# K-CL co-transport plays an important role in normal and β thalassemic erythropoiesis

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*Acknowledgments: we thank Prof. Luca Baldini and Dr, Lucia Nobili from the University of Milan for their helpful discussion and technical support.*

*Funding: this study was supported by FIRB grant RBNE01XHME to MDC, AI and LDF, a Telethon (Prog. GGP02202) grant to AI and NIH grant HL077765 to SLA.*

*Manuscript received March 26, 2007.*
*Manuscript accepted July 24, 2007.*

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## ABSTRACT

### Background and Objectives

Cell volume changes are hallmarks of both cell maturation and apoptosis, and are paralleled by modulation of membrane ion transport pathways. We evaluated the possible role of K-Cl co-transport (KCC) in both normal and β-thalassemic erythropoiesis *in vitro*.

### Design and Methods

We studied the effects of the KCC inhibitor, DIOA, on cell proliferation and differentiation, on expression of KCC mRNA and polypeptides, and on expression of cell cycle and apoptosis genes in *in vitro* liquid-cultures of $CD34^+$ cells from normal and β-thalassemic subjects.

### Results

β-thalassemic erythroid precursors showed increased abundance of *KCC1-3* mRNA and of KCC polypeptides in late erythropoiesis. DIOA markedly modified the composition of normal erythroid precursors, promoting differentiation and arrest at the polychromatic erythroblast stage and resulting in a precursor distribution profile similar to that of untreated β-thalassemic cells. DIOA up-regulated cyclin-D mRNA levels in late erythropoiesis in both cell models, paralleling changes in the percentage of S-phase-cells. Caspase-3 activity in late erythropoiesis declined to similar degrees in both cell models. DIOA did not modify caspase-3 or -8 mRNA levels.

### Interpretation and Conclusions

Ineffective erythropoiesis of *in vitro* cultured β-thalassemic cells is likely related to impaired cell maturation. KCC activity appears to contribute to erythroid cell growth during late erythropoiesis.

Key words: erythroid precursors, DIOA, cyclin-D, p21.

Haematologica 2007; 92:1319-1326. DOI: 10.3324/haematol.11556

Erythropoiesis is a complex multistage process characterized by differentiation of pluripotent hematopoietic progenitors to reticulocytes and then to mature red cells. Although understanding of the mechanisms involved in the maturation of normal erythroid precursors has progressed in the last decade, much remains unknown about erythroid maturation in inherited erythroid diseases. β thalassemia is a congenital red cell disorder characterized by the absence or reduced synthesis of hemoglobin β globin chains. Despite extensive knowledge of the molecular defects causing β thalassemia, less is known about the major factors contributing to β thalassemic anemia: the reduction of β thalassemic red cell life-span and ineffective erythropoiesis.[1-4]

Ineffective erythropoiesis has been shown in β thalassemia *in vivo* by ferrokinetic assays, suggesting a block in erythroid precursors and a precocious cell death in bone marrow.[2-5] Recently, Mathias *et al*. showed that ineffective erythropoiesis in the bone marrow precursors of patients with β thalassemia major is associated with progressively increased apoptosis of polychromatophilic normoblasts of late erythropoiesis and seems to be related to hemoglobin chain synthesis.[6] However, the mechanisms underlying apoptosis of β thalassemic erythroid precursors and the developmental kinetics of β thalassemic ineffective erythropoiesis need further investigation.

Since cell volume change is one of the hallmarks of cell progression towards apoptosis or proliferation, the modulation of ion transport pathways paralleling these cellular events has been proposed to act as a second or even third messenger.[7] In addition, changes in cell $K^+$ content have been described in mouse erythroleukemia (MEL) cells during the transition from proerythroblasts to young reticulocytes, as well as in leukemic cells exposed to glucocorticoid treatment,[8,9] suggesting the involvement of pathways regulating cell $K^+$ content in the maturation of erythroid precursors.

The K-Cl co-transport (KCC) family plays a crucial role in red cell volume regulation and intracellular ion homeostasis.[10-13] Multiple KCC gene products (*KCC1, KCC3* and *KCC4*) have been identified in various non-neuronal cell types.[10-17] Recently, KCC1 and KCC3 have been shown to participate in cellular proliferation events in cervical cancer cells and in KCC3-transfected NIH/3T3 cells, respectively.[10,11] In red cells, the KCC function is modulated by cell swelling, cell acidification, reduced cell magnesium (Mg) content, membrane oxidative damage and cell age.[3,16-20] Abnormal activation of KCC has been reported in sickle red cells, in β thalassemic syndromes and in pathological erythrocytes containing positively charged hemoglobin variants in β6 and β7.[3,18,19,21] Studies in diseased red cells have shown that KCC plays a crucial role in red cell volume regulation, contributing to red cell water and $K^+$ loss and generation of dehydrated red cells.[3,20,22] Recently KCC1, 3 and 4 have been described in human reticulocytes and in human erythroid precursors from sickle cell subjects.[14,15] Although the activity of KCC has been partially characterized in circulating erythrocytes, the role of KCC in cell volume regulation during cell growth in normal and β thalassemic erythroid precursors is still unknown.

