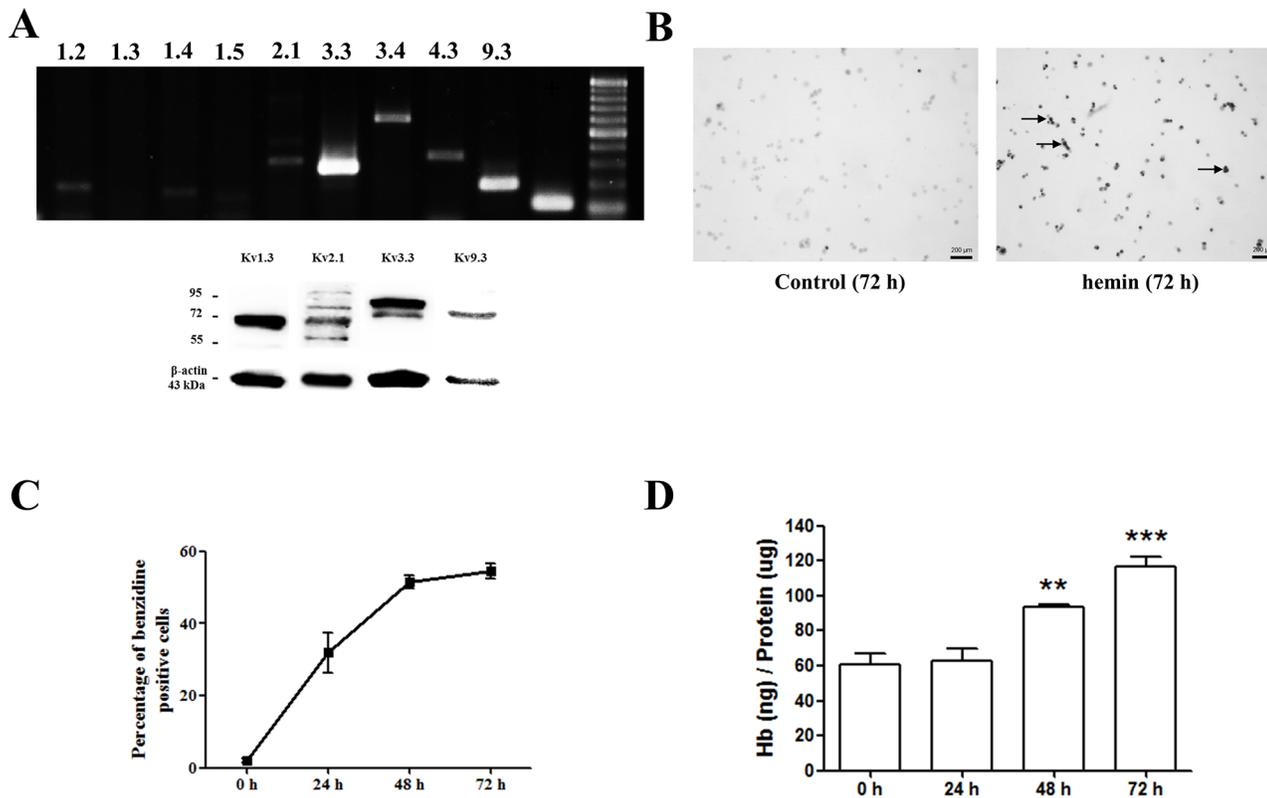


Fig 1. Identification of K_v channels in K562 cells and the erythroid differentiation of K562 cells using hemin. (A) RT-PCR data analysis demonstrated 7 different subtypes of K_v channels ($K_v1.2$, $K_v1.4$, $K_v1.5$, $K_v2.1$, $K_v3.3$, $K_v3.4$, $K_v4.3$, and $K_v9.3$). Western blot demonstrated the protein expression of $K_v1.3$, $K_v2.1$, $K_v3.3$, and $K_v9.3$. (B) K562 cells differentiated into erythroid cells were stained with benzidine after 72 h of differentiation using hemin (magnification $\times 40$). Benzidine-positive cells appeared black, indicated by colored arrows. (C) The percentage of benzidine-positive cells was counted at 4 different time points (0, 24, 48, and 72 h). (D) The hemoglobin content of the differentiated K562 cells was measured at each indicated time point using a modified QuantiChrom Heme Assay. The concentration of hemoglobin at each time point was expressed as nanograms of heme per microgram of total protein.

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3.2. The expression of K_v channels and whole cell patch clamp recording during the late stage of differentiation

The real-time quantitative RT-PCR data demonstrated that the mRNA expression level of $K_v3.3$ was decreased by half after 24 hours of erythroid differentiation and the reduction rate gradually slowed down, whereas $K_v2.1$ increased during the K562 erythroid differentiation (Fig 2A). $K_v3.4$ and $K_v9.3$ showed no change and K_v1 subunits were not detected (Fig 2A). The protein expression level of $K_v3.3$ was also significantly lower during erythroid differentiation as a result of hemin treatment at the indicated time points (24, 48, and 72 h) (Fig 2B). We have tried to perform Western blot analysis for $K_v2.1$ in order to examine protein expression during differentiation; unfortunately, we could not obtain clear Western blot images for $K_v2.1$ in K562 cells. Therefore, we focused on $K_v3.3$ for the following experiments. Fig 2C demonstrated representative current traces recorded from K562 cells. Despite the fact that we detected the mRNA and protein expression of K_v channels, there was no TEA-sensitive current before and after hemin-induced erythroid differentiation (Fig 2C).

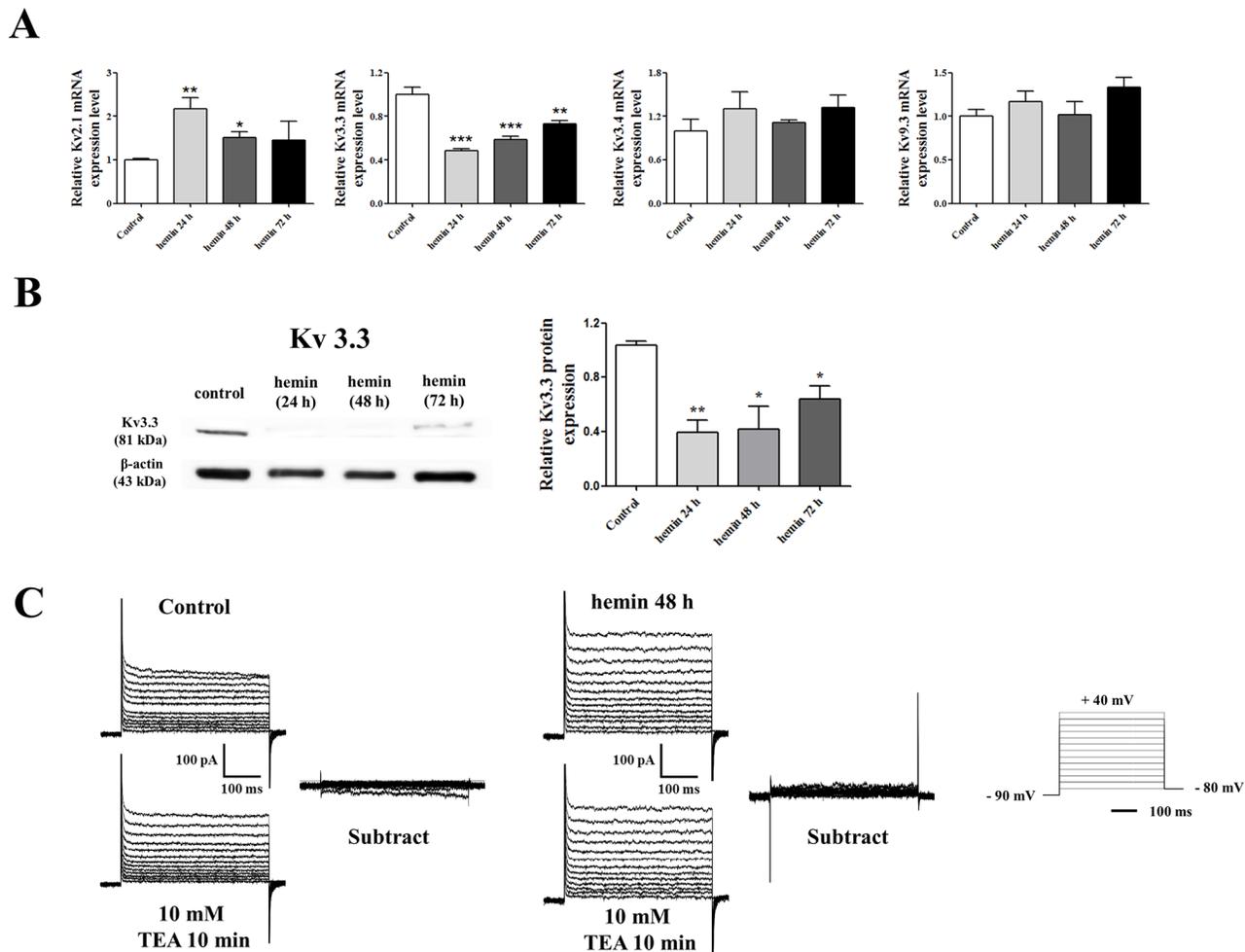


Fig 2. The expression of K_v channels and whole cell patch clamp recording during the late stage of differentiation. (A) After hemin-induced K562 cell differentiation, the mRNA expression levels of K_v channels, including $K_v2.1$, $K_v3.3$, $K_v3.4$, and $K_v9.3$, were compared with those of undifferentiated cells. The mRNA expression level of $K_v2.1$ was increased, whereas the mRNA expression level of $K_v3.3$ was decreased during erythroid differentiation. The mRNA expression of $K_v3.4$ and $K_v9.3$ were not altered during erythroid differentiation. The relative mRNA expressions of the K_v channels were normalized to the GAPDH gene and expressed as a fold change relative to the Mock control group. (B) The protein level of $K_v3.3$ decreased significantly after 24 hours of hemin-induced erythroid differentiation in K562 cells. The relative protein expression of the $K_v3.3$ was expressed as a fold change relative to the control group. (C) Representative current traces recorded from K562 cells. There was no TEA-sensitive current before and after hemin-induced erythroid differentiation. Experiments were performed in triplicate, and data are expressed as mean \pm standard error. ** $p < 0.01$ compared with control value.