Here, we evaluated the expression of *KCC1, KCC3* and *KCC4* mRNA and KCC polypeptides in normal and in β thalassemic erythroid precursors. We then asked whether KCC might be important in erythroid cell proliferation in both normal and β thalassemic cells. We evaluated the effects of the KCC inhibitor [(dihydroindoenyl)oxy]alkanoic acid (DIOA)[3,23] on cell proliferation and differentiation, and on expression of KCC mRNA and protein, expression of the cell cycle genes cyclin-D (*CycD*) and p21 (*P21*), and on expression of the apoptosis genes caspase-3 (*Casp3*) and caspase-8 (*Casp8*).

## Design and Methods

### Cell culture

Peripheral blood from normal volunteers and from homozygous ($\beta^{0cod39}$) β-thalassemia patients was collected into heparinized tubes, after informed consent had been obtained. Blood from β-thalassemic patients was collected before their routine transfusion according to the guidelines established by the local Ethics Committee for studies involving human subjects. We analyzed 20 separate individuals' cultured cells from normal subjects and 20 separate individuals' cultured cells from ten β-thalassemic patients. Light-density mononuclear cells were obtained by centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient, as previously described. The $CD34^+$ cells were positively selected by anti-$CD34^+$ tagged magnetic beads (Mini-MACS columns; Miltenyi Biotech, Auburn, CA, USA) according to the manufacturer's protocol. The recovery was more than 90% $CD34^+$ cells, as determined by flow cytometry. $CD34^+$ cells were grown at a density of $10^5$ cells/mL in α-minimal essential medium (α-MEM; GIBCO, Grand Island, NY, USA) supplemented with 100 U/mL penicillin-streptomycin, 2 mmol/L L-glutamine, $10^{-6}$ mol/L hydrocortisone, $10^{-3}$ g/L nucleotide, $25x10^{-3}$ mg/L gentamycin, $10^{-4}$ mol/L 2-mercaptoethanol, 1% deionized bovine serum albumin [BSA] (all from Sigma, St Louis, MO, USA), 30% fetal bovine serum (FBS, GIBCO) and 1 μg/mL cyclosporine A (Sigma). The following recombinant cytokines were added to the media: 3 U/mL recombinant human (rHu) erythropoietin (rHuEPO, Janssen-Cilag, Milan, Italy), 20 ng/mL rHu stem cell factor (SCF, PeproTech, London, UK), 10 ng/mL rHu interleukin-3 (IL-3, PeproTech).

DIOA was used to inhibit KCC activity. The DIOA was dissolved in DMSO to generate a stock solution. This stock solution was further diluted with the cultured medium, and added to the cells in culture medium at the final concentration of 10 μM or 100 μM as specified in the text or figure legends.[3,18,23] Experiments, which were carried out in parallel cell cultures and to which only DMSO diluted as described above but without DIOA

was added, indicated no effect on cell growth (*data not shown*). Cells were incubated at 37°C in 5% $CO_2$ for 14 days with renewal of the medium after 7 days of culture. Cell samples were collected on days 7 and 14 for cell counting and determination of cell viability.

### Phenotypic analysis of cultured cells

Cell morphology was analyzed on cytospin smears stained with May-Grünwald-Giemsa. Benzidine staining was used to determine hemoglobin-containing cells.

The erythroid cell antigen profile was analyzed with the Becton Dickinson FACScan flow cytometer using Cell Quest software after gating on viable cells, labeled with the following fluorophor-conjugated antibodies: phycoerythrin (PE)-conjugated anti-CD34, perCP-conjugate anti-CD45 (Becton Dickinson, San José, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-glycophorin A (DakoCytomation, Glostrup, Denmark).

### Determination of cell cycle stage by fluorescence-activated cell sorting (FACS)

Cellular DNA content was determined by FACS analysis after staining with propidium iodide (PI; Sigma, St Louis, MO, USA). Cells ($10^6$) were harvested at day 7 and 14 of culture, fixed by slow addition of cold 70% ethanol to a volume of 500 μL, and stored at 4°C. The fixed cells were stained in 1 mL of 20 μg/mL PI, 1 mg/mL RNAase (Sigma), Nonidet P40 1% in 1X phosphate-buffered saline for 1 hour at room temperature. Next, $5\times10^5$ cells were evaluated, and the proportion of cells in S-phase was estimated using the MODFIT cell cycle analysis program (Verity Software House, Topshem, ME, USA) on a FACScan flow cytometer (Becton Dickinson).[10]