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3.3. $K_v3.3$ knockdown using siRNA- $K_v3.3$ increased hemin-induced K562 cell differentiation, whereas $K_v3.3$ overexpression did not decrease the hemin-induced K562 erythroid differentiation effectively

To verify whether $K_v3.3$ directly affects K562 cell differentiation, siRNA- $K_v3.3$ was transfected into the K562 cells. After 24 h of transfection, the K562 cells were cultured with hemin for 48 h (Fig 3A). The degree of siRNA transfection was confirmed by RT-PCR and Western blot analysis (Fig 3B and 3C). The benzidine staining and hemoglobin quantification data demonstrated that decreased $K_v3.3$ expression increased hemin-induced K562 cell differentiation (Fig 3D and 3E). The numbers of benzidine-positive cells, stained black, were increased in siRNA- $K_v3.3$ -transfected cells compared to the control cells. Hemoglobin quantification confirmed an approximately 50% increase in hemoglobin content following transfection with siRNA- $K_v3.3$.

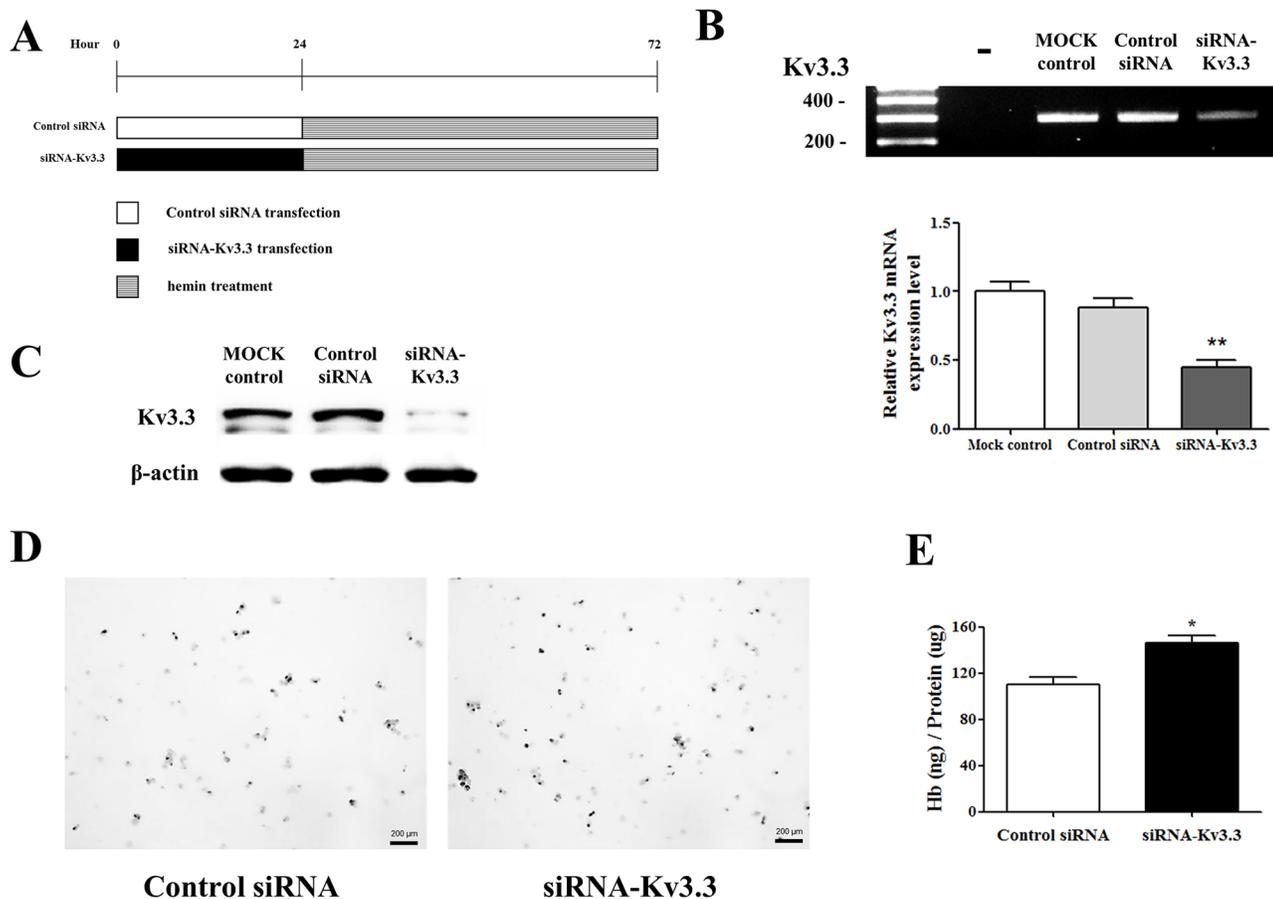


Fig 3. Silenced $K_v3.3$ increased hemin-induced K562 cell differentiation. Hemin-induced K562 cell differentiation was significantly increased by decreasing the $K_v3.3$ expression using siRNA- $K_v3.3$. (A) Protocol for control siRNA and siRNA- $K_v3.3$ transfection and hemin treatment. After 24 h of transfection, the K562 cells were cultured with hemin for 48 h. mRNA (B) and protein (C) expressions were suppressed when the cells were transfected with siRNA- $K_v3.3$. RT-PCR was performed after 48 h of transfection. The relative mRNA expression of the $K_v3.3$ was normalized to the GAPDH gene and expressed as a fold change relative to the Mock control group. (D) Benzidine staining demonstrated greater hemoglobin formation in siRNA- $K_v3.3$ -transfected cells (right) compared to control siRNA-transfected cells (left) during hemin-induced K562 cell erythroid differentiation (magnification $\times 40$). Staining was performed after 24 h of transfection and 24 h of differentiation. (E) The amounts of hemoglobin content were increased by siRNA- $K_v3.3$ transfection compared to control. The hemoglobin content was increased by about 50% by siRNA- $K_v3.3$ transfection. Experiments were performed in triplicate, and data are expressed as mean \pm standard error. * $p < 0.05$ compared with control value.

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Next, we established $K_v3.3$ -overexpressed K562 cells (Fig 4A and 4B) to verify whether $K_v3.3$ overexpression inhibits hemin-induced K562 erythroid differentiation. Benzidine staining data demonstrated that the overexpression of $K_v3.3$ slightly inhibited the hemin-induced K562 erythroid differentiation (Fig 4C). However, when we checked it again using hemoglobin kit assay, the data demonstrated that $K_v3.3$ overexpression did not decrease the hemin-induced K562 erythroid differentiation effectively (Fig 4D), whereas the down-regulation of $K_v3.3$ using siRNA- $K_v3.3$ enhanced the differentiation more clearly (Fig 3E).

3.4. Signaling mechanisms involved in the regulation of K562 erythroid differentiation by siRNA- $K_v3.3$ transfection

Signal cascades involving in K562 erythroid differentiation have been well established [26–29]. We examined p38, ERK1/2, CREB, and c-fos during hemin-induced K562 erythroid differentiation in siRNA- $K_v3.3$ -transfected K562 cells. As shown in Fig 5, the levels of phosphorylated