### Real-time reverse transciptase-polymerase chain reaction (RT-PCR)

Real-time RT-PCR was performed for the following genes: *KCC1, KCC3, KCC4, CycD, P21, Casp3* and *Casp8*. cDNA was generated from total RNA samples with the Reverse Transcription Reagents kit (Invitrogen). Four micrograms total RNA from each sample were incubated at 25°C for 10 min and reverse-transcribed at 42°C for 50 min, using random primers. Real-time SYBR green RT-PCR was performed with the ABI PRISM 7900HT (Applied Biosystems). The following primers (designed using Primer Express Software version 2.1; Applied Biosystems) were used:

*KCC1:* Fov: ACA GCC CCA ACC TTA CGA C
Rev: CTC CAC GAT GTCACC CTT CT
*KCC3:* Fov: GATGATGGAACAAAGGTCCCAG;
Rev: TTCACCAATTGTGCCTCTCTGT
*KCC4:* Fov GTATCACTTGCGCATCAGCG;
Rev GTCCTCTCGTAGGTGAAAGCAGAT
*CycD:* Fov: AGA CCT TTT GGT CCC TCT GT
Rev: AGC TTC GAT CTG CTC CTG AC
*P21:* Fov: AGT GGA CAG CGA GCA GCT
Rev: CCG TGG GAA GGT AGA GCTT
*Casp3:* Fov: TGTGGAATTGATGCGTGATGTT;
Rev: TCACCATGGCTCAGAAGCAC
*Casp8:* Fov: ACACCAGGCAGGGCTCAA;
Rev: CTGGCACTGGCTGTTTGCT
*β-actin:* Fov: CGTGCTGCTGACCGAGG
Rev: GAAGGTCTCAAACATGATCTGGGT

The reaction mixtures (25 μL) contained 5 μL of cDNA solution diluted 10-fold, and 20 μL PCR master mix (PCR SYBER Green master mix, Applied Biosystems) containing 0.6 μM of each primer. The cycling temperature was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 10 min. The ABI PRISM 7900HT instrument (Applied Biosystems) determined the threshold cycle (Ct) representing the cycle number at which fluorescence intensity significantly exceeded background fluorescence. Melting curve analysis was also performed after PCR amplification to verify the absence of primer dimer in the PCR products (95°C for 15 sec, 60°C for 15 min, 95°C for 15 sec). All PCR reactions were performed in duplicate. The target expression values were normalized to the expression value of *β-actin*, used as a housekeeping gene. Since we focused on the effects of cell growth on expression of these genes, we considered the ratio between the mean values of normalized gene expression at day 14 and at day 7 of culture (14d/7d) in both normal and β-thalassemic cells.

### KCC protein expression and cell cation content

KCC protein expression was evaluated by immunoblot analysis using immunospecific anti-KCC-COOH terminus antibody in cells at the different differentiation time points studied;[22,24] anti-actin (Sigma Chemical Co, St Louis, MO, USA) was used as the loading control. Cells were washed in a buffer containing 150 mM choline chloride, 1 mM $MgCl_2$, and 10 mM Tris-MOPS, pH 7.4 at 4°C (CWS). The cells were lysed in ice cold Phosphate Lysis Buffer (LB: 5 mM $Na_2HPO_4$ pH 8.0, containing: protease inhibitor cocktail tablets (Roche, Indianapolis, IN, USA), and 3 mM benzamidine (final concentration). The cell $Na^+$ and $K^+$ content was determined by atomic absorption spectrometry (ANALYST 2000, Perkin-Elmer) of cells washed in CWS buffer. Protein concentration was measured by Lowry's assay.[25]

### Determination of caspase-3 activity

Caspase-3 activity was assayed in cells harvested at day 7 and 14 of culture and lysed by freeze-thawing. The cell lysate was centrifuged at 200 g for 5 minutes and the supernatant assayed for caspase-3 activity using the ENZCHEK caspase-3 assay kit with a DEVD-AMC substrate (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions.[26]

### Statistical analysis

Comparisons of separate variables were performed

using the two-tailed Student's *t* test. Comparisons involving more than two groups were performed by one-way ANOVA with Tukey's test for *post hoc* comparison of means.