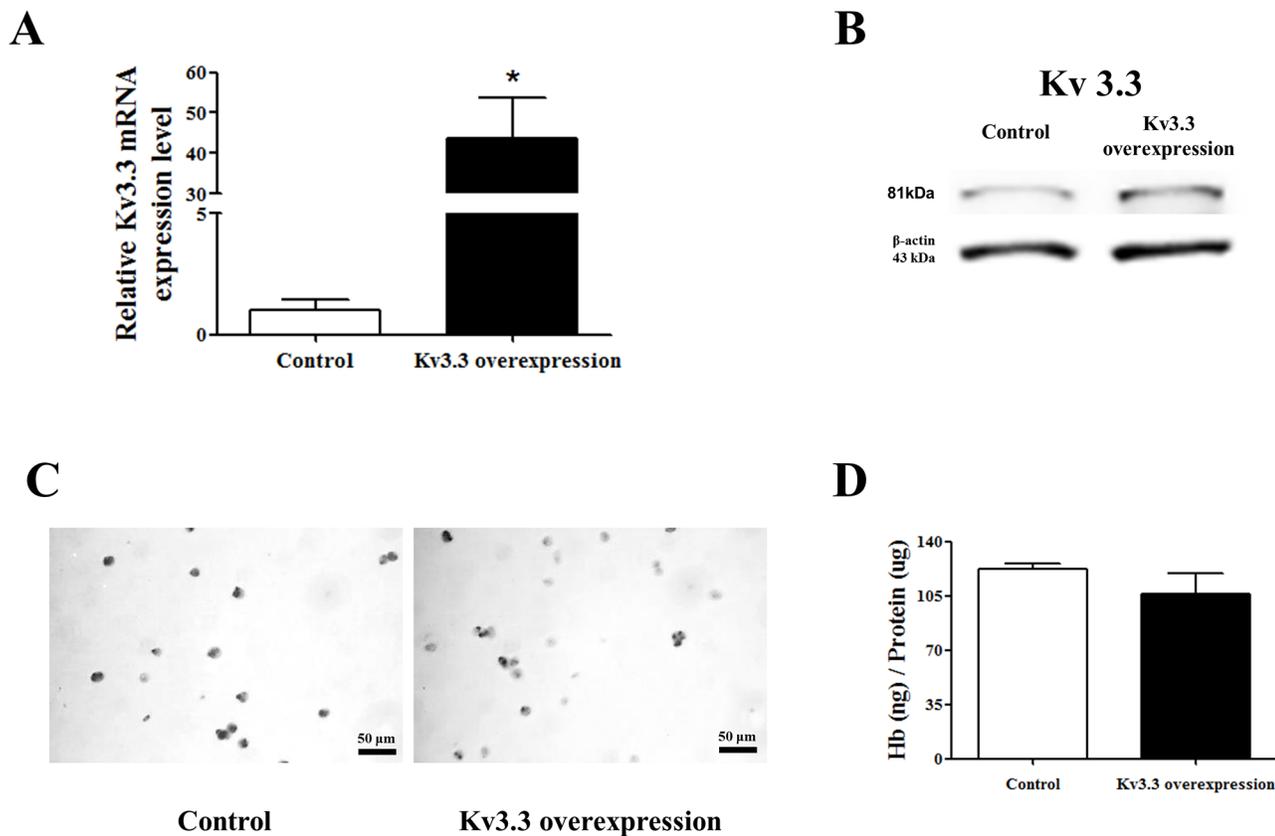


Fig 4. Overexpression of $K_v3.3$ did not have a clear effect on hemin-induced erythroid differentiation. mRNA (A) and protein (B) expression levels of $K_v3.3$ were increased in the K562 overexpressed cell line compared to the normal K562 cell line. The relative mRNA expression of the $K_v3.3$ was normalized to the GAPDH gene and expressed as a fold change relative to the control group. The relative protein expressions of the $K_v3.3$ were expressed as a fold change relative to the control. (C) The degree of hemin-induced erythroid differentiation was measured using benzidine staining in $K_v3.3$ -overexpressed K562 cells or normal K562 cells. There was no significant difference between the two, however. (D) Hemoglobin quantification demonstrated that overexpressed $K_v3.3$ did not have any effect on hemin-induced K562 erythroid differentiation.

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forms of p38 and CREB and the levels of c-fos were reduced significantly by transfection with siRNA- $K_v3.3$ compared to the controls. Although the protein expression levels of the activated (phosphorylated) forms of p38 and CREB were reduced, the total amounts of p38 and total CREB were not significantly reduced. The protein expression levels of total and phosphorylated ERK1/2 (Fig 5) and the phosphorylated form of ERK2 (p-42 MAPK) also decreased; however, the difference was not statistically significant. Phosphorylated ERK1 (p-44 MAPK) was not detected in K562 cells. These changes indicated that decreased $K_v3.3$ expression in K562 cells increased hemin-induced K562 cell differentiation through the reduced activation of p38, p-CREB, and c-fos.

3.5. K_v channels are not involved in the early stage of hemin-induced K562 erythroid differentiation

The mRNA expression levels of $K_v2.1$, $K_v3.3$, $K_v3.4$, and $K_v9.3$ were measured at the presented time points (10 min, 30 min, and 1 h) after inducing differentiation, and we found that the expression levels of K_v channels including $K_v3.3$ were not changed in the early stage of hemin-induced K562 erythroid differentiation (Fig 6A). The protein expression level of $K_v3.3$ was also not changed after 10 min and 30 min of the erythroid differentiation (Fig 6B). $K_v3.3$

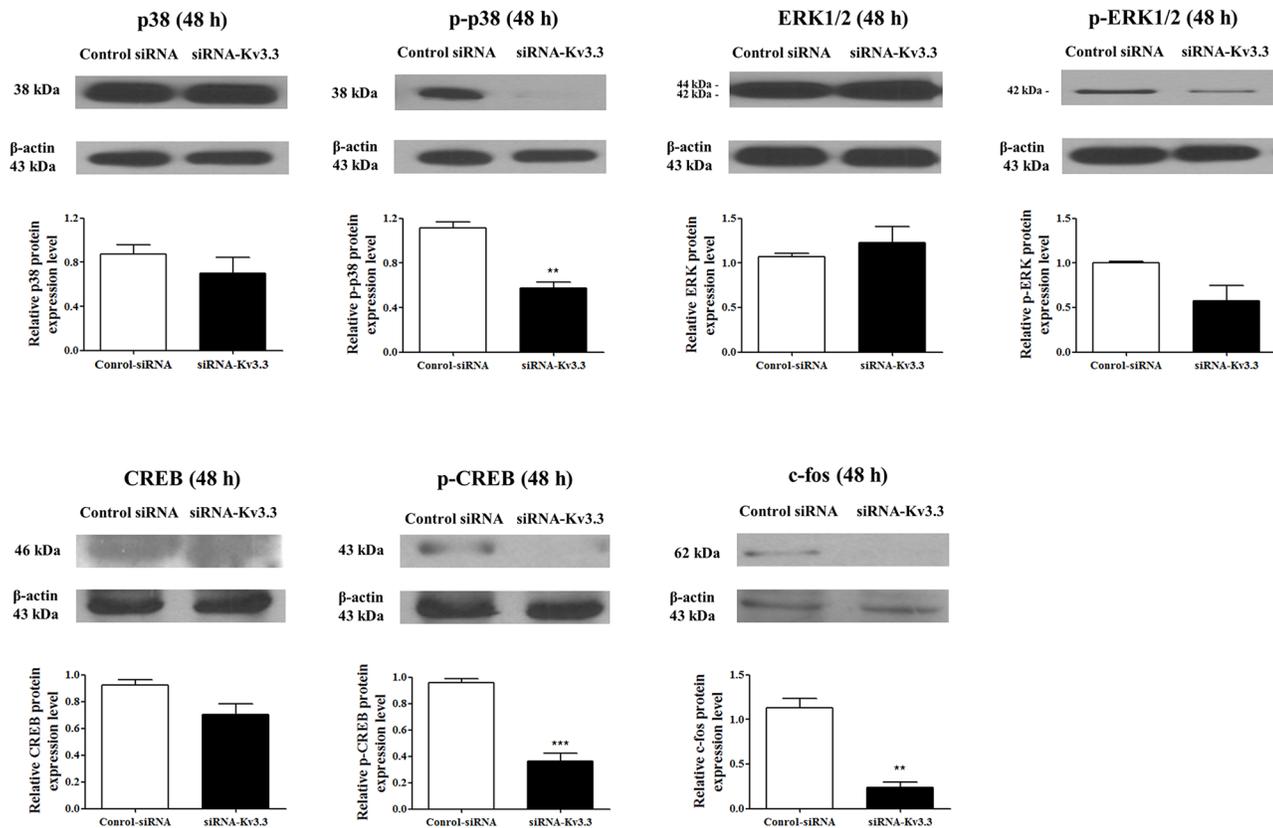


Fig 5. Signaling mechanisms of K562 erythroid differentiation regulation by siRNA- $K_v3.3$ transfection. The expressions of p38, ERK1/2, CREB, and c-fos during cell differentiation changed with siRNA- $K_v3.3$ transfection. The levels of the phosphorylated, activated forms of p38 and CREB were lower in siRNA- $K_v3.3$ transfected cells than in control cells. The levels of c-fos during cell differentiation were also reduced after siRNA- $K_v3.3$ transfection. The levels of phosphorylated ERK2 (p-42 MAPK) also seemed lower; however, the differences were not statistically significant. Phosphorylated ERK1 (p-44 MAPK) was not detected. No changes were noted for the total p38, total ERK1/2, and total CREB. The graphs show the quantitative analysis of each protein. Western blot assay was performed when transfected cells were differentiated for 48 h with hemin; each assay was performed in triplicate, and data are expressed as mean \pm standard error. ** $p < 0.01$ compared with control value. The relative protein expressions of the signal molecules were expressed as a fold change relative to the control group.

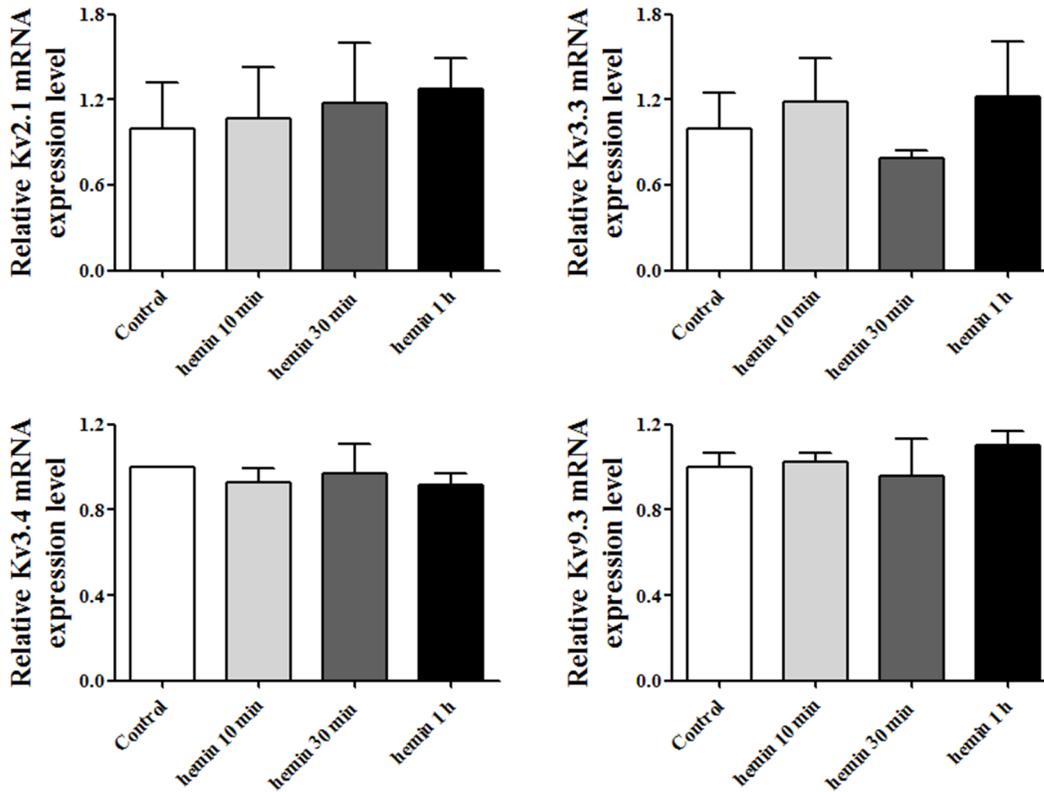
doi:10.1371/journal.pone.0148633.g005

knockdown using siRNA- $K_v3.3$ was also performed during the early stage of hemin-induced K562 erythroid differentiation, and the transfection had no effect on the signaling mechanisms, such as p38 and ERK, which are known to be involved in the early stage of erythroid differentiation (Fig 7).

3.6. $K_v3.3$ knockdown using siRNA- $K_v3.3$ increased cell adhesion in K562 cells

The K562 cells transfected with siRNA- $K_v3.3$ showed interesting morphological changes. Even though K562 cells are suspension and sphenoid cells, we observed the adhesion of a few K562 cells to the bottom of the 6 well plates when we incubated the cells with hemin for one or two days. The adherent cells had spindle-like shapes and more adherent cells were detected when the cells were transfected by siRNA- $K_v3.3$ compared to the control cells (Fig 8A). Real time RT-PCR data demonstrated that the mRNA expression levels for integrins $\beta 3$ (Fig 8B left) and $\beta 1$ (Fig 8B right), which are well-known adhesion molecules, were not statistically changed by siRNA- $K_v3.3$ transfection.

A



B

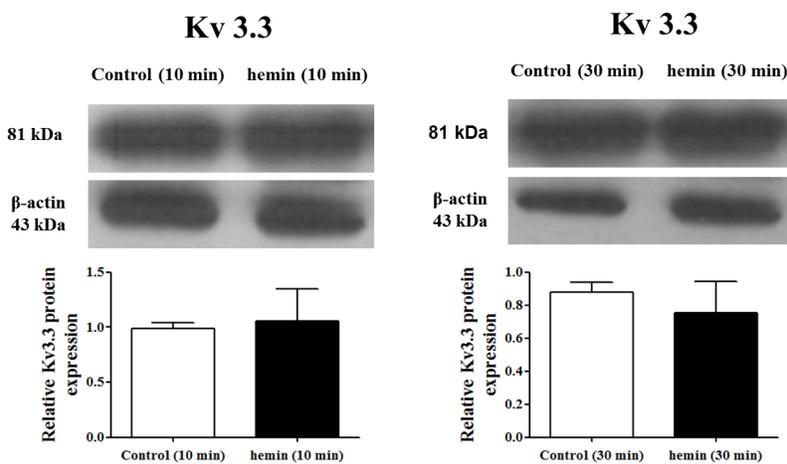


Fig 6. The relationship between $K_v3.3$ and the early stage of K562 erythroid differentiation. (A) The mRNA expression levels of the K_v channels, including $K_v2.1$, $K_v3.3$, $K_v3.4$, and $K_v9.3$, did not correlate with the hemin-induced K562 erythroid differentiation at the indicated time points (10 min, 30 min, and 1 h). The relative mRNA expressions of the K_v channels were normalized to the GAPDH gene and expressed as a fold change relative to the control group. (B) The protein expression level of $K_v3.3$ was estimated after inducing 10 min and 30 min of erythroid differentiation, and there was no change compared to the control cells. The relative protein expressions of the $K_v3.3$ were expressed as a fold change relative to the control.

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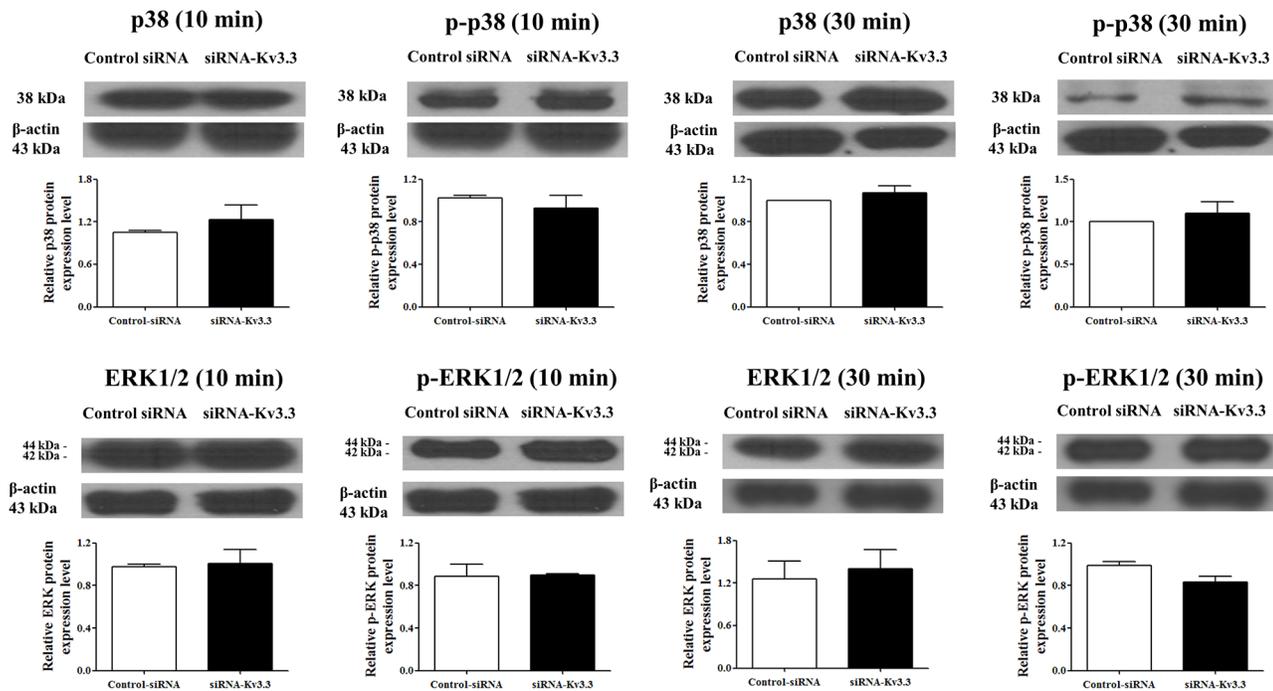


Fig 7. In the early stage of K562 erythroid differentiation, $K_v3.3$ knockdown using siRNA- $K_v3.3$ did not have any effect on the expression levels of signaling molecules involved in K562 erythroid differentiation. The protein expression levels of p38 and ERK were measured after 24 h of siRNA- $K_v3.3$ transfection and 10 min or 30 min of erythroid differentiation. During the early stage of erythroid differentiation, down-regulated $K_v3.3$ had no effect on the protein levels of p38 and ERK. The relative protein expressions of the signal molecules were expressed as a fold change relative to the control siRNA group.

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Next, we incubated the K562 cells in fibronectin-coated plates to enhance cell adhesion because fibronectin binds to integrins [32]. After 24 h of transfection, the K562 cells were cultured with hemin in fibronectin-coated well for 48 h (Fig 8C). As shown in Fig 8D, when siRNA- $K_v3.3$ was transfected, many more cells adhered to the bottom of the plates compared to the controls (Fig 8D). In addition, the mRNA expression level of integrin $\beta 3$ significantly increased in siRNA- $K_v3.3$ -transfected cells (Fig 8E left), whereas integrin $\beta 1$ showed no difference (Fig 8E right). On the other hand, when the cells were cultured in fibronectin-coated plates with hemin, erythroid differentiation was not enhanced by siRNA- $K_v3.3$ transfection. Benzidine staining and hemoglobin quantification indicated no significant differences between control and siRNA-transfected cells (Fig 8F). These results suggest that the erythroid differentiation effect induced by reduced $K_v3.3$ expression in K562 cells was transformed into a cell adhesion-enhancing effect when K562 cells were provided with fibronectin.

3.7. Signaling mechanisms involved in the regulation of K562 differentiation by the siRNA- $K_v3.3$ transfection of cells cultured in fibronectin plates

Cultures in fibronectin-coated wells eliminated the $K_v3.3$ -mediated erythroid differentiation-inducing effect. Therefore, we compared the signaling mechanisms in the presence and absence of fibronectin. As demonstrated in Fig 9, none of the total and activated forms of ERK1/2, p38, and CREB showed any differences in the control and siRNA-transfected cells. These results are consistent with the lack of erythroid differentiation in siRNA- $K_v3.3$ cells cultured in fibronectin-coated wells.