## Results

### Effects of KCC pharmacological inhibition on erythroid cell proliferation

We studied erythroid precursors derived by *in vitro* liquid-culture of CD34⁺ cells from peripheral blood of normal and β thalassemic subjects. As shown in Figure 1A, the morphology of control and β thalassemic cells was compatible with erythroid differentiation, as also supported by FACS analysis of cellular glycophorin-A expression (*data not shown*). We observed an increase in cell number at day 14 of culture only in control cells, whereas in β thalassemic patients, the total number of cells significantly decreased at day 14 of culture, suggesting the presence of ineffective erythropoiesis (Figure 1B). In addition, exposure to the KCC inhibitor – DIOA, significantly reduced the number of total cells proceeding towards maturation in both control and β thalassemic cell cultures at day 14 (Figure 1A and 1B). Since the effect of DIOA on cell number was concentration dependent (Figure 1B), we decided to use 10 μM DIOA in subsequent experiments based on previous *in vitro* pharmacological studies conducted in different cell lines, in which DIOA at similar concentrations maintained KCC inhibition.[10,11,18]

We next evaluated cell population composition at the different time points studied. At day 7 the control cell population comprised 77.5±9.8% (n=10) of proerythroblasts, whereas at day 14, 86.2±2.1% (n=10) of cells were orthochromatic normoblasts and mature erythrocytes (Table 1). The cell populations in β thalassemic patients at day 14 differed from those in their control counterparts. A large proportion of the β thalassemic cells were polychromatophilic erythroblasts, possibly related to a block of cell proliferation shifting towards ineffective erythropoiesis (Table 1). The presence of the KCC inhibitor, DIOA, markedly modified the proliferation profile of control cells, which at day 14 showed a differentiation block somewhat similar to that observed in untreated β thalassemic cells, suggesting that KCC inhibition might contribute to ineffective erythropoiesis (Table 1). In β thalassemic cell cultures, DIOA further amplified the cell proliferation block, as supported by the increase in the basophilic erythroblast population (Table 1).

### KCC expression and cell cation content in normal and β thalassemic erythroid cultured cells

We next asked whether *KCC1, KCC3* and *KCC4* genes were modulated during maturation of erythroid cell precursors. In both control and β thalassemic cells we measured the expression of *KCC1, KCC3* and *KCC4* genes at days 7 and 14 of culture in the presence and absence of the KCC inhibitor DIOA. During cell growth, *KCC1* and KCC3 mRNA levels were higher in β thalassemic cells than in controls, whereas *KCC4* mRNA levels were lower in β thalassemic cells than in controls (Figure 2A). These data suggest that the *KCC1* and *KCC3* genes are up-regulated in β thalassemic erythroid precursors; we did not evaluate the contribution of *KCC1* splice variants to total *KCC1* gene expression.[14] In the presence of the KCC inhibitor DIOA, *KCC1* mRNA levels were up-regulated in both normal and β thalassemic cells, whereas *KCC3* mRNA levels were significantly reduced in both normal and β thalassemic cells. In contrast, *KCC4* mRNA level was unchanged in control cells but up-regulated in β thalassemic cells treated with DIOA (Figure 2A). These data suggest that the pharmacological inhibition of KCC modulates *KCC1-KCC3* gene expression in both cell models, while *KCC4* gene expression is affected only in β thalassemic cells.

We evaluated KCC polypeptide expression by immunoblot analysis with anti-KCC-COOH terminus

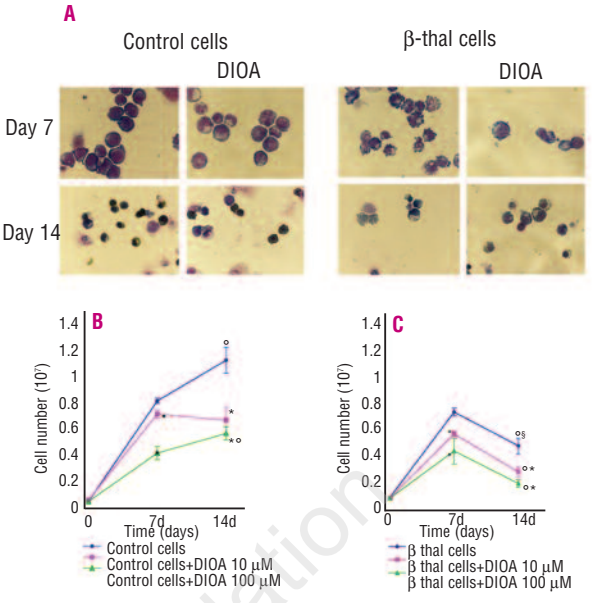


**Figure 1. A.** Morphology of erythroid precursors derived by *in vitro* liquid-culture of CD34⁺ cells isolated from peripheral blood of normal and β thalassemic (β thal) individuals in the absence and presence of the KCC inhibitor, [(dihydroindenyl)oxy] alkanoic acid (DIOA)(10 μM). Cytospin preparations of cells were stained with May-Grünwald-Giemsa and benzidine. Cells were imaged under oil at 10x magnification using a PlanFluor objective with 1.30 numeric aperture on a Nikon Eclipse 80i microscope (Tokyo, Japan). Images were captured with a Nikon DS-5M camera and processed with Digital Slide DS-L1 (Nikon) and Adobe Photoshop 6.0 (Adobe Systems, San José, CA, USA). **B.** Effects of DIOA 10 μM (pink squares) and 100 μM (green triangles) on total cell numbers of control cells (blue squares). **C.** Effects of DIOA 10 μM (pink squares) and 100 μM (green triangles) on the total number of β thalassemic cells (blue circles). Cell viability was monitored by trypan-blue exclusion (0.08%). Data are presented as means ±SD (n=10); *$p<0.05$ compared to untreated cells; °$p<0.05$ compared to day 7 of culture; §$p<0.05$ untreated β thalassemic vs untreated control cells.

**Table 1.** Effects of the KCC inhibitor, DIOA, on the cell population composition of normal and β-thalassemic precursors.

| | *Pronormoblasts (%)* | *Basophilic normoblasts (%)* | *Polychromatophilic normoblasts (%)* | *Orthochromatic normoblasts (%)* | *Erythrocytes (%)* | *White blood cells and macrophages (%)* |
|---|---|---|---|---|---|---|
| *Control cells (n=10)* | | | | | | |
| Day 7 | 59.4±4.6 | 32.3±5.4 | 2.55±1.7 | 1.75±0.5 | 0 | 5.7±2.9 |
| Day 7 + DIOA | 77.5±9.8° | 15.5±3.2° | 0 | 0 | 0 | 7.3±3.5 |
| Day 14 | 0 | 3.7±1.8 | 14.7±1.8 | 58.2±6.8 | 9.1±5.3 | 10.2±4.6 |
| Day 14 + DIOA | 0 | 8.7±3.1° | 35.3±5.7° | 36.7±5.1° | 5.2±2.4 | 14±4.2 |
| *β–thal cells (n=10)* | | | | | | |
| Day 7 | 68.6±8.2 | 24.1±4.4 | 1.4±0.2 | 0 | 0 | 5.8±2.1 |
| Day 7 + DIOA | 82.3±7.8*° | 11.2±2.6*° | 0 | 0 | 0 | 6.7±3.5 |
| Day 14 | 0 | 16.3±4.6 | 36.5±3.7 | 38.1±4.3 | 2.5±0.8 | 7.2±2.6 |
| Day 14 + DIOA | 0 | 38.1±5.2*° | 34.7±2.6 | 16.4±3.1*° | 0 | 9.2±1.2 |

*Data are presented as means ± SD (n of experiments); *p<0.05 compared to untreated cells; °p< 0.05 compared to control cells.*

antibody, which detects all three KCC polypeptide gene products. KCC protein levels were significantly higher in β thalassemic cells than in controls after both 7 days and 14 days of culture (Figure 2B). Moreover, DIOA treatment increased KCC protein abundance in β thalassemic cells, reflecting up-regulation of mRNA levels of two of the three *KCC* genes studied (Figure 2A-B).

In view of the observed changes in *KCC* mRNA and protein levels, we next measured cellular $Na^+$ and $K^+$ contents during differentiation in both control and β thalassemic cells. At day 7, cell $Na^+$ content was slightly higher in β thalassemic cells than in normal control cells (Figure 2C). At day 14, cell $Na^+$ content decreased to similar statistically indistinguishable extents in both cell models (Figure 2C). At day 7, cell $K^+$ content was similar in β thalassemic cells and in normal controls. However, by day 14, cell $K^+$ content increased in both cell types compared to day 7 values, although the β thalassemic cell $K^+$ content increased to a much smaller degree, and was significantly lower than in normal cells (Figure 2C).

In the presence of the KCC inhibitor – DIOA, cell $Na^+$ content at day 7 was markedly lower in both cell types than in untreated counterpart cultures. By day 14, in the presence of DIOA, cell $Na^+$ content had declined still further in control cells, and remained lower than that in untreated control cells. In contrast the $Na^+$ content of untreated cells at day 14 remained essentially unchanged, and was similar to that of untreated β thalassemic cells (Figure 2C). $K^+$ content of control DIOA-treated cells at day 7 was slightly higher than that of untreated control cells, while at day 14 it was significantly lower than that of untreated control cells, reaching values similar to those observed in untreated β thalassemic cells tested at the same time (Figure 2C). In β thalassemic cells, the KCC inhibitor DIOA did not significantly change cell $K^+$ content at day 7 compared to untreated cells, whereas cell $K^+$ content at day 14 was slightly increased compared to that of untreated cells (Figure 2C).