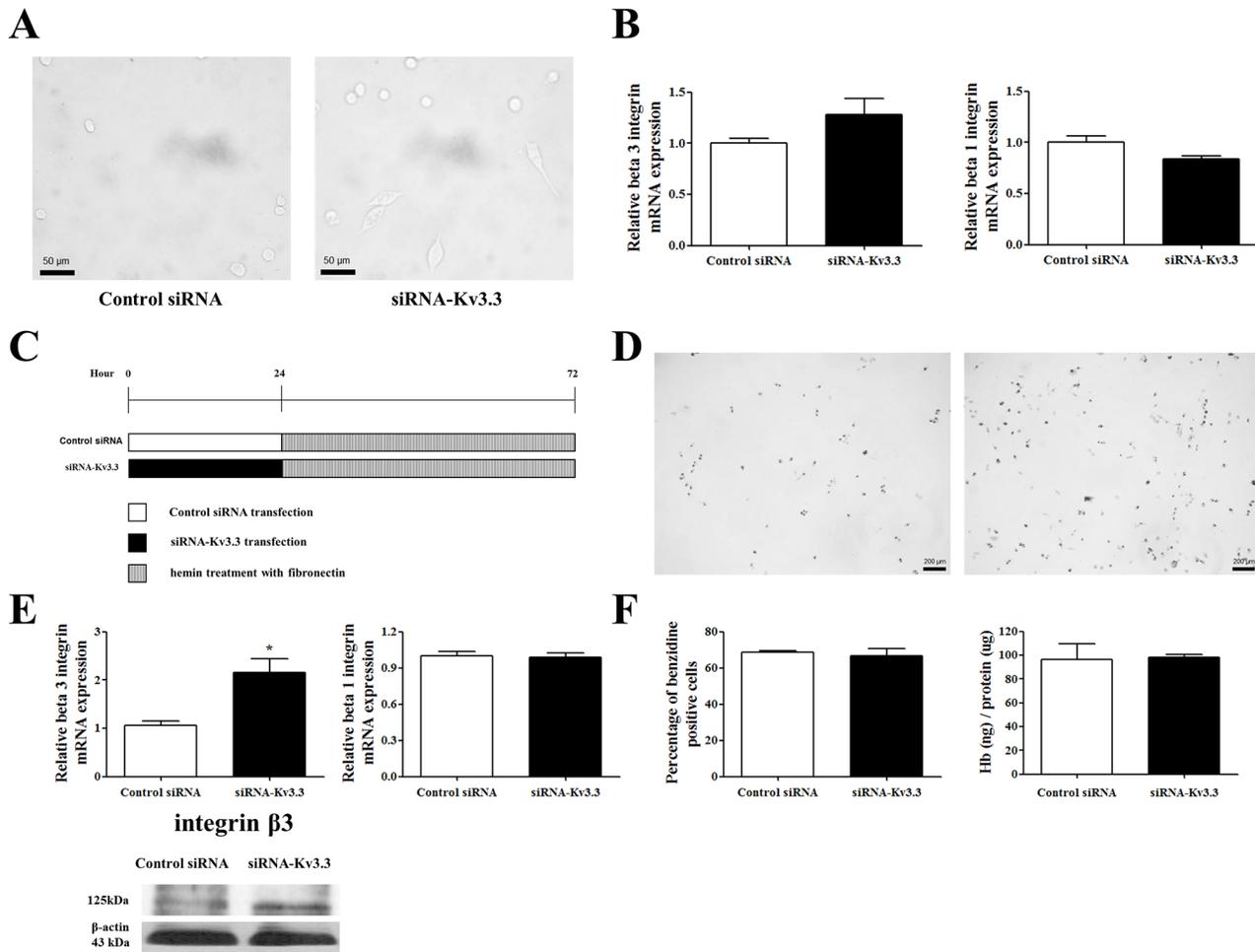


Fig 8. Effects of siRNA-K_v3.3 transfection on cell adhesion. (A) Attached K562 cells were detected in siRNA-K_v3.3-transfected cell cultures (right), whereas fewer attached cells were found in control cultures (left) (magnification ×200). (B) The mRNA expression levels of integrin β3 (left) and integrin β1 (right) during hemin-induced K562 cell erythroid differentiation by transfection of siRNA-K_v3.3. (C) The protocol for control siRNA and siRNA-K_v3.3 transfection and hemin treatment with fibronectin. After 24 h of transfection, the K562 cells were cultured with hemin in fibronectin-coated wells for 48 h. (D) Culturing the cells in fibronectin-coated wells (10 μg/ml) significantly improved cell adhesion during the hemin-induced erythroid differentiation of siRNA-K_v3.3-transfected cells (magnification ×40). (E) Cells cultured in fibronectin-coated wells showed amplified effects of decreased K_v3.3 on integrin β3 levels. The mRNA and protein expression levels of integrin β3 in siRNA-K_v3.3-transfected cells increased much more during hemin-induced K562 cell erythroid differentiation than in the control group (*p<0.05). On the other hand, no differences were noted in integrin β1 expression between the control and siRNA-K_v3.3 transfected-cells when the cells were cultured in fibronectin-coated wells. (F) Benzidine staining (left) and hemoglobin quantification (right) indicated that increased erythroid differentiation by siRNA-K_v3.3 was not detected when the cells were cultured in fibronectin-coated wells. The relative mRNA expressions of the integrins were normalized to the GAPDH gene and expressed as a fold change relative to the control siRNA group.

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Discussion

In the present study, we first identified which K_v channel subunits exist in K562 cells and the roles of K_v channels in K562 cell differentiation. RT-PCR and Western blot analyses demonstrated that K_v3.3 was highly expressed in K562 cells, and that its expression level was down-regulated during the late stage of erythroid differentiation, whereas K_v2.1 was increased and other K_v channels did not show any change. In K_v3.3-silenced cells, erythroid differentiation was significantly increased during the late stage (48 h) via p-p38, p-CREB, and c-fos, whereas in the early stage of differentiation, there was no change compared to the control cells (10 min and 30 min). K_v3.3 overexpression did not decrease the hemin-induced K562 erythroid

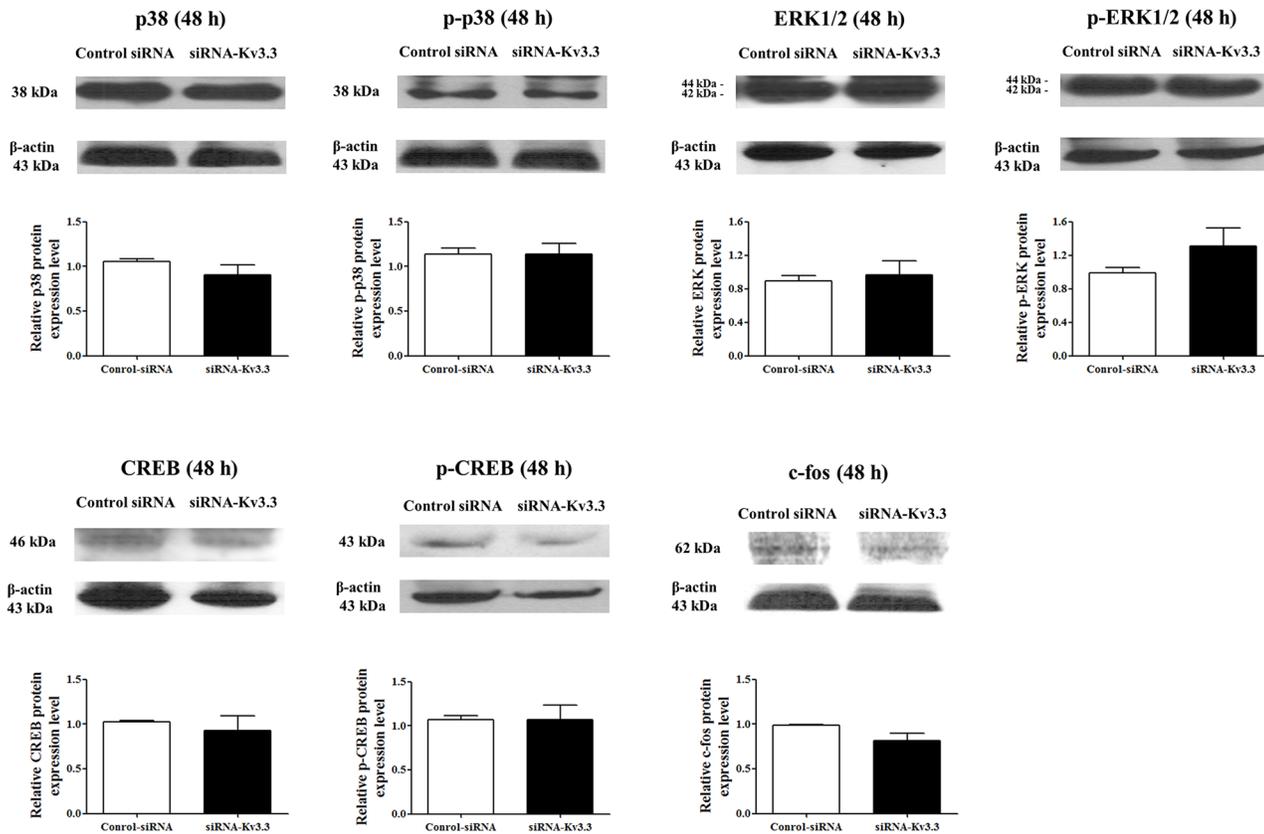


Fig 9. Signaling mechanisms of K562 differentiation regulation by siRNA- $K_v3.3$ transfection and cultures in fibronectin plates. No changes in the expression levels of p38, ERK1/2, CREB, and c-fos were noted in response to decreased $K_v3.3$ levels compared to control cells during cell differentiation in fibronectin-coated wells. No differences were noted in the total amounts or amounts of phosphorylated forms of ERK1/2, p38, and CREB expression in the control and siRNA-transfected cells. No differences were observed in the c-fos levels following siRNA- $K_v3.3$ transfection when the cells were cultured with fibronectin. The graphs indicate the quantitative analysis of each protein. Western blot assays were performed when the transfected cells were differentiated for 48 h with hemin and fibronectin. Each assay was performed in triplicate, and data are expressed as mean \pm standard error. The relative protein expressions of the signal molecules were expressed as a fold change relative to the control siRNA group.