### Effects of pharmacological inhibition of KCC on cell-cycle markers

Cell–cycling was evaluated by FACS-analysis, measuring the proportion of cells in S-phase. Quantitative RT-PCR was used to measure mRNA levels of *CycD* and *P21* in control and β thalassemic cells during cell growth in the absence and presence of the KCC inhibitor, DIOA. In untreated cells, the *Cyc*D mRNA level was down-regulated in β thalassemic cells compared to the level in normal control cells, but the *P21* mRNA level was 7 fold up-regulated in β thalassemic cells (Figure 3A). With chronic exposure to DIOA, *Cyc*D was up-regulated in both cell models (Figure 3A), in agreement with the reduction in the proportion of S-phase cells (control + DIOA 96±1% vs control untreated 140±5%; β thal + DIOA 43±2% vs 63±3% β thal untreated; n=6; $p<0.05$). In contrast, *P21* gene expression in the presence of DIOA was greatly up-regulated in control cells, but substantially down-regulated in β thalassemic cells (Figure 3A).

### Effects of pharmacological inhibition of KCC on cell apoptosis

Caspase-3 and caspase-8 are important in cell apoptosis. Although caspase-3 plays a role in apoptosis in most cells, caspase-3 activation in early-stage erythropoiesis is followed by declining activity in late-stage erythropoiesis. Recent knockdown experiments failed to demonstrate a pro-apoptotic role of caspase-3 in human erythropoiesis, but supported its novel and important role in the enucleation process.[26] The role of caspase-3 in apoptosis and enucleation has not been reported in mouse erythropoiesis.[26,27]

In control cells, *Casp3* gene expression did not change significantly during cell proliferation with or without DIOA. In β thalassemic cells, *Casp3* gene expression greatly exceeded that in control cells during cell proliferation and it was unaffected by DIOA treatment (Figure 3A). Since caspase-3 activity may not reflect modulation of *Casp3* gene expression, we measured caspase-3 activity in both control and β thalassemic cells at days 7 and 14 of cell differentiation. Caspase-3 activity was similar in both cell