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differentiation effectively, whereas the down-regulation of $K_v3.3$ using siRNA- $K_v3.3$ enhanced the differentiation more clearly. According to our results, we assume that the reason $K_v3.3$ overexpression did not reverse the effects of siRNA- $K_v3.3$ is that $K_v3.3$ is already expressed enough to regulate differentiation in native K562 cells. Interestingly, we discovered that the adhesion of K562 cells was increased in $K_v3.3$ -silenced cells and that adhesion was enhanced in the presence of fibronectin.

K_v channels have a known involvement in a range of essential cellular functions, including cell proliferation, wound healing, apoptosis, and oxygen sensing [4–9]. Cell differentiation is another important fundamental event that is regarded as having a close relationship with cell proliferation [14–16]. Several reports have demonstrated the relevance of qualitative and quantitative changes in K_v currents to the differentiation state of peripheral murine CD4⁺ lymphocytes [33]. More recently, K_v channels have been suggested to play roles in cell differentiation [34]. Wild-type *Xenopus* $K_v1.1$ overexpression in *Xenopus* retinal ganglion cells results in morphological differentiation in the form of increased dendritic branching [35]. In particular, You et al. (2013) demonstrated that $K_v2.1$ and $K_v3.3$ may play important roles in the differentiation of human mesenchymal stem cells into adipocytes [36]. According to the results, we also could assume that $K_v2.1$ and $K_v3.3$ are the specific channels involved in cell differentiation in K562

cells. The function and/or expression of $K_v3.3$ are closely related to $K_v2.1$, and further studies need to be performed to answer the question.

To date, many voltage-dependent channels have been identified in hematopoietic stem cells, and these channels function distinctly in proliferation and differentiation [37]. The expressions of $K_v1.3$ and $K_v7.1$ have been identified in $CD34^+/CD45^+/CD133^{\text{high}}$ cells from peripheral blood by RT-PCR. In particular, $K_v11.1$, which is upregulated in leukemic hematopoietic cells, appears to be involved in the physiology of leukemic and stem cells in processes such as cell adhesion and proliferation [37].

The importance of p38, ERK1/2, CREB, and *c-fos* in regulating erythroid differentiation has been demonstrated. K562 erythroid differentiation is well known to involve p38, while ERK1/2 shows an opposite or no effect [26, 29, 38, 39], and CREB protein activation is involved in K562 erythroleukemia cell differentiation [29]. In the present study, the signal molecules p38, CREB, and *c-fos* showed a tendency to decrease during the late stage of hemin-induced erythroid differentiation due to reduced $K_v3.3$. The regulation of CREB by $K_v3.3$ was previously demonstrated. Tong et al. (2010) showed that the blocking of CREB reduced the expression of $K_v3.3$ and *c-fos* in medial nucleus of the trapezoid body (MNTB) neurons [31].

Compared to what is known for other K_v channels, the roles of $K_v3.3$ are poorly understood. Although $K_v3.3$ has its own fast inactivating potassium currents when it is transfected into the HEK cell or CHO cell system [40, 41], there are few reports dealing with the electrophysiological recordings on $K_v3.3$. Similar to the previous results, there is no TEA-sensitive current in K562 cells. We assume that hemin-induced K562 erythroid differentiation-related K_v channels may be not located on cell membranes, or there would be some functions of the K_v channels independent from their potassium currents.

$K_v3.3$ is a known oxygen-sensitive channel; it opens in the presence of oxygen and reversely closes in response to hypoxia [42]. Hypoxia reduces the production of oxygen-reactive intermediates, including H_2O_2 , and $K_v3.3$ is one of the channels that lose its fast inactivation upon external application of H_2O_2 [42–44]. The relationship between the function of potassium channels and oxidative stress has been well established [45], and $K_v3.3$ function can be inferred to have relevance to oxidative stress. On the other hand, differentiated K562 cells produce heme contents, resulting in the increased production of reactive oxygen species [46] and naturally this causes oxidative stress. $K_v3.3$ may be involved in protection against oxidative stress during erythroid differentiation, which may increase oxidative stress as a side effect. Therefore, decreases in $K_v3.3$ expression in K562 cells would induce erythroid differentiation. Furthermore, expressions of signal molecules, such as MAPK and CREB, which are involved in oxidation-sensitive mechanisms [47], were altered during the hemin-induced erythroid differentiation of K562 cells transfected by siRNA- $K_v3.3$.

In the present study, we detected increased adhesion properties in $K_v3.3$ -silenced K562 cells and found that integrins were important in the observed changes. When we used fibronectin, which interacts well with integrins, down-regulated $K_v3.3$ expression during hemin-induced erythroid differentiation resulted in enhanced cell adhesion. At the same time, the hemin-induced erythroid differentiation enhancing effect of siRNA- $K_v3.3$ disappeared, and no differences were seen between control and siRNA- $K_v3.3$ -transfected cells for expressions of the signal molecules. Järvinen et al (1993) demonstrated that differentiation inducers alter the integrin expression of K562 cells [48]. It has been demonstrated that $\alpha5\beta1$ is the only fibronectin receptor integrin expressed in suspension-cultured K562 cells, and differentiation inducers such as TPA (12-tetradecanoyl-13-acetyl-beta-phorbol) or hemin chloride alter the expression levels of integrin; TPA increased the $\beta3$ integrin, whereas hemin chloride did not have any effect on the $\beta3$ integrin and it only decreased the $\beta1$ integrin [48]. K562 cells bind to fibronectin through the $\alpha5\beta1$ integrin receptor when added to wells coated with fibronectin [49]. In Fig

8E, we showed that siRNA- $K_v3.3$ increased $\beta 3$ integrin expression when the cells were incubated in the fibronectin-coated well. From the data, we could assume that siRNA- $K_v3.3$ transfection changed the original cell property of K562. In addition, from the Figs 5 and 9 we found that siRNA- $K_v3.3$ transfection affected signaling pathways were changed due to the cell culture in fibronectin coated wells. It has been suggested that the Src family of tyrosine kinases (SFKs)-receptor tyrosine kinases (RTKs)-MAPK signaling is involved in integrin signaling. Fig 9 demonstrates that the transfection of siRNA- $K_v3.3$ to K562 cells cultured in fibronectin-coated wells does not have any effect on the expression levels of p38 and ERK, which are MAPK families. Therefore, increased $\beta 3$ integrins makes transfection of siRNA- $K_v3.3$ in K562 cells affect signaling pathways other than the MAPK pathway, such as Rho-GTPase, PI3K/Akt or Rac1-related pathways [50–52]. Taken together, our data suggest that in the presence of fibronectin, the erythroid differentiation-inducing effect of a decreased $K_v3.3$ expression level was changed to a cell adhesion-enhancing effect; as a result, there are no increases in hemoglobin content as a potent oxidative stress inducer. $K_v3.3$ appears to enhance cell differentiation, and its enhancing effect may be regulated by providing hemin or hemin with fibronectin. Moreover, $K_v3.3$ may also be involved in the cell adhesion process, similar to the function of hERG potassium channels [53], even if the effect is in the opposite direction; the hERG potassium channels enhance cell adhesion, whereas $K_v3.3$ inhibits cell adhesion. Further studies are warranted to identify the particular mechanisms responsible for the different regulation of these channels.

In summary, we found several K_v channels in K562 cells and determined that $K_v3.3$ is involved in K562 cell differentiation through signal cascades such as the MAPK, CREB, and c-fos signaling pathways. We also confirmed that $K_v3.3$ is also involved in cell adhesion properties through the regulation of integrin $\beta 3$. These results imply that K_v channels, at least $K_v3.3$, function in cell differentiation processes. Therefore, further knowledge of the relationship between K_v channels and cell differentiation mechanisms would open a new paradigm for understanding the regulation of cell differentiation processes.

Supporting Information

S1 Fig. Effects of siRNA- $K_v3.3$ transfection on the mRNA expression of $K_v2.1$, $K_v3.4$, and $K_v9.3$ in K562 cells. The knockdown of $K_v3.3$ using siRNA- $K_v3.3$ increased the expression level of $K_v2.1$, but it did not have any effect on the expression levels of $K_v3.4$ and $K_v9.3$. The relative mRNA expressions of the K_v channels were normalized to the GAPDH gene and expressed as a fold change relative to the Mock control group. (TIF)

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Author Contributions

Conceived and designed the experiments: MSS PDR SYL. Performed the experiments: MSS SYC. Analyzed the data: MSS SYC SYL. Wrote the paper: MSS SYL.