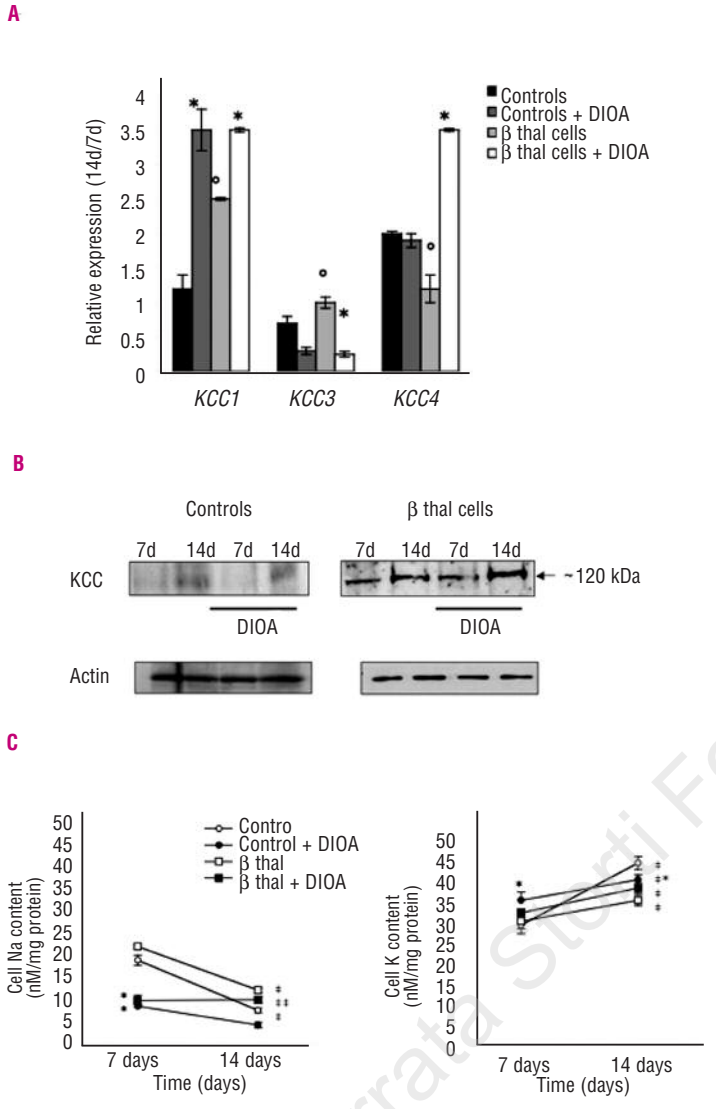


**Figure 2. A.** Effect of cell growth on semi-quantitative RT-PCR expression profiles of *KCC1, KCC3, KCC4* in normal (black bar; controls) and β thalassemic erythroid precursors (light gray bar; β thal cells). Also shown are the effects of the KCC inhibitor DIOA (10 μM), on *KCC1, KCC3* and *KCC4* gene expression in normal (gray bar; controls) and β thalassemic erythroid precursors (white bar; β thal) during cell growth. Data are presented as the ratio between the mean values of normalized gene expression at day 14 and at day 7 of culture (14d/7d) in both normal and β-thalassemic cells; **p*<0.05 compared to untreated cells; °*p*<0.05 compared to control cells (n=10). **B.** Immunoblot analysis of KCC protein expression in normal and β thalassemic erythroid precursors during maturation (7 day and 14 days: 7d and 14 d) in the presence and absence of the KCC inhibitor DIOA (10 μM). Expression of actin was used as a loading control. Representative of six independent experiments with similar results. **C.** Cell $Na^+$ and $K^+$ contents in normal (controls) and β thalassemic erythroid (β thal) precursors during maturation. Data are presented as means±SD (n=6); **p*<0.05 compared to untreated cells; ‡*p*<0.05 compared to baseline.

models and significantly decreased during cell differentiation in both control and β thalassemic cells. DIOA treatment reduced caspase-3 activity observed at day 7 in both cell models compared to the activity in untreated cells (Figure 3B).

*Casp8* mRNA levels in β thalassemic cells were higher than in control cells. DIOA treatment further induced *Casp8* gene expression in both cell types (Figure 3A).

## Discussion

In the present study, we showed that liquid cultures of differentiating erythroid precursors derived from $CD34^+$ peripheral blood cells of β thalassemic subjects mimic the ineffective erythropoiesis characteristic of β thalassemia major (Figure 1, Table 1). In late stages of *in vitro* erythropoiesis, we observed that β thalassemic precursors are mainly represented by polychromatophilic erythroblasts, suggesting a block of cell proliferation contributing to an *in vitro* correlate of ineffective erythropoiesis (Table 1).

Since cell volume changes are hallmarks of both cell maturation events and cell apoptosis and are paralleled by modulation of membrane ion transport pathways,[28] we evaluated the possible role of KCC in both normal and β thalassemic erythropoiesis *in vitro*. KCC activity is involved in cell volume and $K^+$ regulation in both normal and diseased red cells, such as β thalassemic erythrocytes.[3] Recently, *KCC1* and *KCC3* gene products have been reported to play important roles in cell proliferation, growth, and associated events in diverse cell lines.[10,11] In human erythroid precursors the expression of KCC1 splicing variants was found to differ in cells from sickle cell anemia patients compared to red cells from normal subjects.[14,15] In β thalassemic erythroid precursors, the increased abundance of *KCC1* and *KCC3* mRNA and of KCC polypeptides in the late phase of erythropoiesis indicates that KCC expression is modulated during β thalassemic erythroid maturation and that KCC activity is likely mediated principally by *KCC1* and *KCC3* (Figure 2).

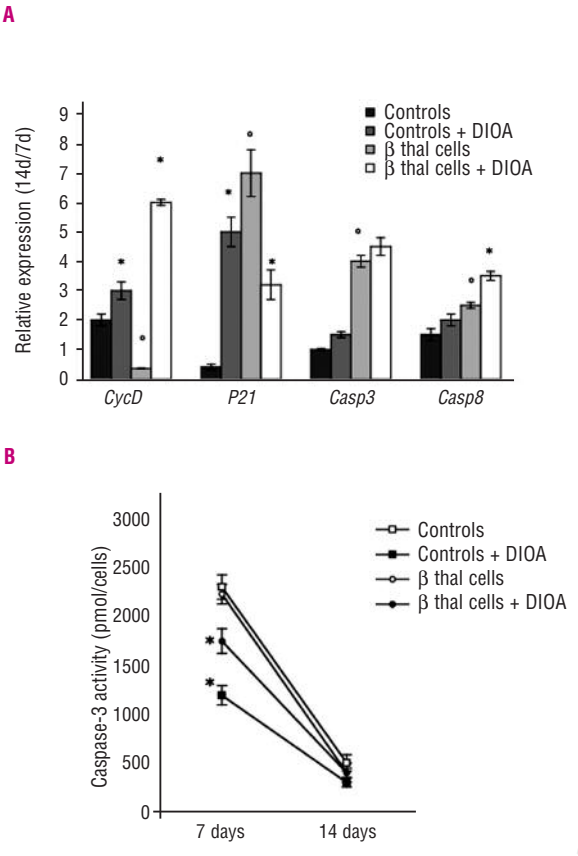


**Figure 3. A. Effect of cell growth on quantitative RT-PCR expression profiles of *CycD, P21*, *Casp3* and *Casp8* in normal (black bars) and β thalassemic erythroid precursors (light bars) during maturation. Also shown are mRNA expression profiles of *CycD, P21, Casp3* and *Casp8* in the presence of the KCC inhibitor, DIOA (10 μM), in normal (black bars) and β thalassemic erythroid precursors (light bars) during maturation. Data are presented as the ratio between the mean values of normalized gene expression at day 14 and that at day 7 of culture (14d/7d) in both normal and β-thalassemic cells; *$p<0.05$ compared to untreated cells; °$p<0.05$ compared to control cells (n=8). B. Caspase-3 activity in normal (controls) and β-thalassemic (β thal) erythroid precursors at 7 and 14 days during maturation in the absence and presence of the KCC inhibitor DIOA (10 μM), *$p<0.05$ compared to untreated cells.**

The relative role of reduced KCC3 and increased KCC4 in differentiation of β thalassemic erythroid precursors remains unclear. The pharmacological inhibition of KCC activity affects cell growth of both normal and β thalassemic cells.