References

1. Hille B. Ion channels of excitable membranes. 3rd ed. Sunderland: Sinauer associates, Inc; 2001.

2. Pardo LA, Suhmer W. Eag1 as a cancer target. Expert opinion on therapeutic targets. 2008; 12(7): 837–43. Epub 2008/06/17. doi: [10.1517/14728222.12.7.837](https://doi.org/10.1517/14728222.12.7.837) PMID: [18554152](https://pubmed.ncbi.nlm.nih.gov/18554152/).
3. Bardou O, Trinh NT, Brochiero E. Molecular diversity and function of K⁺ channels in airway and alveolar epithelial cells. American journal of physiology Lung cellular and molecular physiology. 2009; 296(2): L145–55. Epub 2008/12/09. doi: [10.1152/ajplung.90525.2008](https://doi.org/10.1152/ajplung.90525.2008) PMID: [19060226](https://pubmed.ncbi.nlm.nih.gov/19060226/).
4. Kunzelmann K. Ion channels and cancer. J Membrane Biol. 2005; 205(3):159–73. doi: [10.1007/s00232-005-0781-4](https://doi.org/10.1007/s00232-005-0781-4) ISI:000233908900007.
5. Lang F, Foller M, Lang KS, Lang PA, Ritter M, Gulbins E, et al. Ion channels in cell proliferation and apoptotic cell death. J Membrane Biol. 2005; 205(3):147–57. doi: [10.1007/s00232-005-0780-5](https://doi.org/10.1007/s00232-005-0780-5) ISI:000233908900006.
6. O'Grady SM, Lee SY. Molecular diversity and function of voltage-gated (Kv) potassium channels in epithelial cells. Int J Biochem Cell B. 2005; 37(8):1578–94. ISI:000229709900006.
7. Pardo LA. Voltage-gated potassium channels in cell proliferation. Physiology (Bethesda). 2004; 19:285–92. Epub 2004/09/24. doi: [10.1152/physiol.00011.2004](https://doi.org/10.1152/physiol.00011.2004) PMID: [15381757](https://pubmed.ncbi.nlm.nih.gov/15381757/).
8. Wang H, Zhang Y, Cao L, Han H, Wang J, Yang B, et al. HERG K⁺ channel, a regulator of tumor cell apoptosis and proliferation. Cancer research. 2002; 62(17):4843–8. Epub 2002/09/05. PMID: [12208728](https://pubmed.ncbi.nlm.nih.gov/12208728/).
9. Wang Z. Roles of K⁺ channels in regulating tumour cell proliferation and apoptosis. Pflugers Archiv: European journal of physiology. 2004; 448(3):274–86. Epub 2004/03/30. doi: [10.1007/s00424-004-1258-5](https://doi.org/10.1007/s00424-004-1258-5) PMID: [15048575](https://pubmed.ncbi.nlm.nih.gov/15048575/).
10. Jang SH, Ryu PD, Lee SY. Dendrotoxin-kappa suppresses tumor growth induced by human lung adenocarcinoma A549 cells in nude mice. Journal of veterinary science. 2011; 12(1):35–40. Epub 2011/03/04. PMID: [21368561](https://pubmed.ncbi.nlm.nih.gov/21368561/); PubMed Central PMCID: PMC3053465.
11. Jang SH, Choi SY, Ryu PD, Lee SY. Anti-proliferative effect of Kv1.3 blockers in A549 human lung adenocarcinoma in vitro and in vivo. European journal of pharmacology. 2011; 651(1–3):26–32. Epub 2010/11/23. doi: [10.1016/j.ejphar.2010.10.066](https://doi.org/10.1016/j.ejphar.2010.10.066) PMID: [21087602](https://pubmed.ncbi.nlm.nih.gov/21087602/).
12. Jang SH, Choi C, Hong SG, Yarishkin OV, Bae YM, Kim JG, et al. Silencing of Kv4.1 potassium channels inhibits cell proliferation of tumorigenic human mammary epithelial cells. Biochemical and biophysical research communications. 2009; 384(2):180–6. Epub 2009/04/30. doi: [10.1016/j.bbrc.2009.04.108](https://doi.org/10.1016/j.bbrc.2009.04.108) PMID: [19401188](https://pubmed.ncbi.nlm.nih.gov/19401188/).
13. Hemmerlein B, Weseloh RM, Mello de Queiroz F, Knotgen H, Sanchez A, Rubio ME, et al. Overexpression of Eag1 potassium channels in clinical tumours. Molecular cancer. 2006; 5:41. Epub 2006/10/07. doi: [10.1186/1476-4598-5-41](https://doi.org/10.1186/1476-4598-5-41) PMID: [17022810](https://pubmed.ncbi.nlm.nih.gov/17022810/); PubMed Central PMCID: PMC1621079.
14. Golding B, Pillemer SR, Roussou P, Peters EA, Tsokos GC, Ballou JE, et al. Inverse relationship between proliferation and differentiation in a human TNP-specific B cell line. Cell cycle dependence of antibody secretion. J Immunol. 1988; 141(8):2564–8. Epub 1988/10/15. PMID: [2459217](https://pubmed.ncbi.nlm.nih.gov/2459217/).
15. Langdon SP, Richards FM, Hickman JA. Investigations of the relationship between cell proliferation and differentiation of HL-60 cells induced to differentiate by N-methylformamide. Leukemia research. 1988; 12(3):211–6. Epub 1988/01/01. PMID: [3163397](https://pubmed.ncbi.nlm.nih.gov/3163397/).
16. Volpi N, Petrini M, Conte A, Valentini P, Venturelli T, Bolognani L, et al. Effects of glycosaminoglycans on U-937 leukemia cell proliferation and differentiation: structure-function relationship. Experimental cell research. 1994; 215(1):119–30. Epub 1994/11/01. PMID: [7957660](https://pubmed.ncbi.nlm.nih.gov/7957660/).
17. Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood. 1975; 45(3):321–34. Epub 1975/03/01. PMID: [163658](https://pubmed.ncbi.nlm.nih.gov/163658/).
18. Koefler HP, Golde DW. Human myeloid leukemia cell lines: a review. Blood. 1980; 56(3):344–50. Epub 1980/09/01. PMID: [6996765](https://pubmed.ncbi.nlm.nih.gov/6996765/).
19. Ida C, Ogata S, Okumura K, Taguchi H. Induction of differentiation in k562 cell line by nicotinic acid-related compounds. Bioscience, biotechnology, and biochemistry. 2009; 73(1):79–84. Epub 2009/01/09. PMID: [19129652](https://pubmed.ncbi.nlm.nih.gov/19129652/).
20. Rutherford TR, Clegg JB, Weatherall DJ. K562 human leukaemic cells synthesise embryonic haemoglobin in response to haemin. Nature. 1979; 280(5718):164–5. Epub 1979/07/12. PMID: [95354](https://pubmed.ncbi.nlm.nih.gov/95354/).
21. Yang GH, Wang F, Yu J, Wang XS, Yuan JY, Zhang JW. MicroRNAs are involved in erythroid differentiation control. Journal of cellular biochemistry. 2009; 107(3):548–56. Epub 2009/04/08. doi: [10.1002/jcb.22156](https://doi.org/10.1002/jcb.22156) PMID: [19350553](https://pubmed.ncbi.nlm.nih.gov/19350553/).
22. Yi ZC, Wang Z, Li HX, Liu MJ, Wu RC, Wang XH. Effects of chebulinic acid on differentiation of human leukemia K562 cells. Acta pharmacologica Sinica. 2004; 25(2):231–8. Epub 2004/02/11. PMID: [14769215](https://pubmed.ncbi.nlm.nih.gov/14769215/).

23. Zhang D, Cho E, Wong J. A critical role for the co-repressor N-CoR in erythroid differentiation and heme synthesis. *Cell research*. 2007; 17(9):804–14. Epub 2007/09/05. doi: [10.1038/cr.2007.72](https://doi.org/10.1038/cr.2007.72) PMID: [17768398](https://pubmed.ncbi.nlm.nih.gov/17768398/).
24. Limb JK, Yoon S, Lee KE, Kim BH, Lee S, Bae YS, et al. Regulation of megakaryocytic differentiation of K562 cells by FosB, a member of the Fos family of AP-1 transcription factors. *Cellular and molecular life sciences: CMLS*. 2009; 66(11–12):1962–73. Epub 2009/04/22. doi: [10.1007/s00018-009-8775-5](https://doi.org/10.1007/s00018-009-8775-5) PMID: [19381435](https://pubmed.ncbi.nlm.nih.gov/19381435/).
25. Pettiford SM, Herbst R. The protein tyrosine phosphatase HePTP regulates nuclear translocation of ERK2 and can modulate megakaryocytic differentiation of K562 cells. *Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, UK*. 2003; 17(2):366–78. Epub 2003/02/20. doi: [10.1038/sj.leu.2402767](https://doi.org/10.1038/sj.leu.2402767) PMID: [12592337](https://pubmed.ncbi.nlm.nih.gov/12592337/).
26. Huang HM, Chang TW, Liu JC. Basic fibroblast growth factor antagonizes activin A-mediated growth inhibition and hemoglobin synthesis in K562 cells by activating ERK1/2 and deactivating p38 MAP kinase. *Biochemical and biophysical research communications*. 2004; 320(4):1247–52. Epub 2004/07/14. PMID: [15249224](https://pubmed.ncbi.nlm.nih.gov/15249224/).
27. Sangerman J, Lee MS, Yao X, Oteng E, Hsiao CH, Li W, et al. Mechanism for fetal hemoglobin induction by histone deacetylase inhibitors involves gamma-globin activation by CREB1 and ATF-2. *Blood*. 2006; 108(10):3590–9. Epub 2006/08/10. doi: [10.1182/blood-2006-01-023713](https://doi.org/10.1182/blood-2006-01-023713) PMID: [16896160](https://pubmed.ncbi.nlm.nih.gov/16896160/); PubMed Central PMCID: PMC1895433.
28. Akel S, Bertolette D, Petrow-Sadowski C, Ruscetti FW. Levels of Smad7 regulate Smad and mitogen activated kinases (MAPKs) signaling and controls erythroid and megakaryocytic differentiation of erythroleukemia cells. *Platelets*. 2007; 18(8):566–78. Epub 2007/11/29. doi: [10.1080/09537100701549546](https://doi.org/10.1080/09537100701549546) PMID: [18041647](https://pubmed.ncbi.nlm.nih.gov/18041647/).
29. Di Pietro R, di Giacomo V, Caravatta L, Sancilio S, Rana RA, Cataldi A. Cyclic nucleotide response element binding (CREB) protein activation is involved in K562 erythroleukemia cells differentiation. *Journal of cellular biochemistry*. 2007; 100(4):1070–9. Epub 2006/10/26. doi: [10.1002/jcb.21106](https://doi.org/10.1002/jcb.21106) PMID: [17063485](https://pubmed.ncbi.nlm.nih.gov/17063485/).
30. Kim SJ, Widenmaier SB, Choi WS, Nian C, Ao Z, Warnock G, et al. Pancreatic beta-cell prosurvival effects of the incretin hormones involve post-translational modification of Kv2.1 delayed rectifier channels. *Cell death and differentiation*. 2012; 19(2):333–44. Epub 2011/08/06. doi: [10.1038/cdd.2011.102](https://doi.org/10.1038/cdd.2011.102) PMID: [21818121](https://pubmed.ncbi.nlm.nih.gov/21818121/); PubMed Central PMCID: PMC3263490.
31. Tong H, Steinert JR, Robinson SW, Chernova T, Read DJ, Oliver DL, et al. Regulation of Kv channel expression and neuronal excitability in rat medial nucleus of the trapezoid body maintained in organotypic culture. *The Journal of physiology*. 2010; 588(Pt 9):1451–68. Epub 2010/03/10. doi: [10.1113/jphysiol.2009.186676](https://doi.org/10.1113/jphysiol.2009.186676) PMID: [20211981](https://pubmed.ncbi.nlm.nih.gov/20211981/); PubMed Central PMCID: PMC2876802.
32. Pankov R, Yamada KM. Fibronectin at a glance. *Journal of cell science*. 2002; 115(Pt 20):3861–3. Epub 2002/09/24. PMID: [12244123](https://pubmed.ncbi.nlm.nih.gov/12244123/).
33. Liu QH, Fleischmann BK, Hondowicz B, Maier CC, Turka LA, Yui K, et al. Modulation of Kv channel expression and function by TCR and costimulatory signals during peripheral CD4(+) lymphocyte differentiation. *The Journal of experimental medicine*. 2002; 196(7):897–909. Epub 2002/10/09. PMID: [12370252](https://pubmed.ncbi.nlm.nih.gov/12370252/); PubMed Central PMCID: PMC2194034.
34. Leung YM. Involvement of C-type inactivation gating in the actions of voltage-gated K⁺ channel inhibitors. *Pharmacology & therapeutics*. 2012; 133(2):151–8. Epub 2011/11/15. doi: [10.1016/j.pharmthera.2011.10.005](https://doi.org/10.1016/j.pharmthera.2011.10.005) PMID: [22074796](https://pubmed.ncbi.nlm.nih.gov/22074796/).
35. Hocking JC, Pollock NS, Johnston J, Wilson RJ, Shankar A, McFarlane S. Neural activity and branching of embryonic retinal ganglion cell dendrites. *Mechanisms of development*. 2012; 129(5–8):125–35. Epub 2012/05/17. doi: [10.1016/j.mod.2012.05.003](https://doi.org/10.1016/j.mod.2012.05.003) PMID: [22587886](https://pubmed.ncbi.nlm.nih.gov/22587886/).
36. You MH, Song MS, Lee SK, Ryu PD, Lee SY, Kim DY. Voltage-gated K(+) channels in adipogenic differentiation of bone marrow-derived human mesenchymal stem cells. *Acta pharmacologica Sinica*. 2013; 34(1):129–36. Epub 2012/12/12. doi: [10.1038/aps.2012.142](https://doi.org/10.1038/aps.2012.142) PMID: [23222271](https://pubmed.ncbi.nlm.nih.gov/23222271/).
37. Pillozzi S, Becchetti A. Ion channels in hematopoietic and mesenchymal stem cells. *Stem cells international*. 2012; 2012:217910. Epub 2012/08/25. doi: [10.1155/2012/217910](https://doi.org/10.1155/2012/217910) PMID: [22919401](https://pubmed.ncbi.nlm.nih.gov/22919401/); PubMed Central PMCID: PMC3420091.
38. Liu K, Xing H, Zhang S, Liu S, Fung M. Cucurbitacin D induces fetal hemoglobin synthesis in K562 cells and human hematopoietic progenitors through activation of p38 pathway and stabilization of the gamma-globin mRNA. *Blood cells, molecules & diseases*. 2010; 45(4):269–75. Epub 2010/10/12. doi: [10.1016/j.bcmd.2010.09.004](https://doi.org/10.1016/j.bcmd.2010.09.004) PMID: [20926322](https://pubmed.ncbi.nlm.nih.gov/20926322/).
39. Witt O, Sand K, Pekrun A. Butyrate-induced erythroid differentiation of human K562 leukemia cells involves inhibition of ERK and activation of p38 MAP kinase pathways. *Blood*. 2000; 95:2391–6. PMID: [10733512](https://pubmed.ncbi.nlm.nih.gov/10733512/)

40. Rae JL, Shepard AR. Kv3.3 potassium channels in lens epithelium and corneal endothelium. *Experimental eye research*. 2000; 70(3):339–48. Epub 2000/03/14. PMID: [10712820](#).
41. Fernandez FR, Morales E, Rashid AJ, Dunn RJ, Turner RW. Inactivation of Kv3.3 potassium channels in heterologous expression systems. *The Journal of biological chemistry*. 2003; 278(42):40890–8. Epub 2003/08/19. doi: [10.1074/jbc.M304235200](#) PMID: [12923191](#).
42. Patel AJ, Honore E. Molecular physiology of oxygen-sensitive potassium channels. *European Respiratory Journal*. 2001; 18:221–7. PMID: [11510795](#)
43. Vega-Saenz de Miera E, Rudy B. Modulation of K⁺ channels by hydrogen peroxide. *Biochemical and biophysical research communications*. 1992; 186(3):1681–7. Epub 1992/08/14. PMID: [1380809](#).
44. Ruppertsberg JP, Frank R, Pongs O, Stocker M. Cloned neuronal IK(A) channels reopen during recovery from inactivation. *Nature*. 1991; 353(6345):657–60. Epub 1991/10/17. doi: [10.1038/353657a0](#) PMID: [1922383](#).
45. Liu Y, Gutterman DD. Oxidative stress and potassium channel function. *Clinical and experimental pharmacology & physiology*. 2002; 29(4):305–11. Epub 2002/05/03. PMID: [11985541](#).
46. Ryter SW, Tyrrell RM. The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free radical biology & medicine*. 2000; 28(2): 289–309. Epub 2001/04/03. PMID: [11281297](#).
47. de Nigris F, Lerman A, Ignarro LJ, Williams-Ignarro S, Sica V, Baker AH, et al. Oxidation-sensitive mechanisms, vascular apoptosis and atherosclerosis. *Trends in molecular medicine*. 2003; 9(8):351–9. Epub 2003/08/21. PMID: [12928037](#).
48. Jarvinen M, Ylanne J, Virtanen I. The effect of differentiation inducers on the integrin expression of K562 erythroleukemia cells. *Cell biology international*. 1993; 17(4):399–407. Epub 1993/04/01. doi: [10.1006/cbir.1993.1078](#) PMID: [8318951](#).
49. Danen EH, Aota S, van Kraats AA, Yamada KM, Ruiters DJ, van Muijen GN. Requirement for the synergy site for cell adhesion to fibronectin depends on the activation state of integrin alpha 5 beta 1. *The Journal of biological chemistry*. 1995; 270(37):21612–8. Epub 1995/09/15. PMID: [7545166](#).
50. Huveneers S, Danen EH. Adhesion signaling—crosstalk between integrins, Src and Rho. *Journal of cell science*. 2009; 122(Pt 8):1059–69. Epub 2009/04/03. doi: [10.1242/jcs.039446](#) PMID: [19339545](#).
51. Zeller KS, Riaz A, Sarve H, Li J, Tengholm A, Johansson S. The role of mechanical force and ROS in integrin-dependent signals. *PloS one*. 2013; 8(5):e64897. Epub 2013/06/06. doi: [10.1371/journal.pone.0064897](#) PMID: [23738008](#); PubMed Central PMCID: PMC3667809.
52. Guidetti GF, Canobbio I, Torti M. PI3K/Akt in platelet integrin signaling and implications in thrombosis. *Advances in biological regulation*. 2015; 59:36–52. Epub 2015/07/15. doi: [10.1016/j.jbior.2015.06.001](#) PMID: [26159296](#).
53. Arcangeli A, Becchetti A, Cherubini A, Crociani O, Defilippi P, Guasti L, et al. Physical and functional interaction between integrins and hERG potassium channels. *Biochemical Society transactions*. 2004; 32(Pt 5):826–7. Epub 2004/10/21. doi: [10.1042/BST0320826](#) PMID: [15494025](#).