The presence of the KCC inhibitor, DIOA, during differentiation markedly modified the composition of normal erythroid precursors in the late phase of erythropoiesis. The modified cell profile was characterized by: (i) reduction in the total number of cells; (ii) accumulation of polychromatic erythroblasts and (iii) a reduction in the proportion of S-phase cells, indicating an arrest of cell proliferation at the polychromatic erythroblastic stage similar to that observed in untreated β thalassemic cells (Figure 1, Table 1). In addition, DIOA increased *KCC1* mRNA levels in both control cells and β thalassemic precursors, whereas *KCC4* mRNA was up-regulated and *KCC3* mRNA was down-regulated only in β thalassemic precursors (Figure 2A). The prolonged inhibition of KCC by DIOA during erythropoiesis was associated with a significant reduction in cell $K^+$ content at day 14 in control cells, reaching values similar to those in β thalassemic cells (Figure 2C). However, the changes in cell composition, with a block in cell growth caused by DIOA, might also have contributed to the reduction of cell $K^+$ content compared to that observed in untreated control cells. Thus, in normal erythroid precursors, pharmacological inhibition of KCC activity induces ineffective erythropoiesis with arrested cell expansion similar to that occurring β thalassemic cells, indicating that KCC plays an important role in erythroid maturation.

Cell growth is tightly regulated by cell cycle regulatory molecules, including cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors (Cki). In the current study we evaluated *CycD* expression. In the presence of DIOA, *Cyc*D mRNA levels were increased in both normal and β thalassemic precursors in late phase erythropoiesis. This effect paralleled changes in the percentage of S-phase cells, and supports a role of KCC in the progression of cell growth and differentiation during erythropoiesis.

Although *P21* gene expression increases over time in colonies of all hematopoietic lineages, p21 protein levels increase only in certain cell lineages during cell growth (i.e. megakaryocytes and monocytes-macrophages).[29] Our data confirmed up-regulation of *P21* gene expression in both normal and β thalassemic precursors (Figure 4A). Interestingly, however, *P21* expression appears down-regulated during erythropoiesis of β thalassemic cells in the presence of DIOA.

Since caspase-3 is activated in normal erythropoiesis, playing a pivotal role in both apoptosis and cell maturation towards enucleation,[26] we evaluated *Casp-3* and *Casp-8* gene expression and caspase-3 activity in both cell models of erythropoiesis. In β thalassemic precursors, expression of both *Casp3* and *Casp8* genes was up-regulated in late phase erythropoiesis compared to the expression in normal control cells, whereas caspase-3 activity declined to similar low levels in late stage erythropoiesis (day 14) in both control and β thalassemic precursors (Figure 3). The discrepancy between *Casp3* gene expression and caspase-3 protein activity observed in β thalassemic cells might be related either to the presence of cellular stress factors, inducing *Casp-3* gene up-regulation, or to a ubiquitin-dependent proteolytic pathway, as established for p27 protein.[29] DIOA did not significantly modify *Casp-3* and *Casp-8* gene expression in either cell group. In contrast, DIOA treatment was associated with significantly reduced caspase-3 activity in both normal and β thalassemic cells, but only in the early phase of erythropoiesis (Figure 3B).

In conclusion, this study demonstrates an *in vitro* correlation of ineffective erythropoiesis in cultured β thalassemic cells. The data show that ineffective erythropoiesis of *in vitro* cultured β thalassemic cells is likely related to

impaired cell maturation. The study also strongly suggests that KCC activity may contribute to erythroid cell growth in the late phase erythropoiesis. Further studies are needed to characterize the roles of KCC expression and activity in normal and pathological erythropoiesis.

### Authors' contributions

*All authors meet the criteria for being contributing authors. LDF, AI, MDC, ASL outlined the study and wrote the paper. LR performed liquid cultures, FACS analyses, and caspase-3 activity assays and organized the data for Figure 1, Table 1 and Figure 3B. CF, SV carried out molecular studies (Figures 2A and 3A), AS and CP performed protein studies and determined cell cation content. All authors were involved in discussion and interpretation of the data; all revised the manuscript and approved the final version.*

### Conflicts of Interest

*The authors reported no potential conflicts of interest.*

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